Exploring the Leucine-Proline Binding Pocket of the Src SH3 Domain Using Structure-Based, Split-Pool Synthesis and Affinity-Based Selection

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Abstract: Structure-based, split-pool synthesis was used to discover non-peptide binding elements for the leucine-proline binding pocket of the Src SH3 domain. Binding characteristics of the protein pocket were then explored by comparing a series of ligands that contains subtle variants of the parent non-peptide binding structure. Further insights into this receptor−ligand interaction were provided by multidimensional NMR structure determination of one of the non-peptide ligands.

The study of small molecules that bind protein receptors can provide insight into the molecular forces that govern receptor−ligand interactions. Important binding requirements are often revealed when molecules with very different structures use similar binding contacts to adhere to the protein receptor. We have chosen SH3 domains to test our ability to discover novel structures that have an affinity for the same or similar protein surfaces. Furthermore, analysis of the dependence of the protein affinity on ligand structure reveals much about the intricacies of ligand−receptor interactions.

In previous reports, we have demonstrated how multidimensional NMR analysis and split-pool synthesis can be used to discover high affinity ligands for the Src SH3 domain.[1] Initial explorations[2] used biased peptide libraries wherein the bias was used to ensure that a large percentage of the library met a putative requirement[3] for peptide binding to SH3 domains. NMR analysis was then used to reveal the structural basis for peptide binding to the protein domain.[4] Subsequently, on the basis of the NMR structure of the receptor−ligand complex, we chose a bias for a combinatorial library of non-natural amino acids wherein the bias was used to direct elements of the library to a specific binding surface on the protein receptor. Screening this library led to the discovery of non-peptide elements that occupy the "specificity pocket" of the Src SH3 domain.[5] In this paper and the preceding one,[6] we describe the synthesis and screening of two structurally biased non-natural amino acid libraries. The library reported here is used to explore a second SH3 binding pocket, namely the "leucine-proline (Leu-Pro)" binding pocket, whereas the preceding paper describes the synthesis and assay of a highly homologous library directed toward the specificity pocket.[6]

SH3 domains are small, independently folded receptors that often act as modular components of larger signal transducing proteins.[7] The SH3 fold is composed of two antiparallel β-sheets packed against one another at right angles. Additional loops or helices can be appended to this core framework allowing for variation of SH3 tertiary structure. The structural basis for peptide binding to Src family SH3 domains has been elucidated by both NMR[4] and crystallographic techniques.[8] All structurally characterized peptide ligands have been shown to occupy two hydrophobic "Leu-Pro" pockets and the specificity pocket. These pockets constitute a shallow groove approximately 25 Å long and 10 Å wide. As shown in Figure 1, SH3 peptide ligands adopt a polyproline type II helical conformation that positions two leucine-proline dipeptide segments into adjacent Leu-Pro pockets. The central Leu-Pro binding pocket is formed by protein side chains W118, D117, and Y136. The terminal Leu-Pro pocket is surrounded by SH3 residues Y136, N135, and Y90. The specificity pocket of Src family SH3 domains is filled by an arginine-containing sequence from the ligand wherein the guanidine functional group makes a salt bridge with aspartic acid residue D99 of the protein.

In an earlier report, we used the leucine-proline repeat (PLPPLP) portion of the known SH3 peptide ligand VSLARRPLPPLP (**VSL12**) as a bias to direct elements of a chemical library to the specificity pocket of the protein. The polymer-bead supported library was constructed by attaching the N-terminus of PLPPLP to a sequence of four variable non-natural

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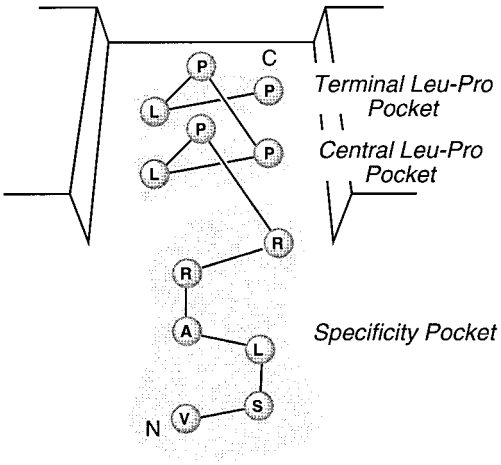

Figure 1. Schematic representation of the dodecapeptide, VSLARRPLPPLP as it binds to the Src SH3 domain.

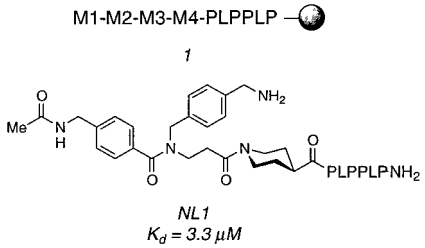

Figure 2. Ligand NL1 was selected from a PLPPLP biased library and contains non-peptide elements that occupy the specificity pocket of the Src SH3 domain.

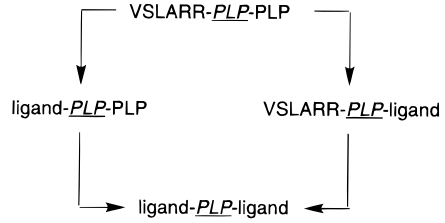

Figure 3. Specificity surrogates might be combined with leu-pro surrogates to provide a new binding epitope for the Src SH3 domain.

amino acids to give **1** (Figure 2). SH3 ligands were then identified by treatment of the entire collection of beads with labeled Src SH3 domain and followed by decoding of colored beads. In this manner, SH3 ligand **NL1** was discovered and, by fluorescence perturbation experiments, shown to have a dissociation constant of 3.3 μM for the Src SH3 domain. Subsequent structure−binding studies, as well as solution structure determination by NMR spectroscopy, revealed the mode of binding of **NL1** to the Src SH3 domain.[5b]

We considered that the above-described procedure might be used to find suitable surrogates for the leucine-proline elements of the peptide ligand. That is, we decided to use VSLARRPLP (**VSL9**) as a bias to direct a library of compounds to the terminal leucine-proline binding pocket. Screening this library would hopefully reveal non-natural binding elements that fill the terminal leucine-proline pocket and thus restore the affinity lost upon truncation of the dodecapeptide **VSL12** to **VSL9**. Subsequently, because the central PLP is conserved in both this library and the PLPPLP biased library, we expected that these independently discovered binding elements might be fused to provide a new binding epitope for SH3 domains (Figure 3).

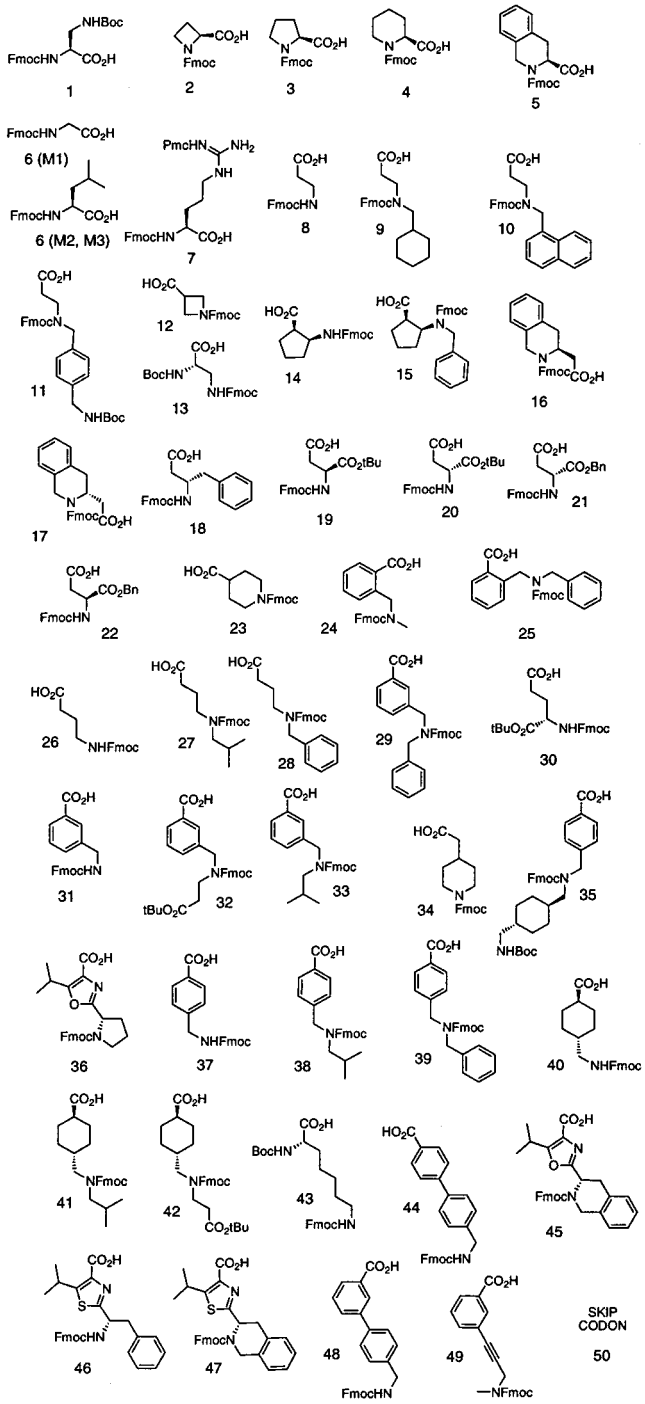

Figure 4. Monomers (M1, M2, and M3) used in the construction of library VSLARRPLP-M1-M2-M3.

Affinity-Based Selection of Ligands for the Src SH3 Domain

Using split-pool library synthesis techniques,[9] an encoded[10] trimeric library of 125 000 compounds was prepared from the 50 monomers (including a skip codon[11]) depicted in Figure 4. Since the Leu-Pro pocket is composed of hydrophobic residues, a preponderance of hydrophobic, as compared to charged,

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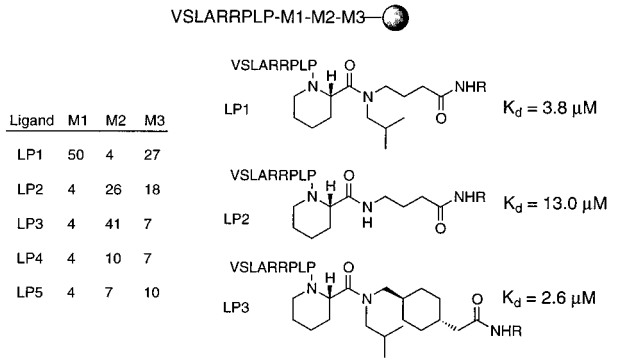

Figure 5. Sequences and binding constants for compounds selected from an on-bead colorimetric screen of library VSLARRPLP-M1-M2-M3.

protected amino acids were used. To the N-terminus of the library was then added the elements Ac-VSLARRPLP, and the library was deprotected and subjected to a colorimetric binding assay.[5a] The data shown in Figure 5 are the combined results of two library assays. While many colored beads were selected that display no consensus sequence (data not shown), five of the sequences appeared to be related. As may be seen in Figure 5, sequences **LP1**-**LP5** all have monomer 4, L-pipecolinic acid, attached to the C-terminus of the bias (50 is the skip codon, thus **LP1** = VSL9-4-27). Of these five ligands, four have purely aliphatic amino acids in the second position of the sequence (10, 26, 27, and 41), two of which are *N*-isobutyl amino acids (41 and 27). The two N-isobutyl substituted ligands, **LP1** and **LP3**, display significant structural homology and differ only in the size of the aliphatic appendage at the C-terminus. The third monomer position shows no consensus and was deemed unnecessary for binding since the selected sequence 50-4-27 has a skip codon and is thus composed of only two monomer positions.

Three of the ligands, **LP1**-**LP3** were resynthesized on solid support. After cleavage and purification, fluorescence perturbation experiments were performed to determine the affinities of the ligands for the Src SH3 domain. In all three cases, addition of ligand to a solution of the Src SH3 domain resulted in a positive shift in fluorescence intensity (at 330 nM) as is typical for arginine-containing Src SH3 ligands. While the secondary amide ligand **LP2** has a 13 μM dissociation constant with the SH3 domain, the tertiary amide ligands **LP1** and **LP3** were found to have somewhat stronger affinities for the SH3 domain with K_d values of 3.8 and 2.6 μM respectively.[12]

(11) The skip codon refers to a process whereby no reagents are added to a single reaction vessel (out of 50 in the current synthesis) in a given step in a split-pool synthesis. See ref 5a.

(12) The observation that the library assay led to the selection of pipecolinic acid in position 3, but not proline, indicates that subtle changes in conformation might engender large changes in binding of bead-bound ligands to the Src SH3 domain. However, we and others (Liang, R.; Yan, L.; Loebach, J.; Ge, M.; Uozumi, Y.; Sekanina, K.; Horan, N.; Gildersleeve, J.; Thompson, C.; Smith, A.; Biswas, K.; Still, W. C.; Kahne, D. *Science* **1996**, *274*, 1520−1522) have noticed discrepancies between the protein-binding ability of support-bound ligands and ligands in solution. It has been argued that these differences arise from the multidentate nature of ligands on solid phase versus monodentate nature of ligands in solution. We believe, that in this case, a more likely scenario is that certain ligands are more able to project themselves from the surface of the bead and are therefore able to more effectively bind to a protein while attached to the bead. This presentation ability is not relevant or is at least less relevant to the case of solution binding. This would explain the observation that **VSL9-Pro-27** has only ~2-fold lower affinity for the SH3 domain as compared to **VSL9-4-27** (K_d = 7.7 mM vs K_d = 3.8 mM) yet **VSL9-4-27** was not selected in the library assay.

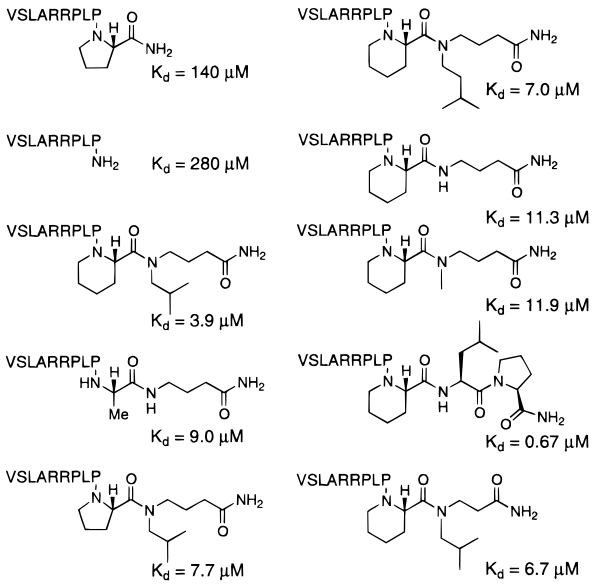

Figure 6. Binding constants for a series of homologous ligands derived from ligand VSLARRPLP-4-27.

Structural Analysis

To address the relative contribution to binding of various elements of ligand LP1(VSL9-4-27), a number of homologues were prepared and analyzed by fluorescence perturbation. As shown in Figure 6, the bias itself, VSLARRPLP, binds the Src SH3 domain with a 280 μM dissociation constant. This precipitous loss in binding affinity, compared to that of VSLARRPLP-4-27 (K_d = 3.8 μM), indicates that the elements 4−27 provide significant binding energy to SH3 ligands. The essential contribution that monomer 27 makes to the overall binding energy is revealed by comparing the VSL9-Pro-27 to VSL9-Pro-NH_2 deletion (K_d = 7.7 μM → K_d = 140 μM). Experiments show that there is some tolerance in position M1 as proline can be used in place of pipecolinic acid with only a slight decrease in K_d (K_d = 7.7 μM versus 3.8 μM). Replacement of monomer 4 with alanine leads to a slightly larger loss in binding affinity as compared to the 4 → Pro substitution (K_d = 9.0 μM versus 7.7 μM). This is consistent with the expectation that pipecolinic acid plays the same bridging role in VSL9-4-27 that proline plays in **VSL12** (VSL9-P-LP). The bridging group serves as a scaffold, linking elements in the Leu-Pro pocket with the rest of the ligand, but makes no contact with the protein surface. The small drop in affinity upon substitution to alanine is indicative of the ability of pipecolinic acid to stabilize the PPII-helical conformation of the biasing element. Such perturbations are not surprising and have been noted before in the case of peptide ligands binding SH3 domains.[13]

While the importance of monomer 27 in the M2 position is readily apparent from the K_d of VSL9-Pro-NH_2, the GABA side chain is not a strict structural requirement. Alteration of the GABA backbone of monomer 27 results in only small changes in the dissociation constant as demonstrated by the *N*-isobutyl-GABA → *N*-isobutyl-β-Ala substitution (K_d = 3.8 μM → K_d = 6.8 μM). The observation that ligand **LP3** (VSL9-4-41) binds to the protein somewhat tighter than ligand **LP1** (VSL9-4-27) indicates that there may be some additional hydrophobic contact with the larger side chain of 41 as compared to the GABA side chain.

(13) In a previous report, it was found that changing the corresponding bridging proline of RKLP**P**RPSK to alanine results in a decrease in binding affinity from 9.1 to 16.5 μM for the PI3K SH3 domain. See ref 4b.

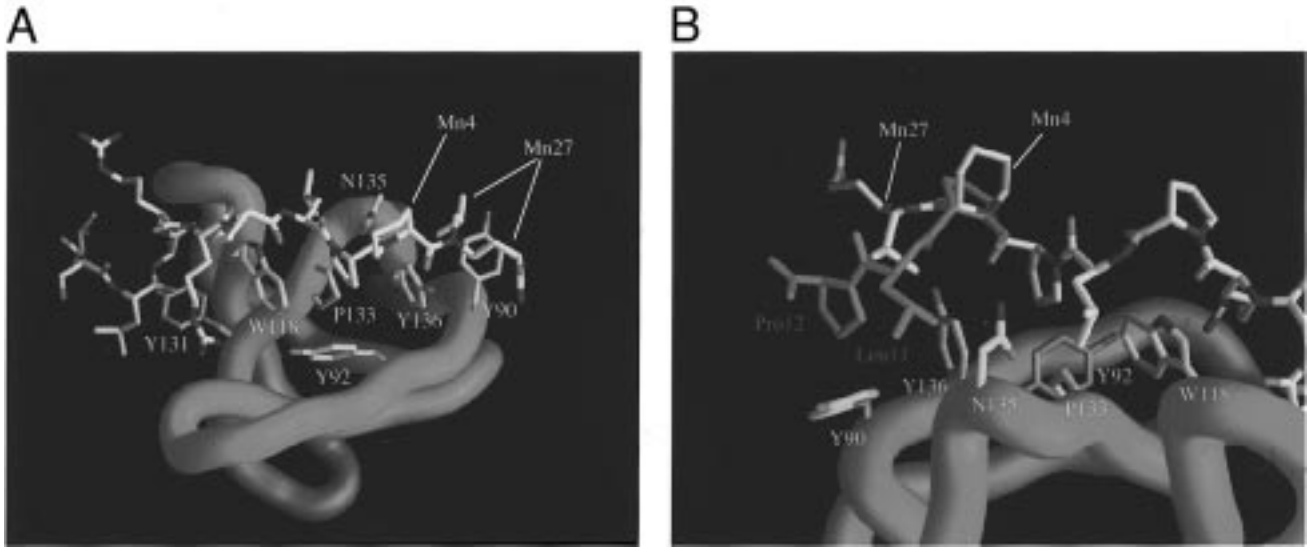

Figure 7. Solution structure of the non-peptide portion of VSLARRPLP-4-27 bound to the Src SH3 domain. The C_α trace of the SH3 domain is shown as a red worm. The SH3 residues are labeled yellow, and the VSLARRPLP-4-27 residues are white. (A) VSLARRPLP-4-27 at the binding site. (B) Comparison of VSLARRPLP-4-27 and VSL12. Only the last three residues, Pro10-Leu11-Pro12, of VSL12 are shown in green.

As described above, nitrogen substitution on monomer 27 appears to be required for highest affinity as removal of the *N*-isobutyl group of 27, to give 26, leads to a drop in protein binding affinity ($K_d = 3.8\ \mu M \rightarrow K_d = 13.0\ \mu M$). However, it does not appear that a simple secondary amide is the minimal binding requirement as VSL9-4-N(Me)GABA is actually a slightly weaker binding ligand that the corresponding VSL9-4-26 (VSL9-4-N(H)GABA). These data indicate that the function of the *N*-isobutyl group *N*-alkyl group is not to simply stabilize an amide conformer, but that it actually makes contact with the protein surface.

Previous structural studies indicate that the three binding pockets of the SH3 domain interact with distinct binding elements in the ligand linked by two scaffolding prolines. To locate the binding site of elements 4−27 and 4−41, the $^{15}N-^{1}H$ HSQC spectra of the two ligand−SH3 (1:1) complexes were acquired using the ^{15}N-labeled SH3 domain. Comparing the spectra with that of the SH3−**VSL12** complex indicates that both **LP1** and **LP3** interact with the protein at the same site as **VSL12**. More specifically, the two HSQC spectra for **LP1** and **LP3** were virtually identical, except for the small difference in chemical shift for the side chain NH of N135 which is located near the terminal Leu-Pro pocket. The difference in the perturbation pattern suggests that the VSLARRPLP bias directs the non-peptidic moiety to the binding site. The structure of the **LP1**-SH3 complex was determined by multidimensional NMR.

As shown in the structure of **LP1**−SH3 (Figure 7), the first nine residues of the ligand adopt the same bound conformation as their counterparts in **VSL12**. Monomer 4 is functionally similar to Pro10 in **VSL12** and serves as the scaffolding element that bridges monomer 27 and a Leu-Pro dipeptide to occupy the terminal and central Leu-Pro pockets, respectively. As evidenced by two small coupling constants between H_2 and its vicinal protons H_7 and H_7', the amide carbonyl substituent of the monomer 4 piperidine ring assumes the sterically favorable axial position. The strong NOEs between H_2 of monomer 4 and H_6 of monomer 27 set the configuration for the amide bond connecting the two amino acids. This places the isobutyl group of monomer 27 at a position reminiscent of the Leu11 side chain in **VSL12**. Most of the NOEs to the SH3 domain from the non-peptidic fragment are between the two isobutyl methyls of monomer 27 and protein residues Tyr136 and Tyr90. The GABA group on monomer 27 has no NOEs to the SH3 domain, presumably because it points away from the binding site to avoid unfavorable *syn*-pentane-type interactions with the nearby isobutyl.

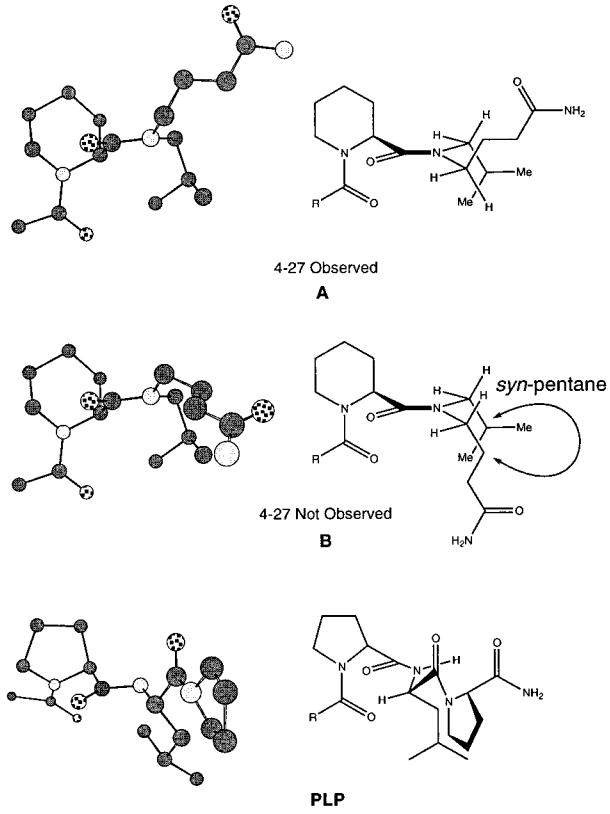

Figure 8. Conformational analysis of the bound conformation of the LP1 elements 4−27 compared to PLP. While structure B might fill the Leu-Pro pocket in a similar manner as PLP, an incipient *syn*-pentane interaction likely disfavors this conformation.

Figure 8 depicts a conformational analysis of the receptor-bound non-peptidic fragment of **LP1**. With both the amide configuration and the *N*-isobutyl position set by observed NOEs it might be expected that, for optimal binding, the GABA chain would be situated as shown for conformer B. The hydrophobic

region of the Leu-Pro pocket that is normally occupied by proline would be filled by the alkyl chain of conformer B. However, conformation A is preferred upon binding as determined by NMR studies. It is likely that this results from incipient *syn*-pentane interactions that disfavor B and result in A being the observed binding conformer. In contrast, side chains of both Leu11 and Pro12 in **VSL12** contact the protein extensively, and **VSL12** binds the SH3 domain 10-fold tighter than **LP1**.

Despite significant deviation in backbone connectivity, **VSL12** and **LP1** both fill the leu-pro pocket by taking advantage of similar binding opportunities. Both the leucine side chain and the isobutyl of monomer 27 occupy the hydrophobic pocket formed by residues Y90, N135, and Y136. However, variations in the structure of **VSL12** and **LP1** lead to accordant differences in affinity toward the Src SH3 domain. Because it is attached to the amide nitrogen as opposed to the C_α, the *N*-isobutyl of 27 does not reach as deeply into the pocket as the Leu11 side chain. By comparing the NMR structures, it is also clear that the proline residue of **VSL12** occupies a much larger portion of the leu-pro pocket than does the GABA chain of monomer 27. The respective differences in the dissociation constants for **LP1** and **VSL12** may be attributed to these factors. While an attempt was made to remedy this situation by extending the isobutyl group further in the pocket, as an *N*-isovaleryl derivative, no gain in binding affinity was observed.

In stark contrast to the position of the C-terminal proline of **VSL12**, the GABA side chain of **LP1** is directed away from the protein. While this helps explain the difference in affinity between **LP1** and **VSL12**, it also reveals the underlying reason for the minor affinity difference between **LP1** and **LP3**, despite a very different hydrophobic group attached to the nitrogen in monomer 41 of **LP3**. The structure indicates that any additional residue following monomer 27 would be too far away from the binding site to contact the protein. As a result, there is no selection at the M_3 position from library screening.

While the bridging pipecolinic acid of VSL9-4-27 appears to play the same role as the proline of **VSL12** (VSL9-Pro-Leu-Pro), analysis of structural data indicates that the pipecolinic acid in VSL9-4-27 deviates significantly from what one would expect. The Φ value for pipecolinic acid residue in VSL9-4-27 is $-113°$, whereas the corresponding bridging proline in **VSL12** (VSL9-**Pro**-Leu-Pro) has a Φ value of $-76°$. In the absence of structural data for either VSL9-4-LP or VSL9-Pro-27, we cannot speculate as to whether the conformation of pipecolinic acid relative to proline influences the conformation of 27 or *vice versa*. With inference from the structure of **VSL12**, one might predict that the proline of VSL9-Pro-27 would more suitably position the *N*-isobutyl substituent in the leu-pro pocket and lead to a stronger binding ligand. However, fluorescence perturbation experiments indicate that VSL9-Pro-27 binds with weaker affinity than VSL9-4-27 to the SH3 domain.

Concatenation

Because the central PLP is conserved in both the library reported here (VSLARR-**PLP**-M1-M2-M3) and in the library reported in the accompanying paper (A-B-(isonipecotic acid)-**PLP**-PLP),[6] it was anticipated that the two independently discovered binding solutions might be fused to create a chimeric binding epitope for SH3 domains.[14] Non-peptide elements A7-B22-(isonipecotic acid) were discovered from the PLPPLP biased library and the resulting ligand, Ac-A7-B22-(isonipecotic acid)-PLPPLP-NH_2, binds to the Src SH3 domain with a 1.1 μM dissociation constant (Figure 9). Fusion of these non-peptide specificity elements to the non-peptide elements 4−41, through a PLP linkage, provided chimera Ac-A7-B22-(isonipecotic acid)-**PLP**-4-41-NH_2 (K_d = 1.6 μM). In the chimera, all but three of the original 12 amino acids of **VSL12** have been replaced by non-peptidic residues. Fluorescence perturbation experiments show that the chimera binds the Src SH3 domain better than VSL9-4-41 (K_d chimera = 1.6 μM versus 2.6 μM for VSL9-4-41). Surprisingly, whereas the bias from **VSL12** (K_d = 0.45 μM) attached to 4−41 gives ligand VSL9-4-41 with a 2.6 μM dissociation constant, attachment of the bias from the *weaker* binding Ac-A7-B22-(isonipecotic acid)-PLPPLP (K_d = 1.1 μM; bias = A7-B22-(isonipecotic acid)-**PLP**) affords a *tighter* binding ligand. This cooperative effect between Leu-Pro elements and specificity elements in this system may be attributed to the structural intricacies described above for ligand **LP1**. Such a subtle effect may serve to better position elements A7-B22-(isonipecotic acid) in the specificity pocket and make the chimera a tighter binding compound than **LP3**.

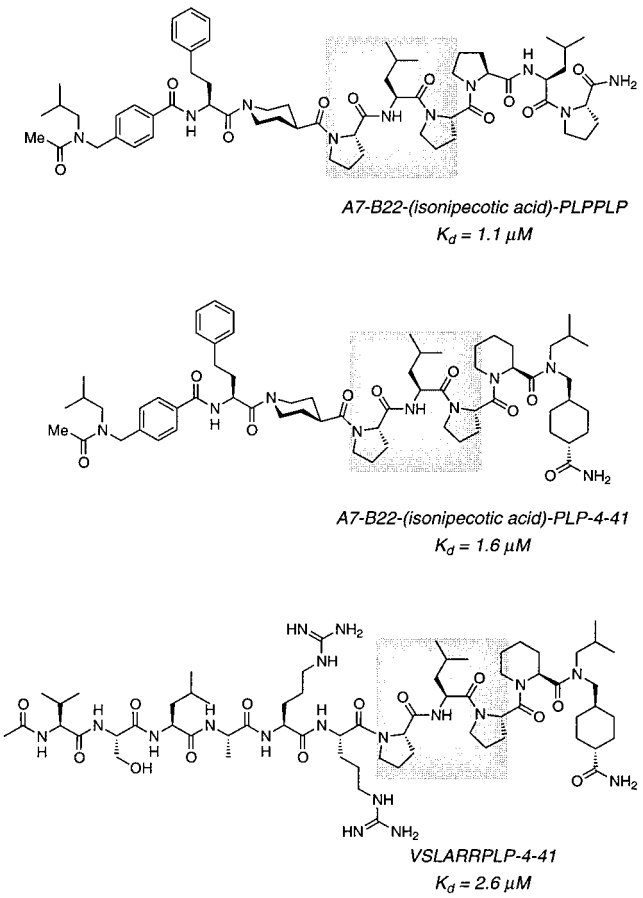

Figure 9. Concatenated ligand A7-B22-(isonipecotic acid)-PLP-4-41 derived from A7-B22-(isonipecotic acid)-PLPPLP and VSLARRPLP-4-41.

Conclusion

Combining diversity-based ligand identification and NMR structure determination, we have uncovered new binding elements for the Leu-Pro pocket of the Src SH3 domain. Whereas earlier reports[5] show that non-peptide SH3 ligands have different interactions than those of known peptide ligands, in this paper we illustrate an example where non-peptide elements have peptide-like interactions.

(14) For a relevant example of an approach to concatenation, see: Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531−1534.

The small differences in hydrophobic contact that **LP1** makes in comparison to **VSL12** with the protein receptor lead to a significant difference in binding affinity (0.47 μM versus 3.8 μM). This observation illustrates the relative importance of hydrophobic contacts in the leu-pro pockets of SH3 domains. Collectively, these results also highlight the difficulties one might encounter when trying to replace a large peptide or protein ligand with a small molecule ligand, as many additional binding contacts would likely be required to significantly improve affinity.

The techniques used to explore protein−ligand interactions in this and the preceeding article have provided insights into the interactions of small molecules with SH3 domains. However, in their current state, they have also been found to have limitations. These relate primarily to the simplicity of the molecules thus far synthesized and the deficiencies of on-bead, affinity-based assays. The development of split-pool synthesis of small molecules bearing greater resemblance to those found in nature and of improved assays will likely enhance the impact of ligand discovery research considerably.

Experimental Section

Monomer Synthesis. Monomers 1−8, 12−14, 19−23, 26, 30, 31, 37, 40, and 43 were either commercially available or were prepared by Fmoc protection of commercially available amino acids (Aldrich, Synthetech, and BaChem) by treatment with Fmoc succinimidyl ester in 1:1 THF/9% Na_2CO_3. Monomers 9−11, 15, 24, 25, 27−29, 33, 35, 38, 39, and 41 were prepared by $NaCNBH_3$, mediated reductive amination[15a] of commercially available amino acids with aldehydes in 1:1 methanol/H_2O at pH = 6, followed by Fmoc protection. Monomers 16,17,18 and 34 were prepared from commercially available Boc-amino acids by Arndt−Eistert synthesis[15b] followed by deprotection and Fmoc protection. Monomers 32 and 42 were prepared by conjugate addition of the corresponding amino acids to *tert*-butyl acrylate followed by Fmoc protection. Monomers 36 and 45−47 were converted from commercially available amino acids to the oxazole or thiazole in analogy to known procedures[15c,d] and followed by Fmoc protection. Monomers 44 and 48 were synthesized by palladium-mediated cross-couping[15e] of commercially available aryl iodides and synthesized aryl stannanes[15f] followed by introduction of the Fmoc group. Monomer 49 was synthesized via Castro−Stephens reaction[15g] followed by Fmoc protection.

Library Synthesis. Using split-pool library synthesis techniques,[9] three copies of an encoded[10] library of 125 000 compounds was prepared from the 50 monomers (including a skip codon[6]) depicted in Figure 4. Synthesis was carried out on 2 g of TentaGel S NH_2 resin (Rapp Polymere, 80 μM beads, 0.25 mmol/g loading) with a spacer element consisting of H_2N-Gly-Cap-βAla-resin. Fmoc protection was used for the N-terminus, Boc protection for side chain amines, *tert*-butyl ester protection for side chain acids, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl protection for the arginine side chain. Monomer coupling was accomplished with one 2 h and one 24 h subjection to 1.5 equiv of each monomeric acid, 1.5 equiv of HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluranium hexafluorophosphate], and 3.3 equiv of DIPEA (diisopropylethylamine) in 400 μL DMF. Deprotection of the N-terminal Fmoc group was accomplished with two 5 min subjections to 20% piperidine in DMF. Each monomer in a given position was encoded with 5%, relative to the resin loading, of either one, two, or three of seven possible tags in a binary encoding format. The photocleavable halogenated tags were attached *after* coupling and deprotection of each monomer. After attachment, deprotection and tagging of the last monomer position, the beads were pooled and stored at −20 °C for further use.

Attachment of the nonapeptide bias, VSLARRPLP, to 132 mg of the trimeric library was accomplished using FastMoc small scale coupling cycles on an Applied Biosystems 431A automated peptide synthesizer. After acylation of the N-terminus (20% Ac_2O in CH_2Cl_2 for 2 × 30 min), the library was deprotected by treatment with a solution of 95% TFA, 2.5% H_2O, and 2.5% Et_3SiH for 4 h. The library was then washed with CH_2Cl_2, MeOH, DMF, MeOH, H_2O/MeOH, H_2O, phosphate-buffered saline (PBS), and finally screening buffer (PBS + triton-X + BSA).

Library Screening.[5a,6] Screening was accomplished by treatment of the biased library (132 mg) with 890 μL of a premixed (30 min) solution of biotinoylated Src SH3 domain (1.92 μM) and streptavidin−alkaline phosphatase conjugate (200 nM). After 14 h incubation at room temperature, the beads were filtered and washed briefly (10 s) with 4 × 1 mL of screening buffer. The beads were then shaken with a nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3′-indolyl phosphate (BCIP) solution (Pierce) for 11 min, drained, washed with 0.01% triton-X and stored in 6 N guanidinium hydrochloride. Colored beads were selected from the library, washed with DMF until colorless, and decoded.

Ligand Resynthesis. Using standard Fmoc chemistry, the following compounds were synthesized on Rink amide 4-methylbenzhydrylamine resin. Unnatural residues were coupled manually using 4 equiv of HATU, 5 equiv of suitably protected amino acid, and 6 equiv of Hunig's base in DMF for 4 h. Unreacted N-terminal amines were capped with 30% acetic anhydride in CH_2Cl_2 for 30 min and then N-terminal protecting groups removed by two 10 min subjections to 20% piperidine in DMF. The bias was attached using an Applied Biosystems 431A automated peptide synthesizer. After cleavage and side chain deprotection with Reagent K, the ligands were purified by HPLC and characterized by mass spectroscopy.

For Ac-VSLARRPLPPLP-NH_2 LRMS calcd for $C_{63}H_{109}N_{19}O_{14}$ 1356, found 1356. For Ac-VSLARRPLPP-NH_2: LRMS calcd for $C_{52}H_{91}N_{17}O_{12}$ 1146, found 1146. For Ac-VSLARRPLP-NH_2: LRMS calcd for $C_{47}H_{84}N_{16}O_{11}$ 1049, found 1049. For Ac-VSLARRPLP-Pip-N-(isobutyl)-GABA-NH_2: LRMS calcd for $C_{61}H_{108}N_{18}O_{13}$ 1301, found 1301. For Ac-VSLARRPLP-Pro-N(isobutyl)-GABA-NH_2: LRMS calcd for $C_{60}H_{106}N_{18}O_{13}$ 1287, found 1287. For Ac-VSLARRPLP-Pip-LP-NH_2: LRMS calcd for $C_{64}H_{111}N_{19}O_{14}$ 1370, found 1370. For c-VSLARRPLP-Pip-N(isobutyl)-βAla-NH_2: LRMS calcd for $C_{60}H_{106}N_{18}O_{13}$ 1287, found 1287. For Ac-VSLARRPLP-Pip-N(isovaleryl)-GABA-NH_2: LRMS calcd for $C_{62}H_{110}N_{18}O_{13}$ 1315, found 1315. For Ac-VSLARRPLP-Pip-N(methyl)-GABA-NH_2: LRMS calcd for $C_{58}H_{102}N_{18}O_{13}$ 1259, found 1259. For Ac-VSLARRPLP-Pip-N-(isobutyl)-*trans*-4-(aminomethyl)cyclohexanecarbozamide: LRMS calcd for $C_{55}H_{98}N_{18}O_{13}$ 1355, found 1355. For Ac-VSLARRPLP-Pip-GABA-NH_2: LRMS calcd for $C_{57}H_{100}N_{18}O_{13}$ 1245, found 1245.

Dissociation Constant Determination. Binding constants were determined using fluorescence perturbation spectroscopy on a Hitachi fluorescence spectrophotometer. A solution of ∼0.15 μM Src SH3 domain in PBS was added to a quartz cuvette, and the fluorescence emission at 330 nm (excitation at 280 nm) was measured for a series of ligand concentrations. The dissociation constant was then calculated as described previously.[2]

NMR Methods and Structure Determination. The NMR samples contained 2−3 mM of a 1:1 complex of the Src SH3 domain and ligand, in a D_2O or 90% H_2O/10% D_2O buffer containing 50 mM potassium phosphate (pH 6.0) and 150 mM KCl. All spectra were recorded at 27 °C using a Bruker DMX500 spectrometer. The assignment of the SH3 domain was accomplised by standard two- and three- dimensional $^{15}N/^{13}C$ edited experiments as described before.[5b] The assignment of the ligand resonances was done by a series of two-dimensional correlation spectroscopy, total correlation spectroscopy, and nuclear Overhauser enhancement spectroscopy (NOESY) filter experiments similar to those reported earlier. The intermolecular NOEs between the SH3 domain and the ligand were obtained by two- and three-dimensional half-filtered NOESY experiments in which the ^{13}C and their attached protons on the SH3 domain are correlated with protons

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attached to the ^{12}C on the ligand. Distance and dihedral restraints derived from these experiments were used to calculate the structures as described before.[5b]

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