Exploring the Specificity Pockets of Two Homologous SH3 Domains Using Structure-Based, Split-Pool Synthesis and Affinity-Based Selection

Tarun M. Kapoor, Amy Hamilton Andreotti, and Stuart L. Schreiber*

Contribution from the Department of Chemistry and Chemical Biology, Howard Hughes Medical Institute, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

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Abstract: Split-pool synthesis was used to prepare large numbers of spatially-separated molecules and thereby to investigate the specificity pockets of similar SH3 domains found in the tyrosine kinases Src and Hck. By taking into account the structure of the Src SH3 domain complexed to a ligand containing non-peptide-binding elements, the molecules were designed to complement the topography of the protein's binding pocket. This procedure led to the discovery of ligands having greater affinity and enhanced selectivity for the Src SH3 domain. It also yielded non-natural ligands that bind selectively to the Hck SH3 domain. Insights gained from this strategy may facilitate the discovery of molecules useful for evaluating the cellular function of SH3 domain-containing proteins.

Insights into the cellular function of proteins can be gained through the use of small molecule ligands in analogy to the use of mutations.[1] Although the majority of such ligands have been either natural products or their synthetic variants, it seems unlikely that natural products alone will allow the exploration of the function of all proteins. The strategy of split-pool synthesis,[2] involving a simple mechanical step imposed at strategic times during a synthesis, has the potential to bridge this gap by providing the means to synthesize vast numbers of natural product-like molecules. Realizing this goal will require developing assays and methods in asymmetric synthesis compatible with split-pool synthesis. With this as our ultimate objective, we selected the Src Homology 3 (SH3) domain[3a] as a test system to work out the steps required for this approach to ligand discovery.

SH3 domains are noncatalytic protein modules that mediate inter- and intramolecular protein interactions in molecules ranging from components of the cytoskeleton to transducers of cellular signaling.[3,4] To study the molecular recognition of these receptors, we have used protein structure to design diverse libraries of potential ligands attached to solid supports.[5,6] Although conventional assays for screening immobilized ligands based on receptor affinity have limitations,[7] we anticipated that their compatibility with split-pool synthesis would allow their use in screens involving millions of compounds.

Early in our work with SH3 domains, we demonstrated the importance of biasing the structures of ligands prepared using split-pool synthesis.[6] Biased peptide libraries yielded two sets of ligands subsequently shown by structural studies to bind to the Src SH3 domain in opposite orientations.[8] The insights gained from these studies guided the split-pool synthesis of 1.1 million compounds designed to direct non-peptide elements to the specificity pocket of the Src SH3 domain.[5] The chemical structures of two ligands, **NL-1** and **NL-2**, discovered from this library are shown in Figure 1. Solution structures of these ligands complexed with the Src SH3 domain[9] have provided insights into the molecular recognition of this receptor and are the basis of our current efforts to improve the affinity and specificity of non-natural SH3 ligands.

This and the following manuscript[10] describe split-pool syntheses and affinity-based screening of two libraries aimed at investigating the specificity and Leu-Pro pockets of SH3 domains. The library described in this paper consists of 2499 compounds structurally related to a previously reported Src SH3 ligand containing non-peptide elements. By screening these molecules with two highly homologous proteins, the Src and the Hck SH3 domains, we demonstrate the ability to generate unique and specific ligands for structurally related binding sites.

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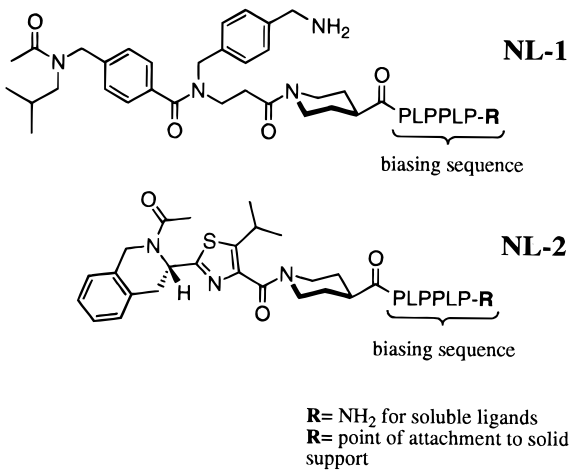

Figure 1. Ligands for the Src SH3 domain previously discovered using structure based design of large collections of molecules using split-pool synthesis.

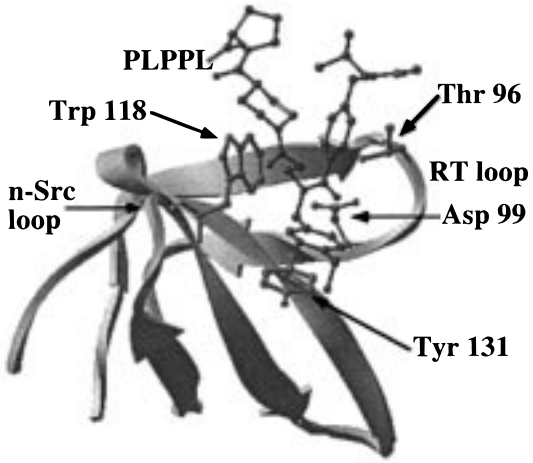

Figure 2. Structure of NL-1 bound to the Src SH3 domain. Important residues in the specificity pocket of the SH3 domain are labeled.

Receptor−Ligand Complexes

The solution structures of **NL-1** and **NL-2** bound to the Src SH3 domain, combined with systematic studies of ligands related to **NL-1** and **NL-2**, have helped define the contributions of functional groups in these ligands.[9] The binding mode of the non-peptide elements differs markedly from that of peptide ligands bound in the same pocket. The characteristic salt bridge between an arginine in the peptide ligands and an aspartic acid (Asp-99) in the protein is altogether absent. In contrast to the peptide ligands, **NL-1** and **NL-2** rely predominantly on hydrophobic contacts on the floor and RT loop (see Figure 2) of this pocket. The divergence in the interactions between the receptor and its ligands underscores the difficulties associated with the design and discovery of ligands in general.

Key interactions between the non-peptide elements of **NL-1** and residues in the Src SH3 specificity pocket are highlighted in Figure 2. The aromatic ring of the N-terminal benzoyl group in **NL-1** interacts with the methyl group of Thr-96 in the receptor. The benzoyl carbonyl is proximal to the carboxylate of Asp-99 and is located at a sharp turn defined by the ligand structure. Also observed is an intermolecular aromatic−aromatic interaction between the aminomethyl-substituted benzyl group in **NL-1** and the phenyl ring of Tyr-131. The piperidine ring of the isonipecotic acid is in a chair conformation and exits the specificity pocket close to Trp-118. **NL-1** does not make significant contacts with the n-Src loop. This **NL-1**/Src SH3 structure has guided the design of new libraries of potential SH3 ligands in the current study.

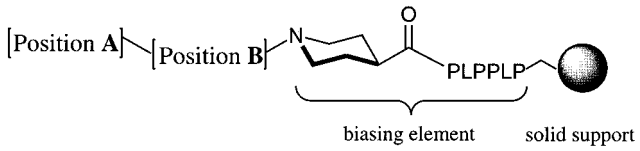

Figure 3. Outline of a new library.

Design of the Library

Recently, parallel synthesis[11] used in conjunction with the structural analysis of receptor−ligand complexes led to collections of 100,[12a] 348,[12b] and 1000[12c] non-natural compounds, several of which having protein-binding properties. Since split-pool synthesis is capable of generating far greater molecular diversity, even 1.1 million compounds in the earlier study,[5] we view it as an important element of our current and future synthetic efforts. To explore the large SH3 surface spanned by **NL-1** systematically, we prepared an encoded[13] library having two diversity positions (Figure 3). Adjacent to the biasing element (position B), we used a set of 50 monomers, 32 of which have a β-alanine backbone (Figure 4). This set of compounds permutes a large subset of functional and conformational variations for monomer B29. Twenty percent of the monomers have an α-amino acid backbone while the remainder have longer backbone spacers. Sixty-four percent of the monomers were designed to retain the potential aromatic−aromatic interaction observed between **NL-1** and Tyr-131 in the receptor. Several variants of monomer B29, including ones having different para substitutions on the aromatic ring, were reported earlier[9] and have not been used here.

At the terminal position we have also used a set of 50 compounds (position A in Figure 3). The relatively weak selection for monomer A7 in our first library[5] suggested the use of more structurally varied monomers at this position, and so we included ones containing amide, urea, and carbamate functionalities (Figure 5). To gain a possible salt-bridge with the carboxylate of Asp-99 in the SH3 binding pocket, we used monomers predicted to be positively charged at pH 7.5. Finally, monomer A7 and a subset of aromatic compounds were included to retain the observed hydrophobic interaction with the threonine side chain in the RT loop.

Modifications of the isonipecotic ring, such as substitutions α to the ring nitrogen, were also considered. Conformational analysis, however, predicted that allylic strain due to the proximal amide would force the substituent to adopt an axial

(11) Parallel synthesis involves the preparation of individual compounds in isolated reaction vessels (Hobbs Dewitt, S.; Czarnik, A. W. *Acc. Chem. Res.* **1996**, *29*, 114−122). It is differentiated from a split-pool synthesis that generates all combinations of coupling partners in a discrete manner, usually on solid support. In mixture synthesis, multiple components are used as mixtures in each reaction (Carell, T.; Wintner, E. A.; Sutherland, A. J.; Rebek, J., Jr.; Dunayevsky, Y. M.; Vouros, P. *Chem. Biol.* **1995**, *2*, 171−183). A large library of compounds can be synthesized with fewer individual steps by the split-pool and mixture synthesis methods than by parallel synthesis.

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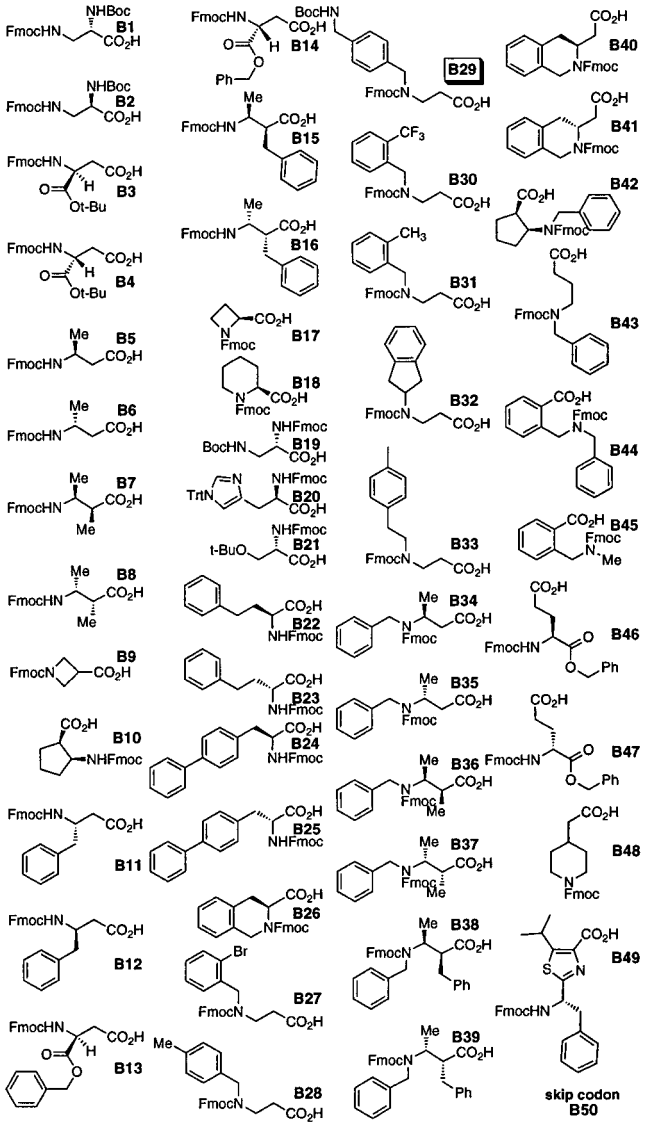

Figure 4. Monomers used at position B. B29 is the monomer at position B in NL-1.

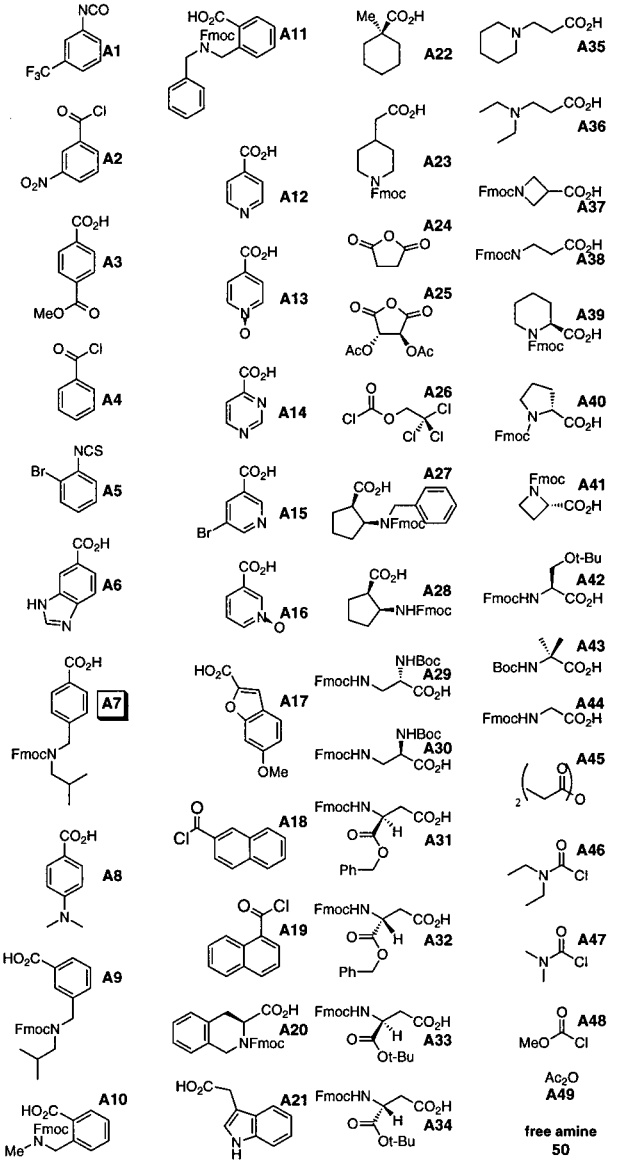

Figure 5. Monomers used at position A. A7 is the monomer at position A in NL-1.

orientation, resulting in the substituent pointing away from the receptor. Thus, each molecule in the library retained a constant bias consisting of (isonipecotic acid)-Pro-Leu-Pro-Pro-Leu-Pro.

Monomers B29, B49, A7, and A49 were included to test the library synthesis efficiency and the stringency of our assay. We expected that if ligands with monomers other than B29, B49, A7, or A49 were selected, their dissociation constants should be lower than those of known ligands containing these monomers.[14]

In addition to the described monomers, the library was designed with a "skip codon" at position B and a "free amine" at position A. The skip codon represents a deletion that has been introduced to generate a sublibrary of the form (position A)-(isonipecotic acid)-PLPPLP-solid support with all of the monomers used at position A directly attached to the biasing element. This strategy increases the molecular diversity at position B. It also serves as a control for failed couplings of position B monomers to the secondary nitrogen of the isonipecotic acid. During encoded split-pool synthesis, failure to couple a monomer does not preclude the incorporation of its tag, since the coupling and encoding steps are independent. If beads are selected that have a skip codon at position B, we analyze all of the sequences that have some other monomer at position B followed by the same compound at position A. It is possible that the encoded position B monomer did not couple efficiently and therefore may be absent in these ligands. The free amine at the N-terminus represents no compound coupled at position A and similarly checks for the fidelity of the coupling chemistry at this position.

Selection of the Highest Affinity Ligands for the Src SH3 Domain

After two coupling steps using encoded[13] split-pool synthesis, we generated a total of 2499 distinct compounds on TentaGel polymer support. Each compound could be uniquely identified by its associated tags and our tag-based code. To evaluate the relative binding of all of the ligands in these pools simultaneously, we used a colorimetric assay. High fluorescence background due to the nature of the ligands combined with effects of the TentaGel support prohibited the use of the previously reported fluorescence assay.[7]

In the current assay, the N-terminal residue of the SH3 domain of interest is attached covalently to a biotin molecule

(14) The K_d values for Ac-(B49)-(isonipecotic acid)-PLPPLP-NH_2 and HN-(A7)-(B29)-(isonipecotic acid)-PLPPLP-NH_2 with the Src SH3 domain are 5.4 and 4.1 μM, respectively.

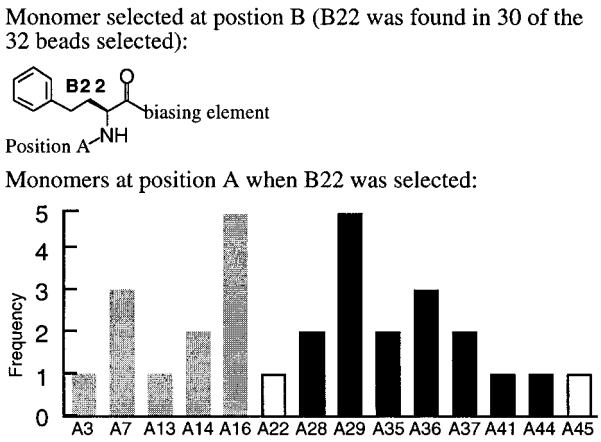

Figure 6. Monomers selected by the Src SH3 domain. The dark shading in the histogram highlights the positively charged ligands. The lighter shading represents the monomers that are structurally related to A7. Other monomers are not shaded.

using the previously described method.[5] This biotinoylated receptor is preincubated with a conjugate of streptavidin and alkaline phosphatase to block the biotin binding site on streptavidin. This receptor−phosphatase complex is incubated with the library of potential ligands. After stringent washes, the library is exposed to a solution of 5-bromo-4-chloro-3′-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT). The beads that contain a ligand for the SH3 domain have a high local concentration of the alkaline phosphatase noncovalently attached to the receptor via the streptavidin−biotin interaction. The phosphatase dephosphorylates BCIP, precipitating the dehydroindigo product that results from indoxyl tautomerization and dimerization. This concomitantly reduces the NBT to an insoluble, deep blue diformazan that precipitates on the bead.[15] These colored beads are microsyringed out of the pool of thousands of beads and decoded[13] after a series of washes that remove the contaminating dye.

Using this assay, we screened two batches of ∼12 500 beads with a biotinoylated Src SH3 domain. Of the 32 beads that were selected, 30 beads had monomer B22 at position B (Figure 6). For these 30 beads two sets of monomers were selected at position A. The set including A7 has a benzoyl substituent at position A. This set includes a ligand that exhibits 3.8-fold greater affinity for the Src SH3 domain than the parent ligand, **NL-1**. The second set of ligands have a β-alanine core with at least one positive charge. The dissociation constants (K_d) of the ligands shown in Figure 7 are lower than 4 μM, the limit defined by the K_d of (A7)-(B29)-(isonipecotic acid)-PLPPLP-NH_2 present in the library.

These results highlight some of the limitations of screening for ligands attached to solid support.[7] The use a colorimetric assay requires that we avoid using monomers that generate colored beads independent of the SH3 domain. It was found that monomers A14 and A16 produced a pale yellow color that could not be washed away completely. This led to a slightly higher selection frequency for these two monomers. (The K_d for the ligand (A16)-(B22)-(isonipecotic acid)-PLPPLP-NH_2 was determined to be 3.5 μM.)

Another effect that we have observed during this screen and others is the greater selection of charged ligands. The doubly charged monomer, A29, is selected with a greater frequency than A7. However, the K_d for the ligand (A29)-(B22)-(isonipecotic acid)-PLPPLP-NH_2 is 5-fold higher than that for

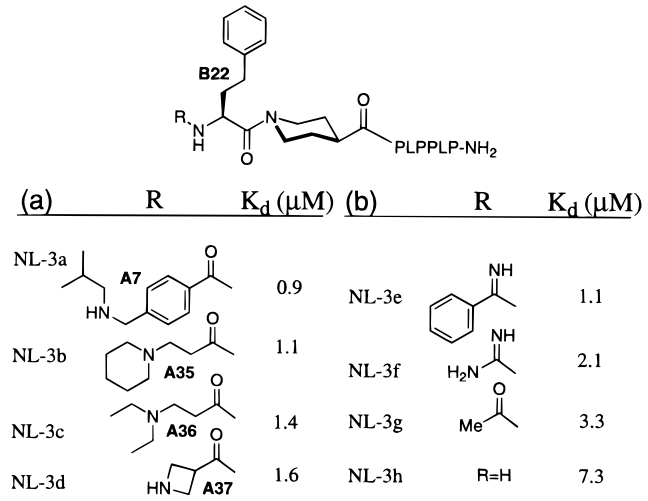

Figure 7. Binding affinities of representative ligands for the Src SH3 domain. (a) Ligands selected from library screens. (b) Rational modifications of selected ligands.

NL-3 (Figure 7). Vágner et al.[16] have demonstrated that large soluble receptors are unable to penetrate the core of beads such as those used here. It is expected that a greater percentage of charged ligands are surface exposed and therefore more accessible to the receptor complex. Hydrophobic ligands, being buried in the core of the bead, may be less accessible. This difference in ligand distribution could result in the selection of weaker binding, charged ligands.

To understand the mode in which monomer B22 interacts with the receptor better, we synthesized a series of ligands, **NL-3e** through **NL-3h** (Figure 7). If the **NL-3** ligands bound the specificity pocket in a conformation similar to **NL-1**, the amidine of **NL-3e** or the guanidine of **NL-3f** would be expected to interact with the carboxylate of Asp-99 in the specificity pocket and, thus, contribute favorably to the free energy of binding. No significant change in the binding constants for these ligands was observed, implying that the ligands may not bind in the same mode as **NL-1**. The weaker binding of **NL-3g** and **NL-3h** with the Src SH3 domain is consistent with the fact that monomers A49 and A50 were not selected at position A.

Deconvolution strategies can be more attractive than encoding strategies for libraries having two diversity sites. However, since the current library has many similar monomers, we expected that the number of assays required for deconvolution would be too encumbering. A positional scan of a library A7-[variable position B]-PLPPLP did, however, select B22.

Identification of Ligands That Are Selective for a Homologous SH3 Domain

Studies with peptide ligands for SH3 domains have shown that distantly related SH3 domains exhibit distinct ligand preferences.[17] However, rather weak ligand specificity is observed for SH3 domains of kinases in the Src family.[18] Src and Hck belong to this family of tyrosine kinases,[3b] and their SH3 domains have a 55% sequence identity.[18] While most of the key structural features of the binding pocket are conserved

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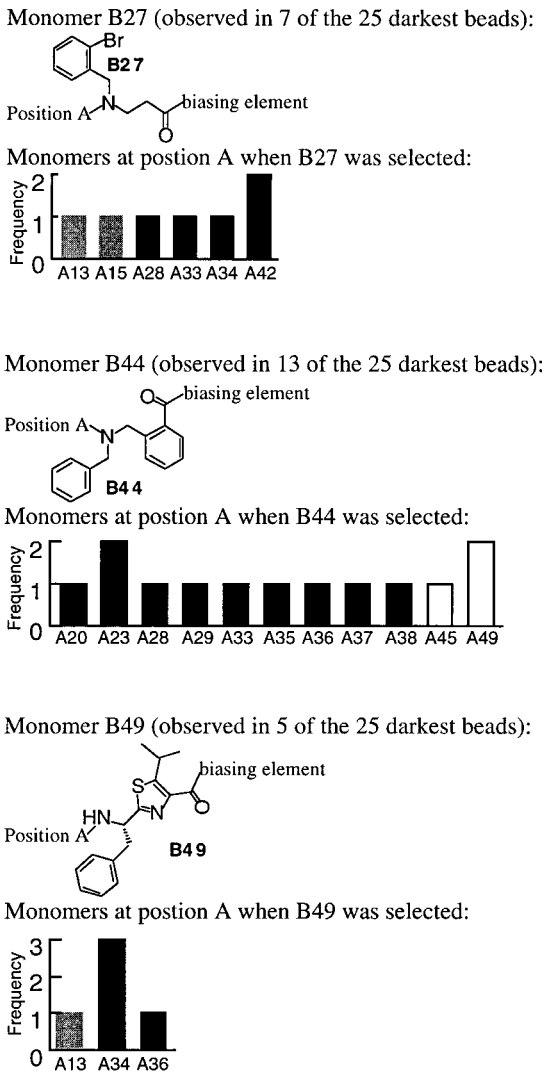

Figure 8. Monomers selected by the Hck SH3 domain. The dark shading in the histogram highlights the positively charged ligands. The lighter shading represents the monomers that are structurally related to A7. Other monomers types are not shaded.

in both domains, there are some differences in amino acid sequence. Hck contains an isoleucine and histidine in its RT loop while Src contains arginine and threonine. This amino acid substitution alters the functionality in the SH3 binding pocket but does not appear to affect the overall peptide backbone conformation in this region.[4c] We have used the Hck SH3 domain to test our ability to identify ligands that are selective for structurally related binding sites.

Two sets of five copies of the new library were screened with a biotinoylated Hck SH3 domain. Decoding the 25 darkest beads showed a strong selection for monomers B27, B44, and B49 at position B. For each of these monomers at position B, a range of monomers was selected at position A (see Figure 8); 76% of the monomers selected at position A carried at least one charged functional group. Cognizant of the effect of charged groups on selection statistics, we determined the affinities of representative compounds with each of the three monomers selected at position B (see Figure 9).

Preliminary studies with the Hck SH3 domain had established that this domain has a greater affinity for the Ac-PLPPLP-NH_2 peptide than the Src SH3 domain (K_d = 160 μM for Hck SH3 and K_d = >1000 μM for Src SH3). However, this difference translated to only a 2-fold greater affinity for **NL-1** (K_d = 2.0 μM) for the Hck SH3 domain. Screens with the Hck SH3 domain did not select monomer B22 at position B, and **NL-3** binds the Hck SH3 domain with a lower affinity than the Src SH3 domain (K_d = 2.2 μM).

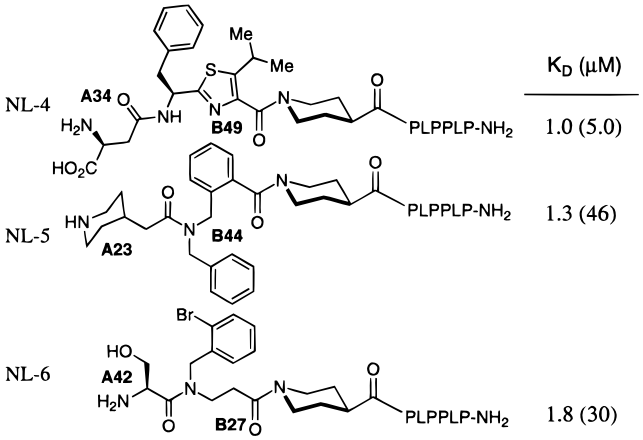

Figure 9. Binding affinities of representative ligands for the Hck SH3 domain. The binding affinity of the same ligand for the Src SH3 domain is reported in parentheses.

NL-5 is structurally divergent from the other SH3 ligands and is the most selective binder for the Hck SH3 domain, having a 30-fold higher affinity for the Hck SH3 domain relative to the Src SH3 domain. The dissociation constant of **NL-5** for the more distantly related PI3K SH3 domain was determined to be 215 μM, which is a 165-fold greater affinity for the Hck SH3 domain over the PI3K SH3 domain.

Conclusion

In this and the following paper, we describe the use of split-pool synthesis to study receptor−ligand interactions. We have used the solution structure of the Src SH3 domain to prepare biased libraries that have yielded new ligands containing non-peptide elements. The ligands generated using this approach have chemical structures that would have been difficult to predict. The ligands discovered for the highly homologous Hck SH3 domain show selectivity for that domain. These results suggest that the use of protein structure to design biased libraries can be an effective method for ligand discovery. As we describe in the following paper, we have concatenated the non-peptide elements discovered from different libraries to generate a ligand with high affinity for the Src SH3 domain. This ligand has only 3 of the 12 amino acids that are present in the peptidic ligand (VSLARRPLPPLP) whose receptor-bound structure guided the design of our libraries.

The discovery of highly specific, cell permeable ligands will assist in the elucidation of protein function *in vivo*. For the SH3 domains that we have discussed here, such ligands could clarify the specific role of the Src SH3 signaling module in mitogenesis and oncogenesis. Additionally, selective Hck SH3 ligands could be used to establish more clearly the relevance of the Hck SH3 interaction with the HIV Nef protein.[19] Such ligands may also be used to resolve subtle differences in the overlapping functions of Src family kinases.[20] The work reported herein illustrates one step toward these goals.

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Experimental Section

Monomer Synthesis. Monomers A1−A6, A8, A12−A22, A24−A26, A28−A49, B1−B4, B9, B10, B13, B14, B17−B26, B46, and B47 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 A7, A9−A11, A27, B29, and B42−B45 were prepared by $NaCNBH_3$-mediated reductive amination[21a] of commercially available amines with aldehydes in 1:1 methanol/H_2O at pH = 6, followed by Fmoc protection. Monomers A23, B11, B12, B40, B41, and B48 were prepared from commercially available Boc-amino acids by Arndt−Eistert synthesis[21b] followed by deprotection and Fmoc protection. Monomers B27, B28, and B30−B33 were prepared by conjugate addition of the corresponding amino acids to *tert*-butyl acrylate followed by Fmoc protection. Monomer B49 was converted from commercially available Boc-phenylalanine to the oxazole in analogy to known procedures[21c] and protected with the Fmoc group. Monomers B5−B8, B15, and B16 were synthesized as Boc-protected amino acids according to known procedures[21d] and subsequently converted to the Fmoc-protected derivatives. Intermediates in the synthesis of these monomers were reductively aminated with benzaldehyde and $NaCNBH_3$ followed by Fmoc protection to yield the monomers B34−B39.

Library Synthesis. The library was synthesized on TentaGel S NH_2 (RAPP Polymere, 0.25 mmol/g, 80 μm) using standard Fmoc chemistry. A linker ((β-alanine)-(6-aminocaproic acid)-gly) and the biasing element ((isonipecotic acid)-Pro-Leu-Pro-Pro-Leu-Pro) were synthesized on 1.0 g (0.25 mmol) of resin using FastMoc small scale coupling cycles on an Applied Biosystems Peptide Synthesizer (Model 431A); 0.375 g of this resin was used to synthesize the library. A modified split and pool strategy,[2] allowing for the incorporation of "skip codons" was used. The entire library was split into 50 portions by transferring a 1 mL volume of a suspension of the resin in *N*,*N*-dimethyformamide (DMF) (50 mL total volume of resin and solvent) to a reaction vessel (BioRad Bio-Spin column no. 732-6008). The solvent from each vessel was then drained. The resin in all 50 vessels was encoded[13] using a combination of nine distinct halogenated tags (not more than three tags for any one monomer, 0.04 equiv of each tag). The resin was then washed with CH_2Cl_2 (5 mL × 3)/DMF (5 mL × 3). One vessel (skip codon) was kept aside, and monomers were coupled to the 49 remaining portions. Fmoc protection was used for the N-terminus, BOC protection was used for side chain amines, and *tert*-butyl ester protection was used for side chain acids. Each vessel was treated with a specific monomer (3 equiv, 0.0056 mmol), HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (2.5 equiv, 0.0047 mmol), and *N*,*N*-diisopropylethylamine (DIPEA) (4 equiv, 0.0074 mmol) for 3 h in DMF (0.4 mL). The resin was washed with DMF (5 mL × 3)/CH_2Cl_2 (5 mL × 3)/DMF (5 mL × 3) and then resubjected to monomer coupling reagents (monomer, HATU, and DIPEA) for 12 h. The resin was again washed with DMF (5 mL × 3)/CH_2Cl_2 (5 mL × 3)/DMF (5 mL × 3) and pooled with all but the resin in the skip codon vessel. The combined resin was treated with 20% Ac_2O/CH_2Cl_2 (7.5 mL) for 30 min and then drained. The Ac_2O treatment was repeated, and the resin was then washed with DMF (5 mL × 3)/MeOH (5 mL × 3)/CH_2Cl_2 (5 mL × 3)/DMF (5 mL × 3). The resin was then subjected to 20% piperidine/DMF (7.5 mL) for 10 min, the solvent was drained, and the resin was retreated with 20% piperidine/DMF (7.5 mL) for another 5 min. The resin was then extensively washed with several volumes of DMF, MeOH, and CH_2Cl_2.

The skip codon vessel was recombined with the resin at this point, and the entire library was again split into 50 equal portions. Another set of nine distinct tags was used to encode the 50 positions. The resin in 49 of the 50 vessels was appropriately treated to react with the respective monomers. All position A acids were coupled using the monomer (3 equiv, 0.0056 mmol), HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (2.5 equiv, 0.0047 mmol), and *N*,*N*-diisopropylethylamine (DIPEA) (4 equiv, 0.0074 mmol). The remaining reagents were coupled under the following conditions: monomer (5 equiv, 0.0094 mmol) and DIPEA (15 equiv, 0.028 mmol) in CH_2Cl_2 (0.4 mL) for 2 h. The solvent was drained, and the resin was washed with CH_2Cl_2 (5 mL × 3) and resubjected to the reaction conditions for another 24 h. The "free amine" fraction was recombined with the total resin, and the protecting groups were removed using 20% piperidine/DMF (7.5 mL) for 10 min and then for 5 min after the solution was washed with DMF (5 mL × 3)/CH_2Cl_2 (5 mL × 3)/DMF (5 mL × 3) followed with a treatment of 100% CF_3CO_2H for 2 h. The solvent was drained, and the entire library was washed with CH_2Cl_2 (5 mL × 3)/DMF (5 mL × 3)/CH_2Cl_2 (5 mL × 3). The library was then dried under a stream of dry nitrogen and stored at −20 °C for further use.

Protein Expression. Expression and purification of the Src and PI3K SH3 domains using the expression vectors pGEX-2T and pLM1, respectively, have been described.[6b] The modified Hck SH3 domain, with the N-terminal serine, was constructed by the recombinant polymerase chain reaction (PCR) technique, analogous to the procedures used for the Src SH3 domain. The 5′ primer (5′-GCG GGA TCC ATC GAA GGT CGT TCT GAG GAC ATC ATC GTG GTT GCC CTG-3′) encoded a BamHI restriction site and a factor Xa proteolytic site, fused in-frame with the mutated N-terminus of the Hck SH3 domain. The 3′ oligonucleotide (5′-GCG GAA TTC TTA GTC AAC GCG GGC GAC ATA GTT GCT TGG-3′) created the termination codons and a EcoR1 restriction site. After digestion with EcoR1 and BamHI restriction endonucleases (GIBCO BRL), the purified PCR-generated DNA fragment was subcloned into the pGEX-2T expression vector (Pharmacia). The sequence of the Hck SH3 fragment was confirmed using chain termination methods as described in the Sequanase protocols by its supplier (U.S. Biochemicals).

A 2 L culture (LB medium, 50 μg/mL ampicillin) of pGEX-2T-SH3 in transformed *Escherichia coli* BL21 (Novagen) was then grown at 37 °C to an OD_{600} of 0.6, induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and harvested 10 h later. The lysed bacterial mixture was centrifugated at 12 000 × *g*, and the GST-SH3 fusion protein was purified by affinity chromatography and cleaved with factor Xa (New England Biolabs). Purified Hck SH3 was obtained by Sepharyl S-100 HR gel filtration chromatography [PBS, 0.02% NaN_3 (pH 7.4)].

Protein Derivatization. In a 200 μL reaction, the SH3 domain (∼0.5 mM) was incubated at room temperature for 20 min with $NaIO_4$ (1.5 mM) in phosphate-buffered saline (PBS) (pH 7.4). After periodate was removed by gel filtration (Sephadex G-25), the oxidized protein (∼150 μM) was mixed with 6 mM 6-((6-((biotinoyl)amino)hexanoyl)-amino)hexanoic acid, hydrazide (Molecular Probes) in 50 mM NaOAc buffer (pH 5.0). (The rather insoluble hydrazide required predilution in 3:1 buffer/CH_3CN to prepare a 60 mM stock solution.) The coupling reaction was incubated at room temperature for 12 h and then fractionated on a Sephadex G-25 column equilibrated with PBS (pH 7.4). Fractions containing the biotinoylated SH3 domain were combined and stored at 4 °C and used within 7 days. HPLC analysis (LDC Analytical; Vydac C18 column, linear gradient from 0 to 60% CH_3CN in 30 min) indicated that the oxidation and coupling reactions were nearly quantitative. The derivatized proteins were also analyzed by SDS-PAGE and electrospray ionization mass spectrometry (ESIMS).

Library Screening. In a typical screen, the N-terminally biotinoylated Src SH3 domain (Src-B SH3) and streptavidin-alkaline phosphatase (SAAP) (Pierce ImmunoPure streptavidin, alkaline phosphatase conjugate) were incubated at the following concentrations: 200 nM Src-B SH3 with 5 nM SAAP in 0.2 mL screening buffer (10 mM Tris (pH 7.4), 0.5 M NaCl, 0.1% Triton X-100, 0.1% deoxycholic acid, 1% bovine serum albumin (BSA)) for 1 h. The complex of the SH3 domain with SAAP was then diluted with screening buffer to 0.8 mL. Five copies of the library (attached to the resin) were typically incubated with 0.8 mL of screening buffer for 1 h to reduce nonspecific binding. The solution was drained, and the resin was incubated with all of the preformed Src-B SH3/SAAP (200 nM, 5 nM) complex for 12 h. The resin was then washed with 4 × 1 mL of screening buffer. Treatment of the resin with the substrates for alkaline phosphatase (5-bromo-4-

(21) (a) Borch, R. F.; Bernstein, M. D.; Durst, H. D. *J. Am. Chem. Soc.* **1971**, *93*, 2897−2904. (b) Plucinska, K.; Liberek, B. *Tetrahedron* **1987**, *43*, 3509−3517. (c) Wipf, P.; Miller, C. P. *J. Org. Chem.* **1993**, *58*, 3604−3606. (d) Estermann, H.; Seebach, D. *Helv. Chim. Acta* **1988**, *71*, 1824−1839.

chloro-3′-indolyl phosphate, *p*-toluidine salt (BCIP) and nitroblue tetrazolium (NBT)) for a few minutes deposited varying amounts of color on the beads. The solution was drained, and the staining reaction was quenched by washing with three volumes of 20 mM EDTA in PBS buffer followed by three volumes of PBS buffer. The darkest beads were physically removed with the aid of a microscope and a 50 mL gas-tight syringe and placed into a new vessel. The beads were washed three times with 6 N guanidinium hydrochloride, H_2O, DMF, and PBS buffer. These beads were then reassayed under identical condition, except without the N-terminally biotinylated Src SH3 domain, in order to eliminate false positives. The darkest beads were placed in capillary tubes, individually destained and prepared for GC-ECD analysis by washing five times with H_2O, DMF, and decane. Decoding was performed by GC-ECD as described by Ohlmeyer et al.[13]

Ligand Resynthesis. All ligands used in solution studies were synthesized on Rink amide 4-methyl(benzhydryl)amine (MBHA) resin (Nova Biochem) using the Applied Biosystems peptide synthesizer (model 431A). Smaller scale syntheses (less than 0.1 mmol) of ligands were carried out by first synthesizing the common core (NH-(isonipecotic acid)-PLPPLP) via automated solid phase synthesis and then completing the synthesis by manual solid phase synthesis of the remaining sequences under conditions identical to those used during library synthesis. All ligands were cleaved from the supports using 5% H_2O/CF_3CO_2H (5 mL × 2 for 30 min) and HPLC purified and characterized by mass spectroscopy. Ligand **NL-3e** was synthesized reacting the free amine of the compound HN-B22-(isonipecotic acid)-PLPPLP-NH_2 with 2 equiv of methyl benzimidate hydrochloride and 2 equiv of DIPEA in ethanol at 50 °C for 24 h followed by HPLC purification. Ligand **NL-3f** was sythesized by reacting by 5 equiv of *N*,*N*′-bis-Boc-pyrazole-1-carboxamidine and 7 equiv of DIPEA with HN-B22-(isonipecotic acid)-PLPPLP-(MBHA resin) in CH_2Cl_2 for 12 h followed by standard cleavage and purification. Ligand **NL-3a**: FAB LRMS calcd for $C_{60}H_{88}N_{10}O_9$ = 1092, found [M + H] = 1093 and [M + Na] = 1115. Ligand **NL-3b**: FAB LRMS calcd for $C_{56}H_{86}N_{10}O_9$ = 1042, found [M + H] = 1043 and [M + Na] = 1065. Ligand **NL-3c**: FAB LRMS calcd for $C_{55}H_{86}N_{10}O_9$ = 1030, found [M + H] = 1031. Ligand **NL-3d**: FAB LRMS calcd for $C_{52}H_{78}N_{10}O_9$ = 986, found [M + H] = 987. Ligand **NL-3e**: FAB LRMS calcd for $C_{55}H_{78}N_{10}O_8$ = 1006, found [M + H] = 1007. Ligand **NL-3f**: FAB LRMS calcd for $C_{49}H_{75}N_{11}O_8$ = 945, found [M + H] = 946. Ligand **NL-3g**: FAB LRMS calcd for $C_{50}H_{75}N_9O_9$ = 945, found [M + H] = 946 and [M + Na] = 968. Ligand **NL-3h**: FAB LRMS calcd for $C_{48}H_{73}N_9O_8$ = 903, found [M + H] = 904. Ligand **NL-4**: FAB LRMS calcd for $C_{57}H_{83}N_{11}O_{11}S$ = 1129, found [M + H] = 1130 and [M + Na] = 1091. Ligand **NL-5**: FAB LRMS calcd for $C_{60}H_{86}N_{10}O_9$ = 1090, found [M + H] = 1091. Ligand **NL-6**: FAB LRMS calcd for $C_{51}H_{77}N_{10}O_{10}$-Br = 1068, found [M + H] = 1069, 1071.

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 as described previously.[6a]

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