Improving SH3 domain ligand selectivity using a non-natural scaffold

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Background: Src homology 3 (SH3) domains bind sequences bearing the consensus motif PxxP (where P is proline and x is any amino acid), wherein domain specificity is mediated largely by sequences flanking the PxxP core. This specificity is limited, however, as most SH3 domains show high ligand cross-reactivity. We have recently shown that diverse *N*-substituted residues (peptoids) can replace the prolines in the PxxP motif, yielding a new source of ligand specificity.

Results: We have tested the effects of combining multiple peptoid substitutions with specific flanking sequences on ligand affinity and specificity. We show that by varying these different elements, a ligand can be selectively tuned to target a single SH3 domain in a test set. In addition, we show that by making multiple peptoid substitutions, high-affinity ligands can be generated that completely lack the canonical PxxP motif. The resulting ligands can potently disrupt natural SH3-mediated interactions.

Conclusions: Peptide–peptoid hybrid scaffolds yield SH3 ligands with markedly improved domain selectivity, overcoming one of the principal challenges in designing inhibitors against these domains. These compounds represent important leads in the search for orthogonal inhibitors of SH3 domains, and can serve as tools for the dissection of complex signaling pathways.

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Introduction

Eukaryotic signal transduction involves the assembly of transient protein–protein complexes. The formation of these complexes is mediated by modular protein–protein interaction domains such as SH2, SH3 and PDZ domains [1,2]. An important goal in chemical biology is the design of small molecule ligands that might selectively disrupt these interactions. Such inhibitors would be powerful tools in the mechanistic dissection of complex signaling pathways, and could serve as therapeutic agents in diseases caused by signaling-based defects.

Src homology 3 (SH3) domains are found in numerous signaling proteins and are attractive targets for drug design as their interactions have been implicated in a number of diseases such as cancer, osteoporosis and inflammation [3,4]. SH3 domains recognize peptide sequences containing the core binding motif PxxP (where P is proline and x is any amino acid) [5]. Residues that flank the PxxP motif provide additional binding energy and most of the selectivity involved in recognition: for example, the SH3 domains from the protein Grb2 require an arginine residue to be carboxy-terminal to the PxxP core (consensus motif: PxxPxR) for binding [6–10].

SH3 domains, however, have proved to be difficult targets for drug design. First, most SH3 ligand interactions are of relatively low affinity, with dissociation constants ranging from 1 to 100 μM. Second, and perhaps most problematic, SH3 ligands show high cross-reactivity with several SH3 domains. For example, the SH3 domains from Grb2, Crk, Hck, Src and others all show overlapping interaction with peptides bearing flanking basic residues (consensus motif: PxxPxR/K) [6–10]. How then can one obtain SH3 ligands with improved affinity and selectivity?

Previously, the most successful efforts to increase SH3 ligand specificity have focused primarily on replacing sequences flanking the PxxP motif with natural or non-natural moieties [8,11–13]. These flanking residues interact with the so-called specificity pocket of the SH3 domain fold, which is composed of the variable 'RT' and 'n-Src' loops. Although these efforts have been successful, they have not eliminated cross-reactivity between domains. In contrast, little effort has been expended on altering the core PxxP motif, which is common to all SH3 ligands and is thus thought to be a poor source of specificity.

Recently, however, we reported a promising strategy for the design of improved SH3 ligands that focuses on the

PxxP core [14]. This strategy is based on the unusual mechanism of SH3 domain recognition. These domains recognize proline solely because it is substituted on the backbone amide nitrogen. Thus, SH3 domains select proline because it is the only endogenous *N*-substituted amino acid. By focusing on a chemical property that is orthogonal to other naturally occurring amino acids rather than optimizing complementarity to the full proline sidechain, these domains can recognize their ligands with high discrimination but low affinity, properties that are advantageous in signaling interactions.

As a result of this mechanism, *N*-substituted glycines, or peptoids [15,16], function as ideal proline mimetics as they maintain the required backbone *N*-substitution pattern. Moreover, the diversity of the *N*-substituted peptoid sidechains can potentially provide improved complementarity to the SH3 surface. Thus a hybrid peptide–peptoid backbone, in which prolines of the PxxP motif are replaced with peptoids, can function as a scaffold from which different sidechains can be sampled for improvement in affinity and specificity. Peptoids have added advantages in that they are modular and easy to synthesize [16], are protease resistant [17] and are more membrane permeable [18] than natural amino acids.

We previously screened a small aliphatic and aromatic hybrid peptide–peptoid library and identified several of the most promising inhibitors against SH3 domains identified so far (dissociation constant, K_d = 40 nM) [14]. These first generation inhibitors show up to a 100-fold improvement in affinity and selectivity over natural peptides. In contrast, most other peptide and non-peptidic libraries have failed to yield ligands that bind significantly better than the wild-type peptide [19–24].

Here we focus on how peptoid substitutions can be used to enhance ligand selectivity. We have explored the effects of combining peptoid substitutions with various flanking sequences on the energetics of SH3 ligand discrimination. First, by screening a new, more chemically diverse peptide–peptoid library, we have identified individual peptoid residues that give rise to significantly improved affinity and specificity. Second, by making multiple peptoid substitutions, we have generated an SH3 ligand that completely lacks the PxxP motif. Finally, starting with a parental peptide that displays cross-reactive binding to a test set of three SH3 domains, we show that, by combining multiple peptoid substitutions with specific flanking sequences, we can fine tune specificity so that binding is only observed for a single SH3 domain in the test set. These new ligands can out-compete natural SH3 interactions, as shown by the inhibition of the Crk–Abl interaction and the activation of the Src-family kinase Hck. These data show that the PxxP core of SH3 ligands can be varied considerably, and that it is an untapped source of specificity. These data also show that cross-reactivity — previously one of the principal obstacles in the design of selective inhibitors against SH3 interactions — can be overcome by combining different specificity elements, spread throughout the entire SH3 ligand.

Results

Library synthesis and screening

A library of 12 new hybrid peptide–peptoid compounds was synthesized and screened for binding to three different SH3 domains. Single peptoid substitutions were made within a 12-residue peptide derived from the protein Sos (Figure 1). Whereas previous libraries were predominantly composed of hydrophobic aliphatic and aromatic sidechains, peptoids of the current library were chosen for diversity of shape and chemical properties. We concentrated mainly on sidechains containing oxygen and nitrogen, reasoning that these groups could interact with residues on the surface of the SH3 domain through polar interactions, which might potentially give rise to higher specificity. We tested this library against SH3 domains from three proto-oncoproteins, Crk, Src and Grb2 (amino-terminal domain, N-Grb2), because they all bind the wild-type Sos peptide with similar affinities (K_d = 6 μM, 25 μM and 5 μM, respectively) [14]. Binding of the ligands to the three SH3 domains was assayed by measuring perturbation of tryptophan fluorescence upon binding.

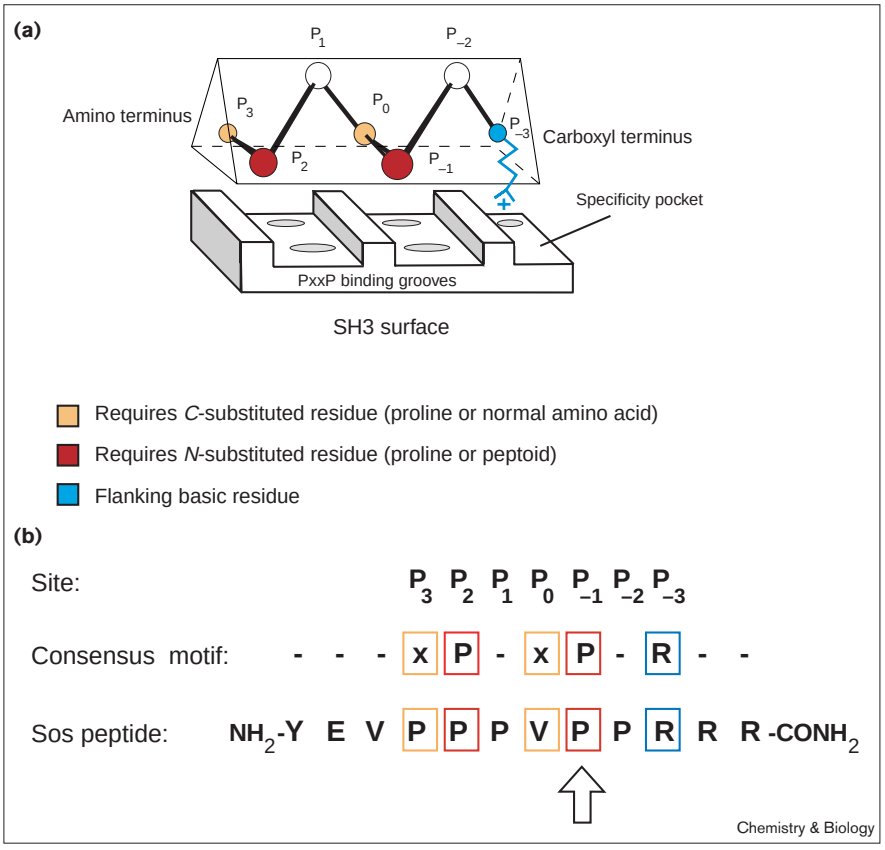
Improved selectivity and affinity

The higher chemical diversity of this new library is reflected in the larger range of affinities observed for SH3 binding. Whereas most of the compounds in the previous libraries bound with similar affinities to the wild-type peptide, most peptoid compounds in this library showed either marked increases or decreases in affinity (Figure 2). As expected, most peptide–peptoid compounds bound with reduced affinity compared with wild-type peptide. Those with peptoid sidechains containing positively charged amino groups showed the least favorable binding.

From this library we identified two compounds that showed significant improvement in affinity and specificity compared with wild-type peptide. Compound **4**, which contains a difluorobenzyl (Ndfb) sidechain, bound N-Grb2 with a K_d of 300 nM, a 1.67 kcal/mol increase in binding energy over the wild-type peptide. More markedly, compound **5**, which contains a dimethoxybenzyl (Ndmb) sidechain, bound N-Grb2 with a K_d value of ~30 nM. This represents more than 100-fold increase in affinity ($\Delta\Delta G \leq -3$ kcal/mol). The improvements were specific to N-Grb2, as both compounds showed slight decreases in affinity when tested against Crk and Src. As a result, compound **5** showed a more than 300-fold specificity for N-Grb2 compared with Crk, and close to a 1500-fold specificity compared with Src (Figure 2). The improvement in affinity and specificity is remarkable, given that they result from a one-residue substitution.

Figure 1

Design of hybrid peptide–peptoid library. **(a)** Representation of a bound SH3 ligand showing the backbone requirements for SH3 recognition [11]. Designation of ligand-binding sites from Yu *et al.* [40]. **(b)** Synthesis scheme for peptide–peptoid hybrid library. Peptoid substitutions in the current library were made at the second proline of the PxxP motif (P_{-1} site, arrow) in a 12-residue peptide derived from the protein Sos. Biochemical and crystallographic data indicate that peptoid substitution locks the ligand into only one possible binding registration, such that the peptoid sidechain must bind at the site that accepts *N*-substitution (P_{-1}) [11]. In any other binding registration, the peptoid sidechain would be placed at a site that requires Cα-substitution, which is disallowed in SH3 recognition.



Two other compounds (**7** and **13**) showed significant improvement in specificity for the SH3 domain of Crk. Both compounds had slight decreases in affinity for Crk, but they had much larger decreases for N-Grb2 and Src SH3 domains. This result illustrates the important concept that specificity can be achieved not only by increasing affinity to a particular target domain, but also by decreasing affinity to competing domains.

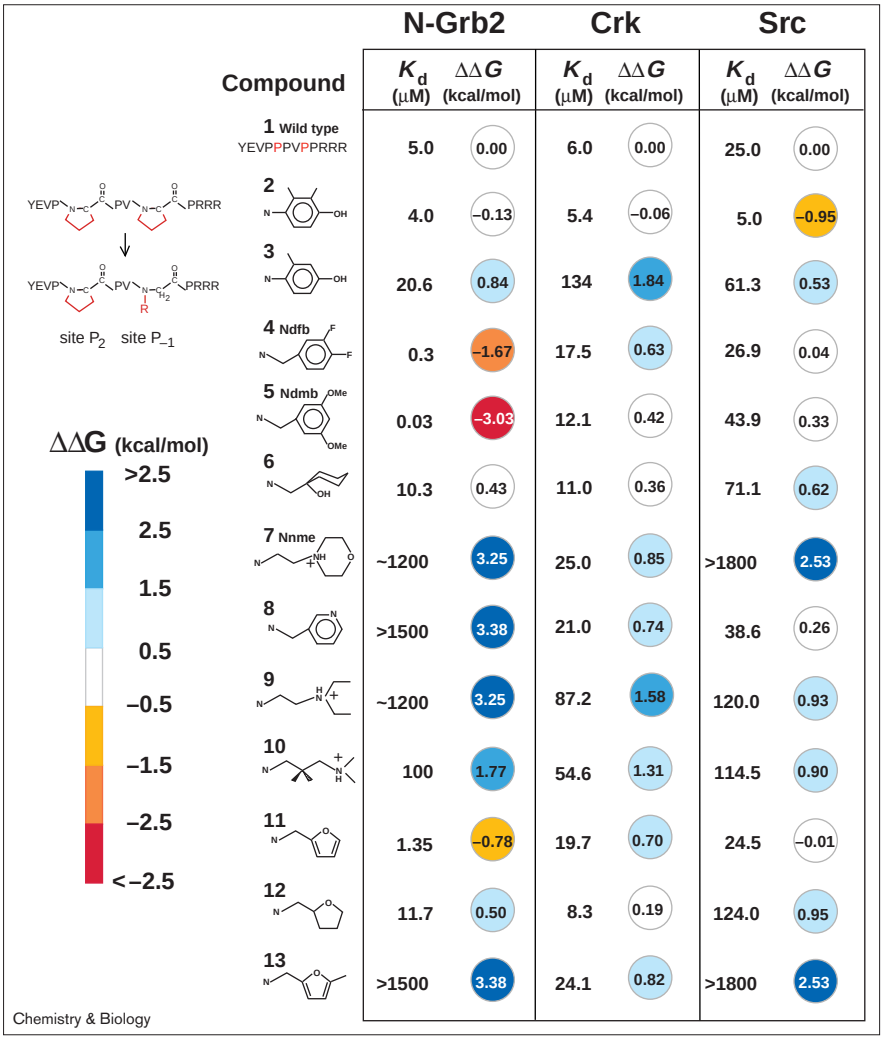
The PxxP core can be completely replaced by nonproline residues

From this and previous work, we have found that the prolines of the PxxP motif can be individually replaced by peptoids to yield improvements in affinity [14]. Here, we have synthesised compounds in which both prolines of the PxxP motif have been replaced by peptoids (Table 1). To our knowledge, this is the first report in which the entire consensus motif has been replaced. The doubly substituted peptoid ligands bind as well as or better than wild-type peptides. Compound **14**, for example, bound to N-Grb2 with a K_d of 100 nM, a 50-fold improvement over the wild-type peptide ($\Delta\Delta G = -2.3$ kcal/mol). Notably, most double peptoid substitutions do not show simple additivity in the energy of binding. For example, substitution of the *N*-(*S*)-phenylethyl peptoid (Nspe) [14] at the P_{-1} site resulted in a~100-fold increase in affinity when tested against N-Grb2, and substitution of the same peptoid at the P_2 site resulted in a fivefold increase. The doubly substituted peptoid (compound **14**) showed only a 50-fold increase in affinity (Table 1). This was the case for most of the doubly substituted peptoids tested (compounds **14**–**17**), wherein double substitution resulted in an affinity intermediate between the affinities of the two singly substituted sites.

This lack of additivity suggests that there is conformational coupling between the two sites requiring *N*-substitution. Indeed, the crystal structures of several peptoid–SH3 complexes show that replacement of one proline with bulky peptoid sidechains can result in a displacement of the ligand backbone atoms relative to the SH3 surface of up to 1.5 Å [14]. Thus, it is not surprising that sidechain preferences at the first site will depend on the exact substitution at the second site. These findings suggest that the most effective peptoid replacements might be obtained by combinatorially varying the two positions simultaneously.

We also tested the effects of the removing other proline residues in the ligands that were not part of the PxxP core. SH3 ligands bind in the poly-proline type II (PPII) helical conformation, and noncritical prolines might be involved

Figure 2



Structure and affinity of hybrid peptide–peptoid library. Effects of peptoid replacement at the second proline-requiring site (site P_{-1}) of SH3 ligand tested against N-Grb2, Crk and Src SH3 domains. The changes in free energy relative to the wild-type Sos peptide are color coded according to $\Delta\Delta G$ (red, favorable; blue, unfavorable). K_d values were determined by fluorescence perturbation and reported values are the averages of at least two independent experiments. For low-affinity compounds wherein the change in fluorescence (I_0) does not approach the maximum change in fluorescence (I_∞) even at the highest ligand concentrations, the dissociation constants were derived using the I_∞ for the wild-type Sos peptide and are therefore presented as estimates.

in maintaining the PPII helical scaffold [6,14]. Mutations of these prolines, even those that do not contact the SH3 surface, can result in a modest decrease in affinity [14]. In compound **18**, we replaced three of the five prolines in the wild-type peptide with either peptoids or natural amino acids, and in compound **19** we replaced four of the five prolines. These compounds bound with an affinity close to that of the wild-type (Table 1). Compound **19** contains no PxxP motifs, only one proline, and no longer resembles a canonical SH3 ligand. Nonetheless, compound **19** still binds N-Grb2 and Src with wild-type affinity (Figure 3). This finding shows very clearly that the cyclic proline sidechain and its resulting conformational constraints are unnecessary for SH3 domain recognition.

Tuning SH3 domain selectivity

SH3 domains contain a 'specificity pocket' that is formed by the RT and n-Src loops proximal to the main PxxP-binding site. This pocket is often lined with acidic residues that interact with a basic residue flanking the PxxP motif on the ligand. Several groups have shown that individual SH3 domains have unique preferences for flanking sequences. For example, the Crk SH3 domain prefers a lysine as the flanking basic residue (PxxPxK) [8,9,13], whereas the Src SH3 prefers the sequence PxxPxRNRPRA [7,22].

We wanted to test whether affinity and specificity can be enhanced by combining specific flanking sequences with peptoid substitutions. On the basis of other studies, we synthesized two high-affinity peptides specific to Src [7] and Crk [13] and measured their affinities for the SH3 domains in the test set. Compound **20**, which contains a Src-specific flanking sequence, bound to Src SH3 with a K_d of 0.3 μM (80-fold higher affinity than that of the Sos peptide). The peptide with a Crk-specific flanking sequence (compound **22**) bound to Crk SH3 with a K_d of 20 nM (300-fold higher affinity than that of the Sos peptide; Figure 4a,b). Significantly, compound **22** was highly specific for Crk SH3,

showing more than 1000-fold selectivity over N-Grb2 and 4000-fold selectivity over Src (Figure 4b).

We then made selected peptoid substitutions in the context of these Src-specific and Crk-specific peptide backgrounds (Table 1). Substitution of the Nspe peptoid at site P_{-1} in the Src-specific peptide background (compound **21**) and at site P_2 in the Crk-specific peptide background (compound **23**) resulted in further increases in affinity. In the case of Crk, we estimate the K_d of compound **23** to be 8 nM (see the Materials and methods section), to our knowledge the tightest interaction reported for an SH3 domain.

Nonetheless, although both compounds **22** and **23** showed a remarkably high preference for the Crk SH3, they still showed weaker but significant binding to the N-Grb2 and Src SH3 domains. Thus, these compounds are not truly orthogonal and may not be ideal inhibitors for selectively blocking the interactions of one SH3 domain. Given that the intracellular concentrations of Crk and other SH3 containing proteins are thought to be fairly high (0.1–10 μM, especially at specific cellular sites), stoichiometric inhibition of these adapter proteins may require greater than micromolar concentrations of inhibitor. At these high concentrations, the effective *in vivo* selectivity of a low nanomolar affinity inhibitor for Crk would be greatly diminished, because the concentration would approach or exceed the dissociation constants of the inhibitor for other competing SH3 interactions.

Ideal SH3 inhibitors should have orthogonal binding properties — that is, they should bind to only one SH3 domain even under conditions of high concentration — if they are to be effective *in vivo*. We therefore wanted use peptoid substitutions to generate ligands that could bind Crk with at least micromolar affinities, but that showed no detectable binding (K_d > 1 mM) to either Src or Grb2.

Looking at the data from the library screen, we saw that substitution of the Nnme peptoid at site P_{-1} resulted in a modest decrease in affinity for Crk SH3 but a significantly larger decrease in affinity for N-Grb2 and Src. Substitution of this peptoid into the Crk peptide background (compound **24**) shifted the K_d for Crk to 0.5 μM, but more importantly it increased the K_d for N-Grb2 to close to 1 mM and abrogated detectable binding to Src (Figure 4c). A doubly substituted compound (**25**) with the Nspe peptoid at the P_2 site and the Nnme peptoid at the P_{-1} site bound to Crk with a K_d of 2 μM, but showed no detectable binding to either N-Grb2 or Src (Figure 4d). Thus, by combining specific flanking sequences and multiple peptoid substitutions, we have generated a ligand with orthogonal selectivity for a single SH3 domain within the test set. These results show that it is possible, in principle, to exploit the multiple specificity elements in SH3 ligands to generate orthogonal inhibitors.

Table 1

Dissociation constants for doubly substituted specific peptoid compounds.

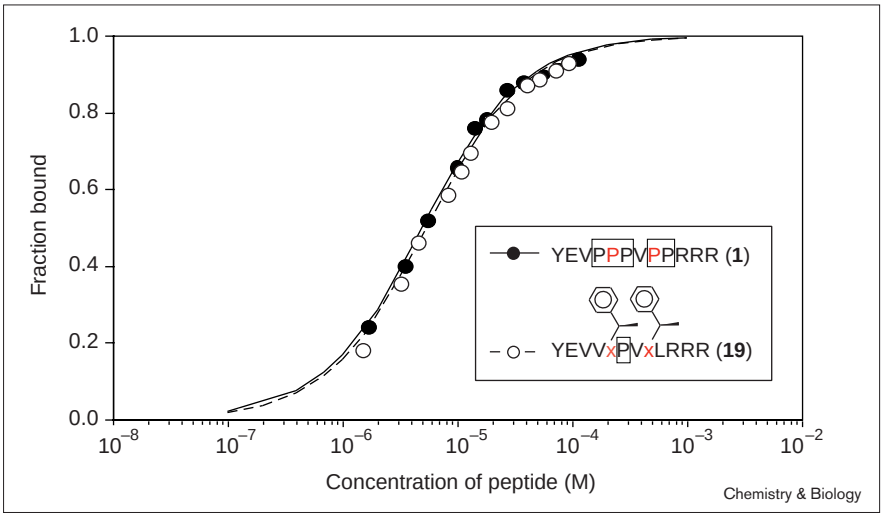
		N-Grb2	Crk	Src
	Doubly substituted peptoids	K_d (μM)	K_d (μM)	K_d (μM)
	site P_2 P_{-1}			
14	YEVPxPVxPRRR	0.1 (1.0/0.04)	2.5 (0.8/4.0)	2.2 (19.0/2.0)
15	YEVPxPVxPRRR	0.41 (3.0/0.04)	4.3 (2.2/4.0)	3.6 (63.0/2.0)
16	YEVPxPVxPRRR	0.24 (2.0/0.04)	2.8 (2.0/4.0)	1.6 (23.0/2.0)
17	YEVPxPVxPRRR	0.55 (2.0/0.04)	4.2 (1.0/4.0)	3.7 (57.0/2.0)
18	YEVLxPLxPRRR	1.3	9.8	21.2
19	YEVVxPVxLRRR	5.3	21.7	21.5
		N-Grb2	Crk	Src
	Domain-specific peptoids	K_d (μM)	K_d (μM)	K_d (μM)
	Src			
20	YAPPLPPRNRPRA	2.03	0.45	0.3
21	YAPPLxPRNRPRA	0.1	2.4	0.14
	Crk			
22	YPPPALPPKRRR	24.9	0.02	86.8
23	YPPxALPPKRRR	13.7	0.008	45.6
24	YPPPALxPKRRR	>904	0.5	No binding
25	YPPxALxPKRRR	No binding	1.98	No binding

Values in parentheses are the dissociation constants for single substitutions of the peptoid residues shown at sites P_2 and P_{-1}, respectively, as determined previously [11]. Important residue changes are highlighted in red. No binding means there was no significant changes in fluorescence even at the highest ligand concentrations (0.8–1 mM).

Potent inhibition of the Crk–Abl interaction

To determine the effectiveness of the Crk-specific peptide–peptoid ligands in disrupting natural Crk SH3 interactions, we determined their abilities to inhibit the interactions between Crk and the kinase Abl. Crk is a well-characterized substrate of the Abl nonreceptor

Figure 3



Full replacement of the PxxP core without loss in affinity. Binding isotherms for the Sos wild-type peptide (closed circles) or compound **19** (open circles) to the N-Grb2 SH3 domain. Required prolines are shown in red, and the peptoid residue is designated as an 'x' showing the corresponding sidechain. Solid and dashed lines show the nonlinear least-squares fit to the data for the wild-type peptide and compound **19**, respectively. Compound **19** has only one of the starting five prolines remaining from the Sos wild-type peptide.

tyrosine kinase. The amino-terminal SH3 domain of CrkII interacts with proline-rich sequences in the carboxy-terminal region of Abl *in vitro* and *in vivo* [25,26], leading to the phosphorylation of Crk on Tyr221 by Abl. Phosphorylation might regulate the ability of Crk to engage in downstream signaling interactions [27].

In order to assay for inhibition of the Crk–Abl interaction, a fusion protein of glutathione-*S*-transferase (GST) and full-length CrkII was immobilized on glutathione-agarose. Lysates from human kidney 293T cells overexpressing Abl were passed over immobilized Crk in the presence of peptide or peptide–peptoid compounds. Bound proteins were separated on sodium dodecylsulfate (SDS)–polyacrylamide gels and visualized by anti-Abl western blotting. As shown in Figure 5, the Sos wild-type peptide was not able to inhibit the Crk–Abl interaction even at 100 μM concentration. But both the Crk-specific wild–type peptide (**22**) and the Crk-specific Nspe peptide–peptoid (**23**) were highly effective at inhibiting Crk binding to Abl. Remarkably, compound **23** at 10 nM concentration showed significant inhibition of the interaction between Crk and Abl, and at 100 nM completely abolished binding. From the gels, we estimate the inhibition at half-maximal concentration (IC_{50}) for compound **23** to be ~50 nM, about tenfold more effective than the Crk-specific wild-type peptide and at least 200-fold more effective than the Sos wild-type peptide. These data show that the peptide–peptoid compounds are highly effective at inhibiting natural SH3 interactions.

Peptide–peptoid activation of a Src-family kinase

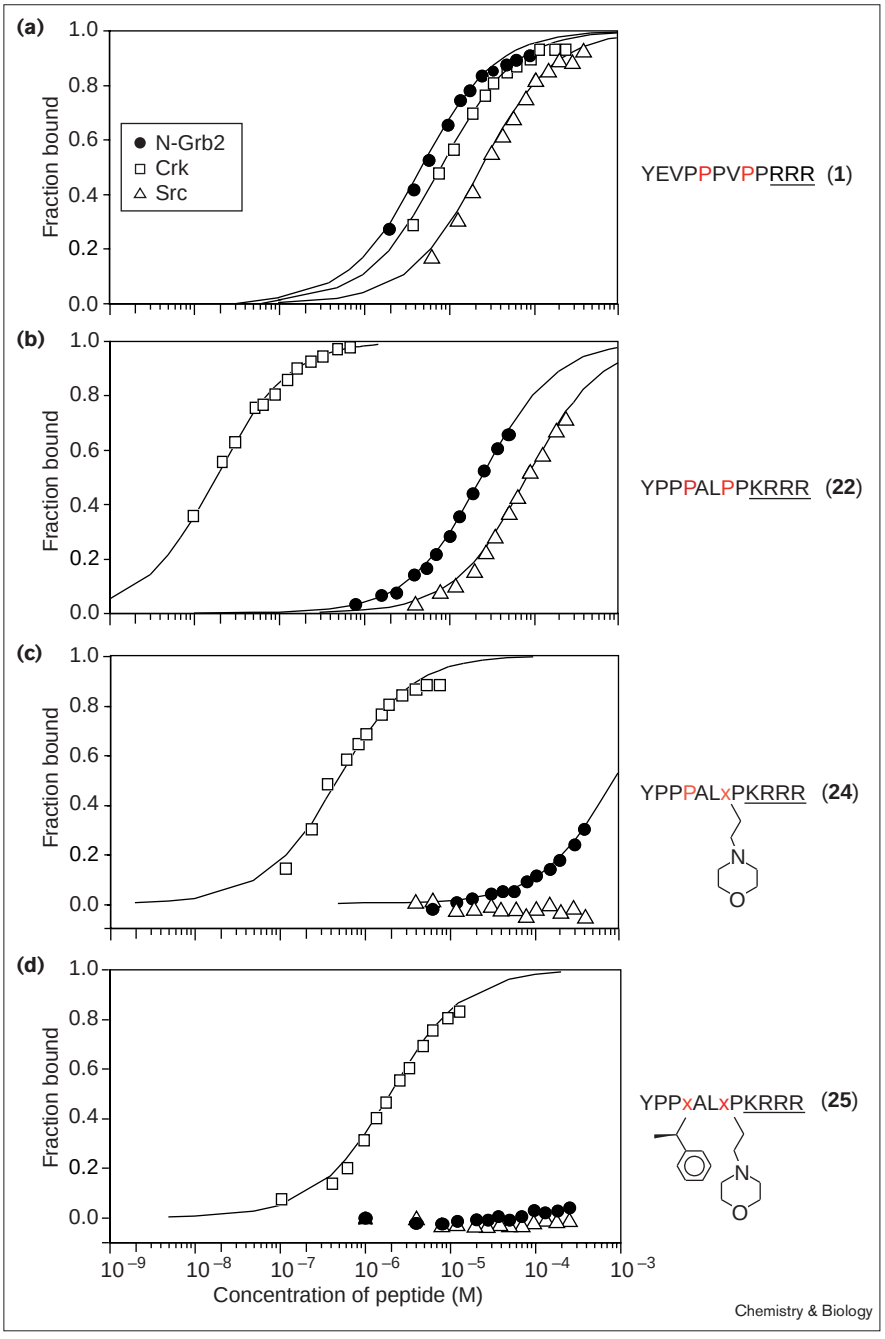
Previous work has demonstrated that Src-family kinases can be activated through SH3-mediated interactions [28]. We wanted to investigate whether the peptide–peptoid compounds identified in our library were effective in activating Src-family kinases. The ability to selectively activate different members of Src-family kinases would greatly aid in understanding their roles in complex signaling pathways.

The domain topology of Src-family kinases consists of SH3, SH2 and kinase domains [29,30]. Biochemical and structural studies revealed that the SH3 domain helps maintain the kinase in the repressed state by interacting with an intramolecular proline-rich sequence. In the crystal structures of the repressed forms of Hck and Src, the intramolecular SH3 interaction appears to lock the kinase domain in a catalytically incompetent state (Figure 6a) [31,32]. Disruption of the intramolecular SH3 interaction by competing intermolecular ligands is therefore proposed to provide a mechanism for kinase activation. Nonetheless, small SH3 peptide ligands make poor activators of the Src-related kinase Hck [28]. In contrast, the intact HIV protein Nef — a 27–30 kDa protein that has been identified as the highest affinity ligand for Hck SH3 [33] — has been shown to potently activate the kinase [28]. Nef binds the Hck SH3 domain through a PxxP motif, but also has extensive tertiary interaction extending beyond the PxxP core, which allows it to bind with an affinity that is two orders of magnitude greater than that of small peptides [34]. Moarefi *et al.* [28] showed that the intramolecular SH3 interaction can be outcompeted by the high-affinity Nef–SH3 interaction, resulting in full activation of the kinase.

We therefore wanted to test whether the small peptide–peptoid ligands identified here were capable of activating Src-family kinases. We chose Hck kinase as a test case rather than Src for two reasons: first, the assays for Hck activation were well-established; and second,

Figure 4

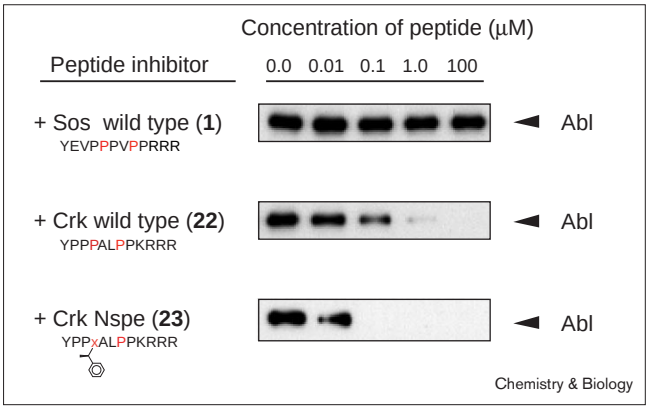
Enhanced domain selectivity from combined peptoid substitutions and specific flanking sequences. Isotherms for the binding of **(a)** Sos wild-type peptide, **(b)** Crk-specific peptide, **(c)** Crk-specific Nnme peptide–peptoid (compound **24**) and **(d)** Crk-specific Nspe–Nnme peptide–peptoid (compound **25**) to the Crk (open squares), N-Grb2 (closed circles) and Src (open triangles) SH3 domains. Flanking sequences are underlined.



there is a natural high-affinity ligand (Nef) for Hck SH3 that has been shown to potently activate the kinase. The published data on the affinity of Nef for the Hck SH3 and its ability to activate Hck enable a direct comparison with the results from our peptide–peptoid studies. From the library above, we chose several high-affinity compounds and measured their affinities for the isolated Hck SH3 domain. The best compounds were then tested for their ability to activate full-length Hck (Figure 6b).

Selected peptides and peptide–peptoid compounds were incubated individually in a kinase reaction with Hck and the rate of phosphotransfer was measured as a function of ligand concentration. The ability of a ligand to activate Hck was quantitated as K_{act}, the concentration of ligand required for half-maximal activation. We found that K_{act} values correlated well with the dissociation constants of the peptide–peptoid compounds for isolated Hck SH3 domain. The strong correlation between K_{act} and K_d is

Figure 5



Potent inhibition of Crk–Abl interaction by a Crk-specific peptide–peptoid. Inhibition of Crk SH3-mediated binding to Abl by the Sos wild-type peptide, Crk-specific wild-type peptide and Crk-specific Nspe peptide–peptoid. Bound Abl was separated by sodium dodecylsulphate (SDS)–PAGE and visualized by anti-Abl antibody.

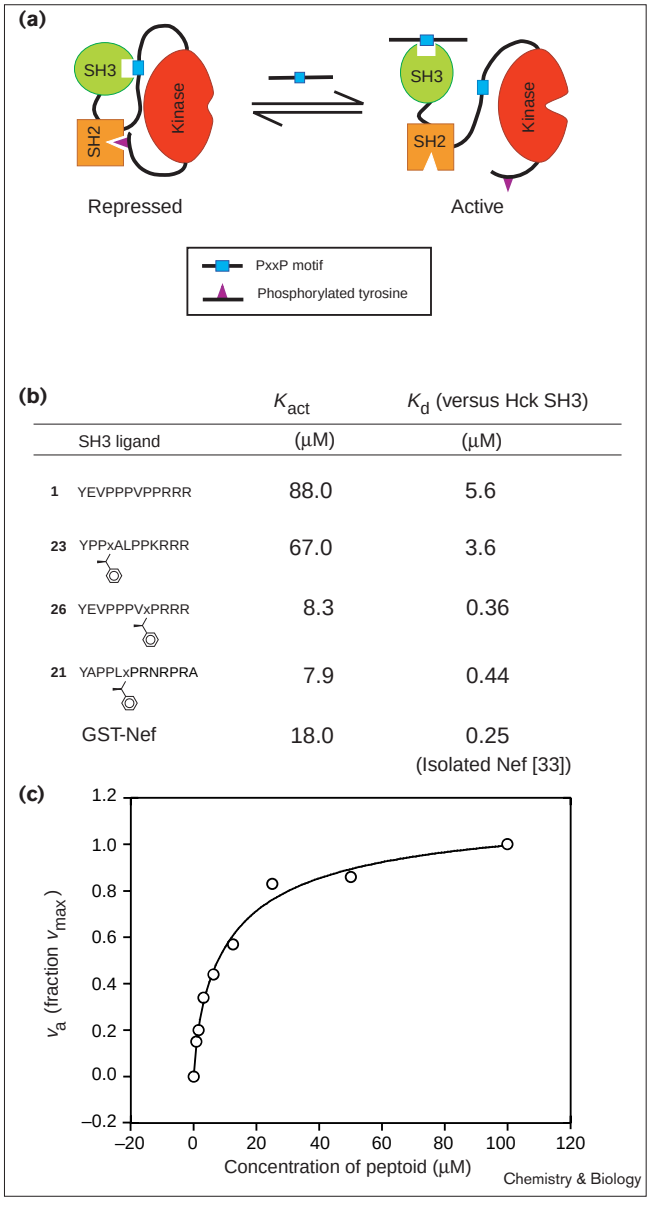
consistent with the model that SH3 displacement is a mechanism for kinase activation, suggesting that the level of activation is dependent on the affinity of the SH3 domain for different PxxP containing ligands. In the best case, the Src-specific Nspe peptide–peptoid (compound **21**) activated Hck with a K_{act} of 7.9 μM (Figure 6c), tenfold better than the wild-type peptide and twofold better than GST–Nef. Thus, the much smaller peptide–peptoid compounds can activate Src-family kinases with comparable potency as intact protein activators. These compounds represent important leads in the generation of small kinase-specific activators which could prove useful in understanding the complex roles of the various members of this important family of kinases.

Discussion

It has been proposed that, owing to the relatively small SH3–ligand interface (~400 Å^2) and the small number of hydrogen bonds, peptide recognition by SH3 domains is highly promiscuous [35]. Interaction with the core PxxP motif provides very little specificity, as the residues that contact the core motif are highly conserved within the SH3 family. To date, most work on ligand specificity in SH3 recognition has focused on nonconserved residues flanking the PxxP core. Biased combinatorial libraries, phage-display libraries and rational mutagenesis have been the most informative in identifying flanking sequences that optimized interactions outside the core, but have been less successful in achieving specificity within the PxxP core [7,12,21,22,36].

Our results indicate that strong specificity determinants exist within the PxxP binding core, but that these were inaccessible to peptide and phage-display libraries. Structural studies have shown that the contacts within the PxxP

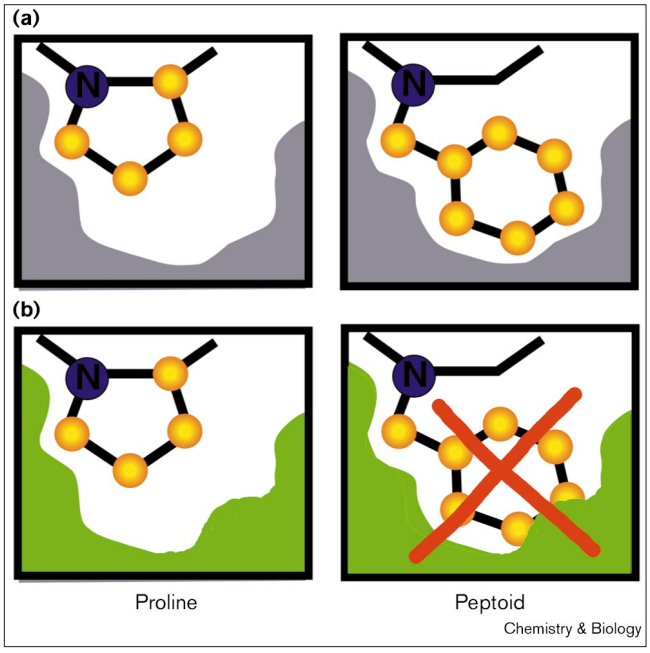
Figure 6



Activation of a Src-family kinase by peptide–peptoid compounds. **(a)** Mechanism of activation of Src-family kinases by SH3 displacement according to Moarefi *et al.* [28]. Src-family kinases are maintained in the repressed state by intramolecular SH3 and SH2 interactions. Displacement of the SH3 domain by a competing intermolecular SH3 ligand results in maximal activation. **(b)** Activation and dissociation constants for the peptide–peptoid compounds tested against full-length autophosphorylated Hck kinase and the isolated Hck SH3 domain, respectively. **(c)** Activation curve for the Src-specific peptoid (compound **21**) tested against Hck kinase.

core are mediated by pairs of Cα- and *N*-substituted amino acids (–xP– dipeptides) on the ligand [6,14]. Phage-display and peptide libraries reveal that different SH3 domains have slight preferences for amino acids at the Cα-substituted position, but they determine that the *N*-substituted position is invariantly proline, simply

Figure 7



Proposed mechanism for peptoid selectivity. Proline recognition pockets of two different SH3 domains **(a)** and **(b)** nominally require proline simply because it is the only *N*-substituted amino acid. Peptoids fulfill the minimum requirement for *N*-substitution, but selected peptoid sidechains are able to exploit slight variations in the structures of different SH3 domains.

because proline it is the only available *N*-substituted amino acid [21,36]. By replacing prolines with *N*-substituted peptoids, we have in effect increased the working 'vocabulary' of *N*-substituted residues, allowing us to expose specificity determinants that were inaccessible to previous studies (Figure 7).

We can begin to assess characteristic preferences for each SH3 domain by going to larger and more diverse peptoid libraries. Taken together, the libraries reported here and elsewhere [14] show, on the one hand, that the N-Grb2 SH3 domain has a strong preference for bulky aromatic peptoid sidechains — the best ligands contain the Ndmb, Nspe and Ndfb peptoids — and that it has a very low tolerance for positively charged amino sidechains. On the other hand, the Crk SH3 domain seems to be considerably more tolerant to various polar peptoid substitutions. We used this to our advantage to design selective ligands for Crk by choosing sidechains that are tolerated by Crk but not by other SH3 domains. By combining such specific peptoid sidechains with a Crk-specific flanking sequence, we have been able to generate an inhibitor that shows orthogonal selectivity for the Crk SH3 domain, within our test set of normally cross-reactive SH3 domains. Development of a deeper understanding of peptoid preferences for individual SH3 domains will aid in the design of directed inhibitor libraries.

We have shown that single peptoid substitutions can have profound effects on affinity and specificity, providing a greater than 100-fold increase in affinity and a roughly 300-fold increase in specificity. The optimization of PxxP core interactions might result in minimized, low molecular weight inhibitors that could still bind with high specificity. Furthermore, we show that combinations of specific peptoid substitutions and flanking sequences identified by phage-display and peptide libraries can enhance both affinity and specificity for SH3 interactions. Thus, by combining specificity-determining elements from inside and outside the PxxP core, ligand specificity can be tuned to a particular SH3 domain.

Significance

Selectively disrupting protein–protein interactions is a principal goal of chemical biology. Src homology 3 (SH3) domains are an important class of biological targets, as they mediate interactions in many regulatory pathways. The identification of selective small-molecule inhibitors has been hampered by the fact that SH3 domains bind their natural peptide ligands with low affinity and with high cross-reactivity for different domains. We have demonstrated that by replacing core proline residues within an SH3 ligand with *N*-substituted glycine residues, or peptoids, and by combining these substitutions with other specificity elements, we can markedly increase both domain affinity and selectivity. The peptoid–peptide ligands described here are some of the most promising inhibitors against SH3 domains to date, and they represent a significant step towards the design of small-molecule SH3 inhibitors that could be used either to dissect signaling pathways or as therapeutic agents targeted against diseases arising from signaling defects.

Materials and methods

Materials

Fmoc amino acids were purchased from Novabiochem (San Diego, Ca) or Anaspec (Santa Clara, CA). Amines were purchased from Sigma (St. Louis, MO) or Fluka (Buchs, Switzerland) and used without further purification. HATU was purchased from PerSeptive Biosystems (Hamburg, Germany).

Peptoid synthesis

Peptoid-containing compounds were synthesized in parallel on a Zymark robotic synthesizer using standard peptide and peptoid chemistry as published elsewhere [14,16] with the following exception: to couple an amino acid onto the peptoid residue, we used 10 equivalents Fmoc amino acid, 10 equivalents HATU and 20 equivalents DIEA. The amino acid was double-coupled for 4 h at room temperature. Compounds were cleaved from resin with 88% (v/v) TFA, 5% (w/v) phenol, 5 % (v/v) H_2O and 2% (v/v) triisopropylsilane. After ether extraction, compounds were purified by reverse-phase high performance liquid chromatography (HPLC) and resuspended in water. Concentrations of stock solutions were determined by tyrosine absorbance and amino acid analysis.

Protein expression and purification

SH3 proteins were expressed as glutathione-*S*-transferase (GST) fusions using a modified pGEX4T-1 plasmid encoding a six-histidine

tag at the carboxyl terminus. Proteins were expressed in TG1 *Escherichia coli* cells by inducing with 1 mM IPTG for 3–5 h. Cells were harvested and sonicated and the lysates were run over a glutathione-agarose column. After washing, bound proteins were cleaved with 10 μg thrombin at 4°C overnight. Released proteins were eluted and bound to a Ni-NTA column, washed and eluted in 100 mM sodium phosphate pH 4, 300 mM NaCl. Purified proteins were dialyzed into 20 mM sodium acetate pH 4, 50 mM NaCl, 1 mM dithiothreitol (DTT), and concentrated to ~5 mg/ml for storage. Proteins were typically more than 90% pure as judged by SDS–PAGE.

Peptoid binding

Dissociation constants (K_d) were assayed by titrating increasing concentrations of ligand and measuring tryptophan perturbation. Assays were performed using a Photon Technology International fluorometer and fit by nonlinear least-squares analysis with the program Sigmaplot or Profit as detailed elsewhere [37]. Total protein concentration (P) for all experiments was fixed at 0.05–1 μM, well below the dissociation constant for most ligands. For high affinity ligands where $K_d \approx P$, data were fit to the following equation:

$$F_b = (K_d + P + L \pm ((K_d + P + L)^2 - 4PL)^{1/2}) / 2P$$

where F_b = fraction bound and L is the total peptide ligand concentration. Data presented are the averages of at least two independent measurements. Error for all measurements ranged between 5 and 20%.

Crk–Abl binding assay

The retroviral vector encoding c-Abl (pGDN-Abl) and the bacterial expression vector encoding GST–Crk were gifts of Bruce Mayer (Harvard Medical School). Human kidney 293 cells expressing the adenovirus large T antigen (293T) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics and antimycotics. 293T cells (4×10^6) were seeded in 100 mm diameter tissue culture dishes 24 h before transfection. Calcium phosphate transfection was carried out according to Pear *et al.* [38]. Immediately before transfection, cells were treated with 25 μM chloroquine and cells were transfected with 0.5 ml of DNA–calcium phosphate coprecipitate containing 5 μg of pDGN-Abl. After 8 h incubation at 37°C, the medium was changed and incubation was continued for another 48 h. Cells in each plate were lysed in 1 ml of buffer containing 25 mM Tris–HCl pH 8.0, 140 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin and 2 μg/ml leupeptin, and then incubated with occasional mixing for 30 min at 4°C. The lysates were cleared by centrifugation at 12,000*g* for 10 min, and the protein concentrations of the postnuclear supernatants were determined by the Bradford method (Bio-Rad).

GST–Crk fusion protein (1 μg) bound to glutathione-agarose beads was pre-incubated with various concentrations of peptoid competitors in 400 μl at 4°C for 10 min. Abl transfected cell lysate (50 μl,10 μl) was added to each tube and incubated for an additional 2 h at 4°C. The glutathione-agarose beads were recovered by centrifugation and washed three times in 1 ml of lysis buffer. Bound proteins were fractionated by SDS–PAGE under reducing conditions and then electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked overnight in buffer containing 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 1% bovine serum albumin. The amount of Abl associated with GST–Crk protein in each case was detected by incubation with anti-Abl monoclonal antibody 8E9 (Pharmingen), followed by HRP-conjugated goat anti-mouse IgG (Amersham), and developed with enhanced chemiluminescent (ECL) western blotting reagent according to the manufacturer's instructions (Amersham).

Kinase activation assay

Down-regulated Hck was produced in Sf9 cells by co-infection with Csk and purified as described [28]. Hck was autophosphorylated by pre-incubation of (1–10 μM) Hck with 0.5 mM ATP for 30 min at room temperature. SH3 ligands were pre-incubated with autophosphorylated Hck for 10 min. The reaction was initiated by the addition of substrate peptide (Ala–Glu–Glu–Glu–Ile–Tyr–Gly–Glu–Phe–Glu–Ala–Lys–Lys–Lys–Lys–Gly) and components of the coupled assay. Initial rates were measured for autophosphorylated wild-type Hck by a coupled spectrophotometric assay in which the production of ADP was coupled to the oxidation of NADH measured as a reduction in absorbance at 340 nm.

The activation constant, K_{act}, was determined by nonlinear regression analysis of the rates as a function of ligand concentration (SH3 binding peptoid or peptide) using the equation,

$$v_a = V_{act}[L]/(K_{act} + [L])$$

where v_a = velocity measured in the presence of ligand minus the velocity measured in its absence, V_{act} = the maximal activated velocity minus the velocity measured in the absence of ligand, and $[L]$ is the concentration of SH3 binding peptoid/peptide [39]. All K_{act} determinations were performed at substrate concentrations of 500 μM ATP and 600 μM peptide.

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