From Consensus Sequence Peptide to High Affinity Ligand, a “Library Scan” Strategy*

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A wide variety of proteins have been shown to recognize and bind to specific amino acid sequences on other proteins. These sequences can be readily identified using combinatorial peptide libraries. However, peptides containing these preferred sequences (“consensus sequence peptides”) typically display only modest affinities for the consensus sequence-binding site on the intact protein. In this report, we describe a parallel synthesis strategy that transforms consensus sequence peptides into high affinity ligands. The work described herein has focused on the Lck SH2 domain, which binds the consensus peptide acetyl-Tyr(P)-Glu-Glu-Ile-amide with a $K_d$ of 1.3 $\mu$m. We employed a strategy that creates a series of spatially focused libraries that challenge specific subsites on the target protein with a diverse array of functionality. The final lead compound identified in this study displayed a 3300-fold higher affinity for the Lck SH2 domain than the starting consensus sequence peptide.

There exist an impressive array of biological phenomena that are regulated by protein-protein interactions. Perhaps nowhere is this more evident than in the formation of coherent signal transducing cascades, which are required to drive such diverse processes as cell motility (1) and division (2), the immune response (3), apoptosis (4), and neuronal activity (5). The dependence of these and many other phenomena on highly specific protein-protein interactions has generated considerable interest in elucidating the molecular basis of these interactions. Much of the initial work in this field focused on the ability of proteolyzed peptide fragments from one component of a known protein-protein pair to interact with the intact binding partner. Such studies have led, for example, to the acquisition of a number of peptide-based protein kinase substrates and inhibitors (6, 7). More recent work has relied on the use of peptide libraries (8–10) for the identification of “consensus sequences” (11) for a wide variety of protein-interacting species, including the SH2 (Src homology 2) domain (12). The SH2 module consists of ~100 amino acids and has thus far been identified in >100 different proteins (13–15). SH2 domains recognize and bind to amino acid sequences that encompass a Tyr(P) moiety. Although Tyr(P) is required for SH2 recognition, specificity is conferred by neighboring residues. In general, simple SH2-directed peptide ligands recapitulate these properties. Peptides lacking a phosphorylated Tyr moiety display little or no affinity for SH2 domains, and SH2 selectivity can be realized by the simple expediency of incorporating Tyr(P) into the appropriate amino acid sequence. However, the use of peptides as ligands for SH2 domains in particular and as agents that can modulate protein-protein interactions in general is fraught with limitations. First and foremost is the general phenomenon that the affinity of peptides for specific binding sites on proteins tends to be weak by comparison with small low molecular species. For example, although a number of ATP analogs have been described for tyrosine-specific protein kinases with affinities in the $\mu$m range, peptides that target the protein-binding site of these enzymes typically display affinities in the high $\mu$m range (16). One possible explanation for this dichotomy is the fact that peptides are limited to an array of 20 standard amino acid residues, whereas the inherent structural diversity possible with low molecular weight compounds, such as ATP analogs, is virtually limitless. High diversity allows one to identify synthetic compounds that are able to engage in an assortment of productive interactions with the target protein, interactions that are otherwise unavailable to conventional peptides.

The use of combinatorial chemistry to generate libraries of high molecular diversity has had a profound impact on the fashion by which biologically useful compounds are synthesized and identified. Indeed, a high premium has been placed on the importance of diversity since seemingly trivial modifications in molecular structure can alter the potency of small molecule enzyme inhibitors by as much as 3–4 orders of magnitude (17, 18). Unfortunately, conventional combinatorial peptide libraries are not designed to identify these comparatively subtle structural factors. Although a variety of synthetic methods (e.g. the one bead/one peptide approach) have been used to generate million member peptide libraries, the “local” diversity associated with these libraries is limited to the 20 standard amino acids employed at each position along the peptide chain. One approach that has been used to enhance local diversity in peptide libraries is to employ additional unnatural amino acid residues, which necessitates the use of molecular encoding (19). Although parallel synthesis (i.e. spatially separated peptides (20)) obviates the need for encoding, the size of these libraries is, by necessity, significantly smaller than what is feasible using the “split-and-pool and related methods.

In this report, we describe a strategy to transform consensus sequence peptides, with modest affinities for target proteins, into high affinity ligands. This approach employs peptide libraries with two key attributes: moderate size (~10$^3$ members each), yet high structural diversity. Due to their small size, these libraries can be synthesized in parallel, which allows each library member to be individually evaluated and elimi-
nates the requirement for subsequent structural deconvolution. Furthermore, since these libraries possess a high structural diversity focused within narrow spatial windows on the target protein, small regions of the protein can be challenged with a multitude of functionality containing structural differences that vary from subtle to gross. We have targeted the peptide-binding region of the Lck (lymphoid T-cell tyrosine kinase) SH2 domain to illustrate the utility of this strategy.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were obtained from Aldrich, except for piperidine, protected amino acids, amino acid derivatives, 1-hydroxybenzotriazole (HOBt), benzotriazol-1-yloxytri(dimethylamino)phosphonium hexafluorophosphate (BOP), and TentaGel resin, which were obtained from Advanced Chemtech and Bachem California. Biotinyl-N-carbonyl; HPLC, high performance liquid chromatography; DMF, dimethylformamide; NeutrAvidin; GST, glutathione S-transferase; Fmoc, N-(9-fluorenylmethoxycarbonyl); HPLC, high performance liquid chromatography; GST, glutathione S-transferase; Fmoc, N-(9-fluorenylmethoxycarbonyl); HPLC, high performance liquid chromatography; DMF, N,N-dimethylformamide; DTT, dithiothreitol; Dap, L-2,3-diaminopropionic acid; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; BSA, bovine serum albumin.

**Peptide Synthesis**—All peptides were synthesized on an automated Fmoc solid-phase peptide synthesis protocol. Crude peptides were purified on a preparative HPLC column using three Waters radial compression modules (25 cm, 10 cm).

**Synthesis of Libraries**—All peptides were synthesized on an automated Fmoc solid-phase peptide synthesis protocol. Crude peptides were purified on a preparative HPLC column using three Waters radial compression modules (25 cm, 10 cm).

**Synthesis of Consensus Sequence Peptides**—Coumarin-Tyr(P)-Dap(R)+-Dap(R)+-ICONHCH2CH2SH and Tyr(P)-Dap(R)+-Dap(R)+-ICONHCH2CH2SH were synthesized as described above, except that Fmoc-Dap(1-[(9′-adamantyl)-1-methylethoxy carbonyl]) was employed at position P+1. The 1-[(9′-adamantyl)-1-methylethoxy carbonyl] protecting group was removed by shaking the resin with 5% trifluoroacetic acid in CH3CN for 3 min. This step was repeated three times or until the protecting group was completely removed. The resin was washed thoroughly using CH3CN, MeOH, DMSO, and 10% piperidine/DMF. 5-Sulfo-salicylic acid was then coupled to the peptide using the benzotriazol-1-yloxytri(dimethylamino)phosphonium hexafluorophosphate/HOBt method. After washing step, the benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate/HOBt group was removed by using 50% trifluoroacetic acid/CH3CN (with 5% H2O, 30 min). The washing steps mentioned above were applied to the resin before the appropriate carboxylic acid was coupled to the peptide. The final washing and cleaning steps were the same as described above. The collected mixture was purified by HPLC. The structures were confirmed using matrix-assisted laser desorption ionization mass spectrometry and NMR. 1H NMR (Me2SO-d6) for peptide 17: δ 0.07–0.12 (m, 2H), 1.92 (d, J = 7.7 Hz, 1H), 8.22 (d, J = 1.4 Hz, 2H), 8.72 (d, J = 1.4 Hz, 1H), 8.54 (d, J = 6.9 Hz, 1H), 8.38–8.44 (m, 2H), 8.35 (d, J = 1.7 Hz, 1H), 8.21 (d, J = 7.8 Hz, 1H), 7.91 (bs, 1H), 7.82 (dd, J = 1.7, 8.5 Hz, 1H), 7.31 (d, J = 8.5 Hz, 2H), 7.26 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 8.7 Hz, 1H), 7.04 (d, J = 7.04 Hz, 1H), 6.82 (dd, J = 8.7, 2 Hz, 1H), 6.69 (d, J = 1.7 Hz, 1H), 4.50 (m, 1H), 4.39 (s, 1H), 4.70 (d, J = 5.7 Hz, 2H), 3.92 (m, 1H), 3.82–3.45 (m, 2H), 3.10–3.25 (m, 1H), 2.85–2.95 (m, 1H), 2.57–2.75 (m, 2H), 2.45 (bs, 1H), 1.85–1.95 (m, 1H), 1.50–1.70 (m, 1H), 1.10–1.30 (m, 1H), 0.97 (d, J = 6.6 Hz, 3H), and 0.91 (t, J = 7.3 Hz, 3H).

**Determination of Peptide Concentration**—Peptide concentrations were determined by the intensity of coumarin absorption (ε = 1.187 × 104 M−1 cm−1). The protein-tyrosine phosphate-catalyzed hydrolysis reaction of phosphotyrosine was also performed to determine the peptide concentration by monitoring the increase in absorbance at 282 nm (ε = 970 M−1 cm−1) at pH 7.0 in buffer containing 50 mM 3,3-dimethylglutarate, 1 mM EDTA, and 1 mM DTT (J = 0.15 m) at 30 °C. The concentrations obtained via the phosphatase method were close or identical to concentrations obtained from UV.

**Screening of the Peptide-Nonpeptide Conjugate Library**—An ELISA was employed to screen the library for SH2 affinity (21). 100 μl of biotinyl-N-aminocaproyl-EPQpYEEIPIYL (10 ng/ml in Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl (pH 7.5))) was added to each well of NeutrAvidin-coated 96-well microwell plates. The plates were shaken overnight at 4 °C and rinsed with TBS (2 × 200 μl) followed by BSA-T-TBS (0.2% BSA, 0.1% Tween 20, and TBS, 2 × 200 μl). Each well was then blocked with 100 μl of SuperBlock blocking buffer (1× buffer, 1×) at room temperature. Two wells in each plate were reserved for standards (one well contained the starting peptide (i.e., either peptide 8 or 9) that had not been acylated, and the other well contained the starting peptide that had been acylated). A 50-μl solution of each member of the P+1 and P+2 libraries (50 μl in TBS) and a 50-μl solution of the Lck SH2-GST fusion protein (3.2 ng/ml in BSA-T-TBS) were added to the wells of 96-well plates. The plates were subsequently shaken for 1 h at room temperature. The solutions were removed, and each well was rinsed with 4 × 200 μl of BSA-T-TBS. 100 μl of polyclonal rabbit anti-GST antibody (100 ng/ml in BSA-T-TBS) was then added to each well, and the plates were incubated for 1 h at room temperature. Following subsequent washing steps with BSA-T-TBS (4 × 200 μl), 100 μl of horse radish peroxidase-conjugated goat anti-rabbit antibody (200 ng/ml in BSA-T-TBS) was added to each well, and the plates were subsequently incubated for 1 h at room temperature. After a series of final wash steps (2 × 200 μl of BSA-T-TBS and 2 × 200 μl of TBS), 100 μl of peroxidase substrate (1-Step Turbo Trithiophenyl benzidine ELISA) was added to each well and incubated for 5–15 min. 100 μl of 1 M sulfuric acid solution was introduced to stop the peroxidase reaction, and absorbance was measured at 450 nm with a plate reader. IC50 values were determined using the ELISA screening method around a 130-fold range of ligand concentrations.

**Determination of Kd Values**—With the exception of compounds 12 and 15–17, peptides of the general structure coumarin-Tyr(P)-Gln-Dap(R)+-ICONHCH2CH2SH are highly fluorescent and exhibit little or no change in fluorescence upon coordination to the SH2 domains of Lck. From the Hill plots (Fig. 1B), Kd values were determined for the 42 complexes that were determined via equilibrium dialysis (21). All samples were prepared in buffer containing TBS and 1 mM DTT at pH 7.5. Slide-A-Lyzer dialysis slide cassettes (0.1–0.5-ml capacity) were employed and contained 3–5 ml SH2-GST fusion proteins. The cassettes (400 μl final volume) were placed in a beaker containing a volume of buffer solution (TBS and 1 mM DTT at pH 7.5) that was at least 250-fold greater than that of the
sample volume in the dialysis cassette. As a consequence, concentrations of non-SH2-bound peptide were held constant in the dialysis slide cassette over the course of the experiment. Equilibrium dialysis experiments were performed over a period of 12 h and maintained at 4 °C. Differences in fluorescence between the solution in the slide cassette and that in the beaker were measured. The excitation wavelength employed for the peptides was 330 nm. Emission was monitored at 460 nm. Equation 1 was used for the determination of $K_D$.

$$K_D = \frac{[E]_i - [E-L]_i}{[E-L]}$$

where $[E]_i$ = total SH2 domain concentration, $[L]$ = total ligand concentration, and $[E-L]$ = SH2/ligand concentration.

**RESULTS AND DISCUSSION**

Combinatorial peptide libraries offer a relatively straightforward method for identifying consensus recognition sequences of proteins that interact with other proteins. As an initial first step, a consensus sequence furnishes invaluable information concerning potential endogenous substrates and/or binding partners and serves as a starting point for the generation of synthetic species that can modulate protein-protein interactions. Although it is relatively straightforward to acquire consensus sequences via the application of peptide libraries, the subsequent conversion of peptide templates into species that display high affinities (i.e., $K_D$ values in the nM range) for the desired protein target often requires a more tortuous route.

Once a consensus sequence has been acquired, the binding contributions of individual residues in the sequence can be assessed via the “alanine scan” method (22, 23). This approach employs a series of peptides containing an Ala residue positioned at each site along the peptide chain. Analogous “phenylalanine scans,” “glycine scans,” and other variations (24) have also been reported. We describe herein a “library scan” that replaces specific residues in a consensus sequence peptide with $10^3$ different amino acid moieties. The overall strategy is outlined in Fig. 1. One or more peptides are synthesized that contain a Day moiety at specific sites along the peptide chain. Once the Dap-containing peptides have been prepared, the side chain Dap amine is deprotected, and the resin-appended peptide is transferred in equal amounts to individual wells of multilwell plates. The free amine-containing Dap functionality on the peptide-bound resin in each well is subsequently condensed with one of $\approx 10^3$ different carboxylic acids (which vary by molecular weight, charge, polarity, hydrophobicity, sters, etc.). Following removal of any remaining protecting groups, the peptide is released from the resin and delivered to a receiving multilwell plate in an assay-ready form. These libraries can be constructed in a linear, iterative fashion (Fig. 2a) or in parallel (Fig. 2b). The former strategy requires the identification of a lead residue (Dap-COR) from an initial library scan, which can then be used as a biasing agent in the construction of subsequent sublibraries. This approach is useful if the residues to be replaced on the peptide bind to the target protein in an energetically coupled fashion. Alternatively, a series of libraries can be synthesized simultaneously (Fig. 2b), an approach that is synthetically more expedient since the nonbiased libraries prepared via this method are not dependent upon the acquisition of lead residues from any other library(ies) (Fig. 2a).

The design of the libraries in this study was based upon the following considerations. (a) The consensus peptide, acetyl-Tyr(T)-Glu-Glu-Ile-amide (peptide 2), displays a $K_D$ of 1.3 ± 0.2 μM for the Lck SH2 domain (21), a value consistent with those obtained for Src SH2 domain-targeted peptides in general. We have recently found that the coumarin-appended peptide 3 (Fig. 3 and Table I) displays a 37-fold higher affinity ($K_D = 35 ± 7$ nM) for the Lck SH2 domain than peptide 2 (21). Consequently, the coumarin moiety was employed as a biasing element in the construction of the SH2 domain-targeted libraries.

(b) The Glu residues in Tyr(T)-Glu-Glu-Ile, which only modestly interact with the SH2 domain, were substituted with Dap-based libraries (14, 25). The Tyr(T) and Ile residues were not replaced because they participate in high affinity interactions with residues on the SH2 domain (14). Our decision to
employ Dap rather than some other diamine-containing species (e.g., lysine) was based on the comparatively short Dap side chain, which limits the conformational mobility (i.e., entropy) of substituents attached to the side chain amine moiety. Under these circumstances, the amine-bound substituents should be more favorably positioned to engage SH2 functionality that encompasses the peptide-binding region of the SH2 domain.

(c) Our initial objective required the preparation of libraries of moderate size (≈10^3) in an assay-ready form. We have recently described the preparation of a cystamine-linked TentaGel resin (peptide 1) and its use in peptide library synthesis (21, 26). The disulfide linkage between the peptide and the TentaGel resin is stable to the conditions of Fmoc-based solid-phase peptide synthesis. Furthermore, the disulfide moiety is cleaved in essentially quantitative yield by conditions (TBS and 10 mM dithiothreitol at pH 7.5) that are virtually the same as and therefore compatible with the subsequent ELISA screen (TBS and 1 mM dithiothreitol at pH 7.5).

(d) Dap-based moieties will ultimately replace both Glu residues in -Tyr(P)-Glu-Glu-Ile-. However, the initial libraries employ only a single Dap insertion, thereby requiring a transient “caretaker” residue at Xaa (i.e., -Tyr(P)-Dap-Xaa-Ile- and -Tyr(P)-Xaa-Dap-Ile-). We chose Gln for this purpose since its non-nucleophilic character will not interfere with the subsequent Dap acylation step. Furthermore, we found that the Gln-for-Glu substitutions in the parent peptide (coumarin-FIG. 2. Iterative (a) and parallel (b) strategies for the preparation of Dap-containing peptide libraries. The iterative strategy (a) employs the stepwise identification of optimal Dap-containing residues at each position along the peptide chain. This approach holds the advantage that side chain residues that are “energetically coupled” can be readily identified. The parallel strategy (b) ignores the latter issue since the acylated Dap residues are acquired independently of each other. However, the parallel approach is synthetically more expedient. AA, amino acid.

FIG. 3. Acyl moieties R1–R5 identified using the library scan strategy against the Lck SH2 domain.

TABLE I

| Peptide                          | IC_{50} | K_D   |
|----------------------------------|--------|-------|
| Acetyl-Tyr(P)-Glu-Glu-Ile-NH₂   | 660 ± 20 | 1300 ± 200 |
| R₁-Tyr(P)-Glu-Glu-Ile-NH₂        | 33 ± 2  | 35 ± 7   |
| R₁-Tyr(P)-Glu-Gln-Ile-NH₂        | 106 ± 4 | 49 ± 14  |
| R₁-Tyr(P)-Gln-Glu-Ile-NH₂        | 260 ± 35 | 116 ± 24 |
| R₁-Tyr(P)-Dap(R²)-Gln-Ile-NH₂   | 13 ± 2  | 3.6 ± 0.4 |
| R₁-Tyr(P)-Gln-Dap(R²)-Ile-NH₂   | 4.2 ± 0.7 | 2.1 ± 0.2 |
| R₁-Tyr(P)-Gln-Dap(R⁵)-Ile-NH₂  | 5.2 ± 0.8 |     |
| R₁-Tyr(P)-Dap(R²)-Dap(R⁵)-Ile-NH₂ | 3.0 ± 0.6 |     |
| R₁-Tyr(P)-Dap(R²)-Dap(R³)-Ile-NH₂ | 0.2 ± 0.02 |     |
Tyrr(P)-Glu-Glu-Ile-amide (peptide 2; $K_D = 35 \pm 7 \text{ nM}$) furnished peptides that display only slightly reduced affinities for the Lck SH2 domain (coumarin-Tyr(P)-Glu-Glu-Ile-amide (peptide 4; $K_D = 49 \pm 14 \text{ nM}$) and coumarin-Tyr(P)-Glu-Glu-amide (peptide 5; $K_D = 116 \pm 24 \text{ nM}$)). Consequently, the TentaGel-appended peptides coumarin-Tyr(P)-Dap-Gln-Ile-amide (peptide 6) and coumarin-Tyr(P)-Gln-Dap-Ile-cystamine-TentaGel (peptide 7) served as the starting species for the synthesis of the P+1 and P+2 libraries, respectively (Fig. 1).

The TentaGel-appended peptides 6 and 7 were prepared using a standard Fmoc protocol (Fig. 1). The $N$-butoxycarbonyl-protected Dap side chain in these resin-bound peptides was subsequently removed with 50% trifluoroacetic acid in CH$_2$Cl$_2$ to furnish peptides 8 and 9, respectively. These TentaGel-appended peptides were then introduced, in 5-mg quantities, into the individual wells of solvent-resistant 96-well filter plates. One of 900 different carboxylic acids (20 $\mu$mol dissolved in 50 $\mu$l of DMF) was subsequently added to each well in an ~500-fold molar excess relative to peptide to ensure complete acylation of the free Dap amine moiety. The acylation reaction was initiated by the addition of 200 eq of BOP, 200 eq of HOBt, and 1000 eq of N-methylmorpholine in 50 $\mu$l of DMF to each well. The plates were gently shaken for 12 h, and each well was subsequently subjected to a series of wash steps to remove excess reagents (see “Experimental Procedures”). All plate washings were conducted via vacuum filtration using a 96-well filter plate manifold. Libraries 10 and 11 were then released from the resin with three washings with a DTT-based solution (TBS and 10 mM DTT at pH 7.5). These washings were filtered into receiving 96-well plates, and these peptide-containing solutions were directly used, without purification, in the subsequent ELISA-based assay.

We selected four compounds from each library (i.e. peptides derivatized at the P+1 and P+2 Dap positions with 7-hydroxycoumarin-4-acetic acid, 3-nitrocinnamic acid, 2-phenoxypropionic acid, and 3,5-dibromo-4-hydroxybenzoic acid) to examine the extent of amine acylation and the efficiency of DTT cleavage. In all cases, we were unable to detect the presence of any free amine following treatment of the peptide-bound resin with the 500-fold molar excess of carboxylic acid. In addition, >90% of the peptide was cleaved from the resin with the first DTT wash step. The final two DTT washings removed the residual resin-bound material. Finally, all compounds were found to be >90% pure by HPLC, and the HPLC-purified compounds (i.e. removal of Tris buffer and DTT) furnished the expected mass profiles by mass spectrometry.

The P+1 library ELISA-based screen identified peptide 12 as the single lead, whereas three clear leads (peptides 13-15) were obtained from the P+2 library (Fig. 3 and Table I). We employed the ELISA to obtain an initial assessment of the affinity of these compounds for the Lck SH2 domain (i.e. IC$_{50}$ = ligand concentration that blocks 50% of the ELISA readout at 450 nm). Values in the low nM range were obtained for all four peptides (Fig. 3 and Table I). We noted that, in the ELISA, the modified peptides created in libraries 10 and 11 directly competed for the Lck SH2 domain with a known SH2-directed standard (brotinyl-$\epsilon$-aminocaproxy-EbQyYEEIPYLYL) bound to the wells of the 96-well plates. Consequently, the lead peptides depicted in Table I most likely associate with the same site on the SH2 domain as brotinyl-$\epsilon$-aminocaproxy-EbQyYEEIPYLYL.

The inherent fluorescence associated with the N terminus-appended coumarin moiety was unaltered in the presence of the Lck SH2 domain. This behavior allowed us to acquire dissociation constants on select compounds via equilibrium dialysis using Slide-A-Lyzer cassettes. Unfortunately, the coumarin fluorescence was partially quenched in peptides 12 and 15. Consequently, we were unable to acquire $K_D$ values for these species. However, as is apparent from Table I, the ELISA-based IC$_{50}$ values correlated well (within 2-fold) with the experimentally derived dissociation constants. Clearly, the P+2 library derivatives 13-15 displayed a substantial enhancement in Lck SH2 domain affinity relative to the parent peptide 5 (~50–100-fold). By contrast, the lead compound from the P+1 library (peptide 12) exhibited only an 8-fold higher affinity for the SH2 domain compared with its parent peptide 4.

Based on the results described above, we prepared the double-substituted Dap-derivatized peptides 16 and 17 (see “Experimental Procedures”). Since both peptides 16 and 17 contain the Dap(R$^2$) residue present in peptide 12, we were not surprised to find that, like peptide 12, the coumarin fluorescence was partially quenched in the double-substituted Dap-derivatized peptides. Consequently, we were able to obtain IC$_{50}$ values for only peptides 16 and 17. Peptide 16, which contains the acylated Dap residues present in peptides 12 and 14, displayed an IC$_{50}$ of 3.0 ± 0.6 nM. The similar Lck SH2 affinities of the monosubstituted (peptide 14) and disubstituted (peptide 16) ligands clearly indicate that the separate SH2 energies of interaction of the Dap(R$^2$) and Dap(R$^3$) moieties are not additive when contained within the same peptide. This result illustrates one of the potential hazards associated with combining lead substituents from separate libraries (Fig. 2b), a hazard common to many combinatorial peptide library strategies: lead residues obtained independently of one another are not necessarily energetically additive in the final consensus sequence (27). Despite this potential difficulty, peptide 17, which contains the acylated Dap residues present in peptides 12 and 13, did display an enhanced affinity for the Lck SH2 domain (IC$_{50}$ = 200 ± 20 pm). The latter result implies that the two acylated Dap residues in peptide 17 do bind independently of one another to the Lck SH2 domain. Insertion of Dap(R$^2$) into the P+2 position of peptide 5 furnished peptide 13 and resulted in a 30-fold enhancement of SH2 affinity. By comparison, incorporation of Dap(R$^3$) into the same position of peptide 12 (to provide peptide 17) resulted in a 65-fold greater affinity for the Lck SH2 domain. In an analogous vein, Dap(R$^2$) insertion at P+1 in peptide 4 generated a 4-fold improvement in affinity (cf. peptides 5 and 12), whereas the incorporation of Dap(R$^3$) into the same position in peptide 14 furnished a 20-fold binding enhancement. The IC$_{50}$ value of 200 ± 20 pm displayed by peptide 17 for the Lck SH2 domain represents a 3300-fold enhanced affinity relative to the simple consensus peptide 2.

The individual members of the Srk kinase family are generally limited to specific cell types. For example, the Lck protein-tyrosine kinase expression is restricted to T-cells. However, a number of SH2 domain-containing proteins are ubiquitously expressed, including phospholipase C, phosphatidylinositol 3-kinase, and Grb2 (growth factor receptor-bound protein-2). Consequently, we examined the release of Lck SH2 selectivity using the SH2 domains from three universally expressed proteins as controls. The coumarin-substituted peptide 3 displayed a >2 orders of magnitude selectivity for the Lck SH2 domain ($K_D = 35 \pm 7 \text{ nM}$) versus the SH2 domains of phospholipase C$_Y$1 ($K_D = 4.9 \pm 0.7 \mu$m), Grb2 ($K_D = 11.3 \pm 3.1 \mu$m), and the p85a subunit of phosphatidylinositol 3-kinase ($K_D = 9.3 \pm 0.9 \mu$m). As noted above, we were unable to acquire dissociation constants for peptide 17 due to the partial quenching of fluorescence of the coumarin moiety in this compound. However, the IC$_{50}$ values obtained from the ELISA screen revealed that peptide 17 was even more selective than peptide 3 for the SH2 domain of Lck (IC$_{50}$ = 200 ± 20 pm) versus those of phospho-
lipase Cyt1 (IC\textsubscript{50} = 7.4 ± 2.5 μM), Grb2 (IC\textsubscript{50} = 0.90 ± 0.09 μM), and phosphatidylinositol 3-kinase (IC\textsubscript{50} > 30 μM).

With the advent of combinatorial peptide libraries, it is now a relatively straightforward matter to obtain consensus recognition sequences for proteins that bind to and/or process other proteins. Unfortunately, a not uncommon trait among consensus sequence peptides is their comparatively low affinity for protein targets. Typically, these peptides contain only a few residues that participate in key interactions with their protein-binding partners. Many if not the majority of residues in any given consensus peptide can often be replaced with little or no impact on overall binding affinity. We have described the use of a parallel synthesis strategy to identify high affinity replacements for these noncritical residues. The attributes of this strategy include the generation of a high diversity library (i.e. 50-fold diversity greater than what is available using standard amino acid residues) in an individual well format. The latter allows one to separately assess the efficacy of each library member without the need to resort to subsequent structural deconvolution. Furthermore, the synthetic methodology delivers the library in an assay-ready solution format, which provides a seamless transition between library synthesis and the subsequent library screen. We have applied this strategy to the SH2 domain of the Lck protein-tyrosine kinase, an enzyme that plays a key role in T-cell activation. The lead ligand, which was identified via a series of sublibraries, displayed 3 orders of magnitude higher affinity for the Lck SH2 than the standard consensus peptide. Indeed, with an IC\textsubscript{50} of 200 ± 20 pm, peptide 17 is among the tightest binding peptide-based ligands described for any protein-protein interaction site.

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