Identification of D-Peptide Ligands Through Mirror-Image Phage Display

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Genetically encoded libraries of peptides and oligonucleotides are well suited for the identification of ligands for many macromolecules. A major drawback of these techniques is that the resultant ligands are subject to degradation by naturally occurring enzymes. Here, a method is described that uses a biologically encoded library for the identification of D-peptide ligands, which should be resistant to proteolytic degradation. In this approach, a protein is synthesized in the D-amino acid configuration and used to select peptides from a phage display library expressing random L-amino acid peptides. For reasons of symmetry, the mirror images of these phage-displayed peptides interact with the target protein of the natural handedness. The value of this approach was demonstrated by the identification of a cyclic D-peptide that interacts with the Src homology 3 domain of c-Src. Nuclear magnetic resonance studies indicate that the binding site for this D-peptide partially overlaps the site for the physiological ligands of this domain.

For the purposes of drug discovery, there are potential advantages in the use of genetically encoded libraries, such as phage display (*1*, *2*), "peptide on plasmid" (*3*), and in vitro translation-based systems (*4*), compared to the use of synthetic small molecule libraries (*5*, *6*). The genetic encoding of libraries allows the resynthesis and rescreening of molecules with a desired binding activity. The resulting amplification of interacting molecules in subsequent rounds of selection can lead to the isolation of extremely rare, specific binders from a large pool of molecules. However, a major drawback of biologically encoded libraries is that the resultant ligands are subject to degradation by naturally occurring enzymes. Furthermore, because of their sensitivity to cellular proteases, peptides composed of naturally occurring L-amino acids are efficiently processed for major histocompatibility complex class II–restricted presentation to T helper cells (T_H cells). As a result, L-peptides can induce a vigorous humoral immune response that impairs the activity of such drugs (*7*). We describe here a general approach that uses a genetically encoded library for the identification of D-peptide ligands. This approach takes advantage of the fact that the three-dimensional structures of proteins composed of D-amino acids are the exact mirror images of the corresponding L-proteins. The D-peptide ligands identified through this method may provide useful starting points for the design or selection of novel drugs.

Around 1850, Louis Pasteur demonstrated through his experiments with tartrate that the forces that create or convert natural products (that is, enzymes) are stereospecific in their actions (*8*). From the principles of van't Hoff–Le Bel stereochemistry (*9*), it follows that this chiral specificity is inverted for "mirror-image proteins" (proteins composed of D-amino acids). The recent syntheses of two D-enantiomeric proteins permitted direct demonstration that these proteins do indeed have optical properties, substrate specificity (*10*), and a structure (*11*) that mirrors those of the naturally occurring L-proteins.

In our method (which we call mirror-image phage display), the D-enantiomer of a protein is prepared by chemical synthesis and used to isolate L-peptide ligands that interact with it from a phage display library. The selection process is performed in an achiral solvent (water), and the interaction between the L-peptide and the D-protein is unlikely to require any chiral cofactors. Consequently, the D-enantiomeric form of the isolated L-peptide ligands should interact with the protein of the natural, L-amino acid configuration (Fig. 1).

To test the validity of our approach, we sought to obtain D-peptide ligands for the Src homology 3 domain (SH3 domain) of c-Src. SH3 domains are 55- to 70-residue protein domains that are found in a variety of intracellular effector molecules (*12*). Be-

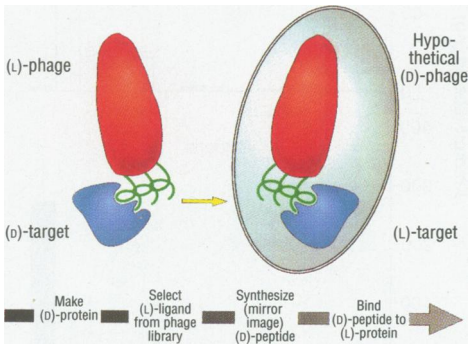

Fig. 1. Identification of D-peptide ligands through mirror-image phage display. Because of the mirror-image relation between ligands for L- and D-enantiomeric proteins, the identification of phage-displayed L-peptides that bind to the D-enantiomer of a target molecule also provides the sequence of D-peptides that bind to the L-protein.

Table 1. Sequences of phage-displayed peptides that interact with the D-SH3 domain (*25*). These peptides were isolated through "biopanning" (*22*, *24*). Semiconserved residues are underlined; all other residues are conserved except for those at positions 3 and 11. Note that for all of these sequences the positions of the conserved residues relative to the Cys residues are preserved. Individual clones were analyzed after four and five rounds of selection. In subsequent rounds, the incubation time between washes was increased (times of 0, 3, 5, 10, and 10 min, respectively, for rounds 1 through 5). After four rounds of selection, 29 clones were sequenced, of which only 7 are within the sequence class described in the table. To ensure that the selected phages were not binding to streptavidin or to a composite surface formed by the streptavidin–D-SH3 complex, a fifth selection round was performed with neutravidin (Pierce) as a matrix. Sequence analysis of clones after this fifth round of selection revealed only sequences of the fdSrc-2 type. The corresponding D-peptide (Pep-D2) has been characterized only slightly, but preliminary experiments suggest that the affinity of this peptide is similar to that of Pep-D1. The other phage isolates obtained after four rounds of selection expressed one of the following two sequences: CKRFVWRGQALC (10 isolates) and CWYLGYWPGQEC (12 isolates) (*25*). The first of these sequences resembles the background sequences that are isolated with a variety of biotinylated ligates (*22*) and is also similar to a sequence (CRFVWC) that was isolated previously with a monoclonal antibody against myoherythrin, although it does not conform to the recognition motif for this antibody (*1*). This sequence is therefore likely to bind to some component in the system other than the SH3 domain. Indeed, a D-amino acid version of this sequence fails to bind to the L-SH3 domain, as judged by ELISA and NMR studies (*41*). The other sequence that was picked up after four rounds of selection shows limited similarity to the first sequence and has not been examined further.

Type	Sequence	Number of isolates	
		Round 4	Round 5
fdSrc-1	CLSGLRLGLVPC	2	—
fdSrc-2	CLMGLRLGLLPC	4	12
fdSrc-3*	CAYGFKLGLIKC	1	—

*This phage clone has an Ala to Arg substitution directly NH_2-terminal to the insert region (*26*).

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cause c-Src activity is essential for osteoclast-mediated bone resorption, interference with Src function may be of value in the treatment of osteoporosis (*13*). SH3 domains interact with sequence elements in their cellular targets that form type II polyproline helices of 8 to 10 residues (*14–17*). Although ligands for a variety of SH3 domains have been isolated from phage display libraries (*15*), the identification of such sequences from a synthetic L-amino acid peptide library was possible only with prior knowledge of the sequences of the preferred ligands (*14*). Thus, the identification of D-peptide ligands for SH3 domains from synthetic peptide libraries is unlikely to be successful in the absence of prior sequence or structure information about potential ligands.

The L- and D-enantiomers of the chicken c-Src domain were prepared by bacterial expression and chemical synthesis, respectively. The synthetic, 60–amino acid D-SH3 domain (*18*) was purified by affinity chromatography with a D-amino acid version of a known peptide ligand for the SH3 domain (*14*). As expected, bacterially expressed L-SH3 (*19*) was retained on an affinity column with the L-enantiomer of this peptide but not with the D-enantiomer, which indicates that the interaction of the SH3 domain with its substrates is stereospecific (*20*).

A phage library was constructed in which random, 10-residue peptide sequences were expressed at the NH_2-terminus of the pIII protein of the bacteriophage fd (*1*). Because many natural bioactive peptides, such as the immunosuppressant cyclosporin and the tumor promotor microcystin, are cyclic, the library was designed to include a large number of sequences that have a propensity for disulfide bond formation (*21*, *22*). When the L-SH3 domain was used to screen this phage display library for interacting peptide sequences, we isolated the disulfide-free polyproline-type sequences that have been identified by others (*14*, *15*, *23*).

When the same phage display library was screened with the D-SH3 domain (*24*), we isolated a series of peptide sequences that showed no obvious sequence similarity to the L-SH3–binding sequences (Table 1). These phage-displayed peptides that bind to the D-SH3 domain are characterized by a combination of conserved Leu and Gly residues and a conserved Arg or Lys residue. In contrast to the L-peptide ligands for the L-SH3 domain (*14*, *15*, *23*), the positively charged residues in the ligands for the D-SH3 domain are located in the middle of a stretch of conserved residues, which suggests that the mode of ligand binding is different. Furthermore, all ligands for the D-SH3 domain contain a pair of Cys residues, a property that is not observed for the L-peptides that interact with the L-SH3 domain (*14*, *15*, *23*). The disulfide bond may increase the affinity of these peptides for the D-SH3 domain by reducing the number of possible conformers.

A D-peptide denoted Pep-D1 [(D)-RCLSGLRLGLVPCA] (*25*), which is the mirror image of one of the phage-displayed peptides that bind to the D-SH3 domain, was synthesized and its interaction with the bacterially expressed L-SH3 domain examined (*26*). Competition binding experiments indicated that the disulfide-bonded form of this peptide binds to the L-SH3 domain with a dissociation constant (K_d) of 63 μM. This affinity is comparable to that of most of the proposed physiological ligands for SH3 domains and about one-tenth that of the optimal L-peptide ligands that have been identified (*14*, *27*, *28*). The reduced form of Pep-D1 shows no detectable binding activity in this assay ($K_d \gg$ 800 μM), which indicates that the formation of the disulfide is required for efficient binding (*28*).

Heteronuclear magnetic resonance (NMR) experiments were performed on the ^{15}N-labeled SH3 domain in the absence and presence of Pep-D1 to determine the binding site of this D-peptide in the SH3 domain. Residues in the SH3 domain that interact with Pep-D1 were identified

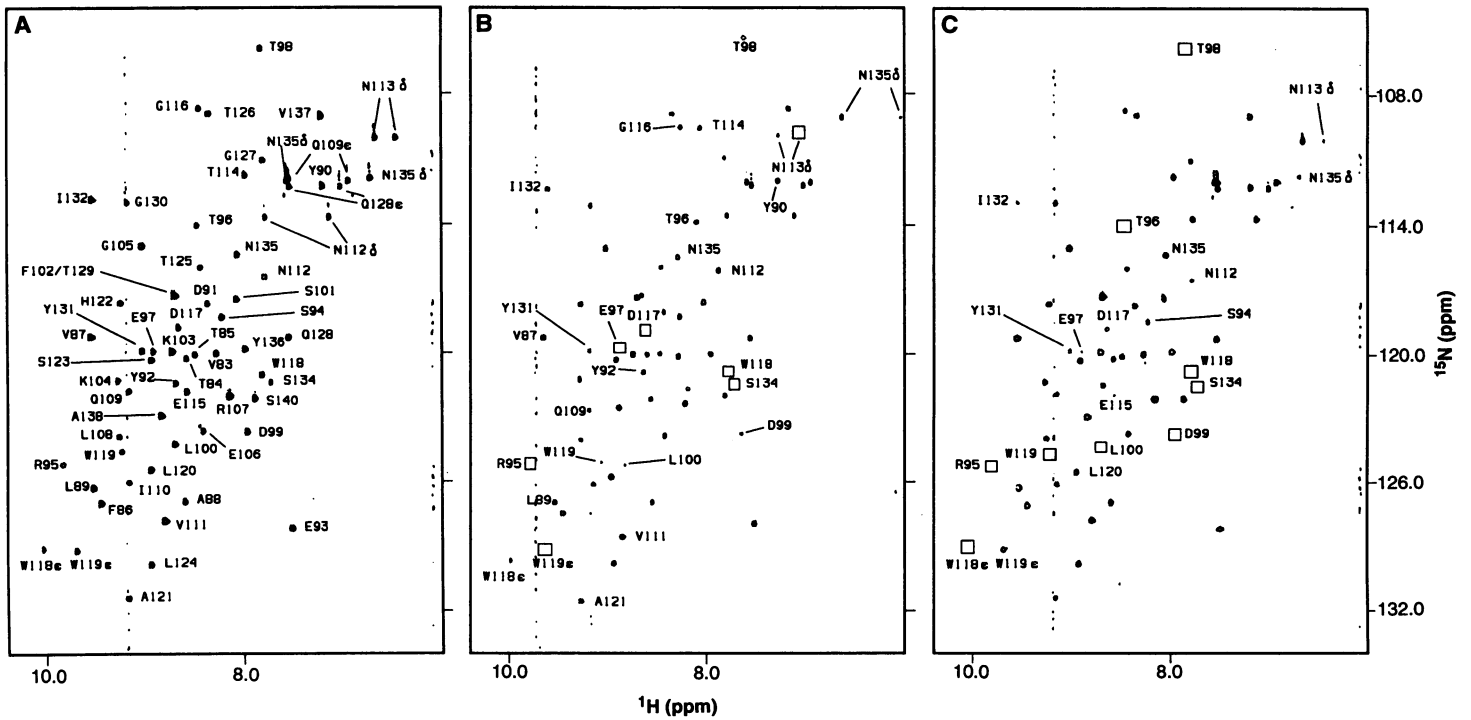

Fig. 2. Heteronuclear NMR spectra of the liganded and unliganded L-SH3 domain (*25*). ^1H-^{15}N correlation spectra (*40*) are depicted (10 mM sodium phosphate, pH 6.0 at 25°C). (**A**) The isolated L-SH3 domain. (**B**) The L-SH3 domain in the presence of the peptide (L)-YGGRELPPLPRF-amide (*36*) at a peptide:protein ratio of 1.2:1. (**C**) The L-SH3 domain in the presence of the D-peptide ligand, Pep-D1, at a peptide:protein ratio of 1.5:1. Residues that change in intensity or chemical shift in the presence of peptide are boxed or numbered (*29*). Assignments for the unliganded SH3 domain agree with those published previously (*39*), except that Asn^{113} could not be assigned unambiguously. No changes were observed in spectra obtained with a 1:1 ratio of peptide to protein and the ratio depicted for either complex.

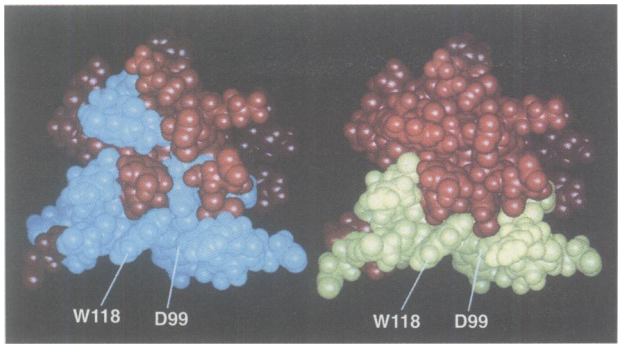

Fig. 3. Binding site of a D-peptide ligand for the L-SH3 domain. Space-filling diagrams of the SH3 domain structure are depicted (*16, 30*). The positions of the residues that do not undergo chemical shift perturbation are indicated in red. Residues that change in intensity or chemical shift (*29*) upon binding of the L-peptide ligand YGGRELPPLPRF-amide are indicated in blue (left). Residues that change in intensity (*29*) upon binding of the D-peptide ligand Pep-D1 are indicated in yellow (right). The structures were generated with the program Insight II (Biosym). Coordinates for the SH3 domain were from (*16*).

through changes in amide ^{1}H or ^{15}N chemical shifts upon the addition of the D-peptide ligand (*29*). The ligand-binding site of the SH3 domain for its natural, L-amino acid ligands consists of three pockets that together form a relatively shallow groove on one side of the molecule (*16, 30*). Pocket A, which is formed by the side chains of Asp^{99} and Trp^{118}, accommodates the conserved Arg residue, whereas pockets B and C form a hydrophobic surface that accommodates the aliphatic and Pro residues in SH3 ligands (*16, 30*).

The binding of Pep-D1 results in the perturbation of the chemical shifts of the residues that form pocket A, as well as a patch of adjacent residues (Fig. 2C). Most of these residues also undergo changes in their chemical shifts upon binding of the L-peptide (Fig. 2B). Pocket A is likely to interact with the conserved Arg or Lys residues in the D-peptides in a manner that is analogous to the recognition of Arg residues in L-amino acid ligands. The interaction of this site with both the L- and D-amino acid ligands explains the competition observed for the binding of these two ligands.

Pep-D1 appears to occupy only part of the binding site that is contacted by the polyproline-type ligands for the SH3 domain (Fig. 3). Residues that form part of pocket B and pocket C (Tyr^{90} and Tyr^{92}), or that are adjacent to this pocket (Val^{87} and Leu^{89}), are not perturbed upon binding of Pep-D1 (Figs. 2 and 3). Mutational analysis suggests that for L-amino acid ligands, interactions at these sites are required for high-affinity binding (*16*). D-Peptide inhibitors of higher affinity could therefore potentially be obtained by the design or selection of analogs of Pep-D1 or Pep-D2 (Table 1) that extend further along the groove into pocket C of the SH3 domain.

Although the syntheses of the D-enantiomeric form of both rubredoxin (45 amino acids) and human immunodeficiency virus protease (99 amino acids) have been described (*10, 11*), for most proteins the synthesis of the full D-enantiomeric form will not be feasible because of size limitations on the likelihood of successful chemical synthesis. However, both intracellular and extracellular proteins are often composed of autonomously folding domains of 100 amino acids or less (*31*). This size range is within reach of current solid-phase peptide synthesis technology, and recent advances in chemical ligation strategies for unprotected protein fragments hold promise for the synthesis of even larger protein domains (*32*). Ligands for multidomain proteins may thus be isolated through the screening of one or more of their constituent domains, as for the SH3 domain (*33*).

Finally, our approach is not restricted to genetically encoded peptide libraries (*1–4*). Because ribonucleotides and deoxyribonucleotides also contain chiral centers (which are recognized by nucleases), this approach applies equally to RNA libraries (*34*) and DNA libraries (*35*). Examination of the large amount of structural space represented in these libraries may yield new ligands of biological and medical importance.

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18. The D-amino acid SH3 domain (sequence GGVTTF-VALYDYESRTETDLSFKKGERLQIVNNTEGDWWL-AHSLTTGQTGYIPSNYVAPS, COOH-terminus, residues 81 to 140 of chicken c-Src) (*25*) was synthesized on HMP resin (ABI/Perkin-Elmer) with an ABI 431A peptide synthesizer and ABI fastmoc cycles Protected D-amino acids were obtained from Bachem California, Bachem Bioscience, Advanced Chemtech, and Novabiochem. For D-Ile and D-Thr, the side chain enantiomers were used in which the chirality of the side chain is also inverted relative to naturally occurring L-Thr and L-Ile. After completion of the synthesis, the NH_2-terminus of the protein was modified with NHS-LC-biotin II (Pierce). After cleavage, the protein was lyophilized, dissolved in 6 M guanidine-HCl, pH 6.0, and dialyzed against 100 mM $NaHPO_4$ and 100 mM NaCl, pH 6.0, with the use of dialysis tubing with a molecular size cutoff of 3500 daltons (Spectra/Por). After dialysis, the material was spun briefly to remove insoluble debris, and the supernatant was subsequently dialyzed against 5% acetic acid and lyophilized. The protein was dissolved at a concentration of 3.3 mg ml^{-1} in tris-buffered saline (50 mM tris, pH 7.5, and 150 mM NaCl) containing 1 mM biotin. The protein was purified by affinity chromatography with a D-amino acid peptide ligand (*36*) that was biotinylated and immobilized on a streptavidin-agarose column (Pierce). Chromatography fractions were analyzed by laser desorption mass spectrometry on a Voyager mass spectrometer (Perseptive Biosystems). Fractions containing material of the expected mass (expected, 7027 daltons; observed, 7027 to 7035 daltons) were pooled and dialyzed against water for 72 hours, lyophilized, and taken up in water at a concentration of 107 μg/ml.
19. The residue numbering system is that of the full-length chicken c-Src protein. Residues 81 to 140 of chicken c-Src were cloned into the Hind III–Bam HI sites of the plasmid pMMHb. In this plasmid, proteins are expressed as a fusion with a modified form of the TrpLE leader sequence in which the Met residues have been replaced with Leu and the Cys residues have been replaced with Ala (*37*), and a stretch of nine His residues has been inserted into the COOH-terminal region of the leader sequence. Expression of the fusion protein encoded by the plasmid pMMHb-Src-SH3 was induced at an absorbance of 0.6 at 600 nm by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Research Organics) to *Escherichia coli* BL21 (DE3) pLys S cells. After induction for 2 hours, cells were centrifuged and inclusion bodies were isolated. Recombinant protein was purified by resuspension of inclusion bodies in 6 M guanidine-HCl and 0.2 M tris, pH 8.7 (buffer A), and chromatography on a Ni^{2+} column (Ni^{2+}-NTA-agarose; Qiagen). After elution, dialysis against water, and lyophilization, the fusion protein was dissolved in 70% formic acid and cleaved with CNBr (*37*). Dialyzed and lyophilized material was subsequently taken up in buffer A and purified by chromatography on a Ni^{2+} column (after cleavage, the isolated SH3 domain flows through the column, whereas uncleaved fusion protein and the cleaved TrpLE leader sequence are retained). After dialysis [against phosphate-buffered saline (PBS) buffers of decreasing ionic strength and finally against water] and lyophilization, the purity and identity of the SH3 domain were confirmed by high-performance liquid chromatography (HPLC) analysis at neutral pH and by laser de-

sorption mass spectrometry (expected, 6686 daltons; observed, 6683 daltons).
20. T. N. M. Schumacher and P. S. Kim, unpublished results.
21. DNA encoding a 10-residue random insert with flanking Ser or Cys residues (S/C-X_{10}-S/C) was prepared by polymerase chain reaction (PCR) amplification of an 85-residue oligonucleotide 5′-C.TAT.TCT.CAC.TCG.GCC.GAC.GGG.GCT.TSC.$(NNS)_{10}$.TSC.GCC.GCT.GGG.GCC.GAA.ACT.GTT.GAA-3′, where S = C/G and N = A/T/C/G, with biotinylated primers as described (*22*). After purification of the PCR product and digestion with Bgl I, the end pieces were removed with streptavidin-coated agarose beads (Pierce). The library was made by ligation of the random PCR product into Sfi I–cut Fuse 5 vector. The ligation product was transferred into electrocompetent MC1061 cells with a Bio-Rad *E. coli* pulser, yielding an initial library of 3.6×10^8 transformants. The transformation mixture was subsequently diluted to a volume of 400 ml of LB and 20 μg ml^{-1} of tetracycline and grown for an additional 14 hours. A phage stock was prepared by two successive polyethylene glycol precipitations of the culture supernatant. The randomness of the inserts was confirmed by sequence analysis of individual clones. We subsequently used 4×10^{10} transforming units to infect K91-kan cells to generate an amplified library. The quality of the library was confirmed by selection of phages that expressed inserts that interact with the lectin concanavalin A [K. R. Oldenburg, D. Loganathan, I. J. Goldstein, P. G. Schultz, M. A. Gallop, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5393 (1992); J. K. Scott, D. Loganathan, B. Easley, X. Gong, I. J. Goldstein, *ibid.*, p. 5398].
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23. Sequence analysis of a small number of isolates after four rounds of selection with the L-SH3 domain revealed the following two peptide sequences: CLARSRLPAIPS (nine isolates) and SRMSPLVPLRNS (one isolate). The sequences of these peptides have features consistent with those described for class I and class II ligands of the c-Src SH3 domain (*14, 15*).
24. Single wells of a flat-bottom 96-well high-binding styrene plate (Costar) were coated overnight with 10 μg of streptavidin (Pierce) in 100 μl of 100 mM $NaHCO_3$ at 4°C. The wells were washed with water and incubated with 100 μl (10.7 μg) of biotinylated D-SH3 for 1 hour at 20°C, blocked for 2 hours with dialyzed bovine serum albumin (BSA) (30 mg/ml) in 100 mM $NaHCO_3$, and again incubated with 100 μl (10.7 μg) of biotinylated D-SH3 for 1 hour. Unliganded streptavidin was blocked by the addition of 8 μl of 5 mM biotin in tris-buffered saline (TBS) for 30 min. The wells were subsequently washed five times with PBS and 0.1% Tween-20 and incubated with 50 μl of the phage stock (*21*) and 50 μl of TBS, 0.1% Tween-20, BSA (1 mg/ml), and 0.05% NaN_3. Wells were then washed by six additions of 200 μl of TBS, 0.1% Tween-20, and 1 mg ml^{-1} of BSA, with increasing incubation times in the later rounds of the selection procedure (Table 1). Bound phage particles were eluted by the addition of 100 μl of D-SH3 peptide ligand [sequence (D)-YGGRELPPLPRF-amide (*36*)] for 15 min at 4°C, at a final concentration of 700 to 1000 μM peptide. The eluate was used to infect K91-kan cells. Acid elution of phages in the screen gives no detectable preferential binding to D-SH3–coated wells after four rounds of selection.
25. Single-letter abbreviations for the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. Pep-D1 corresponds to the fdSrc-1 insert CLSGLRLGLVPC (Table 1) (*25*), with the COOH-terminal Ala that is present in all flanking sequences. The Arg immediately preceding the first Cys residue was observed in the fdSrc-3 sequence (Table 1). The presence of Arg and Lys residues close to the NH_2-terminus of secretory and transmembrane proteins negatively affects protein translocation [D. Boyd and J. Beckwith, *Cell* **62**, 1031 (1990)]. In addition, a selection against Arg residues in the NH_2-terminal part of phage pIII fusions has been observed [B. C. Cunningham, D. G. Lowe, B. Li, B. D. Bennett, J. A. Wells, *EMBO J.* **13**, 2508 (1994)]. The Ala to Arg mutation in this clone may thus increase the affinity of the insert sequence for the D-SH3 domain and could improve the solubility of the peptide; it was therefore included in the synthetic peptide. For affinity measurements, an NH_2-terminal D-Tyr was added to the peptide for concentration determination (*38*). The peptides with and without the NH_2-terminal Tyr were air-oxidized in 100 mM tris, pH 8.5, for 48 hours at a concentration of 1 mg/ml. Oxidized peptide was purified by reverse-phase HPLC with a C_{18} column and a water-acetonitrile gradient in 0.1% trifluoroacetic acid. The identity of the products was confirmed by laser desorption mass spectrometry.
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28. The affinity of Pep-D1 for the L-SH3 domain was determined by a competitive enzyme-linked immunosorbent assay (ELISA). Single wells of a 96-well plate were coated with 5 μg of the L- SH3 domain (*1, 22*). Wells were blocked with BSA, and phages expressing the L-SH3–binding insert CLARSRLPAIPS (*23*) were allowed to bind in 10 mM $NaHPO_4$, pH 7.2, 15 mM NaCl, 1 mg/ml of BSA, 0.05% NaN_3, and 0.1% Tween-20, in the presence of increasing amounts of competitor peptide. Phage binding was quantified with a rabbit M13 antibody (Stratagene) and alkaline phosphatase–labeled goat antibody to rabbit immunoglobulins (Pierce), with a fresh solution of *p*-nitrophenol phosphate as substrate. Absorbance at 410 nm was determined with a Dynatech microtiter plate reader. Titration curves (means of triplicates) were obtained for the L-peptide ligand YGGRELPPLPRF-amide (*36*) and the D-peptide ligand Pep-D1 [sequence (D)-YRCLSGLRLGLVPCA] in the presence and absence of 25 mM dithiothreitol. Relative values for K_d were obtained as described [D. L. Minor Jr. and P. S. Kim, *Nature* **367**, 660 (1994)]. The K_d of the L-peptide YGGRELPPLPRF-amide was determined to be 6.0 μM by direct tryptophan fluorescence spectroscopy. A solution of the peptide was titrated into 1 μM SH3 solution in 15 mM NaCl and 10 mM $NaHPO_4$, pH 7.2. Tryptophan fluorescence was induced by excitation at 295 nm (5 nm slit width), and emission was measured at 339 nm (10 nm slit width), with a Hitachi F-4500 fluorescence spectrometer. The dissociation constant was determined by Scatchard analysis.
29. Uniformly (≥95%) ^{15}N-labeled SH3 domain was obtained by growing *E. coli* harboring the plasmid pMMHb-Src-SH3 (*19*) in M9 medium supplemented with $(^{15}NH_4)_2SO_4$ (99.7% ^{15}N; Isotec, Miamisburg, OH). Upon reaching an absorbance of 0.6 at 600 nm, cells were induced for 4 hours with 0.4 mM IPTG. The protein was purified as described for the unlabeled material (*19*). Spectra were collected on a Bruker AMX 500 MHz NMR spectrometer. Resonance assignments were made by standard methods [K. Wüthrich, *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986); L. P. McIntosh, A. J. Wand, D. F. Lowry, A. G. Redfield, F. W. Dahlquist, *Biochemistry* **29**, 6341 (1990)] and were consistent with the assignments for c-Src SH3 (*39*). The peptide Pep-D1 was added to a solution containing the ^{15}N-labeled SH3 domain to a ratio of 1.5:1 (peptide:protein) in 10 mM phosphate, pH 6.0, at 298 K; heteronuclear single quantum coherence (HSQC) spectra (*40*) of the uncomplexed and complexed form were compared. There were no resonances with chemical shift differences >0.04 ppm in the ^1H dimension or >0.17 ppm in the ^{15}N dimension. However, a number of resonances were reduced in intensity or completely absent in HSQC spectra of the complex. Residues that had the intensity of their HSQC resonances reduced significantly upon Pep-D1 binding, as compared to the ligand-free spectra, were identified as follows: for individual peaks, the ratio of peak intensities in the absence and presence of peptide was determined and converted to a log scale. The resulting distribution around the median is markedly skewed toward the left. A window that included >90% of the residues with ratios that were higher than the median was applied to residues with chemical shifts below the median. Only residues with a ratio lower than the median and that were not contained within this window were considered to have undergone significant perturbation (according to these criteria, only residues with a ratio that was reduced to less than 0.65 of that of the median were considered to have undergone significant perturbation). These residues include residues 94, 97, 112, 115, 117, 119, 120, 131, 132, and 135, the indole resonance of Trp^{119}, and the side chain amides of Asn^{113} and Asn^{135}. The resonances of residues 95, 96, 98, 99, 100, 118, and 134 and the indole resonance of Trp^{118} were absent in the presence of ligand. Control experiments, using the L-peptide YGGRELPPLPRF-amide (*36*) resulted in 17 resonances that were shifted by ≥0.1 ppm in the ^1H dimension or ≥0.5 ppm in the ^{15}N dimension (residues 87, 89, 90, 92, 96, 98, 99, 100, 109, 111, 114, 116, 119, 121, 131, 132, and 135, the indole resonance of Trp^{119}, and the side chain amides of Asn^{113} and Asn^{135}). Five resonances (95, 97, 117, 118, and 134) were absent in HSQC spectra of the complex. To validate the approach chosen to identify residues that interact with Pep-D1, we applied it to the spectra obtained with the peptide L-YGGRELPPLPRF-amide (*36*). With this approach, no new residues were identified that interacted with this peptide. The effect of peptide binding on the chemical shift of Pro^{133}, which forms part of pocket B, cannot be observed in this type of experiment. Attenuation of chemical shifts was interpreted to indicate sites of peptide-protein interactions. It is formally possible that some of these changes result from an indirect effect of peptide binding. However, the general pattern of the perturbations observed here is consistent with the changes observed by Schreiber and colleagues [S. Feng, C. Kasahara, R. J. Rickles, S. L. Schreiber, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12408 (1995)].
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33. The sequences of the resulting peptide ligands may also be used to guide the design of biased synthetic D-peptide and peptide-based libraries. Because of the structural relatedness of SH3 domains and of their L-amino acid ligands, biased libraries based on the sequence or structure of D-peptide ligands for the SH3 domain may contain ligands for a variety of SH3 domains. Thus, D-peptide ligands for other SH3 domains may be obtained through the direct screening of appropriately biased synthetic D-peptide libraries with other L-SH3 domains.
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