Design of N-substituted Peptomer Ligands for EVH1 Domains*

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Ena/VASP proteins are implicated in cytoskeletal reorganization during actin-dependent motility processes. Recruitment to subcellular sites of actin polymerization is mediated by the highly conserved N-terminal EVH1 domain, which interacts with target proteins containing proline-rich motifs. The VASP EVH1 domain specifically binds peptides with the consensus motif FPPPXP present in all its binding partners, including the Listerial ActA protein. Previous studies have shown that the Phe and first and final Pro residues are highly conserved and cannot be substituted with any other natural amino acid without significant loss of binding affinity. We have incorporated peptoid building blocks (sarcosine derived, non-natural amino acids) into the peptide SEFPPPPPTDEDEL from the Listerial ActA protein and were able to substitute the most highly conserved residues of this motif while maintaining binding to the VASP EVH1 domain with affinities in the range of 45–180 μM. We then used NMR chemical shift perturbations to locate specific domain residues involved in particular interactions. These studies may open up the way for designing selective modulators of VASP function for biological studies and for the development of novel therapeutics for diseases involving pathologically altered cell adhesion or cell motility.

Low affinity, protein-protein interactions mediated by proline-rich motifs (PRMs),¹ and PRM-binding domains are becoming increasingly recognized as important players in signal transduction pathways (1, 2). Well known examples include the interactions of Src homology 3 (SH3)-, WW-, and Ena-VASP homology 1 (EVH1) domains with targets containing PxxP, PxxY/PxxL, and FPCP motifs, respectively, which typically bind with affinities in the range of 5–100 μM. Finely tuned interactions of this type are vital for the accurate regulation of important signal transduction processes involved in proliferation, migration, or differentiation of cells in the adult organism or during its development. The high degree of conservation found in the target PRMs can be rationalized on the basis of high resolution structural data now available for many of these complexes. Proline is the only natural N-substituted amino acid, and its unusual backbone structure gives rise to a unique mechanism for highly specific recognition (3). The low affinities often involved make protein-protein interaction modules difficult targets for drug design. However, the importance of these interactions has already triggered several attempts to overcome the principal problems involved in designing ligands for protein surfaces (4).

Ena/VASP proteins are important components of signaling cascades that modulate actin cytoskeletal dynamics in response to extracellular stimuli (5). They are involved in various cellular and subcellular actin-based motility processes that are dependent on highly organized, transient, microfilament networks that lead to the formation of plasma membrane protrusions such as lamellipodia or filopodia and have also been found to be crucial for supporting intracellular motility of the bacterial pathogen Listeria monocytogenes (6). Mechanistically, Ena/VASP proteins are thought to promote local actin filament growth either by recruitment of polymerization-competent proline-rich pinactin to Ena/VASP-binding proteins or by steric exclusion of polymerization-inhibiting actin-binding proteins from growing actin filament ends (for review see Refs. 7 and 8). Ena/VASP proteins are known to be involved in a diverse range of activities, including formation of epithelial sheets, spreading and aggregation of thrombocytes, migration of fibroblasts or neurons, and polarization of lymphocytes or macrophages, as well as being essential for the virulence and cell to cell spreading of Listeria. Recruitment of Ena/VASP proteins to subcellular sites of actin polymerization is mediated by their conserved, N-terminally located EVH1 domain via interactions with target proteins containing PRMs (9).

The EVH1 domain of Ena/VASP proteins (residues 1–115) interacts directly with several target peptide sequences containing the FPCP consensus core motif (where Φ is a hydrophobic residue). Binding partners to date include the focal adhesion proteins, vinculin (10, 11) and zyxin (12, 13), the axon guidance proteins, Roundabout (Robo) (14) and Semaphorin 6A-1 (15), the Fyn-binding/SLP-76-associated protein (Fyb/SLAP) (16), the lipoma preferred partner protein (17), and SAX-3 (18) proteins, as well as the Listeria surface protein, ActA (19). Because the three-dimensional structure of the EVH1 domains of several Ena/VASP proteins are now known (20–22), this opens the way for the rational design of modulators which would act by binding specifically to the EVH1 domain. Small-molecule modulators of Ena/VASP proteins, delivered to cells in a dose-dependent manner, could then be used to obtain valuable information complementary to genetic knockout data (4, 23) by providing means to perturb protein interactions in a specific, rapid, and tunable manner (for a recent
review see Ref. 24). Such inhibitors would be useful in a number of different ways: (i) to selectively inhibit the interaction of EVH1 domains with their natural ligands, allowing controlled and detailed studies of EVH1-mediated signaling events; (ii) in appropriately modified forms, as molecular tags to monitor the localization, distribution, and rates of formation/dissociation of EVH1-mediated interactions within the cell; and (iii) as potential precursors/lead molecules in the development of future generations of novel therapeutics for treatment of diseases where partial inhibition of EVH1-mediated events would be desirable (for example, in modulating pathologically altered cell adhesion or cell motility in inflammatory and metastatic diseased states and in combating virulence of intracellular pathogens).

Here we make use of the available high resolution structural data on complexes of several EVH1 domains with peptides containing FPxP motifs to guide the design of novel EVH1 inhibitors. Specifically, we decided to take advantage of ActA peptides to derive VASP EVH1 inhibitors. The Listerial surface protein, ActA, was found to be singularly necessary for VASP recruitment even in the presence of native VASP binding partners (13, 19, 25–27) by efficiently and competitively binding to the EVH1 domain of VASP via FPPP motifs in the ActA sequence thus enabling the bacterial pathogen to successfully hijack the host cell actin polymerization machinery to support its own motility (19, 22, 28). Although ActA contains four tandem repeats of short sequences containing the FPxP motif, each capable individually of binding to one EVH1 domain (27), it was found that the third of these repeats, corresponding to residues 332–344 of the ActA sequence (numbered in this work as SFEPPPPPPEDEL,13) bound the EVH1 domain most strongly (19). We therefore used derivatives of this peptide for our studies. Our previous work in vitro showed that the proline residues at positions Pro5 and Pro6 were absolutely necessary for EVH1 domain binding (22). When either of these prolines was replaced by any other natural amino acid, complete loss of VASP EVH1-binding affinity was observed in almost every case. The high resolution structures of the EVH1 domains from Mena, Evi, and VASP proteins, in complex with FFXPP-containing peptides (20–22), showed that this conservation was due to the highly efficient packing of the N-substituted Pro5 and Pro6 pyrrolidine rings into hydrophobic grooves between a small cluster of closely spaced, aromatic side chains on the EVH1 domain surface (22, 29). Our first aim was therefore to study the effects of replacing these prolines with alternative N-substituted amino acid peptoid building blocks that would maintain the required N-substituted backbone structure. Previous workers used alternative N-substituted amino acids in place of prolines to create a number of peptomers for both SH3 and WW domains with affinities comparable to the natural peptides (3, 4, 23). We applied a similar approach to the study of EVH1-domain:peptide interactions to help us to understand more about the specific roles of the conserved prolines in EVH1-recognition and to what extent variations in this N-substituted side chain can be tolerated at these sites. We also identified other conserved residues in positions flanking the FPxP core motif using the SPOT method (30). The effects of non-natural amino acid substitution at these positions was then also investigated.

MATERIALS AND METHODS

Preparation of the VASP EVH1 Domain; VASP-(1–115)—The DNA sequence of VASP-(1–115) was cloned into the plasmid pGEX-4T-1 (Amersham Biosciences). Protein for SPOT scan was expressed in 2xYT medium (16 g of bactotryptone (Difco), 10 g of bacto yeast extract (Difco), 5 g of NaCl in 1 liter, 2% glucose, 10 μg/ml ampicillin) and purified as described previously (22). Protein molecular masses were verified by mass spectrometry. Uniformly 15N-labeled VASP (1–115) for NMR titrations, was expressed in an MOPS minimal medium (31) containing 15NH4Cl as the sole nitrogen source. All samples for NMR spectroscopy were prepared at pH 6.0, in a buffer containing 20 mM KH2PO4, 50 mM KCl, 0.2 mM Na3cit.

Preparation of Peptomers on a Planar Cellulose Support—Cellulose membranes (Whatman 50) were prepared as described previously (32). Cellulose-bound peptomers were semi-automatically prepared using an automated ASP222 robot. The peptide building blocks were synthesized according to the standard SPOT synthesis protocols (30) as described in detail previously (33–35). Peptoid building block 1, sarcosine, was used in the Fmoc-protected form and treated in the chemical synthesis like proline, not as a peptoid building block. Reactions were monitored by bромphenol blue staining of membranes after each Fmoc deprotection step.

Screening of the Peptomer Array—The membrane was soaked in methanol and washed twice with 50 ml of methanol, three times with 50 ml of TBS (prepared from a 10× solution comprising 80 g of NaCl, 2 g of KCl, and 61 g of Tris in 1 liter of water and adjusted to pH 8.0), and blocked with blocking reagent overnight (5 ml of blocking buffer from Roche Diagnostics, Mannheim, Germany, 5 ml of TBS, 250 μl of Tween 20 (10% in water), and 2.5 g of saccharose in 50 ml of MilliQ water). The membrane was then washed once with 50 ml of T-TBS (TBS plus 0.05% Tween 20 (added from a 10% Tween solution in water)) and incubated with a solution of GST-VASP EVH1 (50 μM in TBS) for 3 h. Following incubation the membrane was washed three times with 50 ml of BBS and incubated for a further 2 h with a monoclonal rabbit GST antibody (Sigma). After further washing with 50 ml of TBS, the membrane was then incubated for 0.5 h with the second anti-rabbit IgG peroxidase-labeled antibody (Sigma, Deisenhofen, Germany). Analysis and quantification of binding was carried out using a chemiluminescence substrate (SuperSignal West Pico, Pierce) and LumilImage™ (Roche Diagnostics). Images were processed using the program CorelDraw.

Larger Scale Peptomer Synthesis—For the larger scale preparation (several milligrams) of each peptomer, syntheses were carried out in parallel on an Abimed AMS 422 multiple peptide synthesizer. A microtiter plate with 96 wells was used. Each well was filled with 20 mg of TentaGel S RAM resin (from TentaGel, Germany). The resin was derivatized at degree of 0.25 mmol/g) suspended in a 3:7 mixture of dimethylacetamide:dichloromethane. To obtain sufficient yields for our studies, 8 wells were used for each of the desired peptomers, giving a maximum theoretical yield of 40 μmol for each peptomer. For chain elongation with natural peptide residues, Fmoc-protected amino acids with free carboxyl groups were used, and coupling was achieved with standard DIC/HOBT chemistry. Chain elongation with N-substituted peptoid residues was achieved by reacting the free amine-groups first with bromoacetic acid-dinitrophenylester and then with the desired primary amine. For coupling of the residue N-terminal to a peptoid residue, the amino acids to be added were pre-activated as anhydrides with O-(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Cleavage of the complete peptomers from the resin, as well as cleavage of side-chain protecting groups, was achieved by adding 500 μl of a solution of 1% (v/v) phenol, 2% (v/v) water, 5% (v/v) dichloromethane, and 3% (v/v) trisobutylsilane in trifluoroacetic acid to each well. The eight separate reaction vessels for each compound were pooled and diluted with a hundred times excess of cold diethylther to facilitate precipitation of the reaction product. The suspensions were centrifuged with a Heraeus Megafuge 1.0 R, using a BS4542A/4 rotor at 14,000 rpm for 5 min. The supernatants were discarded, and the pellets were resuspended in cold ether. This procedure was repeated three times.

All peptomers were analyzed by reversed phase-high performance liquid chromatography on a analytical Vydac C18 column using a linear gradient of 5–60% acetonitrile:water (0.05% (v/v) trifluoroacetic acid) for 20 min at 1.2 ml/min flow rate (detection at 214 nm) and MALDI-TOF mass spectrometry using a cyano-4-hydroxycinnamic acid matrix (LaserTec BenchTop II mass spectrometer, PE Biosystems, Weiterstadt, Germany) and purified by preparative high performance liquid chromatography on a preparative Vydac C18 column (Dionex).

Biacore Measurements—Biacore measurements were carried out on a Biacore system using CM5 chips. VASP-(1–115) was coupled to the chip using standard protocols provided by the manufacturer, at pH 3.0 in 10 mM glycine buffer. Putative ligands were measured in seven steps, at concentrations: 15.625 μM, 31.25 μM, 62.5 μM, 125.0 μM, 250.0 μM, 500.0 μM, 1.0 M, 3.0 M, 5.0 M, 7.0 M, 10.0 M, 12.0 M. All ligands were then also investigated.
**RESULTS**

We have shown previously that substitution of either of the proline residues at positions 5 or 8 in the ActA peptide $^{S}$SFE-$^{P}$PPPTEDEL$^{13}$ by any other natural amino acid results in loss of EVH1 binding (22). However, by replacing these prolines with non-natural N-substituted peptoid building blocks, we found it was possible to create peptomer ligands that maintained moderate EVH1-binding affinities ($90 - 400\ \mu M$) comparable to some of the natural ligands ($20 - 400\ \mu M$). We would expect, therefore, that replacement of Phe$^{4}$ with a peptoid building block should generally reduce packing efficiency and hence EVH1 binding affinity. With all substitutions except for peptoid building block 7 this was observed. The very faint spots for peptoid building blocks 1, 4, 5, 8, and 13. The lane marked “WT” contains a control containing peptides Trp23, Phe79; VASP numbering) of the VASP EVH1 domain. Conserved residues chosen for substitution are highlighted. $b$, results of the single substitutions of peptide residues 2, 4, 5, 8, and 13. $c$, results of simultaneous, double substitution of peptide residues Pro$^{5}$ and Pro$^{8}$. Numbers 1–24 at the border of the grid correspond to the numbers in Table I; letters D–M within the grid correspond to the peptomers shown in Table II.

**Fig. 1.** Substitution analyses of the peptide ligand from ActA (SFEFPFFFPPITEDEL) known to bind the VASP EVH1 domain. $a$, structure of the ActA ligand. Conserved residues chosen for substitution are highlighted. $b$, results of the single substitutions of peptide residues 2, 4, 5, 8, and 13. $c$, results of simultaneous, double substitution of peptide residues Pro$^{5}$ and Pro$^{8}$. Numbers 1–24 at the border of the grid correspond to the numbers in Table I; letters D–M within the grid correspond to the peptomers shown in Table II.

peptide to intercalate the exposed aromatic side chains (Tyr$^{16}$, Trp$^{23}$, Phe$^{79}$, VASP numbering) of the VASP EVH1 domain surface (20–22, 29). We would expect, therefore, that replacement of Phe$^{4}$ with a peptoid building block should generally reduce packing efficiency and hence EVH1 binding affinity. With all substitutions except for peptoid building block 7 this was observed. The very faint spots for peptoid building blocks 1, 4, 14, and 21 suggest that alternative N-substituted ring systems create Phe mimics that bind very weakly, but that the ortho- and para-Cl atoms of peptoid 7 raise the affinity dramatically. This demonstrates that not only steric factors, but also chemical properties such as polarity are critical in modifying the binding affinity.

Both of the conserved proline residues investigated, Pro$^{5}$ and Pro$^{8}$, could be readily substituted by several of the peptoid building blocks in Table I without loss of EVH1 binding ability. Not surprisingly, hydrophobic substituents are favored, as these two prolines are known to pack tightly into a hydrophobic groove on the EVH1 domain surface. Interestingly, the two proline residues do not show identical behavior, highlighting differences due to the asymmetry of the FPx$^{+}$P:EVH1 interface. Pro$^{5}$ appears to be slightly more tolerant to a wider range of our substituents than Pro$^{8}$, but in general both positions require ring-based side-chain groups similar to those of the

Brook DRX 600 spectrometer in standard configuration, with a triple resonance probe equipped with triple axis self-shielded gradient coils. Uniformly $^{15}$N-labeled VASP EVH1 in phosphate buffer (20 mM KH$_{2}$PO$_{4}$, 50 mM KCl, 0.2 mM Na$_{2}$CO$_{3}$, 10% D$_{2}$O; pH 6.0) was used at a concentration of 0.1 mM. Peptomers were dissolved in the same buffer (with the pH adjusted to 6.0 before titrations) and added stepwise to the protein samples. After each addition of ligand, a $^{15}$N-HSQC spectrum with eight scans was recorded. Measurements of $\delta_N$ were all calculated from CSPs of the EVH1 domain residue Ala$^{75}$, which showed large perturbations in response to all ligands titrated in this work. Data were processed using the XWIN-NMR program (version 2.6) of Bruker Analytik GmbH (Reinsestetten, Germany) and the AZARA program (version 2.1) of W. Boucher. Assignment was carried out on Silicon Graphics O2 workstations, using the interactive program ANSIG 3.3 (37, 38). AZARA and ANSIG are both available by anonymous file transfer protocol from ftp.bio.cam.ac.uk in the directory ftp/pub.

**Modeling of Complexes of VASP EVH1 with Peptomers—** Complexes of the VASP EVH1 domain with peptomer ligands were modeled in a 250-ps molecular dynamics simulation at 300 K using Amber 5.0 (39). Simulations were performed under periodic boundary conditions using explicit water molecules. The periodic boundary conditions box was equilibrated by using constant pressure dynamics. The use of the SHAKE option constraining all heavy atom-hydrogen bond lengths allowed a step width of 2 fs. The non-bonded cutoff was set to 12 Å, and the non-bonded pair list was updated every 10 fs. During the calculation only those residues that showed CSPs greater than 0.1 ppm were allowed to move. The starting structure for each complex was obtained by docking of the FPx$^{+}$P motif of the peptide onto the EVH1 domain surface with the same contacts as reported in the crystal structure of Mena EVH1 (20). The structures of the complexes after 250 ps were minimized until a root mean square deviation gradient of 0.005 was reached. The AMBER program package is available from www.amber.ucsf.edu/amber/amber.html.
Design of N-substituted Ligands for EVH1 Domains

TABLE I
Structures of the 24 primary amines used in the SPOT synthesis and the following solid-phase synthesis of peptomers

| Peptoid building blocks | Structure |
|-------------------------|-----------|
| 1                       | ![Structure 1](image1) |
| 2                       | ![Structure 2](image2) |
| 3                       | ![Structure 3](image3) |
| 4                       | ![Structure 4](image4) |
| 5                       | ![Structure 5](image5) |
| 6                       | ![Structure 6](image6) |
| 7                       | ![Structure 7](image7) |
| 8                       | ![Structure 8](image8) |
| 9                       | ![Structure 9](image9) |
| 10                      | ![Structure 10](image10) |
| 11                      | ![Structure 11](image11) |
| 12                      | ![Structure 12](image12) |
| 13                      | ![Structure 13](image13) |
| 14                      | ![Structure 14](image14) |
| 15                      | ![Structure 15](image15) |
| 16                      | ![Structure 16](image16) |
| 17                      | ![Structure 17](image17) |
| 18                      | ![Structure 18](image18) |
| 19                      | ![Structure 19](image19) |
| 20                      | ![Structure 20](image20) |
| 21                      | ![Structure 21](image21) |
| 22                      | ![Structure 22](image22) |
| 23                      | ![Structure 23](image23) |
| 24                      | ![Structure 24](image24) |

In the case of peptoid building block 9, the long aliphatic chain has the conformational freedom to fold back onto itself and fill the hydrophobic pocket normally occupied by the proline. Peptoid building block 21 was very well tolerated in place of Pro8 but not in place of Pro5. The three-carbon aliphatic chain of this peptoid building block provides sufficient flexibility to allow the ring to bend around into the position normally occupied by the Pro5 pyrrolidine side chain. When in the proximity of Phe4, which provides a strong hydrophobic “anchor,” this substitution does not substantially weaken the interaction with the VASP EVH1-domain.

Simultaneous P5 and P8 Double Substitution—The two proline residues Pro5 and Pro8, known to be crucial for EVH1 binding (22), were investigated in a double substitution analysis to measure the effects of changing these two side chains simultaneously. The results are shown in the two-dimensional 24 × 24 SPOT scan in Fig. 1c wherein the same general trends are observed as in the single substitution analysis of Fig. 1b. The scan shows again that substitution of Pro5 is better tolerated than substitution of Pro8. Given a “good” substitution for Pro8 most other side chains are allowed at Pro5 while maintaining EVH1 binding. The most strongly VASP EVH1-binding doubly substituted peptomers were those for which Pro8 was replaced by peptoid blocks 4, 7–10, 14, 19, 21, or 23, and Pro5 replaced by peptoid building blocks 7 or 21. From these data, ten of the most strongly EVH1-binding peptomers (circled in red in Fig. 1, b and c, and labeled D to M in Table II) were selected for quantitative measurements of EVH1-binding affinity.

Quantitative $K_D$ Values of EVH1-binding Peptomers from Biacore Measurements—Milligram quantities of the peptides A–C (Table II) and each of the ten circled peptomers, D–M (Fig. 1c) were synthesized using classic solid support peptide chemistry with appropriate modifications for the incorporation of peptoid building blocks (see “Materials and Methods”). Binding affinities to the VASP EVH1 domain were measured using the Biacore technique and yielded the dissociation curves as seen below in Fig. 3 and the $K_D$ values summarized in Table II. It should be noted that the spot intensities in Fig. 1 do not provide accurate quantitative information on binding affinities. The measured $K_D$ values varied over a relatively wide range from 49$\mu$M to 1.7 mM. All N-substituted peptomers showed weaker EVH1 binding than the native ActA peptide SFEFPPPPT-EDEL and its natural amino acid derivative SFEWPPPPT-EDEL. The Biacore measurements showed that peptomer D possessed the strongest EVH1-binding affinity ($K_D$ 120.4 $\mu$M), with peptomer E the second best, already binding more weakly by a factor of 2 ($K_D$ 250 $\mu$M). Peptomers F to K all bound with $K_D$ values less than 1000 $\mu$M, whereas L and M showed only very weak associations with the EVH1 domain.

$^{15}$N–$^1$H Chemical Shift Mapping of the Highest Affinity Ligands: $K_D$ Measurements by NMR and Identification of Interaction Sites—Based on the results of our Biacore measurements, the peptomers D and E were chosen for more detailed studies using NMR chemical shift titrations. Fig. 2 shows the overlaid two-dimensional $^{15}$N-HSQC spectra acquired at the beginning and end of a titration of the $^{15}$N-labeled EVH1 domain with peptomer D. The stepwise addition of the peptomer gave rise to continuous changes in the $^{15}$N and $^1$H chemical shifts of EVH1 domain residues involved in ligand binding, as exemplified for residues Ala75, Gly80, and Gly94. The total $^{15}$N–$^1$H chemical shift perturbations (CSPs) were combined and weighted according to $\Delta\delta_{\text{TOTAL}} = \Delta\delta^{(15)}\text{N} + 0.2\Delta\delta^{(1)}\text{H}$ to normalize the chemical shift scale of $^{15}$N relative to $^1$H (40). These were then plotted against ligand concentration for peptide A and peptomers D and E to calculate the binding constants.
shown in Fig. 3 (also listed in Table II). The $K_D$ values obtained from our NMR titrations correlated well with those obtained from Biacore measurements for each of the ligands studied.

The distribution of perturbed shifts in the EVH1 sequence is shown in Fig. 4 for each of the ligands studied. The overall pattern of EVH1 residues perturbed during binding to the peptomers D and E can be seen to be similar to the pattern observed for EVH1 binding to the *Listeria* ActA peptide (peptide A). Not surprisingly, the more weakly binding ligands cause generally weaker CSPs. Fig. 5 shows the differences in CSPs in response to the addition of different ligands. These differences are localized in specific regions of the protein that make contacts to those side chains in the ligand, which were substituted. The difference in CSP between SFEFPPPPTEDEL and Peptomer D and between Peptomer D and Peptomer N are shown in Fig. 5 (a and b, respectively). The most pronounced CSP cluster to residues in the $\beta$-sheet comprising strands $\beta$5-$\beta$6-$\beta$7, which is involved in native ligand binding (see "Discussion"). The CSPs observed for each ligand were mapped onto the three-dimensional EVH1 domain structure as shown in Fig. 6 (a–c). The binding sites for peptomers D and E are very similar to that of the ActA peptide (20–22).

In an attempt to calculate a structure for the complex of the VASP EVH1 domain with peptomer D, we prepared a 1:1 molar ratio complex of U-[^2H,15N]-labeled EVH1 domain with the unlabeled peptomer in 90%H$_2$O/10%D$_2$O and measured three-dimensional 15N-edited NOESY spectra. Any NOEs seen in the aliphatic or aromatic regions of the indirect proton dimension to the 15N-attached protons detected in this experiment should therefore be exclusively intermolecular between protein and ligand. However, no such intermolecular NOEs were detected in these experiments, probably due to the weakness of the NOE transfer from such a weakly binding ligand ($92.6\mu\text{M}$). Therefore we relied on CSP data to dock peptomer D onto the EVH1 domain.

**Molecular Modeling to Rationalize Peptomer Interactions**—
The overall similarity in the CSP patterns induced by peptomer D to those induced by the natural ActA peptide, for which the structure in complex with the EVH1 domain is already known (20, 21), allowed us to use the complex of the EVH1 domain with SFEFPPPPTEDEL as a starting point for modeling the peptomer complex (22). Where possible, identical interactions were maintained. The results of the molecular simulations are shown in Fig. 6.

The complex with the natural ligand SFEFPPPPTEDEL is
The model of the complex formed with peptomer D, in which Pro5 is replaced with peptomer block 21, is shown for comparison in Fig. 6b. In peptomer D, the N-substituted residue replacing Pro5, contains an aromatic side chain separated from the backbone by a three-carbon aliphatic linker. This provides a sufficient degree of flexibility to enable the linker methylene groups to occupy the Pro5 pocket without steric hindrance from the side chain of Phe4, which in our model is located in its usual hydrophobic groove in the EVH1 domain surface (20–22). This shows that Pro5 can be replaced by a peptoid building block with a large N-substituted side chain with the result that EVH1 binding is maintained. In contrast, replacing Pro5 with any natural amino acid, with a side chain at C9251 rather than N, would create steric clashes that prevent EVH1 binding.

The optimization of rationally designed ligands involves the close inspection of such model structures. In this case, our model showed the N-substituted side chain to be in close enough proximity to the Phe4 binding site so that, in the absence of Phe4, this new ring could occupy the Phe4 binding pocket. If this was the case, we reasoned that it should be possible to design an EVH1-binding peptomer in which the normally essential Phe4 side chain was replaced with a much smaller alternative side chain on condition that a neighboring N-substituted side chain could occupy the Phe4 binding pocket. To test this hypothesis, we therefore synthesized peptomer N (see Table II), a close analogue of peptomer D with the residue Phe4 now substituted for Ala. The absence of the large Phe4 side chain should now allow the N-substituted side chain unhindered access to the Phe4 binding pocket. The CSP distributions (Figs. 4c and 5b) from an 15N-HSQC chemical shift titration of the VASP EVH1 domain with peptomer N showed this model to be feasible. The overall perturbations are very similar (Fig. 4, compare a–d), indicating to a large degree, similar binding contacts of the new ligand to the domain, comparable to those observed with our previous ligands. The dissociation constant was determined as 180 M, only a factor of 2 larger than that for Peptomer D. Hence, by designing a ligand capable of occupying the known Phe4 binding pocket, the usually essential Phe4 residue could be substituted.

Fig. 6c shows the distribution of CSPs over the domain surface. Peptomer N occupies the same overall binding site as shown in Fig. 6a. The model of the complex formed with peptomer D, in which Pro5 is replaced with peptomer block 21, is shown for comparison in Fig. 6b. In peptomer D, the N-substituted residue replacing Pro5, contains an aromatic side chain separated from the backbone by a three-carbon aliphatic linker. This provides a sufficient degree of flexibility to enable the linker methylene groups to occupy the Pro5 pocket without steric hindrance from the side chain of Phe4, which in our model is located in its usual hydrophobic groove in the EVH1 domain surface (20–22). This shows that Pro5 can be replaced by a peptoid building block with a large N-substituted side chain with the result that EVH1 binding is maintained. In contrast, replacing Pro5 with any natural amino acid, with a side chain at C9251 rather than N, would create steric clashes that prevent EVH1 binding.

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Fig. 6c shows the distribution of CSPs over the domain surface. Peptomer N occupies the same overall binding site as
the other ligands studied. Fig. 5 (a and b) shows differences in CSP patterns between the natural ligand and peptomer D and between peptomers D and N, respectively. Fig. 5a shows the changes induced by substituting Pro5 of the natural ligand with peptoid building block 21. The most dramatic CSPs occur for the backbone amides of residues 71–74 of $\text{H9252}$. These are located close to Phe4 in all published complexes and in our models. The CSPs reflect the obvious change in the chemical environment of Phe4 and its EVH1 contacts upon replacement of this side chain and occupation of its binding site by the aromatic ring of peptomer D. The indole NH of Trp23 involved in a hydrogen bond to Pro6 of the ligand also contacts the ligand in all published structures (20–22) and, not surprisingly, shows a significant difference in its CSP with the different ligands. Likewise, the backbone amide NH of Tyr16, known to make a close hydrophobic contact to the Pro5 side chain of the natural ActA ligand, is similarly affected. CSPs in residues 80–90 of $\text{H9252}$ and 95–96, are also seen, all of which make close contacts to the natural ligand in the structures published to date (20–22).

The subtracted CSPs in Fig. 5b show the effects of the F4A substitution in the ligand. Again, the region from residues 70–74 shows the most drastic changes, because this is the region of the EVH1 domain that forms the closest contacts to Phe4. According to our model (Fig. 6c), binding to the substituted ligand causes a small re-orientation of the sheet $\beta3$-$\beta6$-$\beta7$. Residue 96, at the beginning of the $\alpha$-helix, also shows a considerable CSP, which is probably due to small movements of the helix in response to the reorientation of the adjacent $\beta$-sheet. As observed for peptomer D, Tyr16 and Trp23 are again weakly perturbed, indicating small changes in the interactions of the recognition triad (20–22).

The conformational changes within the EVH1 domain upon ligand binding were quantified by comparing the Ca positions of each residue in the unbound EVH1 domain with their positions in each of our models (Fig. 7). The Ca displacement was greatest for residues 71–75 of $\beta5$, residues 80–84 of $\beta6$, and residues 86 and 87 in the center of $\beta7$. The displacement is most pronounced in these regions for the native peptide and peptomer D. Peptomer N shows differences suggesting a more native-like conformation of the domain when bound. This may
be due to weaker contacts in the precise Phe4 binding region due to incomplete or sub-optimal occupation of the Phe4 binding pocket.

DISCUSSION

We have shown that new ligands for small protein domains such as the EVH1 domain can be designed by incorporating peptoid building blocks into otherwise natural peptides. An initial combinatorial approach using the SPOT technique facilitated the identification of lead compounds from which new peptomers could be synthesized that bound with comparable affinities to peptides derived from the natural interaction partners. This was previously shown not to be possible using natural amino acid substitutions (22). The most strongly binding peptomers were studied further, and from these we were able to design a new ligand (peptomer N) that allowed us to replace the completely conserved peptide residues Phe4 and Pro5 simultaneously and still maintain significant binding to the VASP EVH1.

Molecular simulations based on CSP data produced model complexes that showed that the aromatic ring of peptoid building block 21 was able to occupy, but not displace, the ring of Phe4. When Phe4 was substituted for alanine, the new side chain occupied the Phe4 binding pocket, albeit with lower packing efficiency. The strong CSPs and Co displacements observed particularly in β5 upon binding of peptomer D and the lesser effects seen upon binding of peptomer N, combined with a knowledge of the domains three-dimensional structure, allowed us to locate and compare important points of interaction.

This work underlines the importance of gaining an understanding of the detailed interactions of protein domains with their natural ligands from high resolution structural data and shows how this information can be used to explain the conservation of specific residues on domain surfaces, which are not involved in stabilization of the fold. Once we understand why certain properties are conserved, we can then modify the natural ligands to design new ligands in which specific positions are exchanged for non-natural building blocks without abolishing binding ability. One long term aim of this work would be to design ligands with desired binding affinities already in mind. Ligands with stronger binding affinities than the natural partners could be used as highly specific small-molecule modulators for reversibly tuning and conditionally perturbing chosen protein interactions on time scales not possible with other genetic techniques (24).

However, inferring the mechanisms of action of these modulators (e.g. their role as inhibitors, agonists, or antagonists) must clearly await further functional analysis of the ligands. This has to be addressed at biological levels beyond the detailed biochemical and biophysical analysis of isolated compounds as presented in this study. This could be important for the Ena/ VASP proteins to which dual modes of function in both facilitation and inhibition of actin-based processes have been repeatedly assigned, depending on whether sub-cellular or cellular functions have been studied in different cell types (8, 41). New ligands of this type may open the way for the selective modulation of EVH1 domain interactions with their respective binding partners in vitro and in vivo. These compounds are only in part peptidic and hence are less prone to proteolytic cleavage in vivo. As has been observed for mice, salvage pathways for the function of VASP exist in many cell types. Therefore it appears feasible to use VASP ligands like these in the treatment of diseased states that involve pathologically altered cell adhesion or cell motility while maintaining the natural body functions via salvage pathways.

Recently, PRM domains have been shown to be important in processes involved in viral budding, especially that of HIV-1 (42). Eventually this process of viral budding may become a new target in AIDS treatment amenable to designed inhibitors.

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![Fig. 7. The difference in Co position (in Å) between the unbound VASP EVH1 domain and the domain bound to each of the three ligands. The most interesting region from residue 60 to 95 is enlarged for viewing in detail. The inset shows Co displacements over the whole molecule. Note that the unperturbed residues in the figure show very similar background CSP levels thus providing a control.](http://www.jbc.org/Downloaded from http://www.jbc.org)
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