5 Materials and Methods

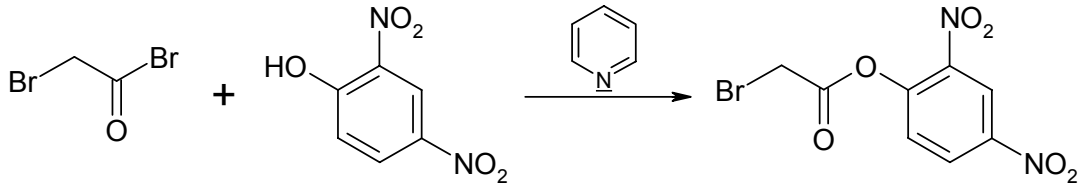
5.1 Synthesis of Peptomers

The peptomers for VASP EVH1 ligand studies were synthesized first on a membrane for screening of a larger number of compounds. Selected peptomers were then synthesized in bulk on a resin.

The peptoidic residues were in each case synthesized in a two-step process from Bromo-acetic acid-2,4-dinitrophenylester and the respective amine.

All chemicals were purchased from Aldrich, unless noted otherwise. Solvents were used as supplied.

5.1.1 Preparation of Bromo-acetic acid-2,4-dinitrophenylester (BADP)



2,4-Dinitrophenol (dry)	9.61 g	(M=184.1 g mol^{-1})	52 mmol
Bromo-aceticacid-bromide	5 ml	(M=201.9 g mol^{-1})	
		(ρ=2.32 g cm^3)	57 mmol
Pyridin	4.3 ml	(M=79.1 g mol^{-1})	
		(ρ=0.98 g cm^3)	53 mmol

2,4-Dinitriphenol(Lancaster) contained 15% water. It was dried *in vacuo* for 4h, the remaining mass was taken. The compound was dissolved in 80 ml dry Dichloromethane, Pyridine is added. The solution was cooled to 0° C on an ice bath. Bromoaceticacid-bromide was dissolved in 25 ml Dichloromethane and added via a dropping funnel during 15 min.

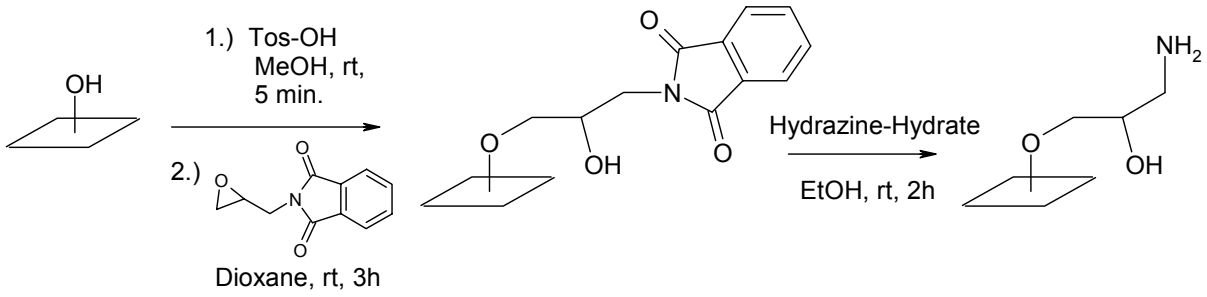
The solution was allowed to warm to room temperature and extracted once with 30 ml water and once with 30 ml 10% citric acid in water. The organic phase was dried over Sodiumsulfate, the solvent is removed *in vacuo*.

Crude Bromoaceticacid-2,4-dinitrophenylester was obtained as a yellow oil that crystallized slowly. It was recrystallized from Diethylether. A moderate yield of 10.39 g (60 %) was achieved.

5.1.2 Preparation of Peptomers on a membrane

5.1.2.1 Preparation of cellulose membrane

A Whatman cellulose membrane was used, 20 x 30 cm. The membrane was soaked in a solution of 2 g p-Toluylsulfonic acid in 50 ml methanol for 5 min. with gentle agitation (all derivatisation, cleavage and washing steps are performed in stainless steel dishes of appropriate size), the solution was removed and the membrane dried by washing with Diethylether and air drying.
N-2,3-epoxypropylphthalimid (7.8 g) was dissolved in 50 ml Dioxane and heated to 80° C. The membrane was soaked in this solution for 4h. It was washed three times with 20 ml Dioxane, two times with 20 ml Ethanol and dried as before.
6 ml Hydrazin-Hydrate were dissolved in 60 ml Ethanol, the membrane was soaked in this solution for 12 - 14h. It was washed twice with 20 ml Ethanol, twice with 20 ml DMF, again twice with 20 ml Ethanol and dried as before. The reactions are outlined in Scheme 1:



Scheme 1
Preparation of cellulose membrane for peptomer synthesis I

To test the derivatisation degree of the membrane, a coupling was performed with 0.6 M Fmoc-β-Ala-OPfp in DMF. 1 µl of the solution was spotted on a sample of the membrane and left to react for 15 min., this was repeated once. The membrane was washed thoroughly with 3 times 5 ml DMF, 2 times 5 ml ethanol and once 5 ml ether. The spot was cut out by use of a whole puncher, the Fmoc group was cleaved by addition of 1 ml 20 % piperidine in DMF and shaking for 20 min. The absorption was measured at 302 nm with 20 % piperidin in DMF as control. Derivatisation was calculated as

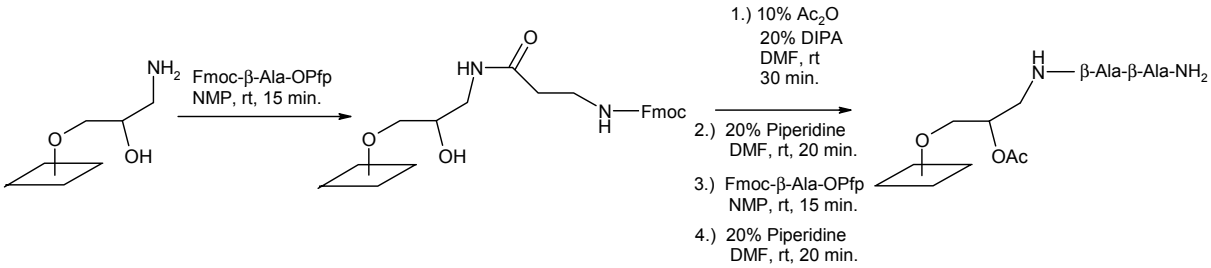
$$\text{Derivatisation} = \text{Absorption} * 1.000 / 8{,}1 \text{ [nM/Spot]}$$

Spot area was assumed to be 0.32 cm². Derivatisation should be in the range of 60 nM/spot.

5.1.2.2 Defining the spots

Spotting was performed using an Abimed ASP222 robot. A grid of the required size was defined by spotting 1 µl of a 0,3 M solution of Fmoc-β-Ala-OPfp onto each desired position, this was repeated once. The membrane was washed and then soaked in acetanhydride for two minutes without agitation. It was then soaked in 2% Ac_2O, 1% DIEA in DMF for 30 min. The membrane was washed as above.

Fmoc-groups were cleaved with 20 % piperidine solution for 15 min. then a second β-Ala residue was spotted. After cleavage with piperidine and washing, the membrane was stained with Bromophenolblue solution. Only spots should be stained due to free NH_2 groups on the amino acids, all free NH- or OH-groups on the remaining membrane should be acylated. These steps are shown in Scheme 2:



Scheme 2
Preparation of cellulose membrane for peptomer synthesis II

5.1.2.3 Spotting of Peptomers

Peptomers were spotted C- to N-terminal. The routine is depicted in Figure 48:

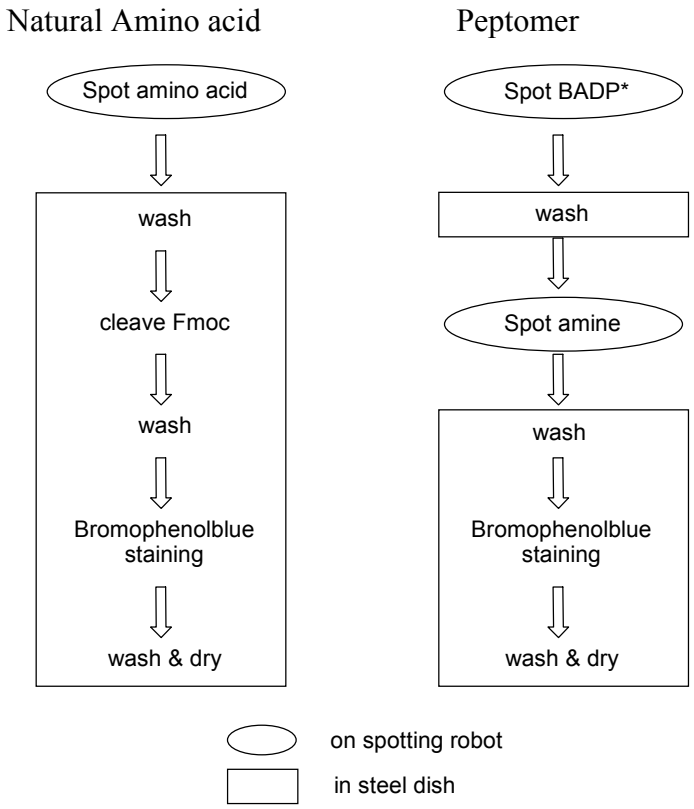


Figure 48
Steps in elongating the chain with a natural amino acid or a peptomer.
*** = Bromoacetic acid dinitrophenylester**

All spotting steps were performed twice, without washing between steps. For natural amino acids, the Fmoc-protected and OPfp-activated forms were used, shown for Alanin in Figure 49. All amino acid solutions were 0.6 M in NMP. Washing consisted of three times washing with 50 ml DMF, twice with 50 ml ethanol, twice with 50 ml Diethylether and air drying.

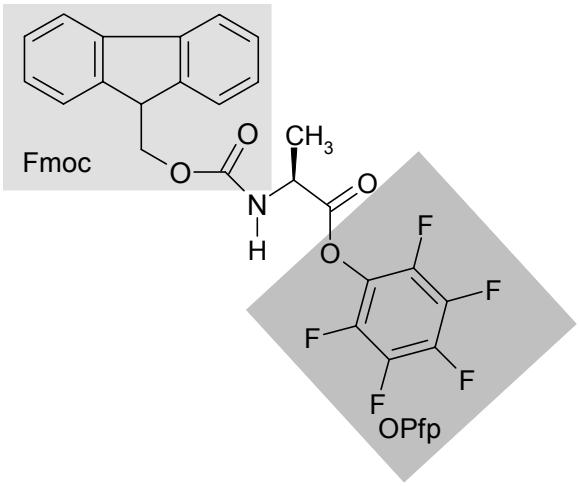


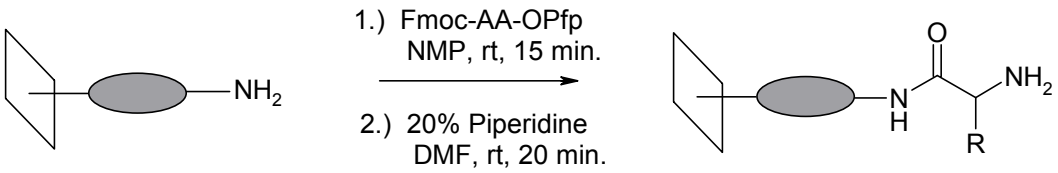
Figure 49
L-Alanin, amino group protected with 9-Fluorenylmethoxycarbonyl (Fmoc); carboxy function activated with Pentafluorphenyl (OPfp)

For the elongation of the peptomer chain, three cases were distinguished. Natural amino acids were coupled directly to the free NH_2-group of the previous natural amino acid. After washing, Fmoc groups were cleaved with 50 ml 20 % pirpridin in DMF, followed by washing and staining with Bromophenolblue solution. After a final washing and drying step one cycle of synthesis is complete.

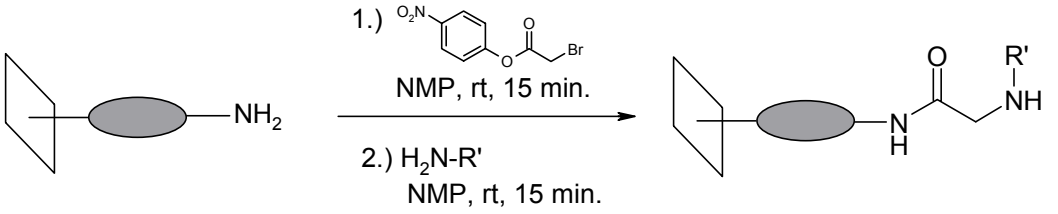
If a natural amino acid followed a peptoid builing block, a slightly different strategy was used. The peptoid building block ends with a secondary amine and will yield a tertiary amine, as opposed to starting with a primary amine from a natural amino acid. Hence the activation of the carboxy group on the amino acid needs to be stronger. For this purpose, amino acids were activated as homo-anhydrides. Twice the necessary amount of the respective amino acid was mixed with ½ equivalent of DCC, resulting in formation of the anhydride as shown in Scheme 3 below. The mixture was left to react at room temperature for 15 min. immediately prior to use. The ensuing washing and deprotecting steps remained the same.

For elongation with a peptoid building block, BADP was spotted first, as a 1 M solution in DMF. After washing, a primary amine was spotted as a 1 M solution in DMF, leading to formation of the peptoid residue.

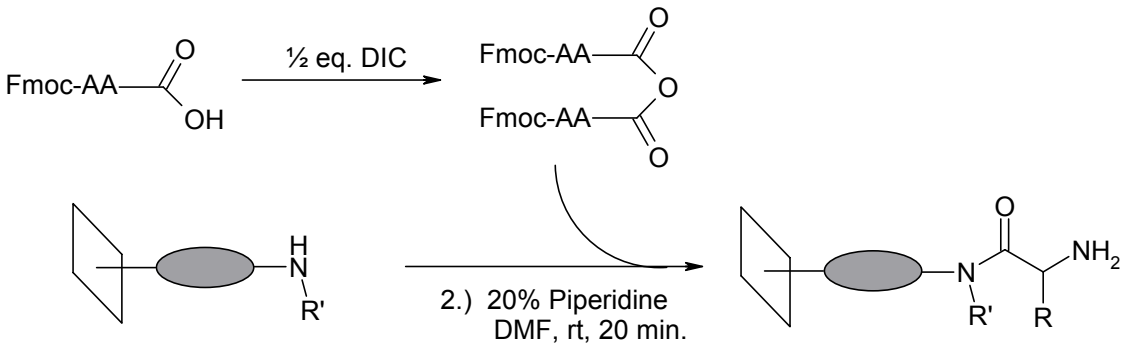
1. Chain elongation with natural amino acid:



2. Chain elongation with peptoid building block:



3. Chain elongation with natural amino acid after peptoid building block:



For two succesive amino acids method 1 is employed.
For two succesive peptoid building blocks method 2 is employed.

Scheme 3
Building of peptomers, three different approaches for natural amino acids, peptomers and elongation after peptoid building block

After completion of the last step in chain elongation, sidechain protecting groups from amino acids need to be cleaved. This is achieved by soaking the membrane in 200 ml of a solution of 90 % TFA, 3 % triisobutylsilane, 2 % water, 1 % phenol and 4 % DCM in a stainless steel dish that is put into a plastic bag and sealed tightly. The membrane is left in this solution for 30 min. without agitation. The membrane needs to be handled carefully during this phase, it becomes instable due to the cleavage solution.

The membrane is washed five times with 50 ml DCM, three times with 50 ml DMF, twice with 50 ml ethanol and twice with 50 ml Diethylether. It is then put into 200 ml 50 % TFA, 45 % DCM, 3 % Triisobutylsilane, 2 % water and 1 % phenol for 2 ½ hours without agitation and finally washed as before.

5.1.2.4 Incubation of the membrane with GST-EVH1

The membrane was washed once in 50 ml methanol for 10 min., then washed three times with 30 ml TBS (Tris buffered saline, prepared as 10 times stock solution from 80 g NaCl, 2 g KCl, 61 g Tris in 1 l water, pH = 8.0). The membrane was pretreated with blocking buffer over night, in order to prevent unspecific binding, e. g. to the cellulose. The buffer contained 3 ml blocking buffer (Roche Diagnostics, Mannheim, Germany), 5 ml TBS, 250 µl Tween 20 (10 % in water), 2.5 g saccharose, filled with water to a final volume of 50 ml.
After blocking the membrane was washed once with 30 ml TBS containing 0.05 % Tween 20. It was next incubated with the GST-fusion protein. The protein concentration was 50 µM, 30 µl are used per cm² of membrane.
Following incubation the membrane was washed three times with 50 ml TBS and incubated for a further 2 h with a monoclonal rabbit GST antibody (Sigma). After further washing with 50 ml TBS, the membrane was then incubated for ½ h with the second anti-rabbit IgG peroxidase-labelled antibody (Sigma, Deisenhofen, Germany). Analysis and quantification of binding was carried out using a chemiluminescence substrate (SuperSignal West Pico, Pierce, Illinois, USA) and images were detected on a LumiImager^{TM} (Roche Diagnostics). Images were processed using the program CorelPhotoPaint.

5.1.3 Peptomer synthesis in milligram amounts

For the larger scale preparation (several mg) of each peptomer, syntheses were carried out in parallel on an Gilson Abimed AMS 422 Multiple Peptide Synthesizer. A microtiter plate with 96 wells was used. The wells had a volume of ~ 1 ml, with a sieve at the bottom that retained the resin but allowed for removal of solutions by reducing the pressure under the plate. Each well was filled with 20 mg of TentaGel S RAM resin (Rapp Polymere, Germany), with a derivatization degree of 0.25 mmol/g. The resin was suspended in a 3:7 mixture of DMA:DCM and distributed into wells

using a biochemical pipette. To obtain sufficient yields for our studies, eight wells were used for each of the desired peptomers, giving a maximum theoretical yield of 40 μmol for each peptomer.
The resin is Fmoc-protected, hence the first step is deprotection with 20 % piperidin in DMA, yielding a free NH_2-group. 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 reaction of the free amine-groups first with bromoaceticacid-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 ½ eq. DIC as described in the literature [62]. Cleavage of the complete peptomers from the resin, as well as cleavage of sidechain protecting groups, was achieved by adding 500 μl of a solution of 1% (w/v) phenol, 2% (v/v) water, 5% (v/v) DCM and 3% (v/v) triisobutylsilane in TFA to each well. The eight separate reaction vessels for each compound were pooled and diluted with a hundred times excess (v/v) of cold diethylether, to achieve precipitation of the reaction product. The suspensions were centrifuged with a Heraeus Megafuge 1.0 R, using a BS4402/A rotor at 14.000 rpm for 5 min. The supernatants were discarded and the pellets resuspended in cold ether. This procedure was repeated three times.
All peptomers were analysed by RP-HPLC on an analytical Vydac C18 column using a linear gradient 5-60% acetonitril:water (0.05% (v/v) TFA) for 20 min at 1.2 ml/min flow rate (detection at 214 nm) and MALDI-TOF-MS using α-Cyano-4-hydroxy-cinnamic acid as matrix (LaserTec BenchTop II mass spectrometer, PE Biosystems, Weiterstadt, Germany) and purified by preparative HPLC on a preparative Vydac C18 column if necessary.

5.2 Expression of recombinant, isotope labelled proteins

Compared to other methods, especially optical spectroscopy, NMR spectroscopy requires big amounts of proteins in a rather high concentration. These concentration would be tedious or impossible to achieve by purification from natural sources. Therefore all proteins for NMR studies are expressed in one of several expression systems specially designed for the task of producing recombinant proteins. The most commonly used expression system is *Escherichia coli* (*E. coli*). This bacterium has been engineered to express large amounts of a desired protein, the DNA for this protein is introduced into the cells via specially constructed plasmids. The expression in *E. coli* is often facile and is most useful for NMR purposes because of the ease of isotope labelling, e. g. growing the cells on media containing ^{15}N, ^{13}C and/or ^{2}H (rather then the naturally most abundant isotopes ^{14}N, ^{12}C and ^{1}H). All proteins discussed in this thesis have been expressed in *E. coli*.

Other protein expression systems include yeast, insect cells, mamalian cells and most recently cell free (*in vitro*) expression systems. The advantage of yeast over *E. coli* is the possible inclusion of post-translational modifications (e.g. phosphorylation, glycosylation or attachement of fatty acids), which cannot be achieved in *E. coli*. Still, media for yeast are more complex and thus more expensive. The cost increases significantly with insect and mamalian cells. Labelling is extremely expensive in these systems and is not routinely applied to date. Also *E. coli* cultures are far less prone to infection with opportunistic bacteria etc. and thus the handling is easier.

5.2.1 Media and Chromatography

Salts and macronutrients were from Aldrich unless noted otherwise. Water was always tridest. grade, prepared on a Millipore Milli-Q synthesis system.

LB and M9 Media were prepeared basically according to Sambrook and Russel [63], secction A 2.x ff. Ampicillin was added to a final concentration of 100 µg/ml.

Minimal medium M9

M9 medium was prepared according to [63], and supplemented with trace elements (10 ml/l of a stock solution containing 2.5 g EDTA, 250 mg $FeSO_4$, 25 mg $ZnCl_2$, 5 mg $CuSO_4$ in 1l water at pH = 7.6). Glucose was added to a final concentration of 4 g/l as the sole carbon source. For ^{13}C-labelled samples uniformly ^{13}C-labelled glucose was used. NH_4Cl was added to a final concentration of 0.5 g/l. For ^{15}N-labelled samples uniformly ^{15}N-labelled NH_4Cl was used.
For a uniformly deuterated sample, the medium was prepared in 100% D_2O. This was sterilized by passing through Millipore screw top filters (0.2 µm pore width). Uniformly deuterated and ^{13}C-labelled glycerol (4g/l, Cambridge Isotopes) was used as the sole carbon source in order to prevent ^{1}H-scrambling from non-deuterated glucose.

SDS-PAGE was performed according to protocol A8.40 ff of [63].

Affinity chromatography

Purification of GST-fusion proteins was achieved using a 10 ml column of Glutathion Sepharose 4B (Amersham Biosciences) according to the manufacturers instructions.
GST buffer A contains 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 x 2 H_2O and 1.8 mM KH_2PO_4, the pH was adjusted to 7.3.
GST buffer B contains 308 mg of reduced Glutathion in 100 ml of 50 mM Tris-HCl (pH = 8.0), dissolved freshly before use of the buffer.
The buffer for gel filtration on a 120 ml column with Superdex 75 (Amersham Biosciences) was constituted of 50 mM Tris-HCl and 150 mM NaCl, pH = 8.0.

5.2.2 Expression of the human VASP EVH2 domain

Chemically competent cells were prepared for both the BL21 (DE3) and BL21 (DE3) star strains of *E. coli* (Invitrogen) according to protocol 1.24 and 1.25 in [63].
Cells were transformed using a vector supplied by Thomas Jarchau, Wuerzburg. The human VASP fragment encoding amino acids 336-380 was cloned into the BamHI and EcoRI sites of the pGEX-4T-1 vector (Pharmacia, Acc. No. U13853). The vector encodes for a GST-fusion of the VASP fragment with a Thrombin cleavage site N-terminal of EVH2. It also encodes for Ampicillin resistance.
Five single colonies of the two strains were picked and transferred into 2 ml LB medium with Ampicillin each for an expression test. The best clone (BL21 (DE3) star) was used for large scale expression.

A starting culture was grown in 3 ml LB medium at 37° C, 230 rpm for several hours. It was transferred to 200 ml ^{15}N-labelled M9 medium containing Ampicillin and incubated at 37° C, 180 rpm over night.
50 ml of this pre-culture were mixed with ~ 1000 ml fresh M9 medium with Ampicillin, to a final OD_{600} of ~ 0.12. The culture was grown in two flasks, containing 500 ml each. After 4 hours at 37° C, 180 rpm the OD_{600} had reached ~ 0.84. The cultures were induced with IPTG ad 1 mM. After growing at 37° C for another 2 hours the cultures were transferred to 25° C, 180 rpm and grown over night. The final $OD_{600} \approx 1.8$.
The cultures were combined and centrifuged at 6,000 rpm at 4° C for 10 min. The pellet was resuspended once in 50 ml 0.9% (w/v) NaCl solution and centrifuged as before.
The cells were resuspended in 20 ml GST-buffer A, half a tablet of Complete Protease inhibitor (Roche) was added, and the suspension applied to a French Press two times at 1,100 PSIG. The lysate was centrifuged at 23.000 rpm, 4° C for 30 min.
The supernatant of the centrifugation was applied to a 10 ml GST-column (Amersham Biosciences). The column was washed with 100 ml GST buffer A, then eluted with 50 ml GST buffer B. The combined eluate fractions containing the GST-fusion protein were concentrated using a centrifugal filter (YM-3, Millipore) to 8 ml at a total protein concentration of 5.7 mg/ml. 114 U Thrombin were added to the solution, the cleavage reaction was allowed to progress for 24 h at room temperature. Completeness of cleavage was monitored by PAGE. The solution was applied again to a GST column, collecting the cleaved EVH2 domain in the flowthrough. The flowthrough

was concentrated to 7 ml and subjected to a gel filtration column (Superdex 75, 120 ml, Amersham Biosciences). The relevant fractions were combined and concentrated via a YM-3 centrifuge filter. The final protein concentration was 0.9 mM in 1 ml.
A uniformly ^{15}N,^{13}C-labelled sample was prepared accordingly, using 2 g/l uniformly ^{13}C-labelled glucose as sole carbon source of the M9 medium.
Samples were concentrated and exchanged into NMR buffer (see below) using a YM-3 centrifugal filter (Millipore).

5.2.3 Preparation of uniformly ^{2}H,^{15}N-labelled EVH1

Competent cells of BL21 (DE3) star were transformed with the pGEX-4T-1 vector (Amersham Biosciences) carrying the DNA sequence for EVH1 (human VASP 1-115) as insert. The plsamid was supplied by Thomas Jarchau, Würzburg. The productivity of six transformants was checked by an expression test on LB. The best clone was used to optimize conditions on deuterated M9 medium.
As a result of testing different conditions (temperature, induction cell density), further expression of EVH1 in D_2O was performed at 37° C, with IPTG induction to a final concentration of 0.7 mM. Cultures were induced at an OD_{600} of 0.4, but differences at this temperature were small.
GST-EVH1 was grown in 2 l M9 medium, labelled with 0.5 g/l $^{15}NH_4Cl$ and 4 g/l glycerol-d_8 in D_2O. Cells were harvested at an OD_{600} = 1.3. The culture medium was centrifuged at 6,000 rpm, 4° C, for 10 min. The pellet was resuspended once in 30 ml 0.9 % (w/v) NaCl solution and centrifuged again. Raw cell mass was ~ 3.0 g. Cells were resuspended in 20 ml 0.9 % (w/v) NaCl solution, containing one tablet Complete&EDTA (Roche) and apllied to French Press two times. The lysate was centrifuged for 30 min. at 23,000 rpm, 4° C. The supernatant was applied to a GST column basically as described for EVH2 and concentrated for Thrombin cleavage to 2.5 mg/ml. The fusion protein was cleaved by addition of 2.5 U/mg Thrombin at room temperature for 14 hours. Cleavage was complete as monitored by PAGE. Figure 50 shows a summary of the purification process up to the Thrombin cleavage.

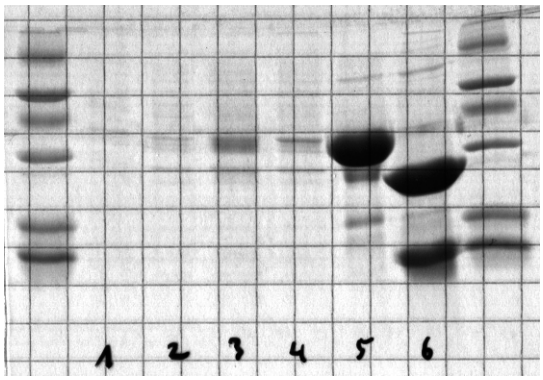


Figure 50 Purification of $^{2}H,^{15}N$-EVH1. Lanes contain: 1 before induction; 2 1h after induction; 3 cell harvest; 4 SN of centrifugation after frensh press; 5 eluate of first GST column; 6 after thrombin cleavage.

The protein was passed over a GST-column again, flowthrough containing EVH1 was concentrated and purified by gel filtration over Sephadex-75 (Pharmacia). The chromatogram of the gel chromatography shows some impurities, which can be separated well from EVH1 as confirmed by PAGE. The relevant fractions containing EVH1 are concentrated via a YM 5,000 centrifugal filter (Millipore) and exchanged into NMR buffer.

5.3 Further methods

5.3.1 Circular Dichroism Spectroscopy

Circular Dichroism (CD) Spectroscopy was performed on a J-720 spectropolarimeter (Jasco, Tokyo, Japan), using a quartz cuvette with 0.1 cm pathlength and a protein concentration of 12.3 μM in NMR buffer (see below). A measurement from 200 to 300 nm was performed to identify a strong signal, averaging 5 scans. Then a temperature scan in the range between 10° and 60° C was performed at 218 nm. Measurements from 200 to 300 nm where performed in the pH range from 1 to 10 at 21° C, averaging 3 scans. Spectra were analysed using the program Origin (Microcal, version 5.0).

5.3.2 NMR Spectroscopy of the human Spred2 EVH1 domain

For all spectra, samples were prepared in aqueous buffer containing 20 mM Phosphate, 50 mM NaCl and 0.1 % NaN_3 at pH 6.0, containing 10% D_2O for the ^2H lock. All spectra were recorded at 300.0 K. Spectra were recorded on a Bruker DRX 600 spectrometer equipped with a cryoprobe, except for the ^{13}C-NOESY-HMQC, which was recorded on Bruker DMX 750, equipped with triple axis self shielded gradient coils.

A CBCACONNH and CBCANNH pair of spectra was recorded for backbone assigment. The assignment of the backbone was further facilitated by several amino acid type selective experiments. These were a G(i+1), G(i,i+1), P(i-1), P(i-1,np), P(i+1,np), P(i+1), DNG(i+1) and DNG(i,i+1). For details of these spectra, refer to Lit. [56, 64-66]. Pulse programs for Bruker machines may be downloaded from the Oschkinat internet homepage. For assignment of carbonyl carbon resonances an HNCO spectrum was recorded, used in TALOS predictions of dihedral angle constraints. Phi angles were experimentally determined via $^3J_{HnH\alpha}$ experiments.

Intensities of the peaks were fitted to Equation 5:

$$I = A \cdot \cos(\pi \cdot J \cdot \tau) \cdot e^{-\frac{\tau}{T_2^*}} \qquad \textbf{Equation 5}$$

were A is the amplitude, J the scalar coupling constant, τ the delay and T_2* the apparent proton transverse relaxation time. Parameters were initialized as A = 40000, J = 6 and $1/T_2$* = -30. (Method by Billeter [59] and adapted by P. Schmieder (unpublished)) using the program Kaleidagraph. Values of $^3J_{HnH\alpha}$ equal to or greater than 7 Hz were assumed to correspond to a ϕ angle between -140° and -100°, according to a β-sheet. Lower values of $^3J_{HnH\alpha}$ were not included in the calculations because of the degeneracy of the Karplus function.

A ^{15}N-HSQC and ^{15}N-NOESY-HSQC (40 ms mixing time) were recorded for identification of NH-spinsystems, a TOCSY-HSQC, HNHA and HNHB were recorded for identification of intraresidue peaks in the ^{15}N-NOESY-HSQC. Two ^{13}C-NOESY-HMQC's were recorded, one in aqueous buffer, for the other a sample was lyophilized and redissolved in 100% D_2O.

For facilitation of sidechain assignments a CCCONNH and HCCCONNH experiment were recorded. In order to determine the relaxation properties and total correlation time of the protein a set of 2-dimensional T_1, T_2 and NOE spectra were recorded and analysed as described in the literature [14]. To guide the assignment of aromatic spin systems a 2D-NOESY, 2D-TOCSY and 2D-COSY in 100% D_2O were recorded.

All spectra were processed with Bruker XWINNMR and analyzed with Azara 2.1 by Wayne Boucher (unpublished) and Ansig 3.3 by Per Kraulis [67] (available via anonymous ftp from ftp://www.bio.cam.ac.uk/pub/). Interactive assignment was performed in ANSIG 3.3 for the complete backbone and almost complete sidechains. The final set of resonance assignments is about 95% complete. NOESY spectra were manually peak picked in ANSIG 3.3.

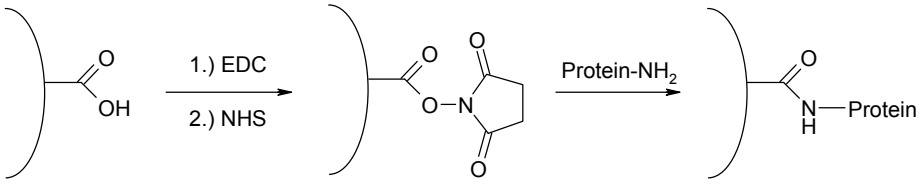
5.3.3 CYANA structure calculations

Structures were calculated with the program CYANA [58]. The program performs an automated assignment of the 3D-NOESY spectra based on peak lists and prior resonance assignment. The ANSIG crosspeaks export file was transformed into XEASY format required by CYANA using the FormatConverter program by Wim Vranken of CCPN, Cambrige, UK. For the final ensemble, 1162 unique distance constraints were defined by CYANA, furthermore 147 dihedral angle

restraints derived with TALOS [46] and from measurements of $^{3}J_{Hn,H\alpha}$ couplings were used. Also, 26 hydrogen bond restraints were included in the calculations. The 3D-^{15}N-NOESY, 3D-^{13}C-NOESY and 3D-^{13}C-NOESY in 100% D_2O were used. The tolerance was set to 0.06 ppm, 0.03 ppm, 0.3 ppm (direct ^{1}H, indirect ^{1}H, heteronucleus) during the calculations for all NOESY spectra. 20.000 steps were performed during the cooling stage of DYANA TAD simulation (Total steps 25.000). 200 frames were calculated in each cycle of CYANA, 20 structures were kept for the final ensemble.

5.3.4 Biacore measurements

Biacore measurements were performed on a Biacore® X machine. All buffers and the chip were used as supplied by Biacore. A CM5 chip was derivatized with VASP EVH1. The chip carries free carboxy groups on a dextrane matrix. These were preactivated with EDC/NHS and coupled to free amine groups on the protein. The reaction is outlind in Scheme 1:



Scheme 4
Activation of Biacore chip and coupling to protein
EDC: N'-(3-Dimethylaminopropyl)-N-ethylcarbodiimid Hydrochlorid
NHS: N-Hydroxysuccinimid

The protein was dissolved in Glycine buffer (10 mM glycine HCl, pH = 3.0, Biacore). After coupling of the protein, remaining activated carboxygroups were blocked with ethanolamine.
The peptides and peptomers were dissolved in HBS-EP buffer (0,01 M HEPES, 0,15 M NaCl, 3 mM EDTA, 0,005 % polysorbate 20 (v/v), pH = 7,4, Biacore). Stock solutions with a concentration of 2 mM were prepared and diluted in 7 steps 1:1 to yield a dilution series of 1 mM, 500 µM, 250 µM, 125 µM, 62,5 µM, 31,25 µM and 15,625 µM.

5.3.5 VASP EVH2 NMR spectroscopy and structure calculation

A 4.5 mM ^{15}N-labelled sample of VASP (336-380) was prepared in standard NMR buffer, containing 10% D_2O for the ^{2}H lock. All NMR spectra were recorded at 300 K, using either Bruker DRX 600 or DMX 750 spectrometers in standard configuration, with triple resonance probes equipped with triple axis self shielded gradient coils. Multidimensional NMR spectra, including 3D ^{15}N-separated TOCSY [68], HNHA, HNHB and NOESY [69] spectra (mixing time 60 ms) were recorded as described in the original references. A 3D ^{15}N/^{15}N-HMQC-NOESY-HSQC spectrum [70] was recorded with ^{15}N evolution in both indirect dimensions to assist with the assignment of short to medium range d_{NN} NOEs. A ~ 4 mM uniformly ^{15}N/^{13}C-labelled sample was used to acquire 3D CBCANNH [71], CBCA(CO)NNH [72] and HNCO spectra for unambiguous backbone assignment. Due to the low level of dispersion in the ^{15}N dimension, digitization in this dimension was optimized using a reduced ^{15}N spectral width of 1282.1 Hz (at 600 MHz).
Relaxation properties of backbone amide H_N-nuclei were determined by recording series of ^{15}N T_1 and T_2 relaxation experiments [73], with randomly ordered relaxation delays to avoid the accumulation of systematic errors. In each case, crosspeak intensities were fitted to a single exponential decay using the program Sparky (version 3.1, T.D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). The mean value of the ^{15}N T_1 and T_2 over all residues was then used for calculation of the correlation time τ_C, using Equation 6 [57]:

$$\tau_C = \frac{\left\{\left[6\left(T_1/T_2\right) - 7\right]\frac{1}{4}\right\}^{\frac{1}{2}}}{\Omega_N * 2\pi} \qquad \textbf{Equation 6}$$

where T_1 and T_2 are the mean ^{15}N relaxation times and Ω_N is the Larmor frequency of ^{15}N at the field strength used (60.827865 MHz in the present study).

Spectra for RDC measurements were recorded twice, once without and once with addition of phage to the sample. Pulse sequences for the non-decoupled HNCO, HNCA and J-NCO experiments were used as in Lit. [42], modified by Peter Schmieder (unpublished).
All NMR data were processed using the XWIN-NMR program (version 2.6) of Bruker Analytik GmbH (Rheinstetten, Germany) and the AZARA (version 2.1) program of W. Boucher (unpublished). Assignment was carried out using the user-interactive program ANSIG 3.3 [67, 74] on a Silicon Graphics O2 workstation.

All calculations were carried out with the program CNS 1.0 [44]. The distribution has been extended by use of a module for integrating Residual Dipolar Coupling restraints kindly provided by James Choy (SANI module).

Calculations were performed on four to eight SGI R12000 processors, 350 MHz, using several setups with different random seeds and combining results after calculations. Standard values were used for force constants, cooling steps and cutoff values. The parameters D_a and R necessary for RDC restraints were recalculated before each run based on a calculation of A_a and A_r in the program Module [45]. The input for Module consists of the previous, preliminary structure and the RDC values (given in Appendix 6.2.2). Values for the last run were:

$$\begin{aligned} R &= \frac{A_a}{A_r} \\ &= \frac{-2.494 \cdot 10^{-4}}{-1.286 \cdot 10^{-4}} \\ &= 0.516 \end{aligned}$$

$$\begin{aligned} D_a &= D_a^0 \cdot A_a \\ &= 1.0785 \cdot 10^4 \cdot \left(-1.286 \cdot 10^{-4}\right) \\ &= -2.689 \end{aligned}$$

Included in the calculations were NOE distance restraints, dihedral angle restraints from TALOS (Appendix 6.2.2), H-Bond restraints (Appendix 6.2.3) and Resiual Dipolar Couplings (Appendix 6.2.4).

200 frames were calculated in total in each run, the average energy of the 15 lowest energy structures in the final run was 3375.13 kcal/Mol, the lowest energy was 3359.91 kcal/Mol. The backbone rmsd for residues 6-42 is 0.49 Å for the 15 lowest energy structures.

Of residues 6-42 in the 15 lowest energy structures, 89.5 % are in the most favoured region of the Ramachandran plot, 10.5 % in the additionally allowed region (determined with Procheck-NMR [47]). When looking at all residues (except for Gly and Pro), 81.6 % are in the most favoured region, 14.3 % in the additionally allowed, 2.6 % in the generously allowed and 1.6 % in the disallowed region.

5.3.6 Analytical ultracentrifugation for VASP EVH2

Sedimentation velocity profiles were recorded at 286 nm at interval of 180 sec. The temperature was 20° C and the speed 45,000 rpm. Sedimentation equilibrium profiles were recorded at 260 nm after 36 hours equilibration at a speed of 28,000 rpm. The curves were fitted using the program POLYMOLE [75].