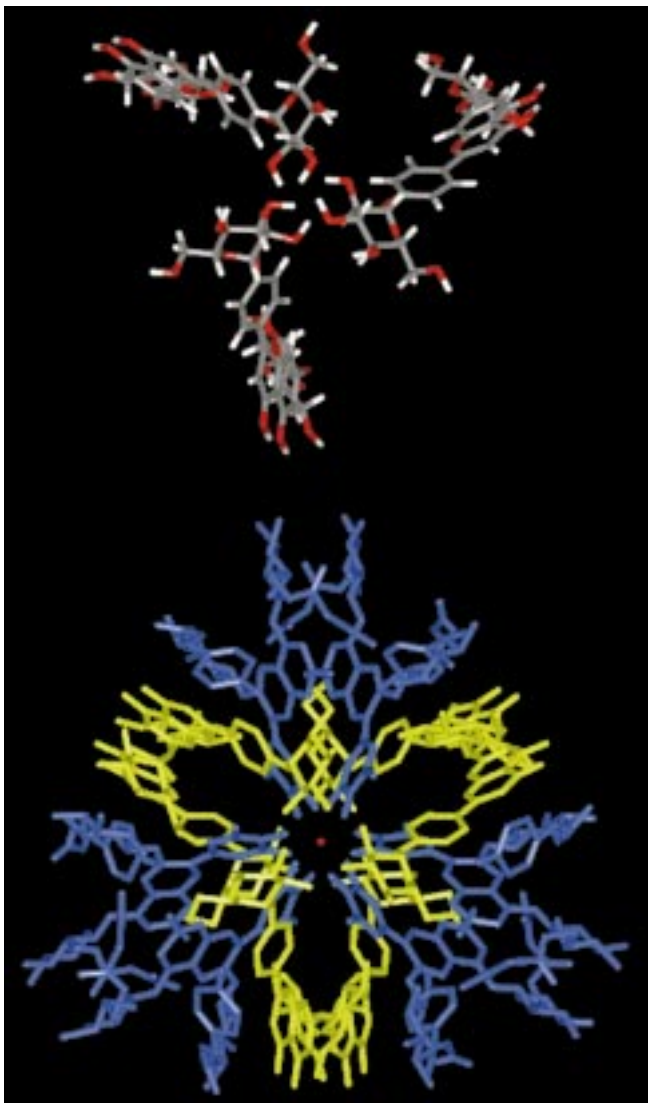

Figure 4. The optimized *M*-helical conformer of three **3a** molecules, similar to a propeller with three blades (top), and the gross structure of **1** (bottom). One set of three F molecules is located at the upper side of the pigment and one at the lower side. The F molecules are intercalated in a face-to-face manner. Mg^{2+} ions exist at both the upper and lower part of the superstructure. The hydrogen atoms are omitted in **1** for clarity. Color scheme: anthocyanin **2**: blue, flavone **3a**: yellow, and Mg^{2+}: red.

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[17] Aqueous trifluoroacetic acid (TFA) was added to the produced pigment, then the solution was analyzed by HPLC using a CHIRALCEL OD-R column (DAICEL CHEMICAL) eluted with TFA/CH_3CN/water 0.3/13/86.7 at RT.

[18] A mixture of **3a** and **3b** (ratio 98:2) gave two peaks completely separated in HPLC.

[19] The starting structure of **1** was built by changing the flavocommelin molecule to **3a** on the basis of the crystallographic structure of commelinin.[3] The local optimization of this structure was performed by QUANTA-97-CHARMm (Ver. 23.2) software.

Synthesis of an Array Comprising 837 Variants of the hYAP WW Protein Domain**

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The elucidation of structure – function relationships of proteins contributes to a better understanding of how they work and also provides clues for the synthesis of agonists and antagonists. Today, variants of the investigated protein required for structure – function analyses are produced almost

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exclusively using recombinant gene technology. The following work sets out to examine whether the SPOT method[1] can be used for the highly parallel synthesis of proteins required for structure–function analyses. The hYAP WW protein domain was chosen as a model due to its short length of about 40 amino acids.[2–7] In addition to being one of the smallest protein modules known this domain is a well-investigated model for studying β-sheet motifs.[4, 6, 8, 9] WW domains have been shown to be of importance in numerous diseases such as Alzheimer's, Chorea Huntington, Liddle's Syndrome, and muscular dystrophy.[10] WW domains bind to short segments of proline-rich sequences to carry out their function.[3, 11] The hYAP WW domain binds to the peptide core motif PPXY (X = L-amino acid).[5, 12]

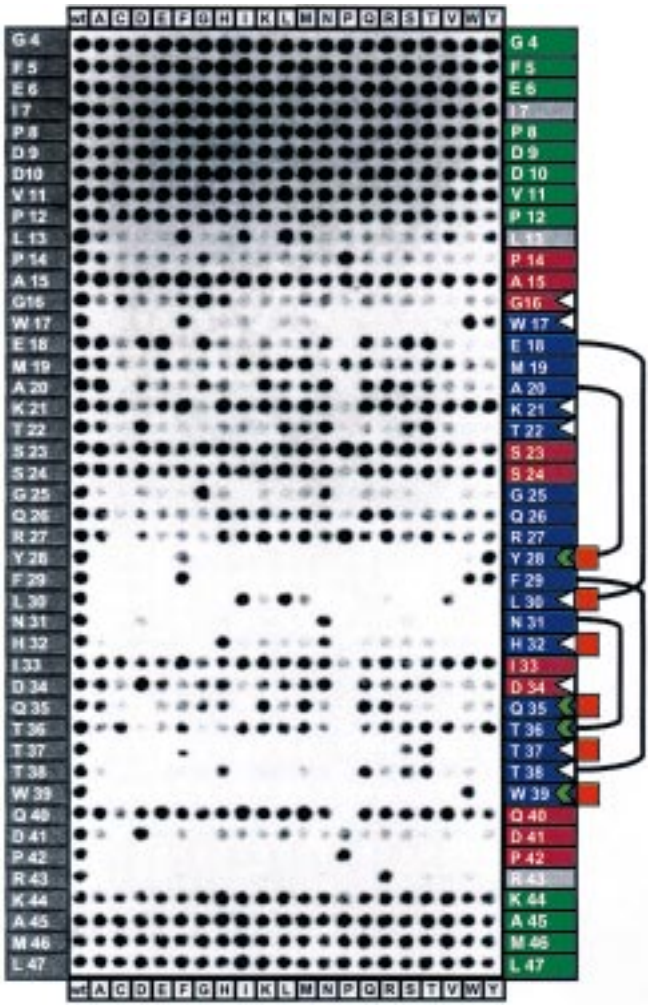

Figure 1. Interaction of the peroxidase-labeled peptide EYPPYPPPPYPSG with a library of hYAP WW variants. All spots in the left column comprise the wildtype (wt) sequence of the hYAP WW domain. All other spots contain single substitution variants that can be read out as follows: Rows define the sequence position that is substituted; columns define the amino acid that is used as a replacement. The figure shows the complete single substitution analysis of the hYAP WW protein domain. Numbering of sequence positions and colored marks on the right side refer to NMR structure analyses of the hYAP WW domain:[4, 13] green rectangles (flexible regions), red rectangles (turn regions), blue rectangles (β-sheet regions), white triangles (chemical shift changes upon ligand binding, approx. 0.2 ppm), green triangles (large changes in chemical shift upon ligand binding, >0.5 ppm), orange squares (domain–ligand contacts) and black brackets (intramolecular hydrogen bonds).

We report the synthesis of an array comprising 837 variants of the hYAP WW domain using stepwise peptide SPOT synthesis on a cellulose membrane and investigation of the binding properties of all variants in the array towards the peptide ligand EYPPYPPPPYPSG (Figure 1). To verify binding studies carried out with the array several WW variants were produced by standard methods, then purified and characterized by mass spectrometry. Subsequently, their affinities for the peptide ligand were measured by surface plasmon resonance spectroscopy (Table 1). Affinities of the purified WW variants range from 1 μM to 100 μM and correlate well with signals obtained at the corresponding array positions. Moreover, the folding conditions of the WW variants synthesized conventionally were analyzed by using NMR spectroscopy in the presence and absence of ligand (Table 1). All variants are folded in the presence of ligand. In contrast, in the absence of ligand no folding was detected in five cases. Our results are in accordance with those from analyses of variants produced by recombinant gene technology (Table 2).

Table 1. Binding constants (K_D) and folding status of hYAP WW variants in the absence (Δ) and presence (Σ) of ligand.[a]

WW variant	K_D [μM]	Δ	Σ
Wt (G4-L47)	2.4	+++	+++
L13F	4.6	++	++
L13W	22.3	0	+++
L13Y	11.9	+	++
W17Y	8.9	0	++
K21Y	2.5	+++	+++
S23P	3.5	++	+++
S24L	1.5	++	+++
R27P	4.3	+++	+++
L30K	48.6	++	++
H32Y	>100	+++	+++
V11-A45	2.9	0	+++
P12-K44	7.3	0	++
L13-R43	20.3	0	++

[a] Ratio of folded to unfolded domain was compared to the wildtype (wt = 100%): +++ (>66%), ++ (33%–66%), + (5%–33%), 0 (<5%). Variants in the last three rows represent truncations of the wildtype sequence (Gly4 to Leu47). First and last residues of the sequence are specified in these cases.

The array comprises 837 individual WW variants, each of which differs from the wildtype (wt) at one position. Each of the 44 residues of the WW domain was systematically substituted by each of the common 19 L-amino acids (Figure 1). A comparison of the overall signal pattern with previous structure analyses[4, 13] reveals correlations between the substitution analysis (Figure 1) and the tertiary structure (Figure 2) of the hYAP WW domain. Insensitivity towards substitutions in the terminal regions of the sequence (positions 4–12 and 44–47) is in accord with a relatively large degree of flexibility of these regions. Furthermore the five positions in the well-ordered central part that are most tolerant towards substitutions (positions 15, 23, 24, 33, 40) are located in turn regions. Positions with a high degree of sensitivity towards substitutions should either have distinct

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Table 2. Results from experiments with hYAP WW variants produced by recombinant gene technology.

WW variant	Ligand	Binding	Folding[a]	Reference
W17F	GTPPPPYTVG	+	nd	[5]
W17F	EYPPYPPPPYPSG	+	+[b]	[6]
Y28F	GTPPPPYTVG	+	nd	[5]
F29Y	GTPPPPYTVG	+	nd	[5]
H32F	GTPPPPYTVG	–	nd	[5]
H32A	GTPPPPYTVG	–	nd	[4]
Q35A	GTPPPPYTVG	+	nd	[5]
W39F	GTPPPPYTVG	–	nd	[5]
W39F	EYPPYPPPPYPSG	–	+[c]	[6]
P42A	GTPPPPYTVG	–	nd	[5]

[a] Affinities of WW variants to the peptide ligand were not quantified in most cases. nd = not determined. [b] Folded only in the presence of ligand. [c] Folded in the presence and absence of ligand.

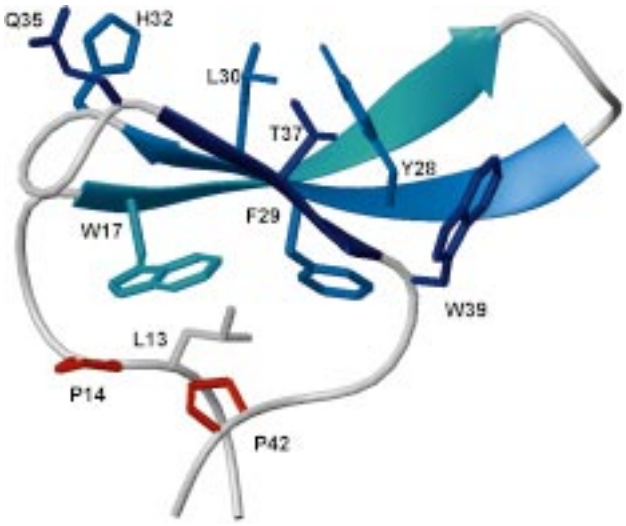

Figure 2. Three-dimensional structure of the hYAP WW domain,[4] residues V11 to R43 are shown. The triple-stranded β-sheet is colored blue (Figure 1 blue rectangles). Side chains displayed above the surface of the β-sheet form contacts with the ligand. Side chains displayed below the β-sheet form a hydrophobic clamp which is important for stabilizing the overall structure of the domain.

importance in constituting the ligand binding site or have an overall influence on the stable folding of the protein. The three-dimensional structure of the domain – ligand complex facilitates the discrimination between these two cases. Thus the high sensitivity towards substitutions within both C-terminal β-sheet strands (positions 25 – 32 and 35 – 39) is feasible since this region constitutes the actual ligand binding site. Also the essential role that Pro 42 has for the folding of the protein as the center of a hydrophobic clamp at the back of the β-sheet is reflected by the fact that this position cannot be substituted at all. Clearly observable is the great importance of tryptophans Trp 17 and Trp 39, accountable for the name of the WW domain family. The structure of the domain ligand complex shows direct contacts between Trp 39 and the ligand, whereas Trp 17 is located at the back of the β-sheet. While substitutions in position 17 may affect the binding activity of the hYAP WW domain primarily by influencing the stability of the overall domain, substitutions in position 39 should change the structure of the ligand binding site directly. Restricting the flexibility of the peptide backbone by incorporation of the amino acid proline should only be possible at relatively few positions in a protein structure. Correspondingly, there are only a few positions in the well-ordered region of the domain (positions 13 – 43) which tolerate the incorporation of proline. In agreement with data from other authors we find that Leu 30, which is involved in the formation of the binding pocket, can be substituted by the other aliphatic residues Ile and Val.[7, 14] Comparison of the sequences of various WW domains revealed that their core motif comprises around 35 – 40 amino acids.[15] The substitution analysis and binding studies with three truncated WW variants (Table 1) show that a ligand-binding hYAP WW variant can be limited to the core region V11 to A45 in our case. This variant however is only folded in the presence of ligand (Table 1).

SPOT synthesis[1] has already proved its value as a tool for the characterization of interactions between peptide ligands and proteins.[16, 17] In the past however, only libraries of peptide ligands were synthesized on the support surface while the protein was produced by recombinant genetic techniques. The stepwise synthesis of a library of variants of a small protein on the membrane surface, presented in this study, expands this concept for the first time. Earlier work has verified that SPOT synthesis and classical solid-phase methods produce peptides of comparable quality.[17, 18] Several spots containing hYAP WW wildtype were analyzed to evaluate the quality of the WW domains synthesized on cellulose membranes. The desired product could be identified by mass spectrometry (not shown). However, it could not be excluded that truncated sequences of nonbinding WW variants would bind the ligand and thus might influence the result. Therefore all the single-deletion variants of the hYAP WW wildtype sequence were synthesized by SPOT synthesis and analyzed for peptide ligand binding (not shown). Almost all deletions resulted in loss of binding activity. Only the regions 4 – 12, 23 – 24, and 44 – 47 tolerated missing amino acids. Thus it appears most unlikely that truncated sequences generate false-positive signals. The reproducibility of the synthesis was good, a number of independently synthesized hYAP WW arrays produced the same signal pattern in binding studies. Further indication for a good reproducibility of the synthesis is the similar signal intensity of the 88 wildtype spots in the array (Figure 1).

The work presented here not only reflects the results of previous mutagenesis studies on the hYAP WW domain, but also reveals a number of new insights into the interconnection between structure and function of this protein domain. A systematic analysis of all single substitution variants would have been extremely difficult with recombinant gene technology, since unfolded proteins of this size are generally degraded in situ. Combining the parallel nature of synthesis and subsequent binding studies, provided in our approach, with information regarding the three-dimensional structure results in an efficient tool for the analysis of folding and specificity in small protein domains. Considering recent advancements in the chemical synthesis of proteins[19] this method for the production of fully synthetic protein arrays should gain increasing significance in the future.

Experimental Section

Synthesis of the WW domain library: The cellulose-bound protein domain library was produced by semi-automatic SPOT synthesis[1, 18] (Abimed, Langenfeld, Germany; software LISA (Jerini AG, Berlin, Germany)) on Whatman 50 cellulose membranes (Whatman, Maidstone, UK), as described in ref. [20].

HPLC-MS analysis of cellulose-bound wildtype hYAP WW domains: Ten spots (approx. 3 nmol peptide per spot) were punched out of the cellulose membrane. The peptides bound to the solid phase through an ester linkage were cleaved off by treatment with NH_3 gas for 16 h. The peptide was then eluted from the membrane with H_2O/trifluoroacetic acid (0.5%) and analyzed by using HPLC-MS. System: Hewlett-Packard series 1100 coupled with a Finnigan LCQ Ion Trap ESI mass spectrometer; column: Vydac C18, 150 × 2.1 mm, 5 μm; flow rate: 0.3 mL min^{-1}; gradient: 5% – 95% B (= acetonitrile) in 17 min. The desired product was identified with a retention time of 10.1 min.

Labeling of the peptide ligand: The peptide ligand C$\beta\beta$EYPPYPPPPYPSG (β = β-alanine) was linked throuhg the cysteine group to maleimide-activated horseradish peroxidase (Pierce, Rockford, IL) according to the supplier's instructions. Remaining maleimido groups were deactivated with a tenfold excess of sulfanylethanol (1 h, RT), followed by dialysis against TBS buffer (50 mM tris(hydroxymethyl)aminomethane (Tris), 100 mM NaCl, pH 8.0).

Incubation and development of the membrane: Membrane-bound WW domains were incubated overnight in blocking buffer (10% blocking reagent (CRB, Norwich, UK), 1% sucrose in TBS buffer). Subsequently, the labeled peptide ligand (2 μg mL^{-1}) was incubated with the membrane in the same buffer at 4 °C overnight then washed five times with TBS. A chemiluminescent substrate (Pierce, Rockford, IL) and a luminescence detector (Lumi-Imager, Boehringer Mannheim, Mannheim, Germany) were employed for detection.

Synthesis of peptide ligands and soluble WW variants for binding and folding studies: Syntheses were carried out according to standard Fmoc protocols (Fmoc = 9-fluorenylmethoxycarbonyl) with an automated peptide synthesizer (Abimed, Langenfeld, Germany). The products were purified by using HPLC and analyzed by MALDI-TOF mass spectrometry.

Measuring of binding affinities of WW variants to peptide ligands: Measurements were made with a BIAcoreX system in HBS buffer (10 mM 2-[4-(2-hydroxyethyl)-1-piperazino]ethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, 3 mM ethylenediamine-N,N,N′, N′-tetraacetic acid (EDTA), 0.005% polysorbate-20). The peptide ligand C$\beta\beta$EYPPYPPP-PYPSG was immobilized on a CM5-sensorchip through the cysteine group with the ligand – thiol method, according to supplier's instructions. The amount of immobilized ligand corresponded to a signal increase of 400 resonance units (RU). An equivalent amount of the nonbinding peptide C$\beta\beta$EAPPAPPPPAPSG was immobilized in the reference cell using the same procedure. Binding experiments were performed with WW variant concentrations ranging from 200 nM to 125 μM (eight different concentrations were applied for each experiment). Binding experiments were performed at 25 °C with a flow rate of 15 μL min^{-1}. Data were evaluated with the software BIAevaluation 3.0 according to the steady-state procedure.

Determining the folding status of WW variants using NMR spectroscopy: ^{1}H NMR spectra of WW variants (1 mM) and WW variants in complexes with the peptide ligand K$\beta\beta$EYPPYPPPPYPSG in a molar proportion of 1:2 were recorded on a 600 MHz DRX Bruker spectrometer. Experiments were carried out in 10 mM aqueous phosphate buffer, pH 6.0, 100 mM NaCl, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA at 15 °C. To determine the folding status of WW variants the proportion of the surface areas of the folding-dependent indole NH signal of W17 (δ = 10.27) and W39 (δ = 9.96) in the folded respectively unfolded states (both δ = 10.05) were compared.[6, 9]

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