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A study on the effect of synthetic α-to-β^3-amino acid mutations on the binding of phosphopeptides to 14-3-3 proteins†

Sebastian A. Andrei, [a] Vito Thijssen, [a] Luc Brunsveld, [a] Christian Ottmann [ab] and Lech-Gustav Milroy [a]

Here we describe the synthesis of a series of α,β-phosphopeptides, based on the phosphoepitope site on YAP1 (yes-associated protein 1), and the biochemical, biophysical and structural characterization of their binding to 14-3-3 proteins. The impact of systematic mono- and di-substitution of α → β3 amino acid residues around the phosphoserine residue are discussed. Our results confirm the important role played by the +2 proline residue in the thermodynamics and structure of the phosphoepitope/14-3-3 interaction.

14-3-3 proteins (a highly homologous family of 7 isoforms with overlapping functionalities) are promising drug targets for treating cancer and neurodegeneration,[1–4] firstly because of their role as hub proteins within a number of signalling pathways critical to pathologies. Secondly, 14-3-3 proteins are eminently druggable in the sense that they preferentially bind to client proteins *via* conserved phosphorylated epitopes,[5,6] which form well-defined drug interfaces[7,8] for studies into 14-3-3 protein–protein interaction (PPI) inhibition or stabilization.[9–11] Such studies have laid foundations for the discovery of small molecule modulators of 14-3-3 PPIs through *e.g.* the discovery of natural product stabilizers,[9,12] the development of peptidomimetics,[13,14] modified peptides,[10] or small molecule inhibitors.[15,16] The peptide epitope of a target PPI serves as an excellent entry point for the structure-based design of novel PPI inhibitors,[13] including for 14-3-3 PPIs.[10] Whereas the amino acid sequence and thus sidechain diversity of the 14-3-3 binding phosphopeptide has been extensively studied,[6,17] the peptide backbone of the phosphoepitope has received less attention despite the clear potential for improved affinity and selectivity between highly homologous PPIs.[18] Backbone modifications can improve both the pharmacodynamic and –kinetic properties of peptide modulators.[19] α,β-Peptides are a particularly promising form of backbone modification.[18,20–23] To the best of our knowledge α,β-peptides have so far not been studied in the context of 14-3-3 proteins. Here we report a comprehensive and systematic biochemical and structural study on the effects of α → β^3 mutations on the binding of the phosphopeptide epitope of the WW-domain protein YAP1 to 14-3-3 proteins.

The yes-associated protein homolog, YAP1, is important for transcription regulation and 14-3-3 regulates trafficking of YAP1 between the cytoplasm and nucleus through binding to phosphorylated Ser127 on the YAP1 protein.[24] YAP1 exhibits tumour promoting properties, which are suppressed by phosphorylation of residue Ser127 through subsequent binding to 14-3-3 proteins.[25] Small molecule modulators of 14-3-3/YAP1 would be useful tool compounds and may serve as potential lead compounds for drug development. The crystal structure of the isoform 14-3-3σ/YAP1 binding interface was solved by our group.[24] The 14-3-3/YAP1 was considered to be an ideal PPI to investigate σ/β-phosphopeptides because of the long extended interface leading to multiple contact points between peptide and 14-3-3 protein allowing to gauge the significance of the corresponding α-amino acids or region of the PPI.

In the original 14-3-3/YAP1 crystal structure [PBD: 3MHR], the 10-mer phosphopeptide sequence, bearing a C-terminal carboxylate and a free N-terminus (Table 1, **YAP1.1**), was co-crystallized with 14-3-3, and all amino acids of the YAP1 peptide could be fitted into the available electron density. A short study using fluorescence polarization (FP) and isothermal calorimetry (ITC) on the binding of the YAP1 peptide to two different 14-3-3 isoforms – ζ & σ, ideally suited for biophysical and crystallization studies[10,11] – with either *N*-acetylation (**YAP1.2**) or *C*-amidation (**YAP2.1**), or both simultaneously (**YAP2.2**) – concluded that such modifications produce only a marginal change in 14-3-3-binding activity compared to **YAP1.1** (Table 1; FP-curves in ESI†). Subsequent mutational studies were performed on **YAP2.2.**

We systematically mutated α → β^3 amino acid residues in **YAP2.2**, except for phosphoserine and histidine residues, due to

[a] *Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems (ICMS), Eindhoven University of Technology, 5600 MB Eindhoven, The Netherlands. E-mail: l.brunsveld@tue.nl, c.ottmann@tue.nl, l.milroy@cantab.net*

[b] *Department of Chemistry, University of Duisburg-Essen, 47057 Essen, Germany*

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Table 1 Sequences of YAP1-derived α- and α/β-phosphopeptides studied. β^3-Amino acids highlighted in bold and underlined

		FP IC_{50}/μM (±)		PDB
		14-3-3ζ	14-3-3σ	code:
α-Peptides:				
H_2N-RAHpSSPASLQ-COOH	**YAP1.1**	3.7 (0.33)[a]	4.4 (0.14)[a]	3MHR
Ac-RAHpSSPASLQ-COOH	**YAP1.2**	1.7 (0.03)[a]	2.9 (0.10)[a]	—
H_2N-RAHpSSPASLQ-NH_2	**YAP2.1**	1.1 (0.05)[a]	1.9 (0.13)[a]	—
Ac-RAHpSSPASLQ-NH_2	**YAP2.2**	0.84 (0.02)[a]	0.90 (0.40)[a]	—
α/β-Peptides:				
RAHpS**S**PASLQ	**βYAP1**	>500[b]	>500[b]	—
RAHpSS**P**ASLQ	**βYAP2**	60.6 (3.8)[b]	49.9 (8.3)[b]	6G6X
RAHpSSP**A**SLQ	**βYAP3**	17.2 (0.4)[b]	12.4 (0.5)[b]	6G8J
RAHpSSPA**S**LQ	**βYAP4**	35.4 (2.3)[b]	29.3 (0.5)[b]	6G8K
RAHpSSPASL**Q**	**βYAP5**	10.0 (0.5)[b]	7.7 (0.4)[b]	6G8L
RAHpSSPASL**Q**	**βYAP6**	5.9 (0.2)[b]	6.7 (0.2)[b]	—
RAHpSSPASLQ	**βYAP7**	5.8 (0.2)[b]	6.8 (0.4)[b]	6G8I
RA**H**pSSPASLQ	**βYAP8**	12.8 (0.3)[b]	14.9 (0.7)[b]	—
RAHpSS**P**AS**L**Q	**βYAP2.2**	61.2 (3.5)[b]	53.8 (3.4)[b]	6G8P
RAHpSSP**A**S**L**Q	**βYAP3.2**	19.0 (0.3)[b]	16.6 (0.8)[b]	6G8Q

[a] 14-3-3 isoform (1 μM), competitor FITC-ERα peptide[9] (0.1 μM), starting concentration of dilution series = (250 μM). [b] 14-3-3 isoform (5 μM), competitor FITC-ERα peptide (0.1 μM), starting concentration of dilution series = (500 μM). **YAP2.1**, **YAP2.2**, **βYAP6**, and **βYAP7** are against the limits of the assay.

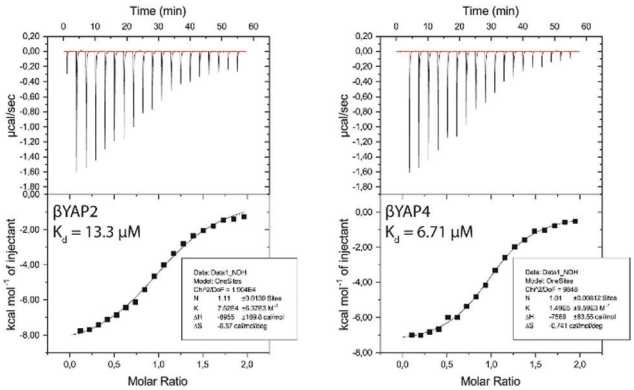

Fig. 1 ITC measurements of the binding of α/β-phosphopeptides **βYAP2** & **βYAP4** to 14-3-3ζ (T = 310.15 K or 37 °C). Additional measurements of these ITC results can be found in the ESI.†

the lack of a suitable commercially available source. Eight α/β-phosphopeptides bearing a single α → β mutation, and two analogues bearing two α → β mutations were synthesized by Fmoc SPPS and purified and characterized by RP-HPLC (Table S1, ESI†). The eight singly mutated α/β-phosphopeptides were first tested in a competitive fluorescence polarization assay and their activity compared against **YAP2.2** (Table 1). The data revealed an increase in the IC_{50} value the closer α → β^3-substitution was made to the phosphoserine residue. As can be observed for **βYAP1**, replacing the α-serine with a β^3-serine residue adjacent to the phosphoserine completely disrupts binding to 14-3-3σ. An α → β^3-substitution one position further either N- or C-terminal to the phosphoserine, *i.e.* **βYAP8** (β^3-alanine) and **βYAP2** (β^3-proline), was correspondingly better tolerated – N-terminal more so than C-terminal. This might perhaps be unsurprising given that the peptides bear more amino acids C-terminal to the phosphate and thus more amino acids are able to interact with the surface of the 14-3-3 protein C-terminal to the phosphoserine. The hypothesis then being that α → β^3-substitution leads to greater flexibility in the peptide backbone and thus a higher potential of disrupting peptide-protein interactions in this region of the peptide. This same principle holds when comparing α/β-phosphopeptides **βYAP3** (β^3-alanine) and **βYAP7** (β^3-arginine), in which the N-terminal substitution is apparently also more tolerated. The only exception to the above trend lies with **βYAP4**, whose activity is less than **βYAP3** despite bearing an α → β^3-substitution one position further away from the phosphoserine. Peptides **βYAP2.2** and **βYAP3.2**, bearing two α → β^3 amino acid mutations – one close and one further away from the phosphoserine – appear to exhibit the same activity as the connected **βYAP2** and **βYAP3** peptides, respectively. α → β^3-mutations in the flanking region of the C-terminus have little effect in both the mono-substituted (**βYAP5** and **βYAP6**) and di-substituted analogues (**βYAP2.2** and **βYAP3.2**).

ITC measurements were performed on **YAP2.2** (ESI†) and representative α → β^3-peptides **βYAP2** and **βYAP4** to understand the biophysical consequences of α → β^3-substitutions for binding to two different 14-3-3 isoforms – 14-3-3σ (ESI†) and 14-3-3ζ (Fig. 1). The binding of **βYAP2** to 14-3-3ζ (K_d = 13.30 ± 1.13 μM) was found to be approximately two-fold weaker compared to that of **βYAP4** (K_d = 6.71 ± 0.43 μM). A similar pattern of behaviour was observed toward the 14-3-3σ isoform (ESI†). The activity of these two α/β-phosphopeptides is approximately 10-fold weaker than observed for the reference peptide **YAP2.2** (K_d = 0.79 ± 0.09 μM). Interestingly, though, this loss of activity is the result of a significantly more positive entropic contribution in the case of **βYAP2** (*i.e.* ΔH = −9.0 ± 0.2 kcal mol^{-1}, $-T\Delta S$ = 2.0 kcal mol^{-1}) in contrast to a significantly less negative enthalpic contribution in the case of **βYAP4** (*i.e.* ΔH = −7.6 ± 0.08 kcal mol^{-1}, $-T\Delta S$ = 0.2 kcal mol^{-1}), relative to **YAP2.2** (*i.e.* ΔH = −9.5 ± 0.08 kcal mol^{-1}, $-T\Delta S$ = 0.8 kcal mol^{-1}). These data suggest that the activity drop observed on introduction of the β^3-amino acid thus might equally be explained by either a disruption to energetically favourable binding interactions between α/β-phosphopeptide and 14-3-3 protein or an increase in the degrees of conformational freedom in the peptide backbone.

A competitive fluorescence depolarization assay of all the α/β-phosphopeptides *versus* **YAP2.2** was performed against five different 14-3-3 homodimer isoforms – σ,ζ,ε,η and τ – to investigate for a possible isoform selective behaviour of the peptides (Fig. S5–S9, ESI†). In general, the affinity of the α/β-phosphopeptides toward 14-3-3ε and 14-3-3η is greater than toward the other isoforms. The preference for 14-3-3η is present in all peptides including the α/β-peptides. Substitution of the proline (**βYAP2**), alanine (**βYAP3**) and serine residues (**βYAP4**) C-terminal to the phosphoserine residue showed a similar pattern of behaviour across the five isoforms (order of affinity, 14-3-3η > 14-3-3ε > 14-3-3σ > 14-3-3ζ or 14-3-3τ). This affinity trend is also shared by the double mutant peptides (**βYAP2.2** & **βYAP3.2**), though less pronounced, but is not observed for the α-peptide lacking β^3-substitution *i.e.* **YAP2.2**.

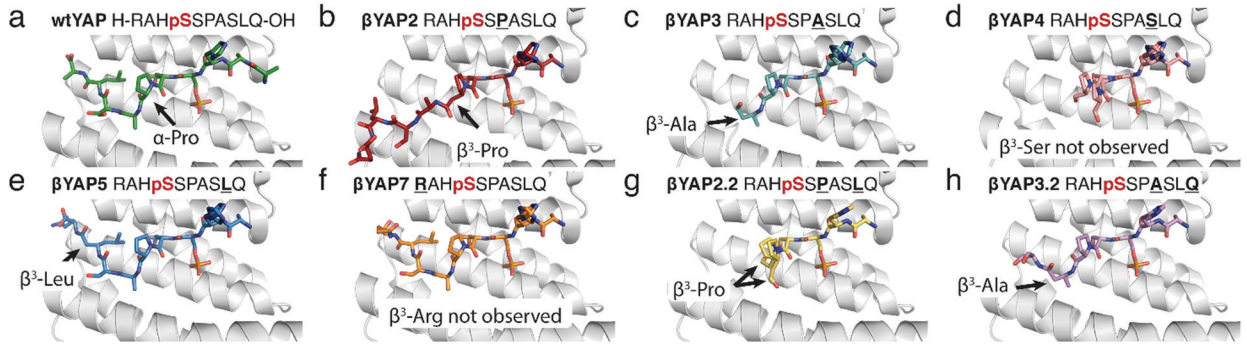

Fig. 2 Overview of the crystal structures of 14-3-3σΔC (grey) in complex with (a) wildtype αYAP 10-mer peptide [PDB 3MHR] and (b–h) βYAP 10-mer peptides from this study.

To gain structural insight into the activity trends observed in the FP and ITC assays, the crystal structure for five of the eight α/β-phosphopeptides bearing a single β³-amino acid mutation – **βYAP2**, **βYAP3**, **βYAP4**, **βYAP5**, **βYAP7** – and both peptides bearing two doubly mutated variants – *i.e.* **βYAP2.2** & **βYAP3.2** was solved (Fig. 2). A non-linear correlation was observed between the position of the α → β³ mutation and the amount of observable electron density C-terminal to the phosphorylation site. The amount of observed electron density decreased the further away from the phosphoserine residue a mutation was situated (**βYAP2** → **4**), only to increase again beyond **βYAP5**. In the case of **βYAP2**, bearing a β³-proline residue +2 to the phosphoserine, despite the mutation, we unexpectedly observe the whole peptide in the structure. On comparison of **βYAP2** with native structure (**YAP1.1**, PDB 3MHR, Fig. 3) we observe that the introduction of the β³-proline residue results in a significant remodelling of the peptide conformation, particularly in the C-terminal region. Therefore, the Pro residue might be considered critical for determining the peptide's preferred bound conformation. For **βYAP3** (β³-alanine) the introduction of the β³-amino acid residue, which is clearly visible in the electron density map, results in a loss of electron density in the C-terminal region beyond the mutated residue, suggesting one conformation for the peptide in the groove that is flexible beyond the mutated site. The introduction of a β³-serine residue by contrast, *i.e.* **βYAP4**, introduces considerable disorder at the peptide C-terminus, starting at the proline residue. The density of the peptide backbone is seen to branch into two conformations, both of which cannot be modelled beyond the proline branching point. For **βYAP5** (β³-leucine) the whole peptide is visible in a very similar conformation to the conformation observed in the native sequence (**YAP1.1**).

In the case of the disubstituted α/β-peptides; **βYAP2.2** introduces more rotational freedom and is slightly less active in FP compared to than **βYAP2**. **βYAP2** plots a "middle road" between the two β³-proline conformations observed in the crystal structures of **βYAP2** and **βYAP2.2**. For **βYAP3.2** the β³-alanine and serine residues N-terminal to the phosphoserine can be observed, thus being slightly more structured in this region than **βYAP3**, but with similar activities observed in FP.

On comparison of the FP data (14-3-3ζ) for **YAP1.1** (IC_{50} = 3,7 μM), **YAP2.2** (IC_{50} = 0,84 μM), **βYAP2** (IC_{50} = 61 μM) and **βYAP4** (IC_{50} = 35 μM), to separate backbone from chain-end effects, the single α → β³ mutation leads to a significant decrease (up to 73-fold) in potency whereas modifications at the C- and N-termini exert only a marginal effect on the phosphopeptide binding affinity. Although the K_d values of **YAP1.1** and **YAP2.2** are similar, the thermodynamic parameters associated with these two α-phosphopeptides are significantly different (Fig. S1, ESI†). Acetylation of the N-terminus and C-terminal amidation (**YAP1.1** → **YAP2.2**) removes two charges from the peptide, resulting in an increase in the enthalpic contribution (ΔH becomes more negative) with a corresponding decrease in the entropic contribution ($-T\Delta S$). The α → β³ mutation in **βYAP2** results in a similar binding enthalpy as **YAP2.2** but a higher K_d value due to a more positive entropic component. This trend suggests more flexibility in the amide backbone due to more degrees of rotational freedom introduced by the β³ amino acid residue. The point mutation disrupts the natural binding mode in the wt sequence (*e.g.* **YAP1.1**), initially disruptive, but introduces backbone flexibility, which enables access to a similar extended high energy binding contact/conformation with the 14-3-3ζ protein, as can be seen in the X-ray crystal structure (Fig. 3). Increased amide flexibility means though more energy that needs to be invested to fix the peptide in the active conformation. Such α → β³ mutational effects in PPIs, are different from effects observed on protein folding.[26] The α → β³ mutation in **βYAP4** does not lead to a net change in entropy but does disrupt the native binding mode (*i.e.* compared to **YAP2.2**) and associated favourable interactions. This can be observed in the corresponding X-ray structure by a decrease in the available electron density in the C-terminus of the α/β-peptide.

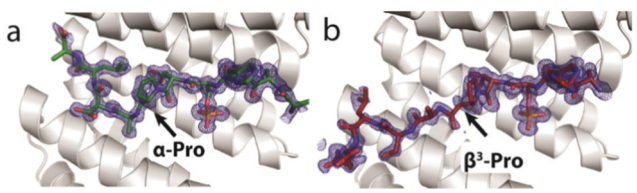

Fig. 3 Crystal structures of **YAP1.1** (a, green sticks, PDB 3MHR), **βYAP2** (b, red sticks, PDB 6G6X). The secondary structure for the 14-3-3σΔC protein (grey cartoon) differs very little between the three co-crystal structures.

The observation that mutating the proline residue in the +2 position relative to the phosphoserine has a large influence on the peptide binding conformation and thermodynamics is also in line with earlier work that shows the evolutionary importance of this residue.[17] The proline residue provides nature with a structural motif that allows the peptide to exit the 14-3-3 binding groove without paying a large entropic penalty. By adding more flexibility at this position through $\alpha \rightarrow \beta^3$ mutation, the conserved structural motif is distorted with major implications for binding mode and thermodynamics. Indeed, of the binding peptides, the peptide bearing only a proline mutation, **βYAP2**, shows the largest decrease in binding affinity and a large shift in binding mode, underlining the structural importance of this residue in 14-3-3 binding interactions.

In conclusion this work reports the first example of α/β-peptides bound to 14-3-3 protein, and their co-crystal structures and, to the best of our knowledge, the first study on α,β-phosphopeptide inhibitors of PPIs. A number of single $\alpha \rightarrow \beta$ mutants – **βYAP5**, **βYAP6**, **βYAP7** – were found to be roughly equipotent with the wild-type α-peptide epitope, **YAP2.2**. Furthermore, mutating the +2 proline residue – *i.e.* **βYAP2** – has a large influence on thermodynamic and structural binding properties, in line with earlier results on conserved 14-3-3 binding epitopes. The crystal structure data verifies the findings of the binding (FP/ITC) data and correlates with the loss in binding affinity and allows to construct a global picture of the impact of these $\alpha \rightarrow \beta$ modifications. The increased flexibility introduced into the peptide backbone by the $\alpha \rightarrow \beta$ mutations tended to decrease peptide binding due to changes in both the entropy and enthalpy of binding, which in some cases translated into well-defined alternative binding modes. These data provide yet further structural proof of the consequences of $\alpha \rightarrow \beta$ modifications for the binding of α/β-peptide to their protein target, and their correlation to the biochemical and biophysical binding data.[27–30] Such insights should help in the design of 14-3-3 PPI modulators and could be used to design peptides having multiple modifications to tune pharmacokinetic properties.

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Conflicts of interest

There are no conflicts to declare.

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