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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†

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Here we describe the synthesis of a series of α,β -phosphopeptides, based on the phosphopeptide 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 $\alpha \rightarrow \beta^3$ 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 phosphopeptide/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 phosphopeptide 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 $\alpha \rightarrow \beta^3$ 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 14-3-3 σ /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 [PDB: 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 $\alpha \rightarrow \beta^3$ amino acid residues in **YAP2.2**, except for phosphoserine and histidine residues, due to

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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 code:
		14-3-3ζ	14-3-3σ	
α-Peptides:				
H ₂ N-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 ₂ N-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:				
RAHpSSPASLQ	βYAP1	> 500 ^b	> 500 ^b	—
RAHpSSPASLQ	βYAP2	60.6 (3.8) ^b	49.9 (8.3) ^b	6G6X
RAHpSSPASLQ	βYAP3	17.2 (0.4) ^b	12.4 (0.5) ^b	6G8J
RAHpSSPASLQ	βYAP4	35.4 (2.3) ^b	29.3 (0.5) ^b	6G8K
RAHpSSPASLQ	βYAP5	10.0 (0.5) ^b	7.7 (0.4) ^b	6G8L
RAHpSSPASLQ	βYAP6	5.9 (0.2) ^b	6.7 (0.2) ^b	—
RAHpSSPASLQ	βYAP7	5.8 (0.2) ^b	6.8 (0.4) ^b	6G8I
RAHpSSPASLQ	βYAP8	12.8 (0.3) ^b	14.9 (0.7) ^b	—
RAHpSSPASLQ	βYAP2.2	61.2 (3.5) ^b	53.8 (3.4) ^b	6G8P
RAHpSSPASLQ	βYAP3.2	19.0 (0.3) ^b	16.6 (0.8) ^b	6G8Q

^a 14-3-3 isoform (1 μM), competitor FITC-ER α peptide⁹ (0.1 μM), starting concentration of dilution series = (250 μM). ^b 14-3-3 isoform (5 μM), competitor FITC-ER α peptide (0.1 μM), starting concentration of dilution series = (500 μM). **YAP2.1**, **YAP2.2**, **β YAP6**, and **β YAP7** are against the limits of the assay.

the lack of a suitable commercially available source. Eight α/β -phosphopeptides bearing a single $\alpha \rightarrow \beta$ mutation, and two analogues bearing two $\alpha \rightarrow \beta$ 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 IC₅₀ value the closer $\alpha \rightarrow \beta^3$ -substitution was made to the phosphoserine residue. As can be observed for **β YAP1**, replacing the α -serine with a β^3 -serine residue adjacent to the phosphoserine completely disrupts binding to 14-3-3 σ . An $\alpha \rightarrow \beta^3$ -substitution one position further either N- or C-terminal to the phosphoserine, *i.e.* **β YAP8** (β^3 -alanine) and **β YAP2** (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 $\alpha \rightarrow \beta^3$ -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 **β YAP3** (β^3 -alanine) and **β YAP7** (β^3 -arginine), in which the N-terminal substitution is apparently also more tolerated. The only exception to the above trend lies with **β YAP4**, whose activity is less than **β YAP3** despite bearing an $\alpha \rightarrow \beta^3$ -substitution one position further away from the phosphoserine. Peptides **β YAP2.2** and **β YAP3.2**, bearing two $\alpha \rightarrow \beta^3$ amino acid mutations – one close and one further away from the phosphoserine – appear to exhibit the same activity as the connected **β YAP2** and **β YAP3** peptides, respectively. $\alpha \rightarrow \beta^3$ -mutations in the flanking region of the C-terminus have little

Fig. 1 ITC measurements of the binding of α/β -phosphopeptides **β YAP2** & **β YAP4** to 14-3-3 ζ ($T = 310.15$ K or 37°C). Additional measurements of these ITC results can be found in the ESI†

effect in both the mono-substituted (**β YAP5** and **β YAP6**) and di-substituted analogues (**β YAP2.2** and **β YAP3.2**).

ITC measurements were performed on **YAP2.2** (ESI†) and representative $\alpha \rightarrow \beta^3$ -peptides **β YAP2** and **β YAP4** to understand the biophysical consequences of $\alpha \rightarrow \beta^3$ -substitutions for binding to two different 14-3-3 isoforms – 14-3-3 σ (ESI†) and 14-3-3 ζ (Fig. 1). The binding of **β YAP2** to 14-3-3 ζ ($K_d = 13.30 \pm 1.13$ μM) was found to be approximately two-fold weaker compared to that of **β YAP4** ($K_d = 6.71 \pm 0.43$ μM). A similar pattern of behaviour was observed toward the 14-3-3 σ isoform (ESI†). The activity of these two α/β -phosphopeptides is approximately 10-fold weaker than observed for the reference peptide **YAP2.2** ($K_d = 0.79 \pm 0.09$ μM). Interestingly, though, this loss of activity is the result of a significantly more positive entropic contribution in the case of **β YAP2** (*i.e.* $\Delta H = -9.0 \pm 0.2$ kcal mol⁻¹, $-T\Delta S = 2.0$ kcal mol⁻¹) in contrast to a significantly less negative enthalpic contribution in the case of **β YAP4** (*i.e.* $\Delta H = -7.6 \pm 0.08$ kcal mol⁻¹, $-T\Delta S = 0.2$ kcal mol⁻¹), relative to **YAP2.2** (*i.e.* $\Delta H = -9.5 \pm 0.08$ 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 14-3-3 ϵ and 14-3-3 η is greater than toward the other isoforms. The preference for 14-3-3 η is present in all peptides including the α/β -peptides. Substitution of the proline (**β YAP2**), alanine (**β YAP3**) and serine residues (**β YAP4**) C-terminal to the phosphoserine residue showed a similar pattern of behaviour across the five isoforms (order of affinity, 14-3-3 η > 14-3-3 ϵ > 14-3-3 σ > 14-3-3 ζ or 14-3-3 τ). This affinity trend is also shared by the double mutant peptides (**β YAP2.2** & **β YAP3.2**), 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 14-3-3 $\sigma\Delta$ C (grey) in complex with (a) wildtype α YAP 10-mer peptide [PDB 3MHR] and (b–h) β YAP 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 β^3 -amino acid mutation – **β YAP2**, **β YAP3**, **β YAP4**, **β YAP5**, **β YAP7** – and both peptides bearing two doubly mutated variants – *i.e.* **β YAP2.2** & **β YAP3.2** was solved (Fig. 2). A non-linear correlation was observed between the position of the $\alpha \rightarrow \beta^3$ 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 (**β YAP2** $\rightarrow$ 4), only to increase again beyond **β YAP5**. In the case of **β YAP2**, bearing a β^3 -proline residue +2 to the phosphoserine, despite the mutation, we unexpectedly observe the whole peptide in the structure. On comparison of **β YAP2** with native structure (**YAP1.1**, PDB 3MHR, Fig. 3) we observe that the introduction of the β^3 -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 **β YAP3** (β^3 -alanine) the introduction of the β^3 -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 β^3 -serine residue by contrast, *i.e.* **β YAP4**, 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 **β YAP5** (β^3 -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; **β YAP2.2** introduces more rotational freedom and is slightly less active in FP compared to than **β YAP2**. **β YAP2** plots a “middle road” between the two β^3 -proline conformations observed in the crystal structures of **β YAP2** and **β YAP2.2**. For **β YAP3.2** the β^3 -alanine and serine residues N-terminal to the phosphoserine can be observed, thus being slightly more structured in this region than **β YAP3**, but with similar activities observed in FP.

On comparison of the FP data (14-3-3 ζ) for **YAP1.1** ($IC_{50} = 3.7 \mu M$), **YAP2.2** ($IC_{50} = 0.84 \mu M$), **β YAP2** ($IC_{50} = 61 \mu M$) and **β YAP4** ($IC_{50} = 35 \mu M$), to separate backbone from chain-end effects, the single $\alpha \rightarrow \beta^3$ 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** $\rightarrow$ **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 $\alpha \rightarrow \beta^3$ mutation in **β YAP2** 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 β^3 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 14-3-3 ζ 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 $\alpha \rightarrow \beta^3$ mutational effects in PPis, are different from effects observed on protein folding.²⁶ The $\alpha \rightarrow \beta^3$ mutation in **β YAP4** 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), **β YAP2** (b, red sticks, PDB 6G6X). The secondary structure for the 14-3-3 $\sigma\Delta$ C 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, **β YAP2**, 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 – **β YAP5**, **β YAP6**, **β YAP7** – were found to be roughly equipotent with the wild-type α -peptide epitope, **YAP2.2**. Furthermore, mutating the +2 proline residue – *i.e.* **β YAP2** – 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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