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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 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 $\alpha \rightarrow \beta^3$ 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 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 $\alpha \rightarrow \beta^3$ 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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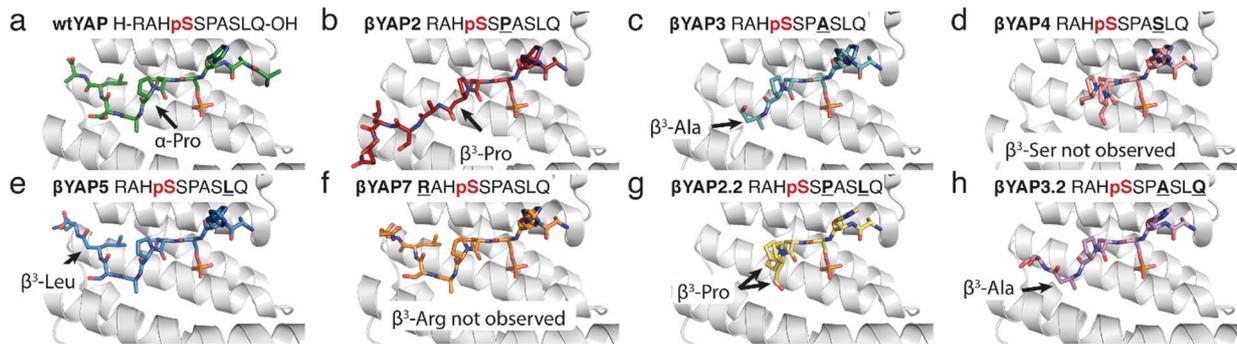


Fig. 2 Overview of the crystal structures of 14-3-3 $\sigma\Delta$ C (grey) in complex with (a) wildtype α YAP 10-mer peptide [PDB 3MHR] and (b–h) β YAP 10-mer peptides from this study.

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** ($IC_{50} = 3.7 \mu\text{M}$), **YAP2.2** ($IC_{50} = 0.84 \mu\text{M}$), β YAP2 ($IC_{50} = 61 \mu\text{M}$) and β YAP4 ($IC_{50} = 35 \mu\text{M}$), 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 ($-\Delta S$). 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.

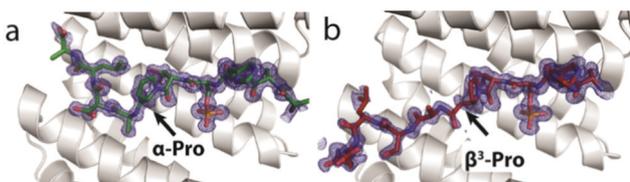


Fig. 3 Crystal structures of **YAP1.1** (a, green sticks, PDB 3MHR), β YAP2 (b, red sticks, PDB 6G6X). The secondary structure for the 14-3-3 $\sigma\Delta$ C protein (grey cartoon) differs very little between the three co-crystal structures.



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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