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A TPX2 proteomimetic has enhanced affinity for Aurora-A due to hydrocarbon stapling of a helix

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Abstract

Inhibition of protein kinases using ATP-competitive compounds is an important strategy in drug discovery. In contrast, the allosteric regulation of kinases through the disruption of protein-protein interactions has not been widely adopted, despite the potential for selective targeting. Aurora-A kinase regulates mitotic entry and mitotic spindle assembly, and is a promising target for anti-cancer therapy. The microtubule-associated protein TPX2 activates Aurora-A through binding to two sites. Aurora-A recognition is mediated by two motifs within the first 43 residues of TPX2, connected by a flexible linker. To characterize the contributions of these three structural elements, we prepared a series of TPX2 proteomimetics and investigated their binding affinity for Aurora-A using isothermal titration calorimetry. A novel stapled TPX2 peptide was developed that has improved binding affinity for Aurora-A and mimics the function of TPX2 in activating Aurora-A's autophosphorylation. We conclude that the helical region of TPX2 folds upon binding Aurora-A, and that stabilization of this helix does not compromise Aurora-A activation. This study demonstrates that the preparation of these proteomimetics using modern synthesis methods is feasible and their biochemical evaluation demonstrates the power of proteomimetics as tool compounds for investigating PPIs involving intrinsically disordered regions of proteins.

Aurora

Introduction

The mitotic spindle is a molecular machine built from microtubules and associated proteins that carries out the segregation of chromosomes during cell division. Assembly of the mitotic spindle is regulated by reversible phosphorylation of microtubule-associated proteins by Aurora-A and other protein kinases.[1,2] In humans, there are two other members of the Aurora family (Aurora-B and Aurora-C), all members of which are serine-threonine protein kinases with very similar catalytic domains and highly variable N-terminal regions. Aurora-A is concentrated at the poles of the mitotic spindle and along microtubules, and functions in centrosome maturation, spindle assembly, maintenance of spindle bipolarity and mitotic checkpoint control.[3,4]

The catalytic activity of Aurora-A is stimulated by phosphorylation and interactions with other proteins.[5-7] Many activating binding partners have been identified, however the interplay between them is unclear. The best characterized of these pathways, and arguably the most important for establishing high Aurora-A kinase activity in early mitosis, involves the microtubule-associated protein TPX2 (Targeting Protein for Xenopus kinesin-like protein 2).[2,8-11] Chromatin signals to the spindle assembly machinery using small GTPase RAs-related Nuclear protein (RAN), highly concentrated around the chromatin. RAN, in turn, releases central spindle assembly factors, including TPX2 from transport factors (importin α/β) in the vicinity of chromatin.[12] TPX2 then localises, binds to and activates the autophosphorylation of Aurora-A on Thr288.[13] Aurora-A promotes spindle assembly, organisation and stabilization via phosphorylation of microtubule (MT) related proteins such as transforming acidic coiled-coil-containing protein 3 (TACC3). TACC3 is also an activator of Aurora-A, and this mechanism serves to fine-tune the rate of spindle assembly through the regulation of its complexes with ch-TOG and clathrin.[14]

The crystal structure of the Aurora-A catalytic domain (aa122-403) phosphorylated on Thr287 and Thr288 in complex with the minimal domain of TPX2 (aa1-43), which is sufficient to bind and activate Aurora-A *in vitro,* shows how TPX2 stabilizes the active conformation of the kinase (Figure 1A).[15] TPX2 binds to Aurora-A with two separate segments: the upstream stretch (residues $Ser7^{TPX2}$-$Ser21^{TPX2}$) which is in an extended conformation (red), binds to Aurora-A's N-terminal lobe and stabilizes the position of the C-helix; the downstream stretch (residues $Asn30^{TPX2}$-$Asn43^{TPX2}$) which is in an α-helical conformation (blue), binds between the N- and C-terminal lobes and stabilizes the activation loop to form a platform for substrate binding (Figure 1B). The region (pink)

between these segments does not appear in the crystal structure, and the contribution of this region of TPX2 to the interaction with Aurora-A, if any, is unknown (Figure 1C).

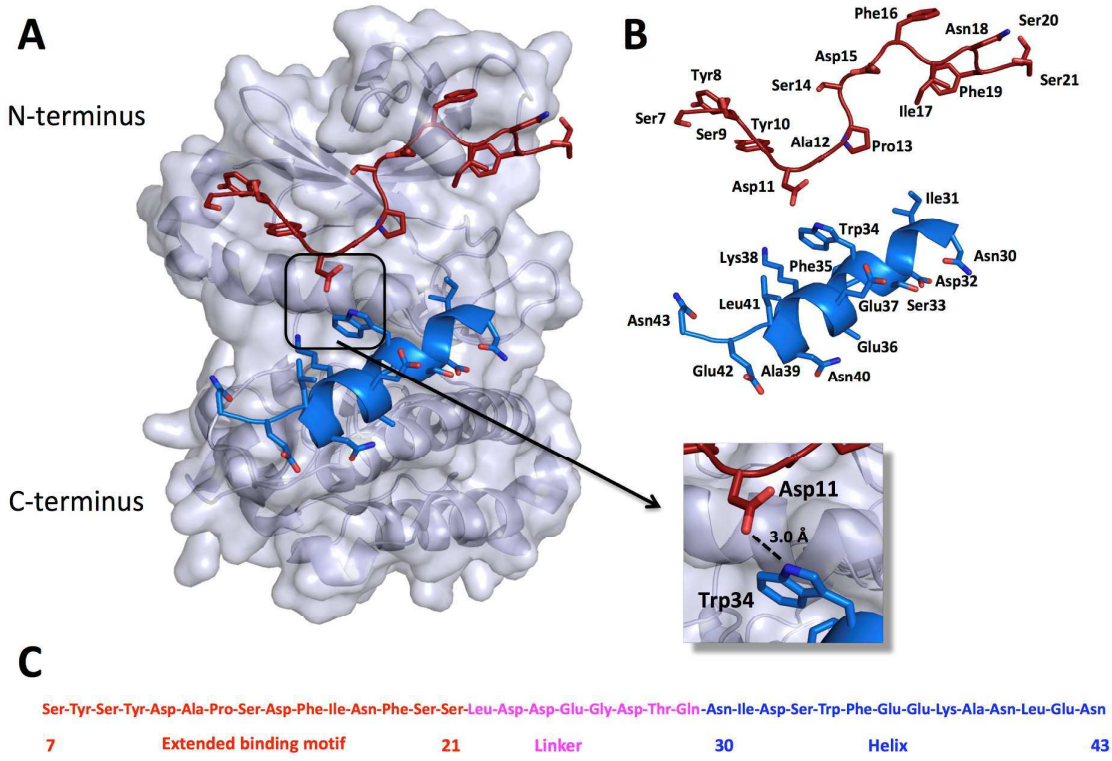

Figure 1. A. X-ray crystal structure of the Aurora-A - TPX2 complex (PDB 1OL5).[15] TPX2 non-structured binding motif (red) and TPX2 α-helix (blue). **B.** X-ray crystal structure of TPX2 minimal binding domain complex. **C**. Sequence of the TPX2 *N*-terminal domain. TPX2 upstream stretch (extended sequence (red)), TPX2 downstream stretch (α-helix (blue)) and the flexible linker (pink).

The insertion of two aromatic side chains on the helix of TPX2 (Trp34, Phe35) into a pocket between the N- and C-lobes of Aurora-A locks the activation loop into a conformation in which the side chain of phospho-Thr288^{AUR} is buried. This stabilizes Aurora-A to dephosphorylation by protein phosphatase 1 (PP1) and increases kinase activity further. In the absence of TPX2, the activation loop is dynamic and, unusually for a protein kinase, activating phosphorylation is not sufficient to stabilize the conformation of the activation loop. Indeed, a point mutation within the helical region (W34A) fails to protect Aurora-A from dephosphorylation by PP1.[16] However, the helix does not appear to play a major role in the ability of TPX2 to stimulate the autophosphorylation of Aurora-A, and the helix was unresolved in the crystal structure of unphosphorylated Aurora-A catalytic in complex with TPX2 (aa1-43).[17] This raises the question of whether the helix is present in TPX2 alone, or if it forms upon binding to phosphorylated Aurora-A.

Regulation of PPIs is a significant challenge in chemical biology and medicinal chemistry. A diverse array of peptidomimetic molecular scaffolds that mimic peptide secondary structures (e.g. α-helix, turn and β-sheet) and have improved physiochemical properties have been developed to disrupt PPIs.[18] A peptidomimetic can be defined as a compound that mimics a short peptide with a single secondary structure binding motif. Compounds that mimic the structure and function of extended regions of protein surfaces incorporating more than one secondary structure or binding epitope are proteomimetics.[19]

The structure of Aurora-A/TPX2 has provided key insights into the nature of the protein-protein interaction and mechanism of activation of this essential kinase. However, many aspects of this dynamic complex have yet to be resolved. What is the role of the TPX2 linker sequence not observed in the crystal structure? Does the helical region of TPX2 form upon binding to Aurora-A and, if so, can the entropic penalty of helix formation be partially overcome using a helix conformational constraint? To address these questions, we have designed and synthesized a series of proteomimetic chemical probes and investigated their binding affinity to Aurora-A. We generated a hydrocarbon-stapled TPX2 proteomimetic that recreates the activity of native TPX2, but with higher affinity binding, and determined the crystal structure in complex with Aurora-A.

RESULTS AND DISCUSSION

TPX2 interacts with Aurora-A through two separate motifs: an upstream stretch in an extended conformation (residues $Ser7^{TPX2}$–$Ser21^{TPX2}$) and a downstream stretch in an α-helical conformation (residues $Asn30^{TPX2}$–$Asn43^{TPX2}$). The middle section of TPX2 that links these two motifs (residues $Leu22^{TPX2}$–$Gln29^{TPX2}$) is not resolved in the crystal structure and so is believed to be flexible and disordered.

To investigate whether the side-chain residues of this linker region contribute to the binding affinity of Aurora-A/TPX2 PPI an analogue was prepared incorporating a polyglycine chain in place of the linker domain (Table 1). Although recombinant expression would have provided the appropriate molecule, we require the ability to incorporate non-native amino acids going forward and so chose to prepare the peptide synthetically. Peptides of greater than 20 residues in length are known to be difficult to synthesize due to problems with on resin aggregation resulting in deletion sequences and/or complete failure of the synthesis.[20] Microwave assisted solid-phase peptide synthesis overcomes this problem simply through heating the coupling and deprotection reactions.[21] Using this

method and a Fmoc/^{t}Bu protection strategy the TPX2 proteomimetics were prepared in satisfactory yields.

Quantitative analysis of binding affinity (K_d) of the synthetic analogues, compared with the native TPX2 sequence, to the catalytic domain of Aurora-A (122-403) was achieved using isothermal titration calorimetry (ITC) (Figure 2). The measured Kd of the interaction between Aurora-A and synthetic native peptides (1-43) **1** and (7-43) **2** were similar to the published value (Table 1).[17] TPX2 analogue **3** incorporates an eight residue (and therefore equivalent length) polyglycine linker sequence that maintained binding affinity relative to the native sequence **2**, which suggests that the side-chain functionality of the amino acids in this linker region are not required for binding to Aurora-A. The *Xenopus* and puffer fish homologues of TPX2 incorporate a shorter linker sequence with three residues Gly26^{TPX2}-Thr28^{TPX2} missing.[15] A synthetic TPX2 analogue **4** with a shorter, five-residue polyglycine linker that is comparable to these other isoforms was prepared to investigate whether binding to Aurora-A would be compromised. This shorter analogue **4** gave comparable binding affinity to the native sequence **2**. To further probe the structural requirements of this linker an analogue incorporating an eleven-residue polyglycine linker **5** was prepared. An 8-fold weaker binding affinity was observed for this longer analogue which suggests that shorter but not longer linkers are tolerated. This would be expected based on the entropic penalty of folding the conformationally more flexible longer sequence **5** versus the shorter peptide sequence **4**.

Table 1. Sequences of TPX2 proteomimetics prepared and used in binding studies.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	
1 Native TPX2	Ac	M	S	Q	V	K	S	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	L	D	D	E	G	D	T	Q	N	I	D	S	W	F	E	E	K	A	N	L	E	N	NH_2
2 Native Linker							Ac	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	L	D	D	E	G	D	T	Q	N	I	D	S	W	F	E	E	K	A	N	L	E	N	NH_2
3 Gly_8 Linker							Ac	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	G	G	G	G	G	G	G	N	I	D	S	W	F	E	E	K	A	N	L	E	N	NH_2
4 Gly_5 Linker							Ac	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	G	G	G	G	G	-	-	-	N	I	D	S	W	F	E	E	K	A	N	L	E	N	NH_2
5 Gly_{11} Linker							Ac	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	G	G	G	G	G	G	G	$(G)_4$	N	I	D	S	W	F	E	E	K	A	N	L	E	N	NH_2
6 Extended motif							Ac	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	NH_2																							
7 Extended motif							Ac	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	L	D	D	E	NH_2																				
8 Stapled Helix																													Ac	N	I	D	S	W	F	E	S_5	K	A	N	S_5	E	N	NH_2
9 Native Helix																													Ac	N	I	D	S	W	F	E	E	K	A	N	L	E	N	NH_2
10 Stapled TPX2	Ac	M	S	Q	V	K	S	S	Y	S	Y	D	A	P	S	D	F	I	N	F	S	S	L	D	D	E	G	D	T	Q	N	I	D	S	W	F	E	S_5	K	A	N	S_5	E	N	NH_2

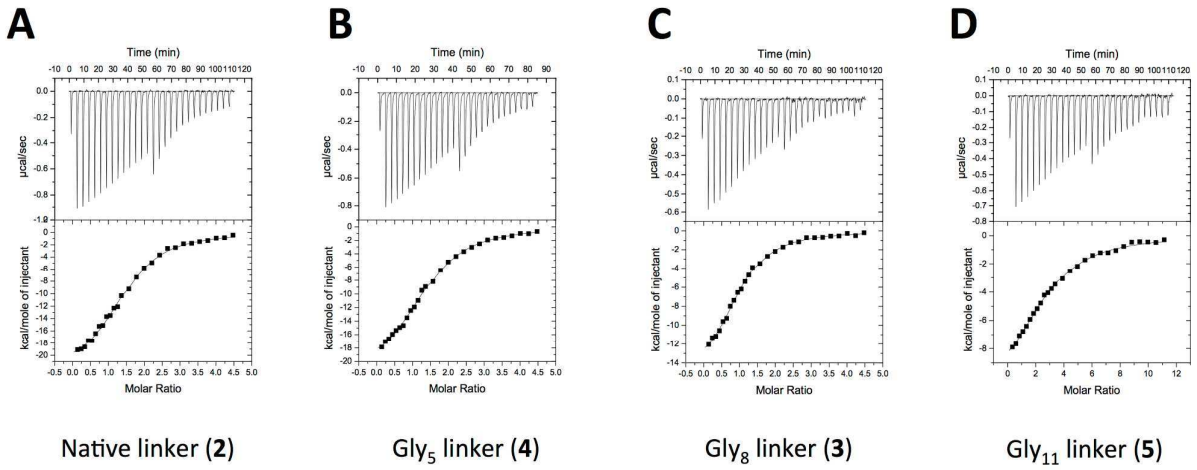

Figure 2. Representative ITC traces of the binding between Aurora-A and different TPX2 variants; native linker **2** (*A*) Gly_5 linker **4** (*B*) Gly_8 linker **3** (*C*) Gly_{11} linker **5** (*D*)

To probe the structural requirements of the extended region of TPX2 $Ser7^{TPX2}$-$Ser21^{TPX2}$ region further we synthesized a TPX2 peptide analogue **6** of this domain. Unfortunately this motif proved to be insoluble in aqueous solution and not amendable to use in ITC experiments. To overcome this solubility issue we chose to extend the sequence by four residues to include charged/polar residues $Leu22^{TPX2}$-$Glu25^{TPX2}$. This analogue **7** demonstrated good aqueous solubility, however did not bind to Aurora-A with significant affinity.

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Table 2. Thermodynamic parameters for binding of different TPX2 variants to Aurora-A as determined by ITC.

TPX2 variant	ID	Residues	K_a (10^3 M^{-1})	ΔH (kcal mol^{-1})	TΔS (kcal mol^{-1})	ΔG (kcal mol^{-1})	K_d (μM)
Native linker	**2**	7-43	468.67 ± 55.81	-23.00 ± 0.83	-15.37	-7.63	2.16 ± 0.28
Gly_5 linker	**4**	7-43*	377.00 ± 52.31	-18.38 ± 3.74	-10.89	-7.49	2.73 ± 0.38
Gly_8 linker	**3**	7-43	410.33 ± 22.03	-18.57 ± 1.58	-11.03	-7.54	2.46 ± 0.13
Gly_{11} linker	**5**	7-43#	68.20 ± 7.00	-15.92 ± 1.17	-9.43	-6.49	14.82 ± 1.52
Stapled helix	**8**	30-43¥	No binding detected				
Native helix	**9**	30-43	No binding detected				
Extended motif	**7**	7-25	No binding detected				
Native TPX2	**1**	1-43	308.33 ± 79.98	-26.19 ± 2.88	-18.81	-7.38	3.54 ± 1.15
Stapled TPX2	**10**	1-43¥	5505.00 ± 49.50	-42.57 ± 1.09	-33.55	-9.02	0.18 ± 0.00

K_a, binding constant; ΔH and ΔS, enthalpic and entropic terms; T = 293 K; ΔG, Gibbs' Free Energy change; K_d, dissociation constant. All experiments were performed at least in duplicate other than for Gly_{11} linker which was performed once. Values quoted were given by Origin software upon curve fitting and errors are the standard deviation between replicate values, or the errors given by Origin curve fitting for Gly_{11}.

* This peptide contains native residues 7-21 and 30-43, with 5 glycine residues linking the two domains
# This peptide contains native residues 7-21 and 30-43, with 11 glycine residues linking the two domains
¥ Glu37 and Leu41 replaced by unnatural α-methyl, α-alkenyl amino acid 'S_5'

Stapled peptides have recently come of age as tool compounds to disrupt PPIs mediated by an α-helix. First proposed by Grubbs[22] and then developed by Verdine and co-workers,[23] these constrained peptides have been designed to target a range of different biologically relevant PPIs including the AKAP complex.[24-27] These peptidomimetics have also been demonstrated to overcome a number of the physicochemical problems associated with peptides such as poor bioavailability, limited protease stability and a lack of membrane permeability.[28-30]

The downstream TPX2 domain forms an α-helical conformation (residues $Asp30^{TPX2}$–$Asp43^{TPX2}$) in the Aurora-A/TPX2 crystal structure and makes a series of key interactions that contribute to binding affinity. In solution TPX2 30-43 adopts a random coil, and the helix must therefore fold upon binding to Aurora-A (Figure 3). Incorporating a conformational constraint into the peptide sequence to induce an α-helix in this region of TPX2 should therefore overcome some of the entropic penalty of folding and provide a proteomimetic with increased binding affinity.

We initially designed a stapled peptide **8** based only on the helix region ($Asp30^{TPX2}$-$Asn43^{TPX2}$). Examination of the Aurora-A/TPX2 crystal structure provides the structural information required to select two residues on the solvent-facing side of the helix that are not involved in the binding event. Careful design of the staple was required because we predicted that stabilization of the helix C-terminal loop at residues $Trp34^{TPX2}$-$Phe35^{TPX2}$

would result in a steric clash with Aurora-A. As such, the *i*, *i* + 4 residues Glu37^{TPX2} and Leu41^{TPX2} were replaced with an α-methyl, α-alkenyl amino acid (S_5) in order to conformationally constrain the *N*-terminal loop of the helix. Macrocyclisation was accomplished on solid support using ring-closing olefin metathesis.

Circular dichroism spectroscopy confirmed that the native TPX2 (30-43) peptide **9** is a random coil with no defined secondary structure, but that the stapled TPX2 (30-43) peptide **8** is helical (Figure 4). Native TPX2 peptide (30-43) **9**, lacking the constraint, has a negative band at 199 nm, characteristic of random coil.[31] The stapled peptide **8** has a negative bands at 208 nm and 222 nm, characteristic of an α-helical conformation. However, neither the stapled peptide **8** nor corresponding native peptide **9** demonstrated significant binding affinity for Aurora-A. This suggests that the TPX2 helix region alone does not bind significantly to Aurora-A but requires the upstream-extended sequence motif (Ser7^{TPX2}–Ser21^{TPX2}) to enhance binding.

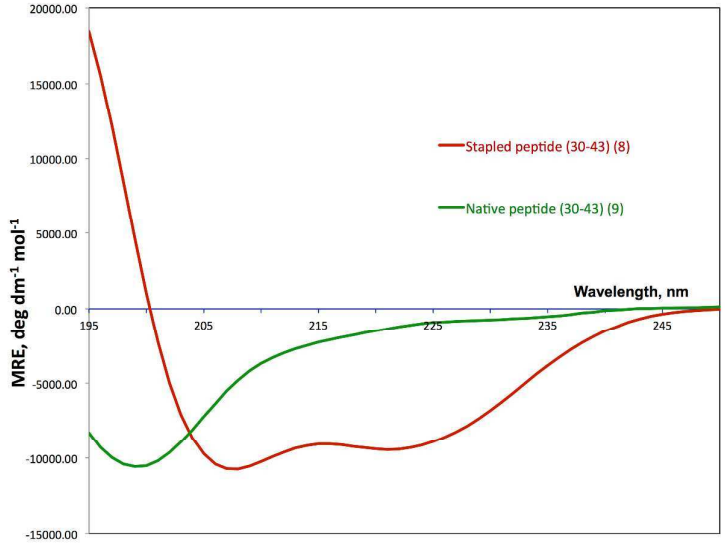

Figure 3. CD spectra of stapled TPX2 helix (30-43) peptide **8** and native TPX2 (30-43) peptide **9**.

With this knowledge, we decided to investigate if a full length stapled TPX2 peptide would bind with increased affinity when compared to the native sequence. The synthesis of this TPX2 stapled analogue **10** was achieved using microwave assisted solid phase synthesis and pseudoprolines to prevent on resin aggregation of the peptide (see Sup Info). In our initial attempt to synthesize this peptide we also observed aspartimide formation as a major side reaction. This was overcome by adding organic acid (e.g. oxyma) to the deprotection solution, generating piperidinium ion which suppresses aspartimide formation.[32]

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Gratifyingly, the stapled TPX2 peptide **10** was observed by ITC to bind with higher affinity than the corresponding unconstrained native peptide **1** (Figure 4A). The thermodynamic parameters determined from ITC measurements of native TPX2 peptide **1** and stapled TXP2 peptide **10** indicate that the Gibbs free energy of binding is predominantly driven in both cases by a favorable enthalpic term (ΔH –26.19 & –42.57 kcal/mol). This suggests that the conformationally constrained peptide **10** makes more favorable interactions with Aurora-A than the native peptide (*vide infra*).

Interestingly, the entropic term for the constrained peptide **10** is more unfavorable when compared to the native TPX2 peptide **1** (TΔS –18.81 to –33.55 kcal/mol). Two major terms contribute to the entropy of binding, the conformational entropy change and the desolvation entropy change.[33] We have demonstrated that the entropic penalty of folding the helix region can be overcome by the conformational constraint. However, the constraint may restrict the peptide from adopting the correct conformation for binding, which may be subtly different to the conformation of the unbound constrained peptide. This data can also be rationalized by considering the positive entropic contribution made by desolvation of water molecules coordinated to the native TPX2 peptide **1** backbone amide functionality. When constrained, this amide functionality is involved in the intramolecular H-bonding network of the α-helix and so is not available to coordinate water.

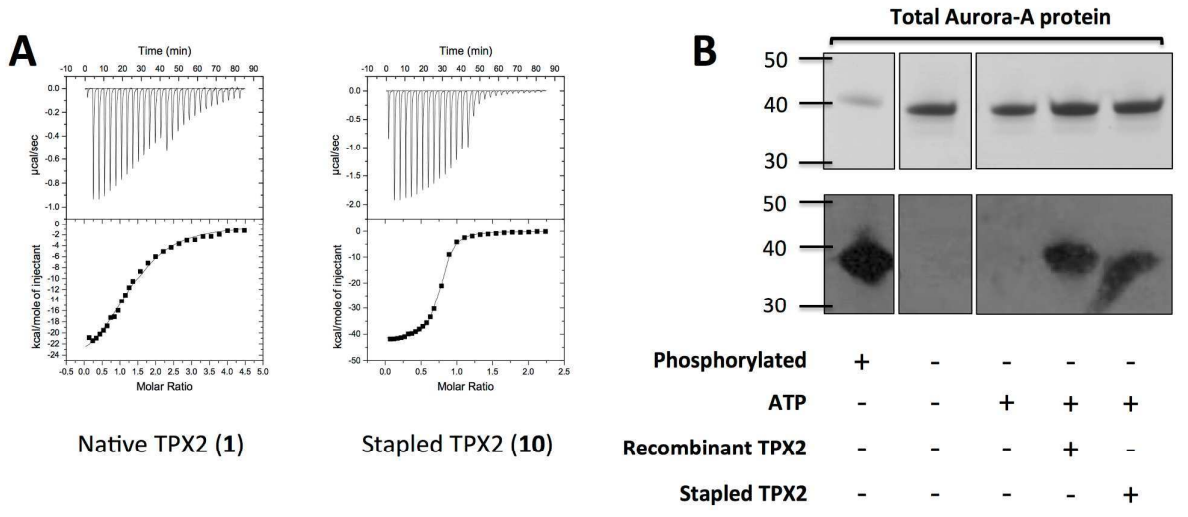

Figure 4. A. Representative ITC traces of the binding between Aurora-A and different TPX2 variants; native 1-43 (**1**), stapled 1-43 (**10**); **B**. Comparison of the effect of recombinant TPX2 1-43 and stapled TPX2 1-43 (**10**) (20 µM) on the autophosphorylation of Aurora-A (2.5 µM) on Thr288 through ATP (160 µM) turnover. Samples were separated by SDS-PAGE, analysed by Coomassie staining (top) and their phosphorylation state probed by Western blot using an antibody specific for phosphorylated Thr288 (Cell Signalling) (bottom). Aurora-A prepared in the absence of phosphatase, which is fully phosphorylated on Thr-288, is included as a positive control in the first lane and labelled “Phosphorylated”.

TPX2 stimulates the autophosphorylation of Aurora-A on Thr288, an event that can be detected using a site-specific antibody. We confirmed that the TPX2 1-43 proteomimetic **10** retained the ability to induce Aurora-A phosphorylation, similar to the recombinant, native 1-43 peptide (Figure 4B).

Encouraged by the relatively high binding affinity value found between the stapled TPX2 peptide and Aurora-A, we crystallized and solved the structure of the complex (PDB: 5LXM, Figure 5A). Electron density was visible for four more TPX2 residues in our model than in that of native TPX2; Ser6 at the N-terminus of the peptide, Leu22 at the start of the flexible linker region and Thr28 and Gln29 directly after the linker, suggesting a lower degree of flexibility in the stapled peptide compared to native TPX2. To model the hydrocarbon staple, we exchanged residues Glu37 and Leu41 for the unnatural amino acid 2-methyl-L-norleucine (PDB: MK8) and formed the double bond between the two alkyl chains using geometric and planar restraints (Figure 5B).

As with the previous structures of the Aurora-A/TPX2 complex, the interactions of the other two regions of TPX2 with Aurora-A are well-resolved. The region from $Ser7^{TPX2}$ to $Ser21^{TPX2}$ adopts an extended conformation characterized by minimal intramolecular contacts and extensive main and side chain interactions with Aurora-A. Residues $Tyr8^{TPX2}$, $Tyr10^{TPX2}$ and $Ala12^{TPX2}$ sit tightly in hydrophobic pockets between the β–$sheet^{AUR}$, helix αB^{AUR} and helix αC^{AUR}. Whereas segment $Phe16^{TPX2}$-$Phe19^{TPX2}$ tightly nestle in an adjacent hydrophobic pocket of Aurora-A. $Phe16^{TPX2}$ also forms a cation-π interaction with $Arg126^{AUR}$. Aromatic residues $Trp34^{TPX2}$ and $Phe35^{TPX2}$ in the helical region of TPX2 interact with $His187^{AUR}$ and $His280^{AUR}$. $Ala39^{TPX2}$ interacts with the activation segment at $Pro282^{AUR}$ (Figure 5C).

The staple itself clearly does not interact with the surface of Aurora-A (Figure 5C). The nearest Aurora-A residue, His187, is more than 9 Å from the staple. TPX2 residues known to be crucial for binding to Aurora-A (Trp34 and Phe35) remain in identical conformations as found in the native TPX2 structure.

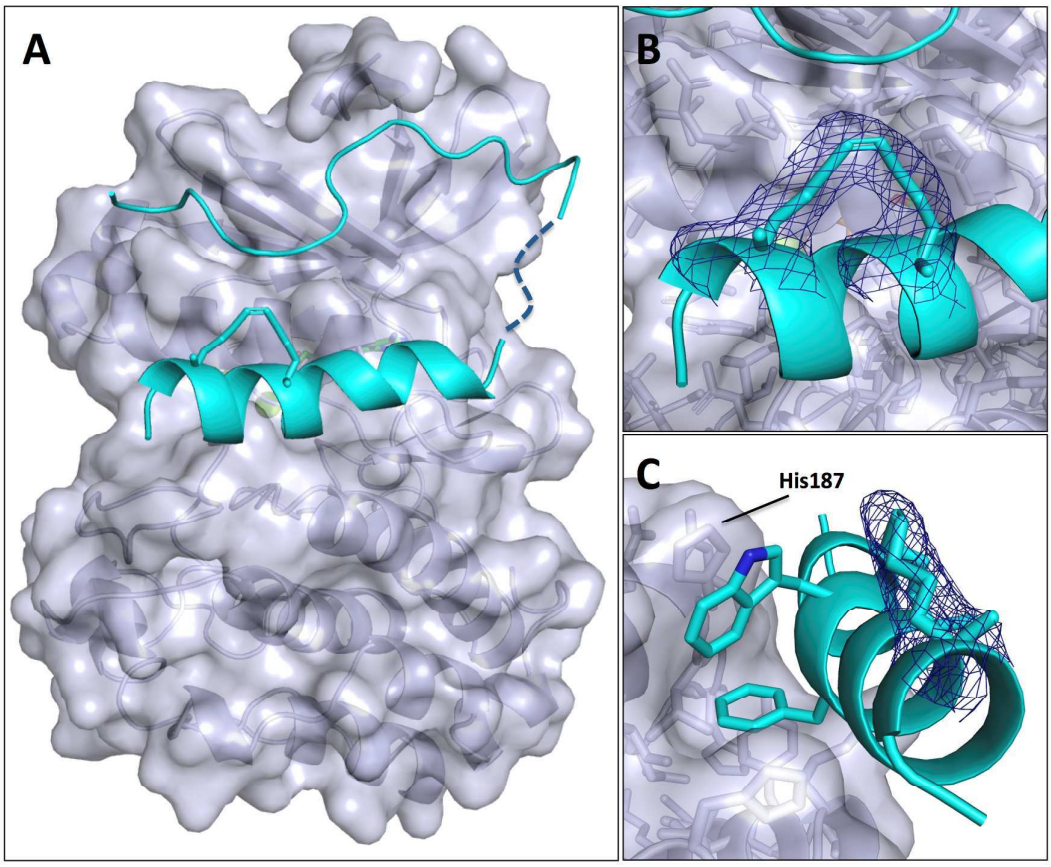

Figure 5. A. Aurora-A (grey) in complex with stapled TPX2 protein **10** (cyan) (PDB: 5LXM). The hydrocarbon staple is shown as sticks with the rest of TPX2 shown as cartoon. The flexible region linking the extended sequence and α-helical domains of TPX2 (not visible in the crystal structure) is represented as a dark blue dashed line. **B**. Zoomed in view of the hydrocarbon staple with the final 2mFo-DFc electron density map shown as wire-mesh contoured at 1.0 σ. **C.** Side view of the TPX2 helix showing the distance of the staple from the Aurora-A surface. The closest residue, His187, is 9.1 Å from the staple. Trp34 and Phe35 of TPX2, known to make crucial interaction with Aurora-A residues, are shown as sticks.

By superposing the existing structure of Aurora-A in complex with native TPX2 (PDB: 1OL5) onto our structure, we were able to easily visualize the influence of the hydrocarbon staple on the binding mode of the stapled TPX2 and its two domains (Figure 6). The N-terminal extended sequence motif of our stapled peptide overlays remarkably closely to that of native TPX2 (RMSD: 0.38) with residues known to be crucial for binding to Aurora-A in almost identical conformations between the two structures (Figure 6B and 6C). This indicates that the introduction of the staple has no effect on the binding mode nor conformation of the extended region of TPX2.

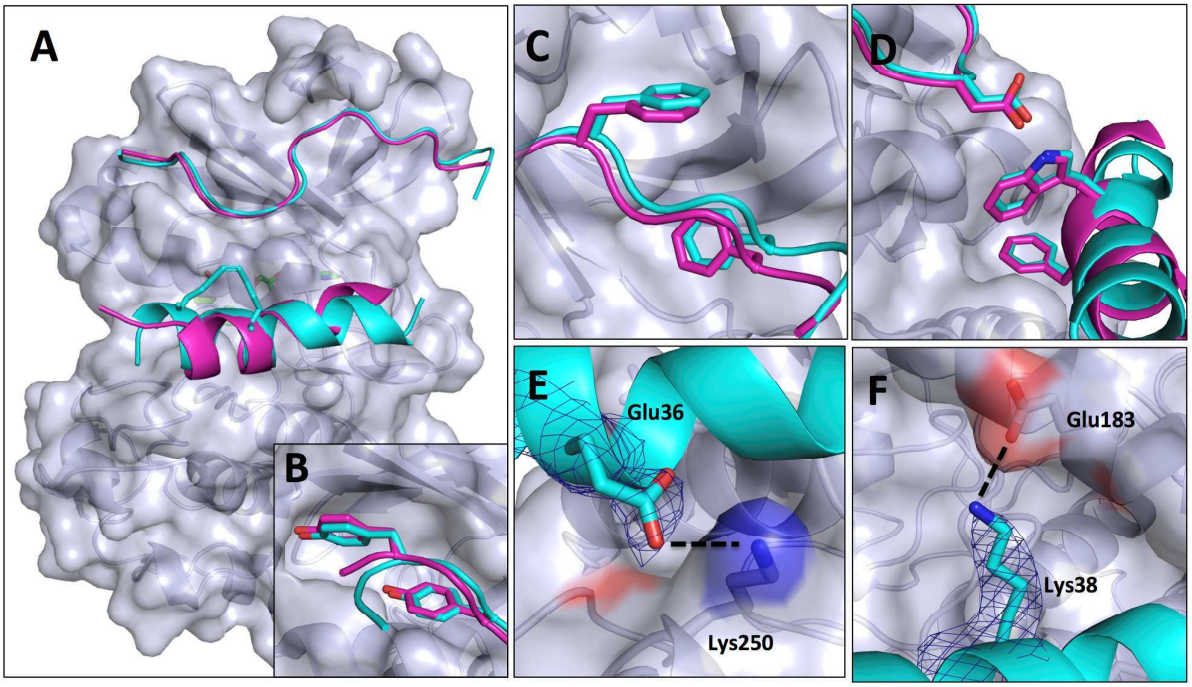

Figure 6. A. View of Aurora-A (grey) bound to stapled TPX2 (cyan) with native TPX2 (magenta, PDB: 1OL5) overlaid. TPX2 residues known to be crucial for binding to Aurora-A are shown as sticks to highlight the conserved binding mode between stapled and native TPX: **B.** Tyr8 and Tyr10, **C**. Phe16 and Phe19, **D.** Asp11 and Trp34, **E.** Potential salt bridges can be seen between Glu36^{TPX2} and Lys250^{AurA} and **F.** Lys38^{TPX2} and Glu183^{AurA}.

The conformation of the α-helical region of TPX2, in contrast, varies between stapled and native TPX2 structures (Figure 6A). The staple extends the length of the helix by an additional turn and the buried surface area at the interface with Aurora-A is increased by over 25% (RMSD: 1.63). The final turn is kinked and follows the contour of the surface of Aurora-A. This flips round the position of TPX2 Glu42, orienting this side chain towards Aurora-A in the stapled TPX2 structure. Despite these differences in the shape and length of the TPX2 helix, the residues on the helix known to be crucial for binding of TPX2 to Aurora-A, namely Trp34 and Phe35, overlay very well between stapled and native TPX2 (Figure 6D). In the context of the stapled TPX2 helix, electron density is clear for two additional charged side chains, Glu36 and Lys38, unlike in the native peptide. These side chains contribute to salt-bridge and/or electrostatic interactions with Aurora-A, which might explain why the enthalpic contribution of the interaction is increased (Figure 6E and 6F). Most of the water molecules at the interface are conserved between the two structures. However, in the structure of Aurora-A bound to stapled TPX2, there is a clearly defined molecule of MES from the crystallization buffer nestled between the C-terminus of the helix and the Aurora-A surface. In the native TPX2 structure, a sulfate ion is present instead, but in almost the same position as the sulfate moiety of the MES molecule (see supplementary

Aurora

information, Figure S1). This is likely due to the different crystallization conditions, which both contained 100 mM MES buffer. However the condition used for the native crystals has 200 mM sulfate, which might therefore be the dominant binding ligand. Alternatively, the slightly different binding mode of stapled TPX2 may generate a surface that complements that of MES more than the native TPX2.

From this data we can infer that the gain in affinity for interaction with Aurora-A seen with the stapled TPX2 peptide **10** over the native TPX2 peptide **1** is mainly due to more favorable enthalpic interactions. The data is also in agreement with the recently reported conformational selection binding pathway model.[34] Remarkably, neither of the two individual motifs in native or stapled TPX2 have measurable binding affinity, and yet the interaction is in the low micromolar range when they are tethered through a linker. We are currently investigating the basis of this effect using biophysical approaches.

The genes encoding Aurora-A kinase and its protein partner TPX2 are frequently co-amplified in cancers, and this complex, and both proteins individually, have been proposed as targets for cancer drug discovery. Due to the inherent difficulty in developing selective active site kinase inhibitors, targeting this protein-protein interaction (PPI) with allosteric small molecule ligands provides a novel strategy to develop Aurora-A inhibitors with enhanced selectivity.[36-38] Indeed, a recent study reported a small molecule, allosteric inhibitor of Aurora-A that binds in the hydrophobic pocket between the β sheet^{AUR}, helix αB^{AUR} and helix αC^{AUR} and blocks the interaction with TPX2.[39] Similarly, a synthetic single domain antibody, vNAR-D01, was shown to bind to the same pocket and inhibit Aurora-A through stabilization of a distorted conformation of the αC-helix.[40] It is very interesting that the same pocket can be used to positively and negatively regulate Aurora-A activity, and this opens up exciting avenues of research to investigate the consequences of manipulating Aurora-A activity in cancer cells.

In conclusion, as part of our investigations into the allosteric regulation of Aurora-A kinase we synthesized and characterization a conformationally constrained TPX2 proteomimetic spanning residues 1-43. ITC data revealed that the constrained TPX2 peptide binds Aurora-A with higher affinity than the corresponding native peptide and mimics the function of TPX2 in activating Aurora-A's autophosphorylation. More generally, this investigation provides further insights into the thermodynamic effects of pre-organizing peptides using conformational constraints and demonstrates that proteomimetics are useful tool

compounds for investigating interactions between intrinsically disordered domains of proteins.

Associated Content

Supporting Information Available: Describing experimental methods, results and analysis, and figures and tables. This material is available free of charge via the Internet.

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