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Switching Aurora-A kinase on and off at an allosteric site

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Abbreviations: PKA – protein kinase A, ATP – adenosine triphosphate, PPI – protein-protein interaction, AGC – protein kinase A, G, C family, sdAb – single domain antibody, HF – hydrophobic motif, vNAR – variable domain new antigen receptor, PDB – protein data bank, HV2 – hypervariable loop 2, CDR1 - complementarity-determining region 1, PP6 - protein phosphatase 6, PP1 – protein phosphatase 1, TPX2 – targeting protein for Xklp2, TACC3 – transforming acidic coiled-coil containing protein 3, PDK1 – phosphoinositide-dependent kinase-1, PKB/Akt – protein kinase B, RSK – ribosomal s6 kinase, CDK – cyclin-dependent kinase, EGFR – epidermal growth factor receptor, ADP – adenosine diphosphate.

Abstract

Protein kinases are central players in the regulation of cell cycle and signalling pathways. Their catalytic activities are strictly regulated through post-translational modifications and protein-protein interactions that control switching between inactive and active states. These states have been studied extensively using protein crystallography, although the dynamic nature of protein kinases makes it difficult to capture all relevant states. Here we describe two recent structures of Aurora-A kinase that trap its active and inactive states. In both cases, Aurora-A is trapped through interaction with a synthetic protein, either a single domain antibody that inhibits the kinase or a hydrocarbon-stapled peptide that activates the kinase. These structures show how the distinct synthetic proteins target the same allosteric pocket with opposing effects on activity. These studies pave the way for the development of tools to probe these allosteric mechanisms in cells.

Introduction

Many of the key signalling and regulatory events inside cells involve protein phosphorylation, a reversible and ubiquitous post-translational modification carried out by protein kinases. The human genome encodes more than 500 protein kinase domains, most of which are found in the context of a multidomain polypeptide chain, and many are components of multi-protein complexes [1]. The catalytic activities of protein kinases are regulated through interactions with these other domains and also by post-translational

modifications [2]. Protein-protein interactions also serve to localise signalling pathways to specific sub-cellular localisations to defined multi-protein complexes that assemble at the sites of kinase activity.

Protein kinases have conserved sequence and structural features that are organised into a similar conformational arrangement when the kinase is in an active state (Figure 1a,b). In contrast, these features are displaced in many different ways when a kinase is inactive. The catalytic domain of a protein kinase comprises two sub-domains or lobes, between which a molecule of ATP binds. The N-lobe is formed from a β-sheet and at least one α-helix, usually called the C-helix based on the canonical PKA structure [3]. The C-lobe is mostly helical and has an extended loop structure called the activation loop, activation segment or T-loop, which is the site of regulatory phosphorylation and forms part of the binding site for protein substrates. The structural basis of protein kinase activation has been the subject of many research articles, and has been extensively reviewed elsewhere [2, 4-7]. Therefore, for reasons of brevity we will focus on a subset of concepts relevant to the allosteric regulation of Aurora-A. Three features in particular are hallmarks of kinase active or inactive states: a Lys-Glu salt-bridge, the regulatory (R-) spine and the activation loop (Figure 1a,b). The Lys-Glu salt bridge brings together the β-sheet and C-helix and helps to position the phosphates of ATP for catalysis [2, 5]. The R-spine is a set of four hydrophobic residues that connect these same two major structural elements of the N-lobe with the C-lobe. The side chains of the R-spine residues form a connected, roughly linear pattern in active kinases, but are displaced from this arrangement in inactive kinases [4, 6].

Structural and biochemical studies have defined the molecular basis of protein-protein interactions (PPIs) involving kinases. As well as kinase-substrate interactions, regulatory interactions of kinases may involve symmetric or asymmetric dimerization or interactions with other binding partners. Studies of protein-protein interactions typically rely on genetic approaches. Small molecule inhibitors offer greater flexibility in terms of application to different cell types and the ability to deliver a known dose at a specified time point. However, the development of small molecule PPI inhibitors has considerable time and monetary costs. Moreover, the expertise required to carry out chemical synthesis is not usually available in a typical molecular, cell or structural biology laboratory. We therefore began to exploit the potential of peptide and protein-based reagents to target the protein-protein interactions of a model Ser/Thr protein kinase, Aurora-A, a key regulator of cell division [8].

Phosphorylation and interactions coordinate to activate Aurora-A

Aurora-A is related to the AGC family of Ser/Thr kinases, which are activated by phosphorylation of a site in their activation loop and, in many cases, one or more sites in a region C-terminal to the catalytic domain [9]. A hallmark of AGC kinases is their activation by a hydrophobic (HF) motif within a C-terminal extension to the kinase domain that fits into a hydrophobic surface on the N-lobe between the β-sheet and the C-helix called the PIF pocket (Figure 1c) [9, 10]. In addition, some AGC kinases have a helical N-terminal extension from which Trp and Phe (F/W) side chains protrude into a groove between the C-helix and activation loop (Figure 1c) [11]. PDK1 is an AGC kinase that lacks a HF motif and, instead, its

PIF pocket is used to recruit other AGC kinases, such as PKB/AKT and RSK, which are then phosphorylated and activated [9]. This property has been exploited in the development of small molecules that bind to the PIF pocket and block interactions and, interestingly, this same pocket can be used to activate or inhibit PDK1 [12-14]. Aurora-A has a similar core catalytic domain to AGC kinases, but lacks the N- and C-terminal extensions (Figure 1d). Instead, the HF motif and F/W side chains are provided by a binding partner, the microtubule-associated protein TPX2 (Figure 1e,f) [15]. HF motifs and the region of TPX2 that interact with the hydrophobic pocket are short extended peptides. However, the same surface area of the catalytic domain of many other kinases is involved in regulatory interactions with an α-helix on the surface of a folded partner protein, for example the binding of cyclins to CDKs, and the asymmetric dimerization of EGFR. The hydrophobic pocket of kinases is therefore a versatile binding site, able to accommodate an extended peptide, an α-helix or an organic compound of low molecular weight.

Aurora-A has an intrinsically low level of catalytic activity that is boosted through autophosphorylation on Thr288, one of a pair of adjacent threonine residues in a region of the kinase called the activation loop [16]. The mechanism of autophosphorylation remains undecided, with biochemical evidence in support of both intramolecular and intermolecular mechanisms [17, 18]. Aurora-A activity is also promoted through interaction with protein binding partners, such as the microtubule-associated proteins TPX2 and TACC3, the centrosomal protein Cep192, the ciliary protein Pifo and the transcription factor N-Myc [15, 19-23]. These regulatory partners stimulate autophosphorylation and, at least in the case of TPX2 *in vitro*, these two activation steps can combine synergistically to produce a hyperactive form of the kinase (Figure 2a) [24]. Crystal structures of Aurora-A in different

states of activity suggest a pathway for step-wise kinase activation (Figure 2b). In its unphosphorylated form, Aurora-A has been captured in an inactive conformation in which the R-spine and C-helix are distorted, disrupting a salt bridge between Glu181 and Lys162, and the activation loop is disordered or rearranged [18, 25]. Interestingly, the R-spine of Aurora-A is unusual in that the C-helix component is not hydrophobic, but is polar (Gln185). The side chain of this residue forms a H-bond with the main chain in the β-sheet, is critical for the activity of the kinase, and may contribute to the selectivity of Aurora-A inhibitors [26, 27]. Phosphorylation of Aurora-A on Thr288 or Thr287, reinforces the proper position of the C-helix and activation loop through interactions with a set of basic residues, interactions that can be mimicked in crystal structures by inorganic phosphate [15, 28, 29]. Alternatively, TPX2 binding on its own is sufficient to stabilize an active conformation of Aurora-A, which is then able to autophosphorylate [18]. This is because TPX2 binds to and stabilises the C-helix through the interaction of hydrophobic side chains, starting with a pair of tyrosine residues that bind into a so-called Y pocket, which is at the equivalent position in the structure to the PIF pocket of AGC kinases. However, TPX2 dramatically enhances Aurora-A activity even in the absence of phosphorylation, i.e. when Thr288 is mutated to a non-phosphorylatable residue [18, 24]. This is of physiological relevance because, at the location of the Aurora-A/TPX2 complex along spindle microtubules, Aurora-A is maintained in a dephosphorylated state by PP6 phosphatase [30]. TPX2 stimulates the activity of Aurora-A that is phosphorylated on Thr288 and protects this site from dephosphorylation by other phosphatases such as PP1. This is explained by the observation that the conformation of the activation loop matches that found in related kinase-substrate complexes only in the presence of TPX2 [15].

Small molecule inhibitors of Aurora-A have been developed and are under clinical evaluation as potential cancer therapeutics [31]. Because they bind to the conserved ATP site of the kinase, they have off-target activity that may contribute to toxicity. We reasoned that targeting less-conserved allosteric sites may result in compounds with superior selectivity, and would have the potential for diverse mechanisms of action such as inhibition or activation, or disruption of protein-protein interactions. As a starting point, we recently explored proteomimetic approaches to trap Aurora-A in active and inactive states using a single domain antibody (sdAb) or hydrocarbon-stapled peptide.

An allosteric, antibody-based inhibitor of Aurora-A

Single domain scaffolds such as nanobody, monobody and adhiron can be used to block protein-protein interactions and to trap specific conformational states [32]. Because they are genetically encoded, they can be expressed in cell models of disease to investigate signalling pathways, synergy with other inhibitors and resistance mechanisms. These reagents are based on stable protein scaffolds from which loops are displayed that form protein-protein interactions. Libraries can be generated in which the loop length and sequence are varied. For example, varying seven amino acids gives rise to over ten billion distinct proteins and yet screening of these libraries is both feasible and inexpensive using biotechnological methods such as phage display.

There are a few reported studies in which sdAbs have been used to activate or inhibit kinases through interactions with regulatory domains [33, 34]. We recently described a sdAb, vNAR-D01, based on a shark sdAb, that targets the catalytic domain of Aurora-A kinase and competes with the TPX2 interaction [35]. A crystal structure of the Aurora-

A/vNAR-D01 complex shows that the sdAb overlaps with the TPX2 binding site at the C-helix, inserting the indole ring of a tryptophan side chain into the Y pocket (Figure 3a-c). However, in contrast with the binding of TPX2, which stabilizes an active conformation of the kinase, binding of vNAR-D01 stabilizes an inactive conformation that closely resembles that observed with unphosphorylated Aurora-A. Two key interactions between the β-sheet and the C-helix, the salt-bridge formed by Glu181 and Lys162, and the H-bond between Gln185 of the R-spine and Leu194, are both disrupted in the inactive conformation. Therefore, we believe that sdAbs will be useful to trap kinases in an inactive conformation to facilitate structural studies on inactive forms.

The vNAR single domain antibody scaffold has three variable regions that provide opportunities to optimize potency and tailor selectivity by contacting multiple points on the target's surface. For example, in the complex with Aurora-A, the HV2/CDR1 region of vNAR-D01 contacts the activation loop of Aurora-A, an interaction that could be manipulated to further enhance inhibition through stabilization of an inactive conformation in this flexible region of the kinase. The region of Aurora-A to which vNAR-D01 binds is conserved in most other kinases at the level of tertiary structure, if not sequence. It is therefore likely that allosteric inhibitors targeting other protein kinases based on this scaffold could be identified. It is also conceivable that the scaffold could be modified to activate Aurora-A or other kinases. Single domain antibodies that control kinase activity could be useful for the localized regulation of kinase activity within cells, providing unique tools to manipulate signalling pathways.

An allosteric, proteomimetic activator of Aurora-A

Constrained peptides have been generated that target a range of different biologically relevant PPIs [36]. The first example of this applied to a kinase was to block the dimerization and subsequent activation of EGFR using triazolyl-bridged peptides that mimic one component of the dimerization interface [37]. Another way of forming constrained peptides is through hydrocarbon-stapling of an α-helix, which is achieved by replacing the native amino acids at two positions in the sequence spaced 3, 4 or 7 residues apart with unnatural amino acids that form a cross-link (or chemical staple) [38]. We recently applied this method to TPX2, which has an α-helix that forms upon interaction with the activation loop of Aurora-A [39]. A synthetic variant of this region of TPX2, residues 30-43, in which the residues at positions 37 and 41 were replaced with α-methyl, α-alkenyl amino acid (S5) was prepared and macrocyclised using ring-closing olefin metathesis. Circular dichroism spectroscopy confirmed that the stapled peptide was helical, unlike the equivalent peptide based on a native TPX2 sequence. The stapled helix segment was incorporated into a longer peptide comprising residues 1-43 of TPX2 (sTPX2) that had enhanced binding to Aurora-A compared to the native TPX2 sequence as measured by isothermal titration calorimetry.

The synthetic protein sTPX2 activated Aurora-A autophosphorylation to the same extent as recombinant TPX2 1-43 and therefore retained the principal function of this fragment of the native protein. The crystal structure of the complex revealed a similar yet more extensive interface between the stapled TPX2 (sTPX2) and Aurora-A (Figure 3d-f). As expected, the staple itself does not interact with the Aurora-A surface. The stapled helix has an extra half turn, resulting in the formation of additional salt bridges between Glu36 and Lys38 and Aurora-A, which might contribute to enhanced binding.

Future prospects

The inhibition of Aurora-A by vNAR-D01 provides compelling evidence that the kinase can be targeted through this allosteric mechanism. Moreover, the structure of the complex provides a good starting point for computational design of small molecule ligands that bind to this pocket. Conformational stabilization has the advantage of generating higher resolution data on the inactive state of Aurora-A, improving from 2.79Å (PDB 4C3P) in the absence of sdAb to 1.67 Å/ 1.79 Å (PDB 5L8L/5L8K) in the presence of vNAR-D01.

The first small molecule inhibitors that target the Y pocket of Aurora-A have recently been reported [40]. The most advanced compound that has been disclosed, AurkinA, binds Aurora-A with a Kd of 3.77 μM and, consistent with its binding mode, competes with TPX2 but not ATP. Interestingly, the crystal structure of the Aurora-A/AurkinA complex appears to be very similar to that of Aurora-A in a pre-active conformation with the Lys162-Glu181 salt-bridge, the Gln185-Leu194 H-bond and all secondary structure elements in their expected positions (Figure 3g-i). There are subtle shifts in the positions of the side chains around the inhibitor binding site, but the kinase is not locked into an inactive conformation, unlike the vNAR-D01 structure (Figure 3h). It will be very interesting to see how these compounds develop, and whether optimized compounds stabilise an inactive conformation of Aurora-A similar to that recognised by vNAR-D01.

The mechanism of Aurora-A inhibition by vNAR-D01 is potentially relevant to other kinases because activation mechanisms often involve movements of the C-helix [2]. Furthermore,

cancer mutations in this region of the kinase structure promote activation [41]. Allosteric kinase inhibitors that exploit this property include PDK1 inhibitors that bind to the PIF pocket, equivalent to the Y pocket of Aurora-A [14]. Protein-protein interactions involving kinases represent a rich, virtually untapped source of targets for therapeutic intervention in disease. We believe that sdAbs and stapled peptides could be very useful tools for the investigation of protein-protein interactions during the target validation phase of a project.

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Figure Legends

Figure 1. Schematic model of the Aurora-A activation pathway

(a) Crystal structure of protein kinase A (PKA, PDB 1JBP) shows the N- (NTE, teal) and C- (CTE, pink) terminal extensions to the catalytic domain, the activation loop (green) that harbours an activating phosphorylated threonine (green spheres), ADP (white spheres), the R-spine (orange surface), the C-helix (αC) and the Lys-Glu salt-bridge (blue and red spheres and lines, respectively). (b) Schematic illustration of the active conformation of PKA. Features coloured as in (a). (c) Magnified view of the hydrophobic grooves on the surface of the catalytic domain into which hydrophobic residues from the NTE and CTE are inserted (teal and pink spheres, respectively). (d) Crystal structure of Aurora-A/TPX2 complex (PDB 1OL5) shows how two stretches of TPX2 sequence (teal and pink) mimic the NTE and CTE of PKA. Residues coloured as in (a). (e) Schematic illustration of the Aurora-A/TPX2 complex. Features coloured as in (d). (f) Magnified view of the hydrophobic grooves on the surface of Aurora-A into which hydrophobic residues from TPX2 are inserted. Features coloured as in (d).

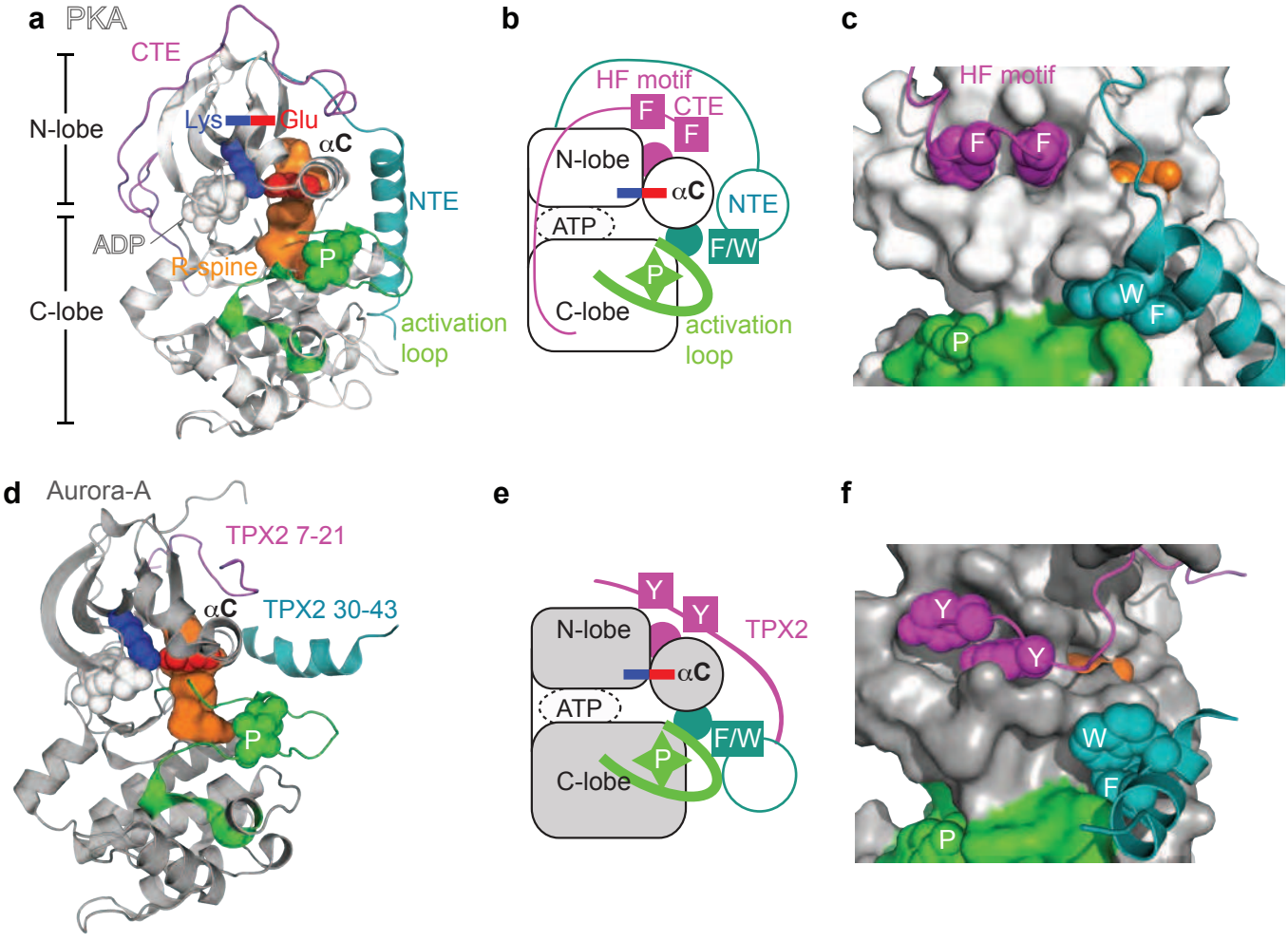
Figure 2. Multi-step activation of Aurora-A

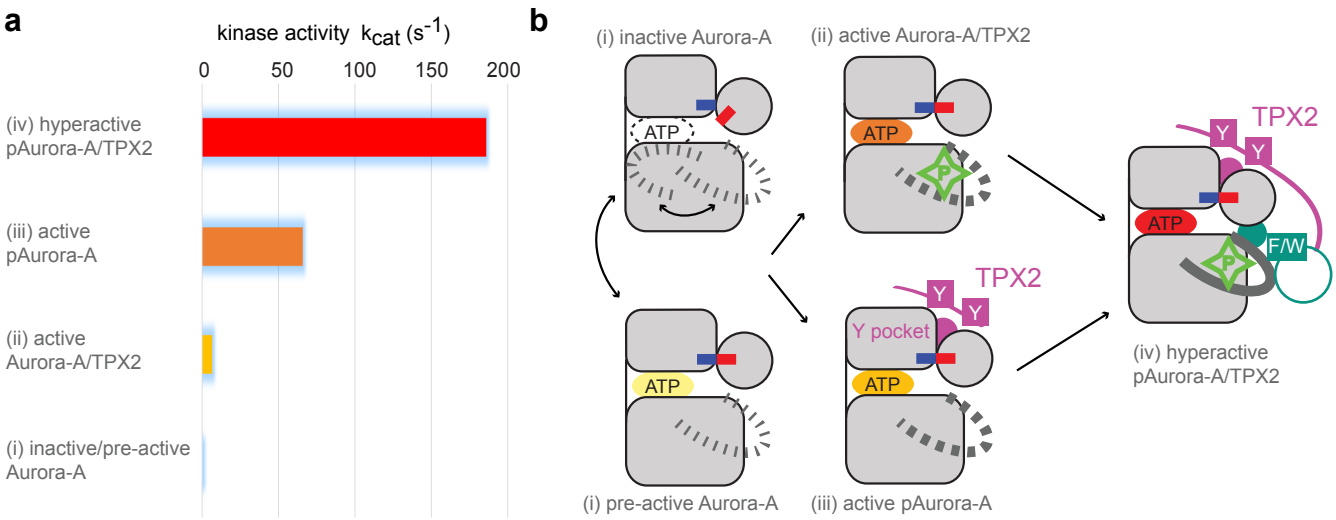
(a) The step-wise increase in Aurora-A activity upon TPX2 binding and phosphorylation is shown from bottom to top using published values [24]. (b) The step-wise activation of Aurora-A is shown schematically from left to right, with the relative activity indicated by the colour surrounding the ATP, which matches the corresponding bar in the chart in (a). Unphosphorylated Aurora-A has been captured in both partially (or pre-) active and inactive conformations, as indicated by the presence or absence of a Lys-Glu salt-bridge (indicated by joined or disconnected blue-red lines), as observed in the crystal structures with PDB 1MQ4/1OL6 and 4C3R, respectively. The activation loop is usually disordered (as shown by the thick, dashed grey line) or trapped in a flipped conformation in the presence of specific

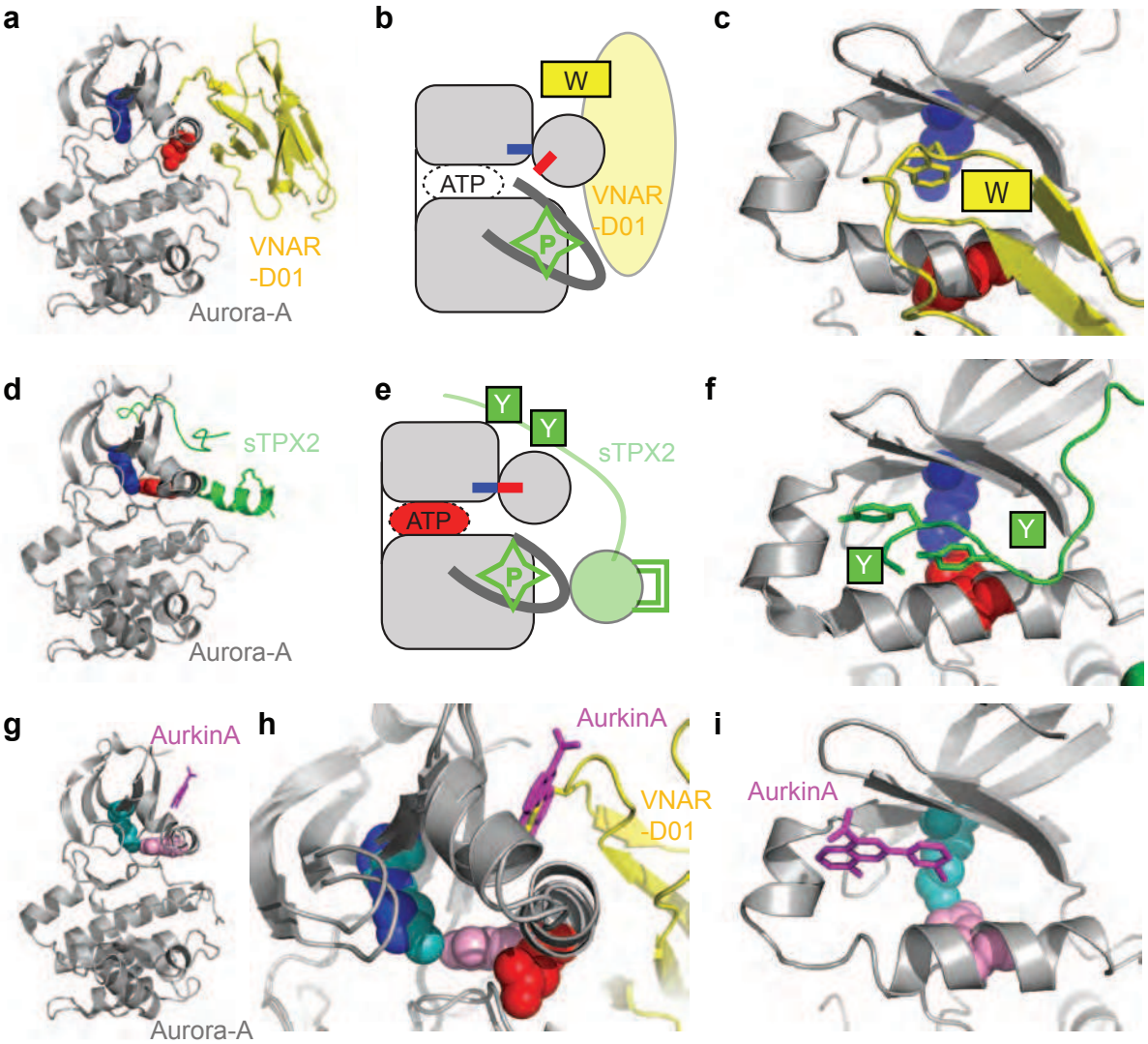
ATP-competitive ligands, as found in PDB 1MUO and 2WTV. Crystal structures of Aurora-A phosphorylated on Thr288 (green star; e.g. PDB 1OL7 and 4CEG) or bound to TPX2 (pink and teal; PDB 4C3P) show a pre-active conformation with a clear Lys-Glu salt-bridge, and the activation loop is more ordered but is not fixed in a conformation compatible with substrate binding. The combination of TPX2 and phosphorylation on Thr288 generates an active conformation with all structural elements in place for substrate binding and catalysis (PDB 1OL5).

Figure 3. Crystal structures of Aurora-A in complex with synthetic regulators

(a) Phosphorylated Aurora-A (grey) in complex with vNAR-D01 (yellow), a synthetic shark single domain antibody, is trapped in an inactive conformation as shown by the broken Lys-Glu salt-bridge (blue and red spheres, respectively). PDB 5L8L. (b) Schematic illustration of the Aurora-A/vNAR-D01 complex. (c) Magnified view of the Y pocket in the Aurora-A/vNAR-D01 complex. (d) Phosphorylated Aurora-A (grey) in complex with sTPX2 (green), a synthetic hydrocarbon-stapled peptide of the human TPX2 protein residues 1-43, is found in an active conformation with an intact Lys-Glu salt-bridge. PDB 5LXM. (e) Schematic illustration of the Aurora-A/sTPX2 complex. (f) Magnified view of the Y pocket in the Aurora-A/sTPX2 complex. (g) Aurora-A (grey) in complex with AurkinA (pink), a small molecule allosteric inhibitor, adopts an active conformation with an intact Lys-Glu salt-bridge (teal and pink spheres, respectively). PDB 5DT4. (h) Superposition of Aurora-A structures in complex with AurkinA (coloured as in (g)) and vNAR-D01 (coloured as in (a)). Note the binding of the two inhibitors to the Y pocket. (i) Magnified view of the Y pocket. AurkinA is represented by pink sticks. Kinase features are coloured as in Figure 1.


a
PKA
CTE
N-lobe
Lys
Glu
αC
NTE
ADP
R-spine
P
C-lobe
activation
loop
b
HF motif
F
CTE
F
N-lobe
αC
NTE
ATP
F/W
P
C-lobe
activation
loop
c
HF motif
F
F
W
F
P
d
Aurora-A
TPX2 7-21
αC
TPX2 30-43
P
e
Y
Y
TPX2
N-lobe
αC
ATP
F/W
P
C-lobe
f
Y
Y
W
F
P


a
kinase activity k_{cat} (s^{-1})
0
50
100
150
200
(iv) hyperactive pAurora-A/TPX2
(iii) active pAurora-A
(ii) active Aurora-A/TPX2
(i) inactive/pre-active Aurora-A
b
(i) inactive Aurora-A
(ii) active Aurora-A/TPX2
ATP
P
TPX2
Y
Y pocket
F/W
(i) pre-active Aurora-A
(iii) active pAurora-A
(iv) hyperactive pAurora-A/TPX2


a
VNAR
-D01
Aurora-A
b
W
ATP
VNAR
-D01
P
c
W
d
sTPX2
Aurora-A
e
Y
Y
sTPX2
ATP
P
f
Y
Y
g
AurkinA
Aurora-A
h
AurkinA
VNAR
-D01
i
AurkinA