Activation of Aurora-A Kinase by Protein Partner Binding and Phosphorylation Are Independent and Synergistic*

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Background: Phosphorylation and TPX2 act together to stabilize the active conformation of Aurora-A.

Results: TPX2 activates unphosphorylated Aurora-A, although phosphorylation makes a 2-fold greater energetic contribution to catalysis than TPX2.

Conclusion: Either activator partially stabilizes the active conformation and stimulates kinase activity.

Significance: Our revised model of kinase activation and mechanistic analysis might be applicable to other kinases.

Protein kinases are activated by phosphorylation and by the binding of activator proteins. The interplay of these two factors is incompletely understood. We applied energetic analysis to this question and characterized the activation process of the serine/threonine kinase Aurora-A by phosphorylation and by its protein partner, targeting protein for \textit{Xenopus} kinesin-like protein 2 (TPX2). We discovered that these two activators act synergistically and without a predefined order: each can individually increase the activity of Aurora-A, and the effect of both bound together is the exact sum of their individual contributions to catalysis. Unexpectedly, the unphosphorylated enzyme has catalytic activity that is increased 15-fold by the binding of TPX2 alone. The energetic contribution of phosphorylation to catalysis is 2-fold greater than that of TPX2 binding, which is independent of the phosphorylation state of the enzyme. Based on this analysis, we propose a revised, fluid model of Aurora-A activation in which the first step is a reduction in the mobility of the activation loop by either TPX2 binding or phosphorylation. Furthermore, our results suggest that unphosphorylated Aurora-A bound to the mitotic spindle by TPX2 is catalytically active and that the phosphorylation state of Aurora-A is an inaccurate surrogate for its activity. Extending this form of analysis will allow us to compare quantitatively the effects of the whole network of kinase-activating partners. Comparison with other kinases showed that kinetic characterization detects those kinases whose activation loops undergo a rearrangement upon phosphorylation and thus whose unphosphorylated state offers a distinct target for the development of Type II inhibitors.

Protein kinases catalyze many of the reactions crucial to cellular control. They often act in complexes with different binding partners, and these protein partners can act as regulatory units or confer substrate specificity \textsuperscript{(1)}. The majority of protein kinases are also regulated by phosphorylation on a region of their sequence known as the activation loop. Phosphorylation may be carried out autocatalytically or by the action of a regulatory kinase as part of a signaling cascade or network of interactions.

Given the ubiquitous nature of these control mechanisms, it is surprising how few quantitative studies there are of kinase activation. Instead, molecular biology, systems biology, and drug discovery programs have all made the approximation of a two-state system: either on or off, active or inactive \textsuperscript{(1–3)}. The first detailed characterization of activation kinetics was of protein kinase A (PKA), which is activated by a single phosphorylation of Thr(160) residue. Phosphorylation of this residue increases the rate of catalysis and also prevents rebinding of the inhibitory PKA regulatory subunit \textsuperscript{(4)}. This was followed by work on CDK2\textsuperscript{3}, but the limitations of detection of the assay system used meant that characterization was only possible for the binding of cyclin A to the phosphorylated kinase and the phosphorylation of cyclin-bound CDK2 \textsuperscript{(5)}. Although many conformations of CDK2 have been characterized structurally, this meant that a full study of the interactions of phosphorylation and cyclin binding could not be carried out. The only kinase we have found for which the interaction of multiple factors has been measured is the mitogen-activated protein kinase ERK2, which requires a double phosphorylation on the activation loop for full activity \textsuperscript{(6)}. Structures are available of un- and doubly phosphorylated ERK2, but the absence of monophosphorylated structures hampers the analysis of the relationship between structure and energetics. Remarkably, we have not been able to find a published characterization of the interaction of phosphorylation and a protein activator for any kinase.

Aurora-A is an oncogene-associated serine/threonine kinase active at the G2/M transition of mitosis and is currently the target of a number of drug discovery programs \textsuperscript{(7, 8)}. It localizes to the centrosome and to the mitotic spindle and binds to a number of different protein partners \textit{in vivo}, the best characterized of which is TPX2. Binding of the N-terminal fragment of TPX2 to Aurora-A activates the kinase by a factor of 2–7

\textsuperscript{3} The abbreviations used are: CDK2, cyclin-dependent kinase 2; TPX2, targeting protein for \textit{Xenopus} kinesin-like protein 2; AIR-1, Aurora/PI3K-related protein kinase 1; TPX1, TPX2-like family member 1; Fps, Fujimani poultry sarcoma; Fes, feline sarcoma.

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depending on the assay used (9–11). The x-ray crystal structure of the complex of TPX2 bound to phosphorylated Aurora-A showed that TPX2 interacts with both the N-terminal lobe and the activation loop, holding the kinase in an active conformation (10).

In this study, we characterized the four possible stages of activation of Aurora-A by two activators: phosphorylation and TPX2 binding. We addressed several outstanding issues such as the contribution of each activator to Aurora-A catalysis and whether TPX2 can stimulate the activity of unphosphorylated Aurora-A through an allosteric mechanism. Our findings led us to a fluid model of Aurora-A activation in which the first activator induces order in the activation loop in addition to having an activator-specific effect. Our data support a revised model of the activity of Aurora-A across the mitotic spindle in which unphosphorylated Aurora-A has a kinase-dependent role. Finally, analysis of the literature in the light of our new data shows that rearrangement of the activation loop upon phosphorylation can be detected using kinetic characterization. This provides an alternative method to x-ray crystallography to select kinases with a distinct inactive conformation that can then be targeted with an inhibitor discovery program (12).

EXPERIMENTAL PROCEDURES

Protein Expression—Point mutants of human Aurora-A kinase domain (residues 122–403) were generated using a QuikChange protocol, and all proteins were expressed with an N-terminal His6,3 tag and linker region in Escherichia coli using a pET30-based vector in LB medium (initial growth at 37 °C followed by 21 °C overnight after induction with isopropyl β-D-1-thiogalactopyranoside). Bacterial pellets were resuspended in 50 mM Tris, pH 7.5, 300 mM NaCl, 5 mM MgCl2, 40 mM imidazole, 10% (v/v) glycerol; lysed; and filtered, and tagged protein was separated from crude lysate using nickel affinity chromatography. The bound fraction was eluted using a step gradient of the same buffer (except with 250 mM imidazole), concentrated, and purified to homogeneity using a buffer of 50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM MgCl2, 10% (v/v) glycerol, 10 mM β-mercaptoethanol on either an S75 or S200 column. Resulting fractions were concentrated to around 10 mg/ml and flash frozen for future use.

TPX2 (residues 1–43) was expressed as for Aurora-A. Bacterial pellets were resuspended in 50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM imidazole for nickel affinity chromatography, and bound protein was eluted with 250 mM imidazole (other buffer constituents were as before). Eluted fractions were concentrated and then gel-filtered on an S75 column in the same buffer as for Aurora-A.

Kinetic Measurements—Kinetic assays were carried out in duplicate in gel filtration buffer using 1.5 μM fluorescein-labeled substrate (Flu-LRRASGL, Caliper peptide 21) in a Caliper EZ Reader II system at room temperature (assumed to be 298 K). Enzyme concentrations were as follows: Km measurements in the absence of TPX2 and TPX2 titration, 3 nM (WT and T287A), 800 nM (T288A), and 3 μM (T287A/T288A); Km measurements in the presence of TPX2, 3 nM (WT and T287A), 222 nM (T288A), and 1 μM (T287A/T288A). Km measurements were made using a 2-fold serial dilution from the highest concentration. The final reaction volume was 25 μl.

Kinetic Analysis—The contribution of a functional group to the energetics of catalysis can be determined by the ratio of kcat/Km in the presence and absence of the group (13, 14) as follows in Equation 1,
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FIGURE 2. Normalized activity of Aurora-A and mutants. a, $K_m$ for ATP determined for enzyme alone. b, TPX2 titration in 2 mM ATP (1 mM ATP for wild type). c, $K_m$ for ATP determined in the presence of 20 μM TPX2 (5 μM TPX2 for wild type). Red solid line, wild type; red dashed line, T287A; blue solid line, T288A; blue dashed line, T287A/T288A. Red graphs are plotted on the left y axis, and blue graphs are plotted on the right y axis. All graphs are normalized for enzyme concentration. Error bars show S.E. of reaction rates (i.e. S.E. of the slope determined by linear regression of reaction time courses with each time course carried out in duplicate and replicate data fitted together).
where $X$ is the enzyme in the presence of the functional group, $Y$ is the enzyme in its absence, and $\Delta G^\ddagger$ is the free energy change between the activated transition state for the reaction and the enzyme + substrate ground state (equal to $\Delta G_{cat} - \Delta G_{na}$ in Fig. 1).

Unlike many textbook examples, a kinase is a two-substrate enzyme, and both ATP and peptide need to bind before transfer of the $\gamma$-phosphate group and release of ADP (Fig. 1). The rate of the reaction shown in Fig. 1b is given in Equation 2.

$$v = \frac{k_{cat}[E][A][B]}{K_{mB} + [A][B]}$$

(Eq. 2)

This can be rewritten as

$$v = \frac{k_{cat}^\text{pseudo}[E][A]}{K_{mB}^\text{app} + [A]}$$

(Eq. 3)

using Equations 4 and 5.

$$k_{cat}^\text{pseudo} = \frac{k_{cat}[B]}{K_{mB} + [B]}$$

(Eq. 4)

$$K_{mB}^\text{app} = \frac{K_{mB}[B] + K_{mB}K_{mB}}{K_{mB} + [B]}$$

(Eq. 5)

In the case where $[B] \ll K_{mB}$, Equations 6 and 7 are used, thus yielding Equation 8.

$$k_{cat}^\text{pseudo} = \frac{k_{cat}[B]}{K_{mB}}$$

(Eq. 6)

$$K_{mB}^\text{app} = \frac{K_{mB}(1 + K_{mB}[B]/K_{mB})}{1 + [B]/K_{mB}} = K_{mB}$$

(Eq. 7)

$$k_{cat}^\text{pseudo} = \frac{k_{cat}[B]}{K_{mB}K_{mB}}$$

(Eq. 8)

Mechanistic analysis of Aurora-A has shown that catalysis proceeds according to a random sequential mechanism (9), and under our assay conditions ([substrate peptide] $\ll K_{mB}$ for substrate) Fig. 1b simplifies to Fig. 1c.

RESULTS

Kinetic Characterization of Aurora-A Kinase and Phosphosite Mutants in Presence and Absence of TPX2—We have previously used an automated kinase assay system based on the phosphorylation of a fluorescein-labeled peptide substrate to investigate the chemical inhibition of Aurora-A (15). The high quality and reproducibility of the data suggested to us that this assay would enable a detailed mechanistic analysis of the kinase including states that might exhibit a very low substrate turnover such as the unphosphorylated kinase.

The activation loop of Aurora-A contains two adjacent threonine residues, Thr-287 and Thr-288, and the phosphorylation of Thr-288 has been shown to increase the activity of the enzyme in cell extracts (16). To distinguish their effects, we mutated each residue to alanine (T287A, T288A, and the double mutant T287A/T288A) and characterized the activities of the resulting enzymes (Fig. 2a and Table 1). Mass spectrometry was unable to distinguish between phosphorylation at positions 287 and 288 of the tryptic digest peptide encompassing these residues but verified that wild-type and T287A mutants were phosphorylated. No phosphorylation was detected for the T287A/T288A double mutant (as expected) or, surprisingly, for Thr-287 in the T288A mutant. All four enzymes were catalytically active, although phosphorylation at position 288 undoubtedly increased the $k_{cat}$ and decreased the $K_m$ for ATP.

Aurora-A is activated at the mitotic spindle by the binding of TPX2 (10, 11). We determined the concentration of TPX2 required to saturate the activation of wild-type and mutant Aurora-A proteins (Fig. 2b) and then characterized the kinetic parameters of Aurora-A under these conditions. Unexpectedly, TPX2 was able to increase the activity of unphosphorylated Aurora-A (Fig. 2, b and c, and Table 1), although this was apparent at a higher concentration of TPX2 than that required for activation of the phosphorylated enzyme. Binding of TPX2 to Aurora-A decreased the $K_m$ for ATP and increased the $k_{cat}$ in all contexts, although the factor change was dependent on the presence or absence of Thr(P)-288.

Phosphorylated, unphosphorylated, TPX2-bound, and TPX2-free constitute four possible states of Aurora-A. The relative catalytic activity of these states (i–iv) are summarized on a relative scale in Fig. 3a, which shows that the most active form of the kinase is roughly 500-fold more active than the least active.

Phosphorylation and TPX2 Binding Act Independently—Two activators of catalysis may be independent of one another (the effect of both is the simple sum of the effects of each alone) or may exhibit cooperativity (the effect of both is greater than the sum of the effects of each individually). These two scenarios can be distinguished from one another by quantifying the energetic

| TABLE 1 |
| --- |
| Activity of Aurora-A constructs $^a$ |
| Values are for the fitting average of duplicate measurements. Errors are fitting errors. |
| | WT | T287A | T288A | T287A/T288A |
| Phosphorylation state | B | B | B | B |
| Enzyme alone | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) |
| | $36 \pm 3$ | $1.1 \pm 0.2$ | $33 \pm 1$ | $1.1 \pm 0.2$ | $121 \pm 9$ | $0.007 \pm 0.001$ | $157 \pm 12$ | $0.0016 \pm 0.0002$ |
| Enzyme + TPX2 $^b$ | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) | $K_m$ for ATP (µM) | $k_{cat}$ (min$^{-1}$) |
| $K_m$ for ATP (µM) | $2.2 \pm 0.1$ | $3.1 \pm 0.4$ | $2.4 \pm 0.2$ | $1.9 \pm 0.3$ | $51 \pm 5$ | $0.31 \pm 0.02$ | $76 \pm 6$ | $0.028 \pm 0.004$ |
| EC$_{50}$ for TPX2 (nM) | $10 \pm 3$ | $82 \pm 17$ | $970 \pm 130^d$ | $520 \pm 30$ |

$^a$ We were not able to measure a $K_m$ for peptide of Aurora-A using our assay system ($K_m > 500$ µM for a selection of peptides including a physiological substrate sequence), and so this value is a pseudo $k_{cat}$ incorporating the effects of substrate binding (full details are under “Kinetic Analysis” under “Experimental Procedures”).

$^b$ Values determined in presence of 5 µM TPX2 (wild type) or 20 µM TPX2 (other constructs).

$^c$ Measured in excess ATP: 1 mM ATP (wild type) and 2 mM ATP (other constructs).

$^d$ Larger error due to curve barely reaching saturation at highest concentration of TPX2.

[1153]
contribution of an activator to catalysis (see “Experimental Procedures” for details).

We calculated the effect of phosphorylation and TPX2 binding on catalysis by Aurora-A both individually and together (Table 2). TPX2 contributes 2.1 ± 0.2 kcal mol⁻¹ to catalysis irrespective of the phosphorylation state of Aurora-A, thus showing that TPX2 and phosphorylation act independently of one another. This compares excellently with a value of 2.3 ± 0.4 kcal mol⁻¹ for TPX2 in the context of the phosphorylated kinase calculated from independent data in Ref. 9. The comparable value for the contribution of cyclin A to phosphorylated CDK2 is 7 kcal mol⁻¹, and the value for unphosphorylated CDK2 is unknown (17).

We calculated the contribution of a single Thr(P) at position 288 to catalysis by comparing T287A and T287A/T288A (these differ by exactly one Thr(P): Thr(P)-288). This compares excellently with a value of 2.3 ± 0.4 kcal mol⁻¹ for TPX2 in the context of the phosphorylated kinase calculated from independent data in Ref. 9. The comparable value for the contribution of cyclin A to phosphorylated CDK2 is 7 kcal mol⁻¹, and the value for unphosphorylated CDK2 is unknown (17).

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**TABLE 2**

| Contribution | −TPX2 | +TPX2 |
|--------------|-------|-------|
| ΔΔG<sub>WT-T287A</sub> | 0.2 ± 0.2 | 0.7 ± 0.1 |
| ΔΔG<sub>WT-T288A</sub> | 3.7 ± 0.2 | 3.8 ± 0.2 |
| ΔΔG<sub>WT-T287A/T288A</sub> | 4.7 ± 0.2 | 4.8 ± 0.1 |
| ΔΔG<sub>WT(TPX2-noTPX2)</sub> | 2.3 ± 0.1 | 2.1 ± 0.2 |
| ΔΔG<sub>T287A(TPX2-noTPX2)</sub> | 1.8 ± 0.2 | 1.8 ± 0.2 |
| ΔΔG<sub>T288A(TPX2-noTPX2)</sub> | 2.1 ± 0.2 | 2.2 ± 0.1 |

**DISCUSSION**

**Fluid Model of Aurora-A Activation**—To better understand Aurora-A activation, we calculated the way in which the ener-
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**TABLE 3**

Contribution of phosphorylated residues to catalysis

| Kinase and context | Residue | Context | Overall contribution to catalysis | Fold decrease in $K_m$ for ATP | Fold decrease in $K_m$ for substrate | Fold increase in $k_{cat}$ | Fold decrease in $k_{cat}$ for substrate | Ref. |
|--------------------|---------|---------|-----------------------------------|-------------------------------|-----------------------------------|----------------------------|-----------------------------------|------|
| **Aurora-A**       |         |         |                                   |                               |                                   |                           |                                   |      |
| WT                 | Thr(P)-288<sup>a</sup> | Activation loop | 4.5 ± 0.1<sup>c</sup> | 3.0 ± 0.2 | 700 ± 100<sup>f</sup> | —<sup>e</sup> | —<sup>e</sup> | This study |
| Bound to TPX2      | Thr(P)-288<sup>a</sup> | Activation loop | 4.2 ± 0.1<sup>c</sup> | 18 ± 2 | 70 ± 10<sup>f</sup> | —<sup>e</sup> | —<sup>e</sup> | This study |
| CDK2               | Thr(P)-160/Thr(P)-157 | Activation loop | 6.5 ± 0.4<sup>d</sup> | 1.4 ± 0.7 | 140 ± 40<sup>d</sup> | 270 ± 130 | 35 ± 11<sup>f</sup> | 17 |
|                   | Thr(P)-160 | Glycine-rich loop (inhibitory) | —± 3.1 ± 0.1 | 1.0 ± 0.5 | 0.4 ± 0.1 | 0.015 ± 0.002 | (decrease of 65 ± 10) | 23 |
| **PKA**            | Thr(P)-197<sup>a</sup> | Activation loop | 3.8 ± 0.3<sup>b</sup> | 90 ± 25<sup>a</sup> | 30 ± 8<sup>a</sup> | 6 ± 1<sup>b</sup> | 1.8 ± 0.5<sup>b</sup> | 4 |
| **v-Fps**          | Thr(P)-1073<sup>a</sup> | Activation loop | 2.1 ± 0.5<sup>a</sup> | 1.1 ± 0.5 | 2.6 ± 0.8 | 42 ± 9 | 0.7 ± 0.4 | 24 |
| **p38α**           | Thr(P)-180<sup>a</sup> | Activation loop<sup>f</sup> | 3.9 ± 0.3<sup>m</sup> | — | — | — | — | 25 |
| WT                 | Thr(P)-182<sup>a</sup> | Activation loop<sup>f</sup> | 1.2 ± 0.1<sup>m</sup> | — | — | — | — | 25 |
|                   | Thr(P)-180<sup>a</sup> | Activation loop<sup>f</sup> | 4.5 ± 0.1<sup>m</sup> | — | — | — | — | 25 |
|                   | Thr(P)-182<sup>a</sup> | Activation loop<sup>f</sup> | 1.8 ± 0.3<sup>m</sup> | 7.9 ± 0.5 | 4 ± 1<sup>m</sup> | 4 ± 1<sup>m</sup> | — | 25 |
| **ERK2**           | Thr(P)-183<sup>a</sup> | Activation loop<sup>f</sup> | 1.9 | 1.2 | 2.5 | 80 | 2.5 | 6 |
| WT                 | Thr(P)-183<sup>a</sup> | Activation loop<sup>f</sup> | 2.3 | 16 | 0.8 | 1000 | 0.8 | 6 |
|                   | Thr(P)-183<sup>a</sup> | Activation loop<sup>f</sup> | 1.1 | 0.9 | 14 | 50 | 120<sup>f</sup> | 6 |
|                   | Thr(P)-183<sup>a</sup> | Activation loop<sup>f</sup> | 1.5 | 13 | 4.8 | 625 | 40<sup>f</sup> | 6 |

<sup>a</sup> Calculated from $k_{cat}/(K_m K_d)$.

<sup>b</sup> Compared with alanine.

<sup>c</sup> Calculated from Table 2.

<sup>d</sup> Increase in pseudo-$k_{cat}$ which combines effect of true $K_m$ and $k_{cat}$.

<sup>e</sup> Cannot be determined from data provided.

<sup>f</sup> Calculated with unphosphorylated residue.

<sup>g</sup> Assuming 10% error on non-linear.

<sup>h</sup> Making the same assumptions for $K_f$ of substrate and equivalence of T197D and T197D-GST as in Ref. 4.

<sup>i</sup> Full analysis of the rate constants for PKA (26) have shown that $K_m$(peptide) does not represent the dissociation constant of peptide and that $k_{cat}$ reflects the dissociation of ATP. Therefore, discussion in the text uses the detailed analysis in Ref. 4, not the numbers shown here.

<sup>j</sup> Compared with phenylalanine.

<sup>k</sup> Making the same assumptions for $K_f$ of substrate for Y1073F as in Ref. 24.

<sup>l</sup> The activation loop of a mitogen-activated protein kinase such as p38α and ERK2 contains a TXY motif, and phosphorylation on both the Thr and Tyr in this motif is required for complete activation.

<sup>m</sup> Calculated using EGF receptor as a substrate. Similar values were obtained using ATF2Δ109.

The energetic contribution of Aurora-A phosphorylation and TPX2 binding is divided between the $K_m$ for ATP and the $k_{cat}$ (Fig. 3). The order of TPX2 binding and Aurora-A phosphorylation in vitro is not known, and either pathway (1–2 or 3–4) may represent the situation at the mitotic spindle in cells. We discovered that although the total energetic contributions of phosphorylation or TPX2 binding are independent of one another (Table 2), the phylogenetic implementations of this are not. For example, phosphorylation of the free kinase (step 3) has a very small effect on the $K_m$ for ATP and a large (~160x) increase in $k_{cat}$, whereas phosphorylation in the presence of TPX2 (step 2) affects both parameters equally. Activation of the unphosphorylated kinase (step 1 or 3) is consistently associated with an increase in $k_{cat}$. Further activation is mostly in $K_m$ (step 4) or equally distributed between the two parameters (step 2). The significance of this will be discussed below.

Our measurements on Aurora-A cannot distinguish between effects on peptide binding and effects on the rate of phosphoryl transfer as our reported $k_{cat}$ is a pseudo-$k_{cat}$ including a contribution from substrate $K_m$. Either an increase in peptide binding or an increase in stabilization of the transition state would increase our measured value of $k_{cat}$. However, we used the data in Ref. 9 to subdivide the value shown in step 4 into a ~3-fold change in $K_m$ for peptide and a 1.2-fold change in true $k_{cat}$. This shows that, in this context, the effect of TPX2 is solely through ATP binding and peptide $K_m$.

How might this be interpreted with respect to the structural and physical states of the kinase? The current model of the interaction of multiple factors in kinase activation was proposed for CDK2. This assigns each activator a well defined physical effect that sum together to give the fully active complex (5,17). Cyclin A increases ATP binding and alignment and also increases substrate binding by stabilizing the activation loop conformation. The main effect of phosphorylation on Thr-160<sup>CDK2</sup> is stabilization of the transition state, but a small part of its role is to increase substrate binding.

In contrast to this, we propose a fluid model of Aurora-A activation based on our kinetic data and the observation from crystal structures that increased levels of Aurora-A activity are associated with increased order in the activation loop of the kinase (Figs. 3b and 4a). The first activation step of free, unphosphorylated Aurora is associated most strongly with ATP that neither can bring about on its own (steps 2 and 4 compared with steps 1 and 3), whereas the majority of the increase of $k_{cat}$ in step 2 is due to stabilization of charge in the transition state by pThr-288 (which we would not expect.
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![Image](https://www.jbc.org/content/jbc/287/2/1156.full.html)

**FIGURE 4. Activation loops of kinases for which activation process has been characterized by kinetic studies.** The crystal structures of activation loops are shown from DFG to APE sequence motifs, which mark the beginning and end, respectively, of the kinase activation loop. *a*, Aurora-A; *b*, PKA; *c*, p38α; *d*, CDK2; *e*, v-Fes, the human viral homologue to avian v-Fps; *f*, ERK2. Residues whose phosphorylation affects kinase activity are shown in stick representation. The phosphate groups of the Thr(P) residues (Tyr(P) for v-Fes) superpose in the fully active kinases when the different molecules are aligned across the kinase domain. Red, phosphorylated kinase; dark blue, unphosphorylated kinase; orange, phosphorylated Aurora-A bound to TPX2 (a only)/phosphorylated CDK2 bound to cyclin A (d only); cyan, unphosphorylated CDK2 bound to cyclin A (d only). Protein Data Bank codes selected are 1OL5, 1OL6, and 1OL7 (Aurora-A); 1BKX and 2UZW (PKA); 3PY3 and 1OUK (p38α); 1B38, 1B39, 1JST, and 1FIN (CDK2); 3CBL and 3CD3 (v-Fes); and 1ERK and 2ERK (ERK2). The activation loop is not visible in the unphosphorylated Aurora-A structure and is assumed to be disordered.

TPX2 to be able to do). The fluid model thus extends previous models of Aurora-A activation by TPX2 to include all four relevant states of the kinase.

**Structural Rearrangements Can Be Inferred from Kinetic Measurements**—The residues of the activation loop form part of the substrate binding pocket, and we thus predict that ordering of the activation loop will increase substrate binding. This prediction is supported by the data in Table 3 and associated references: in those kinases (CDK2 and ERK2) in which there is significant rearrangement of the activation loop upon phosphorylation, there is also an associated change in $K_a$ for substrate. This is not seen for those kinases (PKA and v-Fps) in which there is little or no activation rearrangement (Fig. 4).

Kinetic characterization thus provides a promising tool to detect those kinases in which phosphorylation leads to activation loop rearrangement. We expect such kinases to display a signature of decreasing $K_a$ for peptide and increasing $k_{cat}$ upon phosphorylation. This test can be performed at low enzyme concentrations in solution and provides conformational information in the absence of a crystal structure. This information could help inform decision-making in the drug discovery process as kinases that undergo activation loop rearrangements are likely to be more promising targets for the generation of inhibitors that bind the inactive conformation (e.g. Type II inhibitors).

**Is Spindle-associated Aurora-A Catalytically Active?**—In mitotic cells, Aurora-A is found at the centrosome (TPX2-independent) and on spindle microtubules (TPX2-dependent), and it has previously been assumed that the kinase is both catalytically active and phosphorylated at these locations. However, it has recently been shown that the role of Aurora-A (AIR-1 in *Caenorhabditis elegans*) in chromatin-stimulated microtubule assembly in *C. elegans* embryos does not require phosphorylation on Thr-288 (Thr-201 in *C. elegans*) and that unphosphorylated Aurora-A is localized to the microtubules by TPX2 (TPXL-1) (18). The authors concluded that the mechanism of Aurora-A action was independent of kinase activity, although they did not assess the catalytic activity of the unphosphorylated AIR-1 bound to TPXL-1. We have measured a considerable increase in the kinase activity of unphosphorylated Aurora-A upon binding to TPX2. Although the cellular concentration of TPX2 is not known, its localization in two dimensions along the microtubules means that local concentrations experienced by Aurora-A are expected to be high. This provides an alternative, kinase-dependent mechanism by which TPX2:Aurora-A complexes function to promote chromatin-stimulated microtubule assembly.

**Does Aurora-A Localization and Function along Spindle Microtubules Require Phosphorylation?**—Closer inspection of immunofluorescence studies in human cells discriminating between whole Aurora-A and that phosphorylated on Thr-288 suggests that only the pool of Aurora-A at the poles is phosphorylated (19–21). There could be a technical explanation for these observations, for example if the phosphorylated epitope is masked along spindle microtubules. Arguing against this is the fact that, in *HeLa* cells, phosphorylation of Aurora-A at the spindle becomes apparent in the absence of protein phosphatase 6 (the physiological phosphatase acting against Aurora-A) (22). It therefore seems likely that Aurora-A at spindle microtubules is not stably phosphorylated on Thr-288, but it remains to be confirmed whether the unphosphorylated Aurora-A is functional in human cells. These recent studies, together with our data, show that the regulation of Aurora-A by TPX2 is more complex than previously thought.

Aurora-A catalyzes the phosphorylation of its own activation loop, and consequently the phosphorylation state of Thr-288 on the activation loop of Aurora-A is frequently used as a cellular readout of Aurora-A activity and of the extent of enzyme
inhibition by small molecules. We have shown that unphosphorylated Aurora-A has catalytic activity and that this activity is greatly increased in the presence of TPX2. Because TPX2 does not protect Aurora-A from protein phosphatase 6 activity, a pool of TPX2-bound, active, unphosphorylated Aurora-A will be present in the cell. This pool is distinct from that of the phosphorylated enzyme (18), and studies relying on Thr-288 phosphorylation alone as a biomarker of Aurora-A activity may, unwittingly, limit their scope.

Conclusion—In conclusion, we have dissected the contributions of activation loop phosphorylation and protein partner binding to the activation of the kinase Aurora-A. Unexpectedly, the overall contribution of either activator is independent of the presence or absence of the other. The way in which the energy of activation is distributed between $k_{cat}$ and $K_m$ can be used to predict whether the activation step involves structural rearrangement in the activation loop and thus can be used to test whether pursuit of a Type II inhibitor program would be suitable. Our data show that the kinase activity of unphosphorylated Aurora-A can be increased by the binding of TPX2. This supports a new model of how Aurora-A activity is spread across the mitotic spindle in cells.

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