The oncogenic protein kinase Aurora A is a critical regulator of meiotic and mitotic cell cycles in eukaryotic cells. Aurora A autoactivation by autophosphorylation is promoted by specific non-catalytic binding proteins. One such protein is TPX2, a required spindle assembly factor in higher eukaryotes whose ability to activate Aurora A by direct binding to the kinase catalytic domain has been established by biochemical and structural analysis. In this report we clarify the autoactivation mechanism of Aurora A by demonstrating that of seven amino acids which become autophosphorylated by Aurora A, only Thr-295 is required for activity. Association of Aurora A with TPX2 leads to activation of the kinase, in parallel with phosphorylation of TPX2. We identify the sites as three Ser residues in the N terminus of TPX2; however, mutation of these residues does not affect Aurora A activation by TPX2. In contrast, the mutation of a putative Aurora A-binding motif in TPX2 abolishes both phosphorylation of TPX2 and activation of Aurora A. We have also investigated the interaction between *Xenopus* p53 and *Xenopus* Aurora A. p53 blocks the activity of either full-length Aurora A or the isolated catalytic domain. Interestingly, inhibition is blocked by TPX2, suggesting that the ability of Aurora A to transform cells could be regulated by p53, TPX2, or other binding proteins.

Reversible protein phosphorylation is a regulatory mechanism employed during all eukaryotic cell cycles, and several classes of protein kinase tightly regulate the essential processes of DNA replication, mitosis, and cytokinesis (1). Metazoans contain three classes of Aurora Ser/Thr protein kinase, termed A, B, and C, and each exhibits diverse subcellular localization during the cell cycle, reflecting the discrete role of each kinase during mitosis (2). Aurora was originally described in budding yeast (3) and in the fly (4), where it regulates centrosome separation and is required to generate spindle bipolarity, a precondition for accurate chromosome segregation. More recently, Aurora A homologs in higher eukaryotes have been shown to be critical for multiple steps in cell division, including regulation of the G2/M transition, the centrosome cycle, and spindle assembly and stability (5–8). Significantly, overexpression of either *Xenopus* or human Aurora A causes cell transformation, and Aurora A is overexpressed in numerous tumors, suggesting that its activity is critical in the etiology of cancer (9–15). Aurora A activity is regulated by phosphorylation at a conserved Thr residue (Thr-295 in *Xenopus*, Thr-288 in human) in the activation segment of the catalytic lobe (11, 16–18). Both Aurora A and Aurora B exist in distinct multiprotein complexes that ensure correct spatial localization and activation (2). Moreover, Aurora B has been shown to regulate its own activator, INCENP, by direct phosphorylation (19–21). Activation of Aurora A is thought to occur by an autocatalytic phosphorylation event in which partner proteins stimulate autoactivation by inducing precise alignment of the catalytic residues located between the two clefts of the active site (17, 22). Recently, two putative Aurora A activators have been described, the LIM domain-containing protein Ajuba (8) and the microtubule-associated protein TPX2 (17, 23). Interestingly, because Thr-295 phosphorylation can be rapidly reversed by associated phosphatases, leading to inactivation, TPX2 has also evolved a second regulatory role, the protection of phosphorylated Thr-295 from catalytic attack by protein phosphatases such as PP1 (17, 23). A recent crystal structure has elucidated the mechanism of Aurora A activation by TPX2 at the molecular level (22). TPX2 binding at two sites on Aurora A locks the catalytic domain of the kinase into an active configuration, generating favorable alignment of catalytic residues and unmasking the substrate-binding site. Moreover, TPX2 binding swings the domain containing phosphorylated Thr-295 into a phosphatase-inaccessible conformation by burying the labile phosphothreonine side chain (22).

Several putative physiological Aurora A substrates and binding proteins have been identified. These include Ajuba, TPX2, centrosomin, D-TACC, and p53 (8, 24–27). In some cases the binding of Aurora A to these proteins also localizes the kinase to the correct subcellular organelle, where it presumably phosphorylates substrates required for the orderly progression of mitosis. TPX2 is one of the most efficiently phosphorylated Aurora A substrates identified to date and has been shown to be crucial for Aurora A localization to spindle microtubules (24). TPX2 contains multiple consensus phosphorylation sites for Cdc2 and mitogen-activated protein kinase as well as seven putative nuclear localization signals (28). It is required for spindle assembly in *Xenopus* egg extracts and in HeLa cells and for the generation of stable spindle poles (28–30). During interphase TPX2 resides in the nucleus due to sequestration into complexes containing the proteins importin α and β. However, after nuclear envelope breakdown, the generation of high Ran-GTP levels adjacent to chromosomes stimulates release of TPX2 from the importins and binding to Aurora A (23, 31, 32). The subsequent molecular events driven by activated Aurora A and phosphorylated TPX2 are poorly understood. TPX2 is phosphorylated upon isolation from mitotic cell extracts (28). However, the specific sites and relative importance of these modi-

* § An Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed. Tel.: 303-315-7075; Fax: 303-315-7160; E-mail: jim.maller@uchsc.edu.

† This work was supported by National Institutes of Health Grant DK28353-21 and by the Howard Hughes Medical Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ An Investigator of the Howard Hughes Medical Institute.

§ This paper is available on line at http://www.jbc.org

The abbreviations used are: PP1, protein phosphatase 1; BSA, bovine serum albumin.
fications has not been explored. In this paper we investigate the regulation of Aurora A activity and identify several TPX2 phosphorylation sites. We analyze the role of this phosphorylation in TPX2-mediated Aurora A activation and also identify several mutations in the N terminus of TPX2 that uncouple Aurora A activation and TPX2 phosphorylation. The dynamic association of phosphorylated protein complexes containing Aurora A and TPX2 is likely to be important for both Aurora A and TPX2-dependent processes during the cell cycle.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—Hexahistidine-tagged Xenopus laevis Aurora A or TPX2 was expressed in Escherichia coli BL21 (DE3) as described (17). Site-directed mutagenesis was performed using pET30 Aurora A or pET30 TPX2 plasmid DNA as templates using standard mutagenesis procedures with Deep Vent DNA polymerase (NEB) to generate the required mutant cDNA. All cDNAs were fully sequenced to confirm mutations and to verify the absence of secondary point mutations. Aurora A activation by phosphorylation was investigated by mutation of several recently identified sites of autophosphorylation (18). The regulatory T-loop residue (Thr-295, equivalent to Thr-288 in human Aurora A) was mutated to Ala, Glu, or Asp. Xenopus TPX2 was mutated at three putative Aurora A consensus phosphorylation sites in the N terminus (Ser-48, Ser-90, or Ser-94). Mutation to either Ala or Asp was accomplished by changing the cognate triplet codon to GTA or GAC, respectively. TPX2 and Aurora A deletion mutants (including a catalytic fragment of Aurora A (11), encompassing residues 127–407) were generated by PCR and cloned into the vector pET30, which encodes an N-terminal hexahistidine tag to enable rapid purification. Recombinant His-tagged Aurora A and TPX2 mutants were induced for 16–18 h with 100 μM isopropyl-1-thio-D-galactopyranoside and purified from the soluble bacterial fraction on Talon beads (Clontech). After dialysis into 80% glycerol, the proteins were separated into aliquots and stored at –80 °C. GST-p53 (X. laevis) was purified using glutathione-Sepharose 4B beads (Amersham Biosciences) from BL21 (DE3) cells induced for 20 h with 400 μM isopropyl-1-thio-D-galactopyranoside. The purified protein was >80% pure as judged by Coomassie Blue staining and was stored at –80 °C before assay. X. laevis Survivin (20) was cloned from a Stage VI Xenopus cDNA library, and the cDNA was inserted into the vector pET41, which encodes an N-terminal hexahistidine S-transferase and His6 affinity tags. GST-Survivin was purified from strain BL21 (DE3) using glutathione-Sepharose beads. The antibody that recognizes Aurora A only when phosphorylated at Thr-295 has been described previously as having the corresponding sequence of Anderson- or Laemmli-type gels for analysis of Aurora A or TPX2 after phosphorylation (18).

Assay of Aurora A Activation by TPX2—Inactive (500 ng) or active (200 ng) recombinant Aurora A were preincubated on ice with 2 μg of recombinant TPX2-(1–364) or TPX2-(1–364) mutants in kinase buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM MgCl2, 0.1% (v/v) β-mercaptoethanol, 0.01% Brij 35, 10 mM okadaic acid, 10 mM MgCl2), 0.5 mg/ml histone H3 and then assayed in the presence of 100 μM [γ-32P]ATP for 15 min at 30 °C. Aurora A activity was assessed by TPX2 phosphorylation, histone H3 phosphorylation, or by Western blotting with an antibody that recognizes the active Thr-295-phosphorylated Aurora A. To quantify TPX2 and histone H3 phosphorylation, the Coomassie-stained bands were excised from the gel and analyzed by Cerenkov counting in a scintillation counter. Activity of Aurora A by TPX2-(1–364) or TPX2-(1–364) mutants was assessed using an assay that exhibited linear kinetics with respect to time, permitting direct comparison between the wild-type and mutant proteins. To assay Aurora A activation in the presence of TPX2 and/or histone H3 and GST-p53 or GST-Survivin, 5 μg of p53, 5 μg of Survivin, or 5 μg of BSA were preincubated for 10 min with Aurora A and TPX2 or histone H3 before phosphorylation for 15 min in the presence of 100 μM [γ-32P]ATP. Phosphorylated histone H3 or TPX2 was resolved by autoradiography.

Protection of Aurora A Dепhosphorylation by TPX2—Recombinant, active Aurora A (2 μg) was incubated in phosphatase buffer with 500 ng of PPIγ with or without preincubation with 2 μg of TPX2 or 2 μg of the TPX2-(1–364)/TPX2-(365–715) fragments. After 30 min the PPIγ was inactivated with okadaic acid (10 μM final concentration), and 200 ng of the Aurora A was assessed for activity using histone H3 (and TPX2) as substrate in the presence of 100 μM [γ-32P]ATP. The reaction was terminated with SDS sample buffer, and the TPX2-mediated protection of Aurora A inactivation by PPIγ was demonstrated by the simultaneous phosphorylation of histone H3 and TPX2. 32P-Labeled histone H3 and TPX2 proteins were visualized by autoradiography after SDS-PAGE on an 12% gel.

Determination of Stoichiometry of TPX2 Phosphorylation by Aurora A—To determine the stoichiometry of TPX2 phosphorylation by Aurora A, TPX2 (1–715, full-length), residues 365–715 (C-terminal half) or the N-terminal half of TPX2 containing amino acids 1–364 or mutant TPX2-(1–364) (S48A, S48A/S94A, and S48A/S90A/S94A, 60 pmol each) were incubated with 500 ng of recombinant active Aurora A for the indicated times at 30 °C in kinase buffer containing 100 μM [γ-32P]ATP of known specific activity (400 cpm/pmol). At each time point 10% of the reaction was withdrawn, and the reaction was terminated with SDS sample buffer. Covalent incorporation of phosphate into TPX2 was calculated from Cerenkov counting of the 32P-labeled Coomassie-stained TPX2 gel bands after separation from Aurora A by SDS-PAGE.

RESULTS

Importance of Thr-295 Phosphorylation for Aurora A Activity—Aurora A is as an essential mitotic phosphoprotein (8), and a significant stoichiometric phosphorylation of Xenopus Aurora A occurs at seven Ser and Thr residues after synthesis and autophosphorylation in bacteria (17, 18). The incubation of inactive Aurora A with TPX2, Ajuba, or Xenopus M-phase extracts, which contain a poorly defined array of Aurora A-activating proteins, also promotes the phosphorylation of Aurora A (11, 17, 23). These findings raise the possibility that phosphorylation of multiple amino acids may be required for the activity of Aurora A toward exogenous substrates. To determine the relative importance of these phosphorylated residues, we individually mutated the seven previously determined Aurora A phosphorylation sites (18) to Ala residues and expressed the mutant proteins in bacteria, where Aurora A stimulates its own autophosphorylation and autoactivation. This activation can be conveniently monitored by changes in the electrophoretic mobility of the kinase (17, 18). As a control we expressed kinase dead (D281A) or partially active (K169R) Aurora A mutants (18). As shown in Fig. 1 the only site of phosphorylation that is required for autoactivation is the T-loop residue, Thr-295 (11, 16, 17), because mutation of any of the other six residues to Ala does not abolish the decreased electrophoretic mobility of the kinase or block the activity of the kinase toward histone H3 or TPX2, two Aurora A substrates. Interestingly, mutation of the other Ser or Thr phosphorylation sites to Ala actually generates a minor enhancement of enzyme activity (e.g. compare T122A with wild type Aurora A), possibly due to reduced competition of autophosphorylation sites with the exogenous substrate histone H3. All kinase assays were performed so that substrate phosphorylation followed linear kinetics, to allow an accurate comparison of the activity of each mutant.

The requirement for Thr-295 phosphorylation for Aurora A activity is well documented (11, 17) as is the oncogenic phenotype of cells expressing a T295D mutant of Aurora A, which presumably leads to constitutive (phosphatase-insensitive) activity (Ref. 9, but see Ref. 11). Interestingly T295D or T295E mutants did not exhibit any detectable activity toward a non-specific substrate, histone H3, as previously described (Fig. 1, middle panel, and Ref. 11), but if the same mutants were preincubated with TPX2 and then assayed for phosphorylation of TPX2 (Fig. 1, bottom panel) or histone H3 (not shown), T295D and T295E exhibited significant phosphotransferase activity. This is consistent with recent crystallographic evidence that provides a molecular explanation for the extensive increase of Aurora A catalytic activity generated by association with TPX2 (22). Intriguingly, the loss of activity generated by mutation of T295 to Ala (or Val, not shown) can be partially rescued by binding of TPX2, but only using TPX2 phosphorylation as a marker of activity (compare Fig. 1, middle and bottom panels). A more significant rescue occurs with the partial active K169R mutant. However, no activation is evident
if the D281A (kinase dead) mutant is assayed in the presence of TPX2, confirming the specificity of the reaction (Fig. 1, bottom panel). These findings emphasize the significant enhancement of Aurora A activity that occurs upon TPX2 binding and suggest that Aurora A activity should be examined against several unrelated substrates to assess activity in vitro (17, 23). Ser-53 and Ser-349 are conserved in human and Xenopus Aurora A, and it is likely that Ser-53 phosphorylation regulates Aurora A degradation by the anaphase promoting complex/cyclosome in both species (33). However, Ser-53 phosphorylation is not required for kinase activity (Fig. 1 and Ref. 11). The mutation of Ser-12, Ser-53, Thr-103, Ser-116, or Thr-122 to Asp generates enzymes exhibiting wild-type activity after isolation from bacteria (data not shown); however, we confirmed a previous finding that mutation of Ser-349 to Asp markedly reduces activity (11). This seemingly contradictory result can be rationalized by structural data suggesting that this mutation may disrupt a conserved ion pair in the kinase, thus preventing stabilization of the active conformation (11, 22). Ser-349 phosphorylation may also be involved in the regulation of PP1 binding (11, 22), although this hypothesis requires further evaluation.

The N Terminus of TPX2 Is Sufficient for Aurora A Activation in Vitro—TPX2 stimulates Aurora A activity by both direct (enhancement of catalytic activity) and indirect (prevention of phosphatase-mediated inactivation) mechanisms (17, 22, 23). As shown in Fig. 2A, the N-terminal half of TPX2 (encompassing residues 1–364, NT) is sufficient to prevent PP1-mediated Aurora A inactivation. TPX2 blocks the ability of PP1γ to remove phosphate from Thr(P)-295 in the activation loop, thus preserving Aurora A activity as assessed by both histone H3 and TPX2 phosphorylation. Interestingly, a C-terminal fragment of TPX2 encompassing residues 365–715 (CT) is unable to prevent PP1γ-mediated inactivation of Aurora A nor is it a substrate for the kinase (Fig. 2A). To investigate these findings further two additional TPX2 deletion constructs were prepared encompassing amino acids 1–115 or 116–364 of the N-terminal half of the protein. The N-terminal 115 amino acids of TPX2 are the most highly conserved between human and Xenopus and, therefore, might represent the primary site of interaction between TPX2 and Aurora A (22). As shown in Fig. 2B, these amino acids were sufficient to induce activation of a completely inactive Aurora A molecule (top panel), and this 115-amino acid fragment was as good a substrate for the activated Aurora A as the full-length TPX2 (Fig. 2B, bottom panel). However, amino acids 116–364 were inefficient at activating Aurora A nor was this segment a proficient substrate (Fig. 2B). These data demonstrate that the N-terminal half of TPX2 and, more specifically, the N-terminal 115 amino acids both protect Aurora A from inactivation by PP1γ (Fig. 2A, Ref. 22, and data not shown) and directly stimulates Aurora A activity (Fig. 2B). These experiments also demonstrate that the Aurora A-mediated sites of phosphorylation on TPX2 are located in the N-terminal 115 amino acids.

Determination of TPX2 Phosphorylation Sites—To analyze the phosphorylation of the N terminus of TPX2 by Aurora A and to elucidate the site(s) of phosphorylation we directly assessed the covalent incorporation of [32P]phosphate into TPX2. As shown in Fig. 3A, phosphorylation of TPX2 by active Aurora A reached a plateau after 120 min, with the incorporation of ~1.2 mol of phosphate/mol into either full-length TPX2 (1–715) or TPX2 (1–364). Prolonged incubation did not lead to further phosphate incorporation, suggesting that phosphorylation had reached completion and establishing that fully phosphorylated TPX2 contained >1 mol of phosphate/mol of protein. Consistent with its inability to bind to Aurora A, the C-terminal half of TPX2 was not phosphorylated (Fig. 3A), as previously described (17). To identify the specific amino acids that were modified by Aurora A, we examined the sequence of Xenopus TPX2 and identified three putative Aurora A consensus motifs (RX(S/T); Ref. 34) at positions 48, 90, and 94. As shown in Fig. 3B, all three of these sites become phosphorylated, because sequential mutation to Ala decreased phosphorylation of the mutant proteins. Ser-48 and Ser-94 were phosphorylated more efficiently than Ser-90, although mutation of all three sites was required to completely prevent phosphorylation of TPX2 by Aurora A (Fig. 3B). These data are consistent with Kufer et al. (24), who observed that phosphorylation of human TPX2 by Aurora A occurred principally on Ser residues.

TPX2 Phosphorylation Is Not Required to Activate Aurora A—The Aurora A-related chromosomal passenger protein kinase, Aurora B, regulates its activator, INCENP, by phosphorylation. INCENP phosphorylation then regulates the activation of Aurora B, generating a positive feedback loop (19, 21). These findings suggested that TPX2 phosphorylation might also regulate activation of its cognate kinase, in this case Aurora A.

To examine this possibility mutant TPX2-(1–364) proteins containing either Ala (to prevent Aurora A phosphorylation) or Asp (to introduce negative charge and mimic phosphorylation) at Ser-48, -90, or -94 or combinations thereof were assessed for their ability to reactivate Aurora A, which had been inactivated by PP2A. When inactive Aurora A was incubated with TPX2-(1–364) or any of the phosphorylation site mutants, its ability...
to catalyze Aurora A autophosphorylation at Thr-295 was not diminished regardless of the mutation (Fig. 4A). Fig. 4B demonstrates that equal amounts of recombinant mutant were used in each experiment and illustrates how mutation of different residues affects TPX2 migration after SDS-PAGE and Coomassie staining of an Anderson gel (top panel). Non-phosphorylated recombinant TPX2 (1–364, predicted molecular mass, ~45 kDa) migrates highly anomalously (~60 kDa), and Aurora A phosphorylation induces a further retardation of the protein. This shift is dependent on phosphorylation at Ser-48, because a S48A mutant fails to shift in the gel upon phosphorylation, and TPX2 mutants containing the S48D mutation are constitutively retarded in the gel, even in the absence of phosphorylation (Fig. 4B, middle panel). These findings suggest that Asp mimics Ser-48 phosphorylation. Further evidence that the phosphorylation of TPX2 does not influence its activating ability is shown in Fig. 4B. The mutation of all three phosphoacceptor sites (Ser-48, Ser-90, and Ser-94) to either Ala or Asp does not prevent TPX2-dependent hyper-activation of recombinant Aurora A, as assessed by histone H3 phosphorylation. A robust Aurora A activation was also observed if 4-fold less of each TPX2 mutant was analyzed using either PP2A-inactivated or active Aurora A (data not shown), indicating that phosphorylation does not affect the dose dependence of TPX2 stimulation of Aurora A activity. Interestingly, the same phosphorylation site mutants of TPX2 were also equally effective in preventing PP1-mediated Aurora A inactivation (data not shown), consistent with the regulatory effect of TPX2 on Thr-295 accessibility (22).

To further investigate the binding of Aurora A to the N
terminus of TPX2, we exploited a recent crystallographic analysis of TPX2 and its interaction with the catalytic domain of Aurora A. Three conserved amino acids that closely contact Aurora A are Tyr-8, Tyr-10, and Asp-11, and these residues are conserved between human and *Xenopus* TPX2 (22). We reasoned that mutation of these residues to a small amino acid might prevent TPX2 binding and, therefore, block activation of Aurora A. As shown in Fig. 5, mutation of all three conserved residues to Ala eliminated activation of Aurora A by TPX2, as evidenced by a lack of both increased histone H3 kinase activity and phosphorylation of TPX2 (lanes 13 and 14). The mutation of Tyr-8 or Tyr-10 alone to Ala was sufficient to prevent Aurora A activation, as assessed by histone H3 phosphorylation, although TPX2 still became phosphorylated at a reduced level (lanes 5–8). However, combined mutation of both Tyr-8 and Tyr-10 to Ala severely blocked phosphorylation of TPX2 and completely abolished Aurora A activation, indicating that interaction with the kinase had been prevented (lanes 9 and 10). Mutation of Asp-11 to Ala alone had much less effect on the ability of TPX2 to activate Aurora A (lanes 11 and 12), demonstrating the critical importance of Tyr-8 and Tyr-10 in mediating binding and activation of Aurora A by TPX2.

**TPX2 Prevents Aurora A Inhibition by p53**—We are interested in investigating the regulation of Aurora A by distinct binding partners; one such Aurora A-interacting protein is the tumor suppressor p53 (27). The inhibition of Aurora A activity by p53 may be important for tumorigenesis, as demonstrated by p53-dependent inhibition of aneuploidy and transformation by Aurora A (27, 35). Human p53 has been reported to inhibit the activity of Aurora A through a transactivation-independent binding interaction with the non-catalytic region of the kinase (27). We examined the effect of p53 on Aurora A using purified *Xenopus* proteins. As shown in Fig. 6, *Xenopus* Aurora A activity was inhibited by wild-type *Xenopus* p53; however, inhibition was not dependent on binding of p53 to the N terminus of Aurora A (27), because p53 was still able to inhibit the isolated catalytic domain of *Xenopus* Aurora A. Inhibition was dose-dependent, and we observed maximal inhibition with 5 μg of GST-p53 but no inhibition with 5 μg of either GST-Survivin or BSA as controls (Fig. 6A). Strikingly, the ability of p53 to block either full-length or catalytic domain alone-mediated phosphorylation was blocked by TPX2-(1–364) (Fig. 6B), as assessed by the ability of Aurora A to phosphorylate histone H3 (or TPX2, not shown). These data establish that *Xenopus* Aurora A catalytic domain is inhibited by *Xenopus* p53 and support a model in which TPX2 and p53 compete for similar binding sites within the Aurora A catalytic domain.

**DISCUSSION**

*Xenopus* Aurora A is a functional homolog of human Aurora A as assessed by its ability to generate tumors in nude mice, effects on the centrosome cycle, spindle assembly, and the conservation of several regulatory phosphorylation sites (6, 7, 11). We have shown that sites of autophosphorylation besides Thr-295 are not required for generation of an active kinase. As demonstrated previously (11, 16–18), Thr-295 phosphorylation (Thr-288 in human Aurora A) is required for activation, but we now show that the mutation of Thr-295 to a negatively charged amino acid does indeed mimic activation, albeit only when the kinase is assayed in the presence of TPX2. In cells, Aurora A is likely to be complexed to PP1, Aujba, and/or TPX2, depending upon the status of the cell cycle. It is, therefore, expected that in the presence of an activating protein, Aurora A mutants in which the T-loop Thr is changed to Asp have sufficient activity to generate cellular transformation (9) even though the isolated kinase appears inactive when assayed with a nonspecific substrate *in vitro* (11, 35). In a similar vein, although K169R Aurora A does not cause tumors in nude mice (11), the same mutation does appear to generate aneuploidy in mammalian cells (35), whereas mutation of lysine to methionine blocks the ability of human Aurora A to override the mitotic spindle assembly checkpoint in cells exposed to taxol (15). It has been suggested that tetraploidy and centrosome amplification generated by Aurora A K162R (equivalent to K169R in *Xenopus*) is due to a kinase-independent function of Aurora A in human cells (35). However, we find that K169R Aurora A is partially active either alone or in the presence of TPX2 (Ref. 18 and Fig. 1), making it unlikely that known functions of Aurora A are kinase-independent. These findings could be reconciled by our finding that K169R Aurora A has at least 25% wild-type activity in the presence of TPX2, a physiological kinase substrate (Fig. 1), and this partial activity may be highly enriched at specific intracellular sites during mitosis, causing apparent defects in cytokinesis after overexpression (35).
In this study we have further investigated the regulation of Aurora A by TPX2 (17, 22, 23). We show that the N-terminal 115 amino acids of Xenopus TPX2 are sufficient for Aurora A activation (Fig. 2). These findings are in agreement with those of Bayliss et al. (22), who have identified amino acids 1–43 of human TPX2 as functional for both kinase activation and protection from dephosphorylation. These 43 amino acids, which include Tyr-8, Tyr-10, and Asp-11, are the most highly conserved between several metazoan species (22). A nonphosphorylatable mutant of TPX2-(1–364) containing either Ala or Asp was still able to stimulate Aurora A activity. 2 μg of the indicated recombinant TPX2 mutants (visualized by Coomassie stain, top panel) were incubated in the presence or absence of 200 ng of active Aurora A, 0.5 mg/ml histone H3, and 100 μM γ-32P-ATP. Aurora A-mediated TPX2 phosphorylation was assessed by the decrease in electrophoretic mobility (top panel) and the incorporation of [32P]phosphate label into TPX2 (middle panel). Aurora A activity in the presence or absence of each mutant TPX2 protein was assessed simultaneously by the phosphorylation of histone H3 (bottom panel).

In this study we have further investigated the regulation of Aurora A by TPX2 (17, 22, 23). We show that the N-terminal 115 amino acids of Xenopus TPX2 are sufficient for Aurora A activation (Fig. 2). These findings are in agreement with those of Bayliss et al. (22), who have identified amino acids 1–43 of human TPX2 as functional for both kinase activation and protection from dephosphorylation. These 43 amino acids, which include Tyr-8, Tyr-10, and Asp-11, are the most highly conserved between several metazoan species (22). A nonphosphorylatable mutant of TPX2-(1–364) containing either Ala or Asp was still able to activate Aurora A in vitro (Fig. 4). These mutated residues lie outside the minimal activation motif reported by Bayliss et al. (22). It is, therefore, consistent that phosphorylation of TPX2 at Ser-48, Ser-90, or Ser-94 does not regulate the binding or ability of TPX2 to activate Aurora A (Fig. 4). A similar crystallographic analysis of Aurora B and its activators, Survivin and INCENP, will be required to explain the recent finding that a nonphosphorylatable INCENP C-terminal mutant is a poor activator of Aurora B (19, 21). These studies emphasize fundamental differences between the feedback regulation of Aurora A and B by their substrates, in keeping with other differences between Aurora paralogs.

Our data strongly suggest that phosphorylation of Xenopus TPX2 at Ser-48 causes the marked band shift identified in previous experiments assessing endogenous TPX2 in mitotic extracts (28), since mutation of Ser-48 to Ala abolishes this shift and mutation to Asp constitutively mimics it (Fig. 4). Ser-48 phosphorylation could, therefore, likely cause a major regulatory conformational change in TPX2 during mitosis, perhaps bringing about changes in the conserved NLS or coiled coil domains that may affect binding to the importins or other regulatory proteins (28). Multiple Aurora A phosphorylation sites are also present in human TPX2, and human TPX2 is an efficient Aurora A substrate (17, 24); it is, therefore, likely that...
phosphorylation fulfills similar unidentified functions as with Xenopus TPX2. The importance of TPX2 in the cell cycle has been highlighted by several recent studies in which TPX2 levels were knocked down by degradation of TPX2 mRNA using specific small interfering RNA. In HeLa cells TPX2 is required for spindle formation, because it promotes microtubule assembly around chromatin, presumably by activating Aurora A (30, 31). TPX2 is also required for spindle pole integrity during mitosis (29), although the extent of “cross-talk” between TPX2 and Ajuba, a physiological centrosomal Aurora A activator (8), is currently unknown. TPX2 is an essential target of Ran-GTP for microtubule assembly in Xenopus extracts, raising the possibility that TPX2 phosphorylation mediated by Aurora A may be important for this process. TPX2 phosphorylation might also regulate reversible binding to inhibitory importin molecules, binding to microtubules or to motors that bundle microtubules (32, 36, 37). Interestingly, TPX2 was originally identified as a targeting protein for Xenopus kinesin-like protein 2 (38); therefore, a critical function for TPX2 phosphorylation may be the modulation of motor protein localization or activation. It will, therefore, be interesting to assess the effects of our phosphorylation site mutants on spindle formation and stability in Xenopus egg extracts.

Our finding that TPX2 regulates Aurora A by a phosphorylation-independent mechanism (Fig. 4) led us to investigate the effects of TPX2 on Aurora A in the presence of the regulatory molecule p53. In this study we have demonstrated that p53 inhibits the catalytic activity of Aurora A in vitro, although by a different mechanism than previously described (27). It will be interesting to determine the effect of p53 on Aurora A after immunoprecipitation from cellular extracts, which contain multiple Aurora A-binding proteins such as PP1, TPX2, and

**Fig. 5.** Mutation of the conserved Aurora A-binding element in TPX2 abolishes TPX2-stimulated Aurora A activation and phosphorylation. 2 µg of the indicated mutant TPX2 protein were assayed as described in the legend to Fig. 4. Coomassie stains of the TPX2 proteins (top panel) and autoradiograms showing incorporation of 32P into TPX2 (middle panel) or histone H3 (bottom panel) are presented. pTPX2, phosphorylated TPX2.

**Fig. 6.** TPX2 prevents inhibition of Aurora A by p53. A, 200 ng of active Aurora A or Aurora A active catalytic domain (amino acids 127–407) were incubated with 5 µg of Xenopus GST-p53 and 0.5 mg/ml histone H3 (gray bars) and 100 µM [γ-32P]ATP. Histone H3 phosphorylation was calculated by Cerenkov counting of the excised 32P-labeled bands obtained after SDS-PAGE. Control experiments contained 5 µg of BSA (open bars) or 5 µg GST-Survivin (black bars). B, same as A except 0.1 mg/ml of TPX2-(1–364) was included in the reaction. Histone H3 (or TPX2) phosphorylation was assessed in the presence of BSA (open bars), GST-p53 (gray bars), or GST-Survivin (black bars). The incorporation of phosphate was determined by Cerenkov counting of the 32P-labeled bands excised after SDS-PAGE. Data are presented as the mean ± S.E. from three independent experiments, each performed in duplicate.
Ajuba. TPX2 binds to the catalytic domain of Aurora A, but Ajuba interacts directly with the N-terminal non-catalytic domain (8, 24). The association of p53 with Aurora A in the presence of these molecules could, therefore, change during mitosis. Consequently it will be important to assess Aurora A activity during the cell cycle in cells exposed to a variety of cellular stresses, which increase p53 protein levels, or in cells that are null for the p53 gene. Fibroblasts lacking p53 are more sensitive to transformation by Aurora A than wild-type cells, and the ability of Aurora A to cause tumors is opposed by p53, indicative of a direct functional interaction between the two molecules (27, 35). Given the transforming ability of Aurora A and its consistent overexpression in many aggressive human tumors, chemically designed small molecules that regulate Aurora A function may be useful anti-proliferative drugs.

Acknowledgments—We thank Dr. Christopher Conn for useful suggestions and the University of Colorado Cancer Center Core facility for DNA sequencing (supported by National Institutes of Health Grant CA46934).

REFERENCES
1. Nigg, E. A. (2001) Nat. Rev. Mol. Cell Biol. 2, 21–32
2. Carreroa, M. T., and Earnshaw, W. C. (2003) Nat. Rev. Mol. Cell Biol. 4, 842–854
3. Chan, C. S., and Botstein, D. (1999) Genetics 135, 677–691
4. Glover, D. M., Leibowitz, M. H., McLean, D. A., and Parry, H. (1995) Cell 81, 95–105
5. Andresson, T., and Ruderman, J. V. (1998) EMBO J. 17, 5627–5637
6. Rugh, C., Giet, R., Uzbeckov, R., Morin, N., Charrain, I., Le Guellc, R., Couturier, A., Doree, M., Philippe, M., and Prigent, C. (1998) J. Cell Sci. 111, 557–572
7. Giet, R., and Prigent, C. (2000) Exp. Cell Res. 258, 145–151
8. Hirota, T., Kunitou, N., Sasayama, T., Marumoto, T., Zhang, D., Nitta, M., Hatakeyama, K., and Saya, H. (2003) Cell 114, 585–598
9. Bischoff, J. R., Anderson, L., Zhu, Y., Massie, K., Ng, L., Souza, B., Shryer, B., Flanagan, P., Clairvoyant, F., Gintner, C., Chan, C. S., Navotny, M., Slamon, D. J., and Blowman, G. D. (1998) EMBO J. 17, 3052–3065
10. Zhou, H., Krueg, J., Zhang, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. (1998) Nat. Genet. 20, 189–193
11. Littlepage, L. E., Wu, H., Andresson, T., Deanahan, J. K., Amundadottir, L. T., and Ruderman, J. V. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15440–15445
12. Ewart-Toland, A., Biassouli, P., de Koning, J. P., Mao, J. H., Yuan, J., Chan, F., MacCarthy-Mrrogh, L., Ponder, B. A., Nagase, H., Burn, J., Ball, S., Almeida, M., Linardopoulos, S., and Balmain, A. (2003) Nat. Genet. 34, 412–418
13. Li, D., Zhu, J., Froui, P. F., Abbruzzese, J. L., Evans, D. B., Cleary, K., Friess, H., and Sen, S. (2003) Clin. Cancer Res. 9, 991–997
14. Hamada, M., Yakushijin, Y., Ohitsuka, M., Kakimoto, M., Yasukawa, M., and Fujita, S. (2003) Br. J. Haematol. 121, 439–447
15. Anand, S., Penrhyn-Lowe, S., and Venkitaraman, A. R. (2003) Cancer Cell 3, 51–62
16. Walter, A. O., Seghezzi, W., Kerwer, V., Sheung, J., and Lees, E. (2000) Oncogene 19, 4906–4916
17. Evers, P. A., Erikson, E., Chen, L. G., and Maller, J. L. (2003)Curr. Biol. 13, 691–697
18. Haydon, C. E., Evers, P. A., Avinle-Wolf, L. D., Resing, K. A., Maller, J. L., and Ahn, N. G. (2003) Mol. Cell Proteomics 2, 1055–1067
19. Bishop, J. D., and Schumacher, J. (2002) J. Biol. Chem. 277, 27577–27580
20. Bolton, M. A., Lan, W., Powers, S. E., Mecklenburg, M. L., Kuang, J., and Stakenberg, P. T. (2002) Mol. Biol. Cell 13, 3064–3077
21. Honda, R., Kornner, R., and Nigg, E. A. (2003) Mol. Biol. Cell 14, 3325–3341
22. Baykiss, R., Sardon, T., Vernos, I., and Conti, E. (2003) Mol. Cell 4, 851–862
23. Tais, M. Y., Wiese, C., Cao, K., Martin, O., Donovan, P., Ruderman, J., Prigent, C., and Zheng, Y. (2003) Nat. Cell Biol. 5, 242–248
24. Kufer, T. A., Sillje, H. H., Kornner, R., Gruss, O. J., Meraldi, P., and Nigg, E. A. (2002) J. Cell Biol. 158, 617–623
25. Giet, R., McLean, D., Descamps, S., Lee, M. J., Raff, J. W., Prigent, C., and Glover, D. M. (2002) J. Cell Biol. 156, 437–451
26. Terada, Y., Uejake, Y., and Kuriyama, R. (2003) J. Cell Biol. 162, 757–763
27. Chen, S. S., Chang, P. C., Cheng, Y. W., Tang, F. M., and Lin, Y. S. (2002) EMBO J. 21, 4491–4499
28. Wittmann, T., Wilms, M., Karsenti, E., and Vernos, I. (2000) J. Cell Biol. 149, 1405–1418
29. Garrett, S., Auer, K., Compton, D. A., and Kapoor, T. M. (2002) Curr. Biol. 12, 1955–1959
30. Gruss, O. J., Wittmann, M., Yokoyama, H., Pepperkok, R., Kufer, T., Sillje, H., Karsenti, E., Mattaj, I. W., and Vernos, I. (2002) Nat. Cell Biol. 4, 871–879
31. Gruss, O. J., Carazo-Salas, R. E., Schatz, C. A., Guarguaglini, G., Kast, J., Wilm, M., Le Bot, N., Vernos, I., Karsenti, E., and Mattaj, I. W. (2001) Cell 104, 83–93
32. Schatz, C. A., Santarella, R., Roerger, A., Karsenti, E., Mattaj, I. W., Gruss, O. J., and Carazo-Salas, R. E. (2003) EMBO J. 22, 2090–2070
33. Littlepage, L. E., and Ruderman, J. V. (2002) Genes Dev. 16, 2274–2285
34. Littkepp, L. E., and Ruderman, J. V. (2002) Genes Dev. 16, 2274–2285
35. Cheeseman, I. M., Anderson, S., Jwa, M., Green, E. M., Kang, J., Yates, J. R., Chan, C. S., Drahun, D. G., and Barnes, G. (2002) Curr. Biol. 11, 163–172
36. Meraldi, P., Honda, R., and Nigg, E. A. (2002) EMBO J. 21, 483–492
37. Giet, R., Uzbeckov, R., Cubizolles, F., Le Guellc, K., and Prigent, C. (1999) J. Biol. Chem. 274, 15005–15013
38. Giet, R., and Prigent, C. (2001) J. Cell Sci. 114, 2095–2104
39. Wittmann, T., Boleti, H., Antony, C., Karsenti, E., and Vernos, I. (1998) J. Cell Biol. 143, 675–685