Aurora B Kinase Exists in a Complex with Survivin and INCENP and Its Kinase Activity Is Stimulated by Survivin Binding and Phosphorylation

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Aurora B regulates chromosome segregation and cytokinesis and is the first protein to be implicated as a regulator of bipolar attachment of spindle microtubules to kinetochores. Evidence from several systems suggests that Aurora B is physically associated with inner centromere protein (INCENP) in mitosis and has genetic interactions with Survivin. It is unclear whether the Aurora B and INCENP interaction is cell cycle regulated and if Survivin physically interacts in this complex. In this study, we cloned the *Xenopus* Survivin gene, examined its association with Aurora B and INCENP, and determined the effect of its binding on Aurora B kinase activity. We demonstrate that in the *Xenopus* early embryo, all of the detectable Survivin is in a complex with both Aurora B and INCENP throughout the cell cycle. Survivin and Aurora B bind different domains on INCENP. Aurora B activity is stimulated >10-fold in mitotic extracts; this activation is phosphatase sensitive, and the binding of Survivin is required for full Aurora B activity. We also find the hydrodynamic properties of the Aurora B/Survivin/INCENP complex are cell cycle regulated. Our data indicate that Aurora B kinase activity is regulated by both Survivin binding and cell cycle-dependent phosphorylation.

INTRODUCTION

Defects in chromosome segregation can generate aneuploidy, a condition that is found in almost all human tumors and is a major cause of miscarriages and birth defects. The complex process of chromosome segregation must be highly regulated to ensure fidelity and to prevent aneuploidy. Many of the mitotic events are regulated by the kinetochore, a proteinaceous structure assembled on centromeric DNA that coordinates at least three mitotic functions (for review, see Rieder and Salmon, 1998). First, the kinetochore is the chromosomal site of microtubule attachment and move-

ment. Second, the kinetochore is the major site of cohesion between sister chromatids. This cohesion must be maintained through metaphase and its dissolution is the critical event that triggers anaphase. Third, kinetochores that are not attached to microtubules send signals to the cell cycle machinery to prevent this dissolution of cohesion, a process referred to as the spindle assembly checkpoint. This checkpoint ensures that all chromatids are attached before the onset of anaphase. How the kinetochore coordinates these various functions is a critical unanswered question.

A group of mitotic regulators that includes Aurora B kinase and the inner centromere protein (INCENP) has been given the name chromosomal passengers (Adams *et al.*, 2001a). The passenger proteins are defined by a dynamic pattern of localization (Mackay and Earnshaw, 1993). In G2 and prophase, the passenger proteins have a general chromosomal localization. During prometaphase and metaphase, they move from chromosomes to inner centromeres. When chromosomes move to the poles during anaphase, passenger proteins remain at the spindle midzone where they eventually become part of the midbody (Cooke *et al.*, 1987; Bischoff *et al.*, 1998; Schumacher *et al.*, 1998b; Adams *et al.*, 2001a).

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Abbreviations used: EST, expressed sequence tag; GST, glutathione S-transferase; I, interphase; INCENP, inner centromere protein; IP, immunoprecipitation; MBP, myelin basic protein; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.1% Tween 20; PCR, polymerase chain reaction; Pre-I, preimmune sera; xAurora B and xAurB, *Xenopus* Aurora B; xINCENP, *Xenopus* inner centromere protein; xSurvivin, *Xenopus* Survivin.

The phenotypes of the chromosomal passenger proteins suggest that they are critical regulators of various steps of chromosome segregation. RNAi experiments in *Caenorhabditis elegans* embryos and *Drosophila* cell lines suggest that cells lacking Aurora or INCENP have similar mitotic defects. First, the passenger proteins are necessary for the proper segregation of DNA. During anaphase, the chromosome masses do not properly segregate, leaving a chromatin bridge between the major DNA masses (Schumacher *et al.*, 1998a; Adams *et al.*, 2000, 2001b; Kaitna *et al.*, 2000; Giet and Glover, 2001). Second, cytokinetic furrows begin but fail to fully progress in cells lacking either Aurora or INCENP (Mackay *et al.*, 1998; Schumacher *et al.*, 1998b; Kaitna *et al.*, 2000; Adams *et al.*, 2001b; Oegema *et al.*, 2001). This suggests a second role for Aurora and INCENP in cytokinesis. Third, studies from budding yeast suggest that Aurora is involved in bipolar attachment of microtubules to kinetochores. The Aurora homolog Ipl1 is required during chromosome segregation, as cells without Ipl1 activity have massive nondisjunction and often segregate both sisters' chromosomes to the same pole. Ipl1 is required for kinetochores to release spindle microtubules both in vitro and in vivo (Biggins *et al.*, 1999; Biggins and Murray, 2001; Tanaka *et al.*, 2002). Current models propose that Ipl1 ensures that each chromosome obtains bipolar attachment of microtubules by coordinating the release of kinetochores attached from a single pole, thereby allowing kinetochores to rebind microtubules until proper bipolar attachment is achieved. This model predicts that Aurora B kinase activity is inactivated by bipolar attachment, but we presently know little about how Aurora B activity is regulated.

Some overlapping phenotypes are seen in embryos lacking the Survivin/Bir1 protein (Survivin). Survivin is required in both budding and fission yeast for proper chromosome segregation (Yoon and Carbon, 1999; Li *et al.*, 2000; Morishita *et al.*, 2001). RNAi experiments in *C. elegans* have shown that embryos lacking Survivin display abnormal chromosome condensation, disrupted mitotic spindles, and were ultimately unable to complete cytokinesis, resulting in multinucleate embryos (Fraser, 1999; Speliotes *et al.*, 2000). Survivin-null mouse embryos displayed polyploidy, abnormal mitotic spindles, and failed cytokinesis (Uren *et al.*, 2000). The similarities in phenotypes suggest that Aurora, INCENP, and Survivin could function together.

INCENP and Survivin have both been shown to genetically interact with Aurora B kinases. Chan and colleagues (Kim *et al.*, 1999) first identified mutants of the budding yeast INCENP homolog (Sli15) that are synthetically lethal with temperature-sensitive mutants of the Aurora homolog Ipl1. Moreover, they showed that *sli15* cells had phenotypes identical to those of *ipl1* yeast. As discussed earlier, similar phenotypes are also seen in fission yeast, *C. elegans*, and *Drosophila* cells lacking either Survivin, Aurora, or INCENP (for review, see Adams *et al.*, 2001a). Aurora B kinase is not localized to the kinetochores in fission yeast or *C. elegans* embryos lacking Survivin (Speliotes *et al.*, 2000; Morishita *et al.*, 2001). Similarly, in *C. elegans* embryos and *Drosophila* cells, loss of INCENP by RNAi also leads to the mislocalization of Aurora B kinase (Adams *et al.*, 2001c; Giet and Glover, 2001; Oegema *et al.*, 2001).

Biochemical evidence has shown that Aurora B physically interacts with INCENP. Sli15p and Ipl1p form a complex in

budding yeast (Kim *et al.*, 1999), and a complex containing both INCENP and Aurora B kinase has been purified from *Xenopus laevis* mitotic extracts (Adams *et al.*, 2000). There is no conclusive biochemical evidence that Aurora kinases or INCENP are physically associated with Survivin, although a recent report has shown that human Survivin can interact with either *Xenopus* INCENP (xINCENP) or Aurora B kinase in both two-hybrid and in vitro pull-down assays (Wheatley *et al.*, 2001a). Therefore, some essential questions are whether Aurora, INCENP, and Survivin physically interact in vivo, whether complex formation is cell cycle regulated, and how each subunit interacts in the complex. Moreover, it is critical to identify the molecular function(s) of each protein in the complex.

To understand the interrelationship of the passenger proteins and to further understand how Aurora B kinase is regulated, we have cloned the *Xenopus* Survivin (xSurvivin) gene. xSurvivin is shown to exist in a complex with both xINCENP and *Xenopus* Aurora B kinase (xAurora B) in S-phase (interphase) and mitotic *Xenopus* extracts. Moreover, immunodepletion of xAurora B kinase can completely remove xSurvivin and xINCENP from *Xenopus* extracts, suggesting that all of the xSurvivin and xINCENP is physically associated with xAurora B kinase. We show that the N terminus of xAurora B kinase interacts with the conserved C terminus of xINCENP, whereas xSurvivin interacts with the N terminus of xINCENP. Furthermore, xAurora B activity is stimulated at least 10-fold in mitotic extracts, and this stimulation is shown to be phosphatase sensitive. Adding recombinant xSurvivin protein to xAurora B immunoprecipitations (IPs) stimulates the mitotic kinase activity an additional 10-fold, suggesting that xSurvivin binding to Aurora B plays a regulatory role similar to cyclin binding of CDKs. Therefore, our data suggests that xAurora B kinase is regulated by both complex formation and phosphorylation.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise. All DNA restriction enzymes were purchased from New England Biolabs (Beverly, MA). Adult wild-type *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI).

Xenopus Interphase and Mitotic Extracts

Interphase extracts were prepared as previously described (Stukenberg *et al.*, 1997). Interphase extracts were driven into mitosis by the addition of 66 nM nondegradable glutathione S-transferase (GST)-cyclin B for 20 min at room temperature.

Fusion Constructs and Protein Purification

Based on the sequence of xAurora B (Adams *et al.*, 2001b), primers 1292098 (5'-GCGGATCCATGGAGTACAAAGAGAATCTCAACCC) and 1292100 (5'-CGGCGGCCGCTTTTGATTGGGTGGACTGGTAGAC) were used to amplify xAurora B from a *Xenopus* stage 11.5–14 cDNA library. This polymerase chain reaction (PCR) fragment was subcloned into the *NcoI*-*NotI* sites of pET 28 (Novagen, Madison, WI) to create pET 28-xAurB that expresses C-terminal 6His-tagged xAurora B. xAurora B was also amplified using primers 1292098 and 1292099 (5'-GGCTC-GAGAAGCTTCATTTTGATTGGGTGGACTGG). The resulting PCR fragment was subcloned into the *NcoI*-*HindIII* sites of pET 41 (Novagen) to yield pET 41-xAurB that expresses N-terminal GST-tagged xAurora B.

The gene was sequenced by the University of Virginia Biomolecular Research Facility (Charlottesville, VA), and its sequence was deposited into GenBank (accession no. AY115554). Our isolated gene has only two amino acid changes from the published gene. As both of these changes are also found on each of the GenBank deposited expressed sequence tags (ESTs), they are unlikely to be mutations caused by the PCR isolation. Primers 1292099 and 1984819 (5'-GCCCATGGAATTCCTATGGG-GAAGGGG) were used to amplify the xAurora B kinase domain. The resulting PCR fragment was subcloned into the *NcoI-HindIII* sites of pFast-Bac and was subsequently subcloned into the *NcoI-HindIII* sites of pET 41B to create pET 41-xAurB (99–384) that expresses N-terminal GST-tagged xAurora B kinase domain.

To clone xSurvivin, a BLAST search of the EST database was conducted to find a *Xenopus* EST with high homology to human and mouse Survivin. xSurvivin was then amplified from a *Xenopus* stage 11.5–14 cDNA library using primers 1242204 (5'-CTG-GCCGGCCCCATATGTATTCTGCCAAGAACAGG) and 1242206 (5'-CGCTCGGGTGGTTCGAGATCTATGGAGCACTG). This PCR fragment was subcloned into the *NdeI-XhoI* sites of pET 41 (Novagen) to yield pET 41-xSurvivin that expresses C-terminal 6His-tagged xSurvivin. xSurvivin was also amplified using primers 1242204 and 1242205 (5'-CCGGCGCGCCTCAGTGGTCAA-GATCTATGGAGCAA). The resulting PCR fragment was subcloned into the *NdeI-AscI* sites of pGEXcsFA (a generous gift from Ethan Lee, Harvard Medical School, Boston, MA) to yield pGEXcsFA-xSurvivin that expresses N-terminal GST-tagged xSurvivin. xSurvivin sequence was deposited into GenBank (accession no. AY115553). The modeled crystal structure coordinates were generated by SWISS-MODEL (<http://www.expasy.org/swissmod/SWISS-MODEL.html>). The figures were constructed with RasMol.

A *XhoI* site was engineered into the N terminus of our pCS2⁺xINCENP (Stukenberg *et al.*, 1997) clone using PCR with the oligonucleotides (5'-CCGCGCTCGAGAACGATGCAGAGTCCGTGCCCC) and (5'-CCGGCGGGGCCCTCTAGAGGATCTCGTATTTGAGGCCATAACC). The resulting product was cloned into the *XhoI-Xba* sites of Super GFP-Wee1 (Heald *et al.*, 1993) to form a GFP-xINCENP fusion protein. A *HindIII* to *ApaI* fragment containing GFP and the N terminus of xINCENP was cloned back into pCS2⁺xINCENP, and the remaining PCR product was confirmed by sequence to generate pCS2⁺GFP-xINCENP. The *XhoI* to *XbaI* fragment was cloned into pET 28B in the *XhoI* and *Bpu1103* sites, and finally a *NheI* to *PstI* fragment of pET 28B-xINCENP was removed to generate pET 28-xINCENP (677–874) that expresses N-terminal 6His-tagged xINCENP (677–874).

All proteins were expressed in the *Escherichia coli* strain BL21 (DE3 pLysS; Novagen). 6His-tagged proteins were purified on Ni²⁺-NTA agarose (Qiagen, Valencia, CA) as instructed by the manufacturer. GST-tagged proteins were purified on glutathione agarose (Smith and Johnson, 1988).

Antibody Production, IP, and Immunoblotting

All polyclonal antibodies were made by Covance Research Products (Denver, PA). To make anti-xAurora B antibodies, rabbits 315 and 316 were immunized with purified C-terminal 6His-tagged xAurora B. To make anti-xSurvivin antibodies, rabbits 342 and 343 were immunized with purified C-terminal 6His-tagged xSurvivin. Anti-xINCENP antibodies were produced in rabbits 354 and 355 immunized with N-terminal 6His-tagged xINCENP (677–874) encoding the C-terminal fragment of xINCENP. All antibodies were affinity purified on the corresponding immunizing protein coupled to a cyanogen bromide-activated Sepharose column (Amersham Biosciences, Piscataway, NJ) as described (Harlow and Lane, 1988). After affinity purification, the antibodies were dialyzed into XB no Ca²⁺ (10 mM HEPES, pH 7.7, 1 mM MgCl₂, 100 mM KCl, and 50 mM sucrose), aliquoted, and stored at –80°C. Immunoblotting was performed on Immobilon-P (Millipore, Bedford, MA) and was detected using horseradish peroxidase-conjugated-anti-rabbit second-

ary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) by chemiluminescence (ECL; Amersham Biosciences).

To immunoprecipitate complexes from *Xenopus* extracts, 20 µg of preimmune sera (Pre-I) 1 µg of affinity-purified anti-xAurora B, anti-xSurvivin, or anti-xINCENP antibodies were bound to 50 µl of protein A Sepharose beads (Amersham Biosciences) in phosphate-buffered saline (PBS) for 1 h at room temperature. The beads were washed five times in XB no Ca²⁺, and one-half was added to 40 µl of interphase extract and the other one-half was added to 40 µl of interphase extract driven into mitosis by the addition of GST-cyclin B. Beads were incubated with extracts for 1 h at 4°C and were subsequently washed five times in XB no Ca²⁺ containing an additional 200 mM NaCl and 0.1% Brij 35.

The concentration of xAurora B, xSurvivin, and xINCENP in *Xenopus* extracts was determined by quantitatively comparing interphase extracts with recombinant GST-xSurvivin, GST-xAurora B, and 6His-xINCENP (677–874) levels on immunoblots. For quantification, the amount of INCENP in *Xenopus* extracts was compared with titrations of 6His-xINCENP (677–874) transferred to Immobilon by slot blots rather than by SDS-PAGE followed by transfer. To quantify immunoblots, films were scanned with densitometer hardware (Molecular Dynamics, Sunnyvale, CA) into ImageQuant 5.0. Data were then exported into Microsoft Excel and graphed for analysis.

Cell Culture and Immunofluorescence

XTC cells were obtained from Dr. Douglas DeSimone (University of Virginia, Charlottesville, VA) and were grown at room temperature in 70% L-15 media supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. XTC cells were grown on coverslips and fixed with 4% paraformaldehyde in PHEM buffer (60 mM PIPES, pH 6.9, 25 mM HEPES, 10 mM EGTA, and 4 mM MgCl₂) containing 0.75% Triton-X-100. Coverslips were subsequently washed in PBS containing 0.1% Tween 20 (PBS-T). Fixed cells were blocked in 20% heat-inactivated goat serum (Invitrogen, Carlsbad, CA) and were then incubated for 1 h at room temperature with monoclonal anti-tubulin antibody at 1:500 and either affinity-purified anti-xAurora B (1 µg/ml) or anti-xINCENP (1 µg/ml) antibodies diluted in PBS-T. Coverslips were then washed three times with PBS-T and then incubated with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G at 1:1000 and Cy3-conjugated anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories) at 1:1000, washed three times in PBS-T, stained with 1 µg/ml Hoechst No. 33342 for 30 s, and mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Images were collected at ×100 using an E600 Eclipse microscope (Nikon, Tokyo, Japan) equipped with a CH350 cooled charged-coupled device camera (Photometrics, Tucson, AZ). Images were acquired and analyzed using version 4.5 Meta View software.

Gel Filtration Chromatography and Sucrose Gradient Sedimentation

For gel filtration analysis, 100 µl of interphase extract was diluted 1:5 in XB no Ca²⁺. In parallel, microcystin (5 µM) was added to 100 µl of mitotic extracts and the mitotic extract was diluted 1:5 in 80 mM β-glycerophosphate, 20 mM EGTA, 10 mM MgCl₂, and 5 mM NaF. Clarified interphase and mitotic extracts were prepared by ultracentrifugation at 40,000 rpm for 1 h at 4°C in an S120-AT2 rotor (Sorvall, Kendro Laboratory Products, Newtown, CT). The extracts were then concentrated in a Microcon (Millipore) and were subsequently loaded onto a Superose 6 column (Amersham Biosciences). Thirty-two fractions of 500 µl were collected and trichloroacetic acid precipitated as described (Bensadoun and Weinstein, 1976). Alternating fractions were loaded onto 8 and 15% SDS-PAGE gels and immunoblotted for xINCENP, xAurora B, and xSurvivin.

Similarly clarified extracts were loaded onto 5–30% sucrose gradients and centrifuged at 30,000 rpm for 30 h at 4°C in a SW-40

rotor. Sixteen fractions were collected, trichloroacetic acid precipitated, and equal volumes of sample were separated on 8 and 15% gels for immunoblot analysis. Molecular weight markers were run in a parallel gradient.

Mapping of Passenger Protein-Binding Domains

To identify binding domains among the passenger proteins, the indicated truncated or wild-type proteins were translated *in vitro* in the presence of [³⁵S]methionine using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI). These proteins were allowed to integrate into endogenous complexes by mixing 10 μ l of the *in vitro* translation reaction with 20 μ l of *Xenopus* extract for 30 min, and then the endogenous protein was immunoprecipitated. Labeled proteins were detected by phosphorimager analysis of dried SDS-PAGE gels. Proteins were scored as interacting if the amount of labeled protein immunoprecipitated was fivefold higher than the amount precipitated by Pre-I; typically >80% of a labeled protein was precipitated in these experiments. The deletion set of xINCENP was a generous gift of Susannah Rankin and Marc Kirschner (Harvard Medical School).

Kinase Assays

For each kinase assay, xAurora B kinase was immunoprecipitated from 50 μ l of *Xenopus* extract on ~10 μ l of protein A Sepharose beads (Amersham Biosciences). The beads were washed four times in XB no Ca²⁺ containing an additional 300 mM NaCl and 0.1% Triton-X-100, followed by an additional wash in kinase buffer (20 mM Tris, pH 7.5, 1 mM MgCl₂, 25 mM KCl, 1 mM dithiothreitol, and 40 μ g/ml bovine serum albumin). Beads were aliquoted into tubes containing the indicated recombinant protein in 25 μ l of kinase buffer supplemented with 100 μ M γ [³²P]ATP (2 μ Ci; Perkin Elmer-Cetus Life Sciences, Norwalk, CT) and 1 μ g of myelin basic protein (MBP; Invitrogen). Reactions were incubated in a Mixer 5432 (Eppendorf; Brinkmann Instruments, Westbury, NY) at room temperature, and after 10 min, the reactions were stopped by the addition of sample buffer. One-half of the reaction was loaded onto a gel that was Coomassie stained, dried on 3MM paper (Whatman, Clifton, NJ), and analyzed for ³²PO₄ incorporation with phosphorimager hardware (Molecular Dynamics) and ImageQuant 5.0 software. The other one-half of the reaction was loaded onto a second gel and the amount of xAurora kinase was quantified by immunoblot. In kinase reactions involving lambda phosphatase, beads were washed into lambda phosphatase buffer, treated at room temperature for 30 min with either 200 U of lambda phosphatase or buffer, subsequently washed four times in kinase buffer, and assayed for kinase activity.

RESULTS

Cloning the *Xenopus* Survivin Gene

To biochemically dissect the regulation of passenger proteins, we used embryonic extracts of the frog *Xenopus laevis*. These extracts are excellent tools for studying mitotic regulators for three reasons. First, cell cycle regulators are stockpiled in *Xenopus* eggs and are more abundant than in somatic cells. Second, the cell cycle state of these extracts can be easily controlled and, third, the extracts can recapitulate mitotic events *in vitro*. We have previously cloned the xINCENP gene and demonstrated that the protein is specifically phosphorylated in mitosis (Stukenberg *et al.*, 1997). A gene encoding the xAurora B protein was recently identified (Adams *et al.*, 2000), and a highly related gene was isolated from a *Xenopus* stage 11.5–14 plasmid cDNA library by PCR. The xSurvivin cDNA was cloned by identifying a *Xenopus* EST with high homology to human and mouse Survivin. The

Survivin cDNA was then isolated from a *Xenopus* stage 11.5–14 cDNA library by PCR.

The *Xenopus* Survivin cDNA encodes a protein that is 46% identical to human Survivin (Figure 1A). The predominant motif on the Survivin protein is an inhibitor of apoptosis domain, a motif that has been shown in a related baculoviral protein to inhibit Caspase-3 activity (for review, see Reed and Bischoff, 2000; Verhagen *et al.*, 2001), although the Survivin family does not inhibit Caspase-3 (Yoon and Carbon, 1999; Li *et al.*, 2000; Uren *et al.*, 2000). The xSurvivin protein has the recognizable motifs of Survivin including the inhibitor of apoptosis repeat and a Cdc2 kinase phosphorylation site. Two independent groups have recently published the crystal structure of human Survivin (Chantalat *et al.*, 2000; Verdecia *et al.*, 2000). The *Xenopus* sequence was modeled into these crystal structures by the program SWISS-MODEL. The program predicts that the backbone structures of the *Xenopus* and the human proteins are highly similar (Figure 1, B and C). A prominent motif that is not included on the model is a C-terminal extended α helix (Figure 1, B and C). This is not included because this region is divergent in the primary sequence (Figure 1A); however, a SOPM secondary structure of this region predicted a long C-terminal α helix. Therefore, there is structural similarity between the Survivin homologs throughout the protein. Noel and colleagues (Verdecia *et al.*, 2000) identified two potential interaction regions (an acidic patch and a basic patch) on the surface of human Survivin. To determine if these regions are conserved in xSurvivin, we compared the charged amino acid residues of the xSurvivin model (Figure 1D) with the placement of charged residues in the solved crystal structure of human Survivin (Figure 1E). The placement of surface charge on xSurvivin is predicted to be almost identical to human Survivin.

Characterization of Antibodies

After affinity purification, anti-xAurora B and anti-xINCENP antibodies are specific as they predominantly recognize a band of 41 and 130 kDa, respectively, in a *Xenopus* interphase extract and a *Xenopus* tissue culture cell line (XTC) lysate (Figure 2, B–C). Anti-xSurvivin antibody recognizes a predominant band at 18 kDa (Figure 2A). Even after affinity purification, the anti-xSurvivin antibody recognizes six additional bands; therefore, it was not used for immunofluorescence. The antibodies are highly sensitive as they all recognize <1 ng of recombinant protein (unpublished data, M. Bolton, P.T. Stukenberg). By comparing immunoblot signals of *Xenopus* interphase extracts with known amounts of recombinant protein, we estimate that the concentration of xAurora B protein in these extracts is 500 nM, the concentration of xSurvivin is ~250 nM, and the concentration of xINCENP is 100 nM (unpublished data, M. Bolton, P.T. Stukenberg).

XTC cells stained with the anti-xAurora B antibody (Figure 2, D–O) and anti-xINCENP antibody (Figure 2, P–AA) display typical passenger protein immunolocalization. The concentration of both proteins is low in interphase (Figure 2, D, J, P, and V), but by prophase, the antibodies recognize both chromatin and kinetochores (Figure 2, E, K, Q, and W). Both proteins move to kinetochores at prometaphase-metaphase (Figure 2, F–G, L–M, R–S, and X–Y), and finally to bundles of overlapping microtubules in anaphase (Figure 2,

Figure 1.

H, N, T, and Z) where they remain throughout cytokinesis until they are discarded with the midbody (Figure 2, I, O, U, and AA). These data suggest that the roles of passenger proteins are conserved in *Xenopus* and demonstrate that the anti-xAurora B and anti-xINCENP antibodies are highly specific.

xAurora B, xINCENP, and xSurvivin Are Physically Associated In Vivo

To determine the native molecular weight of the passenger proteins, *Xenopus* interphase and mitotic extracts were size separated by both Superose 6 gel filtration chromatography and 5–30% sucrose gradients, and the location of each of the proteins was detected by immunoblot. In interphase egg extracts, xAurora B, xINCENP, and xSurvivin comigrate in a broad peak from 900 to 600 kDa, whereas all three proteins migrate in a sharp peak at 900 kDa in mitotic extracts (Figure 3A). A slower migrating form of xSurvivin is often seen in SDS-PAGE gels. Detection of this form depends on phosphatase inhibitors and its appearance is not cell cycle dependent (unpublished data, M. Bolton, W. Lan, and P.T. Stukenberg). xAurora B, xINCENP, and xSurvivin also comigrate in a sucrose gradient, sedimenting at 9.5S in interphase and at 4.5S in mitosis (Figure 3B). A number of control proteins, including Eg2, the *Xenopus* Aurora A homolog, migrated similarly in interphase and mitosis, demonstrating that the differences in migration of the passenger protein complex were caused by cell cycle changes and not due to differences in the manipulations of the sucrose gradients (Figure 3B). Because of the contributions of shape, neither gel filtration nor sedimentation can accurately predict native molecular weight. Gel filtration measures the Stokes radius, which is determined by the overall length of the molecule, whereas sucrose gradients measure sedimentation, which is retarded by a long thin shape. Therefore, highly elongated molecules such as myosin migrate faster than spherical proteins of similar molecular weight in gel filtration, whereas they sediment more slowly than spheres in sucrose gradients (Siegel and Monty, 1966). Siegel and Monty (1966) have derived equations using the hydrodynamic characteristics of molecules to overcome the contributions of shape and more accurately measure molecular weight. Such calculations suggest that the mitotic complex is one-half the size (~250 kDa) of the interphase complex (~490 kDa). Moreover, in both complexes, the overall length is comparable because

they migrate similarly by gel filtration. The predicted axial ratio is >20 , indicating a size and shape reminiscent of fibrinogen, but not as elongated as myosin. INCENP has a long coiled-coil domain, which is a motif that often contributes to such elongated shapes in proteins. Thus, the passenger proteins complex has a highly elongated shape in both interphase and mitosis, and the mass of the complex doubles in interphase.

To directly test if the passenger proteins are in the same complex in vivo, we immunoprecipitated each of the passenger proteins from *Xenopus* extracts and immunoblotted the precipitates to determine if other passenger proteins were bound. After IP with xAurora B antibodies, both xINCENP and xSurvivin are also precipitated (Figure 4A). Because equivalent amounts of these three passenger proteins are detected in both interphase and mitotic extracts, it does not appear that their association is cell cycle regulated in the early embryo. Pre-I controls do not precipitate any of the passenger proteins. Moreover, neither xINCENP nor xSurvivin is immunoprecipitated by the xAurora B antibodies if recombinant xAurora B protein is added to the extract before the IP, demonstrating that the interaction is specific to xAurora B antibodies (unpublished data, M. Bolton and P.T. Stukenberg). In xINCENP IPs, both xSurvivin and xAurora B (Figure 4A) are detected by immunoblot. Finally, in xSurvivin IPs, both xAurora B and xINCENP are detected (Figure 4A). Therefore, in *Xenopus* embryos during both interphase and mitosis, there is a physical interaction between xSurvivin, xINCENP, and xAurora B kinase.

Because the three proteins comigrate on a gel filtration column and sucrose gradient (Figure 3), it is possible that the majority of each passenger protein exists in this complex. This hypothesis was directly tested by immunodepleting xAurora B complex from a *Xenopus* low-speed extracts and immunoblotting the depleted extracts for xINCENP and xSurvivin. When 95% of the xAurora B is removed from extracts, xINCENP and xSurvivin are depleted to similar levels (Figure 4B). This experiment demonstrates that in the *Xenopus* embryo, most, if not all, of xINCENP and xSurvivin is physically associated with xAurora B kinase.

Mapping the Chromosomal Passenger Complex Interactions

INCENP Is a Scaffold Protein with xSurvivin Binding Its N Terminus and xAurora B Binding Its C Terminus. An assay was developed to identify the domains on xINCENP that interact with xAurora B and xSurvivin (Figure 5A). ³⁵S-labeled full-length xINCENP protein was translated in vitro and incubated in a *Xenopus* extract. This protein incorporates into the endogenous complex because it can be immunoprecipitated with our anti-xAurora B antibody (Figure 5B) and anti-xSurvivin antibody (unpublished data, M. Bolton, W. Lan, and P.T. Stukenberg). These interactions are specific, as they are not detected after IP with Pre-I controls (Figure 5B). The interaction between xAurora B and in vitro translated xINCENP is quite robust as the xINCENP is quantitatively immunoprecipitated from the extract (unpublished data, M. Bolton and P.T. Stukenberg), and, like the endogenous complex, the interaction is detected in both interphase and mitotic extracts (Figure 5, B and C). We determined whether xAurora B interacted with a series of deletion and truncated xINCENP proteins using this assay

Figure 1 (facing page). Structural and sequence homology of *Xenopus* Survivin. (A) Clustal-W alignment of the amino acids encoding sequenced vertebrate Survivins. Significant residues from the crystal structure of human Survivin are conserved and are indicated by letters or symbols above the alignment: P-cdc2 phosphorylation site; star-residues that coordinate the Zn²⁺ in the Bir domain. (B–E) Comparison of solved human Survivin structure and a structural prediction of *Xenopus* Survivin protein produced by the SWISS-MODEL server (Peitsch, 1996; Guex and Peitsch, 1997). Ribbon diagram of *Xenopus* Survivin (B) and human Survivin (C). Charge distribution in *Xenopus* (D) and human (E) space-filled Survivin models where acidic charged residues are shown in red and basic residues are shown in blue. Note that the long C-terminal α helix is present in the *Xenopus* sequence; however, the homology is too low to fit in the modeled structure.

Figure 2. Specificity of affinity-purified anti-xSurvivin, anti-xAurora B, and anti-xINCENP antibodies in *Xenopus* interphase extracts and XTC cells. (A) Affinity-purified anti-xSurvivin antibody recognizes a band of 18 kDa in an interphase extract. (B) Affinity-purified anti-xAurora B antibody recognizes a band of 41 kDa in an interphase extract and in XTC cells. (C) Affinity-purified anti-xINCENP antibody recognizes a band of 130 kDa in an interphase extract and in XTC cells. (D-AA) Immunofluorescence localization of xAurora B (D-O) and xINCENP (P-AA) in XTC cells. (D-I) Immunolocalization of xAurora B during interphase (D) and throughout mitosis (E-I). (P-U) Immunolocalization of xINCENP during interphase (P) and throughout mitosis (Q-U). Colocalization of either xAurora B (J-O) or xINCENP (V-AA) with DNA and microtubules. Blue, DAPI; green, microtubules; red, xAurora B in (K-M), xINCENP in (W-Y); yellow and orange, overlap of xAurora B or xINCENP with microtubules.

Figure 3. xSurvivin, xAurora B, and xINCENP cofractionate on gel filtration columns and sucrose density gradients during interphase and mitosis. (A) Interphase and mitotic extracts were separated by Superose 6 gel filtration chromatography and equal volumes of alternate fractions were run on SDS-PAGE gels. The fractions were then immunoblotted for xINCENP, xAurora B, and xSurvivin to determine the molecular weight of the passenger protein complex. The molecular weights of the void (2000 kDa), thyroglobulin (670 kDa), bovine γ globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa) are shown. (B) xINCENP, xAurora B, and xSurvivin cofractionate on a sucrose density gradient, but the interphase complex migrates faster than the mitotic complex. Interphase and mitotic extracts were sedimented on 5–30% sucrose density gradients. Equal volumes of each fraction were run on SDS-PAGE gels and immunoblotted for xINCENP, xAurora B, xAurora A (Eg2), and xSurvivin. Thyroglobulin (19S), bovine γ globulin (7S), chicken ovalbumin (3.5S), and equine myoglobin (2S) were sedimented in a parallel gradient as markers.

(Figure 5C). xAurora B interacts with all of the constructs that contain the C terminus of xINCENP except for the construct xINCENP Δ (119–242). We do not understand why the xINCENP Δ (119–242) construct does not interact with Aurora B, but it is likely that either the C terminus is misfolded or a regulatory region is missing. The simplest interpretation of the data is that there is an xAurora B interaction domain in the C-terminal 200 amino acids of xINCENP that is both necessary and sufficient for xAurora B binding. This region contains the IN-box that was previously shown to be an xAurora B binding domain in *C. elegans* and mouse (Kaitna *et al.*, 2000). More recently, it was shown that yeast

Figure 4. xSurvivin, xAurora B, and xINCENP are physically associated in vivo during both interphase and mitosis. (A) xAurora B (xAurB), xINCENP, and xSurvivin were immunoprecipitated from interphase and mitotic *Xenopus* extracts. To determine if the chromosomal passenger proteins are physically associated, the immunoprecipitated samples were loaded onto 8 and 15% gels and were immunoblotted for xINCENP, xAurora B, and xSurvivin. Note that xINCENP is phosphorylated during mitosis and therefore exhibits retarded mobility. (B) In *Xenopus* extracts, the majority of xINCENP, xAurora B, and xSurvivin is physically associated. Interphase and mitotic extracts were immunodepleted with anti-xAurora B antibodies. Samples of the immunodepleted extract (xAurB Depleted Extract), beads, and interphase and mitotic whole cell extracts (WCE) were run on 8 and 15% gels and were immunoblotted for xINCENP, xAurora B, and xSurvivin. xINCENP and xSurvivin were depleted below detection levels (<1 ng) and were detected on the beads.

Aurora (Ipl1p) binds directly to the C terminus of the INCENP homolog Sli15 (Kang *et al.*, 2001).

Using this assay, we also mapped the xSurvivin interaction domain to the N-terminal 119 amino acids of xINCENP (Figure 5C). The three constructs that contained the first 119 amino acids of INCENP could be immunoprecipitated by xSurvivin antibodies, whereas the two constructs that lacked this region do not. Again, we see no cell cycle differences in the xINCENP/xSurvivin interaction. The N terminus of chicken INCENP has been implicated in centromere targeting as well as chromosome and midzone binding (Mackay *et al.*, 1993; Mackay *et al.*, 1998). We are currently testing if these biochemical activities are mediated by xSurvivin.

xSurvivin Binding to the Complex Is NaCl Sensitive. The salt sensitivity of the interaction between the endogenous xSurvivin and xAurora B complex was examined. xAurora B IPs were washed with buffers containing 0.1% Triton-X 100

Figure 5. xAurora B, xINCENP and xSurvivin complex interactions. (A) Schematic of an assay used to study xAurora B, xINCENP, and xSurvivin interaction. Plasmids encoding full-length xINCENP as well as xINCENP deletion constructs (pictured in C) were in vitro translated in the presence of [³⁵S]methionine and were subsequently incubated with *Xenopus* interphase and mitotic extracts. Anti-xAurora B antibodies, anti-xSurvivin antibodies, and Pre-I were used to immunoprecipitate the immunogenic protein and interacting xINCENP fragments from the extract. (B) An example of the assay where full-length xINCENP was immunoprecipitated with anti-xAurora B antibodies, but not with Pre-I. (C) The xINCENP constructs pictured were tested for their ability to coimmunoprecipitate with xAurora B and xSurvivin. Precipitation greater than fivefold above the amount detected by Pre-I controls is indicated by a + whereas less than fivefold was indicated by a -. Full-length xINCENP includes the following domains (N-terminal to C-terminal): the centromere-interacting domain, the chromosome-binding domain, the coiled-coil/microtubule-binding domain, and the IN-box (see Figure 7). (D) The interaction of xSurvivin with the complex is NaCl sensitive. xAurora B was immunoprecipitated from a mitotic extract and was washed in XB buffer containing the indicated amount of additional NaCl. After washing, the concentration of xAurora B and xSurvivin in the IPs was determined by quantitative immunoblot. Densitometric analysis of this experiment indicates that after washing with 300 mM NaCl, there is 10-fold more xAurora B kinase than xSurvivin. (E) The N terminus of Aurora B is

and varying NaCl concentrations and were subjected to quantitative Western blot (Figure 5D). The endogenous complex of xAurora B and xINCENP is stable in high salt. However, the interaction between xAurora B and xSurvivin is salt sensitive. There is twofold more xAurora B kinase in *Xenopus* extracts than xSurvivin, but after washing with only 100 mM NaCl, the ratio of the two proteins is 6:1. This ratio increases to 10:1 after a 300 mM NaCl wash, and no xSurvivin is detected after washing the complex with 1 M NaCl (Figure 5D). These data indicate that the interaction between xINCENP and xAurora B does not require xSurvivin because xAurora B and xINCENP remain tightly associated after removal of xSurvivin.

Reciprocal IPs were performed by in vitro translating xAurora B, incubating the translation mix with *Xenopus* interphase extracts to allow labeled xAurora B to incorporate into the endogenous complex, and immunoprecipitating with either anti-xSurvivin or anti-xINCENP antibodies. The resulting IPs were washed with buffers containing 0.1% Triton-X 100 and varying NaCl concentrations. The amount of xAurora B bound was quantified by phosphorimager and plotted (Figure 5E). As seen in Figure 5D, the xAurora-xINCENP interaction is highly stable; however, the xAurora-xSurvivin interaction is NaCl sensitive. These data confirm two conclusions made from Figure 5D: the Survivin interaction is salt sensitive, suggesting that it is not tightly associated with the complex; and the interaction between xINCENP and xAurora B does not require xSurvivin.

The N Terminus and Kinase Domain of xAurora B Both Interact with INCENP and the N Terminus May Weakly Bind xSurvivin. The first 98 amino acids of xAurora B define an N-terminal region of unknown function and the rest of the protein is mostly kinase domain. We demonstrate that the N terminus of xAurora B is required for the highly stable interaction with xINCENP (Figure 5E). A construct encoding the C-terminal amino acids 99–384 of xAurora B was radioactively translated in vitro and was mixed with interphase *Xenopus* extracts. Unlike full-length xAurora B, which efficiently immunoprecipitates with xINCENP, the xAurora B kinase domain construct is not quantitatively precipitated. Whereas 70% of the full-length xAurora B coimmunoprecipitates with xINCENP, only 30% of the xAurora kinase domain coimmunoprecipitates. After washes with increasing concentrations of NaCl, the amount of xAurora kinase domain bound to xINCENP falls from 30 to 5%. The weak, salt-sensitive interaction between xAurora B kinase domain and xINCENP suggests that the N terminus of xAurora B provides the majority of the binding to xINCENP, whereas the kinase domain only weakly interacts.

It has been reported that xAurora B kinase interacts with both xINCENP and human Survivin in both two-hybrid and in vitro pull-down assays (Wheatley *et al.*, 2001a). Therefore,

required to interact with xINCENP. Full-length xAurora B (circles) and xAurora B (99–384) (triangles) were in vitro translated in the presence of [³⁵S]methionine and were subsequently incubated with *Xenopus* interphase extracts. Anti-xINCENP antibodies, anti-xSurvivin antibodies, and Pre-I were used to immunoprecipitate the resulting complex from the extract, and the IPs were washed with XB + 0.1% Triton-X 100 and the indicated extra NaCl (mM). The presence of either full-length or kinase domain xAurora was detected by SDS-PAGE and subsequent phosphorimager analysis.

Figure 6. The activity of xAurora B is stimulated by stoichiometric xSurvivin binding and mitotic phosphorylation. (A) Recombinant GST-Survivin can stimulate an xAurora B IP kinase assay. xAurora B IP kinase assay from either interphase or mitotic extracts was conducted in the presence or absence of 10 ng of recombinant GST-Survivin. The top panel is an autoradiogram of $[^{32}\text{P}]\text{O}_4$ incorporation into a MBP substrate; the bottom panel is a Coomassie-stained gel showing similar amounts of xAurora B in each reaction. (B) Titration of the amount of xSurvivin needed to activate the xAurora B kinase immunoprecipitated from interphase and mitotic extracts. The top panel is a graph of the kinase activity of the immunoprecipitated xAurora B in the presence of the indicated amount of GST-Survivin. The bottom panel is an xAurora B immunoblot analysis demonstrating similar amounts of xAurora B kinase in the xAurora B IP kinase assay. (C) xAurora B kinase was immunopurified from either interphase or mitotic *Xenopus* egg extracts; Pre-I was used as a negative control. The IPs were washed extensively and either incubated in phosphatase buffer (–) or phosphatase buffer and lambda phosphatase (+), and were then washed again extensively and the kinase activity was assayed on MBP. A Coomassie-stained gel of the IP kinase assay demonstrates that there are similar amounts of xAurora B kinase in each reaction (bottom panel). (D) The phosphorylation state of xAurora B directly regulates its kinase activity. The xAurora B IPs were washed extensively, incubated in either phosphatase buffer (–) or phosphatase buffer and lambda phosphatase (+), washed again, and assayed for kinase activity on MBP substrate. Samples were also immunoblotted for xINCENP, xAurora B, and xSurvivin to determine relative amounts of the proteins.

it is surprising that we have identified xINCENP constructs that robustly bind either xSurvivin or xAurora B, suggesting that xAurora and xSurvivin do not interact directly but rather bind opposite ends of xINCENP. If this were true, we would expect that anti-xSurvivin antibodies would pull down xAurora B kinase domain through its interaction with xINCENP (Figure 5E). However, we find that xSurvivin antibodies were not able to precipitate any xAurora B kinase domain, even though at low NaCl concentrations, there was some interaction between the kinase domain and xINCENP. The simplest interpretation of this data is that there may be a weak direct interaction between the N terminus of xAurora B and xSurvivin that stabilizes the complex.

xSurvivin Protein Can Stimulate Mitotic xAurora B Kinase Activity

Because xSurvivin could be washed off xAurora B kinase IPs, the dependence of xAurora B kinase activity on complex formation was examined. xAurora B kinase was immunoprecipitated from interphase and mitotic extracts, washed with a buffer containing 300 mM NaCl so that the ratio of

xAurora B to xSurvivin was 10:1, and the kinase activity was measured by the incorporation of $^{32}\text{PO}_4$ from $\gamma[^{32}\text{P}]\text{ATP}$ onto a MBP substrate (Figure 6A). Kinase activity is readily detected when the IP is performed with Aurora B antibodies, but not if Pre-I is used. In correlation with previous studies, we find that this activity is cell cycle regulated, as at least 10-fold greater activity is detected if the xAurora B is precipitated from mitotic extracts as compared with interphase extracts (Bischoff *et al.*, 1998). The kinase stimulation is due to an increase in specific activity of the enzyme as similar amounts of protein are detected in the IP by Coomassie stain of the gel (Figure 6, A and C) or xAurora B immunoblot (Figure 6B).

We find that adding 10 ng of recombinant GST-Survivin stimulates the mitotic kinase activity ~eightfold, but has limited effect on xAurora B isolated from interphase extracts (Figure 6A). This stimulation of xAurora B kinase activity was titrated in Figure 6B and was saturated at 10 ng of recombinant GST-Survivin. As a negative control, we added 10 ng of GST-cyclin B, which did not stimulate kinase activity (unpublished data, S. Powers, P.T. Stukenberg). We estimate that there is ~20–30 ng of xAurora B in these IPs

(Figure 5D), suggesting that xSurvivin is stoichiometrically required for xAurora B kinase activity.

xAurora B Kinase Activity Is also Regulated by Phosphorylation

The kinase activity of Aurora A is regulated by phosphorylation (Walter *et al.*, 2000). To determine if Aurora B kinases are also regulated by phosphorylation, xAurora B IPs from interphase and mitotic extracts were washed with low-salt buffer, which allows most of the endogenous xSurvivin to remain bound. This reaction was split, and one-half of the reaction was treated with lambda phosphatase, subsequently washed to remove the phosphatase, and assayed for kinase activity (Figure 6C). The mitotically stimulated xAurora B kinase activity is sensitive to treatment with lambda phosphatase, demonstrating that cell cycle-specific phosphorylation regulates the specific activity of xAurora B (Figure 6C; Murnion *et al.*, 2001).

The simplest interpretation of the phosphatase sensitivity of Aurora B kinase activity is that, as in many other kinases, the phosphorylation state directly regulates kinase activity. However, dephosphorylation may also disturb the xSurvivin interaction. To distinguish between these two possibilities, we phosphatase treated an IP of xAurora B and assayed for both kinase activity and for the relative amount of the three subunits by immunoblot (Figure 6D). Phosphatase treatment eliminated both the kinase activity and greatly increased the gel mobility of xINCENP, demonstrating that the phosphatase treatment had worked efficiently. However, the concentration of each subunit in the complex did not change. Therefore, maximum kinase activity of xAurora B requires both interaction with xSurvivin and the mitotic phosphorylation state of the complex.

DISCUSSION

During both interphase and mitosis, xAurora B kinase is in a complex with the majority of xINCENP and xSurvivin protein in the *Xenopus* embryo. The complex is cell cycle regulated as the kinase is activated >10-fold in mitosis, and hydrodynamic characterization suggests that there is a dramatic change in size that corresponds to changes in the cell cycle. We have determined two independent steps for mitotic xAurora B kinase activation: stoichiometric xSurvivin binding and a phosphorylation requirement. Therefore, like the CDK1 mitotic kinase, xAurora B activation can be regulated by both its phosphorylation state and the binding of a regulatory subunit.

Our demonstration that xSurvivin, xINCENP, and xAurora B kinase are in a complex can simply explain the molecular details underpinning a number of *in vivo* observations. First, in *C. elegans* double-stranded RNA-mediated interference (RNAi) of the INCENP homolog can mislocalize the Aurora B kinase and loss of either protein causes both chromosome segregation and cytokinetic defects (Kaitna *et al.*, 2000; Oegema *et al.*, 2001). Second, after RNAi of the Survivin homolog in *C. elegans*, the Aurora B homolog is mislocalized and embryos lacking either protein have similar phenotypes (Speliotes *et al.*, 2000). Our data suggest that these phenotypes could either be caused by mislocalization of the proteins when the complex is not properly formed or

by inactive Aurora B kinase in the absence of the Survivin protein. The N terminus of human INCENP (1–405) has dominant-negative activity and can inhibit the final stages of cytokinesis when it is overexpressed in tissue culture cells (Mackay and Earnshaw, 1993; Mackay *et al.*, 1998). Our mapping of xSurvivin binding to the N terminus of INCENP and xAurora B binding to the C terminus suggests that overexpression of INCENP (1–405) would sequester Survivin away from Aurora B and inhibit its kinase activity.

A Complex of Chromosomal Passenger Proteins in the *Xenopus* Early Embryo

An *in vivo* passenger protein complex of xSurvivin, xINCENP, and xAurora B kinase has been identified in *Xenopus* early embryos. Our mapping data are consistent with a scaffold protein role for xINCENP, which binds xSurvivin on its N terminus and xAurora B on its C terminus (Figure 7A). The N terminus of INCENP has been implicated in centromere targeting in metaphase and midzone localization in anaphase (Mackay and Earnshaw, 1993; Mackay *et al.*, 1998). Our localization of the Survivin interaction to this region suggests that these targeting events could be mediated through the Survivin protein. The C terminus of INCENP contains the IN-box, which appears to be a highly conserved Aurora B interaction motif as it also interacts with Aurora B homologs in *C. elegans*, mouse, and budding yeast (Kaitna *et al.*, 2000; Kang *et al.*, 2001). We have shown that both the kinase domain of xAurora B and the N terminus provide interactions with the C terminus of xINCENP. We have also shown that the N terminus of Aurora B stabilizes xSurvivin binding in high-salt conditions. The simplest interpretation of this finding is that the major interaction between xSurvivin and xAurora is mediated through xINCENP, although there is a weak direct interaction between xSurvivin and the N terminus of xAurora B (Figure 7B).

It has been reported that Survivin and Aurora B interact directly in a two-hybrid assay and that in an *in vitro* pull-down assay, the complex is stable in treatments as harsh as 3 M NaCl (Wheatley *et al.*, 2001a). Perhaps high concentrations of interacting proteins in the two-hybrid and pull-down systems uncovered the weak interaction between N terminus of Aurora B and Survivin. Alternatively, the difference between the two results may be a problem with Aurora B folding in overexpression systems. We have found that soluble recombinant Aurora B can be purified from *E. coli*; however, protein from these preparations migrates in the void fractions of subsequent gel filtration columns (unpublished data, W. Lan, P.T. Stukenberg). We feel that it is likely that recombinant Aurora B purified from *E. coli* is at least partially denatured and therefore the high NaCl sensitivity in the pull-down assay may be an artifact of an *in vitro* experiment. Because of this problem with recombinant protein, we have been forced to design all activity and interaction experiments with endogenous xAurora B. For further biochemical examination of the passenger protein complex, it will be necessary to develop overexpression systems that can generate the complex in its native folded state.

Although a physical interaction between xAurora B, xINCENP, and xSurvivin is detected during both interphase and mitosis, the hydrodynamic properties of the complex change dramatically during the cell cycle. Specifically, the peak off a gel filtration column is much broader in inter-

Figure 7. Model of xAurora B, xINCENP, and xSurvivin interactions and regulation. (A) Interactions within the passenger protein complex. xSurvivin binds the N terminus of xINCENP, whereas the N terminus of xAurora B binds the C terminus of xINCENP. (B) Cell cycle regulation of the passenger protein complex. The interphase complex is hypophosphorylated and its migration in a sucrose gradient is consistent with either additional subunits (X) or a dimerization of the complex. A weak interaction between xSurvivin and xAurora may also exist. In mitosis, all three proteins are both present and phosphorylated, but the complex is smaller. Both the mitotic phosphorylation of the complex and the presence of the xSurvivin subunit are required for high kinase activity (On). (C) We present a highly speculative model in which the interaction between xSurvivin and xAurora is regulated within mitosis to control kinase activity. We propose that during mitosis, xAurora B kinase activity is inactivated by disrupting the interaction of xAurora B with xSurvivin (Off), perhaps through a mechanical force. It is also possible that Survivin binding to the complex could be spatially controlled. Such a mechanism could permit spatial regulation within parts of the same mitotic cell, allowing for pools of active Aurora kinase with Survivin-bound (On) versus inactive Aurora kinase with no Survivin interaction (Off).

phase than in mitosis, but peaks in the same fractions (migrating at 107 Å). Also, our sucrose density gradient data indicate that the interphase complex sediments significantly faster than the mitotic complex (9.5S and 4.5S, respectively). The simplest model to explain such hydrodynamic characteristics is that the active complex in mitosis is a highly elongated molecule that contains at least one molecule of xINCENP, xSurvivin, and xAurora B; in interphase, two of

these complexes dimerize to double the molecular weight (Figure 7B). It is equally possible that there are unknown subunits that are bound in interphase.

There is a 2:1 M ratio of xAurora B:xSurvivin in extracts, and all of the xSurvivin protein is complexed with xAurora B. This finding was surprising because Survivin has been shown to have two functions: one as a mitotic spindle regulator and another as a chromosome passenger protein required for chromosome segregation and cytokinesis. Because Aurora B kinase has not been localized to the metaphase mitotic spindle, we expected xSurvivin to exist in at least two complexes. It is likely that the mitotic spindle requirement of Survivin is not required in the early embryo, but becomes essential during the mitosis of somatic cells. Interestingly, the metaphase spindle/centrosome localization of Survivin has been implicated to regulate apoptosis, a process that is not present in the *Xenopus* early embryo until after the midblastula transition (12th division).

xAurora B Kinase Activity Is Regulated by xSurvivin Binding and Phosphorylation

Mitotic kinases are highly regulated to ensure that their activity is restricted to the proper time and location of the cell cycle. For example, both the activity and localization of CDK1 kinase is regulated by binding of a cyclin subunit (Labbé *et al.*, 1989; Gautier *et al.*, 1990; Draviam *et al.*, 2001). CDK1 kinase activity is also both positively and negatively regulated by phosphorylation (Simanis and Nurse, 1986; Russell and Nurse, 1987; Murray *et al.*, 1989; Solomon *et al.*, 1990; Gautier *et al.*, 1991). Our goal in beginning this study was to describe in molecular detail the mechanisms of Aurora B kinase activation in mitosis. To this end, we have developed a robust IP kinase assay in *Xenopus* extracts to measure xAurora B kinase activity and found that the kinase is stimulated >10-fold in mitosis. Using this assay, we have made two significant contributions to our understanding of xAurora B kinase regulation. First, we have demonstrated that xAurora B kinase activity is stimulated by xSurvivin binding. Second, we have confirmed that the phosphorylation state of the mitotic complex is critical for the stimulation of kinase activity (Figure 7B; Murnion *et al.*, 2000).

Survivin binding could increase Aurora kinase activity by two mechanisms. It could either change the substrate recognition of xAurora kinase or it could increase the rate of catalysis. Our data cannot distinguish between these two models. During the preparation of this manuscript, Chan and colleagues (Kang *et al.*, 2001) published that the *in vitro* activity of the budding yeast Aurora (Ipl1p) is stimulated 10- to 20-fold by direct binding of the INCENP homolog (Sli15p). Thus, it is either possible that Aurora B activity requires binding by both INCENP and Survivin or that Sli15p has acquired Survivin's activation role. We have not detected stimulation of Aurora kinase activity by the addition of the C-terminal fragment of xINCENP to our Aurora B IPs. However, this is not surprising as xINCENP, unlike Survivin, is not washed off in the salt washes. Unfortunately, recombinant xINCENP and xAurora B from both *E. coli* and baculovirus appear highly misfolded after purification, making direct activation experiments in the *Xenopus* system impossible at this time.

We have also shown that activation of the xAurora B kinase depends upon mitotic phosphorylation. This corrob-

orates a recent study of *Xenopus* Aurora B kinase and is consistent with previous work that suggests that Aurora A kinase is regulated by phosphorylation (Walter *et al.*, 2000; Murnion *et al.*, 2001). Currently, it is unknown if the activating mitotic phosphorylation is on the xAurora B kinase or an interacting protein. Recent data from our laboratory indicate that all three proteins are phosphorylated in mitosis, and we have previously demonstrated that the xINCENP protein is specifically hyperphosphorylated in mitosis (Stukenberg *et al.*, 1997). We have extended this knowledge by mapping 26 cell cycle-specific phosphorylation sites on *Xenopus* INCENP (F. White, D. Hunt, W. Lan, and P.T. Stukenberg, unpublished data). Survivin has been shown previously to be a substrate of CDK1 in mitosis (O'Connor *et al.*, 2000), and if phosphatase inhibitors are used, we often see multiple bands in Survivin blots consistent with heterogeneous phosphorylation (Figure 3). We have mapped seven cell cycle-specific phosphorylation sites on the xAurora B protein that are currently being characterized (F. White, D. Hunt, W. Lan, and P.T. Stukenberg, unpublished data). Therefore, the phosphorylation-dependent stimulation of Aurora B activity is likely to be very complex.

Why does Survivin binding regulate kinase activity? The simplest model is that the kinase is not active until the entire complex is properly assembled. The most provocative interpretation of our data is a highly speculative model that we propose in Figure 7C. In this model, the interaction between Survivin and Aurora is regulated within mitosis to control kinase activity. One way to regulate the interaction of Survivin with Aurora could be through a mechanical force, which could separate Aurora and Survivin in the same complex, or Survivin could simply dissociate from INCENP. Such a mechanism could permit spatial regulation within parts of the same mitotic cell, allowing for pools of active Aurora kinase with Survivin bound versus inactive Aurora kinase with no Survivin interaction. However, we have yet to find conditions where xSurvivin binding to xAurora B is used as a regulatory mechanism in the *Xenopus* early embryo. Similar amounts of xSurvivin are bound to xAurora B in both interphase and mitotic extracts (Figure 4). We have also immunoblotted *Xenopus* embryos traversing the early embryonic cell cycles and we do not detect a gross difference of xSurvivin levels (M. Bolton, P.T. Stukenberg, unpublished data). However, the fact that xSurvivin is stable in interphase is probably a unique feature of embryonic systems, as the protein levels peak during mitosis in somatic cells (Li *et al.*, 1998; Figure 2).

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