Aurora-B Phosphorylation in Vitro Identifies a Residue of Survivin That Is Essential for Its Localization and Binding to Inner Centromere Protein (INCENP) in Vivo*

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The chromosomal passengers, aurora-B kinase, inner centromere protein (INCENP), and survivin, are essential proteins that have been implicated in the regulation of metaphase chromosome alignment, spindle checkpoint function, and cytokinesis. All three share a common pattern of localization, and it was recently demonstrated that aurora-B, INCENP, and survivin are present in a complex in Xenopus eggs and Schizosaccharomyces pombe. The presence of aurora-B kinase in the complex and its ability to bind the other components directly suggest that INCENP and survivin could potentially be aurora-B substrates. This hypothesis was recently proven for INCENP in vitro. Here we report that human survivin is specifically phosphorylated in vitro by aurora-B kinase at threonine 117 in its carboxyl α-helical coil. Mutation of threonine 117 to alanine prevents survivin phosphorylation by aurora-B in vitro but does not alter its localization in HeLa cells. By contrast, a phospho-mimic, in which threonine 117 was mutated to glutamic acid, was unable to localize correctly at any stage in mitosis. Mutation at threonine 117 also prevented immunoprecipitation of INCENP with survivin in vivo. These data suggest that phosphorylation of survivin at threonine 117 by aurora-B may regulate targeting of survivin, and possibly the entire passenger complex, in mammals.

Chromosomal passengers are proteins with an essential role in mitosis that localize to the centromeres of prometaphase and metaphase chromosomes, the spindle midzone of early anaphase cells, and the midzone and equatorial cortex of late anaphase, telophase, and dividing cells (1). Three passenger proteins, aurora-B kinase, INCENP, and survivin, are present in an 11 S complex in vivo (2–5). In addition, we have shown previously that survivin can bind directly to INCENP and aurora-B in vitro (6) and that the localization of both proteins to prometaphase centromeres requires survivin (7). The passenger proteins are required for metaphase chromosome assembly and alignment (8, 9), cytokinesis (8, 10), and the operation of the spindle assembly checkpoint in response to the loss of microtubule tension (7, 11, 12).

It is broadly assumed that the active component in the passenger complex is the aurora-B kinase. Although the consensus phosphorylation sites for some kinases are known, a reliable consensus motif for aurora-B remains to be determined. Recently a consensus signature for the yeast aurora-kinase, Ipl1, was determined, as (K/R)X(S/T)(I/L)V (where X is any residue (4)). A different consensus has been observed for phosphorylation of INCENP by aurora-B, where it has been proposed that the phosphoacceptor residue is immediately preceded by two tandem basic residues (13). However, a consensus sequence for aurora-B is still being developed.

INCENP is phosphorylated by aurora-B in its conserved carboxyl IN-box (13, 14). Phosphorylation at these sites also stimulates aurora-B kinase activity, suggesting a positive feedback loop. However, a multiple alanine mutant incapable of being phosphorylated did not act as a dominant-negative mutant (13). Xenopus and fission yeast survivin have also been shown to enhance the activity of aurora-B (3, 15, 16), although this has been questioned for the human protein (13). Furthermore, in Schizosaccharomyces pombe, Ark1 (aurora) can phosphorylate fragments of Bir1 (survivin) in vitro (15). However, to date, there is no evidence that survivin is a substrate of aurora-B in higher eukaryotes. Here we report that aurora-B phosphorylates survivin in vitro at a site that is important for the in vivo localization of survivin in human cells.

EXPERIMENTAL PROCEDURES

Molecular Biology and Preparation of Recombinant Proteins—All constructs were made by PCR with Pfu-turbo (Stratagene) or vent (New England Biolabs) using standard methods and subcloned into pGEX-4T1 (Amersham Biosciences), pCDNA3 (Invitrogen), pEGFP (Clontech) as indicated. Constitutively inactive aurora-B kinase, aurora-BK106R, and the survivin point mutations, survivinT117A and survivinT117E, were created using QuikChange site-directed mutagenesis (Stratagene) according to the manufacturer’s instructions. All constructs were confirmed by automated sequencing.

Recombinant GST fusion proteins were expressed in Escherichia coli strain BL21 by a 3-h induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C (for aurora-B) and a 3-h induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C (for survivin). GST-tagged proteins were purified from bacteria using glutathione-Sepharose 4B beads (Amersham Biosciences) as described in Ref 6 (for survivin) and in Ref. 17 (for aurora-B). GST-survivin and its derivatives were eluted from glutathione-Sepharose 4B beads using 10 mM gluta-
Survivin was phosphorylated by aurora-B. Isolated bacterially expressed GST-aurora-B was incubated with GST-survivin (lane 1) or GST alone (lane 2) in the presence of [γ-32P]ATP. In lane 3, the active GST-aurora-B was replaced by GST-aurora-BK106R and again incubated with GST-survivin. The figure shows a autoradiograph of the resulting gel.

RESULTS

Survivin in Aurora-B Substrate in Vitro—To determine whether aurora-B can phosphorylate survivin, we performed an in vitro kinase assay using recombinant GST-aurora-B and GST-survivin. As shown in Fig. 1, GST-aurora-B phosphorylated GST-survivin (lane 1) but not GST alone (lane 2). To demonstrate that the phosphorylation was due to the activity of aurora-B and not a contaminating kinase, we expressed and purified a kinase-inactive form of aurora-B, GST-aurora-BK106R. No phosphorylation of GST-survivin was detected when GST-aurora-BK106R was substituted for the wild-type protein in the kinase assay (lane 3).

Aurora-B Phosphorylates Threonine 117 in the Carboxyl Terminus of Survivin—To map the aurora-B phosphoacceptor site(s) on survivin, we constructed a number of GST fusions, including two truncation mutants (1–90 and 98–142), and a series of survivin GST-peptides of ~30 amino acids (1–30, 30–67, 60–90, 90–120, 108–142). In an in vitro kinase assay, aurora-B phosphorylated the carboxyl (98–142) half of survivin but not the amino-terminal end (Fig. 2A, lanes 1 and 2). Phosphorylation of survivin was also detected in amino acid stretches 90–120 and 108–142 (Fig. 2A, lanes 6 and 7). These data are summarized schematically in Fig. 2B. Within these regions there are three putative phosphorylation sites, threonines 97, 117, and 127. Common to all phosphorylated constructs, we expressed and purified a kinase-inactive form of aurora-B, GST-aurora-BK106R. No phosphorylation of GST-survivin was detected when GST-aurora-BK106R was substituted for the wild-type protein in the kinase assay (lane 3).

In Vivo 32P/Phosphate Labeling of Cells—Phosphorylation levels were assessed as described in Ref. 21. HeLa cells lines stably expressing survivin-GFP or survivin517GFP were plated at 1 × 106 cells/10-cm Petri dish. The following day, each culture was transfected with 6 μg of cDNA encoding pEGFP, or pEGFP-aurora-B kinase using FuGENE 6, according to the instructions (Roche Applied Science). Transfection was estimated at 40–60% as judged using a parallel HeLa culture that was previously expressing no other GFP construct. Forty-eight hours later, treatments were cultured for 16 h with 100 ng/ml colcemid to induce a mitotic arrest (as indicated). Cells were then washed twice with 10 mM phosphate-free medium (DME, Sigma), incubated at 37 °C for 4 h with phosphate-free medium supplemented with 10% dithizone fetal calf serum (PAA Laboratories), 0.75% sodium bicarbonate, 2.5 mM HEPES, and 1 μCi of [32P]-labeled sodium orthophosphate (Amersham Biosciences). Cells were then harvested, and immunoprecipitation was performed as described above with the exception that phosphatase inhibitors NaF (50 mM), ZnCl2 (2 mM), and β-glycerophosphate (1 mM) were included. Phosphorylation levels were assessed by autoradiography and PhosphorImager analysis (Storm 860 PhosphorImager, Amersham Biosciences). To assess loading, blots were immunoblotted with polyclonal anti-survivin antibody (5100, Abcam), as described above.

RESULTS

Survivin was phosphorylated by aurora-B. Isolated bacterially expressed GST-aurora-B was incubated with GST-survivin (lane 1) or GST alone (lane 2) in the presence of [γ-32P]ATP. In lane 3, the active GST-aurora-B was replaced by GST-aurora-BK106R and again incubated with GST-survivin. The figure shows a autoradiograph of the resulting gel.
MALDI-TOF analysis revealed peptides that corresponded exactly with the non-phosphorylated forms but not with the phosphorylated forms for peptides containing both Thr$_{97}$ and Thr$_{127}$. By contrast, a peptide covering residue Thr$_{117}$ was present only in the phosphorylated form (Thr$_{116}^{\text{E}}$Thr$_{117}^{\text{phospho}}$NNK$_{120}$). This peptide was found in a non-phosphorylated form.
form when the same reaction was prepared using GST-aurora-B kinase instead of GST-aurora-B (data not shown). The MALDI-TOF data were verified using nanoelcrospray ionization with the Q-TOF tandem mass spectrometer (data not shown). We conclude that the primary in vitro aurora-B phosphorylation site on survivin is residue Thr\(^{117}\).

To confirm these data, we mutated threonine 117 to alanine. In agreement with our prediction, aurora-B did not phosphorylate GST-survivin\(^{T117A}\) in vitro, as determined by autoradiography (Fig. 2D, lane 2) and corroborated by mass spectrometric analysis (Table I, Ala\(^{117}\)).

**Threonine 117 Is Critical for Survivin Localization in Vivo**—To begin to determine the functional significance of threonine 117 phosphorylation in the cell, we made GFP fusion constructs of survivin bearing point mutations that prevent (T117A) or mimic (T117E) phosphorylation. When introduced into adherent HeLa cells, GFP-tagged survivin\(^{T117A}\) localized normally at all stages of mitosis (Fig. 3A). By contrast, localization of survivin\(^{T117E}\)-GFP was disrupted throughout mitosis. The protein was dispersed throughout the cytoplasm and was excluded from the chromosomes (Fig. 3B). In the presence of survivin\(^{T117E}\)-GFP, all stages of mitosis were observed, and aurora-B (Fig. 3B) and endogenous survivin (data not shown) localized normally. These data suggest that, to localize correctly, survivin must not be phosphorylated at threonine 117. Furthermore, because the endogenous aurora-B kinase localized correctly in cells expressing the diffusely localized survivin\(^{T117E}\), this suggests that the kinase is not targeted incorrectly by this survivin mutant, i.e. that the mutant does not exert a dominant-negative effect (Fig. 3B).

**Threonine 117 Mutants Cannot Associate with INCENP in Vivo**—Next we used immunoprecipitation to determine whether the association of survivin with its partner proteins INCENP and aurora-B kinase was impaired in threonine 117 mutants. GFP-tagged versions of survivin were immunoprecipitated from mitotic HeLa cells stably expressing survivin-GFP, survivin\(^{T117A}\)-GFP, or survivin\(^{T117E}\)-GFP using anti-GFP antibodies. Blots from these immunoprecipitations were probed with antibodies against INCENP and aurora-B. As shown in Fig. 4A, survivin-GFP formed a complex with INCENP in vivo (lane 2), but survivin\(^{T117A}\)-GFP and survivin\(^{T117E}\)-GFP lysates did not (lanes 3 and 4, respectively). By contrast, aurora-B was present in all immunoprecipitations, although it was noted that some aurora-B associated with the negative control (data not shown). These observations offer an important insight into the role of survivin in the chromosomal passenger complex (see below).

**Survivin Is Phosphorylated in Vivo**—To determine whether survivin is phosphorylated in vivo, we labeled cells expressing GFP-tagged versions of both survivin and aurora-B kinase with [\(^{32}\)P]phosphorus. This enabled us to immunoprecipitate both proteins from the cultures using anti-GFP antibodies and to use aurora-B autophosphorylation as a positive control. As shown in Fig. 4B, phosphorylated survivin-GFP was detected both in cells expressing GFP and in

**DISCUSSION**

INCENP stimulates the activity of its partner aurora kinase in vitro, as shown for Ipl1 in *Saccharomyces cerevisiae* (22),

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**Table I**

Massfinger printing of human survivin carboxyl-terminal peptide sequences

| Peptide sequence | Peptide mass | Identified? | Phosphopeptide mass | Identified? |
|------------------|-------------|-------------|---------------------|-------------|
| Thr\(^{27}\)KKFEELTLGEFLK\(^{103}\) | 1581.8522 | Yes | 1661.8185 | No |
| Thr\(^{117}\)KKFEELTLGEFLK\(^{103}\) | 1533.7236 | No |
| Thr\(^{27}\)KEFKETAK\(^{120}\) | 865.2552 | Yes |
| Thr\(^{117}\)KEFKETAK\(^{120}\) | 1061.4550 | Yes |
| Ala\(^{117}\)AEFKANNK\(^{120}\) | 575.2783 | Yes | 867.4945 | Yes |
| Ala\(^{117}\)IAKEANNK\(^{120}\) | NA* | NA* |

* NA, not applicable.
FIG. 4. Mutation of threonine 117 inhibits the association of survivin with INCENP. In A, using anti-GFP antibodies, immunoprecipitation was performed on HeLa cells (lane 1) or HeLa cells stably expressing GFP-tagged survivin (lane 2), survivinT117A (lane 3), or survivinT117E (lane 4). Immunoprecipitations were probed for INCENP (upper panel) and survivin-GFP (lower panel). Survivin T117A-GFP (lane 3) and T117E-GFP (lane 4) were unable to associate with INCENP. B, upper panel, autoradiogram of anti-GFP immunoprecipitates isolated from 32P-labeled cultures expressing survivin-GFP (svn-GFP) + EGFP (lane 1), survivin-GFP + aurora-B-GFP (lane 2), survivin-GFP + aurora-BK308E-GFP (lane 3), and survivinT117A-GFP + aurora-B-GFP (lane 4). No significant difference was observed between negative controls (lanes 1 and 3) and experimental cultures (lanes 2 and 4). Auto-phosphorylation of aurora-B was detected in the survivin-GFP culture exposed to aurora-B-GFP (lane 2), which provided a positive control for the experiment (asterisk). Lower panel, immunoblot with anti-survivin antibodies to indicate loading.

AIR-2/aurora-B in Caenorhabditis elegans (14), and human aurora-B (13). Furthermore, INCENP is phosphorylated by its aurora partner (14, 22), and this phosphorylation is required for the enhancement of kinase activity (13, 14). These observations suggest the existence of a positive feedback mechanism for regulation of aurora kinase activity.

It has also been reported that Xenopus survivin stimulates aurora-B activity in vitro (3), although this is disputed for the human protein (13). In fission yeast, Bir1/survivin is a substrate of Ark1/aurora (15), and this also enhances Ark1 activity (16). Aside from these effects on aurora kinase activity, no evidence has yet been obtained for a role of INCENP or survivin phosphorylation on the distinctive localization behavior of the chromosomal passenger proteins in mitosis (1).

Here we report that human survivin is a direct substrate of aurora-B kinase in vitro. Using a combination of molecular dissection and mass spectrometry, we demonstrated that aurora-B phosphorylates survivin on threonine 117. Interestingly, KET(N118) shares some similarity with the consensus signature published for budding yeast Ipl1, (K/R)(S/T)(L/V), (where X is any residue (4)). However, alignment of survivin sequences from a number of species revealed that Thr117 is only conserved amongst the mammals, although Xenopus has a serine at this position (KFS117) that could also be targeted by aurora-B. Thus, if phosphorylation is important for the regulation of survivin function or localization (see below), this regulation may differ between mammals and other vertebrates. In fact, chicken survivin-GFP, which lacks a phosphoacceptor residue corresponding to Thr-117, does not localize correctly in human cells.2

Phosphorylation of survivin at Thr117 appears to be important for its localization as a phospho-mimic mutant (survivinT117E) failed to localize to centromeres, whereas a non-phosphorylatable mutant (survivinT117A) localized correctly when expressed in HeLa cells. Interestingly, aurora-B kinase localized normally in cells expressing survivinT117E. As INCENP, aurora-B, and survivin are all mutually dependent for their localization in mitosis (2, 6, 7), this observation suggests that survivinT117E must be defective in its ability to interact with aurora-B and/or INCENP; otherwise, the delocalized survivin would have been expected to be associated with the pool of delocalized aurora-B.

Prompted by these compelling localization data, we investigated the ability of survivinT117A-GFP and survivinT117E-GFP to interact with INCENP and aurora-B in vivo. Immunoprecipitation with anti-GFP antibodies revealed that although wild-type survivin-GFP associated with INCENP, neither mutant form did. Thus, our data suggest that phosphorylation of survivin at residue Thr-117 by aurora-B kinase may regulate the formation of the chromosomal passenger complex, which is required for correct localization of all three proteins. Importantly, it should be noted that endogenous wild-type survivin is still present in these cells. As survivin is thought to function as a homodimer, and introduction of our mutant forms did not have a dominant-negative impact, it may be that T117A-GFP and aurora-B localize normally, because they are carried by the endogenous survivin. Hence, the true effect of mutating these residues may be more severe than we have observed.

We were unable to detect a significant difference in the level of survivin-GFP phosphorylation in vivo in asynchronous or prometaphase arrested cultures co-expressing aurora-B-GFP. However, survivin is also phosphorylated by the mitotic regulator, CDK1 (23), and has putative CK2 sites (24); thus, the contribution that aurora-B makes to the phosphorylation status of survivin may be insufficient for us to detect over the contributions of other kinases. Furthermore, if aurora-B phosphorylates survivin at the metaphase-anaphase transition (as discussed below), the transience of this event may preclude our ability to detect this modification.

Our observations provide support for the notion that the movements of the chromosomal passengers are regulated by phosphorylation. Interestingly, the localization of aurora-B itself to centromeres is independent of its kinase activity, but kinase-inactive aurora-B does not transfer to the anaphase spindle and equatorial cortex (25), and cells expressing aurora-BK308E subsequently fail in cytokinesis (26).

It was recently reported that aurora kinase (Ipl1) activity peaks during spindle formation in mitotic budding yeast (27). As the kinetics of spindle formation and chromosome movements are different between S. cerevisiae and mammalian cells, and Ipl1 is the only aurora kinase in budding yeast, it will be interesting to determine how the levels of aurora-B activity correlate with the movements of the chromosomal passenger complex in mammalian cells. Indeed, from the work of Bischoff et al. (28), aurora-B activity (referred to as aurora-1) was reported to be highest from metaphase through to telophase. Our findings, together with the data from Bischoff et al. (28) suggest a potential explanation for one of the most intriguing aspects of chromosomal passenger behavior, the transfer of the proteins from the centromere to the spindle midzone at anaphase onset. If aurora-B were to phosphorylate survivin at the metaphase-anaphase transition, when its activity is reported to be highest, this might alter the association of survivin with the chromosomes, thereby releasing the chromosomal passengers to target to other subcellular compartments, such as the midzone microtubules and cleavage plane.

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S. P. Wheatley, A. Carvalho, and W. C. Earnshaw, unpublished observation.
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