Survivin enhances Aurora-B kinase activity and localizes Aurora-B in human cells

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Running Title: Survivin enhances Aurora-B kinase activity
The abbreviations used are: IAP, inhibitor of apoptosis protein; BIR, baculoviral IAP repeat; GFP, green fluorescent protein; AS, antisense; MS, missense; GST, Glutathione S-Transferase; RNAi, RNA interference; INCENP, inner centromere protein; His-tagged, histidine-tagged; hr, hour; min, minute.
SUMMARY

Survivin, one of the most tumor-specific gene products, has been implicated in both anti-apoptosis and cytokinesis. However, the mechanism by which survivin regulates these two different processes is still elusive. Here, we show that survivin binds to the catalytic domain of Aurora-B. We demonstrate that in the presence of survivin, Aurora-B phosphorylates histone H3 much more efficiently than in the absence of survivin in a cell free system. Furthermore, we confirm that cells lacking survivin due to survivin antisense oligonucleotide-treatment have lower Aurora-B kinase activity, whereas cells overexpressing survivin have higher Aurora-B kinase activity. We also provide evidence that depletion of survivin by survivin antisense oligonucleotide-treatment causes significant reduction of endogenous phosphorylated histone H3 and mislocalization of Aurora-B. These results indicate that survivin stimulates Aurora-B kinase activity and helps correctly target Aurora-B to its substrates during the cell cycle, thus providing a mechanism as to how survivin exerts its function in human cells.
INTRODUCTION

Survivin was first identified as a novel transcript in the opposite orientation to the effector cell protease receptor-1 (EPR-1) gene. The functions of these two genes are unrelated and survivin has quickly attracted much attention because it is overexpressed in most cancer cells but not in adjacent normal tissues(1). Sequence analysis suggests that survivin contains a domain shared by members of the inhibitor of apoptosis protein (IAP) gene family. The IAP family proteins was first identified in baculovirus and all of them contain variable number of baculoviral IAP repeat (BIR) domains(2). Several human and Drosophila IAPs inhibit apoptosis by directly binding and potently inhibiting caspases(2). However, the evidence for survivin directly inhibiting caspases remains controversial(3,4). Nevertheless, overexpression of survivin provides a survival advantage for cells undergoing IL-3 withdrawal, treatment with Fas, or treatment with chemotherapeutics(1,4).

In normal cells, the expression of survivin is limited to the mitotic stage of the cell cycle. Recent studies suggest that survivin plays an essential role in cytokinesis(5). Down-regulation of survivin in HeLa cells by antisense oligonucleotides leads to cytokinesis defects(6,7). Both immunostaining of endogenous survivin and ectopic expression of GFP-tagged survivin have shown that survivin is sequentially colocalized with Aurora-B and the inner centromere protein, INCENP, to the centromeres, the spindle midzone, and then to the cleavage furrow during mitosis, a typical behavior of chromosomal passenger proteins(7-9).
The genetic evidence for the functional interaction among survivin, Aurora-B kinase and INCENP comes from gene knockout studies(10,11). Survivin knockout embryos are polyploid and show disrupted microtubule formation resulting in lethality at embryonic day 4.5. This phenotype was remarkably similar to that seen in INCENP knockout mice(11), consistent with the idea that survivin and INCENP act together in the same pathway. In budding yeast, mutants of the orthologs of Survivin (Bir1p), Aurora-B (Ipl1p), and INCENP (Sli15) shown similar defects in chromosome segregation(12,13). Moreover, overexpression of Ark1p, a fission yeast ortholog of Aurora-B, can rescue the defects associated with a Bir1p mutant(14), indicating that yeast ortholog of Aurora-B and survivin can functionally complement each other. Similar phenomena have also been observed in *C. elegans*. Ablation of *C. elegans* ortholog of Aurora-B, AIR-2 by RNAi also results in cytokinesis defects, which are indistinguishable from the down-regulation of the ortholog of survivin, BIR-1 by RNAi(15).

Biochemically, yeast and *Xenopus laevis* Aurora-B and INCENP interact with each other and their interaction is required for the completion of cytokinesis(13,16). A recent study has also shown that human survivin interacts directly with Aurora-B and INCENP(9). The migration of these three chromosomal passenger proteins is intriguing. RNAi mediated reduction of BIR-1 in *C. elegans* results in displacement of the Aurora-B ortholog AIR-2 from chromosomes(17), suggesting that survivin is required to target Aurora-B to the chromosomes. Depletion of Drosophila INCENP by RNAi displaces *Drosophila* Aurora-B from the chromosome and the spindle midzone, while RNAi mediated reduction of *Drosophila* Aurora-B prevents INCENP from being transferred to the spindle midzone, indicating that the relocation of these proteins is interdependent(18).
Among chromosome passenger proteins, only Aurora-B is a mitotic serine/threonine kinase. It has been implicated to play key roles in chromosome segregation, cytokinesis and cancer development(8,19,20). *C. elegans* Aurora-B is required for recruiting ZEN-4/CeMKLP, a key kinesin related protein essential for completion of cytokinesis(21). Aurora-B phosphorylates histone H3, a requirement for premitotic chromosome condensation(8,22). The substrates of Aurora-B kinase also include the kinesin-related protein Eg5(23), other chromosomal passenger proteins CENP-A and INCENP(24,25), and the myosin II regulatory light chain, a key component of the actomyosin ring(26). Finally, two recent studies have shown that Aurora-B kinase activity is required for kinetochore-microtubule interactions and microtubule dynamics during mitosis(27,28).

Taken together, the data to date suggest that survivin interacts with Aurora-B physically, genetically, and functionally. Current evidence suggested that these proteins together with INCENP might form a complex (8), however, the role that survivin plays in the complex is not fully understood. Although survivin is overexpressed in most cancer cells, the mechanism which links survivin to tumor development is poorly understood. In this paper we have focused on the role survivin plays with Aurora-B in this complex. We describe that survivin binds to the catalytic domain of Aurora-B, enhancing its kinase activity both *in vitro* and *in vivo*, and targets Aurora-B to its substrates.
EXPERIMENTAL PROCEDURES

Molecular Cloning - An Incyte (Incyte is a registered trademark of Incyte Genomics, Inc.) clone (ID 1272707) containing the complete DNA sequence for human Aurora-B protein was identified from the LifeSeq database (LifeSeq®) using a BLAST homology search. The following primers were designed for PCR amplification and cloning into the pET-30 Ek/LIC vector: 5’-gacgacgacaagatggcccagaaggagaactcctacc-3’ and 5’- gagagagaagcccggttcaggcgacagattgaagg-3’. PCR products were cloned using the Ek/LIC vector kit (Novagen) per manufacturer’s instructions. pET-30Aurora-BK106R was generated using Quick Change Mutagenesis Kit (Stratagen). The mutagenesis primers are: 5’-tcatctgctggctcagggtcctcttcaagtcc-3’ & 5’-gacttgaagaggaccctgagcgccacgatgaa-3’. Primers used for constructing Aurora-B66-344 in pET28 were:
5’-ggggatgccatatggacatcttacggcacttc-3’ and 5’-cgcggatcctcagggagcagattgaagg-3’. Human survivin (residues 1-120) was cloned into a modified pET14b (Novagen) using primers: 5’-acgtgtccatggctggctcgcctgc-3’ and 5’-acgtgtctcgagcttattgttggtttcctttg-3’. GST-survivin constructs were generated as previously described(3,29). All clones were subsequently confirmed by sequencing analysis.

Protein Expression, Purification and Biotinylation - Exponentially growing BL21 (DE3) cells transformed with pET30Aurora-B, or pET30Aurora-BK106R were induced with 1 mM IPTG at 30 °C for 3 hr. The induction condition for His-survivin or GST-survivin is 40 µM IPTG at 23 °C for 16 hr. Cell lysates were incubated with Ni⁺ ProBond beads (Invitrogen) for 1 hr at 4°C. The beads were washed with buffer A (50 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole). The His-Aurora-B and His-survivin were eluted with 250 mM imidazole, dialyzed against buffer B (50 mM Tris pH 8.0, 100 mM NaCl)
with 50 units of thrombin in the dialysis bag at 4 °C for 16 hr. GST-survivin was purified using GST purification kit (Pharmacia) according to manufacturer’s instructions. Purified survivin was first incubated with EZ-Link Sulfo-NHS-LC-Biotin solution (Pierce) at 1:3 molar ratio for 45 min at room temperature, and then dialyzed against PBS with 1mM DTT for 16 hr. The average biotin molecule per survivin is 1.7 determined by HABA solution (Pierce). The proper folding of this biotin-survivin was confirmed later by NMR.

**In Vitro Binding Assay** - Full length Aurora-B and Aurora-B<sub>66-344</sub> were translated in the TNT T7-coupled reticulocyte system (Promega) according to the manufacturer’s instructions. Five microliters of each [³⁵S]-methionine-labeled Aurora-B or p27<sup>kip1</sup> was incubated with 10 µg survivin plus 30 µl streptavidin beads or with streptavidin beads alone for 1 hr at 25 °C. The beads were washed with PBS plus 0.5 M NaCl five times before resolving on SDS gels and analyzing them by a phosphoimager (Molecular Dynamics).

**Cells and Transfections** - HEK 293 cells (ATCC) transfected with pCDNAhis-survivin or pCDNAhis vector were selected in DMEM (Invitrogen) with 10% fetal bovine serum and 0.5 mg/ml G418 (Invitrogen). Single cell clones overexpressing survivin or neomycin control were confirmed by survivin immunobloting. HeLa cells (ATCC) were maintained in DMEM (Invitrogen) with 10% fetal bovine serum. Transfections were performed using Lipofectamine Plus (Invitrogen) according to manufacturer’s instructions. Survivin missense and antisense oligonucleotides were obtained from ISIS Pharmaceuticals and used as previously described(7).

**Immunoblotting** – 50 µg of cell lysate or 1X10<sup>5</sup> cells lysed in Laemmli sample buffer (Sigma) were resolved on SDS gels. Immunoblotting were performed as described
previously(7). The rabbit polyclonal antibody against phosphorylated Ser-10 of histone H3 was purchased from Santa Cruz Biotechnology Inc. The rabbit polyclonal antibody against survivin was purchased from R&D System.

**Aurora-B Kinase Assay** - The kinase reactions were performed in kinase buffer (20 mM Hepes pH7.5, 10 mM MgCl₂, 1 mM DTT, 10 mM KCl) containing 10 µg histone H3 (Sigma), 5 µCi γ-³²P ATP, 5 µM ATP, 140 ng of Aurora-B or Aurora-Bₖ₁₀₆ₚ, and 2 µg of survivin. The reactions were incubated at 30°C for 30 min before resolving on SDS gels, transferring to PVDF membranes, and analyzing them by a phosphoimager.

**Immunoprecipitation and Kinase Assay** – 24 hr after transfection, cells were incubated with 0.4 µg/ml Nocodazole (Sigma) overnight. The next day cells were washed with PBS and lysed in buffer C (20 mM Hepes pH7.5, 10 mM KCl, 1.5 mM MgCl₂, 10 mM NaF, 1 mM NaVO₄, 0.5 % NP40, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma)). Lysates (1mg) were incubated with 2.5 µg of anti-Aim-1 (Aurora-B) antibody (BD Biosciences) at 4 °C for 1 hr, and the immune complexes were precipitated with protein G-agarose beads (Invitrogen) for 2 hr at 4 °C. Immunoprecipitates were washed four times with buffer C and one time with buffer D (10 mM Hepes pH7.5, 10 mM MgCl₂, 1 mM DTT, 10 mM KCl). Each sample was resuspended in 25 µl of kinase mixture (20 mM Hepes pH7.5, 10 mM MgCl₂, 1 mM DTT, 10 mM KCl, 10 µg histone H3, 5 µCi γ-³²P ATP and 5 µM ATP). The kinase reactions were performed and analyzed as described above.

**Immunohistochemistry and Confocal Microscopy** - Cells grown and treated on 8-chamber slides were fixed with 10% phosphate buffered formalin for 10 min, washed 2 times with PBS, and blocked with 2% BSA, 0.2% nonfat dry milk, 0.4% Triton-X-100 in
PBS for 1 hr at room temperature. Cells were incubated with either rabbit anti-survivin IgG (R&D Systems) or mouse anti-Aim-1 (BD Biosciences), both diluted 1:200 in PBS, for 1 hr at 37 °C. Cells were washed 3 times in PBS containing 0.2% Tween 20 and incubated with Alexa 546 conjugated goat anti-rabbit IgG (Molecular Probes) or Alexa 488 conjugated anti-mouse IgG, both diluted 1:1000 in PBS for 30 min at 37 °C. Cells were washed once as described above, stained for microtubules with a FITC-conjugated α-antitubulin IgG (Sigma Chemicals Co.) diluted 1:75 in PBS for 1 hr at 37 °C and then washed one time as described above. The DNA was counterstained with Hoechst (15 µg/ml) for 10 seconds, and the cells were washed two more times. Cells were mounted with Citifluor and imaged with a BioRad MRC-1000 confocal system attached to an inverted Nikon microscope fitted with epi-fluorescence optics and a 60 X (n.a. = 1.4) objective.
RESULTS

Survivin interacts directly with the catalytic domain of Aurora-B.

To characterize the interaction between survivin and Aurora-B, we conducted pull-down experiments using biotin-labeled survivin1-120 and [35S]-methionine-labeled Aurora-B. As demonstrated by a previous study (9), we observed the same interaction between survivin and Aurora-B (Fig 1A). Similar results were obtained using GST-survivin instead of biotin labeled survivin (data not shown). The interaction between survivin and Aurora-B was also confirmed by anti-Aurora-B antibody immunoprecipitaion followed by Western blot analysis with anti-survivin antibody (data not shown). Although a previous study reported that the localization of Aurora-A kinase protein depends on the N-terminal non-catalytic domain of the kinase in *Xenopus laevis* (30), we found that the catalytic domain of Aurora-B was sufficient to bind to survivin-coated streptavidin beads, but not streptavidin beads alone (Fig 1B). A non-related [35S]-methionine labeled protein, p27kip1, was used to confirm that the interaction between survivin and Aurora-B was specific. Under the same conditions, p27kip1 did not bind to survivin-coated streptavidin beads, nor did it bind to streptavidin beads alone (Fig 1C). These results indicate that survivin specifically binds to the catalytic domain of Aurora-B.

Survivin enhances Aurora-B kinase activity *in vitro*.

When a protein binding to the catalytic domain of a kinase, it usually acts as a substrate, a regulator, or an adaptor. Since a recent study claimed that Aurora-B does not phosphorylate survivin (9), we hypothesized that survivin might regulate Aurora-B activity. Indeed, we found that in the presence of survivin, Aurora-B phosphorylated one
of its physiological substrates histone H3 much more strongly than in the absence of
survivin (Fig 2, lane 1, 2). As survivin and histone H3 have similar molecular weights,
we included a control without histone H3 to rule out the possibility that the increase of $^{33}$P
labeling was due to the phosphorylation of survivin. Under the same assay conditions we
did not observe the phosphorylation of survivin by Aurora-B (Fig 2, lane 3). As a
negative control, we constructed a kinase-inactive mutant Aurora-B$_{K106R}$ (31). Using
Aurora-B$_{K106R}$, we did not observe any detectable kinase activity under the same
conditions (Fig 2, lane 4, 5, 6 and 7).

**Overexpressing survivin enhances Aurora-B kinase activity in vivo.**

To determine whether survivin stimulates Aurora-B kinase activity in vivo, we first
generated HEK 293 cells stably transfected with N-terminal His-tagged survivin (Fig
3A). Subsequently, we overexpressed Aurora-B in both HEK293 cells that overexpress
survivin and vector control HEK 293 cells that do not overexpress survivin. Although
similar expression of Aurora-B in both transfected cells was confirmed by immunoblot
(Fig 3B), we found that immunoprecipitated Aurora-B kinase activity was higher in cells
that overexpress survivin using histone H3 as a substrate (Fig 3C).

**Down-regulation of survivin decreases Aurora-B kinase activity in vivo.**

Next, we used survivin antisense oligonucleotides to down-regulate endogenous
survivin in HeLa cells (Fig 4A). We discovered that the immunoprecipitated endogenous
Aurora-B activity in survivin antisense oligonucleotide-treated cells was 9 fold lower
than that in missense oligonucleotide-treated cells (Fig 4B), even though Aurora-B
protein level was unaffected (Fig 4C). This result indicates that survivin regulates
Aurora-B kinase activity rather than its protein stability. We reasoned that regulating
survivin levels might have an impact on the phosphorylation of endogenous histone H3 phosphorylation, since a previous study showed that RNAi mediated AIR-2 reduction led to a defect in histone H3 phosphorylation by immunostaining(17). Using an antibody against phosphorylated Ser-10 of histone H3, we found that the phosphorylated histone H3 in survivin antisense oligonucleotide-treated cells is much lower than that in survivin missense oligonucleotide-treated cells (Fig 4D). These observations further confirmed that survivin enhances Aurora-B kinase activity in vivo.

**Survivin is required for targeting of Aurora-B.**

To test if human survivin, like *C. elegans* BIR1, targets Aurora-B to chromosomes, we used a commercially available monoclonal anti-Aurora-B antibody to detect the localization of Aurora-B in HeLa cells that had been treated with survivin antisense or missense oligonucleotides. In survivin missense oligonucleotide-treated HeLa cells we observed that survivin and Aurora-B colocalized throughout mitosis as previously described(9). For example, both survivin (Fig 5A and B) and Aurora-B (Fig 5C and D) colocalized to the spindle midzone at metaphase. As reported previously by us and others, we observed an obvious increase in the number of multinucleated cells in survivin antisense oligonucleotide-treated cells(7). Survivin staining in the multinucleated cells was below the level of detection and was not observed in the mitotic apparatus (Fig 5E and F, cell shown at metaphase). In addition, Aurora-B staining was not localized to the mitotic apparatus in multinucleated cells (Fig 5G and H, cell shown in metaphase). However, there was some diffuse staining of Aurora-B throughout the cytoplasm (Fig 5G). This diffuse staining is consistent with the result that Aurora-B protein level remains the same in both survivin antisense and missense oligonucleotide-treated cells (Fig 4C).
These findings suggest that human survivin is required for targeting of Aurora-B in mitosis.
DISCUSSION

In summary, we have found that survivin localizes Aurora-B to its substrates and enhances its kinase activity \textit{in vitro} and \textit{in vivo}. These findings may provide a possible mechanism of action of survivin in cells.

First, we speculate that the cytokinesis defects caused by depletion of survivin are through mislocalization of Aurora-B and a decrease in its kinase activity since Aurora-B is known to recruit ZEN-4 and phosphorylates the myosin II regulatory light chain, two essential proteins for the completion of cytokinesis\cite{21,26}. Results from overexpression of the Aurora-B$_{K106R}$ mutant also indicate that Aurora-B kinase activity is essential for normal cytokinesis\cite{32}. Our evidence that ablation of survivin in HeLa cells causes mislocalization of Aurora-B, a decrease of Aurora-B kinase activity, and increased cytokinesis defects further supports our speculation.

Second, survivin has been implicated in the regulation of microtubule stability\cite{33}. We, along with others, could not find evidence that survivin directly binds to microtubules\cite{34}. Two recent studies have shown that Aurora-B kinase activity is required for regulation of microtubule dynamics\cite{27,28}. Therefore, survivin is a positive regulator of Aurora-B may explain how survivin regulates microtubule stability.

Third, we postulate that survivin targets Aurora-B to the chromosome where Aurora-B can phosphorylate substrates such as CENP-A and INCENP\cite{24,25}. Phosphorylated INCENP then moves to the central spindle and brings survivin and Aurora-B with it. This may provide an explanation of the following intriguing results: depletion of INCENP by RNAi displaces \textit{Drosophila} Aurora-B from the chromosome and the spindle midzone,
while RNAi mediated reduction of *Drosophila* Aurora-B prevents INCENP from being transferred to the spindle midzone(18).

Finally, Aurora-B kinase has been implicated in human cancer development and is known to be overexpressed in a variety of human cancers(19). Positive regulation of Aurora-B by survivin may provide a connection between survivin and tumorigenesis or perhaps tumor maintenance. So far, two other kinases (CDK1, CDK4) have been reported to interact with survivin(35,36). A recent study has shown that CDK1 regulates BAD, a pro-apoptotic protein, via phosphorylation(37). Whether survivin exerts its anti-apoptosis function through Aurora kinase or other kinases is unknown. Further investigation is warranted.

In conclusion, our observations in this study show that survivin directly stimulates the kinase activity of Aurora-B and properly localize Aurora-B, thus providing a mechanism as to how survivin exerts its function in cells.
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FIGURE LEGENDS

Figure 1. Survivin interacts directly with the catalytic domain of Aurora-B. Biotin labeled survivin was bound to streptavidin beads and incubated with in vitro-translated Aurora-B, A; Aurora-B_{66-344}, B; or p27kip1, C. Proteins bound to streptavidin beads were resolved by SDS gels and analyzed using a phosphoimager. Lane 1, total input of Aurora-B; lane 2 Aurora-B bound to streptavidin beads alone; lane 3, Aurora-B bound to survivin streptavidin beads; lane 4, total input of Aurora-B_{66-344}; lane 5, Aurora-B_{66-344} bound to streptavidin beads alone; lane 6, Aurora-B_{66-344} bound to survivin streptavidin beads; Lane 7, total input of p27kip1; Lane 8, p27kip1 bound to streptavidin beads alone; Lane 9, p27kip1 bound to survivin streptavidin beads.

Figure 2. Survivin enhances Aurora-B kinase activity in vitro. Bacterial expressed Aurora-B or Aurora-B_{K106R} mutant was incubated with ^{33}P ATP and histone H3 in the presence or absence of survivin. The reactions were resolved on the SDS gel and analyzed using a phosphoimager.

Figure 3. Overexpressing survivin enhances Aurora-B kinase activity in vivo. A, Cell lysates from HEK293 permanently transfected with N-terminal His-tagged survivin (His-Sur) or vector control (Ctrl) were immunoblotted with anti-survivin antibody. B, Overexpressing survivin HEK293 cells and vector control cells were subsequently transfected with Aurora-B. Lysates from these cells were normalized by total protein amount and immunoblotted with anti-Aurora-B antibody. C. Aurora-B
immunoprecipitated-kinase reactions of these transfected HEK293 lysates were resolved on the SDS gel and analyzed using a phosphoimager.

**Figure 4 Down-regulation of survivin decreases Aurora-B kinase activity *in vivo.***

A, Cell lysates from HeLa cells treated with survivin antisense (AS) oligonucleotides or missense (MS) control oligonucleotides were immunoblotted with anti-survivin antibody (B) Immunoprecipitated endogenous Aurora-B kinase activity from survivin MS or AS oligonucleotide-treated HeLa cells was assayed using histone H3 as substrate. C, Same lysates were immunoblotted with anti-Aurora-B antibody. D, HeLa cells treated with survivin MS or AS oligonucleotides were immunoblotted with anti-phosphorylated histone H3 antibody.

**Figure 5. Immunolocalization of survivin and Aurora-B in fixed HeLa cells at metaphase 48 hr following survivin missense or antisense oligonucleotide-treatment.**

Immunolocalization of survivin following survivin missense (A, B) or survivin antissense (E, F) oligonucleotide-treatment and colocalization with DNA and microtubules (B, F). Immunolocalization of Aurora-B following survivin missense (C, D) or survivin antissense (G, H) oligonucleotide-treatment and colocalization with DNA and microtubules (D, H). Red, survivin (A, B, E, F) or Aurora-B (C, D, G, H); green, microtubules; blue, Hoechst stained DNA.
| Aurora-B | 1  | 2  | 3  | 4  | 5  | 6  | 7  |
|----------|----|----|----|----|----|----|----|
| Histone H3 | +  | +  | -  | -  | +  | +  | -  |
| Survivin | -  | +  | +  | -  | +  | +  | +  |

![Histone H3](image)
A  Survivin  Control

B  Aurora-B

C  Histone H3
Survivin enhances Aurora-B kinase activity and localizes Aurora-B in human cells
Jun Chen, Sha Jin, Stephen K. Tahir, Haichao Zhang, Xuesong Liu, Aparna V. Sarthy,
Thomas P. McGonigal, Zhihong Liu, Saul H. Rosenberg and Shi-Chung Ng

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