It has been previously shown that both survivin and the survivin splice variant survivin-2B are localized in mitochondria. Whereas the mechanism involved in blockade of mitochondria-mediated apoptosis by survivin has been extensively studied, the role of survivin-2B in regulation of apoptosis has not been well defined. In the present study, we report that in addition to mitochondria, survivin-2B is also localized in the microtubule organization center (MTOC) and, in contrast to other survivin isoforms (i.e. survivin and survivin-ΔEx3), behaves as a proapoptotic molecule. We show that forced expression of survivin-2B blocks tubulin polymerization, ablates mitotic cells, and induces mitochondria-dependent apoptosis. The mitochondria-mediated apoptosis induced by survivin-2B was indicated by Smac release from mitochondria, activation of caspases 9 and 3, and loss of mitochondrial potential, while caspase-8 remained inactive. Further analysis of the mechanism for the mitochondria-associated events of apoptosis induced by forced expression of survivin-2B revealed down-regulation of the pro-survival factor Bcl-2 and up-regulation of the pro-apoptotic factor Bax in mitochondria, while the apoptosis-inducing factor (AIF) remains unchanged. Our studies further showed that taxol (paclitaxel) treatment of cancer cells not only up-regulates survivin but also down-regulates survivin-2B and that forced expression of survivin-2B sensitizes cells to taxol-induced cell death, while silencing of endogenous survivin-2B transcripts by survivin-2B-specific siRNA made cells resistant to taxol treatment. These findings advance our current knowledge about survivin-2B and may help to develop novel approaches for cancer treatment.

Survivin is highly expressed in human cancers but is undetectable in most normal adult tissues, and is functionally involved in both inhibition of apoptosis (1) and regulation of cell division (2–4). Specifically, survivin plays a critical role in the mitotic survival checkpoint and mitochondria-mediated apoptosis control (3, 5). During mitosis, survivin is up-regulated and binds to the mitotic apparatus to control a mitotic survival checkpoint (1, 2) through interaction with and phosphorylation by the cyclin-dependent Cdc2 kinase on the mitotic apparatus (6, 7). Phosphorylated survivin interacts with and inhibits caspase-9 activation (6, 7). Previous studies indicated that there is a mitochondrial pool of survivin, which is essential for the promotion of tumorigenesis through inhibition of mitochondria-mediated apoptosis by various cell death inducers (8). Whereas the function of survivin has been extensively studied (3–5, 9–11), the role of its splice variant survivin-2B in apoptosis control has not been well defined (9, 10). Islam et al. (12) reported that the expression of survivin-2B is predominant in some benign neuroblastomas, whereas its expression in most malignant tissues is very low. In renal cell carcinomas, unlike survivin and its splice variant survivin-ΔEx3, which show no significant changes during tumor progression, survivin-2B expression is significantly decreased in late stage tumors (13), suggesting a potential inhibitory role of survivin-2B in cancer development. Similarly, in gastric cancer, a significant stage-dependent decrease in survivin-2B expression was observed in disease stages III+IV in comparison with stages I+II, while the expression of survivin and survivin-ΔEx3 remained unchanged (14). In addition, soft tissue sarcomas express both survivin and survivin-ΔEx3, while survivin-2B is undetectable (15). Survivin-2B expression is dominant in benign brain tumors in comparison with malignant tumors of the brain (16) and, in lung cancer cells, relatively high level expression of survivin-2B is significantly associated with the patient category of “no relapse and alive” (17). Collectively, these observations argue that survivin-2B plays an inhibitory role in cancer development. While our recent data showed that, similar to survivin (8), survivin-2B localizes to mitochondria (10), the mechanism by which survivin-2B functions in promotion of apoptosis has not been well defined.

In this study, we report that survivin-2B also localizes to the microtubule organization center (MTOC).^{2} Forced expression of survivin-2B abrogates mitotic cells and induces mitochondria-dependent apoptosis by blockade of tubulin polymerization and modulation of Bcl-2, Bax, and survivin^{*}

Revised manuscript received, July 25, 2007,
Published, JBC Papers in Press, July 25, 2007, DOI 10.1074/jbc.M705161200
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^{*} This work was sponsored in part by National Institutes of Health R01 Grant CA109481 and Research Award/Grant BCTR63806 from the Susan G. Komen Breast Cancer Foundation (to F. L.), and by shared resources supported by National Institutes of Health Cancer Center Support Grant CA16056 (to Roswell Park Cancer Institute). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: MTOC, microtubule organization center; AIF, apoptosis-inducing factor; BIR, baculovirus IAP repeat; IAP, inhibitor of apoptosis; PI, propidium iodide; MTI, 3-[4-(dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; EGFP, enhanced green fluorescent protein.

2 The abbreviations used are: MTOC, microtubule organization center; AIF, apoptosis-inducing factor; BIR, baculovirus IAP repeat; IAP, inhibitor of apoptosis; PI, propidium iodide; MTI, 3-[4-(dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; EGFP, enhanced green fluorescent protein.
expression of survivin-2B blocks tubulin polymerization and induces mitochondria-dependent, caspase-8-independent apoptosis. Survivin-2B down-regulates the expression of the pro-survival factor Bcl-2 and up-regulates the pro-apoptotic factor Bax, while having no effect on the expression of apoptosis-inducing factor (AIF) in mitochondria. We propose that survivin-2B may represent a novel tumor suppressor molecule, and analysis of its regulation and function mechanism may reveal new approaches for cancer treatment.

**MATERIALS AND METHODS**

*Cell Culture and Reagents*—HeLa, HEK293, HCT116, and MCF-7 cells were maintained as previously described (18, 19). Anti-actin, peroxidase-conjugated goat anti-rabbit IgG, and 2% gelatin and monoclonal anti-α-tubulin (Clone: TUB 2.1) were purchased from Sigma. MTT was purchased from USB (Cleveland, OH) Anti-survivin (FL-142), anti-γ-tubulin (H-183), and anti-Bcl-2 (N-19) antibodies were purchased from Santa Cruz Biotechnology. The anti-EGFP AV. monoclonal antibody was from Molecular Probes (Eugene, OR). Lipofectamine™ was from Invitrogen. Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s instructions. For Western blot analysis and flow cytometry, cells were transfected with 1.5 μg of plasmid DNA and 2.5 μl of Lipofectamine™ 2000 in 6-well plates (4 × 10⁵ cells/well). For fluorescence microscopy, cells were transfected with 0.6 μg of plasmid and 1.0 μl of Lipofectamine™ 2000 in 12-well plates (1–2 × 10⁶ cells/well). For cell apoptosis (DNA fragmentation) assays, cells were transfected with 0.12 μg of plasmids and 0.2 μl of Lipofectamine™ 2000 in 48-well plates (3 × 10⁵ cells/well) and for MTT cell growth assays, cells were transfected with 60 ng of plasmid and 0.08 μl of Lipofectamine™ 2000 in 96-well plates (3000 cells/well). Transfection experiments were performed at least in triplicate for each experiment. For double staining of DNA and γ-tubulin, cells were fixed 48 h after transfection with 4% paraformaldehyde in phosphate-buffered saline (PBS, 50 mM phosphate, pH 7.4, 100 mM NaCl, and 10 mM KCl).

**Western Blot Analysis**—Western blotting was performed as previously described (21) except that cells were lysed in 8 M urea solution. Following protein transfer, the membrane was incubated with antibodies for EGFP (1:1000), survivin-2B (1:2000), Bcl-2 (1:500), AIF (1:1000), Bax (1:1000), or MnSOD (1:10,000). Corresponding protein signals were detected using a HRPL kit (National Diagnostics/LPS, Rochester, NY) by x-ray film exposure with various times (20–120 s).

**Tubulin Depolymerization and Repolymerization Assays** (22)—HeLa cells were seeded in 100-mm dishes (1 × 10⁶ cells/dish) and transfected with EGFP or EGFP-survivin-2B vectors as described above. Twenty-four hours after transfection, cells were treated with nocodazole (2 μg/ml) for 2 h to depolymerize tubulin. Cells were then released from nocodazole and lysed at 0, 4, 8, and 12 min in 400 μl of microtubule-stabilizing lysis buffer containing 4 μM taxol (paclitaxel), 0.1 M PIPES, pH 6.9, 2 mM glycerol, 5 mM MgCl₂, 2 mM EGTA, 0.5% Triton X-100, and 5 μg/ml leupeptin. Cell lysates were centrifuged at 40,000 × g for 30 min at 22 °C for sedimentation of the polymeric tubulin. The sediment (containing the polymerized tubulin) was solubilized in 150 μl of buffer containing 25 mM Tris, pH 7.4, 0.4 mM NaCl, 1% Nonidet P-40, 0.5% SDS, 0.1% deoxycholate, and 5 μg of leupeptin. Equal volumes (50 μl) of resulting samples were analyzed by Western blot analysis using anti-γ-tubulin antibody.

**MTT Assay and Taxol Treatment**—The MTT assay was used to determine the effect of survivin-2B on cell growth/viability with or without taxol treatment. In this assay, MTT was used as...
a colorimetric substrate for measuring cell viability. Non-viable cells, with altered cellular redox activity, are unable to reduce the dye. Seventy-two hours after transfection (equivalent to 48 h after taxol treatment (adding taxol 24 h after transfection)] of survivin-2B, MTT assays were carried out by adding MTT to a final concentration of 0.5 mg/ml in 96-well plates and incubation in a 5% CO$_2$ incubator at 37 °C for 4 h. The resultant reactions were then dissolved by adding 100 µl per well of a buffer containing 20% SDS, 50% N,N-dimethylformamide (pH 4.7) for an additional 4 h incubation. Absorbance in each well was measured at 570 nm using an Ultra Microplate Reader (Bio-Tek Instruments). Results are reported as a histogram. The bars are the mean ± S.D. derived from six independent measurements for each transfected vector.

Propidium Iodide (PI) Staining and Flow Cytometric Analysis—HeLa cells with and without transfection with the pEGFPc1-survivin-2B expression vector or the pEGFPc1 empty vector were stained with PI and analyzed by flow cytometry as previously described (17) by gating the transfected green cells from 10,000 events per sample. Flow cytometric data were analyzed using WinList software (Verity Software House Inc., Topsham, ME). Each condition was performed in triplicate to minimize potential testing system variation. Data were from two independent analyses.

Mitotic Index Analysis—Forty-eight hours after transfection (see above), HeLa cells were double stained for DNA and phosphohistone H3 (Ser10), a mitotic marker. Mitotic cells with phosphohistone H3 (Ser10) positivity were identified by fluorescence microscopy and shown as a percentage of total transfected green cells counted from 10 independent microscopic fields in each well. The S.D. was calculated from six independent wells in each group.

Fluorescence Microscopy—HeLa or HEK293 cells were seeded on circular glass coverslips coated with 2% gelatin in 12-well plates. For double staining of DNA and β-tubulin, cells were fixed with 4% paraformaldehyde in PBS, blocked/permeabilized with PBS containing 2% BSA and 0.2% Triton X-100, and then incubated in PBS containing 1% BSA and anti-γ-tubulin antibody (1:1000) for 60 min at 37 °C. After washing with PBS, cells were incubated in PBS containing Texas red/anti-mouse IgG antibodies for 45 min at room temperature, followed by staining with 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.5 µg/ml in PBS for 10 min. Coverslips were then mounted on glass slides with Gel/Mount™ solution (Biomedia, Foster City, CA). For Bcl-2 staining, cells were incubated in PBS containing 1% BSA and anti-Bcl-2 antibody (1:500) as above without DAPI staining. In these experiments, cell morphology was visualized and digitally captured using a Zeiss Axiovert 100 m Fluorescence Microscopy System.

Survivin-2B-specific siRNA Preparation and Transfection—A designed human survivin-2B mRNA-specific RNA oligonucleotides with 3′-TT overhangs were chemically synthesized and purified by HPLC (GeneLink, Hawthorne, NY): 2B-2f (5′-CCUGUAUACCAGCAGUUT263T) and 2B-2r (263AAGUGCUUAGUACAGT245T). Equal moles of 2B-2f/2B-2r were mixed together to a final concentration of 20 µM in annealing buffer (100 mM KAc, 30 mM HEPES-KOH, 2 mM MgAc$_2$, pH 7.4). After denaturation at 90 °C for 1 min, the mixture was annealed at 37 °C for 60 min and stored at −80 °C for transfection experiments. A scramble RNA duplex was also prepared as above for a negative control in this study. The scramble sequence (5′-CAGUCGCCGUUUCCGAUGGT (forward chain) and 5′-CCAGUGCCAAAACGGACUGT (reverse chain)) was not present in mammalian cells by BLAST search at NCBI.

Cells were transfected with survivin-2B-specific siRNA using Lipofectamine™ 2000 following the manufacturer’s instructions (Invitrogen). Briefly, 1 day prior to transfection, 5 × 10^4 MCF-7 cells (in 2 ml of DMEM medium) per well were seeded in six-well plates (corresponding to a density of 40% at the time of transfection) without antibiotics. The transfection mixture was prepared by mixing 100 µl of DMEM containing 6 µl of 20 µM siRNA with 100 µl of DMEM containing 4 µl of Lipofectamine™ 2000 reagent. The mixture was added to the 6-well plate after 20–30 min incubation at room temperature. The transfected cells were returned to CO$_2$ incubator. Twenty-four hours after transfection with survivin-2B-specific siRNA or control siRNA as described above, cells were treated with or without taxol at different concentrations. For semi-quantitative RT-PCR (QRT-PCR), cells were harvested at 48 h after transfection. For MTT assays, cells were transfected with 0.25 µM of control or survivin-2B-specific siRNA and 0.18 µl of Lipofectamine™ 2000 for each well in 96-well plates (3000 cells per well) and MTT assay was performed 72 h after transfection.

Total RNA Isolation and QRT-PCR—Total RNA was isolated 48 h after transfection of survivin-2B-specific and control siRNA using TRI REAGENT following the manufacturer’s instruction (Molecular Research Center, Cincinnati, OH). Endogenous survivin-2B transcript expression was detected by QRT-PCR using the one step RT-PCR system as described by the manufacturer (USB, Cleveland, Ohio). Hsv5′ (5′-GAG-GCTGGCCTCATCCTACG-3′) and 2B-2 (5′-GTCTCTCTCCTCGGTAGCC-3′) were used to amplify survivin-2B PCR products (183 bp). GAPDH3 (5′-CCTTATTAGCCTACATA-3′) and GAPDH8 (5′-GGCCATCCACAGTCTTC-3′) were used to amplify the GAPDH PCR products (467 bp, internal control). Briefly, one µg of total RNA was added into a 50 µl of total volume containing 12.5 µl RT-PCR Master Mix, 600 nM Hsv5′P1 and 2B-2, 200 nM GAPDH3, and GAPDH8 primers. Reverse transcription was carried out at 50 °C for 25 min; PCR parameters were preheating at 94 °C for 2 min, followed by 28 PCR cycles of 94 °C × 30 s, 54 °C × 30 s, and 68 °C × 1 min. A final elongation was at 72 °C for 5 min, and then the PCR reaction was held at 4 °C. PCR reactions were separated on a 2% agarose gel along with a 100-bp DNA marker. PCR products were visualized under ultraviolet light (236 nm) after ethidium bromide staining. Images were obtained using a digital image system.

Isolation of Cytoplasmic and Mitochondrial Fractions—HeLa cells were transfected with pEGFPc1-survivin-2B or the pEGFPc1 (control) as described above. Cytoplasmic and mitochondrial subcellular fractions were isolated as previously described (23). Resultant supernatants (cytosolic fraction) and pellets (mitochondrial fraction) were analyzed for survivin-2B expression by Western blot analysis.
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Determination of Cytosolic versus Mitochondrial Distribution of Smac—Smac distribution was detected using the TiterZyme ELA human Smac/DIABLO ELISA kit (Assay Designs, Ann Arbor, MI) following the protocol recommended by the manufacturer. Forty-eight hours after transfection with or without pEGFPc1 (control) or pEGFPc1-survivin-2B, cells were harvested by trypsinization and centrifugation at 800 × g for 5 min. Cell pellets were resuspended in Digitonin Cell Permeabilization Buffer and incubated on ice for 5 min. After centrifugation at 1000 × g for 5 min at 4 °C, supernatants were saved as the cytosolic fraction. Remaining pellets were then resuspended with radioimmune precipitation assay buffer Cell Lysis Buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS and Protease Inhibitor Mixture 0.5 μl/ml), vortexed and incubated on ice for 5 min. The lysate was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was saved as the mitochondrial fraction. Total protein concentrations for the cytosolic and mitochondrial fractions were detected using Bio-Rad protein detection reagents. From each portion, 2 μg of total protein were diluted in 100 μl of PBS per well for determination of Smac concentration using ELISA. Sample or standard Smac protein was added into the appropriate precoated well and incubated at room temperature on a plate shaker for 1 h at 250 rpm. Smac ELA antibody was added following four PBS washes. After a 1-h incubation and four PBS washes, 100 μl of goat anti-rabbit IgG conjugated to horseradish peroxidase was added to each well, followed by 30 min of incubation and PBS washing. Substrate solutions were then added to each well, and the plate was then shaken on a plate shaker at 250 rpm for 30 min at room temperature for color development. After adding 100 μl of Stop Solution into each well, the plate was read at 450 nm using an Ultra Microplate Reader (Bio-Tek Instruments).

Mitochondrial Potential Assays—Forty-eight hours after transfection, mitochondrial potential was determined using the JC-1 mitochondrial membrane potential detection kit (Cell Technology). In healthy cells, the JC-1 dye accumulates in the mitochondria and appears bright red by fluorescence microscopy, but, in apoptotic cells, the mitochondrial membrane potential collapses and the JC-1 dye cannot accumulate within the mitochondria (no color). Images were digitally captured using a Zeiss LSM510 v2.8 fluorescence microscope with a digital camera.

Survivin-2B-specific Antibodies—A number of peptides were synthesized as haptens and conjugated to keyhole limpet hemocyanin (KLH) as immunogens. One of these peptides successfully produced survivin-2B-specific antibodies. Briefly, two preimmune eggs from each hen were collected before carrying out immunizations. On day 0, 0.02–0.2 mg of antigen (KLH-conjugated peptides) in Freund’s Complete Adjuvant was injected subcutaneously and/or intramuscularly into the breast tissue of a hen at multiple sites. Immunizations were repeated on days 21, 35, and 49 using Freund’s Incomplete Adjuvant. Immune eggs were collected from day 59. IgY antibodies were then isolated from the yolk of immune eggs and purified by the peptide-conjugated affinity column. This antibody was recently characterized (10).

Statistical Analysis—A Student’s t test was used for analysis of the significance of each corresponding group (24), and significance (p value) was set at the nominal level of <0.05 or less.

RESULTS

Forced Expression of EGFP-survivin-2B Strikingly Reduces Mitotic Cells—To delineate the role of survivin-2B in cell cycle regulation, we investigated cell cycle distribution after ectopic expression of pEGFPc1 control vector or pEGFPc1-survivin-2B. Flow cytometric analysis revealed that ectopic expression of
EGFP-survivin-2B decreased G2/M phase cell populations over time (e.g. 18.5%, in pEGFP-survivin-2B-transfected G2/M cells versus 29.2% in pEGFP-transfected G2/M cells at the 48-h time point), accompanied by increased sub-G1 cell populations (dead cells) (Fig. 1A). To further explore whether the survivin-2B-mediated inhibition of G2/M cell populations resulted from the decrease of G2 or mitotic cells, we morphologically examined mitotic cells by phosphohistone H3 (mitotic marker)-labeled fluorescence microscopy and found that increased survivin-2B expression strikingly decreases the population of mitotic cells (Fig. 1B). This indicates that survivin-2B plays a role in blocking cells going into mitosis.

Survivin-2B Colocalizes with MTOC and Interacts with γ-tubulin—To investigate the potential mechanism for survivin-2B-mediated abrogation of mitotic cells, we performed fluorescence microscopy to determine whether survivin-2B is associated with any structures relevant to mitosis. We found that EGFP-survivin-2B colocalizes with the microtubule organization center (MTOC, Fig. 2A, arrow) while EGFP expression was evenly distributed in the cell (Fig. 3A). Because γ-tubulin is the major component in the MTOC, we investigated the potential interaction between survivin-2B and γ-tubulin. We immunoprecipitated (IP) EGFP from EGFP, or EGFP-survivin2B-expressing cells using anti-EGFP antibody and then immunoblotted the IP pellets with anti-γ-tubulin antibody in Western blots (Fig. 2B, upper panel). The IP pellets were also analyzed for EGFP and EGFP-survivin-2B expression vectors and stained with Texas-red-conjugated anti–γ-tubulin antibodies and DAPI for fluorescence microscopy analysis (A) or lysed at different time points, as indicated, for Western blot analysis with anti-β-tubulin antibodies (B).

Survivin-2B interacts with γ-tubulin. A, HEK293 cells were transfected with EGFP-survivin-2B expression vectors and stained with Texas-red-conjugated anti–γ-tubulin antibodies and DAPI 24 h after transfection. (Note: γ-tubulin is considered as centrosome/MTOC-specific tubulin). The MTOC is indicated by arrows. B, HEK293 cells were transfected with pEGFP control vector or pEGFP-survivin-2B. Twenty-four hours after transfection, cells were lysed, and subjected to immunoprecipitation (IP) using anti-EGFP antibodies (Ab). The resultant IP pellets were analyzed with Western blots using the anti-γ-tubulin Ab (upper panel). The same membrane was re-blotted with the anti-EGFP Ab as an internal control (lower panel).

Survivin-2B blocks tubulin polymerization. HEK293 cells were transfected with pEGFP control vector or pEGFP-survivin-2B expression vector. Twenty-four hours after transfection, cells were treated with Nocodazole (2 h) and then released by removal of the drug. Four minutes after release, cells were fixed and stained with Texas red-conjugated anti-β-tubulin antibodies and DAPI for fluorescence microscopy analysis (A) or lysed at different time points, as indicated, for Western blot analysis with anti-β-tubulin antibodies (B).

Survivin-2B Blocks Tubulin Polymerization and Induces Apoptosis—Several lines of evidence support a critical role for γ-tubulin in mediating α/β-tubulin polymerization (25). The localization of survivin-2B in the MTOC and its interaction with γ-tubulin led us to test the hypothesis that survivin-2B plays a role in the regulation of α/β-tubulin polymerization through its interaction with γ-tubulin. Therefore, we utilized the tubulin-depolymerizing agent Nocodazole to transiently depolymerize tubulin in the cells, followed by release in complete medium without Nocodazole. β-Tubulin polymerization in the cells was determined either by fluorescence microscopy (Fig. 3A) or by Western blot analysis (Fig. 3B). As shown in Fig. 3A, 4 min after removal of Nocodazole, polymerized tubulin fibers/filaments appeared around the MTOC in the EGFP-transfected cells (Fig. 3A, upper panel) but not in the EGFP-survivin-2B-transfected cells (Fig. 3A, lower panel). Consistent with this observation, Western blot analysis demonstrated a striking accumulation of polymerized tubulin in lysates from EGFP-transfected cells by 8 min following removal of Nocodazole (Fig. 3B, left). In contrast, polymerized tubulin was not observed in the lysates from EGFP-survivin-2B-transfected cells (Fig. 3B, right).

Forced Expression of Survivin-2B Inhibits Cell Growth and Induces Apoptosis—It has been reported that survivin-2B expression is lost in the later stages of tumor development (14, 16). We, therefore, investigated the effect of survivin-2B on cell growth and cell death. Expression of exogenous survivin-2B was confirmed at the 24-h time point after transfection by Western blot analysis in several cancer cell types with both transfection and infection delivery systems (Figs. 4A and 5A). MTT assays (72 h) showed that forced expression of sur-
Survivin-2B Blocks Tubulin Polymerization and Induces Apoptosis

FIGURE 4. Forced expression of EGFP-survivin-2B inhibits cell growth and induces apoptosis. HeLa cells were transfected with expression vectors as shown. A, expression of the EGFP-fused survivin-2B was confirmed by Western blotting using anti-survivin antibodies 24 h after transfection. B and C, EGFP-survivin-2B inhibits cell growth. Cell growth was determined by MTT assay (B) or by direct cell number counts (C) 72 h after transfection. Data are the mean ± S.D. from five independent assays (B) or from three independent counting experiments (C). D, EGFP-survivin-2B induces nuclear condensation. Experimental conditions are the same as in B and C at the 72-h time point. Images of EGFP (control) or EGFP-survivin-2B-transfected cells are shown. E, EGFP-survivin-2B expression increases the sub-G1 cell population. Cells were transfected at three time points (24, 48, and 72 h) as shown and analyzed by PI staining and flow cytometry by gating the transfected green cells. Data in E are the mean ± S.D. derived from two independent flow cytometry experiments in duplicate.

Survivin-2B in HeLa (Figs. 4, B and C and 5B) and HCT116 (Fig. 5C) cells using pEGFP (Fig. 4, B and C), or recombinant adenovirus (Fig. 5, B and C) delivery systems, inhibited cell growth (Figs. 4, B and C and 5, B and C). Next, we determined whether survivin-2B-induced growth inhibition was accompanied by apoptosis. Ectopic expression of EGFP-survivin-2B induced cell/nuclear condensation at the 72-h time point (Fig. 4D, lower panel) compared with EGFP control (Fig. 4D, upper panel). Further, EGFP-survivin-2B also increased the sub-G1 cell population (dead cells) compared with the EGFP control at various time points (Fig. 4E). These results indicate that cells were undergoing apoptosis. Consistently, complementary experiments in which survivin-2B was silenced, survivin-2B with survivin-2B-specific siRNA revealed that loss of survivin-2B resulted in increased resistance to taxol-induced cell growth inhibition (Fig. 6).

Survivin-2B Induces Apoptosis through a Mitochondria-dependent but Caspase 8-independent Cell Death Pathway—Previously we have shown that survivin-2B is localized in mitochondria (10). This finding prompted us to hypothesize that survivin-2B modulates the mitochondria-dependent cell death pathway. Consistent with this hypothesis, forced expression of EGFP-survivin-2B but not EGFP induced Smac release from mitochondria (Fig. 7A), disrupted mitochondrial potential (Fig. 7B), activated caspase-9 (Fig. 8, A and B) and caspase-3 (Fig. 8, C and D), while caspase-8 remained inactive (Fig. 8E). These observations indicate that apoptosis induced by survivin-2B employs a mitochondria-dependent pathway. As a note, a weak band of active caspases 9 and 3 was detected in EGFP-expressing cells (Fig. 8, C and D). This appears to reflect EGFP-induced nonspecific caspase activation because a similar band was observed for caspase-8 in Fig. 8E (lane 2).

Combination of Survivin-2B Expression and Taxol Treatment Results in a Significant Increase in Cell Growth Inhibition and Cell Death Induction—We have previously shown that taxol-mediated induction of survivin expression is an early event before taxol-mediated G2/M arrest, and that disruption of taxol-mediated rapid induction of survivin expression sensitizes cells to taxol-induced cell death (21). Intriguingly, taxol actually decreased survivin-2B protein, which may reflect an

FIGURE 5. Forced expression of survivin-2B inhibits cell growth. Expression of survivin-2B was confirmed by Western blot analysis using anti-survivin-2B antibodies 24 h after transfection (A). As a note, anti-survivin-2B-specific antibody was previously characterized (10) (also see “Materials and Methods”). HeLa (B) and HCT116 (C) cells were infected with corresponding virus particles as shown. Cell growth/viability was determined by MTT assay 72 h after infection. Data are the mean ± S.D. from five independent assays.

FIGURE 6. Silencing of endogenous survivin-2B transcripts by survivin-2B-specific siRNA increases cell resistance to taxol-mediated growth inhibition. Survivin-2B-specific shRNA-mediated inhibition of survivin-2B transcripts was confirmed using QRT-PCR 48 h after transfection (A). MCF-7 breast cancer cells were transfected with either survivin-2B-specific siRNA or control RNA (B), and followed by taxol treatment as shown. Cell growth inhibition was analyzed by MTT assay 72 h after transfection (equivalent to taxol treatment for 48 h). Data are the mean ± S.D. from five independent assays.
Survivin-2B Blocks Tubulin Polymerization and Induces Apoptosis

additional mechanism for taxol resistance, while increasing survivin (Fig. 9A). Therefore, we hypothesized that forced expression of survivin-2B in combination with taxol treatment should increase taxol efficacy. Consistent with this hypothesis, forced expression of survivin-2B followed by taxol treatment in HeLa (Figs. 9, B and C and 10B), 293 (Fig. 10A) and HCT116 (Fig. 10C) using both transfection (Figs. 9, B and C and 10A) and infection (Fig. 10, B and C) delivery systems, resulted in significant increase in cell growth inhibition and the sub-G1 cell population (Fig. 9D). In contrast, as we show in Fig. 6, silencing of survivin-2B transcripts using survivin-2B-specific siRNA made cells more resistant to taxol-induced growth inhibition. Moreover, these results are also consistent with the role of survivin-2B in the modulation of pro-survival and pro-apoptotic factors revealed below (Fig. 11).

Forced Expression of Survivin-2B Results in Down-regulation of Bcl-2 and Survivin but Up-regulation of Bax in Mitochondria, without Affecting Mitochondria-localized AIF (Apoptosis-inducing Factor) Expression—To further explore the mechanism by which survivin-2B triggers activation of the mitochondrial cell death pathway, we examined whether forced expression of survivin-2B in mitochondria is able to modulate the expression of Bcl-2 family proteins. We found that forced expression of survivin-2B using both transfection (Fig. 11A left panel) and infection (Fig. 11A, middle and right panels) delivery systems in HeLa (Fig. 11A, left and middle panels) and MCF-7 (Fig. 11A, right panel), down-regulated the expression of Bcl-2, while survivin-2B did not affect the expression of mitochondrial AIF. Experimental conditions from A to E are 72 h after transfection. The relative caspase activation in B, D, and E is indicated. Actin expression is used as an internal total protein loading control in Western blot experiments.

![Graph](image1)

**FIGURE 7.** Forced expression of EGFP-survivin-2B induces Smac release from mitochondria and loss of mitochondrial potential. A, HeLa cells were fractionated into cytosolic and mitochondrial fractions 48 h after transfection. Smac protein was determined using an ELISA kit (Assay Designs). Each bar is the mean ± S.D. derived from three independent experiments. B, forced expression of EGFP-survivin-2B disrupted mitochondrial potential (bottom panel) while forced expression of EGFP (control) did not (upper panel). Mitochondrial potential was determined using a JC-1 mitochondrial membrane potential detection kit (Cell Technology) 48 h after transfection. Transfected cells (green, left panel) with the corresponding JC-1 staining profile (red, middle panel) and overlay (right panel) are shown. Note: the white arrow indicates some examples of transfected cells, and the red arrow indicates very late apoptotic cells with nonspecific red label.

![Graph](image2)

**FIGURE 8.** Forced expression of EGFP-survivin-2B activates caspases. A and B, EGFP-survivin-2B activates caspase-9. Activation of caspase-9 was determined by immunofluorescence (A) and Western blot (B) analyses using active caspase-9-specific antibodies (unprocessed form could not be recognized). C and D, EGFP-survivin-2B activates caspase-3. Activation of caspase-3 was determined by immunofluorescence (C) and Western blot (D) analyses using active caspase-3-specific antibodies. E, EGFP-survivin-2B does not activate caspase-8. The status of caspase-8 activation was analyzed using Western blots with anti-active caspase-8 antibodies. Experimental conditions from A to E are 72 h after transfection. The relative caspase activation in B, D, and E is indicated. Actin expression is used as an internal total protein loading control in Western blot experiments.
but sustained decrease of Bcl-2 expression after forced expression of survivin-2B (Fig. 11C).

It is known that translocation of the pro-apoptotic factor Bax to mitochondria is important for the initiation of mitochondrial-mediated apoptosis, and that there is a mitochondrial pool of survivin (8). Therefore, we explored the expression of survivin and Bax in mitochondria after forced expression of EGFP-survivin-2B. Intriguingly, ectopic expression of EGFP-survivin-2B transiently increased survivin expression in mitochondria at early times (i.e. 18 h following transfection) with reversal of this effect by 24 h (Fig. 11C). In contrast, forced expression of survivin-2B up-regulated the expression of Bax in mitochondria (Fig. 11C), which may reflect the increased translocation of Bax from cytosol to mitochondria for apoptosis induction, while forced expression of EGFP had no effect on these molecules (not shown).

Together, these observations provide a sound molecular mechanism for survivin-2B-induced mitochondria-dependent apoptosis, although the mechanism underlying survivin-2B modulation of these pro-survival and pro-apoptotic molecules remains to be investigated (see “Discussion”).

DISCUSSION

A great deal of effort has been made to investigate the function of survivin associated with its unique subcellular localization (3, 10). However, little is known about the roles and underlying mechanisms of the survivin splice variant survivin-2B. In this report, we used multiple approaches to examine the role of survivin-2B in cell cycle regulation and apoptosis induction. We demonstrated for the first time that, in addition to being localized to mitochondria (10), survivin-2B colocalizes and interacts with γ-tubulin in MTOC (Figs. 2 and 3), and blocks tubulin polymerization (Fig. 3). These findings provide a plausible explanation for the observation that forced expression of survivin-2B abrogated mitotic cells (Fig. 1B). While there is no conclusive evidence suggesting a contribution of this phenomenon to survivin-2B-mediated induction of apoptosis, we observed a significant association of sub-G₀ DNA content increase with the decrease of G₂/M cells (Fig. 1A). In any case, we would like to emphasize that although we found a portion of survivin-2B that interacts with γ-tubulin and localizes in the MTOC, we did not find a nucleation defect. However, an inability of tubulin fiber formation surrounding the MTOC in vivo (Fig. 3A) and of microtubule polymerization in vitro was noted (Fig. 3B). These observations strongly suggest that the MTOC-associated survivin-2B molecules block G₂ cells moving into

mitochondria and found that forced expression of EGFP-survivin-2B not only decreased the expression of Bcl-2 in mitochondria, but also impaired its proper localization compared with the EGFP control (67.9% of total survivin-2B-transfected cells were impaired for Bcl-2 localization versus only 14.1% in total EGFP vector-transfected control cells, Fig. 11B). Next, we further examined the time course for survivin-2B-mediated down-regulation of Bcl-2. This experiment showed a gradual

FIGURE 9. Forced expression of survivin-2B sensitizes cell to taxol treatment (I). Taxol up-regulated endogenous survivin and down-regulated exogenous survivin-2B expression determined by Western blot analysis using the corresponding antibodies (A). Forced expression of pEGFP-survivin-2B sensitized HeLa cells to taxol-induced cell growth inhibition (B and C) and cell death induction (increased sub-G₁ cell population, D) compared with the results from the corresponding pEGFP controls. Data are the MTT assay (B and C) and PI staining/flow cytometry (D) at 72 h after transfection (equivalent to taxol treatment for 48 h). Each bar is the mean ± S.D. derived from three independent experiments (B and C). The p values in A, B, and C are indicated.

FIGURE 10. Forced expression of survivin-2B sensitizes cell to taxol treatment (II). Expression of survivin-2B was shown in Fig. 5A. HKE293 (A), HeLa (B), and HCT116 (C) cells were transfected (A) or infected (B and C) with expression vectors or corresponding viruses as shown. Cell growth/viability was determined by MTT assay 72 h after transfection (equivalent to taxol treatment for 48 h). Data are the mean ± S.D. from five independent assays.

Survivin-2B Blocks Tubulin Polymerization and Induces Apoptosis
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Survivin-2B down-regulates Bcl-2 and up-regulates Bax without affecting AIF expression in mitochondria. A, HeLa (left and middle panels) and MCF-7 (right panel) cells were transfected (left panel) or infected (middle and right panel) with control vectors and control viruses (pEGFPc1 and Ad-virus) or targeted vectors and targeted viruses (pEGFPc1-surv-2B and Ad-surv-2B), respectively, as shown. Cells were lysed 48 h after gene delivery. Expression of Bcl-2 and AIF was determined by Western blot analysis. Actin was used as an internal protein loading control. B, cells grown on circular coverslips were transfected as in A and processed for immunofluorescence. C, time course of the modulation of survivin, Bcl-2 and Bax expression after forced expression of pEGFP-survivin-2B. HeLa cells were transfected with pEGFP-survivin-2B. Mitochondrial fractions were then isolated at different time points and examined for the expression of survivin, Bcl-2, and Bax by Western blot analysis over a time course. The expression of Mn-SOD (superoxide dismutase), a mitochondrial-specific protein, was used as an internal control for protein loading and mitochondrial fraction verification.

shown in Fig. 10, forced expression of survivin-2B alone did not significantly induce cell growth inhibition in HKE293 cells (Fig. 10A) while it did in HeLa and HCT116 cells (Fig. 10, B and C). This result is consistent with growing evidence that the regulation and/or function of the survivin gene appear to be different in cancerous cells versus non-cancerous cells (11). For example, AP-2 transcripational factors down-regulates survivin promoter activity in cancer cells but not in normal cells (28).

We further demonstrated in our studies that forced expression of survivin-2B induces mitochondria-dependent apoptosis, at least in part due to the down-regulation of the pro-survival factor Bcl-2 and up-regulation of the pro-apoptotic factor Bax in mitochondria. While the mechanisms underlying the changes in survival factor expression induced by survivin-2B remain to be investigated, it is known that mitochondrial Bcl-2 is essential for blockade of mitochondria-mediated apoptotic events. Thus, decreased expression of Bcl-2 in mitochondria should facilitate mitochondria-mediated apoptosis induction. On the other hand, translocation of Bax from the cytosol to mitochondria is also known to be a critical step for induction of mitochondria-mediated apoptotic cell death. In healthy cells, Bax resides in the cytosol, where it is inactive; during apoptosis, however, Bax undergoes a conformational change involving N-terminal exposure and translocates to mitochondria (29, 30). Thus, the observed increase in mitochondrial Bax expression following forced expression of survivin-2B (Fig. 11C) likely reflects translocation of Bax from the cytosol to mitochondria. Regardless of the molecular events involved in survivin-2B-induced down-regulation of Bcl-2 and up-regulation of Bax in mitochondria, these findings are novel and provide a plausible mechanism for survivin-2B-mediated induction of mitochondria-associated apoptotic events (i.e. Smac release from mitochondria, activation of caspases 9 and 3, and loss of mitochondrial potential).

Dohi et al. (8) previously demonstrated that there is a mitochondrial pool of survivin in cancer cells but not in normal survivin-positive tissues such as testis, and that this pool is essential for the inhibition of mitochondria-mediated apoptosis. These authors further showed that, in response to cell death stimuli, mitochondrial survivin is rapidly released into the cytosol, where it prevents caspase activation and inhibits caspase 9-mediated apoptosis (8). Thus, an alternative mechanism for survivin-2B-induced mitochondria-mediated apoptosis may involve blockade of mitochondrial survivin release into...
the cytosol by the physical interaction between the two molecules. This may subsequently result in the destabilization of survivin protein. Consistent with this speculation, cotransfection of EGFP-survivin-2B and HA-survivin into HeLa cells followed by immunoprecipitation (IP) of survivin-2B using anti-EGFP antibody and Western blots of IP pellets with anti-HA antibody indicates the interaction of survivin-2B with survivin (see additional discussion below). Further, the dynamic modulation profile of mitochondrial survivin resulting from forced expression of survivin-2B (Fig. 11C) is also consistent with this notion. That is, the increased level of survivin in mitochondria at an early time (18h) following forced expression of survivin-2B may reflect blockade of survivin release into the cytosol (Fig. 11C), while the down-regulation of mitochondrial survivin at later times (24 and 48 h) may represent degradation of the protein (Fig. 11C). However, we recognize that further studies are required to explore these possibilities and to determine whether survivin-2B-mediated modulation of survivin levels in mitochondria contributes to survivin-2B-induced mitochondria-dependent apoptosis. Here, we would like to point out that because the current survivin antibodies recognize both survivin and other survivin splice variants, it would be difficult to directly investigate the interaction of survivin-2B with survivin in untransfected cells. Nevertheless, as we discussed above, the interaction between EGFP-survivin-2B and HA-survivin in HeLa cells was also demonstrated in our laboratory.

Additionally, we would like to discuss the detection of γ-tubulin signal not only exists in the MTOC but also in the cytoplasm (Fig. 2A). Several lines of evidence indicate a critical role for γ-tubulin in mediating α/β-tubulin polymerization. In the evidence-derived γ-tubulin-mediated microtubule-assembly model, the γ-tubulin-containing ring complex nucleates microtubule assembly by presenting a row of γ-tubulin subunits or forming a protofilament, which provides a tag/platform for α/β-tubulin subunit assembly (25). Based on this model, γ-tubulin may not be restricted to the MTOC but may also extend outside of it. Of course, an alternative explanation for the γ-tubulin signal in the cytoplasm is that the anti-γ-tubulin antibody may partially recognize α- and/or β-tubulin. A more likely explanation for this observation is a combination of both possibilities.

Finally, we would like to discuss the potential implications of our findings for cancer therapeutics. Studies from Islam et al. (12) demonstrated that, while the expression of survivin is down-regulated during retinoic acid-induced apoptosis in CHP134 neuroblastoma cells, survivin-2B expression is slightly increased during apoptosis. This observation suggests that survivin-2B and survivin could be differentially regulated with potentially different roles during apoptosis. Consistent with this notion, it has been demonstrated that one of the mechanisms for apoptosis induction by the p53 tumor suppressor protein is through differential modulation of survivin and its variants (31). In leukemia cells, doxorubicin-activated p53 up-regulated survivin-2B, but down-regulated both survivin and survivin-ΔEx3 (31). Additionally, based on the observation in this study together with our previous finding (21), it appears that taxol resistance likely involves both up-regulation of survivin and down-regulation of survivin-2B. Together, these findings indicate that survivin and survivin-2B could be differentially regulated. Up-regulation of survivin-2B and down-regulation of survivin may represent a novel approach for cancer treatment.

In summary, this study reveals a novel mechanism by which survivin-2B acts as a potential proapoptotic molecule. Forced expression of survivin-2B abrogates mitotic cells and activates the mitochondria-dependent apoptotic pathway by blockade of tubulin polymerization and modulation of Bcl-2, Bax, and survivin expression.

Acknowledgments—We thank Dr. Bert Vogelstein for the shuttle vector pAdTrack-CMV and Pasha Apontes and Dr. Andrzej Wierzbicki for technical assistance.

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