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Participation of Survivin in Mitotic and Apoptotic Activities of Normal and Tumor-Derived Cells

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Abstract Survivin is a member of the inhibitor of apoptosis (IAP) gene family, containing a single baculovirus IAP repeat (BIR) and no RING finger, that is expressed in many human cancers. Although it has been proposed to be involved in mitotic and cytokinetic processes, its functional subcellular distribution in the cytoplasm and nucleus, and its binding to centrosomes, spindle fibers, and centromeres in relation to these processes, is not fully resolved. We have analyzed the localization of Survivin in normal (Detroit 551, IMR-90) and tumor-derived (HeLa, Saos-2) cell lines, and found that it does colocalize with centrosomes in the cytoplasm during interphase, then moves to centromeres during mitosis, and finally localizes to the midbody spindle fibers during telophase. However, Taxol, a popular microtubule stabilizing agent that is frequently used in the study of these processes, severely disrupted the localization of Survivin. Taxol treatment of cells promoted extensive relocalization of Survivin with α-tubulin on microtubules during either interphase or mitosis. Survivin antisense oligonucleotide markedly sensitized HeLa cells to cell death induced by agents acting at the level of cell surface receptor (Fas pathway) or at the level of mitochondria (etoposide). HeLa cell death induced by Survivin antisense oligonucleotide could be partially complemented by Deterin, the *Drosophila* homolog of Survivin (Jones et al. [2000] J. Biol. Chem. 275:22157–22166). Reciprocally, a chimera of the Deterin BIR domain and Survivin C-terminus could rescue *Drosophila* Kc cells from death induced by transfection of a human caspase-7-expressing plasmid. These results indicate common components of Survivin and Deterin antiapoptotic action in the vertebrate and invertebrate phyla. J. Cell. Biochem. 83: 342–354, 2001.

Key words: Survivin; Deterin; mitosis; localization; cancer

Faithful replication and propagation of genetic material by cells to their progeny through cell division, as well as the delicate balance between cell proliferation and apoptotic cell death, are two important factors in maintenance of cell and tissue homeostasis. Inhibitors of apoptosis (IAP) proteins [Bergmann et al., 1998] were first discovered in baculoviruses [Crook et al., 1993] and then identified in both vertebrate and invertebrate cellular systems [Hay et al., 1995; Rothe et al., 1995; Duckett et al., 1996; Liston et al., 1996; Fraser et al., 1997]. These proteins are typically characterized by 2–3 baculovirus IAP repeats (BIR) and a RING finger motif [LaCasse et al., 1998; Deveraux and Reed, 1999]. However, the role and mechanisms of action of the IAPs in apoptosis and normal cell physiology is poorly understood. Some IAPs such as MIHA/XIAP/HILP, MIHB/C-IAP-1/HIAP-1, MIHC/c-IAP-1/hIAP2, MIHC/cIAP-2/hIAP1 [Rothe et al., 1995; Duckett et al., 1996; Liston et al., 1996; Uren

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et al., 1996] and *Drosophila* DIAP1 [Hay et al., 1995; Meier et al., 2000] are able to inhibit caspase-mediated apoptosis directly or indirectly. On the other hand, the BIR-containing proteins from *C. elegans* and yeast seem to function primarily in mitosis and cell division rather than apoptosis [Fraser et al., 1999; Rajagopalan and Balasubramanian, 1999; Uren et al., 1999; Yoon and Carbon, 1999; Speliotes et al., 2000]. Recently human Survivin and *Drosophila* Deterin, novel members of the IAP family containing a single BIR and no RING finger, were found to be expressed in fetal or embryonic and larval tissues, respectively [Ambrosini et al., 1997; Jones et al., 2000]. Survivin has been detected in most common human cancers but not in most adult differentiated tissues [Ambrosini et al., 1997; Adida et al., 1998]. In both the human and *Drosophila* systems, the respective Survivin and Deterin were shown to be capable of inhibiting caspase-mediated apoptosis in human and insect cells, respectively, and a chimera of Survivin BIR domain and Deterin C-terminal domain was partially active in the insect cells [Jones et al., 2000].

Experimental inquiries into a role of BIR-containing proteins in cell division have assessed immunocytological evidence on the subcellular localization of the proteins with respect to the mitotic and cytokinetic structures. Thus far somewhat contradictory evidence concerning Survivin localization has been reported from the various (primarily tumor-derived) model tissues and cell lines used. In tumor tissues, Survivin has been localized to the cytoplasm of tumor cells of lung adenocarcinoma, lung squamous cell carcinoma, pancreatic adenocarcinoma, high grade non-Hodgkin's lymphomas [Ambrosini et al., 1997], breast carcinomas [Tanaka et al., 2000], neuroblastoma [Adida et al., 1998], BCC tumor cells [Grossman et al., 1999], and gastric tumor cells [Lu et al., 1998]. In contrast, Survivin was detected primarily in the nucleus with only weak cytoplasmic staining in human hepatocellular carcinomas [Ito et al., 2000].

With respect to cancer-derived cell lines, published studies have primarily used the human cervical adenocarcinoma cell line (HeLa). In a series of reports, Survivin localization in mitotic cells was determined to coincide with γ-tubulin on centrosomes [Li et al., 1999] and with β-tubulin on spindle fibers and midbody at prophase, metaphase, anaphase, and telophase [Li et al., 1998; O'Connor et al., 2000]. During interphase, Survivin staining was detected on cytoplasmic microtubules and the microtubule organizing center (MTOC) [Li et al., 1998]. Alternatively, Uren et al. [2000] and Skoufias et al. [2000] reported no detectable Survivin in interphase HeLa cells, no significant colocalization with β-tubulin between interphase and metaphase, and primarily a centromeric localization at metaphase. In another contrasting report, Survivin in human hepatoma cell lines HepG2 and SK-VE2, was reported to be primarily in the cytoplasm [Suzuki et al., 2000a,b], and was either retained in the cytoplasm under serum-free conditions of G1 arrest [Suzuki et al., 2000b] or translocated to the nucleus under conditions of Fas antibody-stimulated cell proliferation [Suzuki et al., 2000a]. It is as yet unclear which of the patterns of Survivin localization, if any, applies to noncancerous human cells. Although, in *C. elegans* staining of the related protein BIR-1 yet further differs from the above cancer cell line outcomes in that BIR-1 staining overlaps with the more general chromosomal staining rather than the specific centromeric (kinetochore) region [Speliotes et al., 2000].

The occurrence of Survivin in so many human cancers has engendered much excitement on the prospects of Survivin as a target for therapeutic strategies in prognosis or treatment of cancer. Possible strategies include generation of Survivin-specific CD^{8+} T effector cells pulsed with Survivin peptides [Schmitz et al., 2000]. Antisense approaches using Survivin antisense cDNA [Ambrosini et al., 1998; Li et al., 1998; Grossman et al., 1999], or Survivin antisense oligonucleotides containing 2′-*O*-methoxylethyl modifications but natural phosphodiester linkages [Chen et al., 2000], or with phosphorothioate linkages instead of natural phosphodiester linkages [Olie et al., 2000], or both modifications [Li et al., 1999] have been shown to interfere with Survivin action. These results have raised the prospect that antisense mediated down-regulation of Survivin may sensitize tumor cells to chemotherapy [Olie et al., 2000], although few studies have yet indicated such sensitization [Grossman et al., 2001].

To further identify the Survivin subcellular distribution and to determine whether the distribution in noncancerous model cells is similar to that seen in cancer-derived human

model cell lines, we have examined Survivin subcellular distribution in each stage of mitosis of cultured normal human cells and human cancer cell lines. We also used Survivin antisense oligonucleotides to further evaluate Survivin interference as a target for inducing cancer cell death or sensitization of cancer cells to other therapeutic agents. Finally, we provide evidence for functional similarity of Survivin- and Deterin-antiapoptotic pathways in human and *Drosophila* cells.

MATERIALS AND METHODS

Cell Culture

Hela cells, Detroit 551 and IMR-90 cells, CHO and NIH3T3 cells (all from American Type Culture Collection), and SaoS-2 (maintained by Dr. Karl Münger, Department of Pathology, Harvard Medical School), were maintained in DMEM (Sigma, St Louis, MD) at 37°C, 5% CO_2 supplemented with 2 mM glutamine, 100 U/ml penicillin-streptomycin solution, 1 × MEM nonessential amino acid solution, 1 × MEM vitamin solution, and 10% heat-inactivated fetal bovine serum (all from Life Technologies, Grand Island, NY) and 2 g/ml D (+) glucose.

Immunocytochemistry

Cells were directly cultured on glass coverslips in a 6-well culture plate, rinsed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 20 min. The cells were then permeated with 2% Triton-X 100 for 15 min and blocked by 10% normal horse serum. These cells were incubated with 1:50 rabbit polyclonal Survivin antibody (Novus Biologicals, Littleton, CO) and 1:300 mouse monoclonal α-tubulin antibody. We only detected a single, apparently full length Survivin protein using this antibody [Conway et al., 2000]. In other experiments the fixed and permated cells were coincubated with 1:50 rabbit polyclonal Survivin antibody and 1:2,000 CREST human anti-kinetochore antibody (kindly provided by Dr. Frank McKeon, Harvard Medical School). Polyclonal antiserum against an insect serum storage protein [arylphorin, Jones et al., 1993] served in some experiments as a negative control. After incubation overnight at 4°C and then washing with PBS, cells were reacted with 1:300 anti-rabbit IgG TRITC (Sigma) and 1:250 anti-mouse IgG FITC or with 1:300 anti-rabbit IgG TRITC and 1:150 anti-human IgG FITC (Vector Laboratories, Burlingame, CA) at 37°C for 2 h. Finally, the cell chromosomes were stained with DAPI for 5 min and the triply stained cells were observed and photographed with a Nikkon E800 Eclipse fluorescence microscope.

Cell Treatment With Taxol Treatment

Cells were directly grown on glass coverslips and treated with 13 μg/ml Taxol (Sigma, St Louis, MD). After 24 h the cells were collected and subjected to indirect immunocytochemistry.

Immunoblotting

Cells were washed and extracted with cell lysis buffer containing 0.1% SDS, 1 mM DTT in PBS. One hundred micrograms of protein per lane were electrophoresed on a 12% SDS–polyacrylamide gel and electroblotted to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) at 15 V for 20 min at room temperature (RT). The membrane was blocked in 5% skim milk for 1 h, washed and incubated with 2 μg/ml rabbit anti-Survivin polyclonal antibody (or the same concentration of rabbit anti-storage protein antibody) and 1:2,000 mouse anti-α-tubulin monoclonal antibody in 5% skim milk at room temperature for 2 h or at 4°C overnight. After washing with PBS, blots were reacted with 1:1,000 alkaline phosphatase goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) or 1:1,000 alkaline phosphatase horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) in 5% skim milk for 1 h. After washing in PBS and blocking with Tris–NaCl–$CaCl_2$ solution (pH 9.5) for 20 min, bands were visualized by NBT/BCIP solution (Boehringer-Mannheim, Germany).

Cell Transfection

Oligonucleotides for interference with Survivin messenger RNA expression were adapted from Li et al. [1999], except that natural phosphodiester bonds were preserved and there were no 2′-*O*-methoxyethyl modifications (Survivin antisense oligonuleotide: 5′-TGTGCTATTCTGTGAATT and negative control oligonucleotide: 5′-TAAGCTGTTCTATGTGTT). HeLa cells (1.5×10^5) were plated onto each cover slip in a 6-well culture plate. After 24 h cells were transfected with 1.8 μg fluorescein-conjugated control or negative control oligonucleotides and/or 1.5 μg of an expression

vector construct. The expression constructs used were either pcDNA3 vector, pcDNA3 encoding the full length Survivin cDNA, pEGFPC1 vector, or pEGFPC1 encoding either Survivin or Deterin. Transfections of HeLa cells were performed using LipofectAmine (Life technologies, Grand Island, NY) according to the instructions of the manufacturer. The cell transfection efficiency was about 60–70% as estimated by FAScan. In some HeLa cells transfections, 24 h after transfection with 300 nM Survivin antisense or control oligonucleotides, cells were treated with 100 ng/ml mouse anti-human Fas (Clone 11) antibody (Upstate Biotechnology, Lake Placid, NY) and/or 100 nM etoposide (Sigma). In all HeLa cell transfections, 48 h after transfection, the supernatant was removed and cells were either stained with 0.4% trypan blue (Life Technologies, Grand Island, NY) or were stained with In Situ Cell Death Detection Kit, TMR red (Boehringer Mannheim, Indianapolis, IN). Briefly, cells were fixed with 2% paraformaldehyde for 60 min at room temperature and rinsed with PBS and incubated in permeabilisation solution (0.1% Triton-X, 0.1% sodium citrate) for 2 min in ice. The cells were washed twice with PBS and incubated with TUNEL reaction mixture for 60 min at 37°C in a humidified box in the dark, and washed twice with blocking buffer PBS/Triton/BSA. Finally the cell nuclear DNA was stained with 0.5 μg/ml DAPI for 5 min.

Three randomly chosen fields of each well were photographed and the ratio of trypan blue-positive or apoptotic cells (chromatic condensation, fragmentation, wrinkling of nuclear membrane) to total counted cells from three wells were calculated under a light microscope or a fluorescence microscope, as appropriate. The experiment was performed with three replications. In other experiments, HeLa cells were transfected with serial concentrations (50, 150, and 300 nM) of Survivin antisense or control oligonucleotides by the above described method. After 48 h, cells were collected with trypsin/EDTA and subjected to immunoblotting to determine the level of endogenous Survivin.

For transfection of insect cells, $\sim 2.3 \times 10^6$ Kc cells (grown in Schneider's medium) were seeded to 6-well dishes in 2 ml medium. The DNA constructs were transfected into the cells using CellFECTIN reagent (Life Technologies, Grand Island, NY) in accordance with company instructions. As a control, 2 μg of empty pIE 1–4 expression vector was transfected alone, and the empty vector was also used to equalize the total DNA amount in each well to 2.3 μg. Other cells were transfected with 1 μg pIE1–4 encoding human caspase-7 and/or 1 μg encoding a chimera of the N-terminal BIR region of Deterin and the C-terminus of Survivin [Jones et al., 2000]. All treatments also included 0.3 μg of pIE1–4 expression vector encoding beta galactosidase to be used as a reporter. Approximately 56 h after transfection, the number of surviving transfected cells in each well was determined by staining the cells with X-Gal and counting the blue cells in a transect across the middle of each well. Or, the cells were stained with an In Situ Cell Death Detection Kit and apoptotic cells counted under fluorescent microscopy. Each treatment was replicated three times and all results are reported as mean and standard error.

RESULTS

Changes in Localization of Endogenous Survivin

First, we determined the mitotic structures on which Survivin was localized during mitosis of human cancer cell lines (SaoS-2 and HeLa). In late interphase or early prophase the strongest Survivin localization was as an adjacent pair of cytoplasmic foci that localized closely to two strongly staining α-tubulin foci (Fig. 1A, inter/pro; see also Fig. 1, inset). Commencing abruptly with prometaphase, Survivin moved into the nucleus and became focussed at numerous spots. At metaphase, the great majority of Survivin staining was on the chromosomes at the metaphase plate. Very little Survivin at metaphase appeared to colocalize with α-tubulin (Fig. 1A, Meta). During anaphase I, a portion of the Survivin staining remained associated with each of the two separating groups of chromosomes, while another portion appeared at the midplate region (Fig. 1A, Ana I). By anaphase II Survivin did not appear to be specifically associated with chromosomes, the staining being at the developing midplate region and, consistently, in part generally dispersed in a pattern not coinciding with chromosomal foci or fibers of α-tubulin staining (Fig. 1A, Ana II). In early telophase Survivin staining was in the interfaces of two separating daughter cells (Fig. 1A, Telo I) and then the midbody between two separated daughter cells

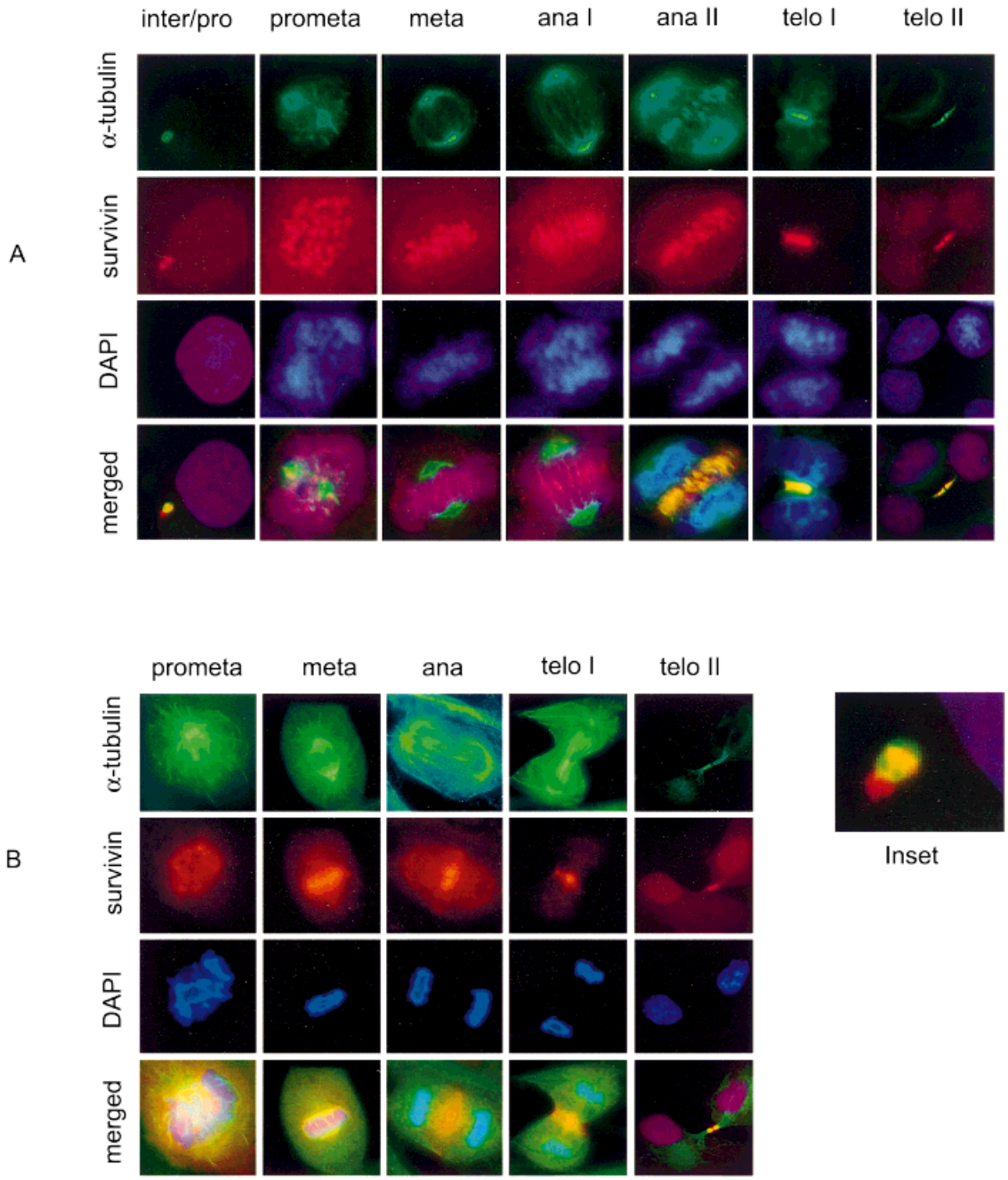

Fig. 1. Survivin localization during the cell cycle. **A:** Cancer cell line (HeLa) and (**B**) normal cell line Detroit 551 cells were stained with anti-Survivin and anti-α-tubulin antibodies, and with DAPI, to visualize Survivin (red), α-tubulin (green), and chromosomes (blue), respectively. Inter/pro (Interphase or early prophase), prometaphase (Prometa), metaphase (Meta), early anaphase I (Ana I), late anaphase II (Ana II), early telophase I (Telo I), and late telophase II (Telo II). Inset shows close up of merged imaged in Figure 1A, inter/pro, showing that the two foci of α-tubulin and Survivin, while very closely juxtaposed, are not coincident.

(Fig. 1A, Telo II). Similar results were observed in another cancer cell line, Saos-2 cells (data not shown).

Next, we assessed whether Survivin exhibited the same localization as above in noncancerous human cell lines, i.e., normal human lung fibroblast-like cells (IMR-90 and Detroit 551 cells). We observed that in Detroit 551 cells most Survivin during prometaphase and metaphase was not distributed on α-tubulin microtubules, but rather as discrete spots coinciding with chromosomes (Fig. 1B, Prometa and Meta).

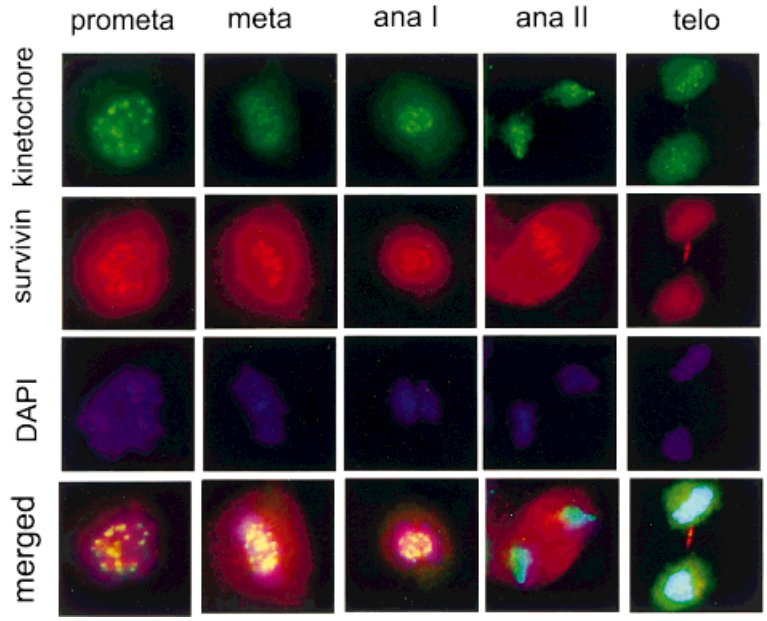

Fig. 2. Co-localization of Survivin and kinetochores during mitosis. Detroit 551 cells were stained with anti-Survivin, anti-CREST (kinetochore), and DAPI to visualize Survivin (red), kinetochores (green), and DNA (blue), respectively. Survivin was colocalized with kinetochores at the prometaphase, metaphase, and early anaphase, and separately localized from the kinetochore at late anaphase and telophase. Prometaphase (Prometa), metaphase (Meta), anaphase I (Ana I), anaphase II (Ana II), early telophase I (Telo I), and late telophase II (Telo II).

(In contrast, we have observed that in mouse NIH 3T3 cells, most Survivin widely colocalized with α-tubulin at metaphase, data not shown). Then, at anaphase Survivin disappeared from the chromosomes and localized toward the region of the midbody, but not localizing with α-tubulin generally (Fig. 1B Ana). During telophase, Survivin specifically localized to the midbody at the interface of two separating sister cells (Fig. 1B Telo I, II). The same distribution during the cell cycle was detected in another human normal cell line, IMR-90 cells (data not shown). No staining was detected in negative controls treated with either rabbit anti-storage protein antibody or by omission of the Survivin or tubulin primary antibodies.

Co-Localization of Survivin and Kinetochore During Early Mitosis

The above results showed that during transition from prophase to metaphase, Survivin stained in numerous foci in the region of the chromosomes, although the Survivin was clearly not generally dispersed along the chromosomes as reported to occur in *C. elegans* [Speliotes et al., 2000]. This distribution suggested a possible centromeric localization, similar to that known for kinetochores, a possibility which we tested with CREST anti-kinetochore antibody. Indeed, staining was partially overlapping with kinetochore staining from prophase through early anaphase in Detroit 551 cells (Fig. 2). Similar patterns of staining were obtained in the HeLa cancer cell line (data not shown).

Relocation of Survivin With Taxol Treatment

The above results showed that subsequent to interphase/early prophase, at no time during the mitotic cycle of two normal or two cancer-derived cell lines did Survivin generally colocalize with α-tubulin. These results appear different than those reported by Li et al. [1998] and O'Connor et al. [2000], who observed marked codistribution of Survivin and β-tubulin microtubules in HeLa cells from prophase through metaphase. One difference in experimental methods is that in those previous studies, HeLa cells were treated with Taxol to stabilize microtubules before the immunocytological analyses, whereas in our studies cells were not Taxol treated. To investigate the distribution of Survivin under conditions of Taxol treatment, we also treated the four cell lines with 13 μg/ml Taxol for 24 h before staining with Survivin, α-tubulin, and DAPI. Under these conditions, ~70% of the cells are in mitosis

with condensed chromosomal DNA, although the nuclear membrane may still be intact [Schiff and Horwitz, 1980]. We observed the strong colocalization of Survivin and α-tubulin in both normal and cancer cell lines, regardless of whether the chromosomal DNA appeared mitotic (DNA condensed) or in interphase (DNA not condensed) (Fig. 3A). Thus, the relationship

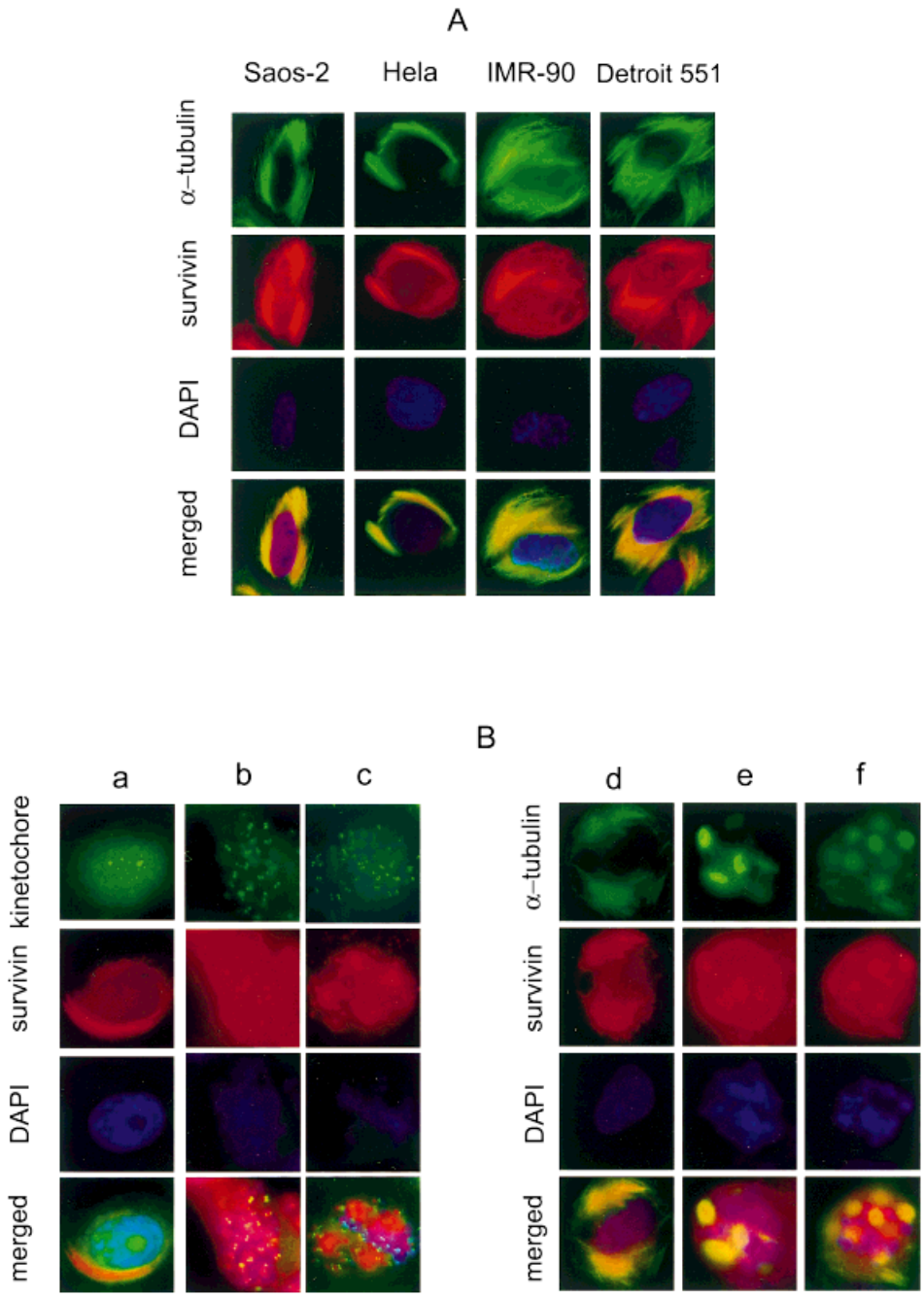

Fig. 3. Subcellular redistribution of Survivin with Taxol treatment. **A:** Cancer cell lines (Hela, Saos-2) and normal cell lines (Detroit 551, IMR-90) were treated with 13 μg/ml Taxol for 24 h and stained with anti-Survivin and anti-α-tubulin antibodies, and with DAPI, to visualize Survivin (red), α-tubulin (green), and DNA (blue), respectively. Strong colocalization of Survivin with α-tubulin was observed in all cells under Taxol treatment conditions. **B:** Survivin and kinetochore localization in Taxol treated HeLa cells during various stages of mitotic condensation of DNA. Panels a, b, and c concern an experiment in which cells were probed with Survivin and kinetochore antibodies, while in d, e, and f the cells were probed with antibodies to Survivin and α-tubulin. Survivin is clearly colocalizing with α-tubulin, and not with kinetochores, under the conditions of Taxol treatment.

between Survivin and α-tubulin, in relation to subcellular localization inside vs. outside the nucleus was not the same in untreated cells vs. those with microtubules stabilized by Taxol. Although quantitative measurements were not performed, it also appeared that the abundance of Survivin under these conditions of Taxol treatment was significantly higher than in untreated cells.

In view of that Taxol apparently altered the relationship between Survivin and α-tubulin, it became necessary to re-examine the spatial relationship between Survivin and kinetochores in HeLa cells under conditions of Taxol stabilization of microtubules. First, we examined the early mitotic situation in which the chromosomal DNA has condensed without concomitant breakdown of the nuclear envelope (Fig. 3B panel a,d). In this case, the kinetochores are associated with DNA inside the nucleus but Survivin is instead essentially cytoplasmic and colocalizing with α-tubulin. In later mitotic stages, when the DNA is more highly condensed and/or the nuclear membrane is dissolved, a minor portion of Survivin localized in a pattern similar to kinetochores, but the majority of Survivin was still colocalizing with α-tubulin. These patterns of cytoplasmic vs. nuclear, and kinetochore vs. α-tubulin, colocalizations of Survivin under Taxol treatment conditions are clearly different than those observed under nontreated conditions.

Increased Sensitivity of Hela Cells to Fas and Etoposide Mediated Cell Death by Survivin Antisense Oligonucleotide

Immunoblotting of proteins extracted from human cancer cell lines and normal cell lines with Survivin antibody yielded a band of 16.5 kDa in each (Fig. 4A). No specific bands were detected with the anti-storage protein antibody. Transfection of HeLa cells with a serial concentration of Survivin antisense oligonucleotide (50, 150, and 300 nM) effected a down-regulation of the 16.5 kDa Survivin protein levels in a dose-dependent manner, whereas the random oligomer had no effect. In addition, no change of α-tubulin expression was observed between the two groups, demonstrating the specificity of the effect to suppress the Survivin protein level (Fig. 4B).

With the above demonstration that the Survivin antisense oligonucleotide we used specifically suppressed Survivin protein levels, we next tested whether the antisense oligomer could sensitize cancer cells (HeLa) to chemotherapy. Hela cells were treated with 100 ng/ml Fas antibody or 100 nM etoposide 24 h after the start of transient transfection with antisense and control oligonucleotides and incubated for another 48 h. Cell death due combined treatment with the antisense oligonucletide and either Fas or Etoposide was markedly increased over that caused by any of the agents alone or either of the agents combined with the control random oligonucleotide (Fig. 4C).

Similarity in Human Survivin and *Drosophila* Deterin Antiapoptotic Activities

It had been previously reported that Survivin could partially complement a cytokinetic effect of loss of BIR-1 activity in *C. elegans* [Fraser et al., 1999], indicating that cytokinetic pathways of the Survivin-type proteins may have similarities between vertebrates and invertebrates. The apoptotic activity of the Survivin antisense oligonucleotide provided an opportunity to assess whether the *Drosophila* Deterin is recognized by pathways in human tumor cells. Transfection of HeLa with the Survivin antisense oligomer plus an expression vector encoding GFP induced significant apoptosis as compared with the control receiving the random oligomer (Fig. 5A). When the cells were instead cotransfected with expression vector producing GFP-Survivin, then apoptosis was significantly decreased. GFP-Deterin showed partial activity, compared to GFP-Survivin, in inhibiting apoptosis in HeLa cells induced by the antisense oligonucleotide (Fig. 5A). We then sought to assess Survivin activity in insect cells. When *Drosophila* K_c cells were transfected with an expression vector encoding human caspase-7, there was a significant reduction in cell survival compared with cells transfected with the empty expression vector (Fig. 5B). However, when the cells were cotransfected with a vector expressing Deterin, the rate of apoptotic death was cut in half. In addition, contransfection with a vector expressing instead a chimeric fusion of the BIR region of Deterin with the C-terminus of Survivin also cut apoptotic death in half.

DISCUSSION

The BIR-containing IAP proteins were initially discovered as antiapoptotic modulators [Miller, 1999]. Prototypically characterized by

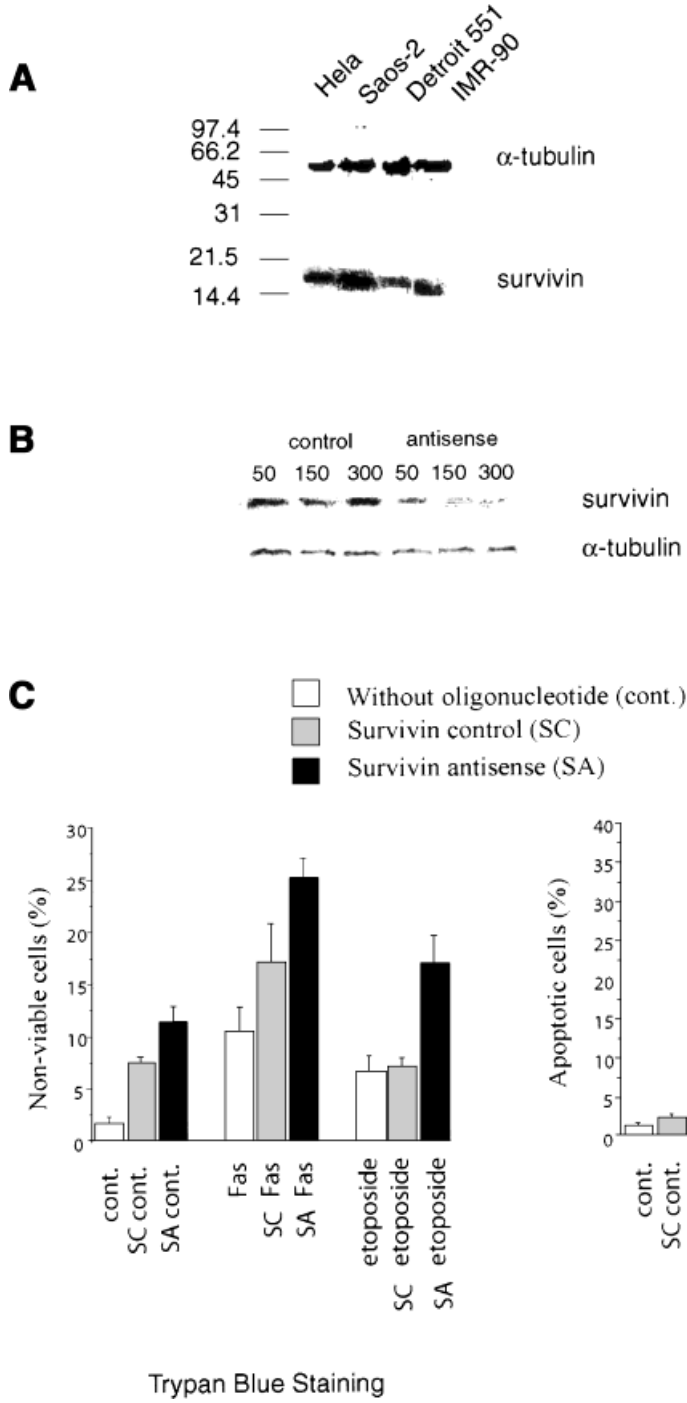

Fig. 4. Sensitivity of HeLa cells to chemotherapeutic agents caused by Survivin antisense oligonucleotide. **A:** Survivin protein expression in human cancer cell lines and normal cell lines. One hundred micro grams of extracted cell protein were fractionated by 12% SDS–PAGE gel and blotted with anti-Survivin antibody. A single band of 16.5 kDa corresponding to Survivin was observed under these conditions in Hela, Saos-2, Detroit 551, and IMR-90 cells by Western blot. **B:** HeLa cells were treated with 50, 150, and 300 nM Survivin antisense and negative control oligonucleotides. A down-regulation of Survivin expression by Survivin antisense oligonucleotide was observed in a dose-dependant manner but not in the control oligonucleotide treatment. No α-tubulin expression changed in two treated groups. **C:** HeLa cells were treated with 300 nM Survivin antisense and negative control oligonucleotides and/or either 100 ng/ml Fas antibody or 100 nM etoposide. After 48 h nonviable cells were stained with trypan blue and counted (left panel) or percentages of apoptotic nuclei were measured by the TUNEL method (right panel). All data are presented as the mean±SE of three independent experiments.

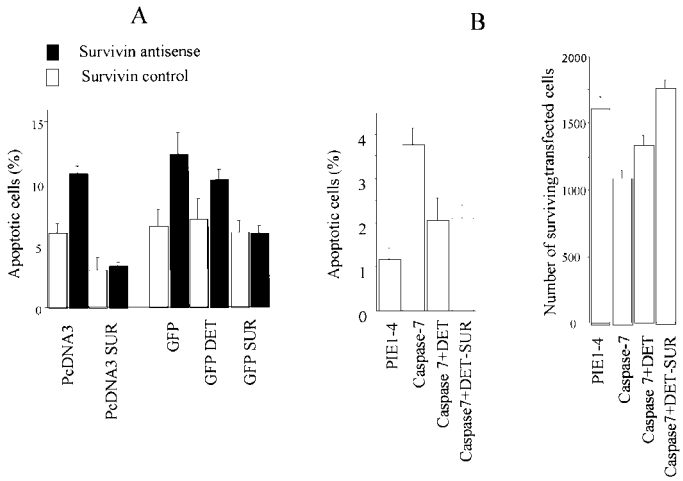

Fig. 5. Antiapoptotic activity of human Survivin and insect Deterin in reciprocal heterologous systems. **A:** HeLa cells were transfected with Survivin antisense oligonucleotide or antisense cDNA expression vector, or with indicated controls, and cotransfected with the indicated expression vectors encoding Survivin or a Deterin-Survivin chimera. Final percentages of apoptotic nuclei were determined by the TUNEL method. **B:** *Drosophila* K_c cells were cotransfected with pIE1-4 vector encoding human caspase-7 and either empty vector or vector encoding either Deterin or Deterin-Survivin chimera. The percentages of apoptotic nuclei were determined by the TUNEL method (left panel) and the number of surviving cells was monitored by cotransfection with β-galactosidase expressing reporter (right panel). All data are presented as the mean±SE of three independent replications.

two or three BIR domains and a C-terminal RING finger, IAPs with these features have been shown to inhibit caspase-dependent apoptosis in a number of vertebrate and invertebrate systems [Bergmann et al., 1998; Deveraux and Reed, 1999]. Interest in therapeutic targeting of apoptotic pathways in cancer cells engendered contemplation of means by which IAPs may be exploited for the control of cancer. The subsequent discovery of Survivin opened a new strategic possibility for cancer research. Survivin, and its homologs in mouse (TIAP) and *Drosophila* (Deterin) have but a single BIR with no RING finger, and are capable of inhibiting caspase-dependent apoptosis [Ambrosini et al., 1997; Jones et al., 2000]. Perhaps even greater interest has arisen in the participation of Survivin-type molecules in regulation of cell cycle and cytokinesis, as evidenced by cell-cycle correlated changes in its subcellular distribution and by outcome of over- or under-expression [Reed and Reed, 1999]. These properties of Survivin-type regulators may provide for a functional link between checkpoint decisions for the mitotic vs. the apoptotic pathways, and as a new target for cancer therapeutic agents.

However, our understanding of Survivin functional mechanisms, and potential exploitation of those mechanisms for cancer prognosis and therapy has been limited by divergent results reported by various laboratories that have used alternative model systems and methodologies. Thus far, most studies on expression of Survivin in tumors have localized Survivin to the cytoplasm [Ambrosini et al., 1997; Adida et al., 1998; Lu et al., 1998; Grossman et al., 1999; Tanaka et al., 2000], although, predominance in the nucleus of some tumors has been shown [Ito et al., 2000]. Studies of cancer-derived cell lines have yielded even more diverse results. The occurrence and location of Survivin during interphase has been much contested. Endogenous Survivin in human heptama cells lines was determined to be primarily cytoplasmic during interphase [Suziki et al., 2000a,b]. In contrast, Uren et al. [2000] did not detect Survivin in interphase HeLa cells and found no marked colocalization with β-tubulin between interphase and metaphase. Conversely, using Taxol-stabilized HeLa cells [Li et al., 1998] found Survivin located during late interphase on cytoplasmic microtubules and the MTOC, and on centrosomes during late interphase and mitosis. Yet alternatively, most cells producing highly overexpressed hemagluttinin-tagged Survivin (HA-Survivin) showed HA-Survivin widely dispersed in the cytoplasm during interphase with no obvious colocalization with β-tubulin, a similar result to that for insect cells overexpressing GFP-Deterin [Jones et al., 2000]. In some reports, the distribution of overexpressed HA-tagged Survivin was

detected solely on chromosomes, and not β-tubulin, at metaphase in Taxol-treated HeLa cells [Skoufias et al., 2000], as was endogenous Survivin [Uren et al., 2000]. However, other reports found that endogenous Survivin was coincident at prophase and metaphase with β-tubulin on spindle fibers in Taxol-treated cells [Li et al., 1998; O'Connor et al., 2000].

In the present study, we have addressed these questions by examining the distribution of endogenous levels of Survivin in both untreated and Taxol-treated HeLa cells, as well as in other tumor-derived and normal cell lines derived from different tissues. We found that during interphase, endogenous Survivin in untreated HeLa cells was reproducibly observed as two strongly staining, closely approximated foci, which localized closely with a pair of tubulin foci on the centrosomes. These observations support the report of Li et al. [1999], at least insofar as the localization of Survivin to centrosomes during later interphase/early prophase. We did not observe the dispersed, general cytoplasmic staining for HA-Survivin reported by Skoufias et al. [2000] or for GFP-Deterin reported by Jones et al. [2000]. We do not believe that these different results arise due to misdistribution on account of interference by the HA or GFP fusion tag, because Jones et al. [2000] demonstrated that the GFP-Deterin retained antiapoptotic activity. It may be that high overexpression leads to saturation of the natural Survivin/Deterin binding sites. Our observation of interphase occurrence of Survivin on cytoplasmic centrosomes suggests a function at that time that is in addition to the function of passenger proteins during mitosis.

We next observed that the subcellular distribution of Survivin in HeLa cells during mitosis was greatly disturbed by microtubule stabilization with Taxol. In untreated human tumor cells (HeLa and Saos-2) and in normal human cells (Detroit 551 and IMR-90) endogenous Survivin changed from its interphase colocalization with α-tubulin on cytoplasmic centrosomes to colocalization at prometaphase and metaphase with kinetochores at centromeres, and finally to midbodies at telophase. The consistency of these results in each of these untreated cell lines supports the proposition that at mitosis one function of Survivin, in addition to checkpoint inhibition of apoptosis, [Li et al., 1998] is as a passenger protein. However, when HeLa cells were treated with Taxol, Survivin vividly decorated α-tubulin fibers in the cytoplasm of interphase cells. In mitotic Taxol-treated cells, only a minor part of endogenous Survivin staining colocalized with kinetochores at centromeres. Instead, Survivin staining generally colocalized with α-tubulin. We interpret that at least some of the different results reported for different studies concerning Survivin localization during mitosis may be due to the use of Taxol in some studies to stabilize microtubules. Under the conditions of our studies here, microtubule stabilization with Taxol strongly promoted the localization of Survivin on microtubules, which may have interfered with the normal movement of Survivin to and from its bindings sites on centrosomes, centromeres, and spindle midbodies. We did detect a portion of the Survivin population binding to spindle microtubules during early anaphase, which was not detected by Uren et al. [2000], but which was detected by the overexpression system used by Skoufias et al. [2000]. Additional studies will be needed to further clarify the significance of Survivin binding to spindle microtubules during anaphase.

The participation of Survivin in both apoptotic and cell cycle/cytokinesis pathways make it attractive as a target for strategies in prognosis or treatment of cancer. Survivin antisense cDNA [Ambrosini et al., 1998; Li et al., 1998; Grossman et al., 1999], Survivin antisense oligonucleotides containing 2′-*O*-methoxylethyl modifications but natural phosphodiester linkages [Chen et al., 2000], or with phosphorothioate linkages instead of natural phosphodiester linkages [Olie et al., 2000], or both modifications [Li et al., 1999] have been shown to interfere with both of Survivin cytokinetic and antiapoptotic activities. Of special interest is whether cancer cells can be sensitized by cell-killing cancer therapeutic agents by Survivin interference methods [Olie et al., 2000]. To test such a potential, we utilized two agents that induce cell death by different mechanisms: (a) etoposide, the topoisomerase II inhibitor that acts at the level of the mitochondria, and (b) the level of the Fas pathway that initiates through cell surface receptors. We observed that the cell death in the presence of Survivin antisense oligonucleotide and either chemical agent was markedly greater than that caused by any of the agents or oligonucleotide alone or of either agent in combination with a

control oligonucletide, all evidencing additional support for Survivin antisense chemotherapeutic approaches.

We have evidenced in this study similarities in the human Survivin and *Drosophila* Deterin antiapoptotic pathways. The Survivin C-terminus, in fusion with Deterin BIR motif, exhibited antiapoptotic activity against human caspase-7 in the heterologous *Drosophila* K_c cells, while Deterin partially complemented Survivin antisense oligonucleotide-mediated apoptosis in HeLa cells. The BIR region of other IAP proteins has been shown to be necessary for antiapoptotic function [Takahashi et al., 1998] and mutation of the BIR domain of Survivin removes its ability to inhibit caspase 7 [Tamm et al., 1998]. There is also evidence that Survivin activity against caspase-3 requires anchoring of the C-terminal domain of Survivin to microtubules [Li et al., 1998]. The activity of the Deterin-Survivin chimera to inhibit human caspase-7 in insect cells suggests that the Deterin BIR region has similar functional structure to Survivin. Survivin has been reported to partially complement RNAi-mediated loss of BIR-1 function in cytokinesis of *C. elegans* embryos [Fraser et al., 1999]. The Deterin–Survivin chimera used in the present study showed similar activity compared to Deterin to inhibit caspase-dependent apoptosis in K_c cells [Jones et al., 2000]. The partial or full complementation detected in these studies strongly indicate the existence of similarities in the participation of Survivin-type IAPs in the antiapoptotic and/or cytokinetic pathways of vertebrates, nematodes, and insects.

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