Molecular Analysis of Survivin Isoforms

EVIDENCE THAT ALTERNATIVELY SPliced VARIANTS DO NOT PLAY A ROLE IN MITOSIS*  

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Survivin is a protein with proposed roles in cell division and apoptosis. Transcripts encoding splice variants of human survivin have been described and their expression correlated with cancer progression. As survivin forms homodimers in vitro, it has been suggested that these isoforms could interfere with wild type function by forming heterodimers. Here we show that survivin-2β and survivin-ΔEx3 can interact with wild type survivin but have reduced affinity for the partner protein of survivin, borealin, and thus do not localize with the chromosomal passenger complex in vitro. Furthermore, we demonstrate that overexpression of survivin-2β-green fluorescent protein (GFP) or survivin-ΔEx3-GFP does not impede cell cycle progression. We also report that wild type survivin, but not survivin-2β-GFP or survivin-ΔEx3-GFP, can rescue cell proliferation inhibited by small interfering RNA-mediated survivin depletion. These data suggest that, despite their ability to interact with wild type survivin, neither of these isoforms acts as its competitor during mitosis nor has an essential function.

Survivin-2β has an additional exon of 23 amino acids, exon 2β, inserted in its BIR domain, which is predicted to alter its tertiary structure and thus to affect its anti-apoptotic function. Survivin-ΔEx3 omits exon 3 causing a frameshift that results in a COOH terminus with no homology to wild type survivin (10). These differences have led to the suggestion that survivin-2β and survivin-ΔEx3 may antagonise wild type survivin by forming heterodimers with reduced capabilities (13). Indeed, when induced to undergo apoptosis, it has been reported that cells transfected with survivin-2β have decreased viability over cells transfected with wild type survivin (14). However, to date there is only indirect evidence that heterodimers can form between these isoforms and wild type survivin (14).

The presence of transcripts of survivin isoforms has been correlated with cancer progression. For example, using reverse transcription PCR Mahotka et al. (9) found that wild type survivin and survivin-ΔEx3 were expressed in cell lines derived from renal carcinomas but not from normal renal cells. Differential expression of survivin-2β was also reported in gastric carcinomas and, in this case, negatively correlated with disease progression (13, 15). Further reports have linked the expression of survivin isoforms with poor patient prognosis (for review, see Ref. 16). Notably, however, with the exception of one recent study (14), the only description of survivin isoforms at the protein level has come from expression of tagged versions, which have revealed that survivin-2β-GFP is cytoplasmic throughout the cell cycle, while survivin-ΔEx3-GFP is nuclear during interphase and diffusely localized in mitosis (10, 14).

In this study we demonstrate that survivin-2β and survivin-ΔEx3 can interact directly with wild type survivin in vitro. Despite these abilities, however, neither survivin-2β-GFP nor survivin-ΔEx3-GFP co-immunoprecipitate or co-localize with endogenous survivin, or the other chromosomal passengers during mitosis. We provide evidence that their differential localization may be due to their altered affinity for borealin. Furthermore, we report that these proteins are present at very low levels in cycling HeLa and U2OS cells and that their overexpression does not interfere with cell proliferation under our experimental conditions. Finally, we reveal that cDNA to wild type survivin, and only wild type survivin, can compensate for depletion of all forms of survivin by siRNA, indicating that these isoforms do not play a vital role in cell division and cannot compensate for loss of the wild type protein. Thus we conclude that the presence or absence of survivin-2β or survivin-ΔEx3 within cultured HeLa or U2OS cells is irrelevant to cell proliferation.

MATERIALS AND METHODS

PCR and Cloning Isoforms—To extract mRNA, exponentially growing cells were lysed in TRizol (Invitrogen) and treated with chloroform...
FIGURE 1. Survivin-2β and survivin-Δ Ex3 can form heterodimers with wild type survivin in vitro. A, recombinant GST, GST-survivin, and GST-aurora-B were expressed in bacteria, bound to glutathione-Sepharose beads and incubated with radiolabeled in vitro translated wild type survivin, survivin-2β, or survivin-Δ Ex3 as indicated. Upper panels show autoradiographs of radiolabeled isoforms bound to the GST preparations. Middle panels show Coomassie Blue staining of the corresponding gel to indicate equality of loading. Lower panels show quantitation of autoradiographs for each sample. B, same as for A, showing interaction between GST or GST-borealin and survivin isoforms. A sample of the TNT reactions in which the isoforms were translated is included to demonstrate levels of expression. All isoforms were pulled down by GST-survivin and GST-aurora-B indicating a direct interaction between these proteins. An extremely strong interaction occurred between GST-borealin and wild type survivin (note difference in scale on graphs). The interactions between GST-survivin and survivin-Δ Ex3 and between GST-borealin and survivin-2β were comparatively weak, and no interaction was observed between GST-borealin and survivin-Δ Ex3.
Analysis of Survivin Isoforms

FIGURE 2. Survivin-2β-GFP can interact with the chromosomal passenger complex in vivo. U2OS cells stably expressing GFP, survivin-GFP, survivin-2β-GFP, and survivin-Ex3-GFP were transfected 24 h prior to harvesting with either pcDNA-aurora-B (B), pcDNA-borealin (C), or pcDNA-survivin (D), arrested in mitosis, then blotted as indicated. Throughout blots of the initial whole cell extracts were probed with the appropriate antibody (left, upper panels) and anti-GFP to indicate equivalence of expression (left, lower panels). Anti-GFP was also used to indicate efficiency of immunoprecipitation (right, lower panels) in each case. Wild type survivin was able to immunoprecipitate all members of the complex, both endogenous and exogenous. By contrast neither of the isoforms could immunoprecipitate the endogenous forms of these proteins (A). However, when overexpressed, aurora-B, borealin, and wild type survivin co-immunoprecipitated with survivin-2β-GFP (B–D). The asterisk indicates IgG heavy chain in immunoprecipitates. WCE, whole cell extract; IP, immunoprecipitation.

(2 min) to remove lipids. After centrifuging (13,000 rpm, 15 min, 4 °C), the aqueous phase was removed and nucleic acids precipitated with ice-cold isopropyl alcohol. RNA was pelleted, washed with 70% ethanol, and re-suspended in RNase-free, diethyl pyrocarbonate-treated water (Ambion) before incubation at 60 °C (5 min). Samples were then incubated with 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in 10 mM dithiothreitol, 10 mM dNTP, 1 × 1 strand (42 °C, 1 h) and then 70 °C for 15 min. cDNA was amplified by PCR using Vent polymerase (New England Biolabs) and cloned into the appropriate vectors.

Real-time PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen), according to the manufacturer’s instructions, and analyzed on an ABI Prism 7900HT Sequence detection system (Applied Biosystems). The common forward primer was GAC CAC CGC ATC TCT ACA TTC, and reverse primers were TGC TTT TTA TGT TCC TCT ATG GG (wild type survivin), AAG TGC TGG TAT TAC AGG CGT (survivin-2β), and ATT GTT GGT TTC CTT TTC ATG (survivin-Ex3). Expression of the splice variants was calculated as a value relative to β-actin.

GSTβ Pull-down Assays—Expression of GST, GST-aurora-B, GST-survivin, and GST-borealin was induced in BL21 cells by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside. After 16 h growth at 18 °C, bacteria were harvested and lysed as described previously (5). Solubility of GST-borealin was increased by an additional 1 h incubation (4 °C) in lysis buffer with 1% Nonidet P-40 and 0.03% SDS. In vitro translation was performed using pBluescript constructs (as indicated) and a TNT T7 kit (Promega), with [14C]methionine (Amersham Biosciences) as tracer as described in Ref. 5.

Tissue Culture, Transient Transfections, and Stable Cell Lines—U2OS and HeLa cells were maintained at 37 °C, with 5% CO2, in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin-streptomycin, and fungizone. Transfections were carried out with the indicated pcDNA3.1 constructs using FuGENE 6 (Roche Diagnostics) and stable cell lines generated by transfection followed by selection with G418 (500 μg/ml). Prior to analysis, GFP-positive cells were sorted using an LSRII fluorescence-activated cell sorter (BD Bioscience) to ensure a homogeneous population. Tissue culture reagents were from Invitrogen unless otherwise stated.

Immunoprecipitation and Immunoblotting—Whole cell extracts and immunoprecipitations were carried out on colcemid (0.5 ng/ml, 16 h; Sigma)-treated cells arrested in mitosis. Approximately 5 × 106 cell equivalents were loaded per lane for whole cell extracts and 3 × 105 cell equivalents per lane for immunoprecipitation, except where indicated. SDS-PAGE analysis and immunoblotting were carried out using normal procedures, with secondary horseradish peroxidase-conjugated antibodies (Dako) and chemiluminescent reagents (Amersham Biosciences). Signals were analyzed by exposure to Hyperfilm (Eastman Kodak Co.) or using a PhosphorImager (Storm 480 PhosphorImager, Amersham Biosciences).

The abbreviations used are: GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; PI, propidium iodide; RNAI, RNA interference.
For immunoprecipitation, whole cell extracts were prepared by incubation for 20 min at 4 °C in RIPA-benzonase buffer (20 mM Tris (pH 8), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate, plus protease inhibitors), with 25 units of benzonase and 2 mM MgCl2, and incubated with a mixture of anti-GFP antibodies (3E1, Cancer Research-UK and 3E6, Molecular Probes), for 1 h at 4 °C with rotation, then with protein G beads (Cancer Research-UK) for 3 h at 4 °C. Beads were centrifuged and washed sequentially in wash I (50 mM Tris, 0.15 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40), wash II (50 mM Tris, 0.15 M NaCl, 1 mM EDTA), and wash III (50 mM Tris, 1 mM EDTA), then boiled in 5 x sample buffer and analyzed by SDS-PAGE.

Isoform-specific Antibodies—Polyclonal antibodies were raised in rabbits by AbCam against the following peptide sequences: C-SWLP-WIEASGRS (survivin-2β), CNTSTLGGRGGRITR (survivin-2). Antibodies were affinity-purified against the peptides and stored at -20 °C. These antibodies are now available from AbCam (www.abcam.com).

Microscopy—Cells were prepared for fluorescence microscopy using standard protocols. Briefly cells were washed with PBS, fixed for 5 min at 37 °C with 4% paraformaldehyde and extracted with 0.2% Triton in PBS (2 min, 37 °C). Samples were then washed with PBS, blocked with PBS containing 10 mg/ml bovine serum albumin for a minimum of 15 min, and immunoprobed sequentially with the appropriate primary and secondary antibodies for 1 h (room temperature). Primary antibodies were polyclonal anti-survivin-δEx3 (1/50, rabbit; now available from AbCam), polyclonal anti-survivin-2β (1/50, rabbit; now available from AbCam), monoclonal anti-aurora-B kinase (1/250, mouse; Transduction Laboratories), and monoclonal anti-tubulin (1/2000; B512, Sigma). Secondary antibodies (Vector Laboratories) were used at 1/200. Samples were mounted in Vectashield containing 4',6-diamidino-2-phenylindole and viewed using a Zeiss LSM 510 Meta confocal microscope or Zeiss Axioplan II.

Growth Curves and FACS Analysis—Cell proliferation was assessed by trypan blue exclusion (Sigma) using a hemocytometer. For FACS analysis cells were pelleted at 750 rpm for 5 min at room temperature, washed in PBS, then fixed for 1 h in 1% paraformaldehyde at 4 °C. Cells were then pelleted, washed in PBS, and resuspended in 500 μl of propidium iodide solution (PI; Sigma) containing 40 μg/ml PI and 100 μg/ml RNase A (MP Biomedicals Ltd. UK) in PBS. Cells were then passed through a 70-μm cell strainer (Falcon) into a 2058 tube (Falcon) and incubated at 37 °C for 30 min before analysis on an LSRII fluorescence-activated cell sorter (BD Bioscience). Cells were initially analyzed by forward scatter versus side scatter to differentiate between live cells and cell debris. The live cell population was selected for by gate P1. P1-gated cells were plotted as PI-width versus PI-area to establish nuclear size. Gate P2 selected for single cells with increased PI intensity, and cell doublets were excluded. Cells from the P2 population were then plotted versus GFP-A. Gate P3 was selected for GFP-negative cells (established using a GFP-negative cell line), and gate P4 was selected for...
GFP-positive cells. To determine the DNA content of the P4 population, PI-A was plotted against the number of cells counted for each cell line.

RNA Interference and Complementation — The RNAi target domain, bases 45–65, within the survivin cDNA sequence was selected as it was common to all human survivin isoforms; hence it should eliminate all splice variants (17). Using site-directed mutagenesis (Stratagene) guanine 54 was substituted for cytosine, thus creating a “wobble” in the RNAi target domain, allowing us to generate isoform specific constructs resistant to “knock down”. These constructs were cloned into the expression vector, pcDNA3.1, and introduced into human cells as described above. RNAi was carried out using 60 pmol of double-stranded RNA per reaction and Oligofectamine as described in Ref. 17 or 1.5 μL of siPORT NeoFx (Ambion) in 24-well plates with 4 × 10⁴ cells/well.

RESULTS

Survivin Isoforms Can Interact Directly with Wild Type Survivin but Have Reduced Affinity for Borealin — To determine whether survivin-2B or survivin-ΔEx3 can interact directly with survivin in vitro, we incubated ³⁵S-labeled, in vitro translated wild type survivin, survivin-2B or survivin-ΔEx3, with GST or GST-survivin bound to glutathione-Sepharose beads. In this assay both isoforms bound to GST-survivin (Fig. 1A, middle panel), albeit survivin-ΔEx3 bound far less efficiently (~3-fold) than wild type survivin. These data suggest that survivin isoforms can form heterodimers with wild type survivin. Similar interactions were also detected using yeast two hybrid pairwise interactions. Interactions of similar intensity were also seen between GST-auorora-B and survivin and GST-aurora-B and the isoforms (Fig. 1A, right panel). Next we investigated the affinities of the isoforms for GST-borealin (Fig. 1B). An intense interaction between GST-borealin and wild type survivin was observed, a comparatively weak interaction was seen between GST-borealin and survivin-2B, and no interaction was observed between GST-borealin and survivin-ΔEx3.

To determine whether these proteins have the potential to interact within the physiological milieu, we generated cell lines stably expressing GFP, survivin-GFP, survivin-2B-GFP, and survivin-ΔEx3-GFP (see

7 S. Tate, P. K. Ferrigno, and S. P. Wheatley, unpublished observations.
below) from the cytomegalovirus promoter. These lines were transiently transfected with aurora-B, borealin, or survivin, then subjected to immunoprecipitation with anti-GFP antibodies (Fig. 2). Using this overexpression method we found that wild type survivin and survivin-2β, but not survivin-ΔE3x3, could immunoprecipitate aurora-B, borealin, and wild type survivin.

Expression of Survivin Isoforms—Reverse transcription PCR performed on four human cancer cell lines, HeLa, U2OS, MCF7, and HT29, revealed three mRNA transcripts corresponding to survivin in each cell type (Fig. 3A). Sequence analysis verified that in each case the predominant band was wild type survivin, while the additional bands above and below the major band were survivin-2β and survivin-ΔE3x3, respectively (data not shown). The signal intensity from the isoform bands varied from line to line. As reverse transcription PCR cannot be used reliably to compare the levels of mRNA expression in a sample, we next used real-time PCR to provide accurate quantitation of mRNA expression of these variants in the cell lines used in this study. As shown in Fig. 3B, real-time PCR revealed that wild type survivin was always the most abundant and survivin-ΔE3x3 the least abundant form in HeLa and U2OS cells.

To detect endogenous survivin isoforms at the protein level, antibodies were raised against the survivin-2β-specific peptide, CNTSTLGGRGGRITR, and a peptide within the COOH terminus of survivin-ΔE3x3, COOH−SWLPWIEASGRS. Immunoblot analysis of recombinantly expressed GST-tagged versions of these proteins revealed that the antibodies specifically recognized the isoforms to which they were targeted and did not cross react with the other forms of survivin (Fig. 4, A and B). When tested on whole cell lysates from HeLa cells transiently transfected with GFP-tagged versions of the isoforms, the survivin-2β antibody recognized a band of the appropriate size for survivin-2β-GFP; however, it also highlighted a higher molecular weight band, circa 100 kDa, in both the untransfected and transfected lanes, indicating that it was not completely specific (Fig. 4C, asterisk). Affinity-purified antibodies to survivin-ΔE3x3 appeared to be relatively specific revealing a single band in the transfected lane of ~40 kDa (Fig. 4D). Despite the reactivity of the antibodies in immunoblots we were unable to detect endogenously expressed protein of either isoform from HeLa or U2OS cells using these antibodies (Fig. 4, E and F, lower panels). Furthermore, we used a commercial polyclonal-survivin antibody that recognizes epitopes present in wild type survivin, survivin-2β and survivin-ΔE3x3, on whole cell extracts prepared from cells either untransfected or transfected with untagged survivin-2β (Fig. 4E) or survivin-ΔE3x3 (Fig. 4F). In each case the untagged exogenously expressed isoform was readily detectable in the transfected extracts with this antibody, however, no corresponding band was observed in the untransfected cell lysates. Together these data suggest that the endogenous expression of the survivin isoforms in these cells is extremely low.

Localization of Survivin Isoforms—It is possible that even very low levels of isoforms could interfere with wild type survivin function; therefore we asked whether localization of isoforms would support this. We used GFP tagging and immunofluorescence with the antibodies described above to detect and localize survivin-2β and survivin-ΔE3x3 (Fig. 5). COOH-terminally tagged CDNA for each variant was transfected into HeLa or U2OS cells and observed 48 h later. Wild type survivin-GFP was found at the centromeres of prometaphase cells, and the midbody of cells executing cytokinesis, as reported previously (Fig. 5A). Using this method, survivin-2β-GFP was found diffuse throughout the cytoplasm at all stages of the cell cycle and was excluded from the mitotic chromosomes and interphase nuclei. Concentration of survivin-2β-GFP was noted at the very center of the midbody (Fig. 5B), in a pattern that was distinct from the bipartite midbody localization of wild type survivin-GFP (Fig. 5A). Examination of control cells revealed that GFP alone localizes to the very center of the midbody (Fig. 5D), suggesting that this localization is a GFP artifact rather than a specific localization of survivin-2β-GFP. Survivin-ΔE3x3-GFP was also found diffusely localized in the cytoplasm and was excluded from the chromosomes in mitosis (Fig. 5C, upper panel). In interphase it was present in the nucleus, as indicated by the G1 cells in Fig. 5C (lower panel). Importantly, at no time did we see localization that corresponded to that of the wild type form as judged by immunostaining for aurora-B (Fig. 5, red panels). Furthermore, aurora-B localization was not disrupted by the overexpression of any of the survivin isoforms.

The peptide-specific antibodies raised against survivin-2β also revealed that it was excluded from the mitotic chromosomes and rarely seen at the midbody. During interphase the signal was diffuse throughout the cytoplasm and absent from nuclei (data not shown). Additionally, using this polyclonal antibody a signal was detected at the centrosome throughout mitosis. However, this signal persisted after RNAi depletion (data not shown), indicating that it was due to a cross-reaction, possibly with the 100-kDa protein in Fig. 4C. Immunolocalization of survivin-ΔE3x3 with the antibodies described in Fig. 4 showed a weak signal of diffuse cytoplasmic staining, exclusion from the chromosomes during mitosis, and no concentration at the midbody (data not shown). In interphase cells it was apparent in both the nucleus and cytoplasm. Thus our immunohistological data confirm the localizations with GFP-tagged forms above.

Survivin Isoforms Cannot Immunoprecipitate Endogenous Chromosomal Passenger Proteins—As shown in Fig. 2, survivin-2β-GFP was able to immunoprecipitate aurora-B, borealin, and survivin when they were overexpressed in the cell. Given that the localization of the isoforms is different from the chromosomal passenger proteins (Fig. 5), we next asked whether it could immunoprecipitate the endogenous forms of these proteins from whole cell extracts in vivo. Fig. 6 shows that only wild type survivin-GFP can interact with the endogenous chromosomal passenger complex, as judged by co-immunoprecipitation of aurora-B, borealin, and wild type survivin.
Analysis of Survivin Isoforms

Overexpression of Survivin Isoforms Does Not Impede Cell Proliferation—Compared with wild type survivin-GFP and survivin-ΔEx3-GFP, we encountered some difficulties in establishing the survivin-2β-GFP expressing lines, suggesting that it may be slightly cytotoxic. Nevertheless, once established, cell lines stably expressing survivin-GFP, survivin-2β-GFP, and survivin-ΔEx3-GFP grew with normal kinetics (Fig. 7A). These lines were subjected to FACS analysis to monitor DNA content (Fig. 7B). No change in distribution of 2N and 4N cells was observed, and no increase in ploidy (8N or 16N) was detected in any of the lines, as compared with a control GFP line, indicating that overexpression of these versions of survivin does not interfere with proliferation in these cell lines.

Survivin Isoforms Cannot Complement for Loss of Wild Type Survivin—To assess whether the isoforms have an essential role in mitosis, we carried out RNAi complementation experiments. The RNAi target sequence, bases 45–65, was selected as it is present in all known splice variants of human survivin; hence its application is predicted to be disrupted by the 23-amino acid BIR domain insertion, and the linker region is absent from survivin-ΔEx3, both variants retain amino acids 6–10, which may be sufficient to permit dimerization. Interestingly, BIR domains are typically present in tandem or multiple repeats, but this domain is singularly present in survivin; thus dimerization may provide a means of duplicating this domain. It should be noted, however, that although crystallographic and NMR studies have described survivin as a homodimer (1–3), it has yet to be determined whether dimerization is critical for its function in vivo.

Survivin Isoforms Do Not Interact with the Chromosomal Passenger Complex in Vivo—During mitosis survivin operates in a complex with aurora-B kinase, borealin, and INCENP, and these “chromosomal passenger proteins” are mutually dependent upon each other for their localization and function (see Ref. 8). Here we report that neither survivin-2β-GFP nor survivin-ΔEx3-GFP is able to immunoprecipitate endogenous chromosomal passengers from U2OS cells. As they do not co-localize with these proteins at any stage of mitosis this may not be wholly surprising. Thus to determine whether the inability of these isoforms to interact with the chromosomal passenger complex is due to their differences in localization, or vice versa, we performed immunoprecipitations on cells in which the chromosomal passenger of interest was also overexpressed. Using this method we discovered that survivin-2β-GFP, but not survivin-ΔEx3-GFP, was able to immunoprecipitate aurora-B and borealin, albeit less efficiently than the wild type protein. Consistent with these data, when investigated in vitro, a strong direct interaction occurred between wild type survivin and borealin; a comparatively weak interaction was detected between survivin-2β and...
borealin, and no interaction was seen between survivin-$\delta$Ex3 and borealin. Previous reports have demonstrated that the interaction between wild type survivin and borealin is mediated by the BIR domain of survivin (7, 18), that most of survivin is bound to borealin in mitotic cells, and that localization to the mitotic centromeres of survivin is borealin-dependent (7). As the BIR domain is altered in survivin-$2\beta$ and truncated in survivin-$\delta$Ex3, our data are consistent with this region being responsible for interaction with borealin. Thus we conclude that the differential localization of these proteins within the cell is likely to be a consequence of their inefficiency/inability to bind directly to borealin.

Survivin-$2\beta$ and Survivin-$\delta$Ex3 Are Low Abundance Proteins—To determine whether survivin isoforms are expressed as proteins in cycling HeLa and U2OS cells, we generated isoform-specific peptide antibodies. The level of expression of these versions of survivin was lower than our detection capabilities by immunoblotting; however, we were able to detect the endogenous proteins within the cell by immunofluorescence microscopy. Importantly, the pattern of localization was similar to that observed for GFP tagged versions of these proteins and no co-localization with wild type survivin was observed during mitosis (10). Using the same antibodies Caldas et al. (14) were able to detect
survivin-\(\delta\)Ex\(\beta\), but not survivin-\(\delta\)Ex3, in HeLa cell extracts. Whether the low abundance of these proteins is simply a consequence of the low level of mRNA available, or rapid turnover at the protein level, remains to be determined; however, accelerated proteosome-dependent clearance of survivin-\(\delta\)Ex3, as compared with wild type protein, has been reported (10) and may be due to an additional degradation signal in its COOH terminus (19). Indeed, in our hands we have also found that survivin-\(\delta\)Ex3 is highly unstable in a number of systems.

If a protein behaves in a dominant or dominant negative fashion, its presence, even at low levels, would be expected to alter cell behavior. To test whether these proteins have a dominant impact on cell cycle progression, we generated stable U2OS cells expressing GFP-tagged forms

### FIGURE 8

Survivin-\(\beta\) and survivin-\(\delta\)Ex3 are non-essential and cannot compensate for loss of wild type survivin. A, immunoblot analysis of U2OS cells stably expressing GFP-tagged versions of survivin (as indicated) 48 h after transfection with control (c) or survivin specific (s) siRNA. Survivin that had not been rendered resistant to siRNA mediated depletion (survivin(nr)-GFP) was eliminated by 48 h, while those with a silent mutation, survivin(s)-GFP, survivin2B(r)-GFP, and survivin-\(\delta\)Ex3(s)-GFP, were still expressed. B and C, HeLa cells expressing survivin-GFP or survivin(s)-GFP were exposed to control (triangles) or survivin specific (open circles) siRNA. Cell growth was arrested in the survivin(nr)-GFP culture 48 h post-treatment with survivin specific siRNA (B), but cells expressing the RNAi-resistant survivin(s)-GFP grew similarly to the control population (C). D, cell number in U2OS populations expressing the constructs indicated were analyzed at 48 and 72 h and normalized to the number of cells in the control population. Cell proliferation was inhibited in all populations except survivin(r)-GFP.

|                | survivin(nr)-GFP | survivin(s)-GFP | survivin2B(r)-GFP | survivin-\(\delta\)Ex3(s)-GFP |
|----------------|------------------|------------------|-------------------|-----------------------------|
| time (h)       | 0                | 48               | 48                | 48                          |
| oligo          | c                | s                | c                 | s                           |
| anti-survivin  |                 |                  | s                 |                             |
| anti-tubulin   |                 |                  |                   |                             |

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**Diagram**

**Figure 8** shows the immunoblot analysis of U2OS cells stably expressing GFP-tagged versions of survivin (as indicated) 48 h after transfection with control (c) or survivin specific (s) siRNA. Survivin that had not been rendered resistant to siRNA mediated depletion (survivin(nr)-GFP) was eliminated by 48 h, while those with a silent mutation, survivin(s)-GFP, survivin2B(r)-GFP, and survivin-\(\delta\)Ex3(s)-GFP, were still expressed. B and C, HeLa cells expressing survivin-GFP or survivin(s)-GFP were exposed to control (triangles) or survivin specific (open circles) siRNA. Cell growth was arrested in the survivin(nr)-GFP culture 48 h post-treatment with survivin specific siRNA (B), but cells expressing the RNAi-resistant survivin(s)-GFP grew similarly to the control population (C). D, cell number in U2OS populations expressing the constructs indicated were analyzed at 48 and 72 h and normalized to the number of cells in the control population. Cell proliferation was inhibited in all populations except survivin(r)-GFP.

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**Analysis of Survivin Isoforms**

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1294 JOURNAL OF BIOLOGICAL CHEMISTRY

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expressed from a cytomegalovirus promoter. Some initial problems were encountered generating the survivin-2β-GFP lines that suggested survivin-2β may be slightly cytotoxic. Once established, however, all lines expressing these isoforms grew normally and did not accumulate cells of increased ploidy, as compared with cells depleted of survivin, previously demonstrated by Lens et al. (20). Thus these cells are clearly able to tolerate high levels of survivin-2β or survivin-δEx3 without any deleterious effects indicating that they do not interfere with the essential role of wild type survivin during mitosis.

Only Expression of Wild Type Survivin cDNA Complements RNAi-mediated Pan-survivin Depletion—Here we have shown that overexpression of survivin isoforms at the protein level is inconsequential to the proliferation of HeLa and U2OS cells in culture. We have also presented complementation experiments to determine whether their expression is required for cell viability. We report that complementation with cDNA to wild type survivin alone is sufficient to restore proliferation indicating that the isoforms are not essential for normal cell cycle progression. In addition, our data reveal that neither survivin-2β cDNA nor survivin-δEx3 cDNA can rescue cell growth, demonstrating that they cannot substitute for the wild type protein.

CONCLUSIONS

We have shown for the first time that the survivin splice variants, survivin-2β and survivin-δEx3, can form heterodimers with the wild type protein and have altered affinity for the chromosomal passenger, borealin. We also demonstrate that expression of survivin-2β or survivin-δEx3 in transformed human cells, even at high levels, is inconsequential to cell proliferation and that neither survivin-2β nor survivin-δEx3 can substitute for wild type function. We conclude that any correlation between patient prognosis and expression of survivin isoforms is unlikely to be due to interference with mitotic events. It will be interesting to determine whether these correlations can be attributed to interference with the anti-apoptotic activities of survivin.

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