[CANCER RESEARCH 59, 6097–6102, December 15, 1999]

Advances in Brief

Survivin-ΔEx3 and Survivin-2B: Two Novel Splice Variants of the Apoptosis Inhibitor Survivin with Different Antiapoptotic Properties[1]

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Abstract

Recently, a novel antiapoptosis gene, *i.e., survivin,* was identified as a structurally unique member of the inhibitor of apoptosis protein family. Survivin expression is turned off during fetal development and not found in non-neoplastic adult human tissues but is again turned on in the most common human cancers. The antiapoptotic properties of survivin might provide a significant growth advantage in tumors and possibly also contribute to chemoresistance of cancer. Therefore, we analyzed the expression of survivin in human renal cell carcinomas (RCCs), known to be largely resistant to chemotherapy. Northern blot analysis and RT-PCR revealed survivin expression in newly established RCC cell lines ($n = 11$) of all major histological types. Moreover, we identified two novel splice variants of survivin, lacking exon 3 (survivin-ΔEx3) or retaining a part of intron 2 as a cryptic exon (survivin-2B). Both sequence alterations cause marked changes in the structure of the corresponding proteins, including structural modifications of the baculovirus inhibitor of apoptosis protein repeat domain. The role of the novel isoforms in the regulation of apoptosis was assessed in transfection experiments, showing conservation of antiapoptotic properties for survivin-ΔEx3 and a markedly reduced antiapoptotic potential for survivin-2B. In conclusion, our observations suggest a complex regulatory balance between the different isoforms of survivin, which might determine the response to proapoptotic stimuli, not only in human RCCs but also in fetal tissues and other types of cancer.

Introduction

Apoptosis plays an important physiological role during embryonic development and in the maintenance of tissue homeostasis. Deregulation of apoptosis has been implicated in carcinogenesis by abnormally prolonging cell survival, facilitating the accumulation of transforming mutations, and promoting resistance to immunosurveillance. Moreover, impairment of apoptotic cell death might significantly affect resistance of cancer to chemotherapy and irradiation (1).

The molecular pathways leading to apoptotic cell death are highly conserved evolutionarily and controlled by proteins that either promote or counteract apoptotic cell death. Regulators of apoptotic cell death identified thus far predominantly include proteins of the Bcl-2 family and the IAP[3] family. First described as baculoviral genes, several cellular homologues of IAPs have been identified in eukaryotes, all of which are characterized by two or three BIRs, a COOH-terminal RING finger domain, and a caspase recruitment domain (2). All mammalian IAPs suppress apoptosis, but their way of function is not yet fully understood and could be different from each other. Some were reported to directly bind and inhibit intracellular cysteine proteases (caspases) activated in apoptosis or, in the case of c-IAP1 and c-IAP2, to bind TRAF1 and TRAF2 (3).

Recently, the gene for a novel member of the IAP family, *survivin,* was found by hybridization screening of a human P1 genomic library with the cDNA of EPR-1 (4). Survivin is structurally unique among mammalian IAPs, containing only a single BIR and lacking the COOH-terminal RING finger domain. Additionally, in contrast to c-IAP1 and c-IAP2, survivin has no caspase recruitment domain, which is necessary for the interaction with TRAFs.

It was shown that survivin inhibits processing of procaspase-3 and procaspase-7 and specifically binds both active caspases *in vitro* (5). Remarkably, caspase-3 has a key role in promoting the apoptosis signal. Thus, caspase-3 is responsible for the cleavage of poly(ADP-ribose) polymerase, PAK2, lamin, gelsolin, and fodrin, resulting in inhibition of DNA repair, formation of apoptotic bodies, nuclear membrane breakdown, and cytoplasmic shrinkage, all of which are hallmarks of apoptosis (6).

Initial studies using Northern blotting, *in situ* hybridization, and immunohistochemistry revealed strong survivin expression in several fetal tissues, whereas no survivin transcripts were detected in a variety of adult tissues (7). Most strikingly, survivin was observed to be expressed in the most common human cancers, including carcinomas of lung, stomach, colon, breast, and prostate as well as high-grade non-Hodgkin's lymphomas (4). These findings suggested that cancer cells return to a fetal pattern of survivin expression to enhance cell viability and thereby possibly also become able to overcome the cytotoxic effects of chemotherapeutic agents. In fact, immunohistochemical studies in neuroblastomas and colorectal carcinomas indicated a correlation between the expression of survivin and a clinically unfavorable course of disease, proposing survivin expression as a potential prognostic factor (8, 9).

To analyze the expression of survivin in cell lines derived from human RCC of all major histological types, we used a survivin-specific RT-PCR procedure, allowing the exclusive amplification of the entire survivin coding sequence. In this report, we describe the identification of two novel alternatively processed survivin transcripts, designated as survivin-ΔEx3 (lacking exon 3) and survivin-2B (retaining a part of intron 2 as a cryptic exon). The expression of both survivin and survivin-ΔEx3 was observed in all RCC cell lines tested, with most RCCs additionally expressing survivin-2B. The function of the novel survivin isoforms was assessed in transfection experiments, showing a largely preserved antiapoptotic function for survivin-ΔEx3 and a marked reduction of antiapoptotic potential for survivin-2B. Our data, therefore, suggest that survivin variants generated by alternative splicing in human RCCs are intrinsically involved in the complex regulation of apoptosis.

Materials and Methods

Cell Lines and Cultures. All cell lines used in this study were derived from typical representatives of the clear cell, chromophilic/papillary, and chromophobe types of RCC, established in our laboratory as described previ-

Received 5/12/99; accepted 10/29/99.

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[1] This work was supported by the Mildred Scheel Stiftung.

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[3] The abbreviations used are: IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; TRAF, tumor necrosis factor receptor-associated factor; EPR, effector cell protease receptor; RCC, renal cell carcinoma; RT, reverse transcription; EGFP, enhanced green fluorescent protein; SD, splice donor; SA, splice acceptor.

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ously (10). The cell lines were maintained with DMEM (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% FCS, penicillin, and streptomycin and cultivated at 37°C in an atmosphere with 5% (v/v) CO_2. Short-term cultures of nonneoplastic renal tubule epithelia were derived from renal cortical tissue samples obtained from nephrectomy specimens. The tissue samples were mechanically macerated, and cultivation in standard growth medium resulted in rapid outgrowth of epithelial cells. Fibroblastic contamination of short-term cultures could be removed by selective trypsinization, as shown by immunocytochemical analysis demonstrating a pure population of cytokeratin-positive epithelial cells.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from cell lines by guanidinium thiocyanate treatment and centrifugation in 5.7 mol/l cesium chloride solution. Poly(A)+ RNA was isolated from total RNA via the Oligotex kit (Qiagen, Hilden, Germany). Northern blotting and hybridization were performed as described previously (11).

cDNA Synthesis. RT reactions contained 2 μg of total RNA, 1× RT buffer (Promega, Heidelberg, Germany), 25 μM of each deoxynucleotide triphosphate, 10 pmol of sequence-specific RT primer (5′-AGG AAC CTG CAG CTC AGA-3′, corresponding to nucleotides 914–931 of the survivin antisense strand) or 0.06 μg of random primer (Stratagene, Heidelberg, Germany), 20 units of RNAsin RNase inhibitor (Promega), and 5 units of AMV reverse transcriptase (Promega) in a final volume of 30 μl. The specific RT reactions were incubated at 50°C and random RT reactions at 37°C for 1 h, respectively.

PCR Assays. PCR amplifications were performed on a PTC-100 Programmable Thermal Controller (Biozym Diagnostic, Hess, Oldendorf, Germany). Three μl of cDNA mixture were subjected to amplification in a 50-μl mixture containing 2.5 units of Taq polymerase, 1× PCR buffer (both from Qiagen), 25 μM each of dATP, dCTP, dGTP, and dTTP, and 25 pmol each of 5′ and 3′ primer. PCR conditions were: initial denaturation for 2 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 1 min at 62°C, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The primer pair used for amplification of the survivin entire coding region was: forward primer, 5′-GCA TGG GTG CCC CGA CGT TG-3′ [corresponding to position 48–67 of the survivin mRNA (GenBank accession NM 001168)]; and reverse primer, 5′-GCT CCG GCC AGA GGC CTC AA-3′ (position 475–494). Glyceraldehyde-3-phosphate dehydrogenase controls were performed using forward primer 5′-ACG GAT TTG GTC GTA TTG GGC G-3′ and reverse primer 5′-CTC CTG GAA GAT GGT GAT GG-3′.

Sequence Analysis. Bands of interest were eluted from agarose gels using the QIAquick gel extraction kit (Qiagen), ligated into the pGEM-T-cloning vector (Promega), and cloned in accordance to standard protocols. Plasmid DNA was recovered using the Plasmid Mini kit (Qiagen), cycle sequenced, and analyzed in a ABI Prism 310 sequencing apparatus (Applied Biosystems, Weiterstadt, Germany) using T7 or SP6 site-specific primers.

S1 Nuclease Analysis. S1 nuclease analysis was performed using the S1 nuclease assay kit (Ambion, Austin, TX) according to the manufacturer's protocol. The sequences of oligonucleotide probes corresponded to exon/exon borders of the complementary strand [survivin-ΔEx3: position 231–270/389–428; survivin-2B: position 3244–3283/4460–4499 (according GenBank accession no. U75285)]. Probes were labeled with [γ-^{32}P]dATP by T4-polynucleotide kinase (Life Technologies) and purified according to standard protocols (1). Samples were analyzed on 8% denaturing polyacrylamide gels (Sequi-Gen GT Sequencing Cell; Bio-Rad Laboratories, Munich, Germany), and dried gels were submitted to autoradiography.

Cloning of survivin Coding Sequences. To generate EGFP-tagged survivin constructs, the coding sequences of the three survivin variants were PCR amplified as described above using the following primer sets: 5′-GTC GTC GGT ACC ATG GGT GCC CCG ACG TTG-3′ (sense); 5′-CAG CAG GGA TCC ATC CAT GGC AGC CAG CTG CTC-3′ (antisense) for survivin and survivin-2B, respectively; and 5′-CAG CAG GGA TCC AGA CAT TGC TAA GGG GCC CAC A-3′ (antisense) for survivin-ΔEx3. PCR reactions were purified using Microspin S-300 columns (Pharmacia Biotech Europe, Freiburg, Germany), digested with *Kpn*I and *Bam*HI, ligated into the mammalian expression vector pEGFP-N3 (Clontech, Heidelberg, Germany), and cloned according to standard protocols.

Transfection of Cultured Cells, Induction of Cell Death, and Assessment of Cell Viability. Eight μg of pEGFP-N3 vector control and survivin constructs were each cotransfected with 2 μg of pCMVβ into HepG2 hepatoma cells according to the DEAE-dextran protocol. After culturing for 24 h, 1×10^5 cells were plated into 24-well plates and incubated with 100 μg/ml of the potent chemotherapeutic drug methotrexate. HepG2 cells had been shown previously to be methotrexate responsive, exhibiting CD95-mediated apoptosis upon exposure to this anticancer drug (12). Cell viability was measured after 24-h incubation by scoring cells as alive or dead by trypan blue staining. Transfection experiments were performed at least three times.

To determine transfection efficiency, control cells were fixed 48 h after transfection (2% formaldehyde/0.2% glutaraldehyde in PBS) and stained for β-galactosidase expression (0.1% 5-bromo-4-chloro-3-indolyl β-D-galactoside/5 mM potassium ferricyanide)/5 mM potassium ferrocyanide/2 mM $MgCl_2$.

Immunoblotting. Protein-normalized aliquots of SDS extracts of HepG2 transfectants were electrophoresed on 15% SDS-polyacrylamide gels and transferred to Optitran BA-S85 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membrane was blocked over night in washing buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.2% Tween 20] plus 3% nonfat dry milk/1% BSA, incubated for 1 h at room temperature with 1 μg/ml rabbit antihuman survivin antibody (kindly provided by Dr. Dario Altieri, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT) or mouse anti-GFP (Clontech), washed, and incubated with a 1: 2000 dilution of horseradish peroxidase-linked donkey antirabbit or sheep anti-mouse antibody (Amersham Pharmacia, Freiburg, Germany) for 1 h at room temperature. After washing, binding of the primary antibody was revealed by incubation with Lumi-Light substrate (Roche, Mannheim, Germany). Equal loading was confirmed by α-tubulin detection with an anti-α-tubulin antibody (Sigma-Aldrich, Deisenhofen, Germany).

Results

Expression of survivin and Two Novel Splice Variants in RCC Cell Lines. By Northern blot analysis, all RCC cell lines ($n = 11$) displayed survivin expression, irrespective of their histological types (Fig. 1*a*). This observation was further confirmed by RT-PCR, demonstrating the expected survivin amplification product of 431 bp, and to our surprise, two other bands of 500 and 329 bp, respectively (Fig. 1*b*). These three survivin-specific amplification products were cloned and sequenced. The largest band was identified as a survivin transcript retaining a 69-bp long part of intron 2 (corresponding to nucleotides 4460–4528 of the published sequence of the *survivin* gene; GenBank accession no. U75285) as a novel exon, termed exon 2B (Fig. 1*c*). The 431-bp product was identified as regularly spliced survivin. The smallest band was a survivin fragment of only 329 bp in length with the 118-bp of exon 3 missing. In addition, formation of heteroduplices occurred but did not influence the ratio between the bands of expected length. Additional amplification of genomic DNA showed no product, confirming the presence of each splice variant on mRNA level only. Short-term cultures of nonneoplastic kidney tubule epithelia showed no expression of any survivin isoform by RT-PCR (data not shown). The existence of the newly identified survivin splice variant survivin-ΔEx3 was further proved by distinctive S1 nuclease analysis, demonstrating the presence of the mRNAs for both regular survivin and alternatively spliced survivin-ΔEx3 transcripts (Fig. 1*d*).

Splice Donor and Acceptor Sites Adjacent to the Novel Exon 2B Are Suitable for Alternative Splicing. Computer analysis with the Signal program of the PC/GENE package revealed that the novel 69-bp exon in survivin-2B is flanked by SD and SA sites (Fig. 2*a*). These sites matched to the consensus sequences of common SD sites ({C/A}AG|GT{A/G}AGT) and SA sites ($\{T/C\}_{11}$N{C/T}AG|G) (13, 14). Thus, it is a well-known fact that the GT bases of SD sites and the AG bases of SA sites are necessary to perform splicing processes on exon-intron and intron-exon boundaries.

The SA site at position 4445–4460 of exon 2B reads as follows: CCTTAATCCTTACAGT. The homology of this SA site to the SA consensus sequence (Fig. 2*b*) seems to be sufficient in some RCC cell lines only to permit alternative exon usage, whereas other RCC cell lines were unable to perform insertion of exon 2B. This observation

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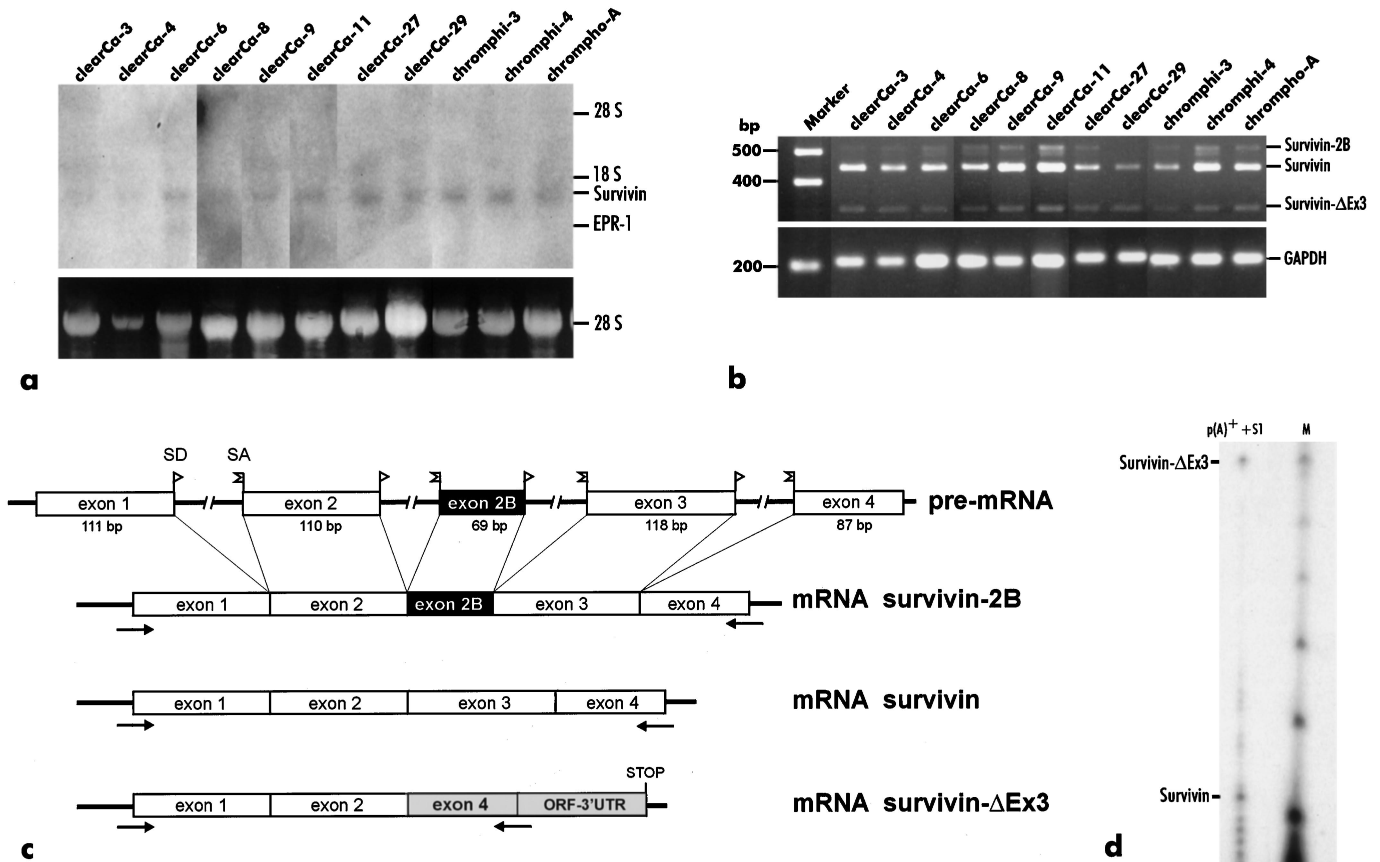

Fig. 1. Expression of different survivin isoforms in human RCC cell lines. *a,* Northern blot hybridization revealed survivin expression in all RCC cell lines, irrespective of their histological subtypes (clear cell, chromophilic, and chromophobe types of RCC). Of note, a band of 1.3 kb indicated EPR-1 expression in clearCa-6. *b,* RT-PCR confirmed the results of Northern analysis showing survivin expression in all RCC cell lines as well as two additional fragments different in length from the survivin band. *c,* sequence analysis of the RT-PCR amplification products identified three different transcripts: The *survivin* transcript with four exons, *survivin-2B* with an additional exon (exon 2B) inserted between the exons 2 and 3, and *survivin-ΔEx3,* showing a loss of exon 3 as well as a frame shift with extension of the reading frame into the open reading frame of the 3′ untranslated region (*arrows,* PCR primer positions; *flags,* SD and SA sites; *black boxes,* the novel exon 2B; *shaded boxes,* the frame shift in exon 4 and the coding part of the former 3′ untranslated region). *d,* S1 analysis of the survivin-ΔEx3 transcript revealed the protected fragments of regular survivin (40 nucleotides) and alternatively spliced (80 nucleotides) survivin-ΔEx3 transcripts.

could indicate patient and/or tumor specificity of the corresponding splicing factors.

The 3′ boundary of exon 2B has a SD at position 4526–4534 (GAGGTCAGG). This SD site (Fig. 2*b*) matches sufficiently to the consensus site and is capable of linking exon 2B to the SA site of exon 3. No splice products, however, were found directly linking exon 2B to exon 4.

In contrast, the SD site of exon 2 is suitable for linking exon 2 directly to three different exons of the *survivin* gene, *i.e.,* exon 2B (survivin-2B), exon 3 (survivin), and exon 4 (survivin-ΔEx3) in human RCC lines (Fig. 1*c*). In conclusion, our data convincingly indicated that the newly identified survivin variants are transcript isoforms generated by alternative splicing of the survivin pre-mRNA.

Differential Alterations of Protein Domains in the Novel survivin Splice Variants. Computational domain analysis of the putative proteins of survivin-2B and survivin-ΔEx3 revealed crucial consequences of the alternative splicing processes (Fig. 3). The chief characteristic of survivin-2B was the modification of the essential BIR domain, which was interrupted by insertion of exon 2B. The inclusion of exon 2B generated a novel potential *N*-glycosylation site at position 84 and two novel potential *N*-myristoylation sites (positions 89 and 92). Inclusion of exon 2B, however, did not result in a frame shift, as became evident in survivin-ΔEx3.

Skipping of exon 3 in survivin-ΔEx3 resulted in a modification of the BIR domain at the same position as in survivin-2B but was followed by an additional frame shift in exon 4. In consequence, the open reading frame of survivin-ΔEx3 ends in the 3′ untranslated region of survivin at position 581 and generates a novel 63 amino acids-long COOH-terminal tail in survivin-ΔEx3 coded by 87 nucleotides of exon 4 and 103 nucleotides of the original 3′ untranslated region. Because of this frame shift, a novel potential *N*-myristoylation domain was generated at position 121.

In summary, both splice variants exhibited truncation of the functionally important BIR domain at the same position but differed in their COOH-terminal regions. Additional posttranslational modifications, *e.g.,* by *N*-glycosylation and/or *N*-myristoylation, could determine differential activity and/or intracellular location of the corresponding proteins.

Differential Antiapoptotic Activity of the Novel survivin Splice Variants. Because RCCs are known to be largely resistant to anticancer drug-induced apoptosis, we used another apoptosis-inducible tumor model for further functional analysis of the different survivin splice variants. Therefore, we selected the HepG2 tumor cell line, because this cell line had been shown previously to respond to methotrexate with marked CD95-mediated apoptosis (12).

Using HepG2 cells, transfection experiments revealed marked differences in the antiapoptotic activity of the novel survivin splice variants (Fig. 4). In accordance with its previously described antiapoptotic potential, transient transfection of HepG2 cells with survivin resulted in a marked increase of cell survival after exposure to

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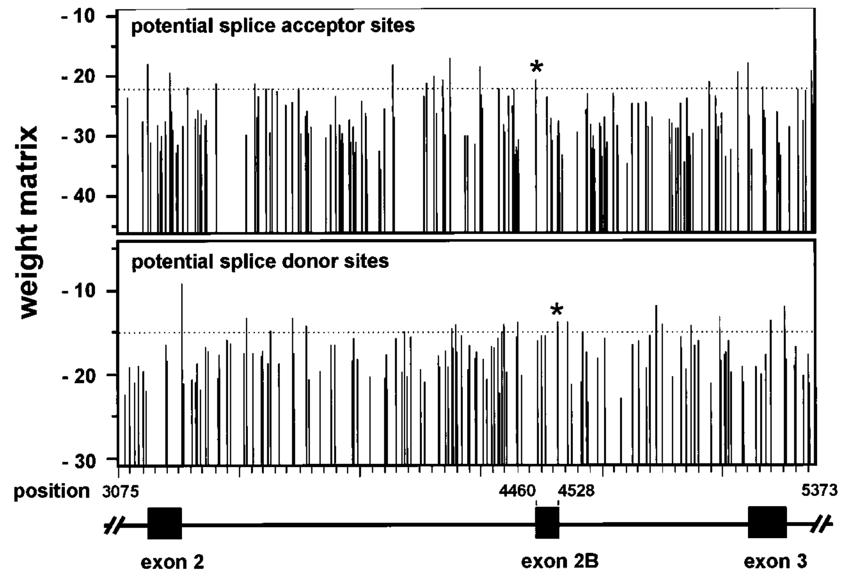

Fig. 2. Analysis of potential SD and SA sites between exon 2 and 3. *a,* computer-aided analysis of SD/SA sites was performed on the *survivin* genomic sequence to confirm the data obtained from RT-PCR and DNA sequencing. *, positions of potential SD and SA sites that correspond to the SD and SA sites used in the newly discovered survivin-2B splice variant. As a result, a part of the intron between exon 2 and 3 is retained as a cryptic exon, termed exon 2B (see Fig. 1*c*). SD and SA sites were analyzed with the Signal program of the PC/Gene Package Ver. 6.60, according to the algorithm developed by Staden (13). *b,* comparison of the SA and SD sites of exon 2B with the consensus sequences for SA and SD sites.

methotrexate when compared with the empty vector control. Closely corresponding cell survival was observed after methotrexate treatment of HepG2 cells transfected with survivin-ΔEx3. In contrast, HepG2 cells transfected with survivin-2B showed a marked reduction of cell survival after exposure to methotrexate (Fig. 4).

These data strongly suggested differential antiapoptotic properties of the novel survivin splice variants, showing a nearly complete preservation (survivin-ΔEx3) or a marked reduction (survivin-2B) of the antiapoptotic effects known from the "regularly" spliced survivin isoform.

Discussion

In this study, we show that human RCC cell lines express the apoptosis inhibitor survivin and extend a recent report by Tamm *et al.* (5) on survivin expression in RCCs of the clear cell type to human RCCs of all major histological types. More importantly, however, we identified for the first time two novel survivin splice variants, which exhibited either largely preserved (survivin-ΔEx3) or markedly reduced (survivin-2B) antiapoptotic properties in transfection experiments. These previously unknown splice variants could not be detected by Northern blot hybridization, because only 118-bp (survivin-ΔEx3) or 69-bp (survivin-2B) differences in length were found when compared with the primarily identified survivin transcript.

Recently, survivin has attracted considerable attention as a novel member of the IAP family. At variance with the ubiquitous distribution of other IAPs in fetal and adult tissues, survivin expression was shown to be developmentally regulated, with intensive expression in fetal tissues and complete down-regulation in most adult tissues (7). Most strikingly, however, reexpression of survivin was found in the most common human cancers, including carcinomas of the lung, stomach, colon, breast, prostate, and high-grade non-Hodgkin's lymphomas (4). Reexpression of survivin in cancer might provide important selective advantages by abnormally prolonging cell survival and promoting resistance to apoptosis induced by immunocompetent cells or anticancer drugs.

The biochemical mechanisms by which survivin suppresses apoptosis are still under investigation. *In vitro* experiments have shown that survivin binds and inhibits the cell death effector proteases caspase-3 and caspase-7 (5), which induce a wide range of cellular degradation processes determining the morphological phenotype of apoptosis. Moreover, survivin has been demonstrated to associate specifically with microtubules of the mitotic spindle (15). Because caspase-3 is involved in proteolysis of mitotic spindle proteins (16), survivin might assist to preserve the integrity of the mitotic apparatus, thereby cooperating with other components of the G_2-M checkpoint. Overexpression of survivin in cancer might negatively interfere with the G_2-M checkpoint and promote the progression of neoplastic cells through mitosis (15).

Although at least some of its antiapoptotic modes of action are common to other IAP family members, survivin additionally exhibits structurally unique features that also have important functional implications. Thus, survivin lacks a COOH-terminal RING finger, which appears to be critical for the antiapoptotic function of baculoviral and some cellular IAPs (2). Moreover, survivin contains a single BIR domain only, in contrast to other human IAP family members identified thus far (2). In this context, it was striking to note that the novel splice variants survivin-ΔEx3 and survivin-2B exhibited pronounced structural alterations of the single BIR domain. Deletion of exon 3 in survivin-ΔEx3 results in a frame shift with truncation of the BIR domain in addition to a new

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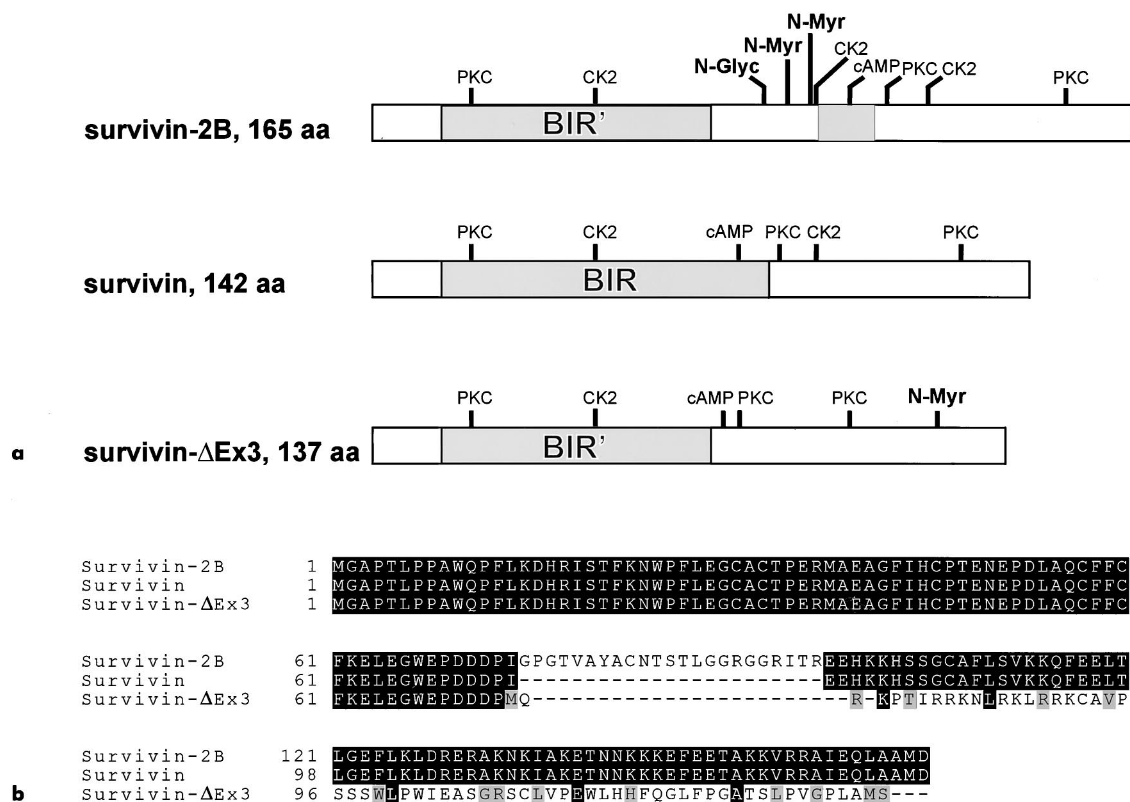

Fig. 3. Differential alterations of protein domains in the novel survivin splice variants. *a,* protein sequences were analyzed with the Prosite program of the PC/Gene package (21). The BIR domain, which is known to be responsible for inhibition of certain caspases, was found to be modified in both alternative splice variants of survivin by computer analysis. Survivin-2B has acquired additional 23 amino acids encoded by the cryptic exon 2B. The novel domain introduces a potential *N*-glycosylation site as well as two potential *N*-myristoylation sites. Skipping of exon 3 in survivin-ΔEx3 results in a frame shift that causes a new COOH-terminal sequence with a potential *N*-myristoylation site. *BIR′,* modified BIR; *PKC,* protein kinase C phosphorylation site; *N-Myr, N*-myristoylation site; *N-Glyc, N*-glycosylation site; *CK2,* casein kinase 2 phosphorylation site; *cAMP,* cyclic AMP-dependent kinase phosphorylation site. *b,* multiple alignment, using Clustal W (22) and enhanced with Boxshade, of amino acid sequences of the three survivin variants. *Darker shading* is of residues that are highly conserved; *lighter shading* is of less well-conserved residues, and residues that are *not shaded* are not conserved.

COOH-terminal protein segment, which in turn might affect functional properties such as subcellular localization (compare Fig. 3). Despite these structural modifications, survivin-ΔEx3 exhibited antiapoptotic properties similar to those of survivin in our experimental system. However, survivin-ΔEx3-mediated antiapoptosis in HepG2 cells might be a cell type-specific phenomenon. Nevertheless, the BIR domain alone was observed to be sufficient for inhibition of apoptosis in many other systems using the cellular IAPs (2). Therefore, we cannot conclusively explain, thus far, why survivin-ΔEx3 still exerted antiapoptotic effects with a single truncated BIR domain. This observation, however, might be related either to preserved antiapoptotic potential of the BIR residues or to as yet unknown antiapoptotic properties in other regions of the survivin-ΔEx3 molecule.

In contrast, the acquisition of an additional "cryptic" exon in survivin-2B resulted in a modification of the BIR domain (compare Fig. 3), which proved to be functionally relevant in transfection experiments. Thus, the potential of survivin-2B to inhibit methotrexate-induced apoptosis in HepG2 cells was markedly reduced, possibly because of a dominant-negative mechanism of competitive binding to the interaction partners of survivin.

Although further experimental work will have to elucidate the functional properties of the different survivin splice variants in more detail, our observations indicated for the first time that alternative splicing might be relevant for the fine tuning of survivin actions and possibly for the actions of other IAPs as well. Alternative splicing has been found previously to play a key role in the regulation of apoptosis, determining the actions of many apoptosis-related genes along all levels of the apoptotic pathway (reviewed in Ref. 17). Thus, members of the CD95 death receptor family exist in membrane-bound and soluble isoforms, which antagonistically affect apoptosis. The Bcl-2-family encompasses genes, *e.g., bcl-x,* the different splice variants of which can antagonistically determine the susceptibility for cell death signals (18). Finally, different isoforms of caspases, the executive proteins of apoptosis, are generated by alternative splicing (19).

Although alternative splicing of survivin considerably adds to the complexity of the systems controlling apoptosis, there might be an additional level of regulation. Thus, the *survivin* gene had been identified by hybridization with the cDNA encoding the EPR-1, and the coding sequence of survivin was found to be largely complementary to that of EPR-1. This observation suggested the possibility of apoptosis regulation by naturally occurring antisense interactions between survivin and EPR-1 transcripts (20). Although it still remains to be proved that these two transcripts actually interact *in vivo*, first evidence for such a mechanism has come from transfection experiments in HeLa cells (20). In these experiments, induction of EPR-1 mRNA suppressed the expression of endogenous survivin, which was followed by increased apoptosis and reduction of cell number. Further investigations will have to show whether the newly identified splice variants might additionally modify these postulated antisense interactions *in vitro* and *in vivo*.

Taken together, the identification of two novel splice variants will have profound implications for our understanding of survivin actions. These splice variants may not only be involved in the regulation of apoptosis during fetal development but may also determine the response of cancer cells to apoptosis-inducing stimuli such as anticancer drugs and irradiation. Elucidation of the mechanisms controlling the

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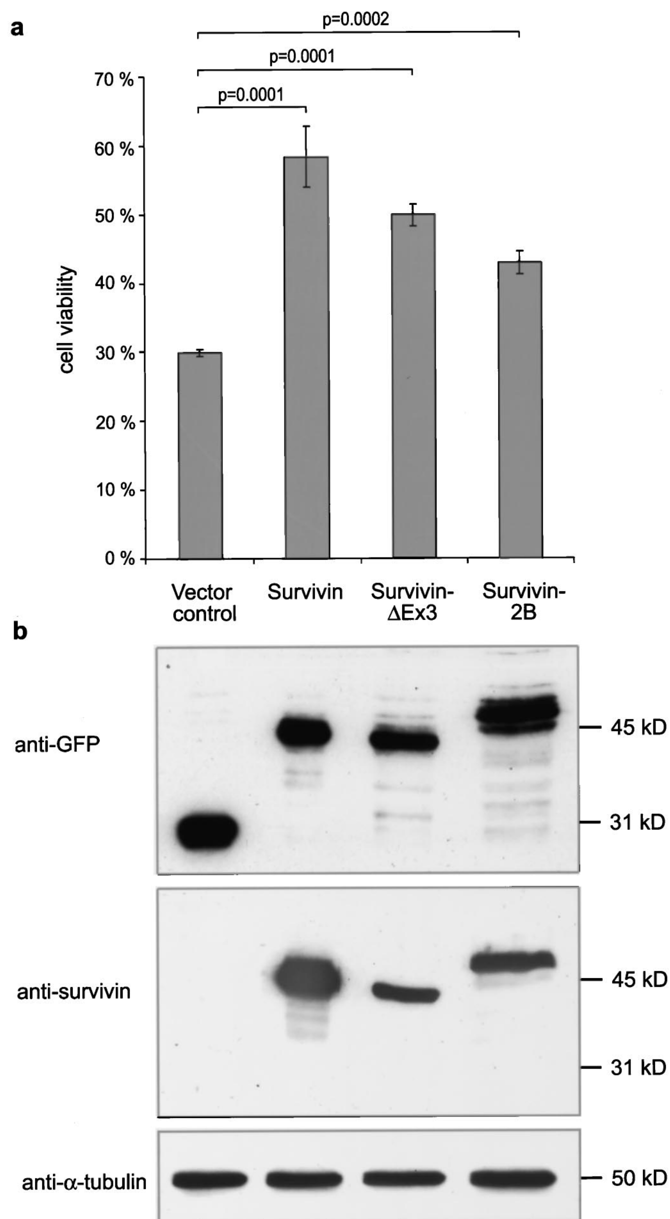

Fig. 4. Differential antiapoptotic potential of the novel survivin splice variants. *a,* coding sequences of survivin and its two splice variants were ligated into the expression vector pEGFP-N3 and transfected into the human hepatoma cell line HepG2. Cell death was induced in transient transfectants by exposure to methotrexate (100 μg/ml). Survivin-ΔEx3 transfectants exhibited cell survival frequencies closely corresponding to that of survivin transfectants. In contrast, survivin-2B transfectants showed a marked reduction of cell survival. The transfection efficiency was $\geq$50% in all transfection experiments. The percentage of transfected cells was determined by microscopic evaluation of β-galactosidase expressing cells. Data are the means of at least three independent experiments; *bars,* SD. Statistical analysis was performed with Dunnett's test. Of note, corresponding results were obtained using untagged pcDNA3.1-survivin/survivin-ΔEx3/survivin-2B constructs (data not shown). *b,* immunoblot analysis with both anti-GFP and anti-survivin antibodies revealed stable and comparable levels of expression for the indicated survivin variants in HepG2 transfectants. Of note, binding of anti-GFP antibody is independent of the survivin component of the fusion protein. Additional survivin-2B bands detected by the anti-GFP antibody might indicate posttranslational modifications of this survivin variant. The polyclonal anti-survivin antibody, which was generated against "regular" survivin, detected survivin splice variants with less intensities, as evident from comparison with α-tubulin controls.

expression and alternative splicing of survivin, therefore, could facilitate the disruption of a potent antiapoptotic mechanism in cancer cells. This intervention could selectively increase the susceptibility of cancer cells to apoptosis-based treatment strategies without affecting viability of nonneoplastic tissues that do not express survivin.

Acknowledgments

We thank Dr. Dario Altieri for providing the antibody to human survivin and Siegrid Khalil, Martina Bellack, and Michael Ringler for excellent technical assistance. The results are part of the Ph.D. thesis of Michael Wenzel.

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