

Survivin Monomer Plays an Essential Role in Apoptosis Regulation*[§]

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Survivin was initially described as an inhibitor of apoptosis and attracted growing attention as one of the most tumor-specific genes in the human genome and a promising target for cancer therapy. Lately, it has been shown that survivin is a multifunctional protein that takes part in several crucial cell processes. At first, it was supposed that survivin functions only as a homodimer, but now data indicate that many processes require monomeric survivin. Moreover, recent studies reveal a special mechanism regulating the balance between monomeric and dimeric forms of the protein. In this paper we studied the mutant form of survivin that was unable to dimerize and investigated its role in apoptosis. We showed that survivin monomer interacts with Smac/DIABLO and X-linked inhibitor of apoptosis protein (XIAP) both *in vitro* and *in vivo*. Due to this feature, it protects cells from caspase-dependent apoptosis even more efficiently than the wild-type survivin. We also identified that mutant monomeric survivin prevents apoptosis-inducing factor release from the mitochondrial intermembrane space, protecting human fibrosarcoma HT1080 cells from caspase-independent apoptosis. On the other hand, our results indicate that only wild-type survivin, but not the monomer mutant form, enhances tubulin stability in cells. These findings suggest that survivin partly performs its functions as a monomer and partly as a dimer. The mechanism of dimer-monomer balance regulation may also work as a “switcher” between survivin functions and thereby explain remarkable functional diversities of this protein.

Apoptosis is a mechanism of programmed cell death that maintains cell homeostasis in multicellular organisms. This process is regulated via a delicate balance between proapoptotic proteins (such as caspases and cytochrome *c*) and antiapoptotic proteins (including Bcl-2, survivin, and XIAP).² Survivin was

initially described as a member of the inhibitor of apoptosis (IAP) family that contains a single baculoviral IAP repeat (BIR) domain (1). It was shown that survivin is one of the most tumor-specific genes in the human genome (2). It forms a complicated interaction network and, being a “nodal” protein, regulates several cell processes (3) such as apoptosis, the spindle checkpoint system (4, 5), microtubule dynamics (6), and cell response to stress (7); moreover, survivin is a part of chromosome passenger protein complex (8, 9) and plays an important role in regulation of mitosis (10). Despite many studies performed to elucidate survivin functions, the precise mechanism of apoptosis inhibition is still a matter of discussion. There are several models of how survivin protects the cell from mitochondria-regulated apoptosis. The most studied antiapoptotic mechanism is based on the ability of survivin to block the proapoptotic protein Smac/DIABLO (11, 12). The second suggested model of survivin antiapoptotic activity is based on its ability to interact with XIAP, protecting it from ubiquitination and increasing its stability, which promotes caspase inhibition (13). Finally, the third and the least studied mechanism of survivin cell protection is connected with inhibition of caspase-independent apoptosis realized by apoptosis-inducing factor (AIF). In response to different apoptosis stimuli, AIF translocates from the mitochondria intermembrane space (IMS) to the nucleus, causing DNA fragmentation. It was shown that survivin decreases the amount of AIF in the nucleus after apoptosis induction, thus protecting cells from death (14, 15). Apart from the hypotheses listed above, there are data that survivin can protect cells from death caused by several anticancer drugs due to its ability to bind to microtubules and increase their stability (16).

Earlier, it was suggested that survivin was present in cells as a dimer, whereas the monomeric form did not have any biological functions (17, 18). Yet, recent research shows that many processes require survivin monomer (8, 9). Moreover, the latest studies revealed a mechanism that regulates the balance between dimeric and monomeric forms of survivin via acetylation of Lys residues by CREB-binding protein (19).

We conducted novel investigations on the survivin monomer role in different pathways of mitochondrial apoptosis. We showed that a survivin mutant unable to dimerize can interact with Smac/DIABLO both *in vivo* and *in vitro*, although before our study it was considered to be impossible (17, 18). Moreover, our results provide evidence that survivin monomer is able to

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² The abbreviations used are: XIAP, X-linked inhibitor of apoptosis protein; AIF, apoptosis-inducing factor; BIR, baculovirus IAP repeat; CFP, cyan fluorescent protein; fmk, fluoromethyl ketone; IAP, inhibitor of apoptosis pro-

tein; IBM, IAP binding motif; IMS, intermembrane space; PE, phycoerythrin; Z, benzylloxycarbonyl.

bind to XIAP as well as protect tumor cells from caspase-independent apoptosis by blocking AIF release from IMS. Finally, we showed that only wild-type survivin, but not the monomer mutant form, enhances tubulin stability in cells, raising the possibility that monomeric and dimeric survivin forms may have different functions.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and RNA Interference—Human fibrosarcoma HT1080 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine in air enriched with 5% (v/v) CO₂ at 37 °C. For microscopy purposes, the cells were cultured on a coverslip-bottomed small chamber. Cells were induced to apoptosis with 50 μM cisplatin (Sigma) for 48 h or 0.5 μM staurosporine (Sigma) for 12 h. In some experiments (see "Results") 20 μM caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-fmk) (Sigma) was added together with apoptosis-inducing agents. The final concentration of vehicle (dimethyl sulfoxide) never exceeded 0.1% (v/v). HT1080 cells were transfected with different plasmids by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were examined 48 h after transfection. Knockdown experiments were performed with siRNA against Smac/DIABLO (siSmac) and luciferase (siLuc) as described previously (20). The oligonucleotide list and description of plasmid construction can be found in the [supplemental material](#).

Expression and Purification of Fusion Proteins—All His₆-tagged proteins were expressed in the *Escherichia coli* strain BL21(DE3) (Novagen) and purified on Ni²⁺-nitrilotriacetic acid-agarose (Qiagen) as instructed by the manufacturer and dialyzed against PBS. 2-Mercaptoethanol or DTT was present at all times to prevent oxidation and subsequent aggregation of survivin and Smac/DIABLO. The purity of the obtained proteins was assessed by SDS PAGE followed by Coomassie Blue staining.

Size Exclusion Chromatography—To determine the molecular mass of the proteins, size exclusion chromatography was performed using a Superose 6 HR 10/300 column (Amersham Biosciences). The system was equilibrated in PBS in the presence of 2.5 mM 2-mercaptoethanol. A flow rate of 0.4 ml/min was used. Elution of samples was monitored by absorbance at 260 nm. Protein markers (Amersham Biosciences) were ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), and ribonuclease A (14 kDa).

Survivin Pulldown Assay—The bacterially expressed and purified survivin or survivin^{F101A/L102A} were incubated with purified mature Smac/DIABLO or Δ63Smac/DIABLO overnight at 4 °C. The mixture was then incubated with survivin mAb bound to protein G-Sepharose (Sigma). After a 1-h incubation at room temperature, the immunoprecipitates were washed five times in PBS, and proteins were eluted in 0.1 M glycine buffer, pH 2.8, and analyzed using Western blotting.

Immunoprecipitation and Western Blot Analysis—Cells were lysed as described previously (12), and immunoprecipitation was performed using a Dynabeads-protein G immunoprecipitation

kit (Invitrogen) as instructed by the manufacturer. Eluted products were subjected to SDS-PAGE for Western blot analysis. Western blot analysis was performed as described elsewhere (12). To detect acetylated α-tubulin, cells were lysed in SDS sample buffer. Monoclonal antibodies against acetylated α-tubulin (clone no. 6A205) were purchased from US Biological. Polyclonal antibodies against Tag(C;G;Y)FP were purchased from Evrogen. To confirm equal protein loading per lane, the membranes were subsequently reprobated with anti-β-actin antibodies (Sigma).

Immunofluorescence Microscopy—The immunofluorescence method was performed as described previously (19). Primary antibodies were against AIF (US Biological) and against cleaved caspase-3 (US Biological). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen) and Alexa Fluor 532 goat anti-rabbit IgG (H+L) (Invitrogen). Images were captured with a Nikon DIAPHOT 300 or a Leica AF6000 LX fluorescent microscope.

Apoptosis Assays—Apoptosis was assayed using two different methods. Detection of membrane externalization of phosphatidylserine was assessed using annexin V-PE conjugate (Invitrogen) according to the manufacturer's instruction. Within 1 h after staining with PE-conjugated annexin V, cells were analyzed by flow cytometry on a FACScan (Becton Dickinson) using Cell Quest software. Cleaved caspase-3 detection was performed as published previously (21), using primary antibodies against cleaved caspase-3 (US Biological) and Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen) as secondary antibodies. After staining, FACScan flow cytometry assay was applied (Becton Dickinson).

AIF Release Flow Cytometry Assay—The cytometry assay was performed according to the method of Waterhouse and Trapani (22). Polyclonal antibodies against synthetic peptide corresponding to 517–531 amino acids of human AIF (US Biological) were used as primary antibodies and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) as secondary antibodies.

Generation of Cell Lines Stably Transduced with Lentiviral Constructs—Replication-defective lentivirus constructs for expression of CFP (control), CFP-survivin, CFP-survivin^{F101A/L102A}, CFP-survivin^{D53A}, and CFP-survivin^{D53A/F101A/L102A} fusion proteins were generated by co-transfecting 293FT packaging cells (Invitrogen) with one of the following plasmids: pLCFP-C-survivin, pLCFP-C-survivin^{F101A/L102A}, pLCFP-C-survivin^{D53A}, pLCFP-C-survivin^{D53A/F101A/L102A}, or pLCFP-C together with viral packaging vectors (pLP1, pLP2, and pVSV-G). Growth medium was changed the following day, and lentivirus-containing supernatants were harvested 72 h later. HT1080 cells were incubated with viral supernatants for 24 h in the presence of 6 μg/ml Polybrene (Sigma). Two days after infection, transduced cells were selected in 2 μg/ml puromycin (Invitrogen).

FRET Microscopy—FRET was measured with the acceptor photobleaching method using CFP and YFP fusion proteins as donors and acceptors, respectively, as described previously (23, 24). Briefly, FRET was performed on a Leica DM IRE2 confocal microscope (Leica Microsystems Inc.) equipped with a 63.0 × 1.40 OIHCX PL APO objective. CFP was excited with a 458-nm argon laser, and emission was detected through a 465–505-nm

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band pass filter. YFP was excited with a 514-nm argon laser, and emission was detected through a 525–600-nm band pass filter. To monitor FRET, the 458-nm line of an argon laser was used to excite CFP. The emission fluorescence was split by a dichroic mirror into separate CFP (465–505-nm band pass) and FRET (525–600-nm band pass) channels, respectively. Photobleaching of the acceptor YFP was performed with a 514-nm laser at 100% power. During the experiment, the acceptor (YFP) was selectively bleached by repeated cell scanning for an indicated period of time. Digital image analysis for quantitative evaluation was performed using LCS image processing software (Leica Microsystems Inc.).

RNA Isolation and Real-time (RT) PCR—Total RNA was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer's protocol. cDNA was written off 0.6 mg of RNA/reaction using the mouse MLV reverse transcriptase (Promega). Quantitative RT-PCRs were run in triplicate on an MX3000P thermal cycler (Stratagene) using the supplied MxPro software. PCR was carried out with primer pair Bcl-Xlfor/Bcl-Xlrev. All target mRNA levels were normalized to GAPDH expression levels. Primer specificity was confirmed by visualizing DNA on an agarose gel following PCR.

Microtubule Fractionation—The extent of tubulin polymerization was measured as described previously (25). Cells were grown to approximately 70% confluence and lysed in a paclitaxel (Taxol)-containing buffer (20 mM Tris-HCl, pH 6.8, 140 mM NaCl, 0.5% Nonidet P-40, 1 mM MgCl₂, 2 mM EGTA, 10 μg/ml paclitaxel, protease inhibitor mixture). Lysates were centrifuged at 12,000 × *g* for 15 min at 4 °C, and pellets containing the polymerized tubulin were solubilized in SDS sample buffer and subjected to SDS-PAGE for Western blot analysis with primary antibodies against α-tubulin (Sigma). To confirm equal protein loading per line, supernatants were subjected to SDS-PAGE for Western blot analysis with primary antibodies against β-actin (Sigma).

Structural Modeling—The structural model of survivin^{F101A/L102A} was built and optimized with MODELER software (26) using the coordinates of survivin (Protein Data Bank ID code 1E31) as a template.

Statistical Analysis—Statistical significance was analyzed using Student's *t* test. Differences were considered significant at *p* < 0.05.

RESULTS

Survivin^{F101A/L102A} Is a Monomer in Solution—To investigate the role of survivin in apoptosis regulation, we chose the previously described mutant form with substituted 101 and 102 amino acid survivin^{F101A/L102A} (8). Earlier, it was shown that survivin dimerization occurs when Leu⁹⁸ of one of the molecules protrudes into a hydrophobic pocket, formed with Leu⁶, Trp¹⁰, Phe⁹³, Phe¹⁰¹, and Leu¹⁰² of the other molecule (9). We used the MODELER program and an earlier obtained survivin structure (Fig. 1A, *left*) (27) as a template to create the model of the survivin^{F101A/L102A} dimerization surface (Fig. 1A, *right*). Fig. 1A, *right*, shows that substitution of Phe¹⁰¹ and Leu¹⁰² by Ala interrupts the hydrophobic interface that is essential for survivin dimerization. It is important to note that substituted

residues were situated beyond the highly conservative BIR domain.

To give evidence that survivin^{F101A/L102A} in solution is a monomer, we expressed recombinant wild-type (WT) survivin and survivin^{F101A/L102A} (Fig. 1B) in *E. coli* and determined molecular masses of the proteins by gel filtration (Fig. 1C). The molecular masses lay in the range of 30–40 kDa and 10–20 kDa for survivin WT and survivin^{F101A/L102A}, respectively. These results were close to the calculated masses of survivin dimer and monomer (37 and 18 kDa, respectively). The data are in good compliance with results obtained by Engelsma *et al.* (8), who showed that survivin^{F101A/L102A} was unable to form homodimers. Even though during gel filtration of WT survivin we registered only one peak corresponding to survivin dimer mass, it is necessary to mention that a small amount of WT survivin (~5%) is present in the solution as a monomer (17). But what is more important, the percentage ratio of survivin monomer and dimer forms in living cells is still unknown.

Survivin Monomer Is Able to Interact with Smac/DIABLO Both *in Vitro* and *in Vivo*—As mentioned above, survivin is able to block different apoptotic pathways. We found it crucial to investigate the role of survivin monomer in the most studied antiapoptotic mechanism, Smac/DIABLO inhibition. Mature Smac/DIABLO exposes N-terminal IBM (IAP binding motif; terminal tetrapeptide AVPI), which makes it able to interact with some members of the IAP family and thereby inhibit their antiapoptotic activity (28). Survivin can compete with other IAPs for binding to Smac/DIABLO and in this way preserve them from inhibition by Smac/DIABLO (11). Deletion of IBM leads to loss of interaction between survivin and Smac/DIABLO (11, 12). To determine whether survivin monomer is able to bind to Smac/DIABLO *in vitro*, we expressed a recombinant mature Smac/DIABLO and Δ63Smac/DIABLO (mature Smac/DIABLO lacking IBM) in *E. coli*. Obtained proteins were used for pulldown assay with purified recombinant WT survivin or survivin^{F101A/L102A}. Fig. 2A demonstrates that mature Smac/DIABLO is able to bind to both WT survivin and survivin^{F101A/L102A} whereas Δ63Smac/DIABLO lacks this ability.

To show that the interaction between survivin^{F101A/L102A} and Smac/DIABLO is possible not only in an artificial system, but also in a living cell, we carried out an *in vivo* investigation using FRET registration combined with an acceptor photobleaching technique. Human fibrosarcoma cell line HT1080 was co-transfected with two plasmids, one encoding mature Smac/DIABLO or Δ63Smac/DIABLO with CFP fused to its C terminus, and the other encoding WT survivin or survivin^{F101A/L102A} with YFP fused to its N terminus (Fig. 2B). The diagram in Fig. 2C shows how CFP fluorescent intensity in cells co-expressing YFP-survivin or YFP-survivin^{F101A/L102A} and Smac/DIABLO-CFP or Δ63Smac/DIABLO-CFP depends on acceptor photobleaching time. An increase in donor fluorescence intensity during acceptor photobleaching indicates that the investigated proteins interact. The obtained results give evidence that both survivin^{F101A/L102A} and WT survivin are able to interact *in vivo* with mature Smac/DIABLO, but not with Δ63Smac/DIABLO, which is exactly what was shown in *in vitro* tests.

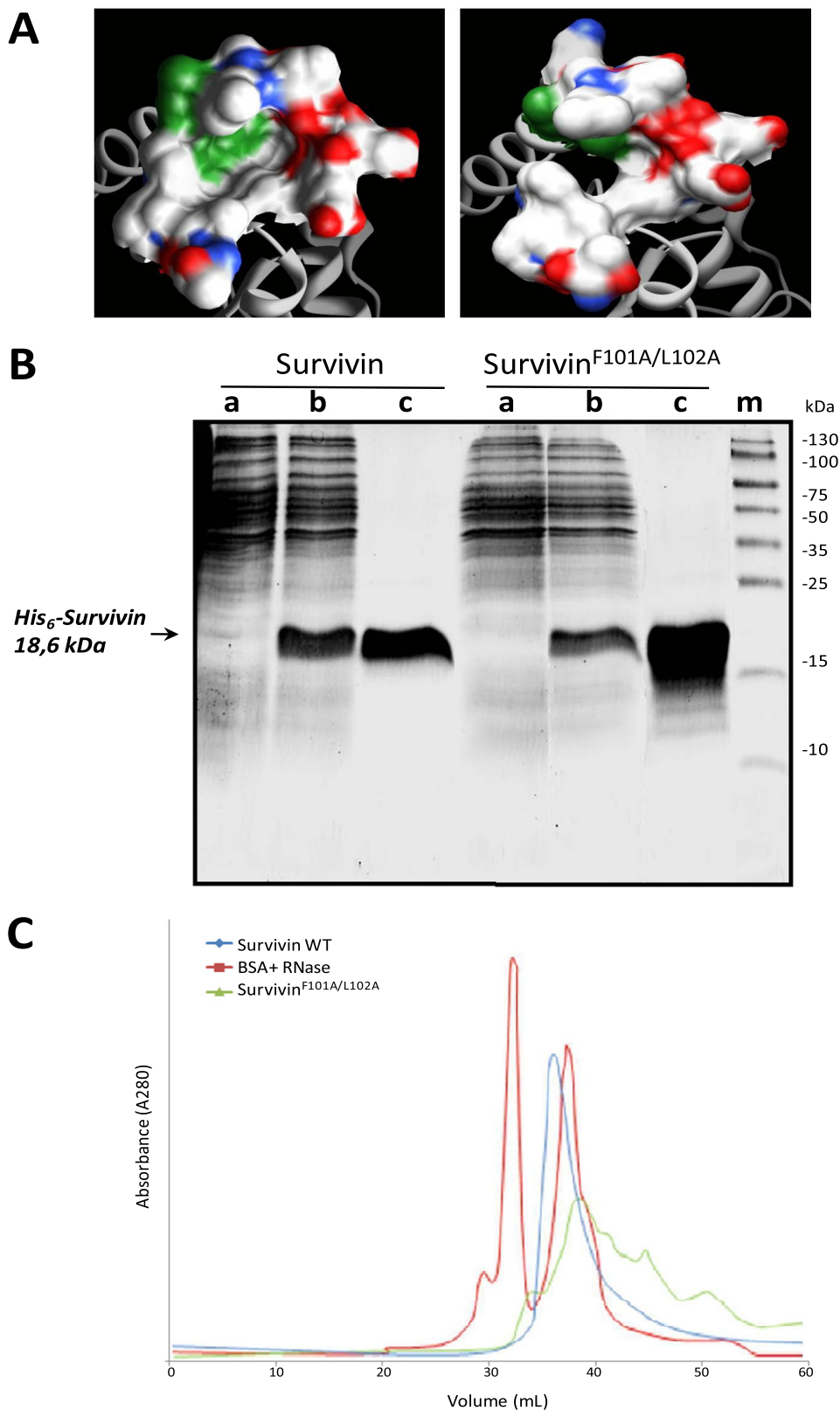


FIGURE 1. Wild-type survivin and survivin^{F101A/L102A} structures. *A*, model of dimerization region electrostatic surface of WT survivin (*left*) (25) (Protein Data Bank ID code 1E31) and survivin^{F101A/L102A} (*right*). Positively charged areas are shown in *blue*, negatively charged regions are in *red*, and 101 and 102 residues are marked in *green*. *B*, Coomassie Blue-stained SDS PAGE: *m*, molecular mass marker; *a*, lysates from *E. coli* cells containing vector pQE80-survivin or pQE80-survivin^{F101A/L102A}; *b*, lysates from *E. coli* cells containing vector pQE80-survivin or pQE80-survivin^{F101A/L102A} after isopropyl 1-thio- β -D-galactopyranoside induction; *c*, recombinant survivin or survivin^{F101A/L102A} purified from *E. coli* strain BL-21 (DE3). *C*, gel filtration profile for purified His-tagged recombinant WT survivin (*blue line*) or survivin^{F101A/L102A} (*green line*). Protein molecular mass markers are shown in *red*: BSA (66 kDa) and RNaseA (14 kDa).

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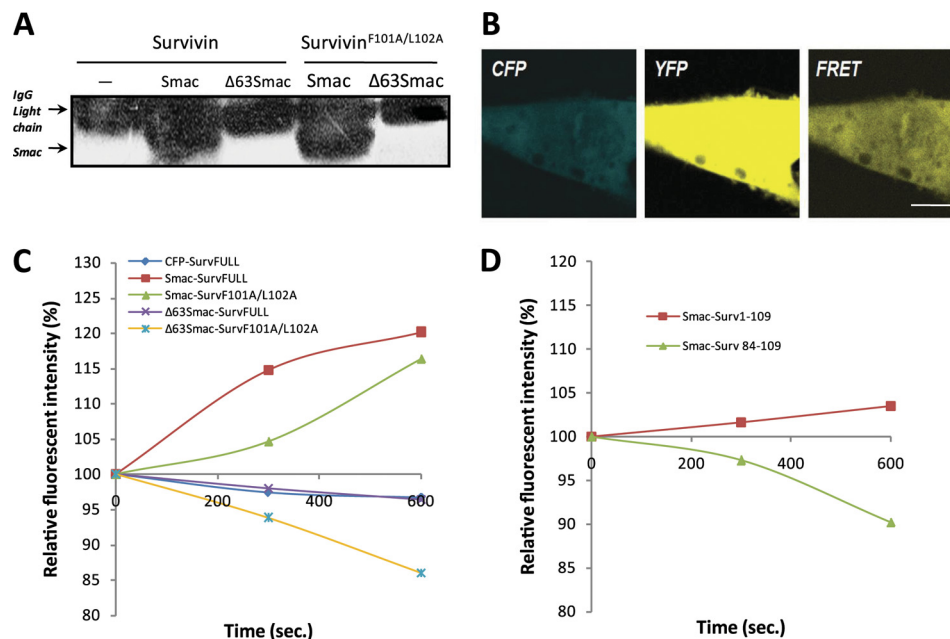


FIGURE 2. Survivin interaction with Smac/DIABLO. *A*, interaction of survivin and survivin^{F101A/L102A} with Smac/DIABLO and Δ63Smac/DIABLO. Recombinant mature Smac/DIABLO-His₆ (Smac) and Δ63Smac/DIABLO-His₆ (Δ63Smac) were incubated with recombinant WT His₆-survivin (survivin) or His₆-survivin^{F101A/L102A} (survivin^{F101A/L102A}), immobilized on protein G-Sepharose with antibodies against survivin or with nonimmune antibodies as a negative control (---). Immunoblotting with primary antibodies against Smac/DIABLO was used to detect bound proteins. *B*, fluorescence images of CFP, YFP, and FRET emission in HT1080 cells co-transfected with pTagCFP-N-Smac/DIABLO and pEYFP-C-survivin plasmids. Scale bars, 10 μm. *C*, acceptor photobleaching on YFP-survivin or YFP-survivin^{F101A/L102A} and CFP, Smac/DIABLO-CFP or Δ63Smac/DIABLO-CFP co-expressing cells. YFP was selectively photobleached at 514 nm. The graph shows the CFP fluorescence intensity versus acceptor photobleaching time. *D*, same as in *C* for HT1080 cells co-expressing fragmented survivin: YFP-survivin 1–109 or YFP-survivin 84–109 and Smac/DIABLO-CFP. The data represent three independent experiments.

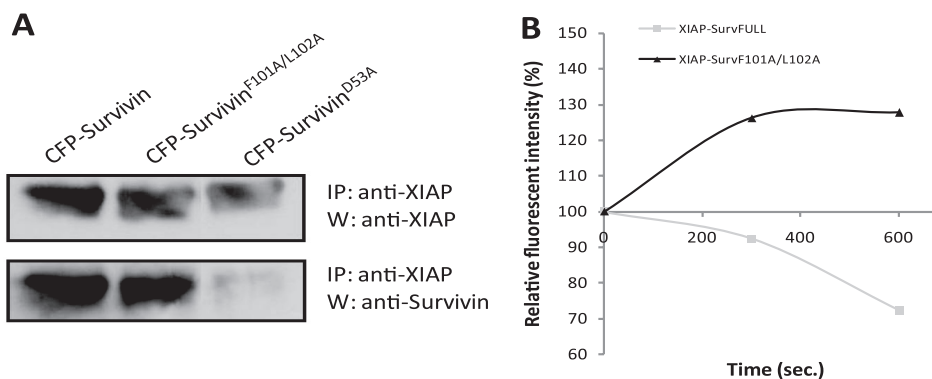


FIGURE 3. Interaction between survivin and XIAP. *A*, co-immunoprecipitation of XIAP from CFP-survivin-, CFP-survivin^{F101A/L102A}-, or CFP-survivin^{D53A}-expressing cells. HT1080 cells were transfected with pTagCFP-C-survivin, pTagCFP-C-survivin^{F101A/L102A} or pTagCFP-C-survivin^{D53A} plasmids. Then, cells were lysed, and endogenous XIAP was immunoprecipitated (IP) with antibodies against XIAP. Precipitated proteins were detected by immunoblotting (W) with primary antibodies against XIAP or survivin. *B*, acceptor photobleaching on YFP-survivin or YFP-survivin^{F101A/L102A} and CFP-XIAP co-expressing cells. YFP was selectively photobleached at 514 nm. The graph shows the CFP fluorescence intensity versus acceptor photobleaching time. The data represent three independent experiments.

Having obtained these results, we decided to investigate the role of survivin dimerization region in binding Smac/DIABLO. Earlier it was shown that the BIR domain was required for this interaction, although the separately expressed BIR domain (1–97 amino acids) without the dimerization region did not bind to Smac/DIABLO (12). Similarly to the investigation of survivin and Smac/DIABLO interactions *in vivo*, we tested the interaction between the survivin 1–109 amino acid fragment, containing BIR domain and dimerization region, and survivin 84–109 amino acid fragment, containing only the dimerization region, with mature Smac/DIABLO. The results (Fig. 2*D*) give evidence that the survivin dimerization region is essential for interac-

tions with Smac/DIABLO, but without the BIR domain it was not able to bind this protein.

Survivin Monomers Are Able to Interact with XIAP in Vivo—Survivin is also known to prevent apoptosis due to interaction with XIAP (13, 29). To test the possible role of survivin monomer in this antiapoptotic pathway we determined whether survivin^{F101A/L102A} is able to bind to XIAP. HT1080 cells were transfected with a plasmid encoding CFP-survivin, CFP-survivin^{F101A/L102A}, or CFP-survivin^{D53A} (mutant survivin form that has an amino acid substitution in the highly conservative BIR domain) (30). Immunoprecipitation of endogenous XIAP shows that it binds to both WT survivin and survivin^{F101A/L102A}, whereas interactions with the mu-

tant survivin form containing impaired BIR domain are much weaker (Fig. 3A). To confirm the obtained results we used a FRET registration method, similar to the procedure of testing interactions between survivin and Smac/DIABLO. The results are shown in Fig. 3B and indicate that survivin^{F101A/L102A} interacts with XIAP *in vivo*. Surprisingly, this method did not reveal interactions between WT survivin and XIAP.

Survivin^{F101A/L102A} Protects Fibrosarcoma Human Cells HT1080 from Caspase-dependent Apoptosis More Effectively Than WT Survivin—To investigate the role of survivin monomer in apoptosis regulation we constructed cell lines HT1080 stably expressing WT survivin or one of the following survivin mutant forms: survivin^{F101A/L102A}, survivin^{D53A}, or survivin^{D53A/F101A/L102A}, all containing N-terminal CFP. Survivin^{D53A} is one of the most studied dominant negative mutants that can form homodimers as well as heterodimers with endogenous survivin (18). In survivin mutant form, survivin^{D53A/F101A/L102A} (constructed in our laboratory), we substituted not only residue 53, which damages the BIR domain, but also residues 101 and 102. These amino acid substitutions, reasoned by analogy with survivin^{F101A/L102A}, would prevent survivin^{D53A/F101A/L102A} from dimerization. To create a stable cell lines, HT1080 cells were infected with lentiviral vectors encoding survivin or one of its mutant forms. To exclude the possibility of nonspecific effects caused by viral infection or CFP expression, we created another cell line stably expressing CFP alone as a control. Fig. 4A shows that all created cell lines expressed the desired proteins at approximately the same level, apart from the cell line expressing CFP. Exogenous CFP is synthesized far more efficiently than other exogenous proteins. This could be explained by the suggestion that survivin, unlike CFP, has a comparatively short lifetime and degrades much more quickly (11).

To determine the effect of different survivin mutants on tumor cell apoptosis, the cell lines obtained in our laboratory were treated with cisplatin. Cisplatin triggers the intrinsic apoptotic pathway via caspase activation and DNA fragmentation mediated by AIF protein (31–33). To estimate the level of caspase-dependent apoptosis we used antibodies against activated caspase-3. Cells were fixed, stained with antibodies against cleaved caspase-3, and analyzed by flow cytometry to quantify the percentage of apoptotic cells. As is depicted in Fig. 4B (gray bars), the highest level of caspase-3 activation in the absence of cisplatin (exceeding the level in control samples >4 times) was detected in cell lines expressing survivin^{D53A} and survivin^{D53A/F101A/L102A}. Notably, the caspase-3 activation level in cells expressing survivin^{D53A/F101A/L102A} was even higher than in cells expressing an earlier described dominant negative mutant survivin, which had only one substituted residue. After the exposure to cisplatin, as shown in Fig. 4B (black bars), the lowest apoptosis level was detected in cell lines expressing WT survivin and survivin mutant unable to dimerize (F101A/L102A). Moreover, the level of apoptosis in cells expressing survivin^{F101A/L102A} was significantly lower ($p < 0.05$) than in cells expressing WT survivin. On the other hand, the highest apoptosis level after cisplatin treatment was

detected in cells expressing mutant survivin with substituted 53 residue. To sum up, the obtained results give evidence that survivin^{F101A/L102A} protects human fibrosarcoma HT1080 cells from caspase-dependent cisplatin-induced apoptosis more efficiently than WT survivin, and both survivin mutants with substituted 53 residue increase the apoptosis level.

Engelsma *et al.* showed that survivin^{F101A/L102A} undergoes more active nuclear export than WT survivin (8), whereas the recent research conducted by Wang *et al.* provides evidence that survivin located in nucleus is able to repress the expression of antiapoptotic proteins Bcl-XL and MCL1 (19). Based on these facts, we wanted to test whether the more efficient antiapoptotic activity of survivin^{F101A/L102A} (compared with WT survivin) is linked to the fact that survivin^{F101A/L102A} is less represented in the nucleus and thereby inhibits the expression of other antiapoptotic proteins less efficiently. To test this suggestion we performed quantitative RT-PCR on the mRNA generated from the stable cell lines created in our laboratory using primers to Bcl-XL. As shown in Fig. 4C, no statistically significant difference ($p > 0.5$) in Bcl-XL expression between cell lines stably expressing WT survivin and survivin^{F101A/L102A} was observed. Interestingly, in cells expressing survivin^{D53A}, the level of Bcl-XL mRNA was higher than in those expressing survivin^{D53A/F101A/L102A}, and all cell lines present in our experiment expressed Bcl-XL at a significantly higher level than control cells (CFP).

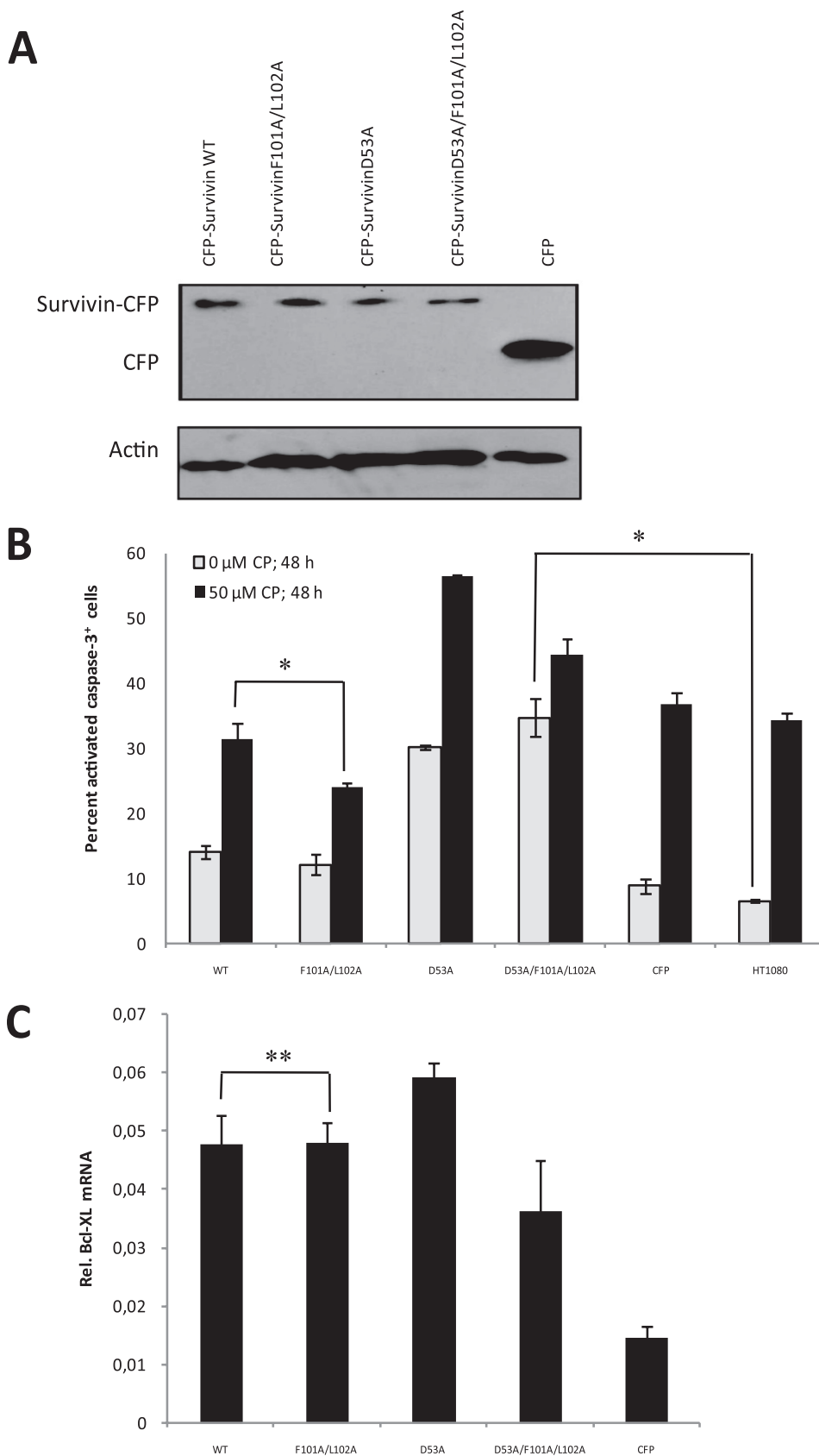
Recently published data show that during apoptosis induction, the endogenous survivin translocates from the cytoplasm to the nucleus, and this significantly reduces its antiapoptotic activity (34). To determine whether the decrease in antiapoptotic activity of WT survivin compared with survivin^{F101A/L102A} is connected to a difference in intracellular localization after apoptosis induction, we examined the localization of various survivin mutant forms before and after cisplatin treatment. Supplemental Fig. S1 shows that under the given experimental conditions no significant changes in localization of CFP-tagged WT survivin and survivin^{F101A/L102A} were observed. After apoptosis induction these proteins remain localized predominantly in the cytoplasm. This corresponds to the previously obtained data for GFP-tagged survivin (34).

Survivin^{F101A/L102A} Inhibits Smac/DIABLO More Efficiently Than WT Survivin—Having stated that survivin monomer protects cells from cisplatin-induced apoptosis more effectively than WT survivin, we wondered what could be the reasons. Therefore, we tested the difference in Smac/DIABLO inhibition by different survivin mutants. We assumed that the more efficiently survivin and its mutants inhibit Smac/DIABLO, the less considerably Smac/DIABLO knockdown would affect caspase-3 activation during apoptosis induction. To test this assumption, cells expressing different survivin mutants were transfected with siRNA against human Smac/DIABLO. Knockdown efficiency was tested by immunoblotting (Fig. 5A). As shown in Fig. 5B, cells expressing survivin^{F101A/L102A} display an insignificant decrease in caspase-3 activation ($p > 0.1$). At the same time, cells expressing WT survivin demonstrated a notable decrease in the amount of the activated caspase-3 ($p < 0.05$). The obtained results made us believe that survivin^{F101A/L102A} inhibits Smac/DIABLO more efficiently than WT survivin. Fig.

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5B demonstrates also that Smac/DIABLO inhibition has little effect on apoptosis in cells expressing survivin^{D53A} and survivin^{D53A/F101A/L102A}.

Survivin Monomer Protects Cells from Both Caspase-dependent and Caspase-independent Apoptosis—To determine whether survivin monomer affects caspase-independent apo-



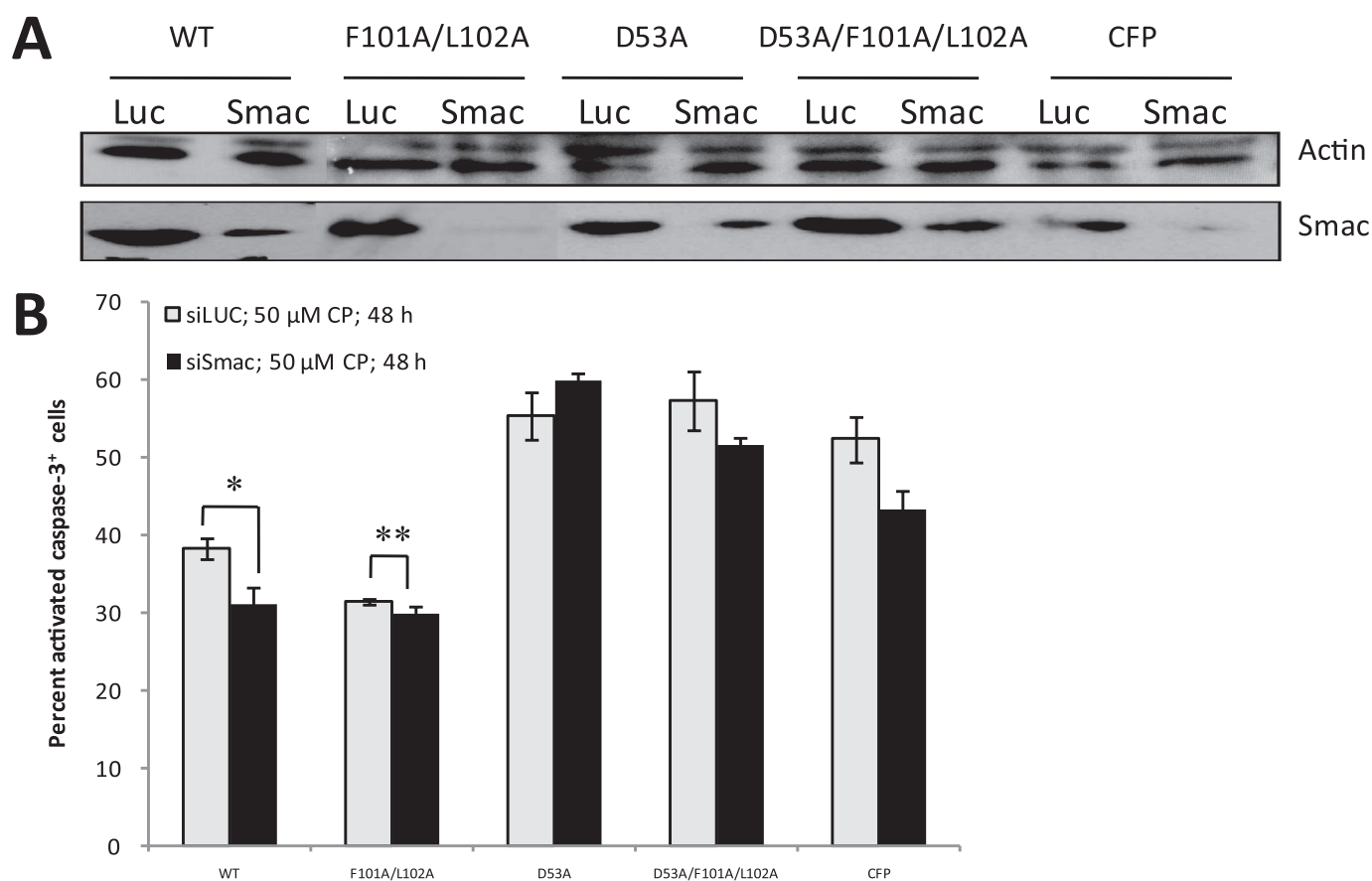


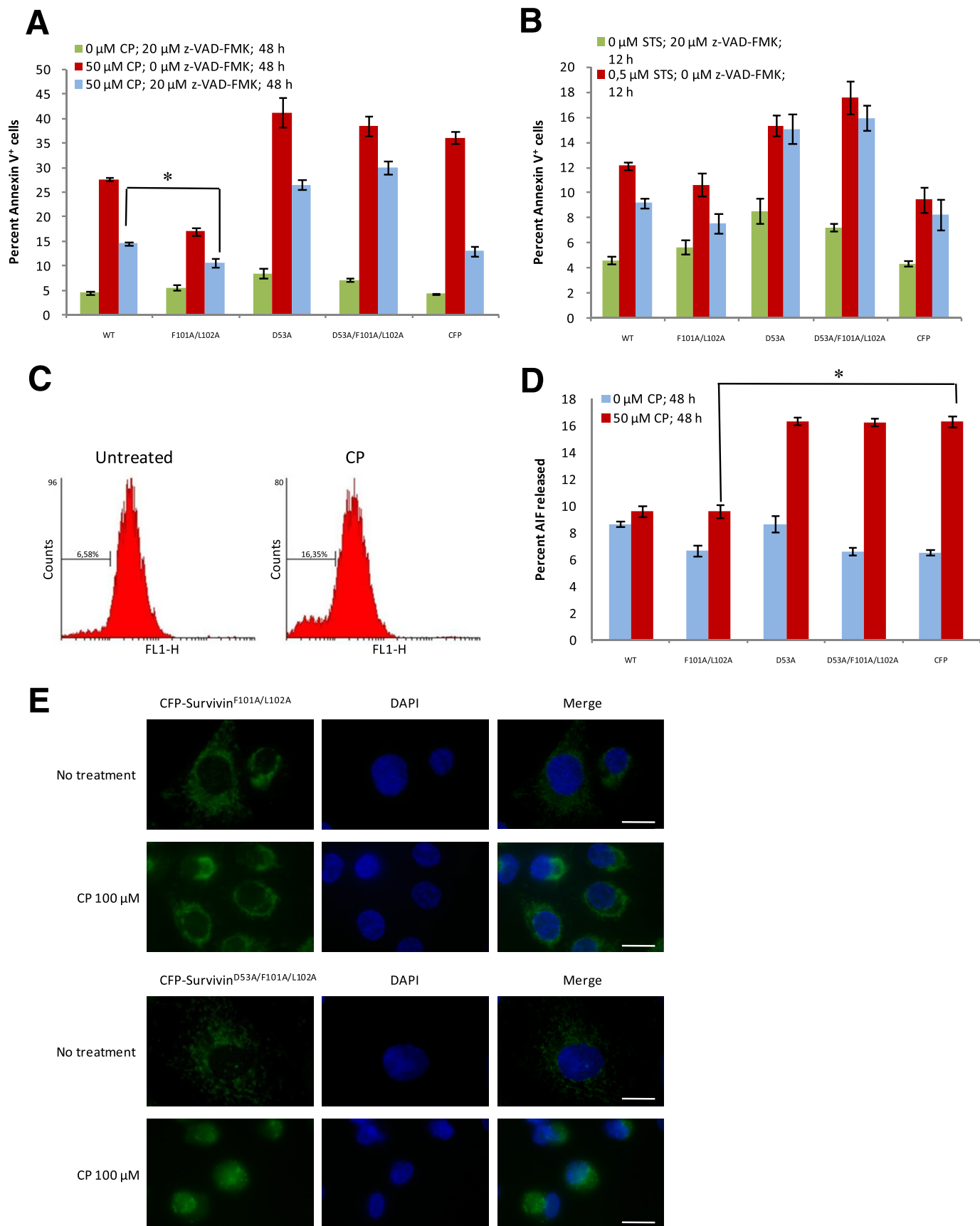
FIGURE 5. Effect of Smac/DIABLO knockdown on caspase-dependent cisplatin-induced apoptosis in HT1080 cells expressing survivin mutants. *A*, siRNA knockdown Smac/DIABLO. HT1080 cells expressing WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP (CFP) were transfected with control siRNA (Luc) or siRNA against Smac/DIABLO (Smac). After a 72-h incubation, Smac/DIABLO expression was analyzed with immunoblotting. *B*, HT1080 cells expressing WT survivin, survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP (CFP) were transfected with control siRNA (gray bars) or siRNA against Smac/DIABLO (black bars). After a 24-h incubation cells were treated with 50 μ M cisplatin (CP). After an additional 48-h incubation the level of caspase-dependent apoptosis was determined as in Fig. 4*B*. The figures represent mean values \pm S.D. (error bars) from three independent experiments (*, $p < 0.05$; **, $p > 0.1$).

ptosis, the response of HT1080 cells toward cisplatin treatment was tested in the presence of Z-VAD-fmk, a pancaspase inhibitor that blocks both the initiator and effector caspases and thereby breaks the caspase-dependent apoptotic pathway. The apoptotic level was determined by staining with annexin-PE followed by flow cytometry. 20 μ M Z-VAD-fmk was sufficient for a notable decrease of apoptotic level in all tested cell lines (Fig. 6*A*, red and blue bars). But even in the presence of the caspase inhibitor the apoptosis level in cells treated with cisplatin was considerably higher than in control samples (Fig. 6*A*, green bars). This result gives evidence that HT1080 cells undergo both caspase-dependent and caspase-independent apoptosis. Fig. 6*A* shows that cells expressing survivin^{F101A/L102A} even in the presence of a caspase inhibi-

tor are less subjected to apoptosis than control cells ($p < 0.05$). This gives evidence that survivin monomer inhibits both caspase-dependent and caspase-independent apoptosis. It is important to note that under the experimental conditions we did not observe any significant difference between the cisplatin-induced apoptosis level in presence of Z-VAD-fmk in the cells expressing WT survivin and the apoptosis level in control samples. Moreover, the obtained data show that in the presence of Z-VAD-fmk the apoptosis level in cells expressing mutant survivin with the substituted Asp⁵³ residue is twice higher than that in other studied cell lines. Together with the fact that Smac/DIABLO inhibition in these cell lines has almost no effect on apoptosis level, the results evidence that survivin^{D53A} and survivin^{D53A/F101A/L102A} in

FIGURE 4. Effect of different survivin mutants on caspase-dependent apoptosis in HT1080 cells. *A*, HT1080 cells were infected by lentiviral vector encoding WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP. Cells were grown on selective puromycin-containing medium. Lysates from cells expressing WT survivin, survivin mutant forms, or CFP were analyzed by immunoblotting with primary antibodies against CFP. Blotting with primary antibodies against actin was used as a control for equivalent protein loading. *B*, noninfected HT1080 cells or cells expressing WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP (CFP) were treated with 50 μ M cisplatin (black bars) or left untreated (gray bars). After 48 h of incubation both attached and detached cells were fixed and permeabilized. To estimate the apoptosis level, permeabilized cells were stained with primary antibodies against cleaved caspase-3 and secondary antibodies conjugated with Alexa Fluor 488, then cells were analyzed on FACScan flow cytometer. The figures represent means \pm S.D. (error bars) from three independent experiments (*, $p < 0.05$). *C*, relative Bcl-XL mRNA levels in HT1080 cells expressing WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP (CFP). RNA was purified, reverse-transcribed into cDNA, and quantitative RT-PCR was performed with primers to human Bcl-XL. The data are mean \pm S.D. for triplicate experiments (**, $p > 0.5$).

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HT1080 cells mostly enhance caspase-independent apoptosis. For an independent validation of the obtained results, a similar experiment was carried out with staurosporine, which inhibits a wide range of kinases and induces both caspase-dependent (35) and caspase-independent apoptosis (36). Fig. 6B shows that the results obtained with staurosporine apoptosis induction correspond to those obtained under cisplatin treatment.

As mentioned above, caspase-independent apoptosis occurs due to AIF release from IMS. Accordingly, we found it interesting to investigate the effect of different survivin mutants on AIF translocation. Because we were not aware of any previously described quantitative methods to analyze AIF release from mitochondria, we modified the Waterhouse and Trapani method aimed at evaluating the release of cytochrome *c* (22). Our method is based on the fact that in the healthy condition AIF is anchored to the inner mitochondrial membrane, but under the influence of diverse apoptotic stimuli C-terminal AIF fragment is released from the inner mitochondria membrane and is able to migrate to the cytoplasm (37). As a result, if a cell where AIF was cleaved is treated with an agent that is able to permeabilize the plasma membrane, a part of AIF diffuses outside the cell, decreasing the amount of AIF left inside. To determine the amount of AIF inside the cells we used antibodies against the C-terminal fragment of AIF (517–531 amino acids). Cells were fixed, stained for AIF, and analyzed on the flow cytometer (Fig. 6C). As shown in Fig. 6D, under the induction of apoptosis in the case of WT survivin and survivin^{F101A/L102A} the level of AIF release from IMS is considerably decreased compared with the control ($p < 0.05$) whereas in cells expressing survivin^{D53A} and survivin^{D53A/F101A/L102A} the level of migrated AIF is mostly the same as in control samples. After increasing cisplatin concentration up to 100 μM , significant differences in AIF release from IMS in cells expressing various survivin mutant forms were observed by immunostaining. Fig. 6E shows that expression of survivin^{F101A/L102A} considerably decreased translocation of AIF to the nucleus compared with survivin^{D53A/F101A/L102A}. All of the above mentioned observations give evidence that survivin^{F101A/L102A}, as well as WT survivin, prevents AIF from leaving the IMS.

Survivin^{F101A/L102A}, Unlike WT Survivin, Does Not Affect Microtubule Stability—Cheung *et al.* showed that survivin, due to its ability to bind to microtubules and stabilize them, preserves cells from microtubule-destabilizing agents (16). We decided to test the effect of WT survivin and survivin

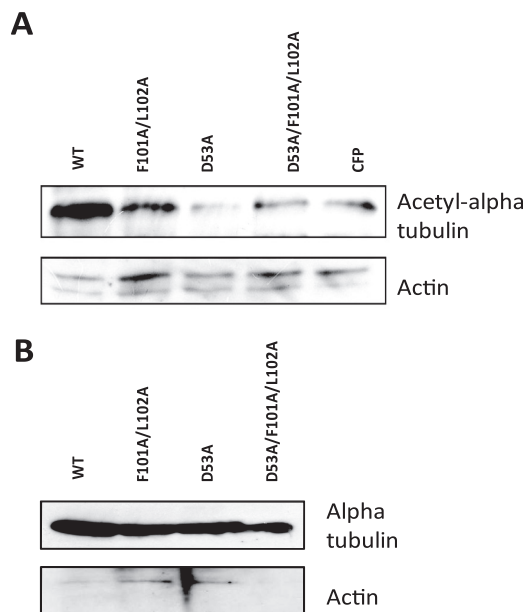


FIGURE 7. Effect of survivin mutants on microtubule stability. A, HT1080 cells expressing WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP (CFP) were lysed and analyzed by immunoblotting with primary antibodies against acetylated α -tubulin. Blotting with primary antibodies against actin was used as a control for equivalent protein loading. B, HT1080 cells expressing WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), or survivin^{D53A/F101A/L102A} (D53A/F101A/L102A) were lysed in microtubule stabilization buffer, and polymerized tubulin was separated from soluble tubulin by centrifugation. Pellet was analyzed by immunoblotting with primary antibodies against α -tubulin, and supernatants were analyzed by immunoblotting with primary antibodies against actin.

mutants on microtubule stability. For this purpose we evaluated the level of acetylated α -tubulin in cells expressing WT survivin and survivin mutants. The level of acetylated α -tubulin is considered to be a hallmark of microtubule stability (16). Fig. 7A shows that in cells expressing WT survivin the level of acetylated α -tubulin is considerably higher than in a control sample (CFP). These results are in good agreement with data obtained earlier by Rosa *et al.* (6). However, the most interesting fact is that survivin mutants with amino acid substitution do not increase the level of acetylated α -tubulin. This gives evidence that only WT survivin stabilizes the microtubule network. To confirm these results, we evaluated microtubule stability by a microtubule fractionation procedure. Fig. 7B shows that only cells expressing WT survivin had an increased amount of assembled tubulin fraction compared with control. This coincides with the results

FIGURE 6. Effect of different survivin mutants on cisplatin-induced caspase-independent apoptosis in HT1080 cells. A, HT1080 cells expressing WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP (CFP) were treated with 50 μM cisplatin (CP) in the presence (red bars) or absence (blue bars) of 20 μM Z-VAD-fmk. Cells treated with 20 μM Z-VAD-fmk without cisplatin were taken as a negative control (green bars). After a 48-h incubation apoptosis level was determined by staining with annexin-PE and flow cytometry. The figures represent mean values \pm S.D. (error bars) from three independent experiments (*, $p < 0.05$). B, same as in A for cells treated with 0.5 μM staurosporine (STS). C, HT1080 cells expressing CFP treated with 50 μM cisplatin (right) or left untreated (left). After a 48-h incubation the level of AIF released from IMS was measured according to the method described above. D, same as C for HT1080 cells expressing WT survivin (WT), survivin^{F101A/L102A} (F101A/L102A), survivin^{D53A} (D53A), survivin^{D53A/F101A/L102A} (D53A/F101A/L102A), or CFP (CFP). The figures represent mean values \pm S.D. from two independent experiments (*, $p < 0.05$). E, HT1080 cells expressing survivin^{F101A/L102A} or survivin^{D53A/F101A/L102A} treated with 100 μM cisplatin or left untreated. After a 48-h incubation cells were fixed, immunostained with antibodies against AIF, and treated with DAPI. Images were taken using a Nikon DIAPHOT 300 fluorescent microscope. Scale bars, 20 μm .

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gained in microtubule stability evaluation assay where the amount of acetylated α -tubulin was determined.

DISCUSSION

Survivin is a unique protein that takes part in many crucial cell processes and is able to bind a wide range of proteins forming a complicated interaction network (3). It appears that different pools of survivin have diverse functions (39). Thus, survivin located in the IMS protects cell from apoptosis (29); cytoplasmic survivin plays an important role in cell cycle regulation (10) and affects microtubule stability during interphase (6, 16); during mitosis some amount of survivin is localized within the chromosome passenger complex and regulates microtubule connections to the kinetochore (4); and yet another fraction of survivin is associated throughout the spindle microtubule network, regulating its stability (40).

For a long time it was considered that within the cell survivin exists only as a dimer, whereas monomeric survivin lacks biological functions (17, 18). However, today there are facts suggesting that some processes require monomer forms of survivin. Thus, only survivin monomer can be a part of the chromosome passenger complex (9), and only in a monomeric form survivin interact with an export receptor CRM1 (8). Finally, Wang *et al.* recently provided evidence that there is a special mechanism that maintains the balance between monomeric and dimeric survivin forms due to acetylation of its various Lys residues (19). Encouraged by these data we were interested in investigating the role of survivin monomer in another crucial process, namely, apoptosis regulation.

As a model of survivin monomer we used a survivin mutant unable to form dimers, survivin^{F101A/L102A}. First, we tested the survivin monomer ability to protect cells from apoptosis through interactions with Smac/DIABLO. We used a pull-down assay and FRET registration method to show that mutant survivin that unable to dimerize interacts with Smac/DIABLO both *in vivo* and *in vitro*. Moreover, we found that the survivin dimerization region is essential for such interaction. Using cell lines expressing survivin mutants and RNA interference we demonstrated that survivin^{F101A/L102A} inhibits Smac/DIABLO more efficiently than WT survivin. We also investigated the role of survivin monomer in protection against apoptosis through increasing XIAP stability and found that XIAP interacts not only with WT survivin, but also with the survivin monomer mutant. Finally, we showed that survivin^{F101A/L102A} inhibits caspase-independent apoptotic pathway in human fibrosarcoma HT1080 cells better than WT survivin and prevents AIF release from IMS. According to the obtained results, we proposed that survivin monomer is more efficient in cell protection against mitochondrial apoptosis. Because WT survivin is present in living cells both as a dimer and a monomer, on the basis of our results we assume that apoptosis protection requires the monomer form of survivin. This hypothesis is in good compliance with the results obtained by the Wang group. Wang *et al.* showed that unacetylated monomer form of survivin located in cytosol protects tumor cells from caspase activation, whereas survivin dimer interacts with STAT3 in nucleus

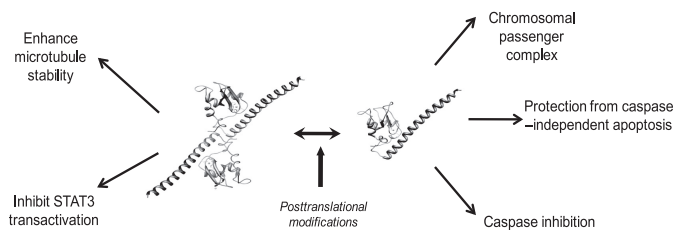


FIGURE 8. Proposed model depicting different function of dimeric and monomeric forms of survivin in cells. Survivin (ribbon representation; Protein Data Bank ID code 1E31) appears to be in dynamic equilibrium between dimeric and monomeric forms, and the balance can be regulated via posttranslational modifications. Survivin monomer prevents cells from caspase-dependent and caspase-independent apoptosis and is a part of the chromosome passenger complex during mitosis. Survivin dimer is required for microtubule stability and takes part in STAT3-dependent repression of transcription.

and acts like a proapoptotic agent decreasing Bcl-XL and MLC1 transcription rates (19).

On the other hand, in the current study we also showed that survivin monomer cannot affect microtubule stability, probably only a dimer is able to bind to polymerized tubulin-mediating interactions between microtubules and other proteins (41), although we cannot reject the possibility that the absence of stabilizing effect of survivin^{F101A/L102A} on microtubules is related not to the monomeric form of the protein, but to a change of spatial structure caused by amino acid substitutions. However, on the basis of our data as well as data obtained previously (42) we can assume that the dimerization region is important for survivin interaction with microtubules.

We suppose that during mitosis survivin monomer is associated with the chromosome passenger complex and takes part in destabilization of microtubules that attached to the kinetochore incorrectly (4), whereas survivin dimer interacts throughout the spindle microtubules, stabilizing them. Based on this assumption we could speculate that survivin acts partly as a monomer and partly as a dimer whereas the balancing mechanism acts as a “switcher” between survivin biological functions (Fig. 8). This model could explain the ability of this protein to perform diverse functions even in a single cell process.

In summary, our finding shows that not only survivin dimer, but also survivin monomer participates in apoptosis regulation. Moreover, according to our data survivin monomer protects cells against apoptosis even more efficiently than survivin dimer. Taking into consideration that the survivin dimerization region has higher affinity to various ligands than the BIR domain (38), we proposed that antitumor drugs targeting survivin monomer could be more efficient than peptide mimetics of Smac/DIABLO. We also showed that a strong proapoptotic effect of dominant negative survivin mutants is mostly based on a caspase-independent apoptotic pathway. Elucidation of the precise mechanism of their action could open new ways to apoptosis induction in tumor cells. Finally, we suggest a new model of how survivin functions in cells. We realize that our hypothesis needs further research, particularly investigation of the effect of posttranslational modifications on the balance between dimeric and monomeric survivin forms is required. However, we hope that the results

presented in this paper will clarify the delicate mechanism of survivin function.

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