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A Single Amino Acid Change (Asp 53→Ala53) Converts Survivin from Anti-apoptotic to Pro-apoptotic

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Survivin is a member of the inhibitor of apoptosis protein (IAP) family that has been implicated in both apoptosis inhibition and cell cycle control. Recently, Survivin has attracted growing attention because of its tumor-specific expression and potential applications in tumor therapy. However, its inhibitory mechanism and subcellular localization remain controversial. Here, we report a novel Survivin mutant Surv-D53A, which displays a function opposite to Survivin and a distinctive subcellular distribution compared with its wild-type counterpart. Surv-D53A was shown to induce apoptosis in a p53-independent manner, indicating that tumor suppressor p53 is not involved in its apoptosis pathway. Surv-D53A was shown to markedly sensitize apoptosis induced by TRAIL, doxorubicin, and RIP3. We also demonstrated that similar to wild-type Survivin, Surv-D53A was localized in cytoplasm in interphase and to midbody at telophase. However, it fails to colocalize in chromosomes with Aurora-B in metaphase as wt-Survivin. Surv-D53A mutant is less stable than wt-Survivin and is degraded more rapidly by ubiquitin-proteasome pathway. Additionally, we found that Surv-D53A interacts with wt-Survivin to form heterodimer or with itself to form mutant homodimer, which may account for the loss of its antiapoptotic function. Finally, unlike Survivin*Survivin, neither Surv-D53A*Survivin nor Surv-D53A*Surv-D53A is able to bind to Smac/DIABLO, which may explain the underlying mechanism for its abolishment of antiapoptotic activity of Survivin.

INTRODUCTION

The members of the inhibitor of apoptosis protein (IAP) family were first identified as negative regulators of programmed cell death characterized by the presence of one to three copies of the baculovirus IAP repeat (BIR) domain (Crook *et al.*, 1993). However, a definite role in physiological cell death has not been established for all IAPs, because some members play roles in cell division, rather than having a direct role in the regulation of apoptosis (Uren *et al.*, 1999, 2000). Survivin is a special IAP protein containing a single BIR domain and lacking the C terminal Ring finger domain (Ambrosini *et al.*, 1997). It is not usually detectable in normal adult tissues, but is prominently expressed in nearly all the common human cancers and most of the transformed cell lines (Ambrosini *et al.*, 1997). Survivin functions as a dimer and is regulated in a cell-cycle–dependent manner, peaking at G2/M, and is associated with the mitotic spindle, centromeres and the midbody in dividing cells (Li *et al.*, 1998; Muchmore *et al.*, 2000). It is a short-lived protein and is degraded by ubiquitin proteasome pathway (Zhao *et al.*, 2000). Recently, it has been reported that wild-type p53 represses Survivin expression at both mRNA and protein levels (Mirza *et al.*, 2002).

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Survivin is also a bifunctional protein that suppresses cell death and regulates cell division (Altieri *et al.*, 1999). However, the mechanism by which Survivin blocks apoptosis has remained controversial. Tamm *et al.* (1998) demonstrated that Survivin could bind to the effector caspase-3 and caspase-7 in vitro and proposed that Survivin may block apoptosis through inhibiting caspase activity in vivo by a similar mechanism. However, comparisons between the x-ray crystallographic structures of Survivin and that of the XIAP (BIR2): caspase-3 complex fail to reveal any clues of how Survivin could directly interact with caspase-3 (Riedl *et al.*, 2001). In addition, Verdecia *et al.* (2000) and Banks *et al.* (2000) have provided contradictory evidence to demonstrate that Survivin is unable to directly bind to caspase-3 in vitro and does not inhibit caspase-3 activity. Therefore more research is needed in order to clarify this issue.

Survivin has been reported as a chromosomal passenger protein that binds to both INCENP and Aurora-B (Skoufias *et al.*, 2000; Wheatley *et al.*, 2001) to form a complex that plays a crucial role in the execution of cytokinesis. However, studies on the biological function and subcellular distribution of Survivin during mitosis became more difficult because of the recent identification of the presence of two novel Survivin splice variants (Mahotka *et al.*, 1999; Conway *et al.*, 2000). Survivin splice variants Survivin-2B and Survivin-deltaEX3 showed reduced antiapoptotic activity and displayed subcellular localization different from that of wild-type Survivin. Moreover, it has been reported that Survivin antisense oligonucleotide or Survivin point mutant (T34A, C84A) was able to spontaneously induce apoptosis

(O'Connor *et al.*, 2000; Grossman *et al.*, 2001), and these dominant negative mutants could provide useful tools for further investigating the functions of Survivin and its downstream events in the inhibition of apoptosis.

In this report, we characterize a new Survivin dominant negative mutant Surv-D53A, which shows proapoptotic effect in a p53-independent manner. Asp (D) at position 53 of Survivin is highly conserved in mammalian BIR domain and substitution of alanine for aspartate results in the conversion of Survivin's apoptotic function and the changes in its subcellular localization. Although wild-type Survivin suppresses apoptosis induced by various stimuli through directly interacting with the caspase activator Smac/DIABLO, mutant Survivin containing Surv-D53A monomer fails to bind to Smac/DIABLO, resulting in the loss of its antiapoptotic activity and the gain of proapoptotic function. Unlike wild-type Survivin, Surv-D53A fails to localize on the chromosomes together with Aurora-B kinase at metaphase.

MATERIALS AND METHODS

Oligonucleotides

The sequences of the oligonucleotides used in this study are listed as follows: P1: 5′ > CGGAATTCCATGGGTGCCCCGACGTTGC < 3′; P2: 5′ > CGCTCGAGTCAATCCATGGCAGCCAGCTG < 3′; P3: 5′ > GAGAACGAGCCAGCCTTGGCCCAGTGTTTC < 3′; P4: 5′ > GGGCCAAGGCTGGCTCGTTCTCAGTG < 3′; P5: 5′ > GCGCCTGCGCCCCGGAGCGGATGG < 3′; P6: 5′ > CCGCTCCGGGGCGCAGGCGCAGCCCT < 3′; P7: 5′ > GCAAGCTTATGGCGGCTCTGAAGAGTTG < 3′; P8: 5′ > GCGATTAATATGGCGGTTCCTATTGCAC < 3′; and P9: 5′ > CGGCGGCCGCATCCTCACGCAGGTAGGCCT < 3′. The underscored nucleotides are used for mutagenesis.

Reagents and Antibodies

The following antibodies were used in this study: polyclonal antibodies antibody-caspase-7, antibody-caspase-3, antibody-survivin, and antibody-actin (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal antibodies: mAb-GFP (MBL, Nagoya, Japan), mAb-Flag (Sigma, St. Louis, MO) and mAb-Smac/DIABLO (Calbiochem, La Jolla, CA). TRAIL was purchased from R&D Systems Inc. (Minneapolis, MN); MG132 from Calbiochem; and doxorubicin, Hoechst 33342 (Somerville, MA), and cycloheximide from Sigma. Restriction enzymes were purchased mostly from New England Biolabs (Beverly, MA). Medium compounds were obtained from Oxid (Basingstroke Hampshire, UK). The majority of biochemical reagents were ordered from Sigma. Trypan blue was purchased from Invitrogen (Carlsbad, CA).

Cell Culture and Transfection

Cell lines HeLa. A549 and H1299 were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 1× nonessential amino acid, 100 μg/ml penicillin, 1× MEM sodium pyruvate, 100 μg/ml streptomycin (GIBCO, Grand Island, NY). Cultured cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Transfection of cells with various mammalian expression constructs by Lipofectamine 2000 (GIBCO) was according to the methods provided by manufacturer's specification.

PCR-mediated Mutagenesis

To obtain the Survivin mutant Surv-D53A or Surv-T34A, we have used the PCR-mediated mutagenesis method. The primer pairs P1/P4 and P3/P2 (for D53A) or pairs P1/P6 and P5/P2 (for T34A) were used to amplify two Survivin fragments. The resultant two overlapping PCR fragments were mixed with equal amounts. This mixture was incubated first at 94 for 4 min, followed by first PCR (PCR1) of 94°C denaturing for 1 min, 56°C annealing for 1 min, and 72°C extension for 1 min. After 10 cycles, the PCR1 products was used as template in a second PCR (PCR2), primed by oligonucleotides P1 and P2 to carry out another 20 cycles of PCR with the same PCR conditions used in PCR1. A prominent band with expected size of 0.42 kb was visible on 1% agarose gel. Mutations was further verified by DNA sequencing.

Plasmids Construction

The cDNA of Survivin or Smac/DIABLO was amplified by RT-PCR from total RNA of HeLa cell line using primer pair P1/P2 or P7/P9. The amplified fragments were cloned into the pGEM-T vector. The cDNA of Survivin and its mutant with a point mutation at amino acid residue 53 (D53A) were subsequently subcloned into *Eco*RI/*Xho*I sites of pGEX-5X-3 vector (for the preparation of GST/Survivin and GST/Surv-D53A) or pEGFP-C1 vector (for the preparation of GFP/Survivin and GFP/Surv-D53A). The cDNA fragment coding for mature Smac (residues 56–239) generated from a PCR reaction using primers P8 and P9 was digested with restriction enzymes *Vsp*I and *Not*I and subcloned into *Nde*I/*Not*I sites of pET-22b (Novagen, Madison, WI) to generate pET-22b/Smac with His6 tag fusion at its carboxyl terminal. The cDNA of XIAP or Survivin mutant Surv-T34A was cloned into pEGFP-C1 vector. The cDNA of Survivin or Surv-D53A was also cloned into pCDNA3-Flag vector.

Expression and Purification of Fusion Proteins

GST, GST fusion proteins, and mature Smac were expressed in *Escherichia coli* cells DH5-alpha or BL21/DE3. The GST fusion proteins were purified through the glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden). The soluble His6-Smac fusion protein was purified by incubating with chelating Sepharose Fast Flow beads (Pharmacia Biotech) according to the procedures specified by manufacturer's instruction. The ultimately elution products were dialyzed with 1× phosphate-buffered saline (PBS) and were used for the in vitro interaction assay.

In Vitro Interaction Assay

The bacterially expressed and purified His6 tagged Smac were incubated with GST or GST fusion proteins immobilized on glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) overnight at 4°C. The mixture was washed three times with 500 μl of 1× PBS. The bound proteins were eluted and were subject to Coomassie staining.

Cell Death Assay

The ability of Survivin or their mutants to affect cell viability was assayed by transfecting HeLa cells (2×10^4 cells/well) in 24-well plates with 0.3 μg mammalian expression vectors. Twenty hours after transfection, cells continued to be incubated with drug, and the viability of the cells was measured with the standard Trypan Blue exclusion method by counting blue dead cells, which are also characterized to be with aberrant nuclei stained by Hoechst 33342. Data are expressed as percentages of control and are the means of three independent experiments.

Assessment of Apoptosis by Annexin V Staining

Annexin V-FITC Apoptosis Detection kit (PharMingen, San Diego, CA) was used in this assay. Forty-eight hours after transfection, cells were harvested and resuspended in binding buffer (0.01 M HEPES/NaOH, pH 7.4; 0.14 mM NaCl, 2.5 mM $CaCl_2$) at the concentration of 1×10^6cells·ml^{-1}. The resuspended solution (100 μl, 1×10^5cells) was transferred to a 5-ml culture tube. After incubation with 5 μl of Annexin V-FITC and 10 μl of PI (50 μg·ml^{-1}) for 15 min at room temperature in the dark, the cells were analyzed by flow cytometry FACS Calibar using the Cell Quest software system (Becton Dickinson, Franklin Lakes, NJ).

Western Blot Analysis

Cells were washed with 1× PBS and resuspended with 5 volumes of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN). The cell lysate was incubated on ice for 30 min and was then centrifuged for 10 min at 4°C. Equal amounts of proteins were loaded onto the gel and separated by SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membrane. After blocking with 5% nonfat milk in TBST overnight at 4°C, the blot was incubated with primary antibody for 1 h at room temperature. The membrane was then probed with HRP-conjugated secondary antibody for 1 h and developed by ECL using the manufacturer's protocol.

Immunoprecipitation

Cells were lysed in a Triton X-100–based lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, protease inhibitor cocktail) for 1 h, and the nuclear and cellular debris was cleared by centrifugation. Then the cytosolic lysis was mixed with Smac mAb bound to protein A/G-Sepharose. After a 1-h incubation at 4°C, the immunoprecipitates were washed five times in lysis buffer, and proteins were recovered either by boiling beads in SDS sample buffer and analysis by Western blot.

Immunofluorescence Microscopy

The immunofluorescence method was performed as described previously by Temme *et al.* (2003). Transfected HeLa cells grown on poly-D-lysine–coated glass coverslips were fixed with 2% paraformaldehyde-PBS for 10 min. After fixation, cells were washed three times with PBS, followed by permeabilization in 0.1% Triton X-100 for 5 min. The fixed and permeabilized cells were blocked in 1% bovine serum albumin in PBS for 15 min followed by a 1-h incubation with the primary antibodies against Flag, Tubulin, Numa, and Aurora-B. After washing three times with TPBS, cells were then probed with secondary antibodies (including FITC-conjugated affinity-purified anti-mouse IgG or rhodamine-conjugated anti-rabbit IgG), and the DNA was stained with DAPI for 10 min. Images were taken on an Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany) by using a 63× 1.3 numerical aperture

PlanApo objective. Figures were constructed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

RESULTS

Survivin Asp53 Is Highly Conserved in Mammalian BIR Domains

Survivin is an IAP family protein containing a single BIR domain that is essential for regulating apoptosis and controlling cell cycle (Ambrosini *et al.*, 1997; Li *et al.*, 1998, 1999). Although some of the results obtained recently from studies on the Survivin and its mutants have been applied to the preclinical research for tumor therapy, the detailed information of Survivin's function still remains obscure (Tamm *et al.*, 1998; Verdecia *et al.*, 2000). Over the past few years, mutational analysis of Survivin has helped provide some insightful clues in characterizing the function of Survivin and dissecting its downstream signaling events (Li *et al.*, 1998; O'Connor *et al.*, 2000). To identify novel mutant that could change the characteristics of wild-type Survivin and contribute to the understanding of the mechanism of Survivin in the inhibition of apoptosis, we have focused firstly on those highly conserved amino acids in Survivin BIR domain. We compared Survivin BIR domain sequence with that of other mammalian IAPs (Livin-BIR, XIAP-BIR1, XIAP-BIR2, XIAP-BIR3, C1-IAP-BIR, C2-IAP-BIR, OP-IAP-BIR1, and OP-IAP-BIR2) using Clustal W program from the European Bioinformatics Institute modified online by Boxshade program from the Pasteur Institute. As shown in Figure 1A, D53 amino acid residue in Survivin was highly conserved in all IAP BIR domains listed, suggesting that D53 may be critical for Survivin's function. Although some other residues such as R18, D71, and C84 are also well conserved, the D53 residue is particularly of importance, because D53 was shown to participate in making up the acidic surface of Survivin by three-dimensional structure analysis (Verdecia *et al.*, 2000). Whether the substitution for Asp53 could affect Survivin's biological function has not been studied thus far; we thereby substituted the Asp53 with Ala using site-directed mutagenesis by the PCR method described in MATERIALS AND METHODS, and the resulting novel Survivin mutant was designated as Surv-D53A. At the same time, we mutated amino acid Thr34 to Ala as a control, because Surv-T34A mutant has been reported to show dominant negative effect (O'Connor *et al.*, 2000; Grossman *et al.*, 2001). We also constructed three GFP fusion plasmids including pEGFP-C1/Survivin, pEGFP-C1/Surv-D53A, and pEGFP-C1/Surv-T34A, and these constructs were used for transfection to compare their ability to induce apoptosis. (Figure 1B). As shown in Figure 1C, the successful expression of the three transfected genes in HeLa cells was detected by Western blot.

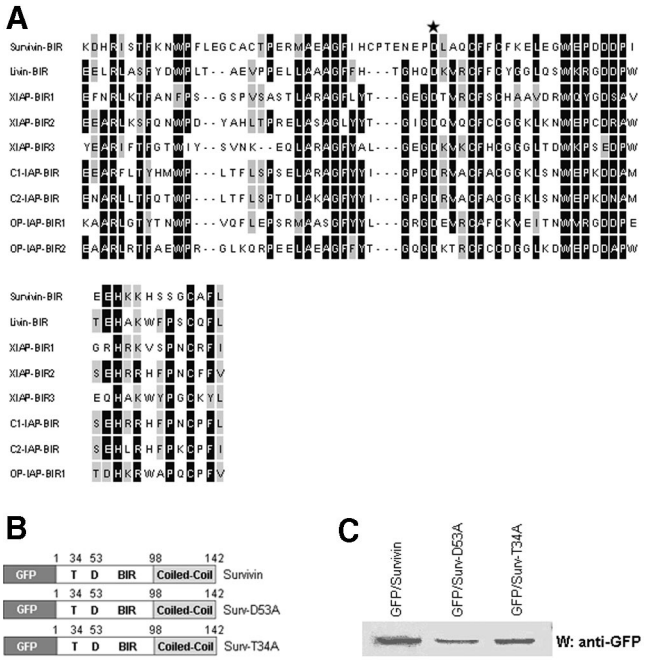

Figure 1. Asp53 of Survivin is highly conversed in BIR domain of mammalian IAP family. (A) Alignment of BIR domains from six IAPs. The amino acids of BIR domains in Survivin, livin, XIAP, C1-IAP, C2-IAP, and OP-IAP were aligned using the ClustalW program from the European Bioinformatics Institute and then were modified online by the Boxshade program from the Pasteur Institute. Asp53 is marked with a asterisk. (B) Schematic diagrams of GFP fusion constructs for GFP/Survivin, GFP/Surv-D53A, and GFP/Surv-T34A. Numbers indicate the position of amino acids in Survivin. (C) The expressions of three transfected plasmids indicated in B were verified by Western blot, and the primary antibody used was anti-GFP mAb.

Surv-D53A Spontaneously Induce Apoptosis in a p53-independent Manner

The antiapoptotic activity of Survivin has been well documented (Ambrosini *et al.*, 1997; Li *et al.*, 1999); however, forced expression of Survivin mutants C84A and T34A resulted in spontaneous apoptosis in several tumor cell types (Li *et al.*, 1998; Grossman *et al.*, 1999; O'Connor *et al.*, 2000). To investigate the function of our novel mutant Surv-D53A, we transfected HeLa cells with various Flag fusion constructs including pCDNA3-Flag, pCDNA3-Flag/Survivin, and pCDNA3-Flag/Surv-D53A 48 h after transfection; only Flag/Surv-D53A was shown to induce significant apoptosis characterized by the typical apoptotic morphological changes of the cells from spindle-like to rounded shapes (Figure 2Ac). To further verify whether the Surv-D53A–induced cell death truly represents apoptosis, we used the AnnexinV-FITC staining method to quantify the apoptotic cell numbers by flow cytometry. As shown in Figure 2Ac′, ~32% of Surv-D53A–induced HeLa cells were Annexin-V positive and PI negative (early apoptotic). Compared with that in Figure 2A, a′ and b′, the numbers of Surv-D53A–induced apoptotic cells were about threefold higher than that of mock-induced apoptotic cells (12%) and about fivefold higher than that of wt-Survivin transfected cells (7%). These data clearly demonstrate that Surv-D53A has been functionally converted from antiapoptotic to proapoptotic. To further compare the apoptotic effect of this mutant with that of T34A mutant, several GFP fusion constructs including pEGFP-C1/Survivin, pEGFP-C1/Surv-D53A, and pEGFP-C1/Surv-T34A were transfected into HeLa cells individually, and the transfectants were then examined under fluorescence microscope at different times (24, 48, and 72 h). Those detached round, membrane-blebbing cells with aberrant Hoechst 33342–stained nuclei were scored as apoptotic cells. Cell viability was measured by the trypan blue exclusion method at different times, and the result clearly showed that apoptotic effect of Surv-D53A resembles that of Surv-T34A (Figure 2B) even after a prolonged period of incubation for 72 h.

Mirza *et al.* (2002) reported recently that human Survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. We realize that the expression of p53 is largely inhibited by HPV-18 E6 protein in HeLa cells; therefore, the induction of apoptosis by Surv-

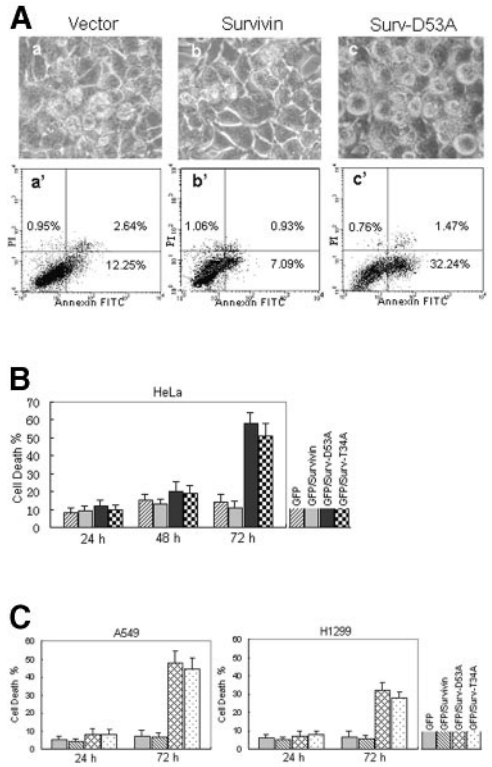

Figure 2. Surv-D53A induce apoptosis in a p53-independent manner. (A) Plasmids pCDNA3-Flag, pCDNA3-Flag/Survivin, and pCDNA3-Flag/Surv-D53A were transfected into HeLa cells separately. Forty-eight hours later, transfected cells were then examined under an inverted light microscope. Cells were stained with Annexin V-FITC and PI for determination of apoptosis by FACS analysis. (B) GFP, GFP/Survivin, GFP/Surv-D53A, or GFP/Surv-T34A was expressed in HeLa cells and the viability of cells was further measured by the trypan blue exclusion method; 72 h after transfection, apoptosis induced by Surv-D53A was up to ~60% and Surv-T34A showed ~50% of the apoptotic effect. (C) The GFP fusion constructs were transfected into A549 (p53+) and H1299 (p53−) separately, followed by the trypan blue exclusion method.

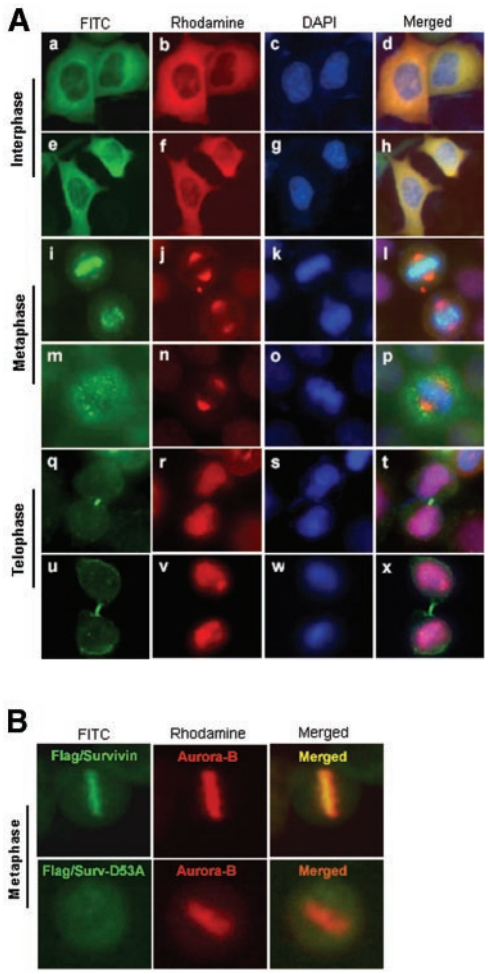

Figure 3. Differential subcellular localization of Surv-D53A vs. wt-Survivin at metaphase. (A) HeLa cells transfected with pCDNA3-Flag/Survivin or pCDNA3-Flag/Surv-D53A were grown on poly-D-lysine–coated glass coverslips for immunofluorescence microscopy as described in MATERIALS AND METHODS. (a, i, and q) The localizations of Flag/Survivin; (e, m, and u) the distributions of mutant Survivin Flag/Surv-D53A; (b and f) the localizations of tubulin; (j, n, r, and v) the localizations of Numa; (c, g, k, o, s, and w) chromosome DNA; (d, h, l, p, t, and v) merged images. Green, FITC; red, Rhodamine; blue, DAPI. (B) The localization of Flag/Survivin, Flag/Surv-D53A, or Aurora-B in metaphase was determined by the immunofluorescence microscopy method as described in A.

D53A may act in a p53-independent manner. To further examine whether Surv-D53A–induced apoptosis requires p53, we transfected plasmids pEGFP-C1/Survivin and pEGFP-C1/Surv-D53A into A549 and H1299 cells to compare their cell viability. These two cell lines are widely used for investigation of the involvement of p53. Human lung cancer cell line A549 expresses wild-type p53, whereas human lung cancer cell line H1299 is a p53 null mutant. As shown in Figure 2C, both Surv-D53A and Surv-T34A resulted in massive cell death in A549 and H1299 cells; this result confirms that wild-type function of p53 is not required for Surv-D53A to induce apoptosis.

Surv-D53A Fails to Colocalize with Aurora-B at Metaphase

Survivin has been reported to act as a chromosomal passenger protein to play crucial role(s) in the execution of cytokinesis (Skoufias *et al.*, 2000; Wheatley *et al.*, 2001). To compare the subcellular localization of Surv-D53A with its wild-type counterpart, we expressed either Flag/Survivin or Flag/Surv-D53A in HeLa cells. The immmunostaining results were shown in Figure 3A. During interphase, both Flag/Survivin and its mutant Flag/Surv-D53A were found to distribute specifically in the cytoplasm but not in the nucleus that was stained by DAPI (Figure 3A, a–h). When cell cycle entered into metaphase, Flag/Survivin strongly associated with chromosomes and were assembled at metaphase plate (Figure 3A, i–l). Unlike wild-type Survivin, mutant Surv-D53A failed to associate with chromosomes and therefore was unable to accumulate at the metaphase plate (Figure 3A, m–p). At telophase, Flag/Survivin was observed to leave the chromosomes and appeared in the midbody (a remnant of the mitotic apparatus) for the mitotic exit (Figure 3A, q–t). Similarly, Flag/Surv-D53A was also found to localize at midbody (Figure 3A, u–x), indicating Surv-D53A did not cause aberrant execution of cytokinesis.

It was reported that functional Survivin exists in a complex with both INCENP and Aurora-B kinase during mitosis (Wheatley *et al.*, 2001). Because Surv-D53A showed distinc-

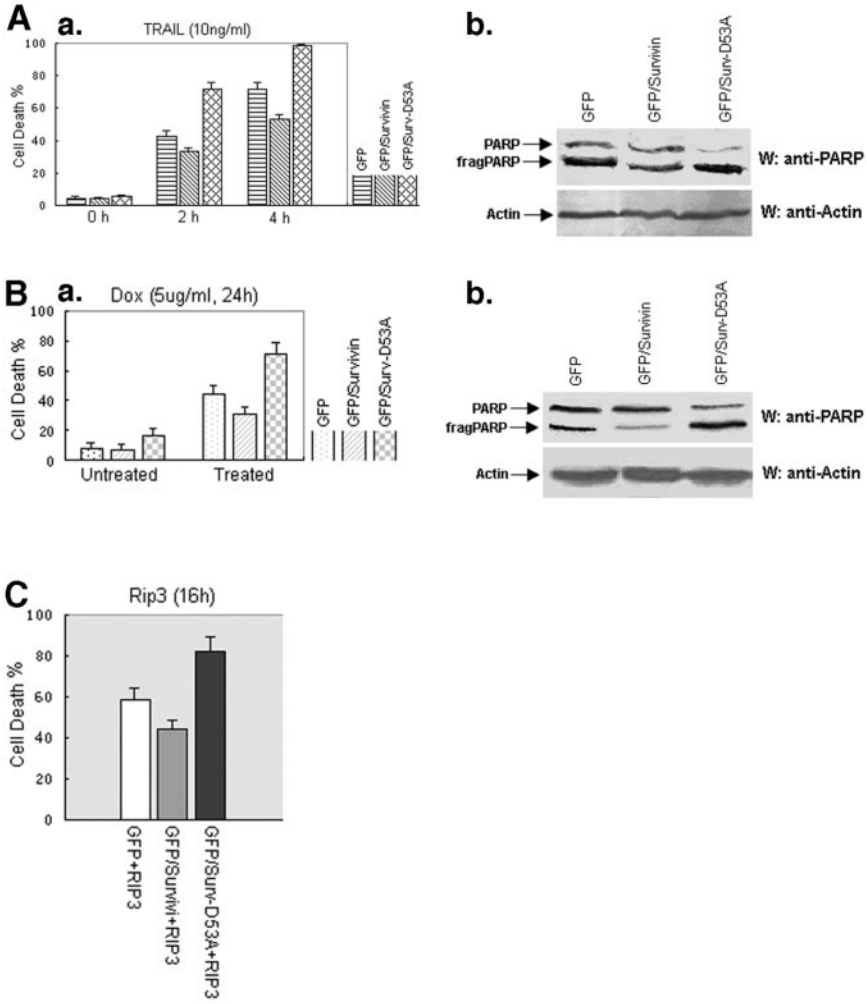

Figure 4. Ectopic expression of Surv-D53A sensitizes HeLa cells to TRAIL-, doxorubicin-, and RIP3-induced apoptosis. (A) HeLa cells were transfected with plasmids pEGFP-C1, pEGFP-C1/Survivin, and pEGFP-C1/Surv-D53A, respectively. Twenty hours posttransfection, cells were exposed to TRAIL (10 ng/ml) for 2 or 4 h. The viability of treated cells was measured by the trypan blue exclusion method (a) and apoptotic processing of PARP was verified by Western blot using anti-PARP antibody (b). (B) After transfection, the transfected cells (described in A) were incubate with doxorubicin for another 24 h, and their viability was measured by the trypan blue exclusion method (a) and the cleavage of PARP was detected by Western blot using anti-PARP antibody (b). (C) The vector pEGFP-C1/RIP3 was cotransfected with pEGFP-C1, pEGFP-C1/Survivin, or pEGFP-C1/Surv-D53A into HeLa cells. Twenty-four hours after transfection, cell viability was determined by the trypan blue exclusion method.

tive subcellular distribution from wt-Survivin at metaphase (Figure 3A, m–p), we next asked whether Surv-D53A is able to colocalize with Aurora-B. As is expected, only wt-Survivin, but not mutant Surv-D53A, was able to colocalized with Aurora-B in the metaphase plate (Figure 3B). The merged image from Survivin (detected by FITC-labeled antibody) and Aurora-B (stained by rhodamine-labeled antibody) showed a strong uniform yellow pattern, whereas Surv-D53A and Aurora-B did not superimpose (Figure 3B). These results indicate that intact BIR motif, in which Asp53 resides, is essential for proper cellular localization of wild-type Survivin.

Ectopic Expression of Surv-D53A Sensitizes HeLa cells to TRAIL-, Doxorubicin-, and RIP3-induced Apoptosis

We have determined that the cytokine TRAIL and chemotherapeutic agent doxorubicin were able to induce apoptosis in HeLa cells (unpublished data). To determine whether Surv-D53A sensitizes the HeLa cells to apoptosis induced by these two agents, we transfected HeLa cells with plasmid pEGFP-C1, pEGFP-C1/Survivin, or pEGFP-C1/Surv-D53A separately. Twenty hours after transfection, cells were exposed to TRAIL (10 ng/ml, 2 and 4 h) or doxorubicin (5 μg/ml, 24 h). The expression of GFP, GFP/Survivin or GFP/Surv-D53A was examined by fluorescence microscopy, and the cell viability was measured by the trypan blue method. pEGFP-C1/Survivin–transfected cells showed reduced apoptosis compared with mock control as shown in Figure 4, Aa and Ba. Because Survivin is a strong antiapoptotic protein that has been reported to inhibit apoptosis triggered by various stimulating factors (Ambrosini *et al.*, 1997; Tamm *et al.*, 1998), this result is expected. In contrast, more significant cell death in Surv-D53A–transfected cells was observed (Figure 4, Aa and Ba), suggesting ectopic overexpression of Surv-D53A promotes TRAIL- or doxorubicin-induced apoptosis in HeLa cells. This conclusion was further supported by the evidence from Western blot analysis shown in Figure 4, Ab and Bb; the accelerated cleavage of PARP, a direct downstream substrate for effector caspases, was found in Surv-D53A–transfected cells. To confirm whether the cell death promoted by Surv-D53A is statistically significant, we performed a Student's *t* test, and the results showed $p < 0.005$, indicating it is statistically significant.

RIP3 is a RIP-interacting protein that contains an N-terminal kinase domain and a C-terminal death domain. It was reported that RIP3 induces apoptosis and activates NF-kappa B in many cell types (Sun *et al.*, 1999). We cotransfected vector encoding RIP3 with vector pEGFP-C1, pEGFP-C1/Survivin, or pEGFP-C1/Surv-D53A separately into HeLa cells, as shown in Figure 4C; cotransfection of RIP3 with Survivin significantly reduced the cell death when compared with cotransfection of RIP3 with empty vector. However, cotransfection of RIP3 with Surv-

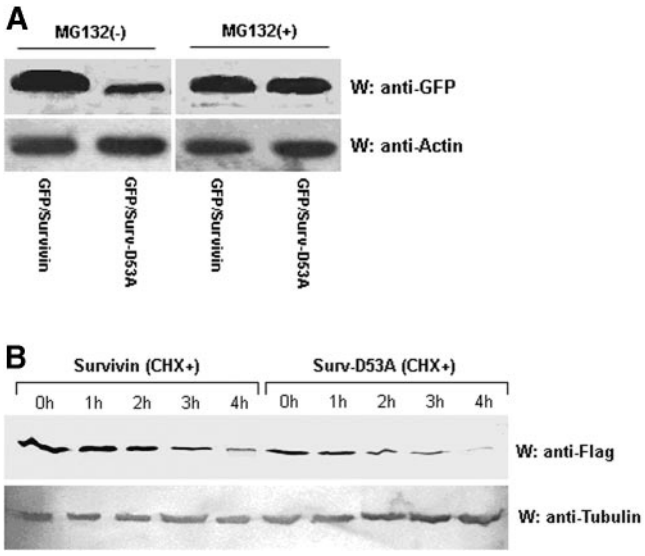

Figure 5. Surv-D53A is less stable than wild-type Survivin. (A) Twenty hours after transfection of Hela cells with pEGFP-C1/Survivin or pEGFP-C1/Surv-D53A, cells were incubated with or without ubiquitin proteasome inhibitor MG132 (20 ìM) for another 16 h. The steady-state levels of wild-type Survivin and mutant Survivin Surv-D53A were then compared by Western blot analysis. (B) Thirty-six hours after transfection of Hela cells with pCDNA3-Flag/Survivin or pCDNA3-Flag/Surv-D53A, cells were treated with CHX (20 μg/ml) for 0, 1, 2, 3, and 4 h before cell extracts were collected. The protein levels of Surv-D53A and Survivin were compared by Western blot analysis using anti-Flag antibody and endogenous tubulin was used as loading control.

D53A in turn resulted in an ~1.3-fold increase for cell death compared with cotransfection of RIP3 with empty vector. On the basis of these findings, we conclude that Surv-D53A sensitizes HeLa cells to TRAIL-, doxorubicin-, and RIP3-induced apoptosis.

Surv-D53A Is Degraded More Rapidly than Survivin

Survivin is mainly expressed during the G2/M phase in cell cycle, followed by rapid decline of both its mRNA and protein at G1 phase (Li *et al.*, 1998). It was reported that Survivin degradation is regulated by the ubiquitin proteasome in a cell cycle–dependent manner (Zhao *et al.*, 2000). Because Surv-D53A has apparently lost the antiapoptotic functions (Figure 4), we then questioned whether mutational change from Asp53 to Ala could accelerate the degradation of Surv-D53A. Overexpression of Survivin under the CMV promoter does not block Survivin degradation (Zhao *et al.*, 2000); we therefore chose pEGFP-C1 vector to express both wild-type Survivin and mutant Surv-D53A by regulation of CMV promoter in HeLa cells. The protein stability between Survivin and Surv-D53A was compared by immunoblotting shown in Figure 5A. The steady-state level of Surv-D53A in transfected cells was much lower than that of wild-type Survivin. Nevertheless, after treatment with ubiquitin proteasome inhibitor MG132 (20 ìM) after transfection, the level of Surv-D53A became comparable to that of wild-type Survivin (Figure 5A). These results suggest that Surv-D53A is less stable than Survivin in HeLa cells. We further performed a cycloheximide (CHX) block experiment to examine whether the degradation of Surv-D53A is more rapid than that of Survivin in the absence of de novo protein synthesis. HeLa cells were transfected with vector pCDNA3-Flag/Survivin or pCDNA3-Flag/Surv-D53A; 36 h after transfection, cells were treated with CHX (20 μg/ml) for the indicated periods before cell extracts were collected and analyzed. As shown in Figure 5B, the level of protein Surv-D53A dropped more rapidly than that of Survivin, and Surv-D53A was hardly detected after 4 h of treatment with CHX. These results demonstrated that Surv-D53A has a shorter half-life ($t_{1/2}$) than Survivin, and its quicker turnover rate may partially help to explain why Surv-D53A has disrupted its antiapoptotic function (see DISCUSSION).

*Surv-D53A Interacts with Either Survivin or Itself To Form Survivin*Surv-D53A Heterodimer or Mutant Homodimer Surv-D53A*Surv-D53A*

It has been demonstrated that the structure of Survivin forms a very unusual bow tie-shaped dimer and reveals an extensive dimerization interface along a hydrophobic surface on the BIR domain of each Survivin monomer (Chantalat *et al.*, 2000; Verdecia *et al.*, 2000). To test whether Surv-D53A monomer could also interact with Survivin monomer to form a heterodimer, we transfected both pEGFP-C1/Survivin and pEGFP-C1/Surv-D53A into HeLa cells; 36 h after transfection; cell lysates were pulled down by GST or GST/Survivin using the in vitro binding assay system, and the eluted proteins were further analyzed by Western blot probing with anti-GFP antibody. As shown in Figure 6A, both Survivin and Surv-D53A could be pulled down by GST/Survivin but not by GST alone, indicating that Survivin was not only able to bind to Survivin to form homodimer Survivin*Survivin, but also could bind to Surv-D53A to form the heterodimer Survivin*Surv-D53A complex.

To further confirm these physical interactions in vivo, HeLa cells were cotransfected with pCDNA3-Flag/Survivin plus pEGFP-C1or pEGFP-C1/Survivin or pEGFP-C1/Surv-D53A separately; 36 h later cellular extracts were prepared and coimmunoprecipitation was conducted. Figure 6Ba showed that Flag/Survivin could immunoprecipitate GFP/Survivin and GFP/Surv-D53A, but not GFP itself, demonstrating that Flag/Survivin does physically interact with Survivin or Surv-D53A in mammalian cells. Finally, to further examine whether Surv-D53A monomer was able to interact with itself to form mutant homodimer, we cotransfected pCDNA3-Flag/Surv-D53A plus either pEGFP-C1 or pEGFP-C1/Surv-D53A into HeLa cells, and coimmunoprecipitation was again performed to detect the interactions. As shown in Figure 6Bb, Flag/Surv-D53A was able to bind to GFP/Surv-D53A but not to GFP per se, demonstrating that Surv-D53A*Surv-D53A homodimer could be formed in transfected cells. Combined, these results showed that monomer Surv-D53A could interact with either Survivin to form the Survivin*Surv-D53A heterodimer or with itself to form the mutant homodimer Surv-D53A*Surv-D53A.

We also compared the relative level of overexpressed GFP-tagged Surv-D53A to that of endogenous Survivin by densitometric measurement using Eagle Eye Jr. Still Video System (Stratagene, La Jolla, CA) by an EagleSight software. The results in Figure 6C showed there is about eightfold more GFP/Surv-D53A than endogenous Survivin, indicating that in transfected cells, virtually all dimers containing endogenous protein would be with GFP partners.

*Survivin*Survivin, But Not Surv-D53A*Survivin or Surv-D53A*Surv-D53A, Is Able To Interact with Smac/DIABLO*

It has been reported that Smac/DIABLO promotes apoptosis through the interactions with IAPs, such as XIAP (Du *et al.*, 2000; Verhagen *et al.*, 2000). However, whether Survivin

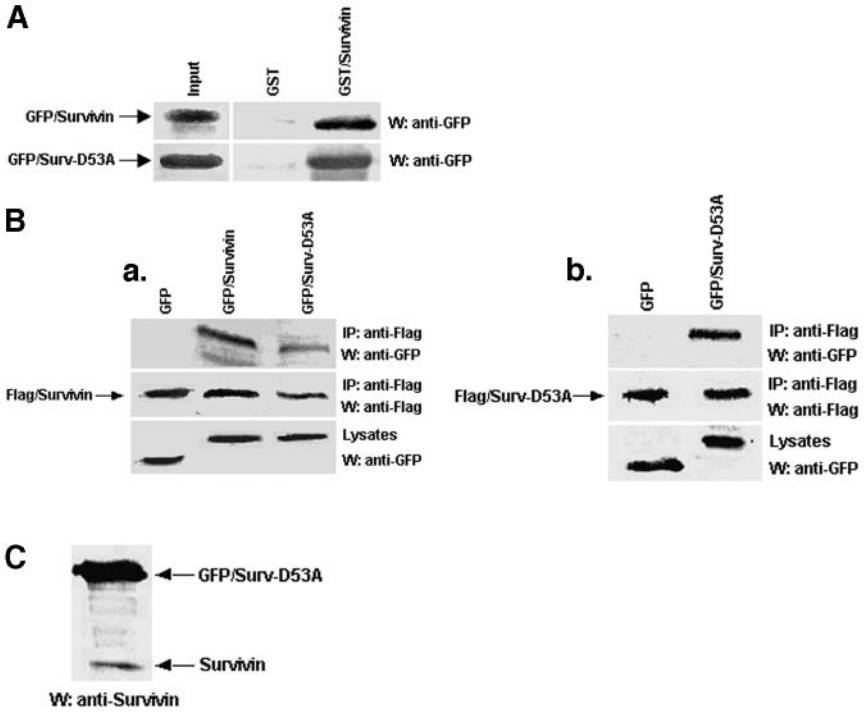

Figure 6. Surv-D53A interacts with Survivin to form Survivin*Surv-D53A heterodimer. (A) Plasmid pEGFP-C1/Survivin or pEGFP-C1/Surv-D53A was transfected into HeLa cells; 36 h after transfection cell lysates extracts were prepared and mixed with GST- or GST/Survivin-coupled beads for GST pull-down assay. The eluted proteins were analyzed by Western blot method using anti-GFP antibody. (B) a, Lysates from the transiently cotransfected HeLa cells expressing Flag/Survivin and GFP, Flag/Survivin and GFP/Survivin, or Flag/Survivin and GFP/Surv-D53A were immunoprecipitated (IP) with anti-Flag antibody and detected for the interactions by Western blot (WB) with anti-GFP antibody. b, HeLa cells were cotransfected with pCDNA3-Flag/Surv-D53A plus pEGFP-C1 or pEGFP-C1/Surv-D53A; 36 h later cell lysates were immunoprecipitated by anti-Flag antibody, and final products were detected by Western blot using anti-GFP antibody. (C) The HeLa cells transfected with pEGFP-C1/Surv-D53A were lysed, and the lysates were subjected to Western blot experiment with anti-Survivin antibody.

homodimer is able to interact with Smac/DIABLO has not yet been characterized. To investigate this issue, we transfected HeLa cells with pEGFP-C1/Survivin, and the cells were then treated with 100 nM Taxol to induce the release of active mature Smac/DIABLO (the first 55 amino acid residues of full-length Smac/DIABLO is cleaved upon activation) from mitochondria to cytosol. Thirty-six hours later, cells were lysed for coimmunoprecipitation using anti-Smac antibody, and the final precipitated products were detected by Western blotting using anti-GFP or anti-Survivin antibodies. As shown in Figure 7Aa, anti-Smac antibody could precipitate GFP/Survivin and endogenous Survivin but not GFP, indicating that Smac/DIABLO was able to bind to Survivin homodimer. Because Surv-D53A has lost the anti-apoptotic activity as was described above (Figure 4), we then asked whether Surv-D53A*Surv-D53A homodimer binds to Smac/DIABLO. The result from the immunoprecipitation experiment demonstrated that anti-Smac was able to precipitate the endogenous Survivin (faint bands seen in Figure 7Aa, panel 2) but not GFP/Surv-D53A (Figure 7Aa), indi-

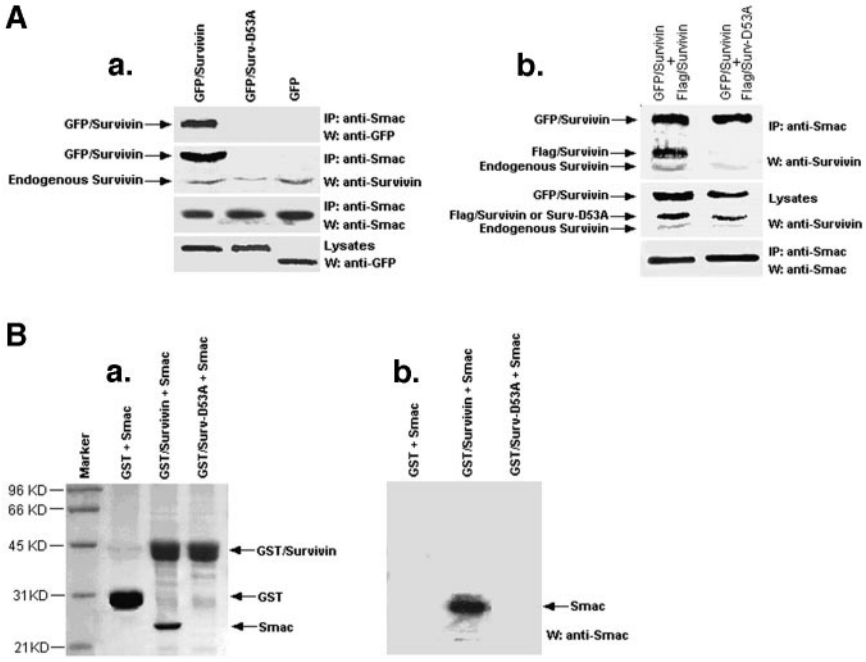

Figure 7. Surv-D53A fails to interact with Smac/DIABLO both in vivo and in vitro. (A) a, Lysates were prepared from GFP-, GFP/Survivin-, or GFP/Surv-D53A-transfected HeLa cells harvested after a 48-h incubation with Taxol to ensure the release of mature Smac/DIABLO from mitochondria to cytosol. Endogenous mature Smac/DIABLO were immunoprecipitated (IP) from the lysates by anti-Smac/DIABLO antibody and examined for the interaction with GFP, GFP/Survivin, or GFP/Surv-D53A by Western blot (W) using anti-GFP or anti-Survivin antibody. b, HeLa cells were cotransfected with pEGFP-C1/Survivin plus pCDNA3-Flag/Survivin or pCDNA3-Flag/Surv-D53A; immunoprecipitation (IP) by anti-Smac was then conducted, and the final pellets were subjected to Western blot using anti-Survivin antibody. (B) a, The interactions between mature Smac/DIABLO and GST/Survivin or GST/Surv-D53A was examined by GST pull-down assay; GST alone was used as a mock control. b, The eluted 23-kDa protein was further verified to be Smac/DIABLO using Western blot with anti-Smac antibody.

cating Surv-D53A*Surv-D53A is by no means binding to Smac/DIABLO. It is worthy to point out that amount of endogenous Survivin precipitated by anti-Smac in the presence of GFP-Surv-D53A is about one third of the endogenous Survivin precipitated by anti-Smac in the presence of GFP/Survivin (Figure 7Aa, densitometry is not shown); this could be explained by the fact that in the presence of Surv-D53A, part of the endogenous Survivin was associated with Surv-D53A to form heterodimer Survivin*Surv-D53A, resulting in either the failure of endogenous Survivin to be precipitated by anti-Smac antibody or the destabilizing of endogenous Survivin.

Next, we asked if Survivin heterodimer Survivin*Surv-D53A is able to bind to Smac/DIABLO. HeLa cells were then cotransfected with pEGFP-C1/Survivin and pCDNA3-Flag/Surv-D53A. As shown in the right panel of Figure 7Ab, Smac/DIABLO only precipitated the GFP/Survivin but not Flag/Surv-D53A, indicating that Smac/DIABLO does not interact with GFP/Survivin*Flag/Surv-D53A heterodimer. This is because if GFP/Survivin*Flag/Surv-D53A could bind to Smac/DIABLO. We would expect that Smac/DIABLO should have precipitated both GFP/Survivin and Flag/Surv-D53A instead of precipitating GFP/Survivin only. In conclusion, we demonstrated that the dimer containing Surv-D53A would lose the ability to interact with proapoptotic factor Smac/DIABLO and further suggest that Asp53 is indispensable for interaction between Survivin and mature Smac/DIABLO. This conclusion was further confirmed by in vitro interaction assay. We successfully expressed and purified fusion proteins GST/Survivin, GST/Surv-D53A, and Smac/His6 from bacteria. The soluble mature Smac/His6 was mixed with GST, GST/Survivin, and GST/Surv-D53A and incubated at 4°C overnight, and the mixture was then used for interaction assay. As shown in Figure 7Ba, only GST/Survivin could bind specifically to the mature Smac/DIABLO, as evidenced by appearance of an eluted 23-kDa mature Smac/DIABLO band, whereas GST/Surv-D53A or GST alone (control) was unable to bind to Smac/DIABLO. To verify that this eluted protein with a molecular weight of 23 kDa is truly Smac/DIABLO, Western analysis using anti-Smac antibody was performed. The result shown in Figure 7Bb confirms that eluted 23-kDa protein shown on the Coomassie blue staining gel is a bona fide Smac/DIABLO. These data indisputably demonstrated that the mutation of Asp53 to Ala nullifies the ability of Survivin to interact with Smac/DIABLO. Additionally, we found that the level of endogenous Survivin was decreased in the presence of Surv-D53A compared with that in the presence of Survivin (Figure 7Ab), indicating that Survivin*Surv-D53A heterodimer was less stable.

Surv-D53A Is Unable To Restore the Prevention of Caspase Activation by XIAP

Smac/DIABLO is a mitochondria protein that is proteolytically processed and released into the cytosol along with cytochrome c and other proapoptotic factors upon apoptotic induction (Du *et al.,* 2000; Verhagen *et al.,* 2000). It was reported that Smac/DIABLO promotes apoptosis through binding to IAPs (Du *et al.,* 2000; Verhagen *et al.,* 2000). We have demonstrated that Survivin*Survivin, but not Survivin*Surv-D53A or Surv-D53A*Surv-D53A, is able to directly interact with Smac/DIABLO. The formation of Survivin*Smac/DIABLO complex may cause more release of XIAP from XIAP*Smac/DIABLO complex to interact with caspases and thus to block cell death. On the contrary, the failure of Surv-D53A to bind to Smac/DIABLO may have disrupted the capability of inhibition of caspase activation.

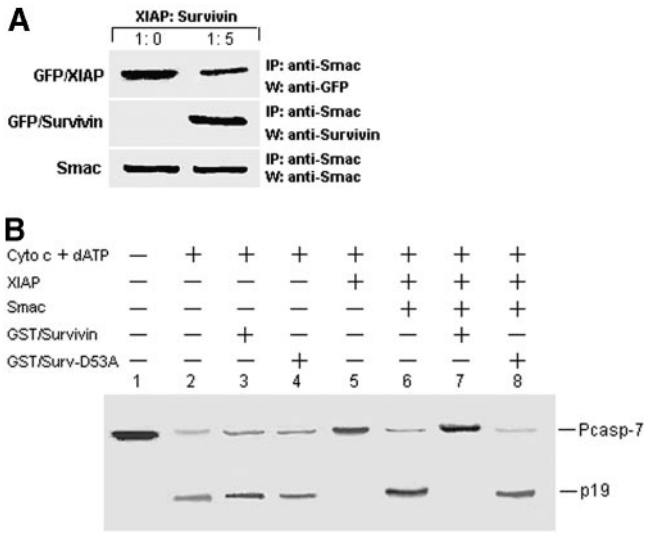

Figure 8. Surv-D53A is unable to inhibit caspase activation. (A) HeLa cells were cotransfected with vectors pEGFP/XIAP and pEGFP/Survivin in ratios of 1:0 and 1:5 followed by Taxol (100 nM) treatment. Equal volume of cell lysates extracted from cotransfected cells was immunoprecipitated by Smac/DIABLO mAb (5 μg) bound to protein A/G-Sepharose. The immunoprecipitates were analyzed by Western blot using anti-GFP (top), anti-Survivin (middle), and anti-Smac (bottom) antibodies. (B) The dATP/Cyto c–dependent caspase-7 processing in cytosolic extracts from mock or stable XIAP-expressed HeLa cells. The purified proteins His-tag/Smac, GST/Survivin, and GST/Surv-D53A were added to cytosolic extracts in different combinations indicated above the Western blot. The concentrations of various purified proteins and compounds used in cell-free system assay were the following: Cyto c (1 μg/ml), dATP (1 mM), and Smac/DABILO (100 nM); GST/Survivin and GST/Surv-D53A were both 300 nM. +, for presence; −, absence.

To confirm this hypothesis, we cotransfected HeLa cells with vectors pEGFP/XIAP and pEGFP/Survivin in different ratios (1:0 and 1:5) followed by Taxol induction in order to release of Smac/DIABLO from the mitochondria to cytosol. Equal volumes of cell lysates extracted from cotransfected cells were used for immunoprecipitation. As shown in Figure 8A, both XIAP and Survivin were able to interact with Smac/DIABLO in vivo. Moreover, with increasing amount of transfection of pEGFP/Survivin, a decreasing amount of XIAP was precipitated from the same sample. Quantitative measurement of anti-Smac precipitated XIAP in the absence of Survivin was at least twofold higher than that in the presence of Survivin (densitometry data not shown). This result confirms that Survivin and XIAP compete with each other to interact with Smac/DIABLO.

The mechanism of antiapoptotic function of Survivin has remained controversial (Tamm *et al.,* 1998; Banks *et al.,* 2000; Verdecia *et al.,* 2000). To further investigate whether the loss of antiapoptotic activity of Surv-D53A is due to its failure of interaction with Smac/DIABLO, we then established a cell-free system to test this hypothesis. We purified mature Smac/DIABLO, GST/Survivin, and GST/Surv-D53A from *E. coli,* and all these purified proteins were used for the cell-free assay system. The effect of Smac/DIABLO, GST/Survivin, or GST/Surv-D53A on caspase activation was evaluated by studying the processing of caspase-7 in cytosolic extracts from empty vector transfected- or XIAP-overexpressed-HeLa cells. As shown in Figure 8B, when Cyto c and dATP were added into cell extracts, the cleaved band (19 kDa) of caspase-7 was detected (Figure 8B, lane 2), which

explains that the cell free system is working well because the activation of caspase-7 could be induced by Cyto c in the presence of dATP. Overexpression of XIAP strongly prevents the appearance of the p19 form of caspase-7, indicating XIAP had blocked the activation of caspase-7 (Figure 8B, lane 5). Addition of GST/Survivin or GST/Surv-D53A alone into mock cytosolic extracts (no XIAP transfected) was unable to block the appearance of the p19 form of caspase-7 (Figure 8B, lanes 3 and 4), suggesting that Survivin or Surv-D53A by itself is unable to inhibit caspase-7.

To exclude the possibility that bacterially expressed Survivin fusion proteins may compromise their inhibitory functions, we used cytosolic extracts instead from cells in which Survivin or Surv-D53A was overexpressed and obtained similar results (unpublished data), indicating that failure of blocking caspase-7 cleavage by Survivin or Surv-D53A alone is not due to its expression from the *E. coli*. In addition, adding Smac/DIABLO protein into the cell free mixture containing XIAP overexpressed- cytosolic extracts abrogated the prevention of caspase activation by XIAP (Figure 8B, lane 6) and resulted in appearance of p19 form, confirming that Smac/DIABLO is able to stimulate caspase-7 activation by removing the inhibition of XIAP. If the GST/Survivin was added to the mixture containing both XIAP and Smac/DIABLO, p19 form will not be generated (Figure 8B, lane 7). In contrast, when the Survivin mutant GST/Surv-D53A was added, the p19 form was detected (Figure 8B, lane 8). From these results we have reached at least two conclusions. First, Survivin is capable of blocking caspase-7 activation in the presence of Smac/DIABLO and XIAP. Second, Survivin mutant Surv-D53A that is unable to bind to Smac/DIABLO fails to inhibit activation of caspase-7 even in the presence of XIAP and Smac/DIABLO. Combined, Survivin was able to rescue inhibition of XIAP through its binding to Smac/DIABLO (Figure 8B), thus to free XIAP from XIAP*Smac complex. In contrast, Survivin mutant Surv-D53A was unable to rescue the inhibitory effect of XIAP, because it was unable to bind to Smac/DIABLO (Figure 7A).

DISCUSSION

Survivin has attracted increasing attention because of its important role implicated in cancer diagnosis and cancer treatment. In this study, we have developed a novel Survivin mutant Surv-D53A, which was shown to spontaneously induce apoptosis in a p53-independent manner. Unlike wild-type Survivin, Surv-D53A fails to colocalize with Aurora-B on the chromosomes in metaphase. In addition to inducing spontaneous apoptosis, when ecotopicly expressed, Surv-D53A sensitizes HeLa cells to TRAIL-, doxorubicin-, or RIP3-induced apoptosis. Surv-D53A is found to be less stable within the cells than wild-type Survivin, and this instability can be reversed by adding ubiquitin proteasome inhibitor. Additionally, Surv-D53A can interact with wt-Survivin to form Survivin*Surv-D53A heterodimer or with itself to form mutant homodimer Surv-D53A*Surv-D53A. Finally, any form of Survivin dimer, as long as it contains Surv-D53A, is unable to bind to Smac/DIABLO, thus abolishing the capability of Survivin to restore the prevention of caspase activation by XIAP.

There is compelling evidence that defects in apoptosis contribute to many types of human cancers. Survivin has been implicated in the control of cell proliferation and regulation of cell lifespan. Survivin has been used as an excellent target for cancer therapy because it is selectively expressed in tumor cells and is required for their viability (Ambrosini *et al.*, 1997). Survivin Asp53 is critical for making up an acidic surface in its three-dimensional structure (Verdecia *et al.*, 2000) and is essential for its antiapoptotic activity (Figure 4). We demonstrated that a single amino acid change D53A completely abrogates Survivin's antiapoptotic function. We believe our Surv-D53A mutant is of particular importance, because enhanced apoptosis may be achieved either by treatment with Surv-D53A alone or by combined treatment with Surv-D53A and therapeutic agents such as TRAIL, doxorubicin, or RIP3 at relatively low concentration. We believe that Surv-D53A could potentially be targeted for preclinical cancer therapy. Conditional expression or adenoviral delivery of Surv-D53A is expected to initiate massive cell death with or without some chemotherapeutic drugs, depending on different tumor cell types.

It was reported that wild-type p53 strongly represses Survivin expression at both mRNA and protein level (Mirza *et al.*, 2002). Data from transient transfection experiments revealed that the expression of wild-type p53, but not of mutant p53, was associated with this repression of the Survivin promoter in various cancer cell types (Mirza *et al.*, 2002). In this study, we demonstrated that Surv-D53A induces apoptosis both in A549 (p53+) and H1299 (p53−) cell lines, indicating wild-type p53 function is not required for proapoptotic activity of mutant Surv-D53A. This p53-independent proapoptotic mutant may have broader applications in cancer gene therapy, because it could induce apoptosis in more different tumor cell types, of which majority are p53 deficient.

During metaphase, Survivin is reported to colocalize with Aurora-B kinase, which is involved in execution of cytokinesis during the later-stage of mitosis (Goto *et al.*, 2003). However, we demonstrated that the dominant negative mutant Surv-D53A fails to colocalize with Aurora-B in metaphase (Figure 3B). Nevertheless, similar to wt-Survivin, Surv-D53A was also found to locate at midbody before the cells were ready for the mitotic exit in telophase. This data clearly suggest that, unlike Surv-T34A, Surv-D53A does not appear to block the cell division during mitosis. Temme *et al.* (2003) reported a phosphorylation-defective Survivin mutant Surv-T34A that causes aberrant execution of cytokinesis. They demonstrated that mutation change of amino acid at position 34 from threonine to alanine eliminates the phosphorylation of Survivin by $p34^{cdc2}$ and is responsible for this defective mitotic exit. The Thr34 phosphorylation site in our mutant Surv-D53A is intact and this may partially explain why Surv-D53A does not affect the cytokinesis. Additional evidence for this conclusion comes from our FACS analysis of Surv-D53A–induced-apoptosis with PI (propidium iodide), in which no peak for G2/M phase arrest was shown (unpublished data).

Although Survivin mutants or Survivin antisense oligonucleotides show proapoptotic activity (O'Connor *et al.*, 2000; Olie *et al.*, 2000), the mechanism by which this occurs has remained largely unknown. In the present study, we have provided some useful clues for Surv-D53A's proapoptotic function. The functional Survivin is known to be a homodimer. Our experiments showed that Surv-D53A was less stable and had accelerated turnover rate by means of ubiquitin-dependent proteasome destruction (Figure 5).

Smac/DIABLO is an important apoptotic factor, which eliminates caspase-inhibitory activity of XIAP during apoptosis. In this report, we have shown that dimers containing Surv-D53A fail to interact with Smac/DIABLO and thus were unable to antagonize Smac/DIABLO, which promotes caspase activation through neutralizing XIAP in the cell free system (Figure 8B). This opposing function displayed by Surv-D53A could be explained the amino acid substitution

Table 1. Characteristics of Surv-D53A

	Dimer formation	Interact with Smac	Stability	Inhibit apoptosis
Survivin*Survivin	Yes	Yes	Stable	Yes
Survivin*Surv-D53A	Yes	No	Less stable	No
Surv-D53A*Surv-D53A	Yes	No	Less stable	No

(Asp to Ala) at position 53 in Survivin, which abrogates its binding capability to Smac/DIABLO, enhancing the chance for XIAP to complex with free Smac/DIABLO to activate the caspases and disrupt the antiapoptotic effect of Survivin.

Survivin mutant that disrupts dimer formation is reported to lose wt-Survivin's activity, suggesting that monomer Survivin does not have biological functions (Muchmore *et al.*, 2000). We report here that Surv-D53A is able to interact with wt-Survivin to form Survivin*Surv-D53A heterodimer and with itself to form mutant homodimer Surv-D53A*Surv-D53A. We have demonstrated that neither heterodimer Survivn*Surv-D53A nor mutant homodimer Surv-D53A*Surv-D53A is able to interact with Smac/DIABLO. Therefore it is reasonable to speculate that the level of endogenous Survivin homodimer in Surv-D53A-transfected cells was reduced, because part of endogenous Survivin has become to interact with Surv-D53A to form heterodimer and the antiapoptotic activity of endogenous Survivin was thus reduced. This may explain why the Surv-D53A is capable of inducing apoptosis. In conclusion, compared with wild-type Survivin, our novel Surv-D53A displays several distinctive properties, which are summarized in Table 1.

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