N-Terminal Deletion Effects of Human Survivin on Dimerization and Binding to Smac/DIABLO in Vitro

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Received: April 23, 2010; Revised Manuscript Received: August 31, 2010

Survivin, as an apoptosis suppressor, exists as a homodimer interfacing at the N-terminal portion (residues 6−13) of its baculovirus IAP repeat (BIR) domain and a linker segment (residues 89−102). Here we expressed full-length human Survivin (SurF) and a series of its mutants, SurΔN7, SurΔN13, and SurΔN18 with significant truncations of the N-terminus, all of which could still dimerize in solution. Single-molecule force spectroscopy (SMFS) was used to quantitate the unbinding forces of full-length and the mutant homodimers and revealed that the N-terminal residues up to Arg18 were not essential for dimerization. Meanwhile, the binding of SurΔN7 to Smac/DIABLO determined by ELISA was as efficient as the wild-type, but that of SurΔN13 was significantly reduced, and that of SurΔN18 was completely lost. Together, these findings provide direct evidence that the N-terminal sequence of Survivin is not critical for dimer formation but may contribute to correct folding and function of BIR.

Introduction

Survivin is selectively expressed in the G2/M phase of the cell cycle and overexpressed in most human neoplasms, embryos, and malignant tissues but not in normal tissues.[1] It is the smallest member of the inhibitor of apoptosis protein (IAP) family and has dual functions in suppressing apoptosis and playing a central role in cell division.[2] Detection of Survivin has prognostic value for some tumor cells and appears to be involved in their resistance to anticancer agents and ionizing radiation. Thus, all these properties render this protein an attractive target for cancer therapy.[3,4]

The Survivin monomer is a 16.5 kDa protein of 142 amino acids. Structurally, it consists of an N-terminal single globular baculovirus IAP repeat (BIR) domain (M1-S88), a linker segment (V89-T97), and a long amphipathic C-terminal coiled-coil α-helix (L98-D142).[5] The Survivin BIR domain contains a zinc-binding fold similar to that found in the X-linked IAP (XIAP) BIR2 and BIR3 domains,[6] including three Cys and a His that bind a zinc, but it lacks the RING finger motif found in XIAP. The secondary structure of the Survivin BIR domain consists of a three-stranded β-sheet and four α-helices. Both X-ray crystal diffraction and NMR spectroscopy of Survivin in solution have confirmed that it exists in two forms which confer different functions in vivo. Survivin participates in cell division as a monomer,[7] associating with Borealin and inner centromere protein (INCENP) to form a complex called the chromosomal passenger complex (CPC) which interacts with Aurora-B kinase as a key regulator of chromosome segregation and cytokinesis during the cell cycle.[8] On the other hand, the bow-tie-shaped homodimer form of Survivin has antiapoptotic functions.[5,9] The dimer interface is extensive, involving residues 6−13 in the N-terminal portion and a 14-amino acid region encompassing residues 89−102 located just in and after the linker segment.[10] Hydrophobic contacts mainly dominate the interaction surface with Leu98 protruding from one monomer and extending into a hydrophobic pocket formed by Leu6, Trp10, Phe93, Phe101, and Leu102 in the other monomer. These contacts have been shown to be important for dimer formation and thus also for protein stability.[9,10]

IAP proteins play an important role in apoptosis as endogenous inhibitors of caspases.[11] This inhibition can be relieved by a mitochondrial protein, Smac/DIABLO, which directly binds to IAPs and suppresses their function.[12] Smac/DIABLO is the second mitochondrial protein, along with cytochrome c, released into the cytosol when cells undergo apoptosis.[13] Unlike other IAPs, Survivin does not bind caspases directly,[14,15] but it has been suggested to exert antiapoptotic effects by physically binding to Smac/DIABLO and neutralizing its effect on other IAPs.[16] NMR analysis has indicated that Smac/DIABLO binds across the third β-strand of Survivin in solution.[10]

Until now, most of the reconstructed Survivin proteins have contained point mutations[5,16−23] or C-terminal sequence truncations.[5,10,16] However, the potential significance of N-terminal sequences both to the dimerization of the molecule and the antiapoptosis function cannot be neglected. For example, residues 6−13 in the N-terminal portion were implicated in dimerization.[10] In particular, Arg18 of Survivin is a key residue with a buried side chain that stabilizes the N-terminal part of the BIR domain.[24] The NMR spectroscopic analysis of the protein in solution clarified the structure of Survivin (residues 6−117) with only a short N-terminal deletion,[10] but these studies were unable to unambiguously resolve the contribution of longer N-terminal residues to the dimerization of Survivin. Therefore, in this study we performed a more extensive evaluation of N-terminal deletions of Survivin by constructing plasmids to express full-length human Survivin (SurF) and a series of its

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10.1021/jp1036603
Published on Web 11/09/2010

mutants, SurΔN7 (7 N-terminal residue truncation), SurΔN13 (13 N-terminal residue truncation) and SurΔN18 (18 N-terminal residue truncation). The four proteins were expressed in *Escherichia coli* and purified using affinity chromatography. After ensuring they could all fold properly and dimerize in solution, we evaluated the dimeric unbinding forces of the various Survivin mutants by using single-molecule force spectroscopy (SMFS), which quantitatively (directly) measured the effect of N-terminal sequences on the strength of the dimerization. In addition, we also investigated the binding ability between Survivin or the mutants to Smac/DIABLO in vitro using a sandwich ELISA. Our findings unambiguously defined the contributions of the N-terminal sequences to the stability of dimerization and function of Survivin in vitro.

Materials and Methods

Construction of Expression Plasmids for Bacterial Survivin Mutants. The *E. coli* codon-optimized gene for full-length human Survivin (SurF) fused with a C-terminal His-6 tag and a Cys was amplified by PCR and subcloned into the T7 expression vector pRSETB (amp^R; Invitrogen, Carlsbad, CA) at the *Nde*I and *Hind*III (Takara Bio Inc., Otsu, JP) cloning sites. Primer pairs with the respective restriction sites were P5′-F, 5′-AATTACATATGGGTGCTCCGACTC-3′ and P3′, 5′-CACGCAAGCTTAGCAATGATGATGATGATGGT-3′. The plasmids expressing the N-terminal truncated mutants, SurΔN7, SurΔN13, and SurΔN18 were generated using the vector containing SurF as a template and the following upstream primers: P5′-8, 5′-ATCTTCATATGCCAGCGTGGCAGCC-3′, P5′-14, 5′-GACACCATATGCTGAAAGACCACCGTATCT-3′, and P5′-19, 5′-CCGACCATATGATCTCTACCTTCAAAAACTGG-3′, respectively, which added a Met at the beginning of each protein. The downstream primer for SurΔN7 was P3′-8, 5′-CACGCAAGCTTAATGATGATGATGATGGT-3′, which did not add a Cys after the His-6 tag especially. The DNA sequences of all inserts were fully verified by sequencing.

Protein Expression and Purification. SurF and the N-terminal deletion mutants were purified from transformed *E. coli* BL21 cells (Novagen, Germany) grown in LB broth at 37 °C under ampicillin selection until the culture density reached $A_{600} = 0.8$. Expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cultures were shaken overnight at 30 °C. Harvested SurF-expressing cells were lysed in 20 mL of buffer A (50 mM Tris-HCl, 300 mM NaCl, pH 8.0) with 20 mM imidazole and 40 μL of 100 μg/mL PMSF (dissolved in dimethylcarbinol) per liter of culture and then sonicated for 30 min. Bacterial lysates cleared by centrifugation were loaded onto a nickel-affinity column (0.5 mL of resin/liter of culture; GE Healthcare Biosciences, PA) at 4 °C for 1 h. The column was then washed with 20 column volumes of buffer A including 100 mM imidazole or 200 mM imidazole, and the soluble SurF proteins were eluted with 10 column volumes of buffer A including 300 mM imidazole.

By contrast, we harvested the cells expressing mutant proteins by lysing in 20 mL of lysis buffer (50 mM Tris-HCl, 300 mM NaCl and 5% Tween-20, pH 8.0) per liter of culture, sonicating for 40 min, and then collecting the inclusion bodies by centrifugation since there was no soluble protein expression. The inclusion bodies were resuspended in 20 mL of buffer B (50 mM Tris-HCl, 300 mM NaCl and 8 M Urea), pH 8.0 per liter of culture and then subjected to sonication for another 30 min. After clearing by centrifugation, the inclusion body extracts were loaded onto a nickel-affinity column at room temperature for 1 h. The column was washed with 20 column volumes of buffer B at pH 7.8, 6.3, and 5.4, sequentially, and the proteins were eluted with 10 column volumes of buffer B at pH 4.0. The purified proteins were detected by Coomassie Brilliant Blue staining (data not shown) and confirmed by Western blotting (Figure 1). The inclusion mutant proteins were renatured by dialysis.

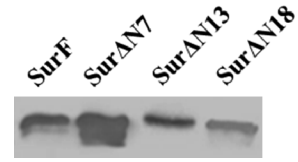

Figure 1. Western blotting analysis of SurF and the N-terminal deletion mutants using a mouse primary monoclonal antibody raised against full-length human Survivin (sc-47750, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by an affinipure goat anti-mouse IgG (H+L) secondary antibody conjugated to alkaline phosphatase (Proteintech Group, Inc., Chicago, IL).

Circular Dichroism (CD) Spectroscopy and Size-Exclusion Chromatography. Before use, the conformational integrity of the purified proteins was confirmed by CD spectrum to ensure their natural structures as expected. Far-UV CD spectra were obtained on a Jasco J-810 spectrophotometer (Tokyo, Japan) at 25 °C using a 0.5 cm path length. The solutions containing SurF and the mutants were prepared with a concentration of 5 μM in buffer C [20 mM phosphate buffer solution (PBS) containing 100 mM NaCl and 50 μM Zn^{2+}, pH 8.0].

The dimerization state of the purified SurF and the extensively dialyzed mutants were determined by size-exclusion chromatography on a Superdex S-75 column (10 × 300 mm, GE Healthcare Biosciences, PA) at 4 °C, using 200 μL of 0.5 mg/mL each protein in buffer C containing 5 mM β-mercaptoethanol (β-ME) at a flow rate of 1.0 mL/min.[25,26] The column was pre-equilibrated also with the buffer C containing 5 mM β-ME. Two proteins, BSA (bovine serum albumin, 67 kDa, Takara Bio Inc., Otsu, JP) and Ad5-knob (human adenovirus type 5 knob protein, 22 kDa, recombinant protein purified from *E. coli*), were chosen as standards.

Single-Molecule Force Spectroscopy. In our experiments, force curves were acquired on a NanoWizard II BioAFM (JPK instrument AG, Berlin, Germany) in contact mode at room temperature. The gold surface and gold-coated Si_3N_4 atomic force microscope (AFM) tip (Olympus, Japan) were both cleaned using freshly prepared piranha solution (H_2O_2/H_2SO_4, 3:7) for 7 min. After washing thoroughly in ddH_2O, the gold surface and tip were dried by a flow of nitrogen. After purification, the SurF, SurΔN13, and SurΔN18 proteins were allowed to adsorb from their inclusion solution [10 μg/mL in buffer D (20 mM PBS, 300 mM NaCl and 8 M Urea, at pH 7.8)] through S−Au linkage between one monomeric C-terminal Cys and the gold-coated surface or AFM tip. The surface characterization of monolayer-like manner was confirmed through QCM-D experiment. Since SurΔN7 construct was used to develop an anticancer vaccine and not cloned with a Cys on the C-terminus, it could not be adsorbed on the gold-surface. After renaturation with gradient Urea/PBS buffer (20 mM PBS, 300 mM NaCl, 50 μM Zn^{2+}, and 6 M, 4 M, 2 M, and 0 M Urea, sequentially, at pH 7.8) on the gold-coated surface and AFM tip directly, the sample was attached to the AFM sample stage for the force measurement. The protein modified AFM tip was brought into contact by 100 pN with the gold-coated surface covered with the corresponding protein and kept in contact for 0.5−1 s in order to allow the formation of the Survivin dimer. When the tip was retracted from the sample

surface, the protein dimer was separated and the corresponding rupture force was recorded. From these curves, we acquired the unbinding force between the two monomers of the dimer and also the contour length of that. These parameters characterized the dimerization interface interactions between homodimer Survivin proteins at the single-molecule level.

Each type of protein (SurF and the mutants) was retracted repeatedly for 600−700 times until about more than 500 rupture events were recorded. However, not all of the measured values were valid. For instance, two or more dimeric molecules may be drawn at one time that will result in an overly large force value, or the monomers may link at an erect direction through disulfide linkage between internal Cys before renaturation that will result also in an overly large length value. Of note is that after renaturation, the proteins on the gold surface located close to each other could possibly form dimers spontaneously. However, the probability of this occurring should be very low because the steric conformation of the monomers immobilized on the surface would make it very difficult. However, in a situation in which such an event could happen, when the tip is brought to the substrate surface, the monomer on it would not be able to form a dimer with the protein on the substrate, and consequently we would not be able to obtain a force measurement. Therefore, the proteins on the surface may spontaneously form dimers that may interfere the interaction with the protein on the AFM tip, but it would not impact the measured force data. As the control experiment, spontaneous dimerizations of purified SurF and the dialyzed mutants were adsorbed from their solution (10 μg/mL in buffer C) through one monomeric C-terminal Cys of the dimer onto the AFM tip and gold-coated surface, respectively. In this case, the procedure of renaturation with gradient Urea/PBS buffer on tip and surface was not needed. And the tip was brought to and retracted from the gold-surface to obtain force signal as above and each type of protein was retracted repeatedly for 600 times.

The spring constants of the employed AFM cantilevers were calibrated using the thermal noise method as described previously.[27−29] The measured values ranged from 6 to 10 pN/nm. Force curves were obtained at a constant stretching velocity of 0.5 μm/s.

ELISA for Binding Ability between Survivin and Smac/DIABLO. The binding ability between Survivin mutants and Smac/DIABLO in vitro were measured by a sandwich ELISA. Ninety-six-well plates coated overnight at 4 °C with 100 μL/well of a mouse monoclonal antibody raised against full-length human Survivin (100 ng/mL; sc-47750) in PBS were blocked with 2% BSA/PBS-T (20 mM PBS containing 0.05% Tween-20). Survivin and the mutants were then added in excess and incubated at 37 °C. The plates were incubated with serially diluted Smac/DIABLO (R&D Systems, Minneapolis, MN) and then with a 1/2000 dilution of rabbit polyclonal antibody (ab23450, Abcam Inc., MA) to Smac/DIABLO at 37 °C. The plates were then incubated with a 1/5000 dilution of a peroxidase-conjugated affinity-purified goat anti-rabbit secondary antibody (Proteintech Group, Inc., Chicago, IL). After every step the plates were washed three times with PBS-T and eventually developed with tetramethylbenzidine (TMB), stopped with 2 M H_2SO_4, and analyzed at double wavelengths 450−630 nm with an EL × 800 Universal Microplate reader (Bio-Tek Instruments, Winooski, VT).

Binding to Smac/DIABLO of each mutant at various concentrations was tested in three replicate wells for each experiment, and this assay was repeated three times as above. Statistical analyses were performed with the GraphPad Prism version 4.01 (GraphPad Software). Comparisons of mean responses among groups were performed by ANOVA with Bonferroni adjustments to account for multiple comparisons.[30,31] In all cases, values of $P < 0.05$ were considered significant. Furthermore, we chose a commercially available protein, BSA and an *E. coli* expressed protein Ad5-knob, as the negative controls to evaluate the background.

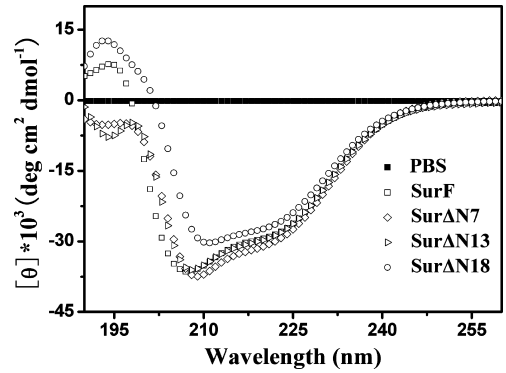

Figure 2. CD spectra of SurF and the mutants at 5 μM in buffer C, including 50 μM Zn^{2+} to stabilize the protein in solution.[19] SurF was soluble in the supernatant solution, and the mutants were renatured by dialyses after extraction from inclusion bodies.

Results and Discussion

Structure Determination and Analysis: Purified SurF and N-Terminal Deletion Mutants Adopt an α-Helical Conformation and Dimerize in Solution. ***CD Spectroscopy.*** Crystallographic studies of human Survivin confirmed that the monomer has a long amphipathic C-terminal coiled-coil α-helix (L98-D142),[5] and the BIR domain is composed of a three-stranded antiparallel β-sheet packed against four short α-helices and is stabilized globally by a Zn^{2+} tetrahedrally coordinated by Cys57, Cys60, His77, and Cys84.[6] Furthermore, the linker segment forms a short β-sheet.

Spontaneous folding of purified SurF and the dialyzed mutants were confirmed by far-UV CD spectra as shown in Figure 2. The CD spectrum of SurF exhibited double minima at 208 and 222 nm and a positive peak tendency at 195 nm, indicative predominantly of α-helical secondary structure and is consistent with the known structural features of the protein. In addition, the mean residue ellipticities of all mutants were similar to that of SurF purified from the supernatant solution, indicating that the mutants formed the natural secondary structure upon renaturation from the inclusion bodies. The quantitative analysis using the spectra data of the 222-nm ellipticity revealed that the helix content of the mutants varied slightly from 43.7% for SurF, to 45.8%, 43.6%, and 42.1% with N-terminal deletions of 7, 13, and 18 residues, respectively. The observed helix content was lower than that determined from the crystal structure of human Survivin.[5] However, other research suggested that the elongated C-terminal α-helix observed crystallographically that are involved directly in crystal packing contacts may not actually exist in solution.[15] In fact, the NMR solution structure of a truncated human Survivin (1−120) showed that the C-terminal helix did not fully extend to the end of the sequences.[10]

Size-Exclusion Chromatography. Survivin is known to dimerize both in crystal form and in solution. Dimerization of Survivin is mediated primarily by hydrophobic interactions between the two N-termini and linker peptides which are thought to be functionally important for association with two BIR domains. Both the crystal and NMR solution structural analyses

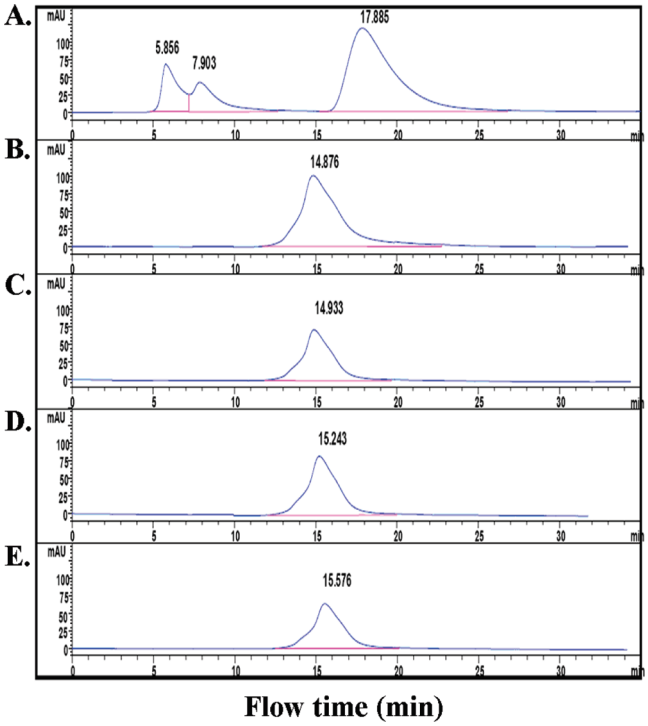

Figure 3. (A) Size-exclusion chromatography profiles for the protein marker: a mixture of BSA (65 kDa, 0.5 mg/mL) and Ad5-knob (22 kDa, 0.5 mg/mL). The BSA produced two peaks: one corresponding to the intact protein and the other to the degraded form. (B−E) Size-exclusion chromatography profiles from SurF to SurΔN18. Purified SurF and the mutants were passed through an Amersham Biosciences Superdex S-75 column. Each protein had a concentration of 0.5 mg/mL in buffer C containing 5 mM β-ME, and the measurements were performed at a flow rate of 1.0 mL/min. Every protein displayed slight differences in flow time as the molecular mass varied.

have suggested that the linker plays a more important key role in homodimerization.[5,9,10] To test the role of the N-terminal sequences in the homodimerization of Survivin, we investigated whether the three truncated human Survivins (SurΔN7, SurΔN13, and SurΔN18) formed dimers in solution through size-exclusion chromatography using a Superdex S-75 column.

The molecular weight of SurF and the mutant dimers ranged from 34.6 to 30.8 kDa. As shown in Figure 3A, we chose two proteins as markers. Ad5-knob, with a molecular weight of 22 kDa and flow time of 17.885 min, was the more important marker since it can be directly used to discriminate the dimer or monomer of Survivin mutants. If the Survivin mutant is a monomer, it should flow more slowly than Ad5-knob. Size-exclusion chromatography showed that in the presence of reducing agent, β-ME, SurF (Figure 3B), and all of the mutants (Figure 3C−E) were entirely dimeric, but the elution time was slightly different as the molecular mass varied. The role of the reducing agent was to prevent the C-terminal Cys from forming a disulfide linkage between the two monomeric Survivin mutants. The flow time of SurF was 14.876 min, which was slightly faster than that of Ad5-knob but slower than BSA. All these observations confirmed that SurF was a homodimer in solution. The flow time of SurΔN7 was 14.993 min. It was reasonable to observe the dimer formation for SurΔN7 in which all of the main residues participated in forming the essential hydrophobic pocket are retained with only the Leu6 missing. A similar result was observed for SurΔN13, although all of the N-terminal residues (6−13) previously suggested being required for dimerization had been removed. The flow time of SurΔN13 was 15.243 min, which demonstrated that the N-terminal dimerization residues were not necessary. Of note, deletion of the N-terminal sequence up to the conserved Arg18, residues determined to be important for stabilization of the BIR domain structure, resulted in a flow time of 15.576 min and confirmed that this protein could still dimerize in solution, although potentially without an intact BIR domain. In the NMR structure of the Survivin dimer,[10] residues 14−18 residues were shown not to participate in dimerization but influenced the BIR domain. Thus, it can be concluded that the N-terminal residues, at least up to Arg18, are not essential for homodimerization, although they may be very important in maintaining the BIR domain and function of Survivin.

Single-Molecule Force Spectroscopic Analysis for the Dimeric Unbinding Forces of Native Survivin and N-Terminal Deletion Mutants. The monomer−monomer interactions in the full-length human Survivin dimer or the mutant dimers were characterized at the single molecule level using AFM-based SMFS.[32] Covalent binding of the recombinant SurF or the mutants to the gold surface and AFM tip, respectively, was achieved by the cysteine tags through S−Au linkage at the C-terminus. The surface characterization of monolayer-like manner at gold-surface was performed by QCM-D experiment, which confirmed it is suitable for SMFS measurement (see the Supporting Information, Figure S1). The extension experiment is depicted schematically in Figure 4A. The protein dimer formed in between the AFM tip and the gold substrate when each were functionalized by protein monomers and brought into contact. Upon separation of the AFM tip and the substrate, the mechanical force applied to the protein dimer caused the sequential extension of the protein leading to an increase in the distance between the AFM tip and gold surface until the dimer structure was broken. During this process, the force versus *Z*-position (or height) signal was obtained, which could eventually be converted to a force−extension curve,[33] as shown in Figure 4B. By measuring the force and the extension length at the rupture point, the unbinding force and contour length of the protein dimer could be obtained.

A typical force versus extension curve is shown in Figure 4B.[34,35] The peak was confirmed to be specific to this dimer interaction, as it was absent when both or either the AFM gold surface and tip remained nonfunctionalized (data not shown). When the dimer was separated into monomers, the force peak curve immediately dropped to zero, as there was no longer a mechanical force between the AFM tip and the substrate. All of the data obtained from SMFS experiment were drew histograms to acquire the distribution of the rupture forces and contour lengths of the SurF and mutants dimers, but the distributions were irregular and they did not match well with the reported value (see the Supporting Information, Figures S2 and S3). Therefore, we screened for valid measurements based on the rule that the maximal unbinding force could not exceed the adhesion between the surface and tip, and the maximal dimeric molecular length of the C-terminal to N-terminal residues covalent binding could not exceed 150 nm. Approximately 200 force curves were screened out and analyzed to extract rupture force, *F*, and the contour length of dimers, *L*, for each case. The data obtained were subsequently pooled into histograms to obtain the distribution of the rupture forces and the contour lengths of corresponding dimers, as shown in Figure 4, panels C and D, respectively. In control, since the AFM tip and gold-surface were both covered with dimeric proteins, only the adhesion force signal could be obtained. As there was few force signals could be recorded between the dimers. After screened according to experimental rules, the rupture events

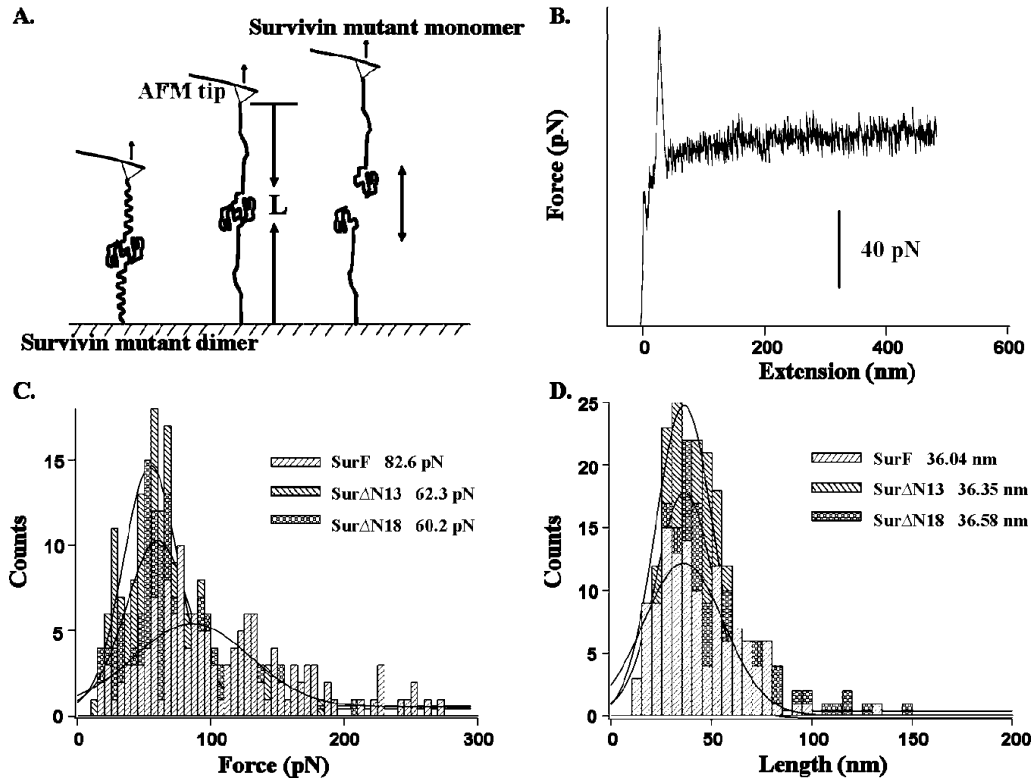

Figure 4. SMFS analysis of SurF and the N-terminal deletion mutants: (A) Schematic of the SMFS experimental setup; the arrow indicates the direction of the applied force; *L* is the contour length of dimers. (B) The characteristic force curve obtained by stretching the dimeric protein showed typical features which were consistent with their modular construction. (C) The distributions of the repeated measurements of rupture forces obtained for SurF and the mutant dimeric proteins. (D) The distributions of the repeated measurements of contour lengths obtained for SurF and the mutant dimeric proteins. The solid lines are Gaussian fits on the corresponding histograms.

acquired in control would be fewer which accounted for only about 0.5% of the total retraction and was obviously much lower than the corresponding experimental one (about 35% to ~40%). In addition, as the very low possibility of the rupture events, the contour length could not be obtained. Therefore, our results of force distribution curves just correspond to the specific dimer unbinding force.

From the histogram shown in Figure 4C, the most probable unbinding force was 82.6 pN for SurF, 62.3 pN for SurΔN13, and 60.2 pN for SurΔN18, respectively. According to both X-ray crystal diffraction and NMR spectroscopy in solution, the dimer complexes between the two monomer units of Survivin are stabilized by the interaction between the protein N-terminal and linker segments. Therefore, it is instructive to compare the values of the rupture forces for this system with the data obtained by a similar experimental technique for pulling linear systems such as α-synuclein dimer.[36] At the similar C-terminus connection and pulling methods, the rupture forces of dimeric α-synuclein were ~71.0, 58.3, and 43.1 pN at pH 2.7, 3.7, and 5.1, respectively. The force values of rupturing the α-synuclein dimer of linear molecular complexes together are the same as the unbinding forces distribution of SurF and the mutants, indicating the specific unbinding force of Survivin mutants dimer are reasonable. From these results shown in Figure 4C, N-terminal sequences obviously contributed to the strength of dimer stability as observed by the large difference in unbinding forces between SurF and two mutants. However, the mutant molecules still had considerable binding forces between the monomers. Therefore, we determined that the N-terminal residues were not requisite, while the linker segment contributed more to the dimer formation, consistent with previous studies.[5,9,10] Compared with SurΔN13, the slightly lower unbinding force of SurΔN18 was determined to be 60.2 pN, which was not significantly different. Hydrophobic contacts mainly dominate the interaction surface of the Survivin homodimer with Leu98 protruding from one monomer and extending into a hydrophobic pocket formed by Leu6, Trp10, Phe93, Phe101, and Leu102 in the other monomer molecule.[5] In addition, there are four hydrogen bonds between main monomer chain atoms (E94O···G99N, L96O···L98N, symmetrical interactions).[9] These contacts have been shown to be important for dimer formation. Therefore, the N-terminal deletions would affect the hydrophobic pocket due to removal of the Leu6 and Trp10 residues. Thus, deleting five more amino acids of SurΔN18 based on SurΔN13 did not further affect the dimerization of Survivin, although it significantly influenced the binding ability to Smac/DIABLO which will be discussed later.

To further validate the unbinding force measurements for the protein dimers, we used the length window of the dimers. These are the contour lengths of the SurF and mutant dimers which should be nearly the same since only the N-terminal sequence were remodeled in the mutants. Upon stretching of the dimer, the molecule strand became tightly oriented along the direction of external load until the dimer was separated. The secondary structure of the C-terminal long α-helix was first unwound;[37] the two consecutive planar units of peptide of the C-terminal α-helical structure in the symmetrical dimer may not be randomly flexible and adopt the maximal distance bond angle; and the distance of Cα−Cα in one planar unit of peptide was under an orientation state of trans-conformation (3.8 Å).[38,39] Therefore, we estimated that the contour length of the dimer was about 38.7 nm, which was highly consistent with that found in the SMFS experimental results (~36 nm) and indicated that our results were reasonable and reliable.

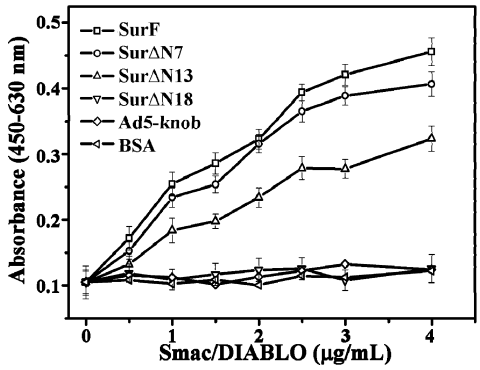

Figure 5. ELISA analysis of the binding ability between SurF or the mutants to Smac/DIABLO in vitro. The commercially obtained BSA protein and the recombinant Ad5-knob protein purified from *E. coli* were negative background controls. The binding ability of SurF or the mutants to Smac/DIABLO were assessed ($n = 3$/group), and the results showed that SurF and the mutants with deletion of 7 or 13 N-terminal residues had functionally significant binding to Smac/DIABLO compared to the control ($P < 0.01$). The mutant with an 18-residues deletion had no significant binding. The error bars represent standard deviations from three replicate measurements for each group, and the data is representative of three repeat experiments.

The experiments above were the first demonstrations of the dimeric unbinding forces of the different species of Survivins at the single molecule level using AFM-based SMFS, which quantitatively showed the effect of N-terminal sequences on the strength of the dimerization. The mutants folded into a proper structure upon renaturation, they could all form dimers. As the HPLC results above did not show the monomeric forms of SurF or its mutants in solution, we were able to evaluate the difference of unbinding force between SurF and the mutants in solution. We further confirmed that the N-terminal residues up to Arg18 are not essential for Survivin dimerization. Further related investigations on longer deletions in the N-terminus of Survivin are underway.

Binding Ability between Survivin to Smac/DIABLO by ELISA. Smac/DIABLO is a mitochondrial protein which is released into the cytosol in response to a number of apoptotic stimuli. Previous reports have clearly shown that Smac/DIABLO physically interacts with the BIR domains of IAPs including Survivin both in vivo and in vitro.[10,16,19] During apoptosis Survivin binds to Smac/DIABLO, then free XIAP is released from the Smac-XIAP complex to directly inhibit caspases, and cell death is blocked.[13,16] The binding ability between Survivin mutants to Smac/DIABLO in vitro was measured by a sandwich ELISA. As shown in Figure 5, the absorbance intensity was Smac/DIABLO concentration-dependent. Smac/DIABLO binding to SurF increased gradually within a concentration range from 0 to 2.5 μg/mL and reached nearly the saturation point at about 2.5 μg/mL in vitro. The absorbance changed very little as the Smac/DIABLO concentration continued to increase up to 4.0 μg/mL. The binding ability of SurΔN7 to Smac/DIABLO was slightly lower but was generally not significantly different from SurF. The interaction of SurΔN13 to Smac/DIABLO was significantly reduced but was still present. However, SurΔN18 completely lost the ability to bind Smac/DIABLO, and the result was the same as that of the negative control.

Many previous studies analyzed point mutations and sequence deletions to reveal certain residues of Survivin which are critical for its binding to Smac/DIABLO, but almost all of them utilized Western blotting[20,24,40] or coimmunoprecipitation methods[16,19,41] which are qualitative and not quantitative. We adopted a sandwich ELISA method to measure the ability of Survivin and the mutants to bind to Smac/DIABLO, and thus we could clearly observe the differences in their binding saturation points.

We found that removing 18 amino acids and including a Met at the N-terminus of Survivin abolished its ability to interact with Smac/DIABLO. The highly conserved Arg18 residue is located near the C-terminus of the first BIR α-helix packed antiparallel to the second helix.[24] The importance of Arg18, whose side chain is buried in the BIR domain may stem from its ability to form multiple distinct types of interactions with other residues. In NMR and X-ray crystal structural analysis, the Arg18 aliphatic side chain was shown to contribute to hydrophobic packing with residues Ile44 and Phe58. The guanidino group was consistently oriented to form a hydrogen bond with the backbone carbonyl oxygen (CO) of Pro12 and Ala39,[5,42,43] also packing against a pair of aromatic residues, Phe43 and Phe58, which are conserved in a majority of BIR sequences, and the positively charged side chain of Arg18 can form attractive cation-π interactions with the aromatic groups.[44,45] These interactions are becoming recognized as important for Survivin protein folding.

The apoptosis stimulator Smac/DIABLO requires only its N-terminal residues 5−10 to bind to the surface groove on the side of the BIR domain opposite to Arg18.[43,46,47] NMR analysis indicated that the N-terminal residues of Smac/DIABLO bind to residues located in a region near β3 and α4, far from Arg18 of Survivin in solution.[10] Previous research had revealed that the entire BIR domain, including the surface groove that binding to Smac/DIABLO, is sensitive to the mutations at Arg18.[24] Although the conserved Arg18 was not included in the BIR polypeptide sequence that forms the Smac/DIABLO-binding site, loss of Smac/DIABLO binding to SurΔN18 indicated that deletion of the N-terminal sequences up to Arg18 may cause slightly tertiary structural changes propagating from one side of the BIR domain to the other. Although drastically reduced, some interaction remained between SurΔN13 and Smac/DIABLO, possibly due to the removal of Pro12 which affected the BIR domain, or to the decrease in dimerization stability which had been verified by SMFS. The results from the ELISA binding assay revealed that the entire BIR domain, including the surface groove which binds Smac/DIABLO, was sensitive to alterations at the N-terminal residues13−18.

Analysis of Survivin and the mutants dimers unbinding force by SMFS and binding ability to Smac/DIABLO revealed the contribution of N-terminal sequences to Survivin dimerization and antiapoptosis activity. Our results confirmed that residues 13−18 in Survivin were necessary for its binding to Smac/DIABLO and the consequent antiapoptosis activity. While SurΔN18 could dimerize in solution, it could not bind to Smac/DIABLO, indicating that the N-terminal domain was requisite for the interaction and the dimer form was not sufficient for antiapoptotic functions. These observations provide us with new insights into the relationship between the mechanical properties and function of Survivin and may help us to design new high-performance inhibitors based on its suprastructure.

Conclusions

Survivin homodimers are overexpressed in most human neoplasms and thus are attractive targets for cancer therapy. Our quantitative SMFS analysis showed that N-terminal deletions of Survivin caused dramatically reductions in the unbinding force of the homodimer. We have also demonstrated that Survivin mutants with truncations of the N-terminal sequence (residues up to Phe13 in SurΔN13 or up to the conserved residue Arg18 in SurΔN18) but retaining the BIR domain active site

lost their ability of binding Smac/DIABLO to different degrees. Thus, we have determined that the N-terminal sequences are not essential for dimer formation, but the N-terminal amino acids 13−18 are critical for Smac/DIABLO binding.

Acknowledgment. The present work was supported by the projects of the Natural Science Foundation of China (Nos. 20934002 and 20973073), Jilin Province Natural Science Foundation (20070926-01), the National Basic Research Program (2007CB808006), the Programs for New Century Excellent Talents in University (NCET), and the 111 Project (B06009). We thank Ningning Liu and Wenke Zhang, at the State Key Laboratory for Supramolecular Structure and Materials, Jilin University, for their help with the SMFS experiment and Bo Peng and Zhaohui Su, at the State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry Chinese Academy of Sciences, for their help with the QCM-D experiment.

Supporting Information Available: QCM-D experiment of SurF was performed to characterize the monolayer-like manner of protein immobilized on gold-surface. All of the measurements obtained from SMFS retracted rupture events were pooled into histograms to obtain the distribution of the rupture forces and the contour lengths of corresponding SurF and mutants dimers before we screened for valid data, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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JP1036603