An Anti-apoptotic Protein Human Survivin Is a Direct Inhibitor of Caspase-3 and -7†

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ABSTRACT: Survivin, an apoptosis inhibitor/cell-cycle regulator, is critically required for suppression of apoptosis and ensuring normal cell division in the G2/M phase of the cell cycle. It is highly expressed in a cell cycle-regulated manner and localizes together with caspase-3 on microtubules within centrosomes. Whether survivin is a physiologically relevant caspase inhibitor has been unclear due to the difficulties with obtaining correctly folded survivin and finding the right conditions for inhibition assay. In this study, recombinant, active human survivin was expressed in *Escherichia coli* and purified to homogeneity. The protein, existing as a homodimer in solution, binds caspase-3 and -7 tightly with dissociation constants of 20.9 and 11.5 nM, respectively, when evaluated by surface plasmon resonance spectroscopy. Consistently, survivin potently inhibits the cleavage of a physiological substrate poly(ADP-ribose) polymerase and an artificial tetrapeptide by caspase-3 and -7 in vitro with apparent inhibition constants of 36.0 and 16.5 nM, respectively. The data suggest that sequestering caspase-3 and -7 in inhibited states on microtubules is at least one mechanism of survivin in the suppression of default apoptosis in the G2/M phase. The localization of survivin on microtubules, which is essential for its function, should increase the protective activity at the action site.

The execution of cell death requires activation of one or more members of the well-conserved caspases, a family of cysteinyl proteases (*1*, *2*). So far, 14 different mammalian caspases have been identified, some of which are involved in the initiation of caspase activation, and others are responsible for disassembly of the cell (*1*−*3*). After proteolytic activation from their proenzyme forms, caspases cleave various protein substrates such as lamins, poly(ADP-ribose) polymerase (PARP),[1] nuclear mitotic apparatus protein (NuMA), and actin regulatory proteins (*4*). There are a number of protein families that inhibit caspases and thus provide a regulatory mechanism against unwanted demise of cells. Of these, inhibitor of apoptosis (IAP) proteins are ubiquitously found in nature ranging from viruses to humans. The IAP protein family members contain 1−3 copies of the baculoviral IAP repeat (BIR) domain composed of approximately 70 amino acids which is essential for the anti-apoptotic activity. Recombinant purified mammalian IAPs, XIAP, c-IAP-1, and c-IAP-2, were shown to directly inhibit the terminal effector proteases, caspase-3 and -7, providing evidence for a mechanism of action for these mammalian cell-death suppressors (*5*, *6*). However, not all BIR-containing proteins (BIRPs) are anti-apoptotic. Recent genetic analysis of *Caenorhabditis elegans* BIR-1 (CeBIR-1) demonstrated an essential role in cytokinesis instead of apoptosis (*7*). The BIRP of African swine fever virus also exhibits no anti-apoptotic function (*8*). In yeasts containing no caspases, BIRPs are found whose gene knockouts result in the defects in meiosis and mitosis (*9*). These results suggest that BIRs have evolved to acquire divergent biological roles.

Human survivin is an IAP protein containing a single BIR domain. Expression of recombinant survivin potently counteracted apoptosis of B lymphocyte precursors deprived of interleukin-3 (*10*), and myc-tagged survivin was co-immunoprecipitated with active caspase-3 and -7, but not with inactive proforms of the two caspases (*11*). It substantially suppressed the cytochrome *c*-induced activity of cleaving the DEVD tetrapeptide in cell lysates, which accumulates in cells undergoing apoptosis and caspase-3 and -7 are primarily responsible for (*11*). In other experiments, co-immunoprecipitation analysis using Fas antibody-treated HepG2 cells demonstrated that survivin interacted with cdk4, rather than

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[1] Abbreviations: AMC, 7-amino-4-methylcoumarin; AFC, 7-amino-4-trifluoromethylcoumarin; BIR, baculovirus IAP repeat; BIRP, BIR-containing protein; CeBIR-1, *Caenorhabditis elegans* BIR-1; BSA, bovine serum albumin; CD, circular dichroism; DMS, dimethyl suberimidate; DTT, dithiothreitol; EXAFS, extended X-ray absorption fine structure; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IAP, inhibitor of apoptosis; MES, 2-(*N*-morpholino)-ethanesulfonic acid; NuMA, nuclear mitotic apparatus protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane.

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caspase-3, while a cell-free experiment using each recombinant protein revealed that survivin interacted with caspase-3 (*12*). Survivin is abundantly expressed during fetal development in humans, but is undetectable in terminally differentiated adult tissues. However, survivin becomes prominently expressed in transformed cell lines and in all the most common human cancers of lung, colon, pancreas, prostate, and breast (*10*). It was shown that survivin associates with microtubules of the mitotic spindle at the beginning of mitosis, and colocalizes within centrosomes with γ-tubulin, caspase-3, and the cyclin-dependent-kinase inhibitor $p21^{Waf1}$, as if to form a supramolecular assembly (*13*). Interference with the expression or function of survivin caused pleiotropic cell-division defects and apoptosis in the G2/M phase of the cell cycle (*13*), indicating that survivin plays a dual role in the control of cell death and the regulation of cell division. Probably, survivin counteracts a default pathway that induces apoptosis during mitosis by preserving the integrity of microtubules (*14*) and the supramolecular assembly of cell-death/cell-cycle regulators within the centrosomes (*13*).

Survivin is the only protein that exhibits anti-apoptotic activity among IAPs containing just one BIR domain. Furthermore, the BIR domain of survivin is more closely related to those of CeBIR-1 and the yeast BIRPs than to mammalian IAPs that inhibit caspases (*15*). For these reasons and difficulties in obtaining correctly folded survivin, it has been elusive whether survivin is a direct inhibitor of caspases or not. Very recently, it was reported that survivin neither inhibits caspase-3 nor binds to the enzyme (*16*−*18*). We present a series of physicochemical data showing that it is indeed a direct and potent inhibitor of caspase-3 and -7. The result enlightens the functional mechanism of survivin.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Recombinant Human Survivin, Caspase-3, and Caspase-7. The survivin cDNA was amplified from a human fetal cDNA library using the polymerase chain reaction (PCR) technique with forward (5′-AATAATGGATCCATGGGTGCCCCGACGTTGCC-3′) and reverse (5′-CTAAGCTTTCAATCCATGGCAGC-CAGC-3′) primers. The PCR products were purified, digested with *Bam*HI and *Hin*dIII, and then ligated into the pQE-30 vector (QIAGEN). The resulting vector was introduced into *E. coli* strain SG13009 containing the plasmid pREP4 (*19*). The expression of N-terminal His_6-tagged survivin was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at an optical density of 0.6−0.8 at 25 °C for 8 h. Bacterial lysates were prepared by sonication in buffer A (20 mM Tris-HCl, pH 7.5, and 300 mM NaCl). After centrifugation at 15 000 rpm for 1 h, the supernatant was applied to a Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose column (QIAGEN), washed with buffer A containing 40 mM imidazole, and eluted with buffer A containing 200 mM imidazole. The eluted solution was passed through a Chelating Sepharose column (Amersham Pharmacia Biotech.), which was necessary for preventing survivin from being precipitated. After concentration using Centriprep-3K (Amicon), the solution was loaded onto a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech.) equilibrated with buffer B [20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 2 mM dithiothreitol (DTT)]. The peak fractions were analyzed by 15% SDS−PAGE, combined, and then dialyzed against buffer B.

Human caspase-3 gene lacking prodomain was cloned into the *Nhe*I and *Bam*HI sites of the pRSET B vector (Invitrogen), and the resulting vector was introduced into *Escherichia coli* strain BL21(DE3) pLysS. The enzyme containing the N-terminal His_6 tag was purified using a Ni-NTA agarose column. Human caspase-7 gene lacking prodomain was cloned into the *Kpn*I and *Eco*RI sites of the pRSET B vector, and the enzyme was purified in a similar way to purify caspase-3. On SDS−PAGE gels, survivin and caspase-3 and -7 showed greater than 95% purity. The purified caspase-3 and -7 exhibited specific activities of 1379 and 35 294 units/1 μg of enzyme, respectively, where 1 unit is defined as the cleavage of 1.0 pmol of the substrate acetyl-Asp-Glu-Val-Asp (Ac-DEVD)-AMC per minute at 25 °C, pH 7.5. The specific activities are comparable or better than those of commercially available enzymes for which Ac-DEVD-derived substrates are used. Concentrations of the purified proteins were determined by the Bradford method (*20*) with bovine serum albumin (BSA) as a standard.

Circular Dichroism Spectroscopy. CD spectra were recorded on a Jasco J-715 spectropolarimeter using 10 μM survivin placed in a 2 mm path-length cell. The CD spectral data points were recorded at room temperature for every 0.1 nm in the wavelength range of 200−260 nm with a scanning speed of 10 nm/min and a 1 s response time.

X-ray Absorption Spectroscopy. X-ray absorption measurements were performed on the EXAFS Beamline 3C1 at Pohang Light Source (Korea) using a Si 111 crystal monochromator which provides ∼1 eV resolution. A passivated implanted planar silicon detection system was used for fluorescence data collection. The energy was calibrated using a Zn metal foil before and after collecting the edge spectrum.

Chemical Cross-Linking. The bifunctional cross-linking agent dimethyl suberimidate (DMS) was used for cross-linking survivin. DMS solution was prepared immediately before use by dissolving 0.13 mg of DMS in 100 μL of ice-cold 0.1 M HEPES buffer (pH 8.0). Cross-linking was initiated by adding DMS (1−10 mM) to survivin (0.5 mg/mL) in the same buffer at room temperature. The cross-linking reactions were stopped after 45 min by adding 0.25 volume of 1 M Tris-HCl (pH 7.0) to the reaction mixtures. The quenched samples were subjected to SDS−PAGE.

In Vitro PARP-Cleavage Inhibition Assay. [^{35}S]Methionine-labeled PARP was generated by coupled transcription and translation in a reticulocyte lysate system (Promega) and was used as a substrate for caspase-3 and -7. After preincubation of caspase-3 for 20 min at 25 °C with or without survivin in the reaction buffer containing 10 mM HEPES (pH 7.4), 80 mM NaCl, and 0.04% Nonidet P40, [^{35}S]-methionine-labeled PARP was added and incubated for an additional 30 min at 30 °C. Caspase-7 was treated similarly in the presence of survivin. The reaction products were analyzed by SDS−PAGE, and PARP-cleavage products were visualized by autoradiography.

In Vitro Caspase Inhibition Assay. Spectrofluorometric assays of caspase-3 and -7 activity were carried out using the CaspACE assay system (Promega) at 37 °C in the reaction buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM DTT. Each of the two enzymes and survivin were preincubated together at 25 °C for 20 min, and the substrate Ac-DEVD-AMC was added to the mixture. After brief mixing in a tube, the reaction mixture was

transferred to a cuvette which was prewarmed at 37 °C. Activities were measured by the release of 7-amino-4-methylcoumarin (AMC) from the substrate using a Quantamaster spectrofluorometer model C-61SE (Photon Technology International) in the kinetic mode with excitation and emission wavelengths of 360 and 460 nm, respectively. No curvature in the initial part of the progress curves of the tetrapeptide hydrolysis indicated that a steady-state was attained before the measurements. Apparent K_i values were determined according to the equation: $(V_0/V_i) - 1 = [\text{survivin}]/K_i$, where V_0 and V_i are the velocity of the reaction in the absence and the presence of survivin, respectively (*21*).

BIAcore Biosensor Analysis. Measurements of the apparent dissociation constants (K_D) between survivin and caspase-3 and -7 were carried out using a BIAcore 2000 biosensor (Biosensor, Sweden). Survivin (30 μg/mL in 10 mM MES, pH 6.0) was covalently bound to the carboxylated dextran matrix at a concentration corresponding to ~1200 response units (RU) by an amine coupling method as suggested by the manufacturer. A flow path involving two cells was employed to simultaneously measure the kinetic parameters from one flow cell containing the survivin-immobilized sensor chip and the other flow cell containing an underivatized chip. For kinetic measurements at 25 °C, caspase-3 and -7 samples, ranging from 50 to 500 nM, were prepared by dilution with HBS buffer [150 mM NaCl, 3 mM EDTA, 0.005% polysorbate, and 10 mM HEPES (pH 7.4)]. For kinetic measurements at 4 °C, caspase-3 samples ranging from 50 nM to 40 μM were prepared with the same buffer. Injection of each sample of 150 μL of caspase solution into the flow cells (association phase) was followed by the flow of the HBS buffer (dissociation phase), both at 30 μL/min. Between cycles, the immobilized ligand was regenerated by injecting 30 μL of 100 mM glycine hydrochloride (pH 2.0) at 10 μL/min.

RESULTS AND DISCUSSION

Expression of Correctly Folded Survivin. Detailed biochemical and structural characterization of survivin requires production of the protein in a functionally active form and ideally in large quantity. Production of active recombinant survivin is not straightforward (*11*). We learned that survivin could be expressed mainly as a soluble form when its expression was induced at 25 °C in *E. coli* SG13009 host using pQE vector based on the T5 promoter system. According to the procedures detailed above, recombinant survivin could be purified in the correctly folded state as indicated by several lines of evidences. First, the recombinant protein was eluted as a sharp single peak from a size exclusion column that was employed as a final purification step, which is an indication of structural homogeneity of the sample. Second, the CD spectrum of the purified survivin sample exhibited two peak minima at 208 and 227 nm, respectively, which indicates that the protein is composed mostly of α-helical structures (data not shown). Third, the protein sample showed a zinc absorption edge at 9659 eV (data not shown) in X-ray absorption spectroscopy, as expected from the conservation of the $CX_2CX_{16}HX_6C$ sequence motif in survivin. This absolutely conserved motif in BIRPs was shown to coordinate a zinc ion with three cysteine and one histidine residues (*22, 23*). Fourth, the purified protein forms a homodimer in solution. On a size exclusion column, survivin was eluted as if the apparent molecular mass of the protein is ~35 kDa, close to the calculated dimeric molecular mass (37 233 Da) (data not shown). Consistently, chemical cross-linking of survivin by DMS yielded a protein band corresponding to ~35 kDa on SDS−PAGE (data not shown). These data accurately reflect the crystal structure of human survivin (*16*−*18*), which is a mostly α-helical homodimer containing a zinc ion per monomer.

Survivin Inhibits Caspase-3 and -7 Directly. Myc-tagged survivin was shown to bind caspase-3 and -7 by immunoprecipitation and to inhibit caspase activity in human cells exposed to apoptotic stimuli (*11*), which does not necessarily mean that survivin is a direct inhibitor of the caspases. With purified survivin, caspase-3, and caspase-7, we investigated whether survivin inhibits the two executioner caspases directly. We first probed the inhibitory effect of survivin on the proteolytic activity of caspase-3 and -7 using PARP, which is a well-defined substrate for these caspases (*24*). [^{35}S]Methionine-labeled PARP was incubated with caspase-3 in the absence or presence of survivin at several different concentrations. As shown in Figure 1A, survivin caused a dose-dependent inhibition of the PARP proteolysis, reaching more than 50% inhibition at 15 nM against 0.8 nM caspase-3. Survivin similarly inhibited caspase-7, reaching apparently complete inhibition at 290 nM (Figure 1B), which is a ~2000-fold molar excess relative to caspase-7. As a next step, we measured the inhibitory potency of survivin against the hydrolytic activity of caspase-3 and -7 using the fluorogenic tetrapeptide Ac-DEVD-AMC as a substrate. As shown in Figure 1C,D, survivin also inhibited the enzyme activity in a concentration-dependent manner. The apparent inhibition constants (K_is) of survivin for caspase-3 and -7 were determined to be 36.0 and 16.5 nM, respectively, on the basis of steady-state kinetic analyses (Figure 1D,F). These K_i values are comparable or superior to those of c-IAPs that are in the range of 30−120 nM when a similar tetrapeptide, Ac-DEVD-AFC, was employed as a substrate (*5, 6*). We noted that the inhibition against caspase-3 or -7 was saturated at ~50 nM concentration of survivin. Beyond this concentration, progress curves were virtually the same even when a 1000-fold molar excess of survivin (500 nM) relative to caspase-3 or -7 (0.5 nM) was added. The maximal inhibitions against caspase-3 and -7 are 55.3 and 75.6%, respectively, relative to a hypothetical complete inhibition. This is in contrast to the complete inhibition of the hydrolysis of the protein substrate PARP (Figure 1B). Similarly, c-IAP-1 and c-IAP-2 were reported to inhibit the hydrolysis of the tetrapeptide by caspase-3 and -7 by ~65 and 75%, respectively, although the proteins were added in a 100−5000-fold molar excess relative to the enzymes (*6*).

At the same assay conditions, a negative control experiment using BSA did not show any inhibitory activity, and a positive control experiment using the caspase-3 inhibitor Ac-DEVD-CHO resulted in a potent inhibition of the enzyme, as observed by others previously (*6*). A complete inhibition of caspase-3 and -7 was achieved at 50 nM concentration of the inhibitor.

Survivin Binds Caspase-3 and -7 with Nanomolar Affinity. We then estimated the actual binding affinity of survivin for the two caspases by employing surface plasmon resonance spectroscopy. Survivin indeed binds caspase-3 and -7

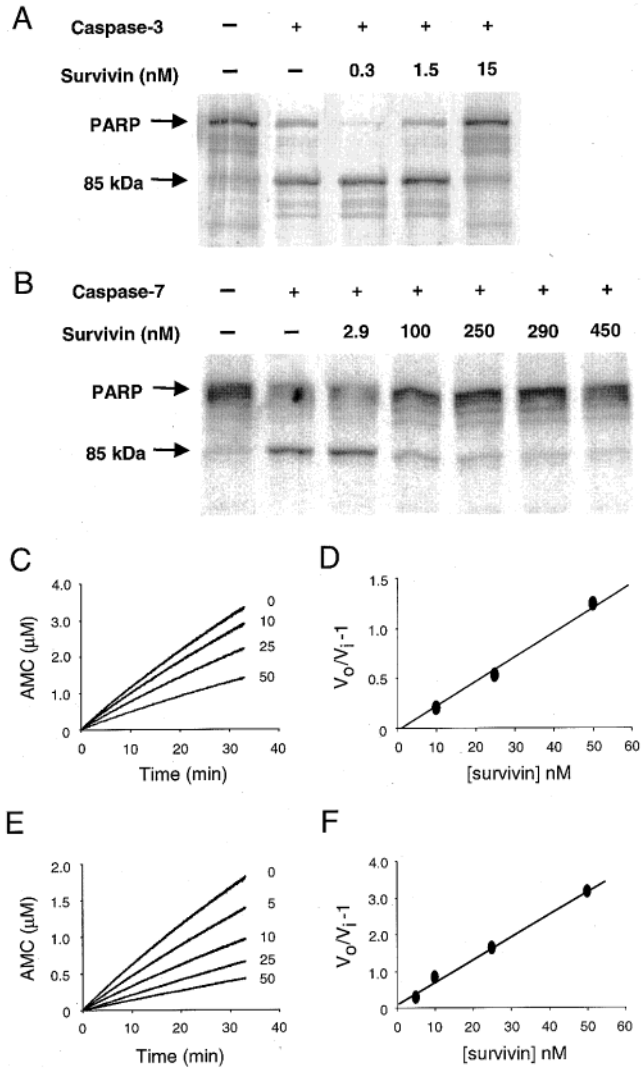

FIGURE 1: Inhibition of caspase-3 and -7 by survivin. (A, B) Inhibition of PARP degradation. Reaction mixtures containing 0.8 nM caspase-3 and [^{35}S]methionine-labeled PARP were incubated in the absence or presence of survivin at various concentrations (0.3−15 nM) for 30 min at 30 °C. A similar inhibition experiment was performed for caspase-7 employing a range of concentrations of survivin (2.9−450 nM) and 143 pM enzyme. The reaction products were separated by SDS−PAGE, and the intact (115 kDa) and the cleaved (85 kDa) PARP were visualized by autoradiography. (C, D) Inhibition of the hydrolysis of an artificial substrate. Survivin at the indicated concentrations (10−50 nM) was incubated for 20 min at 25 °C with 0.5 nM caspase-3 before adding 100 μM Ac-DEVD-AMC. (E, F) A similar inhibition assay was performed for caspase-7 employing the same concentrations of both the enzyme and the substrate as those employed for caspase-3.

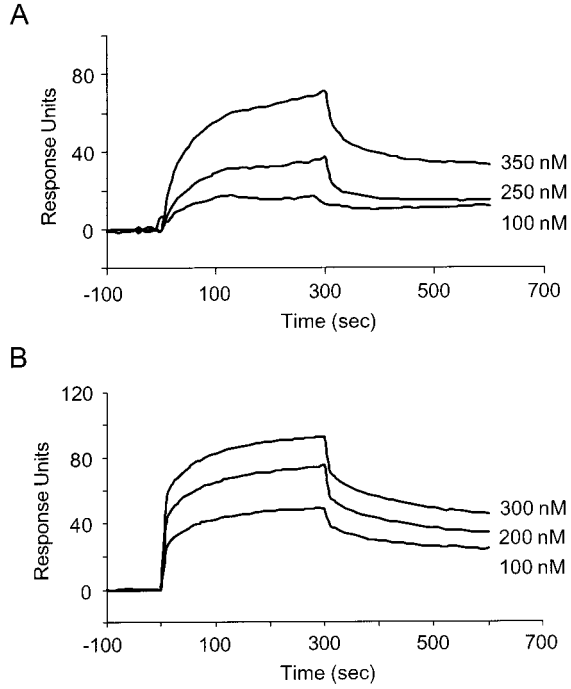

FIGURE 2: BIAcore analysis of binding of survivin to caspase-3 and -7 at room temperature. (A) Survivin was immobilized to the dextran matrix, and caspase-3 samples at the three indicated concentrations were injected for 5 min. (B) A similar binding assay was performed for caspase-7. The kinetic parameters of the binding reactions were determined using the BIAevaluation version 2.1 software provided by the manufacturer and are shown in Table 1. A control experiment using BSA did not show a detectable response of binding to either of the enzymes.

Table 1: Kinetic Parameters of the Binding of Survivin to Caspase-3 and -7[a]

analyte	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
caspase-3	$7.08 \pm 0.90 \times 10^4$	$1.48 \pm 0.24 \times 10^{-3}$	$20.90 \pm 4.26 \times 10^{-9}$
caspase-7	$8.67 \pm 0.29 \times 10^4$	$10.0 \pm 0.16 \times 10^{-4}$	$11.53 \pm 0.42 \times 10^{-9}$

[a] The association rate constant (k_a) was determined from a plot of ln[Abs(dR/dt)] versus time, where R is the intensity of the surface plasmon resonance signal at time t. The dissociation rate constant (k_d) was determined from a plot of ln(R_0/R) versus time, where R_0 is the resonance signal intensity at time zero. The apparent K_D was calculated from the kinetic constants: $K_D = k_d/k_a$.

strongly with an apparent K_D of 20.9 and 11.5 nM, respectively (Figure 2; Table 1), which are similar to the K_i values measured by the kinetic experiment. The results indicate that about 5% caspase would be in the free form when 0.5 nM enzyme and 500 nM survivin are present in a mixture. Therefore, an incomplete block of the active site of the caspases may explain the low maximal inhibition of survivin against the hydrolysis of the artificial substrate. Although survivin is bound to the enzyme, there might be a space between the two, which allows the small tetrapeptide to approach the active site cleft. In the physiological situation, however, a protein substrate would not be able to approach the active site of the enzyme in complex with survivin. This is supported by the nearly complete block of PARP hydrolysis (Figure 1B) when survivin is added in molar excess relative to caspase-7. This binding mode, we propose, is similar to those of c-IAPs, but not to that of XIAP which inhibits the two caspases nearly completely (*6*), and therefore appears to shield the active site of the enzymes from the access of the tetrapeptide. A recent mutagenesis study of XIAP revealed that conserved amino acids in the linker region between BIR1 and BIR2 of the protein are critical for potent inhibition of the activity of caspase-3 cleaving Ac-DEVD-AMC (*23*). The linker segment was proposed to block the active site cleft of caspase-3, as does the β-turn loop of baculoviral anti-apoptotic protein p35 (*25*). Survivin does not contain a corresponding region, but it actually binds caspase-3 and -7 tightly. c-IAPs contain a corresponding region, but this region may not be used to block the active site of the enzymes completely.

Drastic Reduction of the Binding Affinity at Low Temperature. Our result agrees with the direct interaction between

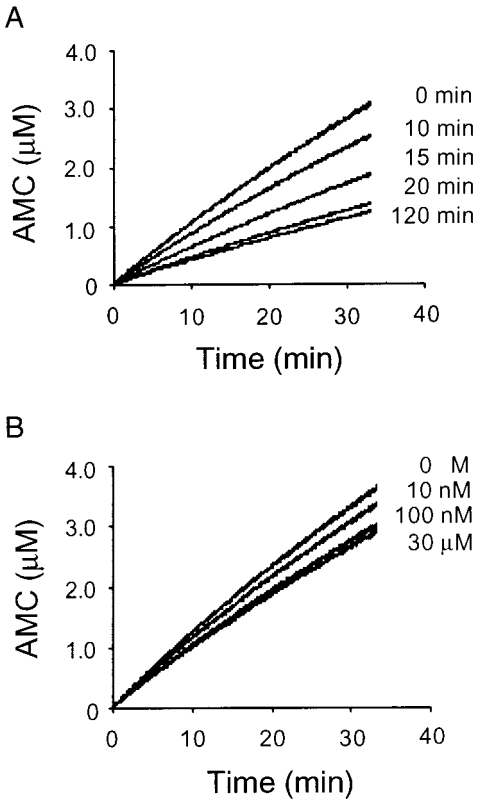

FIGURE 3: Effect of preincubation at 25 °C on the inhibitory potency of survivin. (A) Survivin (50 nM) was preincubated with caspase-3 (0.5 nM) at 25 °C for 5, 10, 15, 20, and 120 min, respectively, prior to the start of enzyme reaction by adding Ac-DEVD-AMC (100 μM). (B) Survivin (0−30 μM) was preincubated with the peptide substrate (100 μM) at 25 °C for 20 min prior to adding caspase-3 (0.5 nM). The enzyme activities were measured by monitoring the release of AMC from the substrate at 37 °C.

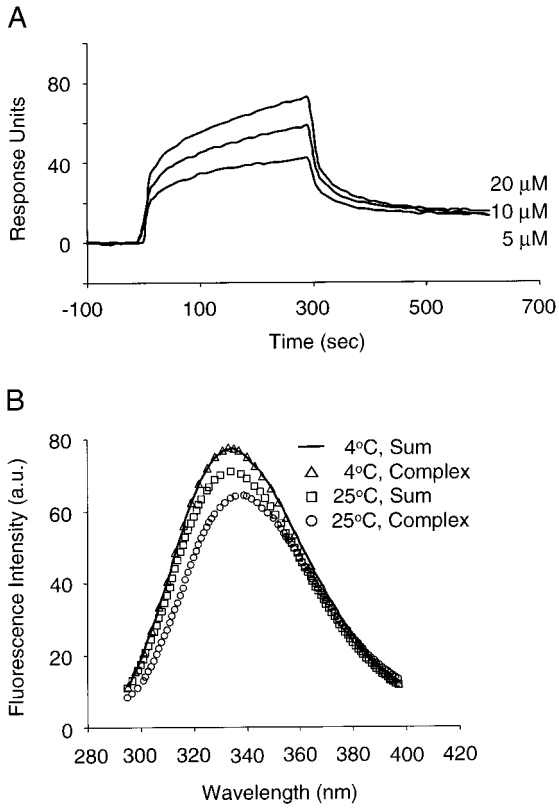

FIGURE 4: Temperature effect on the binding of survivin to caspase-3. (A) BIAcore analysis of binding of survivin to caspase-3 at 4 °C. The instrument was cooled gradually to 4 °C prior to the binding experiment. Binding responses were detected at much higher concentrations of caspase-3 compared to the experiment at 25 °C. The kinetic parameters were analyzed with the three indicated concentrations of the enzyme. (B) Fluorescence spectra of the 1:1 mixture of survivin and caspase-3 and the sum of the spectra of individual survivin and caspase-3. Survivin and caspase-3 were preincubated together for 20 min at 4 (△) and 25 °C (○) or individually at 4 (—) and 25 °C (□), respectively. The sum spectrum at 4 °C is virtually identical to the spectrum of the mixture at the same temperature. Fluorescence spectra were recorded at the respective temperature of preincubation. The emission spectra were recorded with a wavelength of 285 nm to excite tryptophan and tyrosine. The concentration of each of the two proteins was 5 μM. The sample buffer contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM DTT.

recombinant caspase-3 and the fusion protein glutathione-*S*-transferase−survivin detected by immunoblotting with caspase-3 antibody (*26*). However, our result is sharply contradictory to a recent report by Verdecia et al. (*16*), which found no inhibitory and binding activity of survivin against caspase-3, suggesting that the anti-apoptotic activity of the protein is not through the inhibition of this enzyme. The cause of the failure to find association of the two in the previous study was likely due to the preincubation on ice prior to the measurement of enzyme activity and the immunoprecipitation. As shown in Figure 3A, full inhibitory potency of survivin required preincubation of the protein with caspase-3 at least for 20 min at 25 °C. Without the preincubation, we also did not observe the inhibition of caspase-3 by survivin in the same buffer described in the previous report by Verdecia et al., where as high as 30 μM survivin was employed. However, with preincubation of the two proteins for 20 min at 25 °C before the enzyme reaction, we observed a strong inhibition of caspase-3. A kinetic analysis at five different concentrations of survivin yielded a K_i of 13 nM (data not shown), which is similar to that obtained from our experimental conditions. It is not understood why the absence of the preincubation resulted in no inhibition throughout 40 min of reaction at 37 °C. Probably, the presence of the peptide substrate and product (with a combined concentration of 100 μM) in the reaction mixture might have interfered with the binding of the two proteins. In a control experiment, preincubation of survivin with the substrate at 25 °C for 20 min prior to the addition of caspase-3 did exhibit inhibition of the enzyme, but substantially less compared to the preincubation of survivin with caspase-3 (Figure 3B). This indicates that the tight association of the two proteins requires a thermal activation and the presence of the two proteins.

The unusual and dramatic temperature effect on the inhibitory potency of survivin was further investigated by BIAcore biosensor analysis. In contrast to the high binding affinity at 25 °C, K_D between survivin and caspase-3 increases drastically to 2.2 μM at 4 °C (Figure 4A). Depending on the experimental conditions, this low-affinity binding could not be detected by the immunprecipitation method as in the previous study (*16*). The temperature-dependent binding activity of survivin was also probed by fluorescence measurement. At 25 °C, a red shift and a decrease in the fluorescence intensity took place when survivin and caspase-3 were mixed together in a 1:1 molar ratio, both at 5 μM (Figure 4B). However, no appreciable fluorescence change was detected at 4 °C in the same

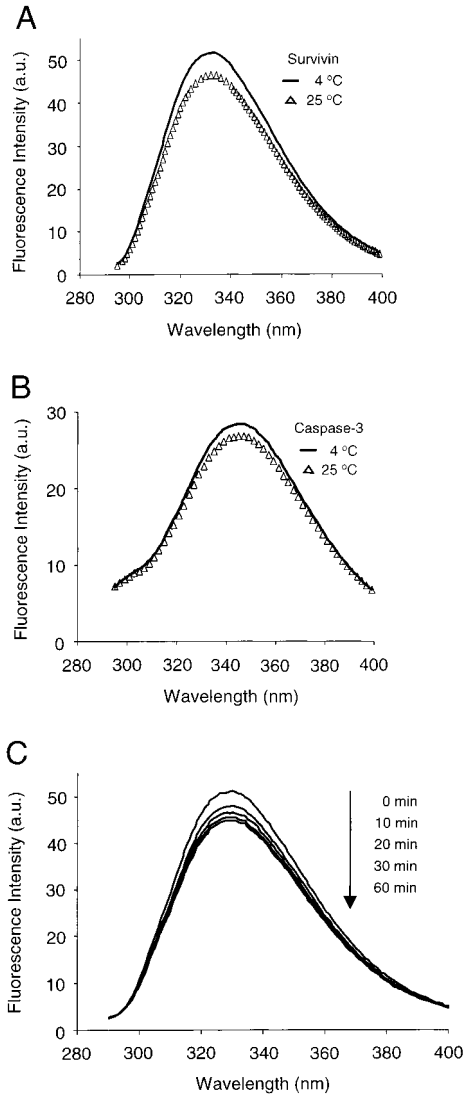

FIGURE 5: Fluorescence spectra of survivin and caspase-3. (A) Spectra of survivin recorded at 4 (—) and 25 °C (△), respectively. (B) Spectra of caspase-3 recorded at 4 (—) and 25 °C (△). (C) Survivin was incubated on ice for 20 min and transferred to a cuvette which was prewarmed at 25 °C. Spectra were recorded at the indicated laps of time after the transfer. After 1 h, the spectrum did not change at all. In the sample holder, the protein sample, whose total volume was 125 μL, reached thermal equilibrium in less than 5 min.

experimental conditions except for the temperature, which is consistent with the result from the BIAcore analysis.

Survivin appears to undergo some degree of temperature-dependent conformational change, because the fluorescence spectrum of the protein exhibits an appreciable change as a function of temperature as shown in Figure 5A. This is in contrast to caspase-3, which displays a smaller spectral difference (Figure 4B). The process of the change is apparently slow, since the fluorescence spectrum of survivin transferred from ice temperature to 25 °C changes gradually as shown in Figure 5C. The observation provides an explanation to why the protein requires a prolonged incubation at 25 °C for the full inhibitory activity. The change in survivin as a result of the temperature shift may account for the minor inhibition of caspase-3 that took place when survivin was preincubated with the inhibitor at 25 °C (Figure 3B). However, it may not be sufficient for the maximal inhibition of the enzyme observed when survivin was preincubated with caspase-3 at the same temperature (Figure 3A). Probably, the tight interaction of the two is accompanied by more significant conformational changes of both or one of the two proteins, which are likely to be greatly facilitated at room temperature.

A Plausible Mechanism of Action of Survivin in the Cell. The expression of mammalian c-IAP-2 is induced via NF-κB signaling after TNF-α stimulation, and c-IAP-1 is constitutively expressed (*27*). On the other hand, the expression of survivin is upregulated in the G2/M phase of the cell cycle. The specific expression suggests that survivin contributes to the regulation of apoptosis during cell proliferation (*26*). It associates with the mitotic spindle through the C-terminal domain (*14*) as indicated by a deletion analysis of the last 50 residues. Since survivin is a homodimer, two possibilities are considered to explain a mechanism of action of survivin. First, survivin interacts with caspase-3 and -7 in the C2 symmetric mode to inhibit the enzymes, that is, the interaction of two identical N-terminal BIR domains of dimeric survivin with the two identical sites near the active sites of the $(\alpha\beta)_2$ dimeric caspases (*28*). Second, survivin anchors on microtubules via the C-terminal domain. Since survivin interacts with caspase-3 strongly, the colocalization of the two at centrosomes (*13*) is likely to be a result of the direct interaction between the two. We suggest that the direct binding of survivin sequesters the executioner caspases in the supramolecular assembly of cell-cycle/cell-death machinery within the centrosomes in the inhibited state. By the suggested mechanism, survivin could be able to protect key cell-cycle regulators such as $p21^{Waf1}$ that colocalizes with caspase-3 and is cleaved by the enzyme when the function of survivin is interfered (*13*). The ability of survivin to bind microtubules could also protect in general the integrity of the mitotic apparatus, at least from caspase-3 that is known to attack structural proteins such as NuMA (*29*) and most likely from caspase-7 that shares a similar substrate profile with caspase-3. Previous immunoprecipitation experiments suggested that survivin in cytosolic lysates binds caspase-3 and -7 consistently less efficiently than XIAP (*11*). The result is in concord with the K_D value of survivin for caspase-3 (20.9 nM), which is higher compared with the K_i value of XIAP against caspase-3 (~0.7 nM) (*5*). However, the inhibitory potency of survivin should be augmented in physiological conditions where the effective concentration of the protein increases by the recruitment to the mitotic spindle, the action site of the protein. This may explain why disruption of the survivin−microtubule interaction results in the loss of the anti-apoptotic activity of survivin. Recently, survivin was proposed to exert anti-apoptotic activity by promoting procaspase-3/$p21^{Waf1}$ complex formation (*12*). Our results suggest that survivin controls apoptosis and preserves the mitotic spindle for progression of cell division at least partly by direct binding and inhibition of the executioner caspases.

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