J. Biochem. **141,** 293–299 (2007)
doi:10.1093/jb/mvm028

W323S Variant of Xiap–Bir3 Binds to SMAC but not Caspase-9

Jamshid Davoodi[1],*, Azadeh Mohammad-Gholi[1], Ali Es-haghi[1] and Alex MacKenzie[2]

[1]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran; and [2]Apoptosis Research Center, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada

Received November 4, 2006; accepted December 11, 2006; published online December 19, 2006

The ability of the wild-type XIAP BIR3 domain as well as its Trp323Ser variant in inhibition of human caspase-9, binding to AVPFVASLPN (SMAC-peptide), SMAC protein, and mature caspase-9 was investigated. In order to investigate the role of W323 on these interactions, this residue was mutated to Serine. Circular dichroism as well as thermal denaturation studies showed that W323S mutation did not hamper proper folding of the protein. The dissociation constants for the interaction of the wild type BIR3 as well as its mutant to Smac-type peptide were found to be 1.8 and 27 μM, respectively. The inhibition of and binding to caspase-9 by wild-type BIR3 and its mutant were also compared. While the wild-type protein potently inhibited the enzyme, the mutant failed to do so. The lack of caspase-9 inhibition was due to absence of interaction of the mutant BIR3 with mature caspase-9. These results indicate that Trp323 of BIR3 plays a pivotal role both in maintaining necessary conformation for caspase-9 interaction and to a lesser extent, recognition of Smac-type peptide. Moreover, decreased stability of the mutant compared with the wild type indicates that W323 is essential for maintaining the stability BIR3–Smac-peptide complex.

Key words: caspase-9 inhibition, Smac, XIAP BIR3.

Abbreviations: BIR, baculoviral IAP repeat; NAIP, neuronal apoptosis inhibitory protein; XIAP, X-linked inhibitor of apoptosis protein.

INTRODUCTION

Apoptosis is a controlled process of cell destruction executed by caspases. In response to internal or external signals, initiator caspases are activated that leads to the activation of downstream executioner caspases (*1*). Unlike executioner caspases, activation of initiator caspases requires the assembly of monomeric forms of the enzyme with the aid of adapter molecules (*2, 3*). In the case of caspase-9, this complex, called apoptosome, is formed by the participation of Apaf-1, cytochrome *c*, ATP and procaspase-9 (*4*). The formation of apoptosome leads to the activation of procaspase-9 as well as its processing to a small and a large subunit. It has been suggested that this processing is not required for the activation of procaspase-9 and that the assembly alone is sufficient to activate the enzyme (*5*). The activity of caspases is inhibited by a class of proteins called Inhibitor of Apoptosis Proteins (IAPs), which are characterized by the presence of one or more Baculoviral IAP repeat (BIR) domains (*6*). X-linked Inhibitor of Apoptosis Protein (XIAP), as the best characterized IAP, has three BIR domains. It has been shown that the BIR2 domain is a potent inhibitor of both caspase-3 and caspase-7 (*7–9*), whereas the BIR3 domain is a potent inhibitor of caspase-9 (*10*). Furthermore, the inhibition of caspase-9 is achieved by the interaction of the IAP binding motif (IBM, AXPX sequence) at the N-terminus of the small subunit of caspase-9 with the BIR3 domain of XAIP (*11, 12*). This tetrapeptide sequence is sufficient for the binding to XIAP-BIR3 and in fact the mutation of this sequence abolishes BIR3 mediated inhibition of caspase-9 (*13*). X-Ray crystallography as well as NMR data suggest an important role for tryptophan 323 residue of XIAP-BIR3 in this interaction (*11, 12, 14*). Naip-BIR3 protein, which is also capable of inhibiting caspase-9 but not binding to Smac, contains cysteine instead of tryptophan in this position (*15*). Thus, we decided to mutate W323 to serine, which has the highest similarity to cysteine, in order to assess whether or not Xiap-Bir3 would attain properties similar to NAIP-Bir3 in both caspase-9 inhibition as well as Smac binding. Due to interference of cysteine with proper protein folding, tryptophan was mutated to Serine instead of cysteine. The caspase-9 inhibition assays were performed in high concentrations of ammonium citrate known to dramatically enhance the activity of the enzyme (*16*). Our results show that XIAP-BIR3 can still inhibit caspase-9 in ammonium citrate buffer potently and that W323S mutation of XIAP affects both caspase-9 inhibition and interaction more than the SMAC binding. Furthermore, in addition to weakening interaction between BIR3 and Smac-type peptide, substitution of tryptophan at position 323 with serine leads to destabilization of BIR3–Smac-peptide complex.

*To whom correspondence should be addressed at: Jamshid Davoodi, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran. Tel: +98-21-6696-9190, Fax: +98-21-6640-4680, E-mail: jdavoodi@ibb.ut.ac.ir

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins—Isolation of XIAP cDNA and the construction of

pGEX-XIAP-BIR3 and –NAIP-BIR3 have been described in detail elsewhere (*15, 17*). Wild type and mutant XIAP glutathione *S*-transferase (GST)-tagged BIR3 proteins were overexpressed in *Escherichia coli* strain BL21-TrxB-DE3 (Novagen Inc.). Overnight bacterial cultures were diluted 1 in 20 in Lauria Bertani (LB) medium and incubated at 37°C temperature. When the absorbance at 600 nM reached 1.2 units, the culture temperature was reduced to 25°C and the cells were induced for the production of the recombinant proteins with 0.5 mM Isopropyl beta-D-1-thiogalactopyranoside (IPTG). Cells were harvested after 1 h induction. Cell paste was re-suspended in buffer A (50 mM Tris-HCl, 0.1 M NaCl, 10 mM β-mercaptoethanol, 0.5% Triton X-100, pH 8.0) and mixed at 4°C for 15 min. After a gentle sonication, the mixture was centrifuged at 20,000*g* for 15 min. The supernatant was incubated with glutathione-Sephadex 4B beads (Amersham Biosciences) and equilibrated with buffer A for 1 h. The resin was washed with buffer A followed by buffer B (buffer A supplemented to 0.2 M NaCl and 10% glycerol). Recombinant XIAP-BIR3 proteins were eluted with 10 mM reduced glutathione in 50 mM Tris- HCl, pH 8.0, 10% glycerol, 125 mM NaCl, 5 mM β-mercaptoethanol. Protein concentrations were measured using Bio-Rad protein assay kit according to the manufacturer's protocol. The purity of the proteins was determined by SDS-PAGE and was generally greater than 95%. Expression of recombinant caspase-9 was based on the procedure described by Stennicke *et al*. (*18*) with minor modifications. Briefly, BL21-TrxB -DE3 (Novagen, Inc) cells were transformed by pET23b-caspase-9 (a generous gift from Dr Guy Salvesen) and grown in 2TYmedium supplemented with 10 μg/ml Kanamycin and 100 μg/ml Ampicillin. Overnight cultures were diluted 1 in 20 and grown to absorbance (600 nm) of 0.8. This was followed by 0.5 mM of IPTG induction for 3 h at 37°C. Purification of recombinant caspase-9 was similar to that of XIAP-BIR3 protein except that Ni-NTA resin was used for affinity purification of the protein. Furthermore, the column was washed with the wash buffer (50 mM tris, pH 7, 500 mM NaCl, 10 mM immidazole, 5 mM β-mercaptoethanol). Finally, caspase-9 was eluted with a Tris buffer, pH 7.5, containing 10% glycerol, and 300 mM immidazole.

Ub-SMAC—A eukaryotic expression plasmid containing the fused ubiquitin and mouse Smac/DIABLO coding regions was a generous gift from Dr Peter Liston (*19*). This fused coding region was amplified by the forward ATCCATGGGCCAGATCTTCGTGAAAACCCTTACC and the reverse primer TGCTCGAGTTAACGACGTTGAACG ACGTTTTTTTACGACCACTATGGTGATGGTGATGATGG TCCCCCCGCAGATCCTCTTCTGAGATGAGTTTTTGTC TTCACGCAGGTAGGCCTCCTC, TOPO-TA cloned into pcr2.1-topo plasmid (Invitrogen), and sequenced. The coding region was then sub-cloned using *NcoI* and *Xho*I sites into pET28a plasmid. The final plasmid construct encodes a 290 amino acid precursor protein consisting of 77 aa of human ubiquitin fused to aa residues 54–237 of mouse Smac followed by a myc and 6XHis tags. Overexpression and Purification of Ubi-Smac from BL21(DE3) cells (Novagen) was performed by transforming cells with pET28a-Ubi-Smac vector. The cells were grown at 27°C in LB media containing 30 μg/ml kanamycin. When the absorbance at 600 nm reached 1.0 unit, cells were induced for the production of Ubi-Smac with 0.1 mM IPTG. Cells were harvested after 4 h induction. Cell paste was re-suspended in Buffer A (50 mM NaH_2PO_4, 0.3 M NaCl, 10 mM βME, pH 8.0) and sonicated. The mixture was then centrifuged at 20,000*g* for 15 min and the supernatant was incubated with Ni-NTA resin (Qiagen), equilibrated with Buffer A, for 1 h. The resin was washed with Buffer A, followed by Buffer B (the same as Buffer A except 20 mM immidazole). Recombinant Ubi-Smac was eluted with 250 mM imidazole in Buffer A. Production of mature Smac (the mature form of the protein that starts with AVPI sequence) was achieved by cleaving the ubiqutin tag with Ubiqutin *C*-terminal hydrolase (Sigma).

Site Directed Mutagenesis of pGEX-XIAP-BIR3—Overlapping PCR strategy was used to mutate W323 to serine using QuikChange XL Site-Directed Mutagenesis kit (Stratagene) with the forward GGGAACAACATGCT AAATCGTATCCAGGGTGC and the reverse GCACCCTG GATACGATTTAGCATGTTGTTCCC primers according to the manufactures instructions. The presence of the mutation was confirmed by sequencing the construct on both strands.

Caspase-9 Inhibition Assays—Caspase-9 assays were performed in 25 mM HEPES, pH 7.4, 1 M ammonium citrate, 10% glycerol, and 10 mM dithiothreitol using 85 units (one unit corresponds to the amount of enzyme required for the hydrolysis of 1 μM of LEHD-pNA substrate per min in 1 M ammonium citrate at 30°C) of caspase-9 and 2 mM Ac-LEHD-pNA as a substrate in a 96 well plate format. The hydrolysis of the substrate was monitored at 405 nm at 30°C for 60–90 min. The inhibitory constants were obtained using a range of inhibitor concentrations while maintaining constant levels of both the substrate and enzyme. To study the effect of AVPFVASLPN peptide (SMAC-peptide) on reversing the inhibition of caspase-9 by the BIR3 domain of XIAP, the BIR3 protein was incubated with the final concentrations of 10 and 50 μM of AVPF-peptide prior to the incubation with caspase-9.

SMAC-peptide Binding Assays—Interaction of the BIR3 proteins with SMAC-peptide was measured by monitoring the quenching of the emission fluorescence of the protein at 300–450 nm as a function of the peptide concentration. The protein samples were excited at 280 and 295 nm using Cary Eclipse Fluorescence Spectrometer.

Thermal Denaturation Assays—Thermal stability of the BIR3 proteins were obtained by heating the samples at the scanning rate of 0.5°C/min using Cary Eclipse Fluorescence Spectrometer equipped with AVS temperature controller. The quenching of the emission spectra at 340 nm resulting from excitation at 270, 275, 280 and 295 nm was plotted against temperature. Similar experiments were performed for the complex of the wild-type XIAP-BIR3 as well as its W323S variant with 25 μM concentration of the protein and 75 μM concentration of SMAC-peptide.

Circular Dichroism Studies—Circular dichroism (CD) studies were performed using AVIV 215 spectropolarimeter. The instrument was calibrated with

ammonium-d-camphorsulfonate. Spectra in the far UV region (200–260 nm) were measured in a 0.1 cm-path-length quartz cell at a protein concentration of 6.5 μM. The spectra of the protein–peptide complex were obtained in a similar fashion except that the sample contained 65 μM of SMAC-peptide.

Smac Pull Down and Western Blotting Experiments. Glutathion-Resin bound to the wild type and mutant gst–BIR3 proteins were incubated with Ubi-Smac in buffer B (50 mM Hepes, 100 mM NaCl, 10 mM DTT, 0.1% Chaps). In order to expose AVPI N-terminal sequence of Smac, half of each sample was treated with Ubiqutin C-terminal hydrolase. Following the incubation of the samples at 37°C for 3 h, they were washed by Buff.B and Buff.B containing 300 mM NaCl. The samples were loaded onto SDS-PAGE and transferred to PVDF membrane. Following incubation of the membrane with a mixture of anti-GST and anti-His monoclonal antibody (Qiagen) overnight at 4°C, it was washed with PBS and probed with anti mouse and anti Rabbit-HRP conjugate (Lumi-Light Western Blotting Kit, Roche-Applied-Science) for 2 h. Following further washes, the membranes were incubated with the substrate and exposed to ECL-plus film according to the manufacturer's protocol. Caspase-9 pull down and western blotting was performed similar to Smac, except that Ubiqutin *C*-terminal hydrolase treatment was absent and the pull down was performed at 4°C instead of 37°C.

RESULTS AND DISCUSSION

Comparison of Caspase-9 Inhibition by the Wild-type XIAP-BIR3 and its W323S Variant in 1 M Ammonium Citrate—Activity of caspae-9 can be greatly enhanced in the presence of high concentrations of kosmotropic salts like ammonium citrate (*16*). However, it is not clear whether BIR3 domain of XIAP can inhibit the enzyme under 1 M ammonium citrate and how potent this inhibition is. Therefore, we decided to investigate the inhibition of kosmotropic salt-activated caspase-9 by XBIR3. Figure 1 shows that the ammonium citrate activated enzyme can also be inhibited by the wild-type XIAP-BIR3 with the IC50 value of 21 nM. XIAP–BIR3 has been shown to inhibit caspase-9 with the IC50 values of 17 nM (*15*) and 10 nM (*20*) for caspase-9 alone and caspase-9 in apoptosome complex, respectively. Taken together, our results indicate that XIAP-BIR3, similar to low salt buffers and apoptosome, is a potent inhibitor of kosmotropic salt-activated caspase-9. In order to assess whether or not XIAP-BIR3 maintained its SMAC-peptide binding activity under high kosmotropic salt concentration, the effect of the peptide on caspase-9 inhibition by XIAP-BIR3 was investigated (Fig. 1). Smac-peptide was indeed capable of reversing the caspase-9 inhibition by XIAP-BIR3 indicating that the affinity of the BIR3 domain to both the enzyme and the Smac-peptide was maintained in 1 M ammonium citrate salt.

The inhibition of caspase-9 by W323S mutant of Xiap-Bir3 was also investigated in ammonium citrate buffer (Fig. 1). This mutant failed to exhibit significant inhibitory activity, which, in this respect, is similar to W323A mutant reported previously (*11*). This finding

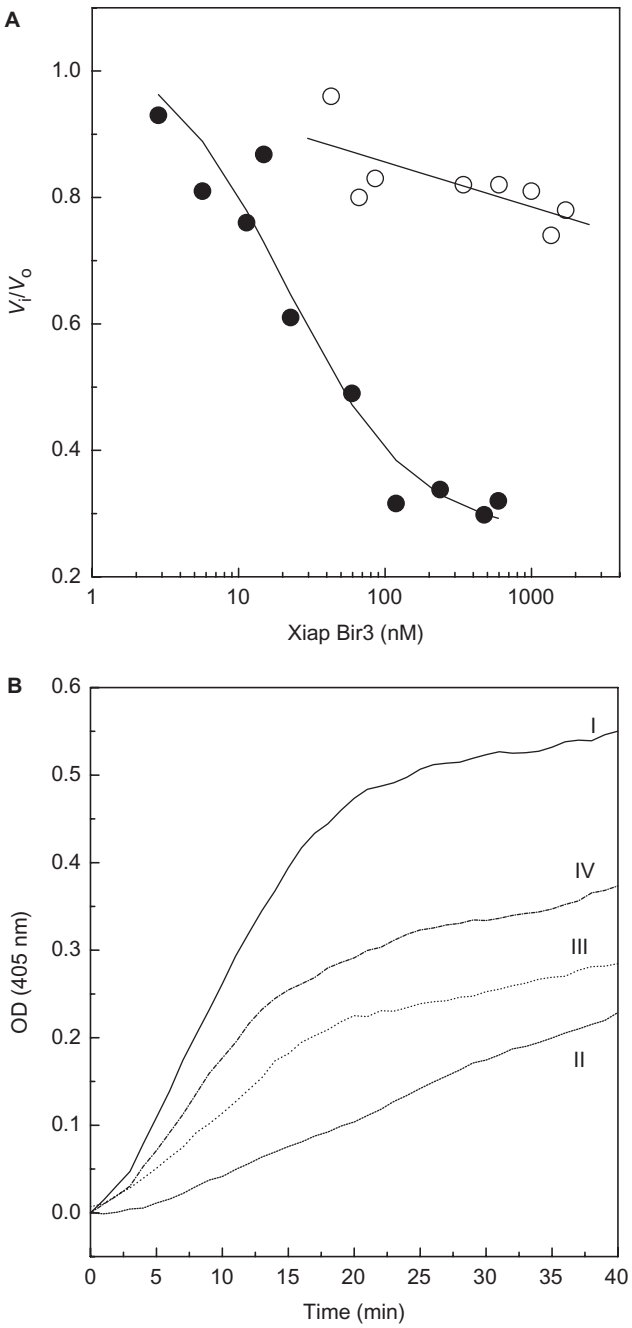

Fig. 1. **(A) IC50 values for the wild type XIAP–BIR3, filled circles, and W323S variant, open circles, in the presence of 1 M ammonium citrate.** (B) The effect of SMAC-peptide on caspase-9 inhibition by XIAP–BIR3, I, caspase-9 in the presence of 50 μM SMAC-peptide, II, caspase-9 in the presence of 0.25 μM XIAP–BIR3, III, caspase-9 in the presence of both 0.25 μM XIAP–BIR3 and 25 μM SMAC-peptide, and IV is the same as the latter except 50 μM SMAC-peptide.

taken with the fact that Naip-Bir3 with cysteine in this position is a potent inhibitor of caspase-9 suggest that Naip and Xiap-Bir3 proteins inhibit caspase-9 using different mechanisms. If their mechanism of interaction with caspase-9 were the same, one would have expected noticeable inhibition of caspase-9 by W323S variant.

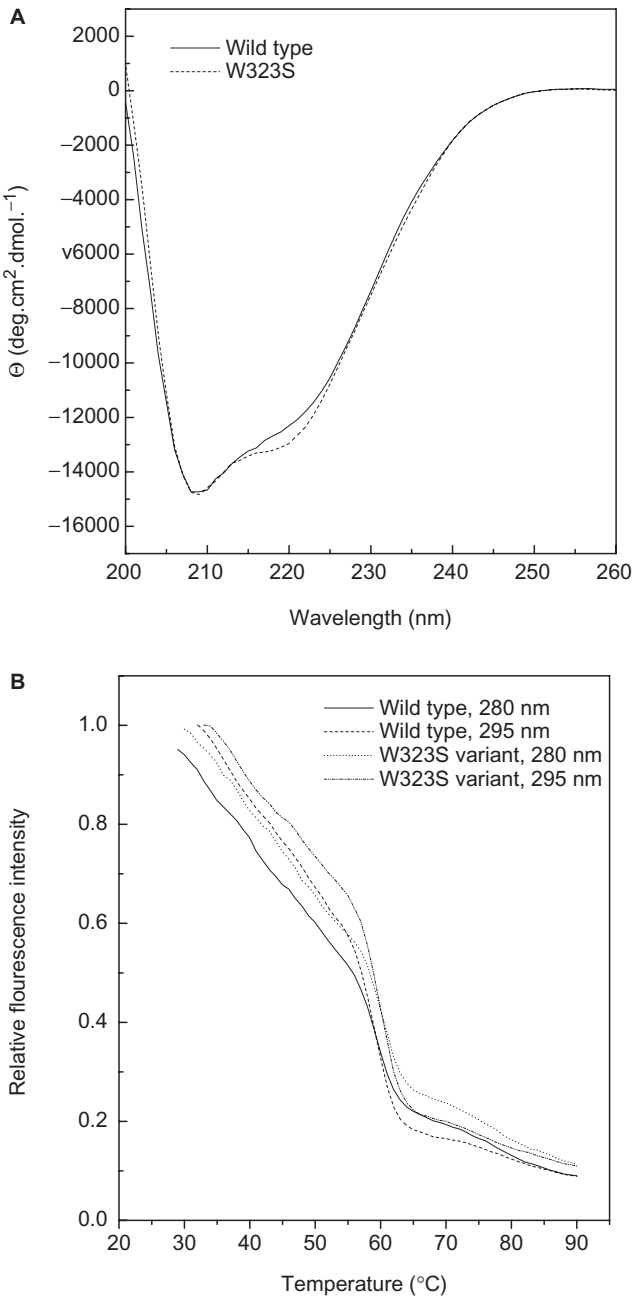

Fig. 2. **(A) Circular dichroism spectra for the wild type XIAP–BIR3 and its W323S variant obtained according to the procedure described in Materials and Methods.** (B) Thermal denaturation profile of 25 μM wild-type XIAP–BIR3 exited at 280, solid line, and 295 nm, dashed line, and 25 μM W323S variant exited at 280, dotted line, and 295 nm, dash-dotted line.

W323S Mutation does not Hinder Proper XIAP–BIR3 Folding—W323S variant of XIAP–BIR3 was found to be deficient of caspase-9 inhibitory activity (Fig. 2). To rule out that this is not due to improper folding of the mutant protein, the Far-UV CD data of the mutant was compared to the wild type protein. As seen in Fig. 2, mutation of W323S did not hinder proper folding of the protein albeit somewhat altered rotation of the circular light at 215–225 nm, indicating slightly altered secondary structure for the mutant compared to the wild-type protein. Heat-induced unfolding of the protein was also performed for both the wild-type protein as well as its W323S variant (Fig. 2). Thus, changes of the fluorescence emission of the proteins were monitored at 340 nm while excited at 280 or 295 nm. A single cooperative transition was observed for both proteins with midpoint transition temperatures of 59°C and 60°C for the wild type and the mutant, respectively. This indicates that the mutation of W323 to Serine did not create unfavourable interactions inside the protein; neither did it create any cavity that would destabilize the structure. Overall, these data indicate that, irrespective of some structural changes, the overall structural properties of the mutant remained similar to the wild type and that the lack of caspase-9 inhibition by W323S mutant did not originate from improper protein folding. Presence of only one co-operative transition during thermal induced unfolding indicated that unfolding of the GST tag and the BIR3 domain occurred simultaneously.

Interaction of BIR3 with Smac-peptides—X-ray and NMR studies have shown that W323 from XIAP directly binds to the AXPX sequence from Smac as well as the small subunit of caspase-9. In order to determine whether lack of caspase-9 inhibition by W323S variant is due to loss of binding to the AXPX sequence at the N-terminus of the small subunit from caspase-9, the binding of SMAC-peptide to both mutant and wild type Bir3 proteins was monitored by fluorescence spectroscopy. The interaction of the peptide resulted in quenching of the intrinsic fluorescence of XIAP proteins (Fig. 3). Thus, using the quenching data, binding constants of the peptide to the proteins were estimated as 1.8 and 27 μM, for the wild type and the mutant, respectively. NAIP–BIR3, which was used as a negative control, did not interact with Smac-peptide consistent with our previous report showing lack of interaction of Naip-Bir3 with Smac protein (*15*). Taking the ability of W323S variant in binding to Smac-peptide and its inability in caspase-9 inhibition strongly argues for the fact that AXPX recognition by IAPs is insufficient for caspase-9 inhibition.

As an additional proof that Smac-peptide indeed interacts with Bir3 proteins, thermal denaturation profile of both wild type XIAP–BIR3 and its W323S variant in complex with Smac-peptide was obtained by monitoring the quenching of the emission at 340 nm as a function of temperature. A single co-operative transition occurred at 59°C for the wild type protein upon excitation at 280 or 295 nm (Fig. 3B). In this respect, the presence of the peptide did not cause a noticeable change in the thermal transition temperature of the wild type protein suggesting that new interactions introduced by filling the IBM binding grove by the peptide as well as increase of the secondary structure due Smac-peptide do not significantly affect the stability of the protein. In other words, forces stabilizing the complex compared to overall forces stabilizing the protein itself do not differ significantly. For the mutant protein however, irrespective of the excitation wavelength,

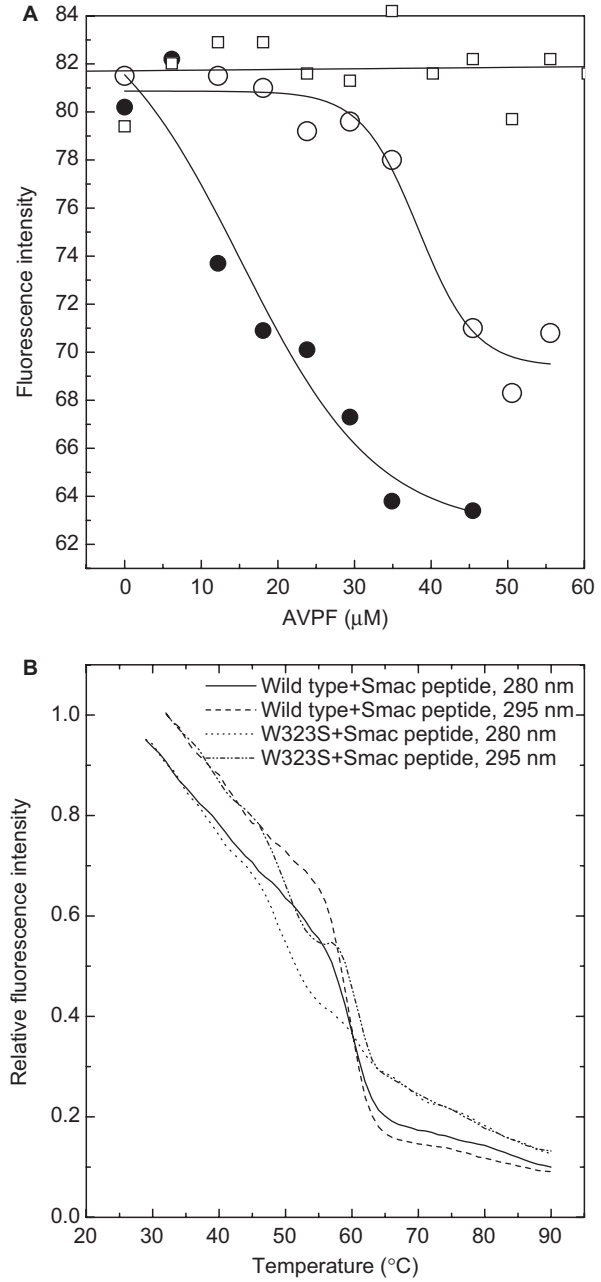

Fig. 3. **(A) Interaction of the wild type XIAP–BIR3, filled circles, W323S variant, open circles, and NAIP–BIR3, open squares, by SMAC-peptide.** Quenching of the fluorescence at 340 nm by SMAC-peptide was used to quantify the strength of the interaction between the protein and the peptide. (B). Thermal denaturation profile of 25 μM Wild type XIAP–BIR3 in the presence of 75 μM Smac-peptide exited at 280, solid line, and 295 nm, dashed line, and 25 μM W323S variant in complex with 75 μM Smac-peptide exited at 280, dotted line, and 295 nm, dash-dotted line.

two co-operative transitions at 50°C and 60°C were observed indicating that the presence of the peptide led to destabilization of the mutant BIR3 protein. This was surprising because this phenomenon was not observed for the wild type protein–peptide complex. Considering lower affinity of the peptide towards the W323S variant,

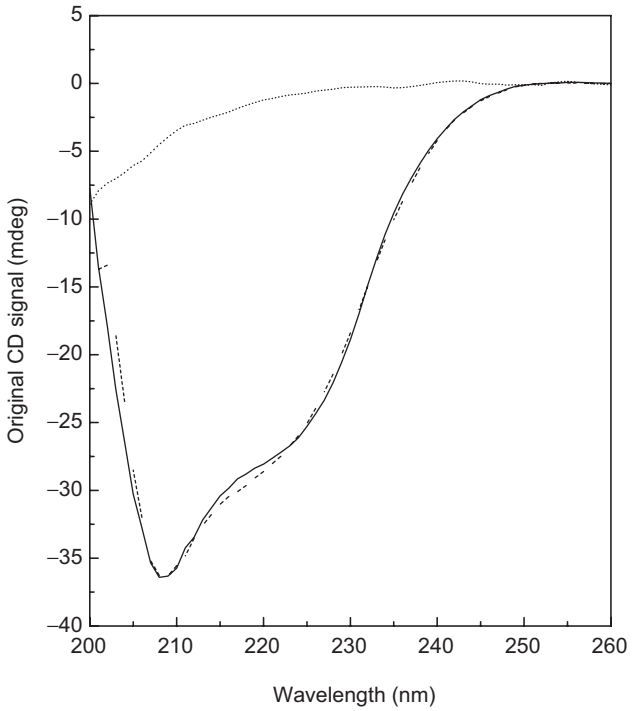

Fig. 4. **Circular dichroism spectra for 6.5 μM wild type XIAP–BIR3, solid line, and its W323S variant, dashed line, in the presence of 65 μM Smac-peptide.** The CD spectrum for the peptide alone is shown as a dotted line.

it is possible that the presence of the peptide forces the mutant protein to alter its conformation in order to accommodate the peptide in the IBM binding grove. To verify this hypothesis, we compared the conformation of the wild type and mutant XBIR3 in complex with Smac-peptide (Fig. 4). Thus, the CD spectra for the two complexes were obtained and compared to the curves of the individual proteins. The CD spectra of the two protein forms in complex with the peptide overlap very well, with minor differences centred around 217 nm. However, the differences for the individual proteins are more pronounced and occur at slightly higher wavelength around 220 nm (compare Fig. 4 with Fig. 2). This suggests that W323S variant adopts a structure more similar to that of wild type BIR3 in complex with Smac-peptide than its absence. Apparently, conformational changes of the W323S variant induced by AVPF binding is accompanied by the loss of stabilizing forces that otherwise would have existed. Alignment of the three-dimensional structures of XIAP–BIR3 alone (PDB access number of 1F9X) or in complex with AVPI peptide (PDB access number of 1G3F) shows that the peptide binding causes an increase in the β-structure of the protein (data not shown). It seems that this increase in β-structure is not favoured for the mutant protein. Therefore, W323, in addition to making direct contact with AXPX sequence, plays a vital role in maintaining the stability of the peptide–protein complex.

Interaction of BIR3 with Smac Protein—In order to dispel the concern that W323S variant may bind to

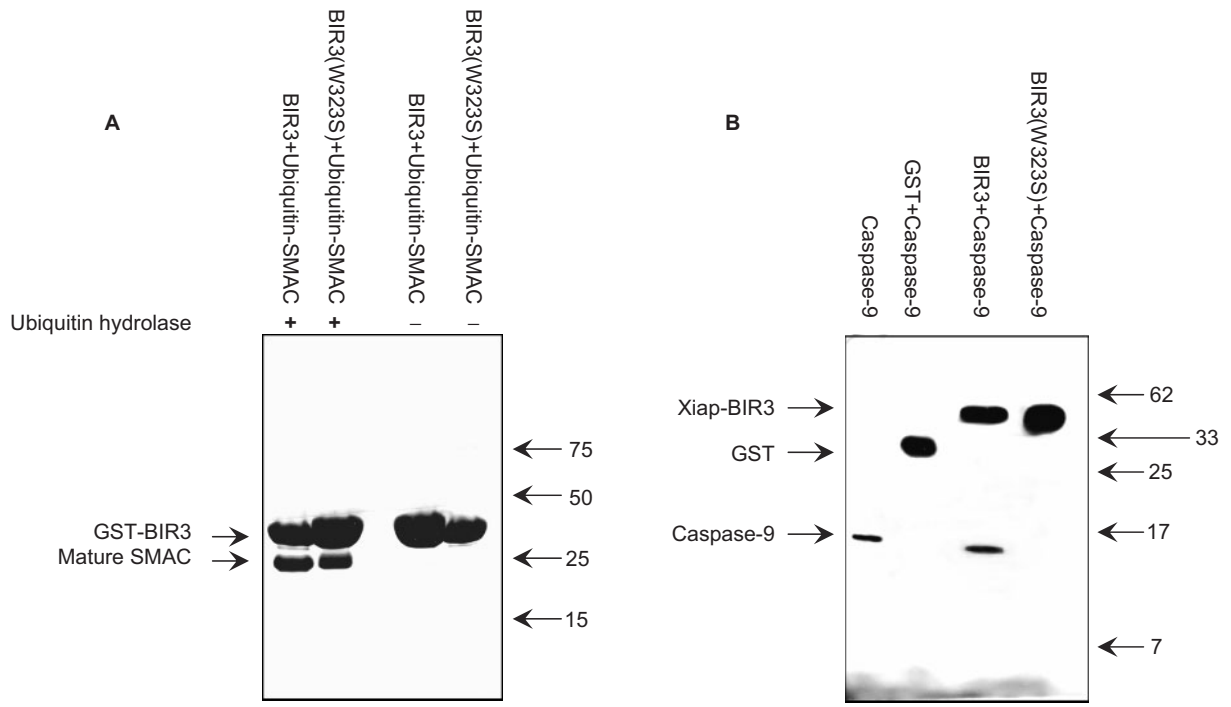

Fig. 5. **SMAC (A) and caspase-9 (B) pull down by the wild type and mutant GST–BIR3.** A. Glutathion–Resin bound to the wild type and mutant gst-BIR3 proteins were incubated with Ubi–Smac and washed. Half of each sample was treated with Ubiqutin *C*-terminal hydrolase in order to expose AVPI *N*-terminal sequence of Smac. Following interaction the samples were analysed by Western blot using a mixture of anti-gst polyclonal and anti-his monoclonal antibodies. B. GST resin alone, or bound to the wild type or mutant BIR3 proteins were incubated with Caspase-9 and washed. A mixture of anti-His monoclonal and anti-gst polyclonal antibodies was used to detect poly-histidine tagged caspase-9 and gst tagged proteins, respectively.

Smac-peptide but not to Smac-protein, we decided to perform Smac pull-down by both the wild type and the mutant Bir3 proteins. To do so, Ubiquitin–Smac fusion protein was produced in *E. coli* and purified. AVPI sequence is not located at the N-terminus of the fusion protein and only treatment with Ubiqutin C-terminal hydrolase removes Ubiquitin, exposing the AVPI sequence. Thus, wild type and mutant Bir3 proteins bound to glutathione resin were used to pull down Ubiquitin–Smac protein in the presence and absence of Ubiqutin C-terminal hydrolase (Fig. 5). Both the wild type and mutant Bir3 proteins affinity isolated the Smac protein when AVPI sequence was exposed (i.e. in the presence of Ubiqutin C-terminal hydrolase). These taken with fluorescence and CD data indicate that both the wild type and mutant Bir3 proteins interact with Smac-peptide as well as Smac protein.

Interaction of BIR3 with Caspase-9 Protein—It has been previously reported that mutation of His343 of Bir3 to Alanine compromised caspase-9 inhibition while caspase-9 binding still remained (*11, 12*). Therefore, lack of caspase-9 inhibition does not necessarily mean lack of caspase-9 interaction. Thus, caspase-9 pull down by wild type as well as mutant Xiap–Bir3 was performed to determine whether or not lack of caspase-9 inhibition by W323S variant reflects lack of caspase-9 interaction. In the pull-down experiment, the wild type, but not the mutant Bir3 protein, could precipitate the enzyme. This indicates that inability of W323S variant in caspase-9 inhibition results from the loss of caspase-9 binding. It is surprising that despite Smac-peptide and protein recognition by mutant Bir3, caspase-9 interaction was lacking. These findings strongly suggest that not every protein carrying IAP binding motif at the *N*-terminus will bind to proteins with AXPX binding grove. Furthermore, the absence of caspase-9 interaction while the presence of both Smac-peptide and protein interaction for W323S variant, leads us to conclude that W323 residue plays a very important role in inhibition of caspase-9 which is not solely due to its role in binding to the AXPX sequence of caspase-9. Considering the involvement of W323 in both the formation of a large protein–protein interface between Xiap–Bir3 and caspase-9 as well as recognition of AXPX motif at the *N*-terminus of the small subunit of caspase-9 (*13, 14*), it appears that the structural alterations of W323S variant mostly affects the interactions between the large protein–protein interface of BIR3–caspase-9 complex. This in turn leads to loss of caspase-9 inhibition as well as interaction of W323S variant without the loss of Smac-peptide binding. In other words, the Smac-peptide binding does not necessarily translate into caspase-9 interaction. Finally, failure of Xiap–Bir3 in becoming more similar to Naip–Bir3 by mutating W323 to Serine indicates that the structural diversity of Bir3 domain of IAPs is rendered through numerous mutations during the course of evolution.

This research was supported by University of Tehran and the Grant 04-358 RG/BIO/AS from The Academy of Sciences for the Developing World.

REFERENCES

1. Fadeel, B. and Orrenius, S. (2005) Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J. Intern. Med.* **258**, 479–517
2. Wang, J., Chun, H.J., Wong, W. *et al.* (2001) Caspase-10 is an initiator caspase in death receptor signaling. *Proc. Natl. Acad. Sci. USA* **98**, 13884–13888
3. Wang, J. and Lenardo, M.J. (2000) Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. *J. Cell Sci.* **113**, 753–757
4. Li, P., Nuhawan, D., Budihardjo, I., Srinivasula, S.M. *et al.* (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479–489
5. Stennicke, H.R., Deveraux, Q.L., Humke, E.W. *et al.* (1999) Caspase-9 can be activated without proteolytic processing. *J. Biol. Chem.* **274**, 8359–8362
6. Liston, P., Fong, W.G., and Korneluk, R.G. (2003) The inhibitors of apoptosis: there is more to life than Bcl2. *Oncogene* **22**, 8568–8580
7. Deveraux, Q.L., Takahashi, R., Salvesen, G.S. *et al.* (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* **388**, 300–304
8. Roy, N., Deveraux, Q.L., Takahashi, R. *et al.* (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo. J.* **16**, 6914–6925
9. Deveraux, Q.L., Roy, N., Stennicke, H.R. *et al.* (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo. J.* **17**, 2215–2223
10. Deveraux, Q.L., Leo, E., Stennicke, H.R. *et al.* (1999) Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *Embo. J.* **18**, 5242–5251
11. Liu, Z., Sun, C., Olejniczak, E.T. *et al.* (2000) Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* **408**, 1004–1008
12. Wu, G., Chai, J., Suber, T.L. *et al.* (2000) Structural basis of IAP recognition by Smac/DIABLO. *Nature* **408**, 1008–1012
13. Srinivasula, S.M., Hegde, R., Saleh, A. *et al.* (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* **410**, 112–116
14. Shiozaki, E.N., Chai, J., Rigotti, D.J. *et al.* (2003) Mechanism of XIAP-mediated inhibition of caspase-9. *Mol. Cell* **11**, 519–527
15. Davoodi, J., Lin, L., Kelly, J. *et al.* (2004) Neuronal apoptosis-inhibitory protein does not interact with Smac and requires ATP to bind caspase-9. *J. Biol. Chem.* **279**, 40622–40628
16. Boatright, K.M., Renatus, M., Scott, F.L. *et al.* (2003) A unified model for apical caspase activation. *Mol. Cell* **11**, 529–541
17. Maier, J.K., Lahoua, Z., Genron, N.H. *et al.* (2002) The neuronal apoptosis inhibitory protein is a direct inhibitor of caspases 3 and 7. *J. Neurosci.* **22**, 2035–2043
18. Stennicke, H.R. and Salvesen, G.S. (1999) Caspases: preparation and characterization. *Methods* **17**, 313–319
19. Hunter, A.M., Kottachchi, D., Lewis, J. *et al.* (2003) A novel ubiquitin fusion system bypasses the mitochondria and generates biologically active Smac/DIABLO. *J. Biol. Chem.* **278**, 7494–7499
20. Zou, H., Yang, R., Hao, J. *et al.* (2003) Regulation of the Apaf-1/caspase-9 apoptosome by caspase-3 and XIAP. *J. Biol. Chem.* **278**, 8091–8098