NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP

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Running Title: Structure and Mutagenesis of the Bir3 Domain of XIAP

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Summary

The inhibitor of apoptosis proteins (IAPs)\(^1\) regulate the caspase family of cysteine proteases which play an important role in the execution of programmed cell death. Human XIAP is a potent inhibitor of caspases-3, -7, and -9. Here we show that the Bir3 domain is the minimal region of XIAP that is needed for potent caspase-9 inhibition. The three-dimensional structure of the Bir3 domain of XIAP, determined by NMR spectroscopy, resembles a classical zinc finger and consists of five \(\alpha\)-helices, a three-stranded \(\beta\)-sheet, and a zinc atom chelated to three cysteines and one histidine. The structure of the Bir3 domain is similar to that of the Bir2 domain of XIAP but differs from the previously determined structure of the Bir3 domain of MIHB. Based on site-directed mutagenesis, we have identified the regions of the Bir3 domain of XIAP that are important for inhibiting caspase-9. Despite the structural similarities of the Bir2 and Bir3 domain of XIAP, a different set of residues were found to be critical for inhibiting the individual caspases. These results suggest that XIAP inhibits caspase-3 and caspase-9 in a different manner.
Introduction

Programmed cell death is a tightly regulated process that is critical for normal development and tissue homeostasis and when dysregulated can lead to a variety of diseases such as neurodegenerative disorders and cancer (1). One class of proteins that negatively regulates cell death signalling is the inhibitor of apoptosis proteins (IAPs). IAPs are highly conserved and have been found in many species (2-6). The members of this family are characterized by having one or more baculovirus IAP repeats called Bir domains. Bir domains consist of approximately 70 amino acids that contain the characteristic signature sequence CX_2CX_{10}HX_6C (7,8). Some IAPs also contain a C-terminal ring finger that contains one zinc atom chelated to three cysteines and one histidine and another zinc ligated to four cysteines (9).

One of the major functions of the IAPs is their ability to bind to and inhibit the cysteine proteases known as caspases (10,11) which play a key role in the execution of programmed cell death (12). Caspase inhibition by the IAPs can directly explain their antiapoptotic activities. For human XIAP, the region responsible for inhibiting caspases-3 and –7 was localized to a fragment containing the second Bir domain (13). Although the Bir domain was necessary for caspase-3 inhibition, residues outside of the Bir2 domain were also found to be critical for inhibiting caspase-3 (14). On the basis of site-directed mutagenesis and NMR studies on the interaction of XIAP with caspase-3, it was postulated that the residues N-terminal to the Bir2 domain of XIAP bind to the active site of this enzyme (14).

XIAP also inhibits caspase-9. However, a different portion of XIAP is involved. Recently, Deveraux et al. (15) have shown that it is the Bir3-ring finger of XIAP that potently inhibits caspase-9. Since neither the ring finger nor the Bir3 domain of XIAP was sufficient to inhibit
caspase-9, it was postulated that the ring finger may function to stabilize the Bir3 domain in a conformation required for caspase-9 inhibition (15).

Three-dimensional structures of the Bir2 domain of XIAP (14) and the Bir3 domain of MIHB (cIAP-1) (16) have been reported. The structure of the Bir2 domain of XIAP resembles a classical zinc finger and consists of four α-helices and a three-stranded antiparallel β-sheet. The structure of the Bir3 domain of MIHB, on the other hand, was reported (16) to be an α-helical protein with a fold not previously observed for zinc-binding proteins.

In this paper, we have identified the mimimal region of XIAP that is required for inhibiting caspase-9. In contrast to earlier studies (15), we find that the ring finger is unnecessary for potent caspase-9 inhibition. The Bir3 domain alone is sufficient. We have also determined the three-dimensional structure of the Bir3 domain of XIAP. The structure of this protein is compared to the previously determined structures of the Bir2 domain of XIAP and Bir3 domain of MIHB. Finally, on the basis of site-directed mutagenesis, we have identified amino acids of XIAP that are important for caspase-9 inhibition.
Materials and Methods

Protein Preparation - XIAP (residues 241-497) was cloned from a Jurkat cDNA library into the pET-28b vector (Novagen). C-terminal deletions were created by introducing a stop codon at the appropriate positions. Site-directed mutants were prepared using the Quick-change mutagenesis kit (Stratagene). The coding region in all of the plasmids was confirmed by sequencing.

The protein (residues 241-356) used in the structure determination was expressed in the *E. coli* strain BL21(DE3) (Novagen). Uniformly $^{15}$N/$^{13}$C and $^{15}$N labeled proteins were prepared by growing bacterial cells in a minimal medium containing a trace amount of Zn(Ac)$_2$ and $^{15}$NH$_4$Cl with or without uniformly $^{13}$C-labeled glucose. Recombinant proteins were purified using a nickel-NTA column (Qiagen) followed by gel-filtration (Pharmacia). The N-terminal His-tag was cleaved with thrombin (Novagen), leaving four extra residues (GSHM) at the N-terminus.

NMR Spectroscopy - NMR samples contained 1.7 mM protein in 50 mM bis-Tris-d$_{19}$ (pH=7.2), 300 mM KCl, 50 uM Zn(Ac)$_2$, and 1 mM DTT. NMR spectra were acquired at 30°C on a Bruker 500, 600, or 800 MHz NMR spectrometer. The $^1$H, $^{15}$N, and $^{13}$C resonances of the backbone and side chains were assigned using a standard set of double and triple resonance experiments (17). The prochiral methyl groups of Val and Leu were stereospecifically assigned by recording an HSQC spectrum of a 15% $^{13}$C -labeled sample of the protein (18). Distance restraints were derived from $^{15}$N- and $^{13}$C - resolved 3D NOESY experiments. Slowly exchanging amide protons were identified from a series of $^1$H-$^{15}$N HSQC spectra after the H$_2$O buffer was exchanged to a buffer containing $^2$H$_2$O. Residual dipolar couplings were measured as
previously described (19, 20) using a $^{13}\text{C}^{15}\text{N}$ labeled sample of the protein dissolved in a solution containing phage at a concentration of 17 mg/ml.

*Structure Calculations* - In the first step of the structure calculations, 577 unambiguous NOEs, 62 hydrogen bond restraints, and 84 $\phi$ or $\varphi$ angular restraints derived from the TALOS program (21) were incorporated into a torsion angle dynamics (22) and simulated annealing protocol (23) using the program CNX (MSI Inc., San Diego, CA). A single family of low energy structures was obtained from these calculations that defined the overall fold. Refinement of these initial structures was accomplished using the ARIA protocol (24). New NOE assignments were accepted from ARIA if they agreed with the manually-derived NOEs. From 8 iterations, 1280 unambiguous and 904 ambiguous NOE assignments were derived. In the final round of refinement, 46 N-H, 26 $C^{\alpha}$-H$^{\alpha}$, 30 $H^{N}$-C', and 43 $C^{\alpha}$ -C' residual dipolar couplings were used along with the NOE-derived distance restraints, angular restraints from TALOS, and hydrogen bond restraints.

*Caspase Inhibition Assays* - Caspase-9 inhibition was measured using a truncated form of the enzyme that lacks the N-terminal CARD domain. This form of the enzyme has the full catalytic activity and the same substrate specificity as the full length protein (Krebs and Wu, unpublished data). LEHD-AMC (Calbiochem) was used as the substrate in the caspase-9 assay. The reaction mixture (200 uL) contained 0.55 nM caspase-9 and 25 uM substrate in the assay buffer (20 mM Hepes, 0.1 mM EDTA, 0.1% Chaps, 10% sucrose, and 5 mM DTT) at pH 6.5. The activity was measured at 30 °C by monitoring the formation of fluorescent AMC over time at 460 nm using an excitation wavelength of 360 nm. IC$_{50}$ values were calculated from the percent inhibition measured using various concentrations of XIAP proteins. XIAP proteins were prepared as 8 $\mu$M
stock solutions in the assay buffer and subsequently serially diluted to give a ten-point curve. In each assay, a control inhibitor (DEVD-aldehyde) was included.

Results and Discussion

Minimal region of XIAP that inhibits caspase-9 - To identify the minimal region of XIAP that inhibits caspase-9, a series of truncated XIAP proteins were expressed in bacteria, purified, and tested for their ability to inhibit recombinant caspase-9 \textit{in vitro} (Fig. 1). We also tested the ability of these proteins to inhibit a panel of caspases (caspase-1, -3, -6, -7, -8, and -9). Consistent with earlier results (15), the Bir3-ring finger was found to specifically inhibit caspase-9. However, in contrast to this earlier study, we found that the ring finger is unnecessary for caspase-9 inhibition. Indeed, much smaller proteins containing only the Bir3 domain were sufficient to inhibit caspase-9 (Fig. 1). The reason for this apparent discrepancy was investigated by characterizing the shorter protein (residues 261-336) used in the previous work by NMR spectroscopy. The amide protons observed in an $^{15}\text{N}-^{1}\text{H}$ correlation spectrum of the uniformly $^{15}\text{N}$-labeled protein appeared in a relatively narrow frequency range. These results strongly suggest that the protein is unfolded and can explain why the short protein was unable to inhibit caspase-9. The smallest protein that was folded and potently inhibited caspase-9 contained residues 241-356 (Fig. 1). This protein was chosen for the structural studies.

[Figure 1] [Table 1]

Structure determination of XIAP (residues 241-356) - The $^{1}\text{H}/^{15}\text{N}$ HSQC spectrum of the Bir3 domain (residues 241-356) was well-dispersed which allowed the signals to be readily
assigned (Fig. 2). A total of 2475 NMR-derived restraints were used in the structure calculations (Table 1). Fig. 3 depicts the ensemble of 20 structures of the Bir3 domain of XIAP that were derived from the NMR data. The r.m.s.d. about the mean coordinate positions in the ensemble for residues 255-345 is 0.70 ± 0.07 Å for the backbone atoms and 1.41 ± 0.16Å for all heavy atoms. The N-terminus (residues 241-254) and C-terminus (residues 345-356) corresponding to the amino acids outside of the Bir3 domain are disordered. However, the Bir3 domain is well-defined except for residues I276-N280 and T308-E314. The amide protons of the ill-defined residues were not observed in any of the NMR spectra, suggesting that these signals are exchange broadened.

[Figure 2 and 3]

The structure of XIAP (241-356) consists of five α-helices, three β-strands, and a zinc atom which is coordinated to the conserved residues C300, C303, C327, and Ne2 of H320 (Fig. 4a, Fig. 5). The first α-helix (residues 265-272) runs antiparallel to the second (residues 282 - 286). These two α-helices are followed by the conserved residues A287 and G288 which form a sharp turn, leading to the three-stranded antiparallel β-sheet. The β-sheet is followed by α3 (residues 316-322), α4 (residues 327-333), and α5 (residues 336-342) which are nearly perpendicular to one other. α5 packs against α3 and forms a hydrophobic cluster involving W317, I339, and I342. These interactions appear to be important and can explain why the shorter protein (residues 261-336) lacking the C-terminal α-helix is unfolded and unable to inhibit caspase-9.

[Figures 4 and 5]
The surface of the Bir3 domain is depicted in Fig. 6. In addition to the conserved hydrophobic residues (F272, L284, F289, V298, L330) that form the core of the Bir3 domain, there are several hydrophobic residues that are exposed on the surface, including F270, W275, V279, and W310. Also noteworthy is a patch of negatively charged residues on the surface which is composed of E314, D315, and E318 (Fig. 6). On the other side of the protein, there is a cluster of positively charged residues (R268, R258, and K299).

[Figure 6]

**Structural comparison to other Bir domains** - The structure of the Bir3 domain of XIAP is very similar to that of the Bir2 domain of XIAP (Fig. 4, 5). They both contain a three-stranded antiparallel β-sheet, α-helices of similar lengths and orientations, a zinc atom chelated by three conserved cysteines and a histidine, and a hydrophobic core made up of highly conserved residues. The r.m.s.d. between the Bir3 and Bir2 domain of XIAP is 1.5 Å for 49 residues located within the conserved secondary structural elements. The only difference is the presence of an extra C-terminal α-helix in the Bir3 domain. The residues that form the C-terminal α-helix are highly conserved in Bir3 domains but not in other Birs, suggesting that the C-terminal α-helix may be uniquely found in Bir3 domains. Indeed, the Bir3 domain of MIHB (Fig. 4c) contains a C-terminal α-helix corresponding to the one found in the Bir3 domain of XIAP. In addition, like the Bir2 and Bir3 domain of XIAP, a zinc is chelated to three cysteines and a histidine. However, unlike the Bir domains of XIAP, the Bir3 domain of MIHB lacks a β-sheet, is missing α4, and has shorter α-helices for α1 and α3 (Fig. 4, 5). These structural differences are surprising in view of the high sequence homology observed for the Bir domains (8).
Amino acids important for caspase-9 inhibition - In an earlier study (14), we found that the residues of XIAP important for caspase-3 inhibition were located in a linker region between the Bir1 and Bir2 domains that contained a sequence (DISD) resembling the residues found in substrates and inhibitors of caspase-3. The substrate specificity for caspase-9 is different and consists of the consensus sequence (I/L/V)-X-X-D. Interestingly, a stretch of amino acids (VSSDRNI) that resembles the substrate consensus sequence for caspase-9 was found N-terminal to the Bir 3 domain of XIAP. To determine whether these residues were important for caspase-9 inhibition, a truncated protein that lacked these residues was prepared. The truncated protein (residues 252-356) potently inhibited caspase-9 (Fig. 1). Furthermore, the mutation of D247 to alanine, which would be predicted to alter caspase-9 inhibition if this region were important, shows no effect (IC50 ~17 nM, Table 2). Therefore, unlike the importance of the residues N-terminal to the Bir2 domain for caspase-3 inhibition, the analogous region N-terminal to the Bir3 domain is not required for the inhibition of caspase-9.

Several other mutant proteins were prepared and tested for their ability to inhibit caspase-9 (Fig. 6, Table 2). Most of the mutant proteins showed no effect and potently inhibited caspase-9. However, mutation of E314 to a serine completely abolished caspase-9 inhibition. In addition, an attenuation of caspase-9 inhibition was observed for W275A and V279A, and a complete loss of caspase-9 inhibition was observed for the W310A and H343A mutant proteins (Table 2).

In order to check whether the mutant proteins were properly folded, $^{15}$N labeled proteins were prepared for those mutants that displayed a large loss of caspase-9 inhibition, and their $^1$H,
\(^{15}\)N-HSQC spectra were compared to a spectrum of the wild-type protein. With the exception of the W275A mutant protein, the spectra of the mutant proteins only displayed minor chemical shift differences compared to the wild-type protein, suggesting that the structures are very similar.

As shown in Fig. 6, the residues that have the largest effect on caspase-9 inhibition (E314, W310, H343) are all located in the same region of the protein. E314 is part of a cluster of acidic residues that make up a negatively charged patch on the surface of the protein (Fig. 6). However, the other acidic residues of this patch (D315 and E318) do not appear to be important for caspase-9 inhibition because when mutated to alanine show no loss of inhibitory potency (Table 2). W310 and H343 are located on the edge of a basic patch that forms a shallow pocket on the protein surface. This site appears to be the region of XIAP that interacts with caspase-9.

Conclusions

The IAPs play a critical role in the regulation of programmed cell death. One of their major functions is to bind to and inhibit the caspases which if left unchecked could lead to massive cell death. XIAP potently inhibits both caspase-3 and caspase-9. The inhibition of caspase-3 is accomplished by binding of the Bir2 domain and residues N-terminal to the Bir2 domain to the enzyme (14). In contrast, as shown here, only the Bir3 domain of XIAP is required to inhibit caspase-9. Residues N-terminal to the Bir3 domain or the ring finger are unnecessary for potent caspase-9 inhibition. The structure of the Bir3 domain of XIAP was found to be similar to the previously determined structure of the Bir2 domain of XIAP (14). Despite these structural similarities, however, the amino acid residues in the Bir3 domain found to be
important for caspase-9 inhibition are different from those necessary to inhibit caspase-3. Thus, XIAP employs a distinctly different mechanism to inhibit different caspases.
Footnotes

1The abbreviations are: IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein; Bir, baculovirus IAP repeat; NMR, nuclear magnetic resonance; AMC, 7-amido-4-methylcoumarin; r.m.s.d., root mean square deviation.

The atomic coordinate (code 1F9X) has been deposited in the Protein Data Bank.

Acknowledgements

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References


Figure Legends

FIG. 1. Schematic representation of the truncated XIAP proteins along with their inhibition constants (IC$_{50}$s) against caspase-9. The Bir3 and ring finger domains are indicated with hatched and cross-hatched boxes, respectively. The amino acids are numbered according to the full length protein. All proteins contain an N-terminal His-tag.

FIG. 2. $^{15}$N-$^1$H HSQC spectrum of the Bir3 domain (residues 241-356) of human XIAP. Assignments for the backbone NH peaks and side-chains of W317 and H320 peaks are labeled.

FIG. 3. Stereoview of the backbone of 20 NMR-derived structures of the Bir3 domain (residues 252-346) of XIAP.

FIG. 4. Ribbons (25) depiction of the structural comparison of (a) the Bir3 domain of XIAP (residues 255-346), (b) the Bir2 domain of XIAP (residues 155-234), and (c) the Bir3 domain of MIHB (residues 268-356). The $\alpha$-helices are shown in red, $\beta$-sheets in green, zinc in magenta, and the side chains of the residues that chelate zinc are colored yellow.

FIG. 5. Sequence alignment of the Bir3 domain of XIAP (XB3), the Bir2 domain of XIAP (XB2), and the Bir3 domain of MIHB (MB3). The secondary structural elements for each protein are color coded in red for the $\alpha$-helices and green for the $\beta$-strands. The circles above the sequence denote the residues that have been mutated in the Bir3 domain of XIAP and tested for caspase-9 inhibition. Open circles indicate no significant change in caspase-9 inhibition, the half-
filled circles indicate more than a three-fold decrease in caspase-9 inhibition, and the filled circles indicate the complete loss (IC₅₀ > 1 µM) of caspase-9 inhibition.

FIG. 6. (a) Two views of the electrostatic potential surface of the averaged minimized NMR structure of XIAP (245-356) prepared using the GRASP program (26). The red areas correspond to the negatively charged regions, and the blue areas represent the positively charged portions of the protein. (b) Surface representation of the Bir3 domain of XIAP in the same orientation as shown in Fig. 5a. Residues with little effect on caspase-9 inhibition are colored green; whereas, those residues that displayed a marked loss in their ability to inhibit caspase-9 (IC₅₀ > 1 µM) are colored magenta.
Table 1. Structural Statistics and rmsds for the 20 NMR-derived structures of the Bir3 domain of XIAP.

<table>
<thead>
<tr>
<th>CNX potential energies (kcal mol(^{-1}))(^b)</th>
<th>(&lt;\text{SA}&gt;)</th>
<th>(&lt;\text{SA}&gt;_r)</th>
</tr>
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<tbody>
<tr>
<td>(E_{\text{tot}})</td>
<td>288.0 ± 19.4</td>
<td>286.8</td>
</tr>
<tr>
<td>(E_{\text{bond}})</td>
<td>9.1 ± 1.2</td>
<td>9.3</td>
</tr>
<tr>
<td>(E_{\text{ang}})</td>
<td>120.2 ± 7.3</td>
<td>127.6</td>
</tr>
<tr>
<td>(E_{\text{impr}})</td>
<td>20.0 ± 2.5</td>
<td>18.4</td>
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<tr>
<td>(E_{\text{vdw}})</td>
<td>27.9 ± 4.2</td>
<td>33.4</td>
</tr>
<tr>
<td>(E_{\text{cdih}})</td>
<td>2.7 ± 0.7</td>
<td>2.7</td>
</tr>
<tr>
<td>(E_{\text{noe}})</td>
<td>30.8 ± 8.1</td>
<td>20.0</td>
</tr>
<tr>
<td>(E_{\text{sani}})</td>
<td>76.4 ± 5.0</td>
<td>73.2</td>
</tr>
<tr>
<td>Cartesian coordinate r.m.s.d. (Å)(^c)</td>
<td>N, Cα, C'</td>
<td>all heavy</td>
</tr>
<tr>
<td>residues 255-345</td>
<td>0.70 ± 0.07</td>
<td>1.41 ± 0.16</td>
</tr>
</tbody>
</table>

\(^a\)<\text{SA}> is the ensemble of 20 NMR-derived structures for XIAP(residues 241-356), \(<\text{SA}>_r\) is the energy-minimized mean structure.

\(^b\) Energies were calculated by using CNX. The \(F_{\text{repel}}\) function was used to simulate van der Waals interactions with a force constant of 4 kcal mol\(^{-1}\) \(Å^{-4}\) with the atomic radii set to 0.8 \(×\) their CHARMM values (27). Torsional restraints were derived from TALOS with 2 \(×\) standard deviation and a minimum error of ±15°. No torsional angle restraints were violated by more than 5° in any of the final structures. No NOE restraint was violated by greater than 0.4 Å in any structure. Residual dipolar coupling restraints were employed using the SANI potential energy function with a force constant of 0.5.
Atomic rmsd between the 20 NMR structure ensemble structures and the mean structure after superposition of all backbone atoms (N, C\(\alpha\), C') or all non-hydrogen atoms.
Table 2. Inhibition constants (IC_{50}) of XIAP mutants against caspases-9.

<table>
<thead>
<tr>
<th>Name</th>
<th>IC_{50} (nM)</th>
<th>Name</th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bir3-RF (241-497)</td>
<td>11 ± 4</td>
<td>E294A</td>
<td>36 ± 10</td>
</tr>
<tr>
<td>Bir3(241-356)</td>
<td>22 ± 9</td>
<td>D309A</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>D247A</td>
<td>16 ± 2</td>
<td>W310A</td>
<td>&gt;2µM</td>
</tr>
<tr>
<td>D264A</td>
<td>31 ± 7</td>
<td>E314S</td>
<td>&gt;2µM</td>
</tr>
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<td>F270K</td>
<td>46 ± 12</td>
<td>D315A</td>
<td>64 ± 18</td>
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<td>W275A</td>
<td>145 ± 3</td>
<td>E318A</td>
<td>31 ± 14</td>
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<tr>
<td>I276A</td>
<td>38 ± 11</td>
<td>E332A</td>
<td>38 ± 9</td>
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<td>Y277A</td>
<td>84 ± 3</td>
<td>E349A</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>V279A</td>
<td>161 ± 21</td>
<td>H343A</td>
<td>&gt;1µM</td>
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<td>37 ± 11</td>
<td>H346A</td>
<td>24 ± 6</td>
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<tr>
<td>R286A</td>
<td>34 ± 10</td>
<td>C351A</td>
<td>19 ± 3</td>
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