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Emmanuele Ambrosi, Stefano Capaldi, Michele Bovi, Gianmaria Saccomani, Massimiliano Perduca, et al.. Structural changes in the BH3 domain of SOUL protein upon interaction with the anti-apoptotic protein Bcl-xL. *Biochemical Journal*, 2011, 438 (2), pp.291-301. <10.1042/BJ20110257>. <hal-00614614>

HAL Id: hal-00614614

<https://hal.science/hal-00614614v1>

Submitted on 13 Aug 2011

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Manuscript BJ2011/0257 Revised

**STRUCTURAL CHANGES IN THE BH3 DOMAIN OF SOUL
PROTEIN UPON INTERACTION WITH THE ANTI-APOPTOTIC
PROTEIN BCL-XL**

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Running title: *Interaction of SOUL protein BH3 domain with Bcl-xL*

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SYNOPSIS

The SOUL protein is known to induce apoptosis by provoking the mitochondrial permeability transition and a sequence homologous to the Bcl-2 homology 3 (BH3) domains has been recently identified in it thus making it a potential new member of the BH3-only protein family. Here we present NMR, SPR and crystallographic evidence that a peptide spanning SOUL residues 147 – 172 interacts with the anti-apoptotic protein Bcl-xL. We have crystallized SOUL alone and the complex of its BH3 domain peptide with Bcl-xL and solved their three-dimensional structures. The SOUL monomer is a single domain organized as a distorted beta barrel with eight anti-parallel strands and two alpha helices. The BH3 domain extends across 15 residues at the end of the second helix and 8 amino acids in the chain following it. There are important structural differences in the BH3 domain in the intact SOUL molecule and the same sequence bound to Bcl-xL.

INTRODUCTION

The Bcl-2 (B-cell lymphoma-2) family are a group of evolutionarily conserved proteins that interact to maintain a balance between newly forming and old, damaged or superfluous dying cells. [1]. They play a central role in the regulation of apoptosis, or programmed cell death, a process that in multicellular organisms leads to the controlled death of unneeded or unwanted cells. A crucial event in apoptosis is the mitochondrial permeability transition (MPT), a drastic increase in the permeability of the mitochondrial inner membrane to low molecular weight solutes [2]. Through the regulation of apoptosis the Bcl-2 proteins have an important function in embryogenesis [3], tissue remodelling [4], and the immune response [5]. Their abnormal behaviour is linked to many diseases such as autoimmunity [6], neurodegenerative disorders [7] and cancer [8].

The effect of the Bcl-2 proteins on the apoptotic process is due to the presence of one or more conserved regions of amino acid sequences, known as Bcl-2 homology (BH) domains named BH1, BH2, BH3 and BH4 [9,10]. The proteins of this family that contain only the BH3 domain are pro-apoptotic and function as initial sensors of apoptotic signals resulting from various cellular processes whereas the pro-survival Bcl-2 family members, like Bcl-2 or Bcl-xL, wield their effect by binding and sequestering their pro-apoptotic counterparts [11]. Peptides spanning the sequence of BH3 domains appear to exert the physiological activity of the intact proteins and their complexes with anti-apoptotic members of the Bcl-2 family have received considerable attention since this interaction is believed to explain the effect at the molecular level. In particular the complexes of peptides with the sequences of the BH3 domains of Bad, Bim, Bak Bid and Beclin1 with Bcl-xL have been examined by X-ray crystallography and NMR and the conserved crucial interactions between the peptides and the protein have been identified [12,13].

Cancer cells frequently over-express the anti-apoptotic members of the Bcl-2 family and small molecules, that incorporate the structural features of the BH3 domains necessary for binding to these anti-apoptotic proteins, have been synthesized and are being tested as specific cancer cell killers [14].

SOUL was first identified at the transcriptional level by suppression subtractive hybridization in chicken retina and pineal gland, and its gene was named *ckSoul* because of the high transcript levels found in the pineal gland, the organ René Descartes hypothesized was the location of the Soul [15]. A few years before this report, human SOUL had been isolated and characterized from saline extracts of human term placentas and had been called placental protein 23 (PP23) [16]. More recently, the protein has also been identified in normal human amniotic fluid [17]. It has subsequently been shown that the gene coding for this protein is very widely distributed in evolution and it has been characterized in many other species including the popular model organism of plant biology *Arabidopsis thaliana*. On the basis of its sequence similarity with the mouse gene p22 HBP, which codes for heme-binding protein 1 or p22HBP, SOUL has also been called with the alternative name of heme-binding protein 2 (HEBP2) [18]. Recombinant mouse SOUL was reported to be a dimer in the absence of heme and to a hexamerize upon heme binding with a dissociation constant in the nanomolar range [19].

A very important observation is that SOUL can induce mitochondrial permeability transition, a condition that leads to mitochondrial swelling and cell death [20]. More recently, analysis of the human SOUL sequence revealed the presence of a putative

BH3 domain of the Bcl-2 protein family whose deletion abolished the apoptotic effects of SOUL [21].

In this paper we provide experimental evidence that SOUL is a BH3-only protein since the sequence spanning amino acids 147 – 172 interacts with the anti-apoptotic protein Bcl-xL. In addition, we have crystallized both intact SOUL and the complex of a peptide that contains its BH3 domain with Bcl-xL and solved their three-dimensional structures to 1.6 and 2.0 Å resolution respectively. The Bcl-xL SOUL BH3 domain-interactions are particularly interesting since the domain adopts a different structure when bound to Bcl-xL.

EXPERIMENTAL

Protein expression and purification

The cDNA coding for human SOUL (IMAGE ID 3445763), obtained from RZPD (Deutsches Ressourcenzentrum fuer Genomforschung GmbH), was amplified by PCR using primers designed to introduce restriction sites for BamHI and HindIII endonucleases and a sequence coding for a digestion site for thrombin in the C terminal end in the amplified fragment. After purification, the fragment and the expression vector pQE50 (Qiagen) were digested with the restriction enzymes mentioned above and incubated with ligase to insert the cDNA in the vector respecting the reading frame. BL21 C41 strain *E. coli* cells were transformed with the resulting vector, grown at 37°C and protein synthesis was induced overnight at 20°C with 0.5 mM IPTG (isopropyl beta-D-1 thiogalactopyranoside). Under these conditions of subcloning in pQE50, the expressed intracellular domain is fused to a histidine tag through its C terminus. The presence of the tag allowed the affinity purification of the fused protein by passing the bacterial extracts through a nickel-sepharose column. The column was equilibrated with 20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 10 mM imidazole and 0.02% NaN₃ and the bound protein was eluted with a linear gradient of imidazole from 10 to 500 mM. After the affinity column, the tag was removed by thrombin digestion and the protein was further purified by gel filtration in a Superdex G-200 column equilibrated with 20 mM Tris-HCl pH 7.5, 0.15 M NaCl and 0.02% NaN₃ and by hydrophobic interaction chromatography (Lipidex1000).

Recombinant human Bcl-xL (the cDNA used has the IMAGE ID 2823498 and was obtained from RZPD) was prepared in a similar way. A truncated form lacking the flexible loop spanning amino acids 27 - 82 and the last 24 amino acids which are the transmembrane domain was inserted in the pET15b vector which introduces an N terminal histidine tag and a thrombin digestion sequence. The purification protocol followed that of SOUL.

Complete removal of the tag was assessed by Western blot analysis using an anti-His-HRP-conjugated antibody (Sigma-Aldrich). The purified protein showed in both cases one band in SDS-PAGE.

UV/Visible Spectra were recorded with a UV/Vis Unicam spectrometer (Cambridge, U.K.). An aliquot of 250 µM of hemin dissolved in DMSO was diluted with 20 mM Tris HCl buffer 0.15 M NaCl pH = 7.5 so that the final hemin concentration was 10 µM. The concentration of the hemin solution was determined as described in the literature [22]. Two other samples were prepared adding, in addition to hemin, appropriate aliquots of SOUL and BSA dissolved in the Tris buffer to bring their final concentration to 100 µM. These samples contained thus a ratio of 10 times the molar concentration of SOUL and BSA with respect to the hemin concentration. The three samples were incubated for 30 minutes at room temperature and their UV-visible spectra recorded.

Nuclear Magnetic resonance measurements

For the production of ^{15}N -labeled human Bcl-xL lacking only the C terminal transmembrane domain, host cells were grown in M9 minimal medium using $^{15}\text{NH}_4\text{Cl}$ as sole nitrogen source. HSQC NMR spectra were recorded on a Bruker Avance spectrometer operating at 600.13 MHz, equipped with a cryoprobe. The labelled protein, dissolved in 20 mM Tris, pH = 7.5, 0.15 M NaCl (in 10 % D_2O) and at a concentration of 85 μM , was titrated with the SOUL BH3 peptide dissolved in the same buffer at a concentration of 600 μM . Nine additions were made so that, after correcting for the peptide precise concentration and taking into account dilutions, the molar ratio BH3 peptide/protein was 0.07, 0.17, 0.26, 0.35, 0.52, 0.69, 1.38, 2.77 and 3.83. After each of the additions the sample was incubated at 20°C for about five minutes and a 1D ^{15}N decoupled- ^1H spectrum and a [2D ^1H - ^{15}N] HSQC spectrum were recorded at the same temperature. Standard sequence schemes with pulsed field gradients were used to achieve the suppression of the solvent signal.

The dissociation constant was calculated from the shifts in two peaks in fast exchange in the methyl region of the decoupled- ^1H spectrum fitting the data with the equation

$$\Delta = \Delta_{\max} \frac{\left[(P + L + K_d) - \sqrt{(P + L + K_d)^2 - 4PL} \right]}{2P}$$

where Δ is the chemical shift change, P is the protein concentration, L is the total ligand concentration and K_d the dissociation constant. Appropriate corrections for the dilution of protein and ligand were made after the addition of each aliquot during the titration.

Surface plasmon resonance studies

Bcl-xL was immobilized on a COOH1 research-grade sensor chip (Nomadics) by amine-coupling chemistry using the manufacturer's protocols, and the SOUL BH3 domain peptide was used as the analyte. SPR measurements were carried out in HBS buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% P-20 surfactant) at 20°C using the SensiQ Pioneer instrument (ICx Technologies, Oklahoma City, U.S.A). Data were analyzed with the Qdat evaluation analysis software.

Crystallization, X-ray data collection, structure solution and refinement

Purified native SOUL was used at a concentration of about 20 mg per ml for the initial screen of crystallization conditions. Molecular Dimensions Structure Screens were used at 20°C with the hanging-drop method, mixing 1 μl of the protein solution with the same volume of the precipitating solution, and equilibrating versus a volume of 0.3 ml of the latter in the reservoir. The conditions yielding small crystals were later refined and the sitting-drop method with larger volumes was also tested until crystals that were large enough for data collection were obtained.

The SOUL BH3 peptide spanning amino acids 147 – 172 was synthesized by TAG Copenhagen A/S. The complex of recombinant human Bcl-xL with the peptide was prepared by mixing the protein at a concentration of about 4 mg per ml with four times the molar ratio of the peptide. The mixture was incubated for about one hour and then concentrated to about 15 mg per ml and used at this concentration for the crystallization experiments.

Two different crystal forms of native SOUL were obtained, both in the presence of 1-butyl-3-methylimidazolium chloride added to a concentration of 0.2 M because it improved drastically the diffraction properties of the crystals. The first crystal form is hexagonal, space group P6_122 with $a = b = 143.9 \text{ \AA}$ and $c = 242.1 \text{ \AA}$. It contains four molecules in the asymmetric unit (see Table 1), diffracts to about 2.85 \AA and appears to

be closely related to another crystal form reported in the literature [23]. The crystals grow by adding to the protein solution equal volumes of 0.1 M Tris-HCl pH 8.5, 2.0 M ammonium sulphate. The second crystal form is orthorhombic, space group $C222_1$, $a = 137.7 \text{ \AA}$, $b = 114.7 \text{ \AA}$ and $c = 67.4 \text{ \AA}$ and grows by mixing equal volumes of the protein solution and 0.85 M NaH_2PO_4 , KH_2PO_4 , 0.08 M Hepes, pH 7.5. These crystals diffract to a better resolution, about 1.6 \AA , contain two molecules in the asymmetric unit and are the crystal form that was solved first using the S.I.R. (single isomorphous replacement) method. The hexagonal crystal form was solved later by molecular replacement.

The best crystals of the complex human Bcl-xL – SOUL BH3 peptide grow by mixing equal volumes of the complex solution and 15 % PEG 6000, 0.2 M sodium sulphate and 0.3 M 1-butyl-3-methylimidazolium 2-(2-methoxyethoxy)ethyl sulphate. They are tetragonal, space group $P4_3$, with $a = b = 66.8 \text{ \AA}$ and $c = 175.2 \text{ \AA}$ and diffract to 2.0 \AA resolution.

The diffraction data were collected from crystals frozen at 100 K after a brief immersion in a mixture of 80% of the mother liquor and 20% glycerol. The data set for a gold heavy atom derivative used for phasing were obtained using copper $K\alpha$ radiation from a Rigaku RU-300 rotating anode X-ray generator with a Mar345 imaging plate area detector. The final data sets used for refinement of this and the other crystal forms were collected at the ID14-2 beamline of the European Synchrotron Radiation Facility in Grenoble ($\lambda = 1.001 \text{ \AA}$). The data were indexed, integrated and reduced using the programs AUTOMAR, MOSFLM and Scala [24]. The diffraction data statistics of the data sets used for refinement are summarized in Table 1.

Initial phases for the orthorhombic crystals to 2.3 \AA resolution were determined by the single isomorphous replacement method with the derivative data collected at the home source. Two gold sites were located in a difference Patterson map using the program SHELXS [25] and entered as input for the program autoSHARP [26] that was used to locate the minor sites of the derivative, and for density modification and final phasing to 1.8 \AA resolution. The electron density map thus produced was of very good quality and could be readily interpreted. The initial model of SOUL was built in this map using the program Coot [27].

Refinement was carried out initially using the program REFMAC [28] and, in a second stage, with the program Phenix.refine [29]. During the process of refinement and model building, the quality of the models was controlled with the program PROCHECK [30]. Solvent molecules were added to the model in the final stages of refinement according to hydrogen-bond criteria and only if their B factors refined to reasonable values and if they improved the R free. The model was finally subjected to a final round of TLS refinement.

The structure of the hexagonal crystal form of SOUL was solved using the CCP4 suite of programs for crystallographic computing. The initial phases were calculated by the molecular replacement method as implemented in the program MOLREP [31], with the coordinates of the orthorhombic model as search probe. The automatic search with data up to a resolution of 2.9 \AA gave a solution that placed in their correct position three out of the four molecules present in the asymmetric unit. Fixing these coordinates the fourth molecule was found by the same program. The score of this solution was 0.544 and its R factor 41.6 %.

A similar procedure was followed to solve and refine the structure of the complex human Bcl-xL – SOUL BH3 peptide using the coordinates of a protomer of Bcl-xL (32, Protein data bank accession code 2YXJ) as the search probe. After the four protomers of

Bcl-xL present in the asymmetric unit had been placed in their correct position, it became evident that they dimerized with domain swapping and at this point the extra electron density for the four BH3 helices present in the asymmetric unit was also very clear. The final refinement statistics for the models of the three crystal forms are summarized in Table 1.

RESULTS

X-ray structure of human SOUL

The SOUL monomer is a single domain structure organized as an open distorted beta barrel with eight anti-parallel strands. The barrel is open in the sense that the first and the last strands (A and E) are not in contact in the sheet (Figure 1). Two alpha helices connect the second to the third strand and the sixth to the seventh. They are both located on one face of the molecule and pack against the curved sheet that forms the barrel. A monomer of SOUL fits into a box with the approximate dimensions 56 x 47 x 40 Å. This fold is quite different from that of the canonical member of the BH3 only protein family, BID (Figure 1).

Examination of the secondary structure (Figure 1B) reveals that the molecule is made up of two repeated units, each with β - β - α - β - β topology related by a pseudo 2-fold axis of symmetry. Two SOUL monomers (A & B) are present in the asymmetric unit of the orthorhombic crystal form (Table 1). The secondary structure assignments of monomer A are, for the beta strands, the following: strand A, residues 39-43, B, residues 46-55, C, residues 89-94, D, residues 103-110, E, residues 127-132, F, residues 135-142, G, residues 174-178 and H residues 190-195. The two alpha helices span residues 58-73 and 148-164. In addition, residues 25 and 26 extend the beta sheet and residues 113-116 form an additional helix turn in both monomers. A minor difference between the two monomers was also observed: strand D of molecule B has one residue less at the N terminal end.

The space within the beta barrel is filled by the side chains of both hydrophobic and hydrophilic residues: Trp48, Met135, Leu137, Leu139 and Trp193 but also Arg41 and Arg132, Asp130 and Asp191, Thr90 and Tyr179. Packing of the first helix takes place through hydrophobic and the following specific contacts: Trp58 (NE1)-Ser91 (OG), Lys68 (O)-Glu124 (OE), Tyr72 (OH)-Pro120 (O), Tyr72 (O)-Gln77 (OE) and Ile73 (O)-Ile83 (N). Packing of the second helix involves the following specific contacts: Gln154 (OE)-Arg140 (NE) and Leu162 (O)-Lys167 (N).

Two NMR structures of murine p22HBP, have been published [33, 34]. The protein has about 28% sequence identity with murine SOUL. Comparison of the two SOUL molecules present in the crystallographic asymmetric unit of the orthorhombic crystal form with the two NMR models of murine p22HBP reveals that the four models differ substantially only in rather limited areas (Supplemental Fig. S-1). The zone where the two SOUL molecules in the asymmetric unit differ more from one another are the region before the first strand of beta sheet and the loop connecting strands C and D. The chains before the first strand are totally exposed to the solvent while in the case of the connection of strands C and D the loop in chain A is in close contact with a symmetry related molecule while the equivalent area in molecule B is in contact with the solvent. These differences are thus probably simply a consequence of molecular packing in the crystal. Although there is more variability in the two NMR structures of murine p22HBP the two models are very similar in the region connecting strands C and D which is also the region where both are most different from SOUL. This particular region of the SOUL molecule appears thus to be more variable than the rest of the molecule.

The BH3 domain predicted by sequence alignment to be present in SOUL, spans residues 158-172, i.e. the last 7 residues of the second alpha helix of the molecule and 8 amino acids in the loop connecting it to strand G. The domain is represented in light blue in Fig.1D. Additional details on this structure have been included as Supplemental Information.

Does SOUL bind hemin?

All our attempts to prepare co-crystals of SOUL and hemin failed. When crystallizations were set up, with molar ratios of hemin to SOUL of even up to five, the crystals obtained, after screening many different conditions, were invariably those of the apoprotein. Soaking the pre-formed crystals did not reveal any electron density other than that of the apoprotein. It was thus suspected that the interaction of the two molecules, if present, was not as strong as expected. For this reason the UV-visible spectrum of hemin was examined alone and in the presence of 10 times the molar ratio of SOUL and bovine serum albumin used as a control (Supplemental Fig S-2A). Whereas the sample containing BSA has, due to the ligand protein interaction, its peak higher and shifted from 390 to 401 nM, as expected for hemin bound with no axial coordination to a hydrophobic cavity [35], the sample containing SOUL shows only negligible differences that can be explained by the absorption of the protein present in the sample and cannot be considered as evidence of a SOUL-hemin interaction.

A second control was carried out recording ^{15}N , ^1H HSQC NMR spectra of ^{15}N labelled SOUL in the presence of increasing amounts of hemin dissolved in Tris buffer. Hemin aliquots corresponding to 0.5, 1, 2, 3 and 4 equivalents of SOUL were added to the protein sample and the spectra were recorded at 25°C (Supplemental Fig S-2B). After the addition of up to four equivalents of hemin per protein molecule the spectrum remains unaltered. Given the time involved to record the different spectra, a kinetic effect can be excluded in this case or at least if there is such an effect it has to be proposed that the reaction is so slow that, even after more than one day of observation, no change in the spectrum is detectable at all. These observations thus lead to the conclusion that, with the methods described here, no interaction between hemin and SOUL, that may be considered of physiological relevance, can be observed.

Interaction of the SOUL BH3 peptide with human Bcl-xL

The BH3 domain predicted by sequence alignment to be present in SOUL spans residues 158-172, i.e. the last 7 residues of the second alpha helix of the molecule and 8 residues in the loop connecting it to strand G. BH3 domains are known to be helical and, since we knew that in the structure of SOUL the helix began before, we decided to examine the interaction with the anti-apoptotic protein Bcl-xL of a 26 amino acids long peptide, spanning residues 147-172, i.e. covering the entire helix and the region predicted to be part of the BH3 domain by sequence homology. The sequence of the peptide studied is the following: SAQKNQEQLLTASILREDGKVFDEK. Figure 1D represents the SOUL monomer with the BH3 peptide colored light blue for the chain predicted to be part of the domain by sequence alignment and yellow for the rest of the peptide corresponding to the N terminal portion of the second alpha helix of SOUL.

The interaction of the peptide and the protein was examined in solution by one and two-dimensional NMR and by SPR. Figure 2A represents the amidic region of the ^{15}N , ^1H HSQC NMR spectrum of ^{15}N labelled human Bcl-xL (lacking the trans-membrane domain after amino acid 209) titrated with increasing amounts of the peptide. Note the significant changes in some of the peaks of the protein as the interacting BH3 peptide is added to the solution. Figure 2B shows the chemical shift displacements in the two peaks in fast exchange in the methyl region of the ^1H spectrum that were used to calculate an approximate dissociation constant of the interaction and Figure 2C shows

the fitting of these displacements as a function of equivalents of the BH3 peptide added. The dissociation constant values calculated using the two peaks are in reasonable agreement with one another and are 47.8 and 41.3 μM (see Figure 2C).

The SOUL BH3 peptide - Bcl-xL interaction was also studied by surface plasmon resonance (SPR). Both intact Bcl-xL and a form lacking amino acids 27-82 ($\Delta 27-82$) that is known to bind BH3 domains like the intact protein [36] were immobilized on a sensor chip and the SOUL BH3 domain peptide was used as the analyte. Similar results confirming the interaction were observed for both variants of Bcl-xL. Figure 2D shows a sensorgram of the $\Delta 27-82$ form of the protein. The dissociation constant estimated with this method is about 5 times the values observed in the NMR experiments. The discrepancy is probably due to errors in the estimate of the peptide concentration used in the experiments. However, we did not detect significant binding between intact SOUL and Bcl-xL (data not shown), which may reflect either a lack of interaction under the conditions tested or more probably the requirement of drastic structural changes in SOUL.

X-ray structure of the SOUL BH3 domain peptide complexed with Bcl-xL

The data collection and refinement statistics of the co-crystals of human Bcl-xL ($\Delta 27-82$) with the SOUL BH3 peptide are summarized in Table 1. The crystals are tetragonal, space group $P4_3$ and present 50% merohedral twinning. The structure was solved by molecular replacement, initially assuming that the space group was $P4_32_12$ but the model could not be properly refined. A standard statistical test of the structure factors revealed that the correct space group is $P4_3$ with the twinning law $h, -k, -l$. The law was introduced in the program Phenix.refine [29] and the model was refined to give the statistics listed in Table 1. The asymmetric unit contains four Bcl-xL protomers organized as dimers that exhibit domain swapping exchanging their $\alpha 1$ N terminal helix (Fig 3A). This kind of domain swapping has been observed in several co-crystals of BH3 domains and human Bcl-xL ($\Delta 27-82$) [13,37,38,39]. It is considered to be an artifact due to the $\Delta 27-82$ deletion but it does not affect in any way the BH3 domain binding activity or anti-apoptotic properties of the protein. In fact, the complex in solution of the Beclin 1 BH3 domain and another truncated form of Bcl-xL, studied by NMR exhibits the same interactions observed in the crystals [40]. The two dimers present in the asymmetric unit of the crystals of the SOUL BH3 complex are very similar to each other, with a root mean square standard deviation of 0.607 Å over 314 alpha carbon atoms of Bcl-xL and the BH3 domains. Their Bcl-xL part is also quite similar to that of the other domain swapped dimers of human Bcl-xL ($\Delta 27-82$) that exchange the N terminal $\alpha 1$ helix. The rms deviations for the 276 alpha carbons are 1.845 Å for the Beclin 1 complex [13, PDB code 2P1L], 2.425 Å for the BIM L12F mutant peptide complex [38, PDB code 3IO8] and 2.216 Å for the helical α/β peptide foldamer complex [39, PDB code 3FDM].

The structures of several BH3 domains in complex with Bcl-xL have been examined. They all reveal that the BH3 sequence forms an amphipathic helix that inserts into a hydrophobic groove on the surface of the anti-apoptotic protein [41,42,43].

The four SOUL BH3 domain peptides observed in the co-crystals with Bcl-xL present an ordered structure which in every case contains more amino acids at the N terminus than those predicted by sequence similarity (residues 158-172 of the SOUL sequence). Of the 26 amino acid long peptide co-crystallized with Bcl-xL, for only the first three (S147-A148-Q149) there is no clear electron density in any of the four peptides present in the asymmetric unit. In all the four BH3 domain peptides examined the helix

observed is at least 18 amino acids long (153 EQLTLASILREDGKVF_D 170). Figure 3B represents in two colours the BH3 domain peptide bound to a protomer of Bcl-xL that more closely corresponds to the prediction (chain E). The extra amino acids at the N terminus are represented in a different colour. Figure 3C shows the electron density of the BH3 domain peptide oriented as in (B), with the Bcl-xL protomer represented as a space-filling model. In intact SOUL, the last amino acid of the second alpha helix is Glu164 and thus 8 amino acids with the sequence DGKVFDEK in the loop following the helix change their conformation upon interaction with Bcl-xL to become the last portion of the BH3 helical domain in the complex.

The interactions of the SOUL BH3 domain peptide with Bcl-xL are mostly hydrophobic (Table S-1 and Fig 4) but they include also other, more specific contacts, due to charged residues: Gln154 (BH3) – Gln111 (Bcl-xL), Ser160 (BH3) – Glu129 (Bcl-xL), Arg163 (BH3) – Glu129 (Bcl-xL), Asp165 (BH3) – Tyr101 (Bcl-xL) and Asp170 (BH3) – Tyr195 (Bcl-xL). Residues participating in hydrophobic contacts are Leu 155, 156, 158 and 162 and Phe172 of the BH3 domain peptide and Leu 108, 112 and 130, Val 126 and 141, Phe 97 and 105, Tyr 101 and 195 and Trp137 of the Bcl-xL molecule.

The amino acid contributions to the free energy of binding of BH3 peptides to Bcl-xL have been calculated using molecular dynamics simulations coupled with the molecular mechanics/Poisson-Boltzmann surface area method [12]. The Bcl-xL residues that give important contributions are: Phe97, Tyr101, Leu112, Val126, Leu130, Arg139, Tyr185 and Phe146. With the exception of the last residue, they all participate in the interactions with the SOUL BH3 domain peptide. Leu 158 and 162 of SOUL BH3 correspond to Leu 112 and 116 of Beclin 1 [13] and to Ile90 and Leu94 of BIM [43], Phe169 corresponds to Phe123 in Beclin 1 and Phe101 in BIM. Arg163 of the SOUL BH3 domain peptide, one of the residues controlling the specificity of binding, corresponds to Lys117 in Beclin 1 and Arg95 in BIM. These three basic amino acids are hydrogen bond donors to Glu129 of Bcl-xL.

Two of the specific charged residue contacts of the SOUL BH3 domain peptide and Bcl-xL are represented in Figures 4A and 4B. They involve Arg163 and Asp170 of the BH3 domain peptide. The first interaction is established with Glu129 of Bcl-xL while the second is with Tyr195. Analogous contacts are found in both Beclin 1 and BIM. The sequence of the peptide is aligned to those of several well known BH3 only proteins in Fig 4C. Note that some amino acids in the N terminal region of the peptide are also present in other members of the family.

Fig 5A superimposes the domain in intact SOUL with the helical conformation bound to Bcl-xL found in the crystals. It is evident that very drastic changes are required to transform the structure of the free domain into the bound one. Fig 5B shows that binding of the BH3 domain peptide of SOUL to Bcl-xL (Δ 27-82) is quite similar to the binding of another recognized BH3 domain protein, Beclin1. Only one important contact is missing in SOUL, that of an Asp (121 in Beclin 1) with Arg139 of Bcl-xL which is conserved in all the BH3 domain peptides studied so far. The absence of this interaction in SOUL might explain the relatively high dissociation constant we have observed for this complex.

DISCUSSION

The BH3-only members of the Bcl-2 protein family play a central role in the process that leads to programmed cell death or apoptosis. Their effect is due to inhibition through binding of their BH3 domain, in the hydrophobic cleft of the anti-apoptotic members of the Bcl-2 family like Bcl-2, Bcl-xL or MCL-1. Indeed one of the criteria to include a protein in this group is a demonstration of its interaction with one of the pro-

survival proteins of the Bcl-2 family, in addition to showing that they have a cellular death-inducing activity. On the basis of these criteria, since the discovery of the first member of the family, BIK [44], many other members have been added to the list [9,10]. In many cases the proteins seem to have additional functions, besides their role in inducing cellular death. Although a very large number of complexes of BH3 peptides with pro-survival proteins of the Bcl-2 family are available [12, 45], only one NMR structure of an intact BH3 only protein is known, BID [46, 47].

Two different mutually non-excluding functions have been attributed to SOUL: heme-transport [19] and a role in apoptosis as a BH3 only protein [20, 21]. We have used NMR, SPR, UV spectroscopy and crystallography to explore both functions and, in addition, we have determined the three-dimensional structure of the protein. Human SOUL is a monomer with a fold which is quite different from that of the other BH3 only protein whose three-dimensional structure is known, BID, which is similar to Bcl-xL. BID contains eight alpha helices, two central, hydrophobic surrounded by six amphipathic with their hydrophilic face exposed to the solvent. The SOUL molecule is similar to murine p22HBP [33,34].

The interaction of human SOUL with hemin was explored by titrating the latter with the former and following the UV spectrum, using as a control albumin. In our experiments we made sure that the protein we used did not contain even traces of the histidine tag used for purification and that no imidazole, residual from the purification in the affinity columns, was present in the samples. We did not find any evidence of an interaction of hemin with SOUL. This result was confirmed by the ^{15}N , ^1H HSQC NMR spectra of ^{15}N labelled SOUL in the presence of increasing amounts of hemin. We do not have an explanation of why our results appear to be at variance with those reported for mouse SOUL [19]. The latter is reported to be a dimer in the absence of heme and a hexamer in the bound state which is different from both the current work and work on the related p22HBP proteins. It is also worth noticing that the histidine residue that Sato et al. found to be essential for heme binding does not seem to be involved in heme binding to p22HBP that appears to bind this moiety through hydrophobic interactions and not metal coordination.

The interaction of a peptide spanning residues 147-172 of human SOUL with human Bcl-xL was also studied with ^{15}N , ^1H HSQC NMR spectroscopy using two different forms of human Bcl-xL, the entire molecule lacking only the hydrophobic trans-membrane domain after amino acid 209 and another truncated form lacking also amino acids 27-82 (Δ 27-82). The results were comparable and indicated in both cases that there is an interaction of the 26 amino acid SOUL peptide with Bcl-xL with a dissociation constant estimated in 40-50 μM . These results were confirmed by SPR measurements and by preparing co-crystals of the complex and solving their three-dimensional structure. The new crystal structure revealed swapping of the first helix of the Bcl-xL dimer, a phenomenon associated with the presence of the Δ 27-82 truncation. The amino acids participating in the interaction of BH3 domain and protein were identified. The interactions are mostly hydrophobic but a significant number of specific charged residue contacts were also found to be present.

When the SPR experiments performed to detect the interaction of the SOUL BH3 domain peptide are repeated using the entire SOUL molecule instead, no interaction is detected between ligand and analyte. This result might be explained by the fact that our data predict a very drastic conformational change in the protein molecule to allow the portion of polypeptide chain involved in the contacts to adopt the conformation required for the contacts to be established. The last eight amino acids of the BH3 domain are not in helical conformation in intact SOUL and, in addition, side chains that are important

for the interaction point towards the interior of the molecule and are not available on the protein surface. Conformational changes in the anti-apoptotic members of the Bcl-2 family upon interaction with BH3 domains have been described [38] as well as changes in the interacting BH3 domains [41,46]. In addition, it has been shown that Bim, Bad and Bmf have intrinsically unstructured BH3 domains that undergo a localized conformational change upon binding to pro-survival Bcl-2 targets [48]. No important changes were found in Bcl-xL but the changes in the BH3 domain are remarkable and suffice to explain why no interaction is observed with the intact SOUL molecule. These drastic modifications might require conditions that have not yet been found but that should be further explored given the importance of this interaction in the functionality of the two proteins.

The mechanism of activation of BID, the prototype of BH3 only proteins, involves cleavage by caspase 8 in a region with the sequence LQTDG [46]. The second amino acid in the sequence can be an E and the last can be any amino acid other than P, E, Q, K or R. This cleavage site is not present in SOUL although the similar sequence LESDV spans residues 123-127 and is exposed to the solvent in the molecule, in the loop connecting strand D to E. However, a test with caspase 8, using human BID as a control reveals that SOUL is non a substrate of this enzyme (data not shown).

The role of SOUL in inducing apoptosis is documented but up to now there was no information at the molecular level on the mechanism through which this function is accomplished. We have shown that its BH3 domain interacts with a pro-survival member of the Bcl-2 family and thus have provided new evidence that SOUL behaves like a novel member of the expanding family of BH3-only proteins.

Two very important questions remain unanswered: the nature of the molecular alteration that intact SOUL must undergo for the interaction to take place and the precise specificity of the interaction of the SOUL BH3 domain with different members of the Bcl-2 family.

ACKNOWLEDGEMENTS

We are grateful to Michael Assfalg and to Pete Simpson for their help in recording and interpreting the NMR spectra and to the staff of the ESRF in Grenoble (Proposal MX-805) for assistance during data collection. This work was supported by grants from the Fondazione Cassa di Risparmio di Verona, Vicenza, Belluno e Ancona. The NMR facility of the University of Verona is funded by Fondazione Cariverona and the Department of Biotechnology. Emmanuele Ambrosi is recipient of a scholarship granted by Fondazione Cassa di Risparmio di Padova e Rovigo and Gianmaria Saccomani was supported by Banca Popolare di Verona e Novara.

The coordinates of the models and the structure factors of SOUL and of the complex of human Bcl-xL with the peptide have been deposited in the protein data bank; accession codes 3R85, 3R8J & 3R8K

Abbreviations used: HEBP, heme-binding protein; PP₂₃, placental protein 23; Bcl-xL, apoptosis regulator Bcl-X, Bcl-2-like protein 1, HSQC, ¹⁵N, ¹H Heteronuclear Single Quantum Correlation; SPR, surface plasmon resonance.

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Figure legends

Figure 1. Overall structure and folding of SOUL

(A) Stereodiagram of the SOUL molecule. The eight-stranded β sheet is shown in two colours, red and blue, to emphasise the presence of two repeated units, each with β - β - α - β topology, related by a pseudo-2-fold axis of symmetry.

(B) A topological diagram of SOUL. The eight antiparallel β -strands form a distorted β -barrel with the two α -helices arranged on one face. Strands are labelled in the order of their appearance from the N terminus to the C terminus using the letters A-H. The 8 strands span the following residues: A, 39–43; B, 46–55; C, 89–94; D, 103–110; E, 127–132; F, 135–142; G, 174–178; H, 190–195. The two helices span residues 58–73 and 148–164.

(C) Ribbon representation of the SOUL monomer viewed in a direction rotated approximately 90° with respect to Fig 1 (A). The two helices are yellow, and the strands of beta sheet have been coloured as in Figs 1 (A & B) to emphasise the presence of the pseudo two-fold axis.

(D) Ribbon diagram of SOUL with the peptide spanning the BH3 domain represented in light blue for the portion of the chain identified by sequence similarity with known BH3 domains and yellow for the rest of the second helix of the molecule.

(E) Ribbon diagram of the NMR model of BID (lowest energy structure, PDB code 2BID) with the BH3 peptide and domain oriented and colour coded as in (D).

The figures of the models were prepared using the program PyMol.

Figure 2. Interaction of the SOUL BH3 peptide with human Bcl-xL

(A) ^{15}N - ^1H NMR correlation spectra of ^{15}N labelled human Bcl-xL titrated with increasing amounts of a 26 amino acid long peptide spanning residues 147–172 of human SOUL. The peptide sequence is SAQKNQEQLLTASILREDGKVFDEK. Arrows indicate the direction of peak shifts. Only four spectra are represented in the figure; the black one is before any addition, the green after the addition of 0.52 equivalents of the peptide, the blue after the addition of 1.38 equivalents and the red after adding 3.83 equivalents of the BH3 domain peptide.

(B) Decoupled- ^1H spectrum showing the shifts in two peaks in fast exchange in the methyl region used to calculate an approximate dissociation constant. The colour of the spectra is the same as in (A) and correspond to the addition of the same amounts of peptide.

(C) Magnitude of the change in the ^1H chemical shift of the two selected peaks plotted as a function of the total number of equivalents of BH3 domain peptide added. The curve best fit is shown along with the coefficient of determination (R^2) and the calculated dissociation constants. The values determined using the two peaks are 47.8 and 41.3 μM . The equation used to fit the data is given in the Experimental section under NMR measurements.

(D) Surface plasmon resonance studies of the same interaction. The sensorgram shows the binding of the BH3 peptide to truncated immobilized Bcl-xL. Relative units (RU, vertical) are plotted as a function of time (in seconds, horizontal).

(E) A plot of the response as a function of the peptide concentration used to estimate the dissociation constant. The diagram is the result of several experiments and higher peptide concentrations could not be used because the BH3 peptide had a tendency to aggregate.

Figure 3. Crystal structure of the SOUL BH3 domain peptide complexed with Bcl-xL.

(A) A dimer of human Bcl-xL ($\Delta 27-82$) showing swapping of the $\alpha 1$ domains. The two helical SOUL BH3 peptides are represented red.

(B) A protomer of Bcl-xL and the SOUL BH3 peptide in contact with it. The portion of the peptide predicted by sequence homology to be the BH3 domain is blue while the yellow part is the additional portion of the peptide, the beginning of the second helix of SOUL.

(C) Electron density of the SOUL BH3 peptide bound to Bcl-xL oriented as in (B). The molecular surface of Bcl-xL shows the negatively charged residues red and those positively charged blue. The $2F_{obs} - F_c$ map was contoured at a 1.2σ level. Selected BH3 peptide amino acids participating in important contacts with Bcl-xL have been labeled. The figure was prepared using the program Pymol.

Figure 4. SOUL BH3 peptide side chains that interact with Bcl-xL.

(A) Diagram representing Arg 163 and other side chains participating in one of the specific contacts of the SOUL BH3 peptide with Bcl-xL. Hydrogen bonds are indicated with green dotted lines whereas the amino acids that make hydrophobic contacts are only indicated but not represented as ball and stick models.

(B) The same type of diagram as (A) but with another specific contact in which the key residue is Asp 170 of the SOUL BH3 peptide.

(C) Sequence alignment of the SOUL BH3 peptide and eight BH3 only proteins. The BH3 domains are boxed and the amino acids that are identical in SOUL and at least two other sequences are red whereas those that are identical in at least three sequences are blue. The leucine conserved in all the sequences is indicated on a green background. Figures 4 (A) & (B) were prepared using the visualization program LIGPLOT [49].

Figure 5. Structural changes in the BH3 peptide of SOUL upon binding to Bcl-xL.

(A) Comparison of the peptide bound to Bcl-xL ($\Delta 27-82$) (blue) and the same sequence in the intact SOUL molecule (red). Eight amino acids change their conformation to extend the BH3 helix towards its C terminus. The coordinates were superimposed by using the CCP4 suit of programs. Three charged amino acids that give specificity to the interaction are represented as stick models in the two conformations.

(B) Superposition of the SOUL BH3 peptide Bcl-xL ($\Delta 27-82$) complex (the peptide is yellow and Bcl-xL is orange) and the same complex of Bcl-xL (light blue) and the Beclin 1 BH3 peptide (green). The PDB code of model the Beclin 1 peptide complex is 2P1L (reference 13). The interactions of the SOUL amino acids Arg163 and Asp170 (shown in Figure 4) are indicated with dotted lines. The Asp is conserved in Beclin 1 and the equivalent of the Arg is a Lys. Note that the important contact of Arg139 of Bcl-xL with an Asp in Beclin 1 (conserved in all BH3 domain Bcl-xL complexes) is missing in the SOUL peptide.

Table 1 Data collection and refinement statistics.

Data set	Human SOUL Native	Human SOUL Native	Complex Human Bcl- XL – Human Soul BH3 peptide
Space group	C222 ₁	P6 ₁ 22	P4 ₃
<i>a</i> (Å)	137.72	143.92	66.83
<i>b</i> (Å)	114.66	143.92	66.83
<i>c</i> (Å)	67.42	242.12	175.22
α	90.0	90.0	90.0
β	90.0	90.0	90.0
γ	90.0	120.0	90.0
Molecules in the asymmetric unit	2	4	4
Resolution Range (Å)	24.9 - 1.60	80.0 - 2.85	53.1 - 1.95
Observed reflections	470,976	723,081	276,588
Independent reflections	69,663	35,317	54,709
Multiplicity*	6.8 (6.5)	20.5 (20.4)	5.1 (5.2)
Rmerge (%) ^a	6.5 (38.1)	8.5 (38.8)	7.3 (32.0)
I/ σ	18.3 (4.6)	25.7 (8.3)	17.6 (6.0)
Completeness (%)	98.9 (98.0)	99.9 (100.0)	98.0 (96.5)
Reflections in refinement	69,648	35,223	54,700
Rcryst. (%) ^b	17.5	23.5	21.1
Rfree (%) (test set 5%) ^c	19.7	26.8	26.3
Protein atoms	2,974	5,631	5,132
Ligand atoms	5 (phosphate)	-	5 (sulphate)
Water molecules	395	-	172
r.m.s.d. on bond lengths (Å) ^d	0.008	0.008	0.009
r.m.s.d. on bond angles (°)	1.250	1.169	1.124
Planar groups (Å)	0.006	0.006	0.004
Chiral volume dev. (Å ³)	0.081	0.074	0.068
Average B factor (Å ²)	21.2	73.3	36.4
Protein atoms	20.1	73.3	36.8
Ligand atoms	26.7	-	49.2
Solvent atoms	29.4	-	27.3

* The values in parentheses refer to the highest resolution shells. For the data collection of the orthorhombic form, the highest resolution interval is 1.69–1.60 Å and for the hexagonal form 3.00–2.85 Å whereas for the cocrystals of the BH3 domain with Bcl-xL it is 2.05–1.95 Å.

The highest resolution shells used in the refinements are: 1.66–1.60 Å, 2.95–2.85 Å and 1.98–1.95 Å for the co-crystals of the BH3 domain.

The ligand of the SOUL crystals is phosphate and that of the complex sulphate.

^a $R_{\text{merge}} = \sum_i \sum_h |I_{ih} - \langle I_h \rangle| / \sum_i \sum_h \langle I_h \rangle$ where $\langle I_h \rangle$ is the mean intensity of the i observations of reflection h .

^b $R_{\text{cryst}} = \sum | |F_{\text{obs}}| - |F_{\text{calc}}| | / \sum |F_{\text{obs}}|$, where $|F_{\text{obs}}|$ and $|F_{\text{calc}}|$ are the observed and calculated structure factor amplitudes, respectively. Summation includes all reflections used in the refinement.

^c $R_{\text{free}} = \sum | |F_{\text{obs}}| - |F_{\text{calc}}| | / \sum |F_{\text{obs}}|$, evaluated for a randomly chosen subset of 5% of the diffraction data not included in the refinement.

^d Root mean square deviation from ideal values.

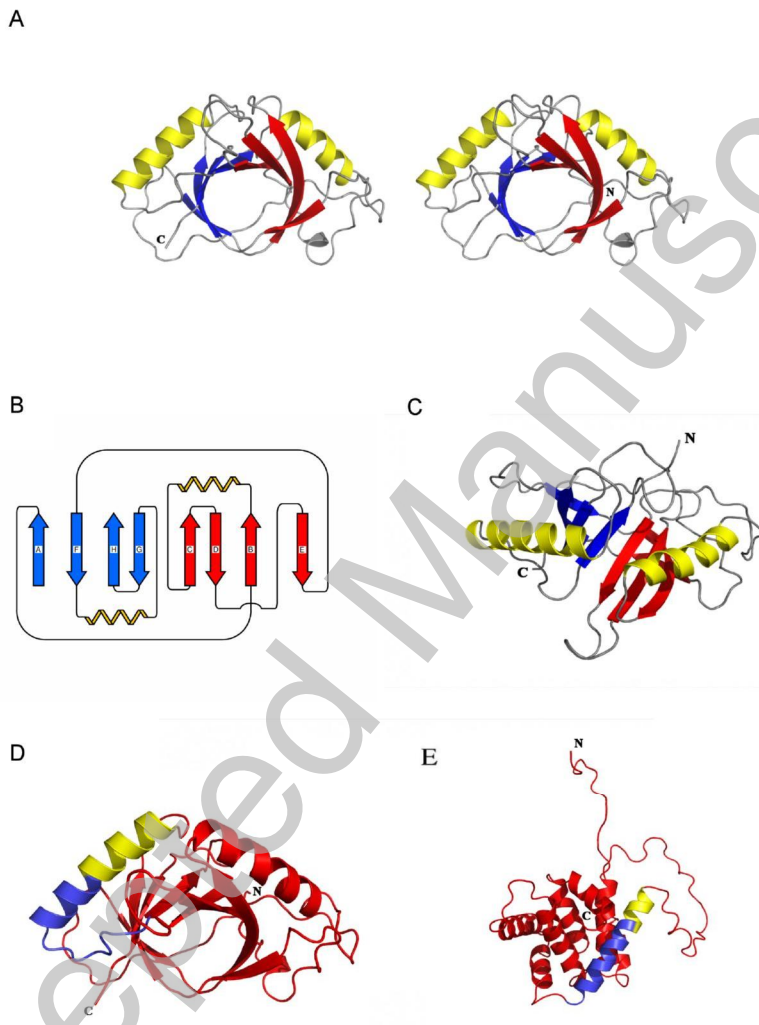


Figure 1

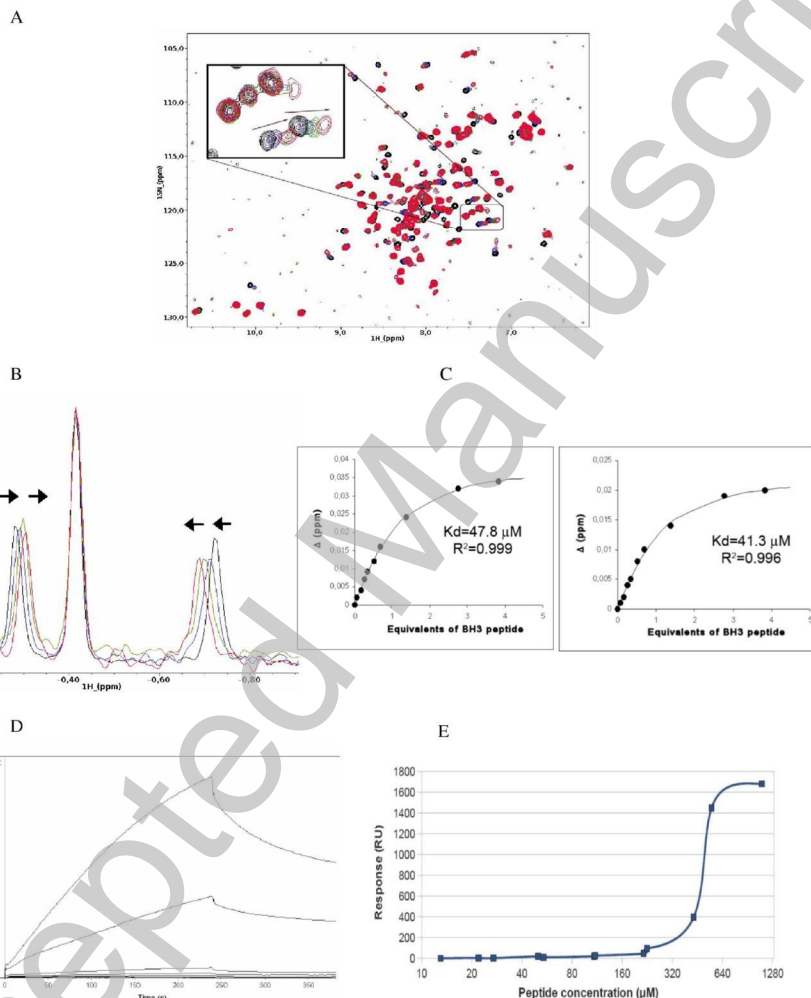


Figure 2

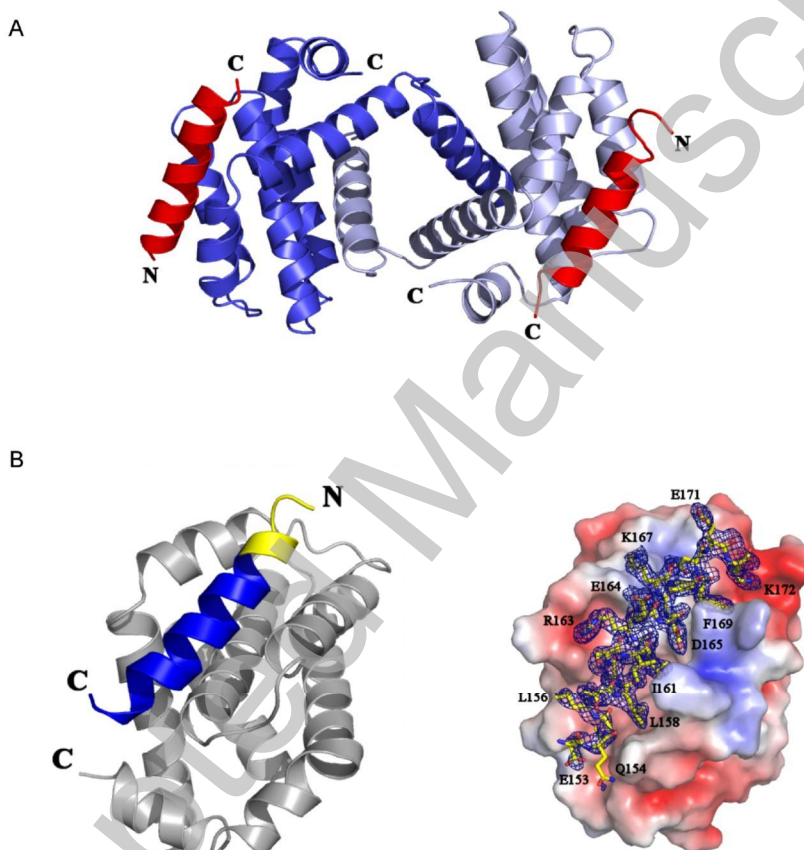


Figure 3

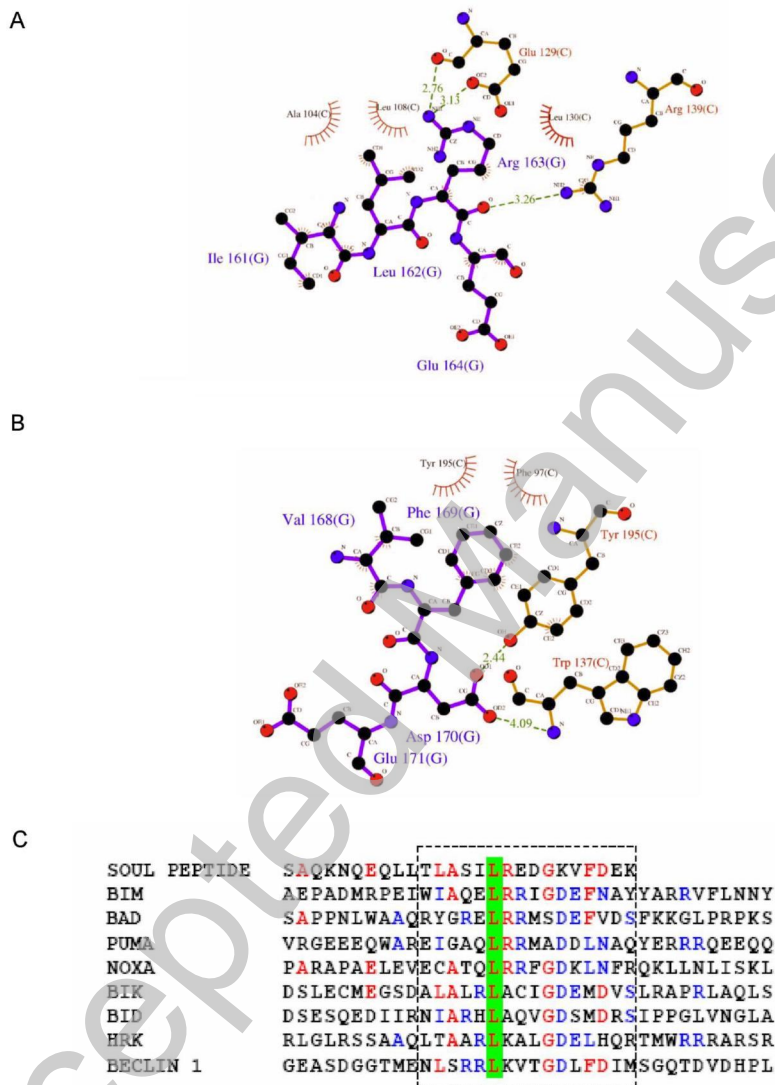


Figure 4

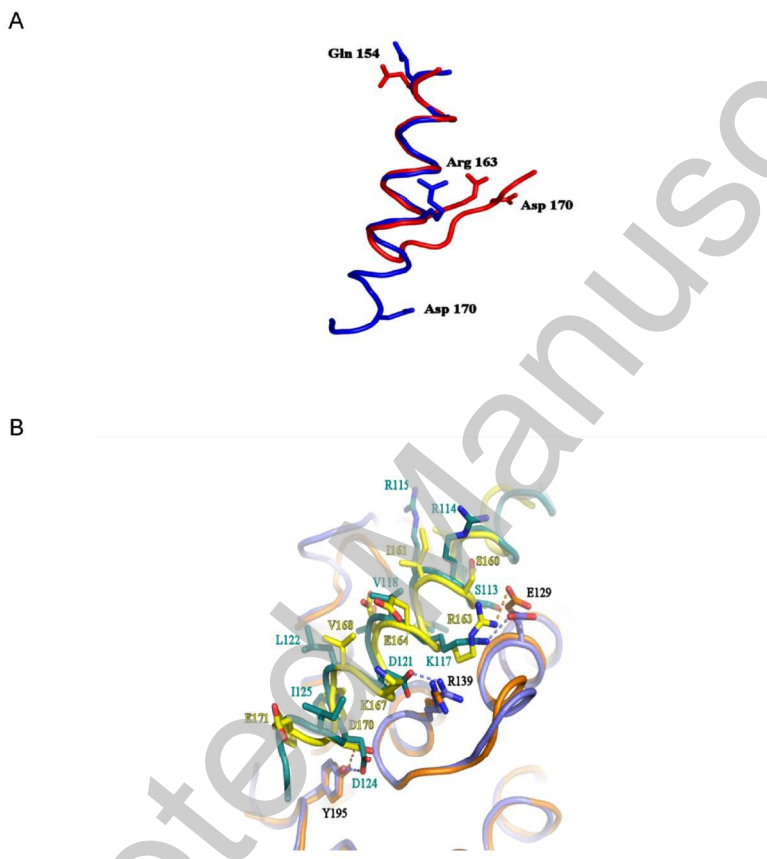


Figure 5