Characterization of a Novel Interaction between Bcl-2 Members Diva and Harakiri

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Abstract

Interactions within proteins of the Bcl-2 family are key in the regulation of apoptosis. The death-inducing members control apoptotic mechanisms partly by antagonizing the prosurvival proteins through heterodimer formation. Structural and biophysical studies on these complexes are providing important clues to understand their function. To help improve our knowledge on protein-protein interactions within the Bcl-2 family we have studied the binding between two of its members: mouse Diva and human Harakiri. Diva has been shown to perform both prosurvival and killing activity. In contrast, Harakiri induces cell death by interacting with antiapoptotic Bcl-2 members. Here we show using ELISA and NMR that Diva and Harakiri can interact in vitro. Combining the NMR data with the previously reported three-dimensional structure of Diva we find that Harakiri binds to a specific region in Diva. This interacting surface is equivalent to the known binding area of prosurvival Bcl-2 members from the reported structures of the complexes, suggesting that Diva could function at the structural level similarly to the antiapoptotic proteins of the Bcl-2 family. We illustrate this result by building a structural model of the heterodimer using molecular docking and the NMR data as restraints. Moreover, combining circular dichroism and NMR we also show that Harakiri is largely unstructured with residual (13%) \(\alpha\)-helical conformation. This result agrees with intrinsic disorder previously observed in other Bcl-2 members. In addition, Harakiri constructs of different length were studied to identify the region critical for the interaction. Differential affinity for Diva of these constructs suggests that the amino acid sequence flanking the interacting region could play an important role in binding.

Introduction

Programmed cell suicide known as apoptosis controls cell homeostasis and is thus central to the life cycle of multi-cellular organisms [1]. Proteins of the Bcl-2 family are key regulators of apoptotic mechanisms by mediating in an intricate network of interactions between pro- and antiapoptotic members that eventually lead to the activation of caspases, the true apoptosis executors [2–3]. Bcl-2 proteins share low sequence homology in small stretches of amino acids named Bcl-2 homology (BH) domains. Members that promote cell survival (e.g. Bcl-2, Bcl-X\(_L\)), Bcl-w, Mcl-1, BFL-1) contain four BH domains (BH1-BH4), whereas members with killing activity can share homology either in three BH domains or solely in the BH3 region (the BH3-only subfamily). As a response to death stimuli, BH3-only proteins form heterodimers with prosurvival members, thus antagonizing their function [4–7]. Reported evidence indicates that peptides of \(\sim 16–25\) amino acids comprising the BH3 domain of BH3-only proteins suffice for heterodimer formation [8]. Therefore, most of the structural information currently known on BH3-only proteins is centered at BH3 peptides. All known three-dimensional (3D) structures of complexes between prosurvival Bcl-2 members and these peptides show that the latter adopt \(\alpha\)-helical structure and are located in a hydrophobic groove of the prosurvival protein surface [8–9]. However, BH3 peptides have been shown to behave like random coils in isolation [9], and experimental evidence together with prediction programs support that several BH3-only proteins are intrinsically disordered [10]. Thus, it has been suggested that additional energetic factors besides specific intermolecular interactions likely play a role in this peculiar binding process [9].

The dysfunction of apoptotic mechanisms has been pointed as a hallmark of cancer. In particular, tumor cells overexpress prosurvival Bcl-2 members and tumor suppressor p53 fails at activating several BH3-only proteins conferring death resistance to cancer cells [11]. These findings have both increased interest in the use of BH3-only proteins as scaffolds for drug design [12–14] and targeted research at the detailed understanding of Bcl-2 interactions. Recent work in this direction has shown that antiapoptotic Bcl-2 members can bind preferentially particular subsets of BH3-only proteins [15–17]. This selectivity has been related to differential apoptotic response [16,17]. However, the conclusions derived from these studies are at variance likely because of the complexity of the molecular mechanisms involved as well as the need to compare in vitro and in vivo data. Additional work is thus necessary to fully understand Bcl-2 interactions and their relation to programmed cell death. To gain insight into the structural and biophysical factors involved in Bcl-2 protein-protein

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binding, we report here the characterization of a novel interaction between the BH3-only protein Harakiri and the Bcl-2 member Diva (also called Boo).

Harakiri localizes in membranes and exerts proapoptotic activity by interacting with survival Bcl-XL and Bcl-2 [18]. Harakiri has not been characterized at the structural level except for its C-terminal sequence (~30 amino acids), which is known from low-resolution techniques to adopt α-helical conformation in model membranes [19]. Diva has also been found predominantly in membranes [20–21]. However, little functional data on Diva is available. Specifically, previous independent reports indicate that Diva can have both pro- or antiapoptotic function [20–21]. Diva has also been reported to bind antiapoptotic Bcl-XL and the proapoptotic Bcl-2 members Bik and Bak, according to co-immunoprecipitation assays [21]. In contrast, binding studies using isothermal titration calorimetry indicate that Diva does not bind peptides comprising the BH3 region of several proapoptotic Bcl-2 proteins, including Bak and Harakiri [22]. On this basis it has been suggested that Diva is not functionally equivalent to other Bcl-2 proteins [22]. However, the 3D structure of Diva is very similar to the known structures of other Bcl-2 members [22].

Here we show using ELISA and NMR that Diva and Harakiri can interact in vitro. Our NMR data combined with the recently reported structure of Diva [22] indicate that the interaction involves in Diva’s surface the same groove previously observed in all other known structures of antiapoptotic/BH3-peptide complexes, indicating that binding is specific. To illustrate the formation of the complex a 3D structural model of the heterodimer is built using molecular docking and the NMR data as restraints. Altogether, these results suggest that at the structural level Diva binds death-inducing Harakiri in a fashion similar to other antiapoptotic Bcl-2 proteins. In addition, structural studies on Harakiri were carried out using NMR and circular dichroism. The data show that Harakiri is largely unstructured with only a small population of residual α-helical conformation. This result indicates that Harakiri is an intrinsically disordered protein like several other members of the BH3-only subfamily [10]. As BH3-derived peptides in isolation show little structure [9] whereas they form a helix when bound to the prosurvival protein, it is plausible that structure formation in the peptide is connected to binding [9,10]. Thus, using NMR titration experiments we estimated an apparent dissociation constant of the complex assuming a simple model that takes into account Harakiri folding upon binding. In addition, by studying the binding to Diva of Harakiri constructs of different length we identify the critical region for binding in Harakiri and observe that affinity increases for constructs longer than this region, suggesting that the flanking sequence can influence binding.

Results

Interaction between Diva and Harakiri detected by ELISA

The interaction between Harakiri and Diva was studied using three Harakiri constructs of different length all including the BH3 region (Fig. 1A); the entire cytosolic domain of Harakiri encompassing residues 1 to 59 (Hrk_ATM) [18], a 32 residue-long peptide comprising the BH3 domain flanked by two segments predicted to have α-helical propensity according to the program PredicProtein [23] (Hrk_medium), and a 16 amino acid-long peptide spanning the BH3 domain alone (Hrk_BH3).

ELISA was used to test the binding capabilities of the three constructs to Diva. Hrk_ATM and Hrk_medium show significant levels of interaction, with the entire cytosolic domain displaying higher binding affinity (Fig. 1B). Interaction levels typically decrease with peptide concentration as expected (Fig. 1B). In contrast, protein-binding levels are negligible for the shortest fragment Hrk_BH3 and do not show any dependence with peptide concentration, suggesting that the observed residual signal corresponds to background levels (Fig. 1B). Altogether, ELISA data indicate that the length of the Harakiri constructs has a significant effect on binding to Diva.

NMR characterization of Diva/Harakiri interaction

We further studied the interaction between Diva and Harakiri by NMR, as this is the technique of choice to identify at the atomic level structural changes associated to protein interactions [24]. Amide 1H,15N-HSQC experiments [25] of 15N-labeled Diva were recorded upon the addition of unlabeled Hrk_ATM. Significant changes in NMR signals were observed, thus confirming the interaction (Fig. 2A). Chemical shift assignments of the backbone amide 1H and 15N resonances were obtained for each titration point (Fig. 2B). Residues with the largest chemical shift perturbations (>0.1 ppm) are mainly located in the regions 44–67, 74–78, 83–98 and 135–160 (Fig. 2C), indicating their participation in the binding interface. According to the secondary structure of Diva [26] these residues belong to helices 2, 3, 4, 5 and 8 (Fig. 2C). In addition, the mapping of the chemical shift perturbation data on the 3D structure of Diva [22] reveals that the binding region is equivalent to that observed in all known 3D structures of complexes between antiapoptotic Bcl-2 members and BH3-derived peptides [8,9,27,28]. This result is illustrated in Figure 3 using as examples the 3D structures of the complexes human Mcl-1/Bid_BH3 peptide [28] and mouse Bcl-XL/Bad_BH3 peptide (pdb ID 2Z2W) (Fig. 3B,C). The 3D structure of human Mcl-1/Bid_BH3 peptide was selected because Mcl-1 is the closest structural analogue to Diva according to the Dali server [29] and the peptide is the longest reported up to date for these complexes (35 residues), thus more similar to the Diva/Hrk_ATM interaction. In addition, the structure of mouse Bcl-XL/Bad_BH3 peptide complex was included to help illustrate that heterodimers of human and mouse species are equivalent at the structural level (Fig. 3). The interaction between prosurvival Bcl-2 members and BH3-only proteins is specific in that binding always involves the BH3 domain of the BH3-only protein and helices 2, 3, 4, 5 and 8 of the prosurvival partner. According to the chemical shift perturbation data the Diva/Hrk_ATM interaction shares the same type of specificity as illustrated in the sequence alignments shown in Figure 3D&E.

The interaction of Diva with the shorter fragments Hrk_medium and Hrk_BH3 was also studied by NMR. For the shortest fragment Hrk_BH3, no chemical shift changes in the 1H,15N-HSQC spectrum of Diva were observed even at a Diva/Hrk_BH3 ~1:15 molar ratio (Diva at 0.1 mM and Hrk_BH3 at 1.5 mM) (data not shown), suggesting the absence of the interaction. In contrast, Diva’s NMR spectrum varies upon the addition of Hrk_medium (Fig. 4). The spectrum of a mixture of Diva and Hrk_medium at ~1:10 molar ratio (Diva 0.1 mM and Hrk_medium 1 mM) shows changes almost identical to those observed for the binding to Hrk_ATM at ~1:2 molar ratio (Diva 0.18 mM, Hrk_ATM 0.37 mM) (Fig. 2C, lower panel). Therefore, there is very good agreement between the NMR and ELISA results on weaker binding observed for Hrk_medium relative to the entire cytosolic domain, and negligible binding for Hrk_BH3 (Fig. 1B). Moreover, the identical NMR spectra obtained for Diva/Hrk_ATM and Diva/Hrk_medium mixtures (Fig. 2C, lower panel) indicate that the binding interface of both complexes is analogous.
Hrk_DTM is largely disordered and shows residual \( \alpha \)-helical structure. Circular dichroism (CD) experiments were performed on Harakiri’s cytosolic domain to obtain information on its conformational behavior. The CD data indicate that Hrk_DTM adopts \( \alpha \)-helical structure with \( \approx 13\% \) population in aqueous milieu that increases to \( \approx 35\% \) in the presence of the secondary structure enhancer trifluoroethanol (Fig. 5). Thus, the cytosolic domain of Harakiri is largely unstructured. Consequently, the NMR spectra of Hrk_DTM show small amide \( ^1H \) chemical shift dispersion (from \( \approx 7.9 \) to 8.7 ppm) and severe signal overlap (Fig. 6). These spectral features together with the absence of methyl signals at \( \approx 0 \) ppm are characteristic of disordered proteins, in agreement with the CD data. Similar conformational behavior has been observed for BH3-derived peptides [9] and other members of the BH3-only subfamily [10]. For instance, the BH3-only proteins Bim, Bad, and Bmf have been found to be unstructured. In particular, Bim undergoes partial folding upon binding to Bcl-2 members [10]. The relation between function and disorder is a distinctive feature of natively unfolded proteins [30], thus several BH3-only members have been suggested

Figure 1. Harakiri constructs and ELISA binding. (A) One-letter code amino acid sequence of human Harakiri and constructs studied. The BH3 region, the transmembrane domain and the predicted \( \alpha \)-helical segments are indicated. (B) Difference in ELISA absorbance relative to the control for the binding of Diva to Hrk_ATM, Hrk_medium and Hrk_BH3 vs. fragment concentration. Shown values are average of two measurements. Thin bars represent the standard deviation. The absorbance value for the control is 0.12 \( \pm \) 0.01.
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Hrk_ATM is largely disordered and shows residual \( \alpha \)-helical structure. Circular dichroism (CD) experiments were performed on Harakiri’s cytosolic domain to obtain information on its conformational behavior. The CD data indicate that Hrk_ATM adopts \( \alpha \)-helical structure with \( \approx 13\% \) population in aqueous milieu that increases to \( \approx 35\% \) in the presence of the secondary structure enhancer trifluoroethanol (Fig. 5). Thus, the cytosolic domain of Harakiri is largely unstructured. Consequently, the NMR spectra of Hrk_ATM show small amide \( ^1H \) chemical shift dispersion (from \( \approx 7.9 \) to 8.7 ppm) and severe signal overlap (Fig. 6). These spectral features together with the absence of methyl signals at \( \approx 0 \) ppm are characteristic of disordered proteins, in agreement with the CD data. Similar conformational behavior has been observed for BH3-derived peptides [9] and other members of the BH3-only subfamily [10]. For instance, the BH3-only proteins Bim, Bad, and Bmf have been found to be unstructured. In particular, Bim undergoes partial folding upon binding to Bcl-2 members [10]. The relation between function and disorder is a distinctive feature of natively unfolded proteins [30], thus several BH3-only members have been suggested
Figure 2. Interaction between Diva and Harakiri by NMR. (A) $[^{1}H,^{15}N]$-HSQC spectra of $^{15}N,^{13}C$-labeled Diva (black), and Diva (0.12 mM) in the presence of unlabeled Hrk$_{D}$TM (0.85 mM) (red). Several residues undergoing large perturbations are indicated. The asterisk indicates a folded peak. Trp side chains are labeled as “s.c.”. (B) Superposition of regions of Diva $[^{1}H,^{15}N]$-HSQC spectra resulting from the titration with Hrk$_{D}$TM. Residues
used in the estimation of the $K_{\text{dapp}}$ are boxed and the linewidth of the corresponding signals has been increased relative to the others for clarity. Shown spectra correspond to 9 titration points out of the 16 measured at the following values of Hrk$_{\text{TM}}$ concentration (mM): 0, 0.08, 0.16, 0.29, 0.44, 0.73, 0.85, 1.60, 1.80. Some panels look particularly crowded because of the superposition of a total of 9 spectra. The noise level of the spectrum corresponding to the titration at 1.80 mM Hrk$_{\text{TM}}$ was reduced for clarity as the threshold level had to be lowered to properly determine the chemical shift of the significantly broadened signals. (C) Upper panel: chemical shift differences ($\Delta \delta = (\Delta \delta^\text{H})^2 + (\Delta \delta^\text{15N})^2)^{1/2}$ between spectra of Diva and a mixture of Diva (0.12 mM) and Hrk$_{\text{TM}}$ (0.85 mM) versus Diva’s residue number. Lower panel: chemical shift changes between spectra resulting from Diva/Hrk$_{\text{TM}}$ (0.18 mM/0.37 mM) and Diva/Hrk$_{\text{medium}}$ (0.1 mM/1 mM) mixtures vs. Diva’s residue number. The position of the $\alpha$-helices and the BH domains is indicated.

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to be intrinsically disordered [10]. Moreover, coupled folding and binding, a mechanism shown to be followed by some natively unfolded proteins [31], has been proposed for BH3-only proteins that are disordered in the unbound state and adopt helical structure when complexed [10]. The residual $\alpha$-helical structure and sensitivity to trifluoroethanol of Hrk$_{\text{ATM}}$ indicate its propensity to fold as a helix, thus suggesting a connection between folding and binding to Diva. The observed changes in the signals of the NMR spectrum of Hrk$_{\text{ATM}}$ upon the interaction (Fig. 6) are small because chemical shifts are averaged by the populations of the bound and unbound forms, and the latter predominates under the conditions used (0.85 mM Hrk$_{\text{ATM}}$ and 0.12 mM Diva). The upper limits for the bound and unbound forms are 0.12 mM and 0.73 mM, respectively. The observed chemical shift changes could result from both the formation of the helix and the interaction itself. However, further analysis of the spectra is significantly complicated by the small chemical shift dispersion and signal overlap (Fig. 6).

Structural model of Diva/Harakiri heterodimer

To illustrate the interaction between Diva and Harakiri we built a model of the complex using molecular docking and the chemical shift perturbation data (Fig. 2C). In the 3D structures of all known heterodimers the BH3-peptide forms a helix [8,9,28]. Therefore, based on the residual $\alpha$-helical structure of the entire cytosolic domain of Harakiri, a 3D model of the helix was obtained using a protein structure prediction program and the structure of the helix formed by a BH3-peptide bound to a Bcl-2 member as template (Materials and Methods). It is noteworthy that the modeled helix spans residues 23 to 59, which includes the BH3 region and the predicted helices (Fig. 1A), leaving the N-terminal 22 residues with no regular secondary structure. This 3D model together with the reported structure of Diva [22] were used in the molecular docking protocol, which also included information from the NMR data on the interacting surface in Diva as experimental restraints (Materials and Methods). The complexity of Harakiri NMR spectra precludes the identification of residues directly involved in the interaction, and thus residues 22–53 (Hrk$_{\text{medium}}$), which are known to be critical for binding from the NMR and ELISA data, were all input as participating residues in the docking protocol. The resulting 3D model of the heterodimer shows a binding cleft formed by helices 2–8 (residues 175–231) and a mixture of Diva (0.12 mM) and Hrk$_{\text{medium}}$ (0.18 mM/0.37 mM) and Diva/Hrk$_{\text{medium}}$ (0.1 mM/1 mM) mixtures vs. Diva’s residue number. The position of the $\alpha$-helices and the BH domains is indicated.

Apparent binding affinity between Diva and Harakiri

NMR titration experiments on Diva upon binding to the cytosolic domain of Harakiri were performed to estimate an apparent dissociation constant of the complex (Fig. 2B). However, we have shown that Harakiri is largely unstructured before binding and as already established for BH3-peptides [8,9], we propose that it adopts helical structure upon complex formation. It has been previously reported that coupled folding-and-binding is not realistically represented by the conventional two-state process [31]. Therefore, in an attempt to obtain an approximate value of the dissociation constant of the Diva/Hrk$_{\text{ATM}}$ complex we have assumed the simplest binding model that takes into account Hrk$_{\text{ATM}}$ folding/unfolding equilibrium:

$$Hrk_{\text{ATM}} + \text{Div} \leftrightarrow Hrk_{\text{ATM}}\text{Div}$$

Where Hrk$_{\text{ATM}}$, and Hrk$_{\text{ATM}}$ are the unfolded and folded forms of Hrk$_{\text{ATM}}$, respectively. Div is the unbound form of Diva and Hrk$_{\text{Div}}$ is the complex (see Materials and Methods).

The NMR titration experiments (Fig. 2B) depend on both the dissociation constant of the complex ($K_{\text{dapp}}$) and the unfolding/folding constant ($K_s$). These two processes cannot be separated in the overall chemical shift change resulting from complex formation. Therefore, for proper fitting of the data the $K_s$ value was fixed based on the structure population obtained from the CD experiments. The values of $K_{\text{dapp}}$ and $K_s$ strongly depend on each other thus, upper and lower limits of $K_{\text{dapp}}$ can be obtained considering two possible scenarios in the folding/unfolding equilibrium of Hrk$_{\text{ATM}}$. On the one hand we assumed that the observed 13% of helical population in the CD spectrum of the 59-residue long construct is localized in the critical binding region
Figure 3. Interacting region mapped in the 3D structure of Diva. Comparison to other prosurvival/BH3 peptide complexes. (A) Ribbon diagram of the 3D structure of Diva (pdb ID 2KUA) showing in orange the position of residues with chemical shift perturbations upon binding to Hrk_TMD. Light and dark orange indicate perturbations between 0.04 and 0.1 ppm, and larger than 0.1 ppm, respectively. Helices involved in the interaction are numbered. (B,C) Ribbon diagram of human Mcl-1/Bid_BH3 (pdb ID 2KBW) (B) and mouse Bcl-XL/Bad_BH3 (pdb ID 2BZW) (C) complexes. The peptides are shown as translucent green cylinders. The orientation of Diva, Mcl-1 and Bcl-XL is equivalent. Helices forming the typical binding pocket are colored in dark orange and numbered. Fig. 3A,B,C were created with PyMOL [43]. (D) Secondary structure and sequence alignment of mouse Diva (Q9Z0F3), human Mcl-1 (Q07820), mouse Bcl-XL (Q64373) and human BFL-1 (Q16548) used in the structural studies of the complexes with Harakiri, Bid (pdb ID 2KBW), Bad (pdb ID 2BZW) and Noxa (pdb ID 3MQP) respectively. Helices are color coded and numbered from 1 to 8. BH domains appear as bars. The amino acid sequences correspond to those of the structural studies, and thus do not show the C-terminal TM helix. Residues involved in the interacting surface with the cytosolic domain of Harakiri and the other BH3 peptides are underlined. Residues involved with the C-terminal TM helix are in red. The conserved small residue (Ser/Gly) and Asp residue in the BH3 domain are shown in red. The four conserved hydrophobic residues in the BH3 domain of Harakiri and those known to form part of the contact area in the complexes are underlined.

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from residues 22–53 (Hrk_medium), which leads to a $K_u$ value of $3.1$. By fixing this parameter, changes in the chemicals shifts of Diva (Fig. 2B) were fitted according to the proposed model (Materials and Methods) (Fig. 8). The obtained upper limit for the apparent dissociation constant is $K_{dapp} = 397 \pm 72$ M. On the other hand, by assuming that the 13% helical content is distributed throughout the entire cytosolic domain, $K_u, 6.7$ and the smallest resulting value of $K_{dapp}$ is $132$ M for V45 (Fig. 8). The upper and lower limits of $K_{dapp}$ are in the order of magnitude of the largest dissociation constant values found for other BH3-peptide/prosurvival protein complexes (nM to $100$ M) [9,16,17].

These results are in contrast to recent studies by isothermal titration calorimetry on the binding capabilities of Diva to peptides comprising the BH3 domain of several Bcl-2 proteins [22]. These studies found that Diva does not show “substantial affinity” for any BH3-derived peptide, including Bak and Harakiri. However, previous work using co-immunoprecipitation indicates that Diva can interact with Bak [21]. Our ELISA and NMR data also show unambiguously that Diva interacts with Harakiri and that the interaction is specific as binding involves a particular region in the 3D structure of Diva (Fig. 3A, D). The observed differences could be related to the systems and conditions used in the different experiments. Particularly, the isothermal calorimetry titrations reached Harakiri BH3-peptide concentration of only $17.5$ M.
The isothermal titration calorimetry results are also in contrast with the structural similarity between Diva and other Bcl-2 proteins [22]. Mutations in Diva relative to other members of the Bcl-2 family in two conserved residues (G88S, R89Q) of the BH1 domain that forms part of the hydrophobic cleft have been suggested to be responsible for the non-detected binding [22]. By contrast, our NMR data clearly show that Diva uses this hydrophobic groove, including the BH1 domain (Fig. 2C), to interact with Harakiri. Particularly, significant changes in the chemical shift of S88 (Fig. 2B, Fig. 8) and Q89 (Fig. 2C) upon the interaction with Hrk_ATM were observed, indicating that these residues are perturbed by the interaction.

Comparison of Diva/Harakiri affinity to other heterodimers in the Bcl-2 family

Dissociation constant values of direct interactions between antiapoptotic Bcl-2 proteins and BH3-derived peptides measured in vitro by fluorescence polarization have been found to correlate with the apoptotic response [17]. Complexes with \( K_d \) in the nanomolar range clearly show stronger killing activity than weaker interactions [17]. However, heterodimers with affinity in the micromolar range (>2.5 \( \mu M \)) are also functional [17]. For instance, the BH3 domains of the BH3-only proteins Noxa A and Noxa B with significant affinity (\( K_d \approx 20–30 \) nM) only for antiapoptotic Mcl-1, show comparable killing activity in the presence of Mcl-1 or Bcl-2, albeit the measured \( K_d \) for the latter is >2.5 \( \mu M \) [17]. Similar results were found for the BH3 domain of the BH3-only protein Bik, which shows equivalent killing activity in the presence of Mcl-1 and BFL-1 although the affinity for both antiapoptotic proteins is significantly different (\( K_d = 109 \) nM for Mcl-1 and >2.5 \( \mu M \) for BFL-1) [17].

More relevant to the Diva/Harakiri complex are the results found for the BH3 domain of Harakiri, which shows killing activity in the presence of Bcl-2 for which the measured \( K_d \) is >2.5 \( \mu M \) [17]. In addition, some discrepant results on the binding capabilities of Harakiri for antiapoptotic Bcl-2 members have been reported. For example, IC\(_{50}\) values ~50 nM, measured by competition assays using surface plasmon resonance, have been found for the interaction between the BH3 domain of Harakiri and the antiapoptotic proteins Bcl-w and BFL-1 [16]. In contrast, \( K_d \) values >2.5 \( \mu M \) in the direct binding affinity studies have been reported for the same proteins [17]. These discrepancies could result from the different techniques, conditions and systems used.

In particular, it is noteworthy that 4, out of the 9 BH3-only proteins known, show a transmembrane domain. Specifically, Harakiri [18] and Diva [21] have been found predominantly in the membrane, and thus they are expected in vivo to interact while anchored to the membrane. The differences between in vivo and in vitro binding conditions will likely be larger for those BH3-only members with a transmembrane domain, resulting in dissociation constants values measured in vitro that are significantly different in vivo. For example, higher protein local concentration at the membrane surface and restricted mobility resulting from membrane anchoring could increase binding affinity. Therefore, the \( K_d \) value measured in vitro for the Diva/Harakiri heterodimer is only a rough estimate of the “real” dissociation constant between these proteins, which most likely decreases under in vivo conditions.

Discussion

The ELISA and NMR data herein reported consistently demonstrate that the Bcl-2 members Diva and Harakiri are able
to interact in vitro. Moreover, the NMR results indicate that the interaction is specific involving in Diva the same hydrophobic cleft observed in all of the reported 3D structures of other Bcl-2 complexes. No information on the interaction between Diva and Harakiri has been previously reported. Thus, further studies are necessary to test whether the Diva/Harakiri complex is functionally relevant in apoptosis. Nevertheless, from the biophysical and structural perspective our results indicate that Diva is structurally suited to function as other negative regulators of cell death, in contrast to recent binding studies suggesting that the structure of Diva reveals a functionally divergent protein [22].

In addition, we show that the full-length cytosolic domain of Harakiri is intrinsically disordered with residual α-helical structure. Therefore, we propose that Harakiri folds as an α-helix upon complex formation, as previously suggested for the interaction between the BH3-only member Bim and the antiapoptotic protein Bcl-w [10]. Our data also indicate that the cytosolic domain of Harakiri binds Diva with higher affinity than the shorter constructs. However, Diva shows the same interacting surface for both Hrk_ATM and Hrk_medium, suggesting that factors other than those pertinent to intermolecular interactions within the interacting area are playing a role in binding. The influence on binding of disordered regions outside the interacting interface has been theoretically predicted [32] and experimentally observed before [33]. Intrinsically disordered proteins can follow different binding mechanisms in which preformed elements of secondary structure, together with concomitant folding and flexibility in the unbound and bound state can play important roles [34]. Thus, further mechanistic studies are necessary to identify the factors responsible for the affinity differences of the Harakiri constructs. Nevertheless, our results suggest that studies on fragments longer than the typically 25-residue BH3 peptides will help to better understand Bcl-2 interactions.

Materials and Methods

Protein production

The c-DNA fragment encoding for residues 1–160 of mouse Diva (lacking its putative 30-residue long transmembrane α-helix) was cloned into the NcoI and HindIII restriction sites of pBAT4 expression vector [35], which was transformed in BL21(DE3) E. Coli strain (Novagen). Bacteria were grown at 37°C and protein expression was induced at OD600 of 0.6–0.7 for 6 h at 30°C by adding 1 mM IPTG (isopropyl-β-D-thiogalacto-pyranoside). Uniformly 15N- and 13C-labeled Diva was produced using 15C6-D-glucose and 15NH4Cl (Spectra Stable Isotopes) as sole carbon and nitrogen sources, respectively. The cells were harvested by centrifugation and resuspended in a buffer containing 50 mM sodium acetate at pH 5.4, 50 mM NaCl, 0.1 mM protease inhibitor cocktail (Sigma) and 1 mM TCEP (Tris(2-carboxyethyl)-phosphine). Cells were lysed by sonication at 4°C and centrifuged at 25000 rpm for 30 minutes. The soluble protein was purified by cation exchange chromatography using an SP sepharose Fast Flow column (GE Healthcare). A second purification step was necessary using reverse phase chromatography in water-acetonitrile mixtures, followed by lyophilization of the protein solution.

Peptide synthesis

The full-length cytosolic domain of human Harakiri, residues 1–59 (Hrk_ATM), and fragments 22–53 (Hrk_medium) and 33–47 (Hrk_BH3) were synthesized and purified by CASLO Laboratory (Denmark). The purity (>95%) and molecular weight were confirmed by liquid chromatography and mass spectrometry, respectively. Hrk_medium and Hrk_BH3 are protected by a C-terminal amide.

Enzyme-linked immunosorbent assays (ELISA)

Microplates (Costar Ltd., US) were coated with 50 µl of Diva in PBS at a concentration of 7.5 µg/ml. After overnight incubation at 4°C plates were washed three times with distilled water and then blocked with 2.5% BSA (Sigma-Aldrich, Spain) in PBS for 2 h at 37°C. Solutions of Harakiri fragments were prepared at fixed initial peptide concentrations (derived from absorbance measurements) containing PBS, 2.5% BSA and 0.1% Tween to avoid aggregation and non-specific binding. Samples were loaded and incubated overnight at 4°C. BSA at 2.5% in PBS was used as negative control. After thorough wash (6 times) with distilled water and 0.1% Tween, bound Harakiri fragments were detected by a polyclonal rabbit anti-Harakiri BH3 domain antiserum (1:1000) (Abcam, UK). Following a streptavidin peroxidase conjugate anti-rabbit Ig (1:1000) (Dako) as secondary antibody, the peroxidase activity was detected by the addition of 3,3′,5,5′-tetramethylbenzidin...
Circular dichroism experiments

Hrk_ATM was dissolved in 20 mM sodium phosphate buffer at pH 5.8 in the presence and absence of 35% (v/v) trifluoroethanol (TFE). The concentration of Hrk_ATM was 23 μM in the buffer without TFE and 44 μM in TFE. The concentration was calculated by measuring absorbance at 280 nm. CD measurements were acquired at room temperature using a JASCO model J-815 spectropolarimeter with a 1 mm cuvette. The CD signal at 222 nm was converted to mean residue ellipticity (\( \theta_{\text{obs}} \)) after subtracting the blank using the equation:

\[
\theta_{\text{obs}} = 100(\theta_{222}/c Nl)
\]

where \( c \) is the peptide concentration (in mM), \( N \) is the number of peptide residues and \( l \) is the path-length (in cm).

The percentage of \( \alpha \)-helical population was determined using the following equation:

\[
\%\text{helix} = (\theta_{\text{obs}} - \theta_{\text{coil}})/[(\theta_{\text{helix}} - \theta_{\text{coil}})]
\]

where \( \theta_{\text{helix}} \) is the mean residue ellipticity of a complete helix, i.e., -42,500(1-(3/N)), and \( \theta_{\text{coil}} \) is the ellipticity of a random coil, i.e. +640 [38,39].

Apparent dissociation constant measurement

Exchange between the free and bound forms of Diva is fast on the NMR time scale and thus the observed chemical shifts (\( \delta \)) are weight-averaged relative to the free and bound fractions:

\[
\delta = F_b\delta_{D_H} + F_f\delta_D
\]

Where \( F_b \) is the fraction of bound Diva, \( F_f \) is the fraction of free Diva, \( \delta_{D_H} \) is the chemical shift of Diva bound to Hrk_ATM and \( \delta_D \) is the chemical shift of free Diva. Since \( F_b + F_f = 1 \), equation (3) becomes:

\[
F_b = [\text{Hrk}_D]/[\text{Div}_T] = (\delta_{D_H} - \delta_D)/[\delta_{D_H} - \delta_D]
\]

where [Hrk_Div] and [Div_T] are the concentration of Diva bound to Hrk_ATM and the total concentration of Diva, respectively. (\( \delta_{D_H} - \delta_D \)) is the chemical shift change at infinite concentration of Hrk_ATM.

Previously reported studies on coupled folding and binding have shown that the binding process might not be adequately represented by two states [31]. Therefore, for a more realistic description of the binding of Hrk_ATM to Diva taking into account that Hrk_ATM is largely unstructured in the unbound form and likely folds upon binding, we have assumed the simplest
model in which Hrk_ATM folding/unfolding equilibrium is related to binding as follows:

\[ \text{Hrk}\_\text{ATM}_u + \text{Div} \leftrightarrow \text{Hrk}\_\text{ATM}_f + \text{Div} \leftrightarrow \text{Hrk}\_\text{Div} \]  
(5)

where Hrk\_ATM\_u and Hrk\_ATM\_f are the unfolded and folded forms of Hrk\_ATM, respectively. Div is the unbound form of Diva and Hrk\_Div is the complex.

The equilibrium constants of the model shown in equation (5) are:

\[ K_u = [\text{Hrk}\_\text{ATM}_u]/[\text{Hrk}\_\text{ATM}_f] \]  
(6)

\[ K_{\text{app}} = [\text{Div}\_\text{Div}]/[\text{Hrk}\_\text{ATM}_u][\text{Div}] \]  
(7)

\( K_u \) and \( K_{\text{app}} \) are the unfolding and apparent binding constants, respectively. In addition, the total concentration of Diva and Hrk\_ATM are:

\[ [\text{Div}_T] = [\text{Div} + [\text{Hrk}\_\text{Div}] \]  
(8)

\[ [\text{Hrk}\_\text{ATM}_f] = ([\text{Hrk}\_\text{ATM}_u] + [\text{Hrk}\_\text{ATM}_f] + [\text{Hrk}\_\text{Div}] \]  
(9)

where \([\text{Div}_T] \) and \([\text{Hrk}\_\text{ATM}_u] \) are the total concentration of Diva and Hrk\_ATM at each titration point. Substituting equation (9) in (6):

\[ [\text{Hrk}\_\text{ATM}_f] = ([\text{Hrk}\_\text{ATM}_u] - [\text{Hrk}\_\text{Div}])/(K_u + 1) \]  
(10)

Substituting equations (4), (8) and (10) in equation (7):

\[ (\delta - \delta_D) = K_{\text{app}} \left\{ \frac{[\text{Hrk}\_\text{ATM}_f][\delta_{\text{Div}} - \delta_D] - [\text{Div}_T][\delta_{\text{Div}}]}{[\text{Hrk}\_\text{ATM}_u] - ([\text{Div}_T][\delta_{\text{Div}}]/(\delta_D - \delta_D))} \right\} \]  
(11)

There are three unknowns in equation (11), i.e., \( (\delta_{\text{Div}} - \delta_D) \), \( K_{\text{app}} \), and \( K_u \). To fit the data properly we fixed the value of \( K_u \) which can be estimated from the CD experiments (Fig. 5). Upper and lower limits of \( K_u \) depend on the number of residues assumed to adopt the helical conformation and derive in upper and lower limits for \( K_{\text{app}} \). For the \( K_u \) lower limit it was assumed that the helical population is localized in the critical binding region in Harakiri (residues 22–53). Thus, out of the 59 residues in Hrk\_ATM, the 32 residues of Hrk\_medium shown to be sufficient for the interaction fold into an \( \alpha \)-helix. This assumption leads to folded and unfolded populations of 24% and 76%, respectively, and thus \( K_u \sim 3.1 \). By fixing this parameter the changes in chemical shift resulting from the titration of the total of 10 signals shown in Fig. 2B were fitted to equation (11) using the software GRACE (http://plasma-gate.weizmann.ac.il/Grace). The individual fits and calculated \( K_{\text{app}} \) are shown in Fig. 8. A global upper limit for \( K_{\text{app}} \) is obtained from the average of the 10 values \( (397 \pm 72 \mu M) \). For the \( K_u \) upper limit it was assumed that all residues populate the helical conformation. Thus, the folded and unfolded populations are 13% and 87%, respectively, and \( K_u \) is \( \sim 6.7 \). Fixing this parameter the lowest \( K_{\text{app}} \) value is 132 \( \mu M \) for V45.

**Molecular docking calculations of Diva/Harakiri complex**

The program I-TASSER (Protein structure and function prediction based on iterative TASSER simulations) [40] was used to produce a 3D model of Hrk\_ATM (59 residues). For a better representation of Hrk\_ATM, the structure of the longest (35 residues) reported \( \alpha \)-helical peptide comprising the BH3 region of a BH3-only protein (Bid) complexed to a prosurvival Bcl-2 protein (Mol-1) [29] was used as template. The five predicted structures with the highest scores consistently show an \( \alpha \)-helix spanning Harakiri residues 23 to 57. In contrast, loops of different lengths are predicted from residues 1 to 22. Molecular docking was carried out with the software HADDOCK (High Ambiguity Driven biomolecular DOCKing based on biochemical and/or biophysical information) [41] using the 3D structure of Diva [22] and the structure of Hrk\_ATM with the highest score from I-TASSER. The online HADDOCK server was used to build a model of the Diva/Hrk\_ATM heterodimer. HADDOCK uses residues involved in the interaction (so-called “active” residues) to create a list of ambiguous interaction restraints used in the docking calculation. These restraints set intramolecular interatomic upper distance limits of 2 Å for all atoms of residues input as “active”. “Passive” residues, which do not directly participate in the interaction and surround active ones, can also be incorporated. Residues in helices 2, 3, 4, 5 and 8 with chemical shift perturbations larger than 0.05 ppm and with \( >20\% \) of the total surface solvent exposed were included as “active” for the molecular docking. No “passive” residues were defined. Solvent accessibility was calculated with MOLMOL [42]. The list of “active” residues used and the corresponding percentage of solvent accessible surface is: R43 (33%), R47 (36%), Q48 (26%), Q51 (38%), H53 (24%), E55 (26%), F56 (42%), S59 (27%), S63 (25%), R64 (51%), K77 (47%), S88 (23%), D152 (37%), R156 (50%), F157 (34%), K159 (53%). For Hrk\_ATM, residues from 22–53 known to be critical for the interaction from the NMR and ELISA data were included as “active” residues. No “passive” residues were defined. HADDOCK default parameters were accepted for the docking protocol. No other restraints were incorporated by the user besides the residues specified above. The three resulting HADDOCK clusters of structures with highest scores out of 1000 calculated were inspected and compared to known antiapoptotic protein/BH3-peptide 3D complexes. The relative orientation of both molecules in the best structure of cluster 3 is closer to other known complexes and thus was selected as the representative structure.

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**Author Contributions**

Conceived and designed the experiments: LS PO EdA. Performed the experiments: LS SB-V PO EdA. Analyzed the data: LS SB-V PO EdA. Wrote the paper: LS PO EdA.
References

1. Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257.
2. Adams JM, Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281: 1322-1326.
3. Chao DT, Korsmeyer SJ (1998) Bcl-2 family: regulators of cell death. Annu Rev Immunol 16: 395-419.
4. Olbryi ZN, Korsmeyer SJ (1994) Checkpoints of dueling dimers foil death wishes. Cell 79: 189-192.
5. Reed JC (2000) Mechanisms of Apoptosis. Am J Pathol 157: 1415–1430.
6. Chao DT, Korsmeyer SJ (1998) Bcl-2 family: regulators of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X(L). EMBO J. 16: 3839–3842.
7. Lomonosova E, Chinnadurai G (2009) BH3-only proteins in apoptosis and beyond: an overview. Oncogene 27: S2-S19.
8. Sattler M, Liang H, Nettesheim DG, Meadows RP, Harlan JE, et al. (1997) Structure of Bcl-XL/Bak peptide complex: recognition between regulators of apoptosis. Science 275: 983–986.
9. Petros AM, Nettesheim DG, Wang Y, Olejniczak ET, Meadows RP, et al. (2000) Rationale for Bcl-X L/Bad peptide complex formation from structure, mutagenesis and biophysical studies. Protein Sci 9: 2528-2534.
10. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, et al. (2004) Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. Cell Death Differ 11: 129-136.
11. Letai A, Parganas E, Lee Y, Yang C, Wang J, et al. (2003) Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 4: 321–328.
12. Fernandez-Luna JL (2008) Regulation of pro-apoptotic BH3-only proteins and its contribution to cancer progression and chemoresistance. Cell Signal 20: 1921–1926.
13. Horne WS, Boersma MD, Windsor MA, Gellman SH (2008) Sequence-based design of beta-Peptide foldamers that mimic BH3 domains. Angew Chem Int Ed 47: 2853-2856.
14. Vogler M, Dinndal D, Dyer MJ, Cohen GM (2009) Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. Cell Death Differ 16: 360–367.
15. Letia L, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, et al. (2004) BH3-only proteins: targets of apoptotic cell death. Curr Opin Chem Biol 8: 383–389.
16. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, et al. (2006) Mitochondria primed by death signals determine cellular transition to antia apoptotic Bcl-2 family members. Cancer Cell 9: 351–365.
17. Inohara N, Ding L, Chen S, Nunez G (1997) Harakiri, a novel regulator of cell death. EMBO J. 16: 1686–1694.
18. Bernabeu A, Guille ´n J, Pe ´rez-Berna´ AJ, Moreno MR, Villalain J (2007) Structure of the C-terminal domain of the prosapptotic protein Hrk and its interaction with model membranes. Biochem Biophys Acta 1768: 1659-1670.
19. Inohara N, Gourley TS, Carrio R, Matuz M, Merino J, et al. (1996) Dia, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. J Biol Chem 273: 32479–32486.
20. Soroji L, de Alba E (2010) Sequence-specific H3, H4, and 15N resonance assignments of Diva (Bou), an apoptosis regulator of the Bcl-2 family. Biomol NMR Assign 4: 65–68.
21. Suzuki M, Youle RJ, Tjandra N (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. Cell 103: 656–654.
22. Liu Q, Moldoveanu T, Sprules T, Matta-Camacho E, Mansur-Azzam N, et al. (2010) Apoptotic regulation by MCL-1 through hetero-dimerization. J Biol Chem 285: 9615–9624.
23. Helno J, Ka Tunnelen S, Rosenstrom P, Schelken A (2008) Searching protein structure databases with DallLite v.3. Bioinformatics 24: 2780–2781.
24. Weinreb PH, Zhen W, Poon AW, Conway KA, Lansbury PT, Jr. (1996) NACP, a protein implicated in Alzheimer’s disease and learning, is natively unfolded. Biochemistry 35: 13709–13715.
25. Sugase K, Dixon HJ, Wright, PF (2007) Mechanism of coupled folding and binding of an intrinsically disordered protein. Nature 447: 1021–1027.
26. Shoemaker BA, Pormann JJ, Wolynes PG (2006) Speeding molecular recognition by using the folding funnel: The fly-casting mechanism. Proc Natl Acad Sci USA 97: 8966–8973.
27. Rautureau GJ, Day CL, Hinds MG (2010) The structure of Boo/Diva reveals a divergent Bcl-2 protein. Proteins 78: 2181–2186.
28. Roy A, Kucikural A, Zhang Y (2010) E-Tasser, a unified platform for automated protein structure and function prediction. Nat Prot 5: 277–293.
29. Meyer JS, Pace CN, Scholtz JM (1997) Helix propensities are identical in volume 2 homologue that binds directly to Apaf-1. EMBO J 18: 167–178.
30. Rost B, Yachdav G, Liu J (2004) The PredictProtein Server. Nucl Acids Res 32 (Web Server issue). W321–W326.
31. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW (1996) Discovering high-affinity ligands for proteins: SAR by NMR. Science 274: 1531–1534.
32. Br Zax A, Grzesiek S (1993) Methodological advances in protein NMR. Accounts Chern Res 26: 131–138.
33. Hites RA, Laskowski RA, Swaminathan S (1996) Automatic protein structure and function prediction. Nat Prot 5: 725–738.
34. Garrett DS, Powers R, Gronenborn AM, Clore GM (1994) A common sense approach to peak picking in two-, three-, and four-dimensional spectra using computer analysis of contour diagrams. J Magn Reson 95: 214–220.
35. Arai K, Watanabe K, Kikuchi M, Hasegawa Y, Kusumoto Y, et al. (2010) Apoptotic regulation by MCL-1 through hetero-dimerization. J Biol Chem 285: 19615-19624.
36. Sugase K, Dixon HJ, Wright, PF (2007) Mechanism of coupled folding and binding of an intrinsically disordered protein. Nature 447: 1021–1027.
37. Shoemaker BA, Pormann JJ, Wolynes PG (2006) Speeding molecular recognition by using the folding funnel: The fly-casting mechanism. Proc Natl Acad Sci USA 97: 8966–8973.
38. Rohl CA, Chakrabartty A, Balwin RL (1996) Helix propagation and N-cap propensities of the amino acids measured in alanine-based peptides in 40 volume percent trifluoroethanol. Protein Sci 5: 2623–2637.
39. Meyers JK, Pace CN, Scholtz JM (1997) Helix propensities are identical in volume 2 homologue that binds directly to Apaf-1. EMBO J 18: 167–178.
40. Rost B, Yachdav G, Liu J (2004) The PredictProtein Server. Nucl Acids Res 32 (Web Server issue). W321–W326.
41. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW (1996) Discovering high-affinity ligands for proteins: SAR by NMR. Science 274: 1531–1534.
42. Br Zax A, Grzesiek S (1993) Methodological advances in protein NMR. Accounts Chern Res 26: 131–138.
43. Hites RA, Laskowski RA, Swaminathan S (1996) Automatic protein structure and function prediction. Nat Prot 5: 725–738.
44. Garrett DS, Powers R, Gronenborn AM, Clore GM (1994) A common sense approach to peak picking in two-, three-, and four-dimensional spectra using computer analysis of contour diagrams. J Magn Reson 95: 214–220.
45. Arai K, Watanabe K, Kikuchi M, Hasegawa Y, Kusumoto Y, et al. (2010) Apoptotic regulation by MCL-1 through hetero-dimerization. J Biol Chem 285: 19615-19624.
46. Sugase K, Dixon HJ, Wright, PF (2007) Mechanism of coupled folding and binding of an intrinsically disordered protein. Nature 447: 1021–1027.