

# Integrated strategy reveals the protein interface between cancer targets Bcl-2 and NAF-1

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Life requires orchestrated control of cell proliferation, cell maintenance, and cell death. Involved in these decisions are protein complexes that assimilate a variety of inputs that report on the status of the cell and lead to an output response. Among the proteins involved in this response are nutrient-deprivation autophagy factor-1 (NAF-1) and Bcl-2. NAF-1 is a homodimeric member of the novel Fe-S protein NEET family, which binds two 2Fe-2S clusters. NAF-1 is an important partner for Bcl-2 at the endoplasmic reticulum to functionally antagonize Beclin 1-dependent autophagy [Chang NC, Nguyen M, Germain M, Shore GC (2010) *EMBO J* 29 (3):606–618]. We used an integrated approach involving peptide array, deuterium exchange mass spectrometry (DXMS), and functional studies aided by the power of sufficient constraints from direct coupling analysis (DCA) to determine the dominant docked conformation of the NAF-1–Bcl-2 complex. NAF-1 binds to both the pro- and antiapoptotic regions (BH3 and BH4) of Bcl-2, as demonstrated by a nested protein fragment analysis in a peptide array and DXMS analysis. A combination of the solution studies together with a new application of DCA to the eukaryotic proteins NAF-1 and Bcl-2 provided sufficient constraints at amino acid resolution to predict the interaction surfaces and orientation of the protein–protein interactions involved in the docked structure. The specific integrated approach described in this paper provides the first structural information, to our knowledge, for future targeting of the NAF-1–Bcl-2 complex in the regulation of apoptosis/autophagy in cancer biology.

Cisd1 | Cisd2 | mitoNEET | Miner1 | CDGSH

Life requires a controlled balance of energy conversion and utilization. These critical processes are governed by an elaborate set of reactions involving numerous protein–protein interactions. Among them is the ability of organisms to control the recycling of high-energy compounds and to control cell proliferation. These processes are, at least in part, under the control of cell survival and programmed cell death (autophagic and apoptotic) processes. Misregulation of these processes leads to many diseases, including cancer. Among the key proteins involved in these processes are Bcl-2 (1, 2) and the more recently identified iron-sulfur (Fe-S) protein nutrient-deprivation autophagy factor-1 (NAF-1) (also known as Cisd2, Miner1, Eris, and Noxp70) (3–5).

NAF-1 is important for human health and disease. Missplicing of NAF-1 causes Wolfram syndrome 2 (6). NAF-1 is also functionally linked to the regulation of autophagy in cancer, and aging (3–5, 7, 8). This protein is a member of the 2Fe-2S cluster NEET family. NAF-1 has a similar backbone fold and 3Cys-1His coordination of the 2Fe-2S cluster as found in the founding member of the NEET family, mitoNEET (mNT). NAF-1 differs from mNT in the distribution of charged and aromatic surface residues (9, 10). These differences alter the 3D shape and electrostatics of the surfaces of mNT and NAF-1, leading to interactions with distinct binding partners. In fact, recent work identified NAF-1 as a Bcl-2/Bcl-XL binding partner (4) at

a branch point between autophagy and apoptosis, life and death, under nutrient-deprived and oxidative stress conditions in vivo (4, 7). This unusual interaction between a 2Fe-2S protein with the apoptotic/autophagic response pathways provides a new target for designing pro- and antiapoptotic therapies. Structural studies of the molecular determinants for these newly discovered protein–protein interactions are important for the specific design of modulators of the apoptotic/autophagic pathways.

Here we report on how a combination of peptide array, deuterium exchange mass spectrometry (DXMS), functional studies, and an unconventional application of direct coupling analysis (DCA) for eukaryotic complexes was used to determine both the dominant docked interface and functional consequences of NAF-1–Bcl-2 interaction. We show that NAF-1 binds to the BH3 and BH4 regions of Bcl-2 and that Bcl-2 binds to the NAF-1 groove formed between the  $\beta$ -cap and iron-sulfur cluster binding domains, with the strongest coupled interactions to the cluster binding domain. Binding of specific Bcl-2 peptides destabilizes the 2Fe-2S clusters of NAF-1 and affects protein functionality. Our unique integrated strategy that combines peptide array, DXMS, and DCA provides a set of data sufficient to obtain a level of structural detail that is not possible from any one individual technique. Furthermore, this robust strategy may be of general use in structural biology, in particular for interactions that may not be easily amenable to crystallography or NMR studies. The results

## Significance

Misregulation of cell growth and proliferation leads to the onset of various diseases, including cancer. Two proteins crucial for proper cellular control that were recently shown to affect cellular proliferation are Bcl-2, well-known for its role in programmed cell death, and the newly identified iron-sulfur protein NAF-1, localized near the mitochondrial outer membrane. In this report, we use a strategy utilizing a combination of experimental and computational techniques that provides valuable information to enable us to determine a molecular picture of the NAF-1–Bcl-2 interaction interface that is more complete than that obtained from any one technique alone. This interaction interface provides the basis from which novel drugs can be developed for the treatment of diseases such as cancer.

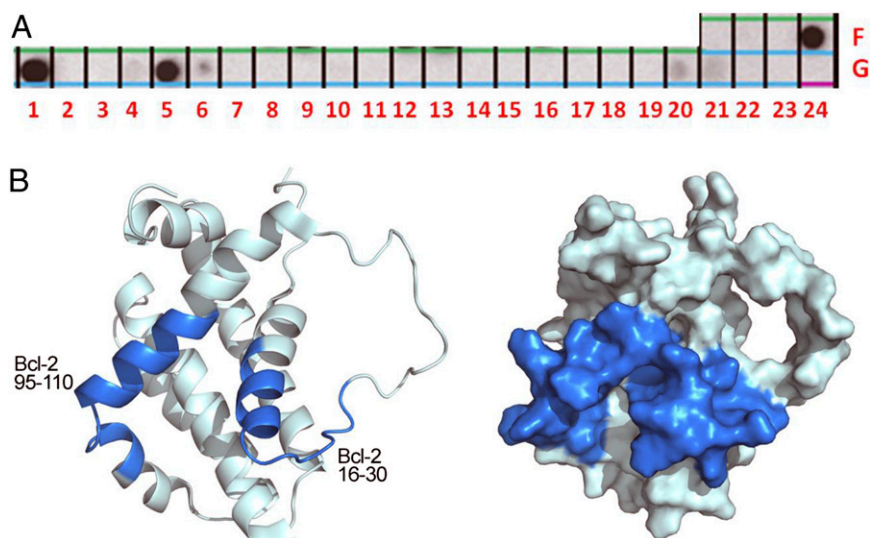
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**Fig. 1.** Mapping the binding sites of the peptides interacting with NAF-1 on the Bcl-2 protein. (A) An array consisting of partly overlapping peptides derived from Bcl-2 was screened for binding to NAF-1. Each dark spot represents binding of NAF-1 to a specific peptide (Table 1). (B) The binding sites of the peptides discovered in the peptide array screening (described in A) are colored on the 3D structure of Bcl-2. The interaction surface involves regions previously shown to be involved in interactions with both pro- and antiapoptotic proteins.

presented provide structural information for future targeting of the novel NAF-1–Bcl-2 protein pair in disease management, such as in the regulation of apoptosis/autophagy in cancer.

## Results

**Identification of the NAF-1 Binding Interface with Bcl-2 by Peptide Array Screening.** We designed an array composed of 27 partially overlapping peptides derived from Bcl-2 to identify the binding interface between regions in Bcl-2 that mediate the protein–protein interaction with NAF-1. Peptide length varied between 8

and 21 residues (Table 1). Peptides were designed based on the secondary and tertiary structures of the Bcl-2 NMR structure [Protein Data Bank (PDB) ID code 1YSW]. NAF-1 (His-tagged NAF-1 57–135 S92C) was expressed, purified, and screened for binding to the peptide array (Fig. 1A and Table 1). NAF-1 bound Bcl-2 peptides from two distinct regions of the full-length protein (Fig. 1B). The first region was composed of peptides derived from the N-terminal  $\alpha$ -helix and from the loop that follows (Bcl-2 peptides 16–30 and 23–44). This site corresponds to the BH4 domain, which is conserved among antiapoptotic Bcl-2 family members, and is essential for apoptosis inhibition. The second region consists of parts of helices 2 and 3, represented by the binding peptide Bcl-2 95–110, part of the BH3 domain. This site overlaps with a known binding site for proapoptotic members of the Bcl-2 family such as Bak and Bad (2). The Bcl-2 interaction surface deduced from the peptide array data is shown in Fig. 1B. The regions are structurally contiguous and overlap both regions associated with known pro- and antiapoptotic binding sites. For studies on the effects of peptide binding on the properties of NAF-1, we initiated our analyses using the strongly interacting Bcl-2 16–30 peptide.

**Table 1. Peptides used in the array screen**

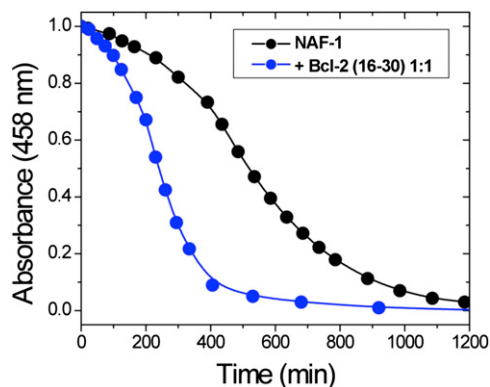
Array no.	Sequence	Bcl-2 residues
F21	HAGRTGYD	1–8
F22	HAGRTGYDNREIVMK	1–15
F23	YDNREIVMKYIHYKLSQR	7–24
<b>F24</b>	<b>YIHYKLSQ<b>RGYEWDA</b></b>	16–30
<b>G1</b>	<b>Q<b>RGYEWDA</b>GDDVEENRTEAPEG</b>	23–44
G2	AGDDVEENRTEAPEG	30–44
G3	EAP <b>EGTESEV</b> VHLTL	40–94*
G4	TESEV <b>VHLTL</b> RQAG <b>DDFSRR</b>	45–104*
<b>G5</b>	<b>RQAGDDFSRRYRRDF</b>	95–108
G6	<b>SRRYRRDFA</b>	102–110
G7	RYRRDFAEMSSQL	104–116
G8	RYRRDFAEMSSQLH <b>LT</b>	105–119
G9	FAEMSSQLH <b>LT</b>	109–119
G10	LH <b>LT</b> PFTARGRFATV	116–130
G11	PFTARGRFATV <b>VEEL</b>	120–134
G12	RFATV <b>VEEL</b> FRDGVN	126–140
G13	LFRDGVN <b>W</b>	134–141
G14	LFRDGVN <b>WGRIV</b> FAFF	134–148
G15	WGRIV <b>FAFF</b> EFGGVMCVESVNR	141–161
G16	FEFGGVMCVESVNR	148–161
G17	GVMCVESVNR <b>EM</b> SPL	152–166
G18	EM <b>SPL</b> VDNIALWMTE	162–176
G19	LVDNIALWMTE <b>YL</b> NR	166–180
G20	EYLN <b>RHLHT</b> WTIQDNG	176–190
G21	RHL <b>HT</b> WTIQDNGG	180–191
G22	NGGWDA <b>FV</b> ELYG	189–200
G23	WDA <b>FV</b> ELYG <b>PSMR</b>	192–204

Residues in bold are interacting regions from overlapping peptides.

\*These peptides are lacking residues 49–88, which is part of a disordered loop of Bcl-2.

**The NAF-1 2Fe-2S Cluster Is Destabilized by Interaction with the Bcl-2 16–30 Peptide.** Small-molecule binding to NEET proteins can either stabilize (11) or destabilize (12) the 2Fe-2S clusters. To test whether the binding of the Bcl-2 peptide affects the stability of the NAF-1 2Fe-2S clusters, we incubated NAF-1 with the Bcl-2 16–30 peptide and measured the absorbance at 458 nm [characteristic of the intact 2Fe-2S cluster of NAF-1 (9)] over time and compared the rate of cluster release with the NAF-1 protein in the absence of added peptide. The absorbance at 458 nm decreased faster in the presence of the Bcl-2 peptide (Fig. 2, blue) than without the peptide (black). This indicates that the NAF-1 protein binds the Bcl-2 16–30 peptide and that its cluster is destabilized by the interaction.

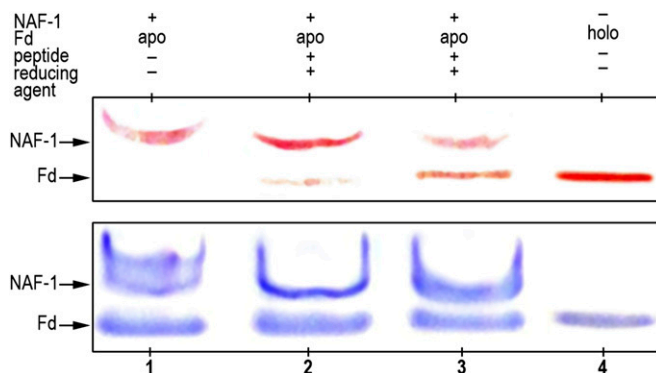
**NAF-1's 2Fe-2S Cluster Transfer Is Accelerated by the Bcl-2 16–30 Peptide.** We established that NAF-1 is a cluster donor protein in vitro and in vivo (11). In an effort to determine whether binding of the Bcl-2 16–30 peptide alters these functional properties, we tested the ability of NAF-1 to transfer its cluster to the apo-acceptor protein ferredoxin (Fd) as described previously (11). NAF-1 and apo-Fd with and without the Bcl-2 16–30 peptide were incubated for 20 min at room temperature with 5 mM DTT (to keep the cysteines of apo-Fd reduced). The progress of the reaction was assessed by native gel electrophoresis, as previously described (11) (Fig. 3). As can be seen in the



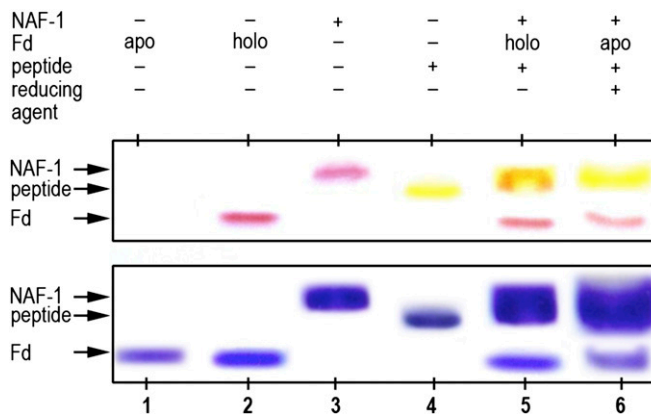
**Fig. 2.** Bcl-2 16–30 peptide destabilizes the 2Fe-2S cluster of NAF-1. UV-vis spectroscopy was used to observe the 2Fe-2S cluster stability of NAF-1 in the absence and presence of stoichiometric amounts of the Bcl-2 16–30 peptide. The Bcl-2 peptide interaction accelerates the cluster loss by a factor of two. All UV-vis spectra were measured from 250 to 800 nm on a Cary 50 spectrophotometer (Varian). Assay conditions were 100  $\mu$ M NAF-1 with or without 100  $\mu$ M Bcl-2 16–30 peptide in 50 mM bis-Tris buffer (pH 6.0) and 100 mM NaCl.

nondyed gel (*Upper*), the intensity of the red band of NAF-1 is decreased concomitant with an increase in the intensity of the red band of Fd in the presence of Bcl-2 16–30 (compare lane 2 with lane 3), suggesting that cluster transfer is accelerated in the presence of the Bcl-2 16–30 peptide. Coomassie staining of this gel (*Lower*) shows that the levels of NAF-1 and apo/holo-Fd do not change during the cluster transfer assay. Holo-Fd was run as a reference (holo).

**The Bcl-2 16–30 Peptide Remains Bound to NAF-1 Following Cluster Transfer.** Fluorescein-labeled Bcl-2 16–30 peptide was used to monitor the fate of the peptide following transfer of NAF-1's 2Fe-2S cluster to the apo-Fd acceptor protein. The results are presented in Fig. 4 and show that the Bcl-2 peptide (yellow band in lane 4) binds to NAF-1 when added to the reaction mix (upper red band in lane 5) as the position of the yellow fluorescent band comigrates with the NAF-1 band. The binding of the Bcl-2 16–30 peptide is specific to NAF-1, as no interactions with apo/holo-Fd are detected. Upon transfer of the 2Fe-2S cluster from NAF-1 to apo-Fd, the yellow Bcl-2 16–30 peptide remains bound to NAF-1



**Fig. 3.** NAF-1's 2Fe-2S cluster transfer is enhanced by the Bcl-2 16–30 peptide. NAF-1 was incubated with apo-Fd for 20 min at room temperature under different conditions, and the products were run on a native gel. Holo-Fd was run as a reference (lane 4). Prereduction of the acceptor Cys ligand residues of apo-Fd with 5 mM DTT ensures transfer from NAF-1 to apo-Fd. Cluster transfer was enhanced by the addition of Bcl-2 16–30 peptide (lane 3). (*Upper*) The gel is not stained (the red is from the 2Fe-2S in the cluster). (*Lower*) Coomassie blue staining of the gel verifying similar protein levels in all lanes. NAF-1 and Fd are indicated.



**Fig. 4.** Bcl-2 16–30 peptide remains bound to NAF-1 following 2Fe-2S cluster transfer. Lane 1, apo-Fd; lane 2, holo-Fd; lane 3, NAF-1; lane 4, fluorescein-labeled Bcl-2 16–30 peptide; lane 5, mixture of NAF-1, holo-Fd, and peptide; lane 6, mixture of NAF-1, apo-Fd, and peptide under conditions to promote cluster transfer (as observed by the presence of the red band at the Fd position). These results show that the Bcl-2 peptide binds to NAF-1 and remains bound following 2Fe-2S cluster transfer to apo-Fd. (*Upper*) The gel is not stained (the red is from the 2Fe-2S in the cluster, and the yellow is from the fluorescein-labeled peptide). (*Lower*) Stained with Coomassie.

(lane 6); the lower red band demonstrates that the 2Fe-2S cluster is transferred to form holo-red-Fd under these conditions.

**Direct Coupling Analysis Identifies Putative Interfacial Residues.** Because the experimental results showed an interaction between NAF-1 and Bcl-2, we used DCA (13) to identify important residues for the interaction based on the coevolution of the amino acid side chains between the two proteins (using sequence data for Pfam domains zf-CDGSH, Bcl-2, and BH4). A distinction with respect to previous applications of DCA to protein interactions (14, 15) is that our criterion for sequence pairing is based on the presence of both proteins (sequences) in the same organism rather than genome adjacency. DCA computes direct information (DI) values for each potential interresidue pair, providing a metric of the most directly coupled residue–residue interactions. DCA found that the top 30 of 1,000 potential interdomain residue–residue interactions involved the 13–29 amino acid peptide region of Bcl-2, consistent with the tight binding observed in the experimental measurements described above (Figs. 1–4). Moreover, the DCA determined that this region of Bcl-2 interacts with the region near the 2Fe-2S clusters of NAF-1; this interaction is shown in Fig. 5. We also verified that the average DI values per residue pair for the interaction region between domains zf-CDGSH and BH4 (amino acids 7–33) are higher than the average DI values for the potential interaction between domains zf-CDGSH and Bcl-2 (amino acids 97–195). This provides additional support that the BH4 region is part of the interaction interface.

**Deuterium Exchange Mass Spectrometry Analysis.** Protein–protein interactions result in changes in the stability of the backbone amide protons to solvent deuterium exchange at the binding interface and, in some cases, distal to the binding interface (16). To assess the changes upon NAF-1–Bcl-2 complex formation, we used a DXMS method. This method is powerful for analyzing protein–protein interactions and also provides an experimental test of the predictions from the DCA presented above. Importantly, DXMS allows an assessment of the role of the signature long loop in helix  $\alpha$ 2 of Bcl-2 that cannot be evaluated by DCA because it is outside of the evolving core sequence. Also, this loop is not present in the solution structure of the protein (17). In the DXMS experiment a fragmentation map is established for the protein, as described (18). The nondeuterated protein is exposed to deuterated buffer for varying amounts of time, and





purified on a Gilson HPLC using a reverse-phase C8 semipreparative column (ACE; Advanced Chromatography Technologies) with varying gradients of acetonitrile in water [both containing 0.001% (vol/vol) trifluoroacetic acid]. The peptides were analyzed by mass spectrometry (Voyager DE-PRO; Applied Biosystems). The Bcl-2 peptides G3 and G4 lack residues 49–88, an unstructured loop that was deleted in the NMR structure.

**Expression and Purification of NAF-1 and Apo-Fd Proteins.** The soluble part of NAF-1 (amino acids 57–135) was expressed and purified as previously described (9). Apo-NAF-1 was produced by removing the cluster by dialysis with 20 mM Tris-HCl (pH 6.0), 100 mM NaCl, and 0.02% (wt/vol)  $\text{Na}_2\text{S}_2\text{O}_8$  at 30 °C for 24–48 h. Apo-Fd was expressed and purified as previously described (24).

**Native-PAGE 2Fe-2S Cluster Transfer in Vitro Assay.** NAF-1 (200  $\mu\text{M}$ ) was incubated and shaken at room temperature with 400  $\mu\text{M}$  apo-ferredoxin (apo-Fd) with and without 500  $\mu\text{M}$  Bcl-2 16–30 peptide in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM DTT, and 0.02%  $\text{Na}_2\text{S}_2\text{O}_8$  for 20 min. DTT was added to keep the disulfide bonds in apo-Fd reduced. Transfer of the 2Fe-2S cluster from NAF-1 to apo-Fd was then analyzed by native gel as previously described (11).

**Cluster Stability Analysis.** An amount of 100  $\mu\text{M}$  NAF-1 was incubated either with or without 100  $\mu\text{M}$  Bcl-2 16–30 peptide in 50 mM bis-Tris (pH 6.0) and 100 mM NaCl at 37 °C. The absorbance at 458 nm was measured every 10 min on a Varian Cary 50 UV-visible spectrophotometer with temperature control (Agilent).

**Direct Coupling Analysis.** DCA was performed using the mean-field formulation of DCA as previously described (13). When using DCA for protein–protein interactions, there is a sequence-pairing procedure that combines the domains of interest. Similar pairings have been used previously to devise interaction interfaces (14, 15). Here the criterion used to pair proteins was to select domain pairs whose proteins belong to the same organism, the goal being to determine the most-coupled coevolving residue pairs between the two domains and hence the two proteins. The input sequences come from the Pfam (15) zinc finger domain zf-CDGSH (PF09360) in NAF-1, the Bcl-2 homology region 4 BH4 (PF02180) domain, and the Bcl-2 (PF00452) domain in Bcl-2 protein. DI values were computed for all interprotein residue–residue pairs and ranked according to their value. The top-ranked pairs have a higher probability of being in contact during protein–protein binding.

The model in Fig. 7 was created by minimizing the length of the green links representing the top coevolving residue pairs obtained from DCA. Rather than a physics-based docking, geometrical constraints were used to

satisfy the DCA contacts but also to include interaction regions obtained experimentally. The docked complex is depicted with a separation amenable for analysis and is not intended to represent an in vivo complex comparable to those obtained via crystallization or NMR. The model was created using UCSF Chimera software (25).

**Deuterium Exchange Mass Spectrometry.** Instrument setup and operation were described previously (26). All frozen samples were thawed and run using the conditions determined during fragmentation optimization.

**Fragmentation conditions.** The initial fragmentation conditions for the sample composition and instrument parameters were determined before starting the exchange time-course experiments. Stocks (1.4 mg/mL) of wild-type proteins were diluted with storage buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 2 mM DTT, pH 7.0) at room temperature and quenched with 0.5% formic acid, 16.6% glycerol, and 3.2 M guanidine-HCl (quench buffer) at 0 °C, and then immediately frozen on dry ice and stored at –80 °C until analysis as described (27).

**Deuterium on-exchange time courses.** The exchange time-course experiments for NAF-1, Bcl-2, and NAF-1 + Bcl-2 were all performed simultaneously at 25 °C with the following procedure. A full time-course experiment was initiated by adding 100  $\mu\text{L}$  of protein in 10 mM sodium phosphate buffer (pH 7.4) with 20 mM NaCl to 300  $\mu\text{L}$  of the equivalent deuterated exchange buffer for a final %  $\text{D}_2\text{O}$  of 75%. The exchange was monitored over intervals of 10, 30, 90, 300, 900, 3,600, and 5,600 s. Aliquots were removed and quenched at 0 °C in a final concentration of 0.5 M Gdn-DCl, 10% glycerol, and 0.5% formic acid. Quenched time points were flash-frozen and stored at –80 °C until analysis. In- and back-exchange controls were performed as previously described (28, 29).

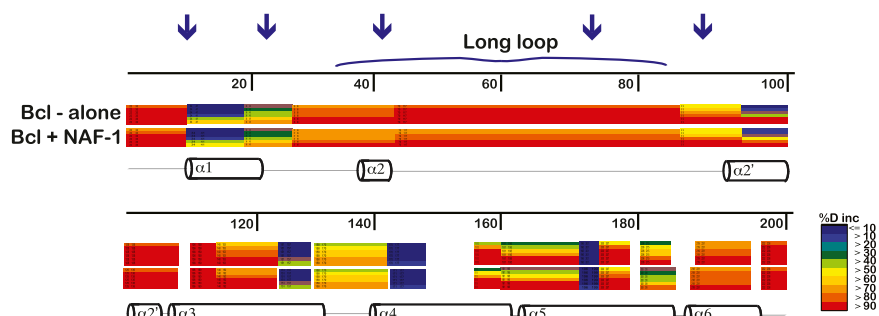
**Sequence identification of peptide fragments.** The identity of the parent peptide ions was determined using the SEQUEST software program (Thermo Finnigan) and MS1 and MS2 data. The quality of each peptide was monitored by individually examining each measured isotopic envelope spectrum for the entire time-course exchange. The deuterium content was calculated for each time point by using specialized software as previously described (26, 30).

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# Supporting Information

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**Fig. S1.** Time-dependent deuterium incorporation into Bcl-2. Deuteration levels of probes as a function of time are colored-coded and assigned to the primary structure in Bcl-2. Regions of secondary structure known from the solution structure data are assigned below the residues. Arrows indicate peptides with significant differences in protection.

**Table S1.** Top 30 DI-ranked pairs computed with DCA for interdomain interactions of Pfam families zf-CDGSH and BH4 mapped to proteins NAF-1 and Bcl-2

DI rank of pairs in domains zf-CDGSH-BH4	Residue in NAF-1	Residue in Bcl-2
1	99	13
2	101	13
3	80	20
4	103	21
5	105	20
6	96	26
7	112	23
8	108	13
9	110	13
10	114	13
11	94	7
12	111	13
13	115	15
14	104	13
15	99	23
16	80	21
17	80	13
18	109	29
19	115	23
20	101	23
21	105	30
22	89	19
23	92	7
24	109	14
25	103	20
26	115	7
27	105	21
28	101	20
29	99	20
30	99	21

DCA, direct coupling analysis; DI, direct information.

**Table S2. Top 30 DI-ranked pairs computed with DCA for interdomain interactions of Pfam families zf-CDGSH and Bcl-2 mapped to proteins NAF-1 and Bcl-2**

DI rank of pairs in domains zf-CDGSH-Bcl-2	Residue in NAF-1	Residue in Bcl-2
1	77	171
2	85	150
3	99	192
4	101	192
5	99	141
6	101	141
7	98	155
8	99	185
9	101	185
10	88	155
11	105	136
12	106	140
13	99	150
14	101	150
15	80	141
16	96	108
17	80	192
18	105	139
19	84	150
20	105	94
21	98	140
22	105	143
23	108	192
24	110	192
25	111	192
26	114	192
27	104	147
28	112	191
29	112	192
30	105	96