B cell lymphoma 2 (Bcl-2) residues essential for Bcl-2’s apoptosis-inducing interaction with Nur77/Nor-1 orphan steroid receptors

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ABSTRACT

Apoptosis is mediated through the extrinsic or intrinsic pathway. Key regulators of the intrinsic apoptotic pathway are the family of B cell lymphoma 2 (Bcl-2) proteins. The activity of the prototypical Bcl-2 protein is usually considered antiapoptotic. However, in some conditions, Bcl-2 associates with the orphan nuclear hormone receptors Nur77 and Nor-1, converting Bcl-2 into a proapoptotic molecule. Expression of Nur77 and Nor-1 is induced by a variety of signals, including those leading to apoptosis. Translocation of Nur77/Nor-1 to mitochondria results in their association with Bcl-2, exposing the Bcl-2 BH3 domain and causing apoptosis. However, the molecular details of this interaction are incompletely understood. Here, through extensive Bcl-2 mutagenesis and functional assays, we identified residues within Bcl-2 that are essential for its interaction with Nur77/Nor-1. Although an initial report has suggested that an unstructured loop region between the Bcl-2 BH4 and BH3 domains is required for Bcl-2’s interaction with Nur77/Nor-1, we found that it is dispensable for this interaction. Instead, we found important interacting residues at the BH4 domain and crucial interacting residues between the BH1 and BH2 domains. Bcl-2 alanine mutants at this region could no longer interact with Nur77/Nor-1 and could not initiate Nur77/Bcl-2-mediated cell death. However, they still retained their anti-apoptotic capability in two different death assays. These results establish crucial residues in Bcl-2 required for Nur77/Nor-1-mediated apoptosis and point to potential new strategies for manipulating Bcl-2 function.

Apoptosis is an essential cell death program for maintaining normal tissue homeostasis by remove unwanted and potential dangerous cells (1-7). Dysregulation of apoptotic pathways could lead to human pathological consequences, such as autoimmunity and cancer(8-11). Two pathways – extrinsic and intrinsic – can mediate apoptosis (12-15). The extrinsic pathway is mediated by death receptors, such as Fas and the tumor necrosis factor receptors (5, 16-18). The intrinsic pathway is mediated by the conserved family of Bcl-2 proteins, which are important for regulating apoptosis through mitochondria(4, 19, 20). The Bcl-2 family share related regions of sequence and structural homology, and can be subdivided into groups by their function and by the presence of one to four conserved Bcl-2 homology (BH) domains(19). The pro-apoptotic BH3-only molecules, such as Bcl-2 interacting mediator of cell death (e.g. Bim, Puma), sense and respond to apoptotic signals and activate the effectors molecules, Bax/Bak(19, 21-23). Bax and Bak contain BH1 to BH3 domains, and can induce permeabilization of the outer mitochondrial membrane to release cytochrome-c, leading to activation of capase-9 and the downstream caspases(6, 24-26). The anti-apoptotic family members, which include Bcl-2 and Bcl-X, contain all four BH domains and can prevent apoptosis by sequestering and inactivating the...
BH3-only proteins(19, 27). This function requires intact BH1, BH2 and BH4 domains(28, 29). Overexpression of Bcl-2 protein is a common mechanism of apoptosis dysregulation(4, 11). Elevated levels of Bcl-2 protein can offer a survival advantage in cells and has been associated with resistance to chemotherapy and poor prognosis(30). Hence, an on-going chemotherapeutic strategy in cancer has been to target Bcl-2 to restore the ability of cancer cells to undergo apoptosis(30-35).

Nur77 and Nor-1 belong to the family of orphan nuclear hormone steroid receptors, and have been implicated as pro-apoptotic factors in developing T cells and cancer cells(36-38). During T cell development, thymocytes expressing T cell receptors (TCR) with high affinity for self-antigens induces Nur77 and Nor-1 expression to a level that correlates with apoptosis accompanying negative selection(36, 37, 39). Prior studies have showed that a constitutive active form of either Nur77 or Nor-1 in thymocytes led to increased apoptosis, whereas the overexpression of a dominate-negative Nur77 protein, which can effectively inhibit the activity of all Nur77 family members, can rescue thymocytes undergoing cell death during negative selection(36, 37, 39-42). How Nur77 and Nor-1 are able to initiate apoptosis is still not completely clear and delineating the mechanism has long been under investigation. We and others have shown that Nur77 may initiate apoptosis by modulating the activity of Bcl-2(38, 39, 43, 44). While Bcl-2 is known as an anti-apoptotic molecule, several reports have highlighted the fact that Bcl-2 can be converted into a pro-apoptotic protein under certain circumstances(38, 41, 44). In thymocytes, strong TCR signals induce Nur77 and Nor-1 translocation to the mitochondria which then associates with Bcl-2, an event originally described in several cancer cells upon apoptotic stimuli(39, 44). This association leads to a conformational change that exposes Bcl-2’s BH3 domain. This may convert Bcl-2’s normal anti-apoptotic activity into a killer, pro-apoptotic protein, possibly through saturation binding of Bcl-x(44, 45). The importance of the Bcl-2 BH3 domain was demonstrated in transgenic mice engineered to express a Bcl-2 protein with its BH3 domain mutated in T cells(46). This Bcl-2 mutant exhibits increased anti-apoptotic activity and rescues more autoreactive T cells when compared to transgenic wild-type Bcl-2. Most strikingly, unlike wild-type Bcl-2 transgenic mice, these mice exhibited accelerated death from multi-organ autoimmunity(46).

Showing the in vivo significance of Bcl-2 conversion by Nur77/Nor-1 interaction has nevertheless been elusive, primarily because the molecular details of this interaction have not yet been fully elucidated. The essential residues within Bcl-2 for Nur77/Nor-1 interaction are unresolved. Early publications have reported that Nur77 associates with Bcl-2 through a linker region between BH4 and BH3 domains (i.e. an unstructured loop domain)(44, 47). However, Nur77 is also capable of interacting with other Bcl-2 family members, Bcl-b and Bcl-2a1(45, 48). Given that neither Bcl-b nor Bcl-2a1 contain a BH4-BH3 linker region(49-51), how Nur77 may convert them into pro-apoptotic molecules is not clear. This also raises the question whether the loop domain in Bcl-2 is even necessary for interaction with Nur77. Recently, the existence of a novel Nur77 binding pocket for Bcl-b was reported(52). However, whether this Nur77-binding pocket pertains to Bcl-2 has not been addressed.

Here, we identify Bcl-2 mutants that abrogate the interaction for both Nur77 and Nor-1 through extensive Bcl-2 mutagenesis. In contrast to prior observations, we report that the Bcl-2 loop domain is dispensable for Nur77/Nor-1 interaction. We also find that mutating a cluster of residues located in an intervening sequence between BH1 and BH2 domain can abolish Nur77 and Nor-1 interaction. Mutations at this site do not affect the Bcl-2 normal anti-apoptotic function but can block Nur77-mediated apoptosis. Taken together, our study further refines the molecular details of Nur77/Nor-1 and Bcl-2 interaction.

RESULTS

Bcl-2 Y18 and Y21 are essential for a truncated but not the full-length Bcl-2 to interact with Nur77 and its family member Nor-1 –To identify amino acids in Bcl-2 required for its interaction with Nur77 and Nor-1, we initially focused our attention on the loop between the BH4 and BH3 domain. This unstructured loop domain of Bcl-2 (amino acids 31 to 92) was reported to be a region where Bcl-2 interacts with Nur77(44, 47).
However, a precise location within the loop necessary for Nur77 interaction has not been defined. To investigate this further, we engineered constructs containing a truncated Bcl-2 fused to GFP with only the BH4 and loop domain (2-92), as well as progressive C- and N-terminal deletions within this BH4-loop fragment (Figure 1A). A tagged Bcl-2 was used because we discovered that the epitope for monoclonal antibodies against Bcl-2 (clone C-2, Santa Cruz Biotechnology) is located within the Bcl-2 loop region (data not shown). For Nur77, we used a FLAG-tagged Nur77 lacking a DNA binding domain (Nur77ΔDBD) as described previously(43-45). This allows us to bypass the requirement to stimulate the cells to initiate Nur77 nuclear to mitochondria translocation(53). These constructs were then transfected into HEK293T cells and FLAG co-immunoprecipitation (FLAG-IP) assays were performed to test for the mutant Bcl-2/Nur77ΔDBD interaction. Briefly, we used anti-FLAG antibodies to immunoprecipitate Nur77 from the cell lysates and the presence of co-immunoprecipitated Bcl-2 was detected on western blot using GFP specific antibodies. Surprisingly and in contrast to previous reports(44, 47), however, the Bcl-2/F(11-60) mutant lacking much of the loop was still able to interact with Nur77 (Figure 1B). Several other C-terminal deletions up to 35 amino acids also interacted with Nur77 (Figure 1B).

To further locate the Nur77 interacting site(s) in this Bcl-2 region, we generated progressive N-terminal deletions (Figure 1A). As shown in Figure 1C, Bcl-2/F(2-92), Bcl-2/F(11-92) and Bcl-2/F(15-92) were able to interact with Nur77, but deletion to amino acid 21 {Bcl-2/F(21-92)} abolished its ability to interact with Nur77 (Lane 7, Figure 1C). Similar results were obtained using Nor-1, with the exception that Bcl-2/F(15-92) consistently exhibited reduced interaction with Nor-1 (Lane 11, Figure 1C). We concluded that Bcl-2 can interact with Nur77 and Nor-1 through the N-terminal region at amino acids 15 through 21 in the BH4 domain, but the loop region between BH4 and BH3 domain is not required for this interaction.

To refine the region within BH4 required for interaction with Nur77/Nor-1, multiple alanine scan mutants along amino-acids 15 to 21 within the BH4-loop fragment were generated. These include Bcl-2/F(Ala(11-14)), Bcl-2/F(Ala(15-18)), and Bcl-2/F(Ala(19-21)), which respectively replace amino acids 11-14, 15-18 or 19-21 with alanines. As shown in Figure 1D, although each alanine scan mutant was able to co-immunoprecipitate with Nur77 to some extent, the largest reduction in interaction was observed with Bcl-2/F(Ala(15-18)) and Bcl-2/F(Ala(19-21)) (Lane 6-7, Figure 1D). Single amino-acid alanine substitutions were then generated for residues 15 to 21, but each was able to co-immunoprecipitate with Nur77 (data not shown). Notably, Y18A or Y21A alone was observed to reduce, but not fully prevent Nur77 interaction (Lane 5 and 6, Figure 1E). A significant loss of interaction with Nur77 was only observed in the Bcl-2 BH4-loop fragment containing both the Y18A and Y21A mutations (Lane 4, Figure 1E). The Bcl-2/F(Y18A,Y21A) protein completely lost its ability to interact with Nor-1 (Lane 9, Figure 1E). To see whether Y18A and Y19A within a full-length Bcl-2 protein was sufficient to abrogate Nur77 family binding, a GFP-fused WT Bcl-2 or Bcl-2 containing the double Y18 and Y21 alanine mutation (Bcl-2/Y18A,Y21A) was generated. In contrast to the Bcl-2/F(2-92) Y18/Y21 mutant, however, an interaction with Nur77 or Nor-1 persisted in the context of full-length protein Bcl-2/Y18A,Y21A (Lane 4 and 7, Figure 2A), suggesting that there might be multiple Bcl-2 regions that interact with Nur77. We also found that deletion of the Bcl-2 loop region (Bcl-2/Δ31-51, Bcl-2/Δ52-71, and Bcl-2/Δ72-92) in the context of the full protein has no effects on its ability to interact with Nur77 (Figure 2B). Taken together, these data provide evidence that the loop domain is dispensable, but Y18 and Y21 are essential for Nur77/Nor-1 interaction in the context of a truncated Bcl-2 protein.

Identification of Bcl-2 mutants that do not interact with Nur77 and Nor-1 – Consequently, we reasoned that Bcl-2 could contain multiple interacting residues with the Nur77/Nor-1 since combined alanine substitution of Y18 and Y21 in a full length Bcl-2 protein was unable to abrogate binding. These putative alternative sites could compensate for the loss of Y18 and Y21 to allow interaction with Nur77/Nor-1. Consistent with this notion, multiple Nur77 binding sites were recently found in Bcl-b, a member of the Bcl-2 family that can also undergo anti- to pro-apoptotic conversion by Nur77(52). Utilizing NMR spectroscopy-based
methods with a Nur77 derived peptide, the authors reported that Nur77 may interact with Bcl-b through several novel interaction sites adjacent to the BAX-binding crevice(52). We sought to test if the observation was also applicable to Bcl-2. Because Bcl-2 anti-apoptotic family members are structurally similar, we performed an amino-acid sequence alignment between Bcl-b and Bcl-2 and highlighted the Bcl-b residues involved with Nur77 interaction (Figure 3A). Interestingly, the Bcl-b residue Y19 involved in Bcl-b-Nur77 interaction aligns to Bcl-2 Y18. This is consistent with one of the two essential residues identified above using the BH4-loop fragment. The alignment also revealed other potential Bcl-2 interacting residues, which were primarily found within or flanking the BH3 domain. In addition, there are two Bcl-b Nur77-interacting residues that align to Bcl-2 residues between BH1 and BH2. To test whether analogous Bcl-2 residues could be involved in Nur77 interaction, we first generated two Bcl-2 mutants akin to the Bcl-b mutants that abolish its interaction with Nur77 (Bcl-b/Y19A,A44L to Bcl-2/Y21A,D102A,D103A & Bcl-b/R47A,E99A to Bcl-2/S105A,R106A,V159A,E160A, see Figure 3B) and performed FLAG-IPs as described before. Surprisingly, however, both Bcl-2 analogous mutants (Bcl-2/Y21A,D102A,D103A and Bcl-2/S105A,R106A,V159A,E160A) were still able to interact with Nur77 (Lane 4, Figure 3C; Lane 3, Figure 3D). We then expanded our search for potential interacting residues within the BH3 domain by generating Bcl-2 alanine scans. Bcl-2 alanine scan mutants within this domain were generated in a fashion that was N- or C-terminal to the core residues (G101, D102, D103) to avoid disrupting the potential pro-apoptotic function of BH3(41). These included Bcl-2/Ala(93-96), Bcl-2/Ala(94-97), Bcl-2/Ala(95-98), Bcl-2/Ala(106-110), and Bcl-2/Ala(110-114) (Figure 3B). To determine whether any of these Bcl-2 alanine mutants were able to abrogate Nur77 binding, FLAG-IPs were performed as described before. Surprisingly, each mutant again interacted with Nur77 although at slightly different efficiency as that WT Bcl-2 (Figure 3C, 3E). Among these mutants, only Bcl-2/Ala(93-96) was able to consistently reduce, but not fully abolish Nur77 or Nor-1 interaction (Lane 4 and 12, Figure 3E).

Finally, we investigated potential Bcl-2 residues found to align with Bcl-b in the intervening sequence between BH1 and BH2 domains. We generated the Bcl-2 alanine scan mutants: Bcl-2/Ala(157-160), Bcl-2/Ala(158-161), and Bcl-2/Ala(159-163) (Figure 3B). Using FLAG-IP as an assay, we found that Bcl-2/Ala(158-161) and Bcl-2/Ala(159-163), and occasionally Bcl-2/Ala(157-160) could no longer co-immunoprecipitate Nur77 (Figure 3E). Similar results were also observed with Nor-1 (Lane 14 and 15, Figure 3E). Hence, amino acids 158-163 of Bcl-2 within the intervening sequences between its BH1 and BH2 domains contains essential residues important for interaction with Nur77 and Nor-1.

**The BH1-BH2 intervening Bcl-2 mutants that no longer interact with Nur77 or Nor-1 still exhibit normal anti-apoptotic function** – The Bcl-2 region between BH1 and BH2 is not known to be important for the Bcl-2 anti-apoptotic function(28). To assess whether this is true, HeLa cells were transiently transfected to express either the control eGFP-C1 vector or vector encoding WT Bcl-2, Bcl-2/Ala(158-161), or Bcl-2/Ala(159-163). For comparison, we also transfected Bcl-2/Ala(93-96) or Bcl-2/Ala(94-97) with alanine mutations affecting the Bcl-2 BH3 domain. Bcl-2/Ala(93-96) but not Bcl-2/Ala(94-97) exhibited reduced interaction with Nur77. Cell viability was evaluated after apoptosis was initiated with either staurosporin (STS) or cisplatin (CIS). Cell viability of cells transfected with wild-type Bcl-2 was set at 100%. Immunoblots with the GFP-tag confirmed that each Bcl-2 mutant was expressed at similar levels to WT Bcl-2 (Figure 4A). As expected, HeLa cells expressing the control vector resulted increased cell death with 0.5 μM or 1 μM concentrations of STS (top panel, Figure 3B). Interestingly, HeLa cells expressing Bcl-2/Ala(93-96) or Bcl-2/Ala(94-97) proteins resulted in cell death indistinguishable to the empty vector control when challenged with 0.25μM, 0.5 μM or 1 μM STS. In contrast, Bcl-2/Ala(158-161) and Bcl-2/Ala(159-163) proteins exhibited protective activity to staurosporin-induced death at a similar or better level to WT Bcl-2 (top panel, Figure 4B). A similar result was obtained when cells were subjected to 30 μM CIS treatment (lower panel, Figure 4B).
We also assessed whether the 158-161 and 159-163 Bcl-2 mutants would affect anti-apoptotic function in a more physiological relevant setting by testing the Bcl-2 mutants in a classical cytokine deprivation assay. LyD9 murine hematopoietic progenitor cell line was utilized for this purpose as LyD9 cells undergo apoptosis in the absence of interleukin-3 (IL-3) (54, 55). Overexpression of Bcl-2 could protect cells from death by cytokine withdrawal (56). To test whether Bcl-2 alanine mutants were able to offer similar protection, LyD9 cells were stably transfected with the MSCV-PIG retroviral vector, MSCV-PIG encoding an HA-tagged WT Bcl-2, Bcl-2/Ala(158-161), or Bcl-2/Ala(159-163) by viral transduction. Successful stable expression of WT Bcl-2 or each Bcl-2 mutant were confirmed by western blot analysis (Figure 3C). To assess the anti-apoptotic activities of the Bcl-2 mutants, IL-3 was withdrawn from the culture medium and cell viability was monitored over time. As expected WT Bcl-2 offered protection when cells were deprived of IL-3 compared to the control vector (Figure 4D). A similar protection was also observed for Bcl-2/Ala(158-161) and Bcl-2/Ala(159-163), although the latter exhibited slightly less protective effect (Figure 4D). Together, these results provide evidence that Bcl-2/Ala(158-161) and Bcl-2/Ala(159-163) mutants impair the interaction with Nur77 and Nor-1, but have minor to no effect on its anti-apoptotic function.

Bcl-2 mutants that do not interact with Nur77 or Nor1 have reduced pro-apoptotic activity – The conversion of Bcl-2 from an anti- to pro-apoptotic molecule is thought to be mediated by direct Nur77 or Nor-1 interaction, which exposes its BH3 epitope (38, 39, 44). We reasoned that the identified Bcl-2 mutants unable to interact with Nur77 family should exhibit reduced death mediated by Bcl-2 conversion. To test this hypothesis, HeLa cells were transiently transfected with WT Bcl-2 or Bcl-2 mutants with or without Nur77 or Nor-1. Cell death was then measured by flow cytometric analysis of cells using Annexin V. The results showed minimal death (<5%) in all single transfectants (top panel, Figure 5). To ensure equivalent loading and expression, immunoblot was used to detect the corresponding FLAG- or GFP-tag (bottom panel, Figure 5). As reported previously by others (44), co-expression of Nur77ΔDBD or Nor-1ΔDBD with WT Bcl-2 in HeLa cells significantly increased cell death. In line with our reasoning, reduced cell death was observed when Bcl-2/Ala(158-161) and Bcl-2/Ala(159-163) to some extent when they were co-expressed with Nur77. Similar data were also observed when these mutants were co-expressed with Nor-1. We concluded that these Bcl-2 mutants that are unable to interact with Nur77 also fail to undergo Nur77-mediated cell death.

DISCUSSION
The ability for Nur77/Nor-1 to associate and convert Bcl-2 from a normally anti-apoptotic to a killer molecule is an attractive mechanism to harness and exploit for anti-tumor chemotherapies, yet the molecular details of this interaction is not completely clear. We therefore initiated this study to determine the precise Bcl-2 interacting residues essential for Nur77/Nor-1 interaction through extensive Bcl-2 mutagenesis. An unstructured Bcl-2 loop region (amino acids 31 to 92) between its BH4 and BH3 domain has been reported to be the site of Bcl-2/Nur77 interaction (44, 47). However, we found that this Bcl-2’s unstructured loop domain is not the primary site for Nur77/Nor-1 interaction. Two observations are consistent with this notion. First, Bcl-2 mutants containing large deletions across the loop region were still able to interact with Nur77. Second, by mutagenizing a truncated Bcl-2 protein containing only the BH4-loop domains, we observed that Nur77 or Nor-1 was able to immunoprecipitate a truncated Bcl-2 protein lacking much of its loop domain. Consistent with our data, a later paper by the same group reported that a Bcl-2/Bcl-x(L) chimera without the Bcl-2 loop region was still able to bind to Nur77 (52, 57). While the chimera contains a Bcl-x loop, it is known that Bcl-x(L) does not bind to Nur77 (48, 52, 57).

In the context of a truncated Bcl-2 protein, we identified two important Nur77/Nor-1 interacting residues (Y18 and Y21) within the BH4 domain. Neither of these BH4 residues have been reported to be essential for the Bcl-2 anti-apoptotic activity (58, 59). Surprisingly, when these residues were replaced in the context of a full length Bcl-2 protein (Bcl-2/Y18A,Y21A), we observed that the mutant protein still interacted with Nur77/Nor-1. This might indicate the presence of additional Nur77/Nor-1 interaction...
sites that can compensate for the loss of Y18 and Y21. In line with this notion, a recent study performed on Bcl-b for its site of interaction with Nur77 has shown that its BH4 domain is one of the Nur77-binding sites. However, a single Bcl-b residue modification in its BH4 domain was also insufficient to affect Nur77 interaction.(52)

The reported Bcl-b mutations that abolished Nur77 interaction were aimed at affecting the Nur77-binding pocket, either by hindering access by increasing Bcl-2 hydrophobicity (Bcl-b/Y19F,A44L) or by eliminating a charge residue (Bcl-b/R47A,E99A).(52) Our mutagenesis on Bcl-2 itself either by progressive alanine scans or by mimicking these Bcl-b mutants suggests that the putative Nur77-binding pocket for Bcl-2 is not entirely similar to that of Bcl-b. This is not too surprising, since there is an apparent structural difference between Bcl-b and Bcl-2, with the latter possessing an unstructured, “linker” region between BH4 and BH3 domains that could shift the location of this Nur77-binding site. The protein alignment between Bcl-b and Bcl-2 revealed one Bcl-b residue involved in Nur77 binding that matched to a critical residue within Bcl-2 BH3 domain for pro-apoptotic function (Bcl-b/A44 to Bcl-2/D102). It would be unlikely for Nur77/Nor-1 to bind Bcl-2 at this residue since an interaction at this site would block the potential pro-apoptotic BH3 domain function. Consistent with this, we observed that the Bcl-2 mutant (Bcl-2/Y21F,D102A,D103A) corresponding to a Bcl-b mutant (Bcl-b/Y19A,A44L) was still able to interact with Nur77. Thus, D102 from Bcl-2 that corresponds to the Bcl-b BH3 site is not a critical interacting residue with Nur77. Furthermore, normal Nur77 binding can still be seen for another Bcl-2 mutant that is analogous to a Bcl-b mutant that cannot interact with Nur77. Moreover, we were unable to identify a location within the Bcl-2 BH3 domain after performing a thorough alanine scan that could lead to a consistent and/or complete loss of Nur77 binding. All these data combined highlight differences between Bcl-2 and Bcl-b in their binding sites for Nur77.

In contrast to Bcl-b, where the essential sites of interaction with Nur77 occurs over multiple domains (i.e. BH4, BH3, and BH1), we identified essential interacting residues within Bcl-2 located in an intervening sequence between BH1 and BH2 domains(52). The mutants Bcl-2/Ala(158-161) and to a lesser extent Bcl-2/Ala(159-163), which correspond to residues CVES or VESVN, respectively, failed to interact with Nur77 and Nor-1. Unlike the Bcl-b mutants, where it was necessary to mutate discrete residues along the putative Nur77-binding pocket, the Bcl-2 mutants identified here are located within a single region. Why these mutations in Bcl-2 were sufficient to abolish Nur77 interaction instead of the mutating multiple sites similar to Bcl-b is not completely clear but might be due to the differences in the Nur77 interaction pocket between Bcl-2 and Bcl-b. One conceivable difference might be that the Nur77 interaction surface area in Bcl-b is larger than Bcl-2. In fact, it has been reported that Nur77 binds the tightest with Bcl-b compared to Bcl-2a1 and Bcl-2 in vitro and by fluorescence polarization assays(48, 52). Hence, mutations in Bcl-2 that affect the Nur77 binding pocket have a greater effect on Nur77-association. Alternatively, the residues are located within the central core helix (α5), a region that may play role in structural and functional importance(60). Although we cannot completely rule out the possibility that mutating four or five residues at a time could affect Bcl-2 tertiary structure, we were still able to observe detectable levels of these Bcl-2 mutants similar to that of WT levels by probing for the GFP-fused tag by immunoblot. In addition, these Bcl-2 alanine mutants exhibited little to no effect on the anti-apoptotic activities compared to WT Bcl-2. The latter datum also demonstrates that the tagged Bcl-2 behaves normally like that of WT Bcl-2. Interestingly, mutating V159 and E160 to alanines together were insufficient to abrogate Nur77 binding, suggesting that this cluster of residues may be working synergistically to interact with Nur77 and Nor-1. We also note that Bcl-x(L) also contains the residues CVES between BH1 and BH2 domains(57). However, Bcl-x(L) does not interact with Nur77, suggesting that this sequence alone would be insufficient to interact with Nur77/Nor-1. These observations taken together suggest that CVES are the required interaction residues in Bcl-2 for it to interact with Nur77/Nor-1.

In summary, we extend our findings to show an important Nur77 interacting residue cluster within Bcl-2 that is located in an
Characterization of Bcl-2 interaction with Nur77/Nor-1

We have provided evidence that these Bcl-2 mutants do not affect the normal Bcl-2 anti-apoptotic function and that these Bcl-2 mutants do not induce Nur77/Bcl-2 mediated death. These findings provide additional insights on the molecular details of the Nur77 conversion death pathway, and provide means to improve targeting Bcl-2 for anti-tumor therapy and to assess the importance of Bcl-2/Nur77 interaction in T cell negative selection.

**EXPERIMENTAL PROCEDURES**

**Plasmid constructs** –Murine N-terminal FLAG-tagged Nur77 and Nor-1 plasmids lacking a DNA binding domain (ΔDBD) (43, 44) were generated by standard deletion mutagenesis by overlap extension PCR protocol using BioRad iProof High-Fidelity DNA Polymerase in pmKate2-N (Evrogen) or pCI (Promega) expression vector. The final PCR products were cloned into pmKate2-N using HindIII and EcoRI restriction sites. Nur77ΔDBD corresponds to the deletion of amino acids 169-466. Nor-1ΔDBD corresponds to the deletion of amino acids 190-460.

Human Bcl-2 mutants were generated in pEGFP-C1 (Clontech) or MSCV-PIG in a similar fashion as described above or by Gibson cloning method (61). All mutations were verified by DNA sequencing (UC Berkeley Sequencing Facility). Primers used to generate the Bcl-2 recombinant plasmids are available upon request.

**Cell culture and transfection** –HeLa or the IL-3 producing X61-IL-3 cell line were cultured and maintained at 37°C in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, non-essential amino acids, 5 x 10^{-5} M 2-mercaptoethanol and 1 mM sodium pyruvate (cRPMI). The mouse multipotent IL-3 dependent LyD9 cells were cultured in cRPMI supplemented with IL-3 enriched media produced by X61-IL3 cells. Each cell line was periodically tested negative for mycoplasma. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacture’s recommendations.

**Co-immunoprecipitation and western blot** –HEK293T cells were transiently co-transfected with Nur77ΔDBD and WT Bcl-2 or Bcl-2 mutants at a 1:1 ratio. To study the interaction between Nur77 family members with human Bcl-2, cells were pelleted after 24 hours of transfection, washed with PBS, and resuspended in lysis buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.5; 20 mM EDTA; 1% NP-40; Protease Inhibitor Cocktail (Sigma); and 1 mM DTT). Lysates were clarified by high speed centrifugation and pre-cleared with Protein G agarose beads (Thermoscientific) prior to incubating with anti-FLAG or isotype with Protein G agarose overnight. Beads were washed with lysis buffer prior to being boiled in SDS sample loading buffer, ran on a 10% SDS-PAGE gel, and transferred onto nitrocellulose membranes. Membranes were blocked with 5% BSA in TBS containing 0.1% Tween and probed using anti-HA (clone HA.C5, Abcam), anti-MYC (clone E910, Clontech), anti-FLAG (clone 5E10, Accurus), anti-GFP (clone FL, Santa Cruz Biotechnology), or anti-GAPDH (clone 14C10, Santa Cruz Biotechnology) antibodies. Quantification of GFP levels were performed using Image Studio Light (LI-CORE) as described (62). Briefly, the rectangle tool was used to outline each protein band with each rectangle having equal areas. Immunoprecipitated FLAG or co-immunoprecipitated GFP band signal values were obtained. Co-immunoprecipitated GFP signals were normalized to immunoprecipitated FLAG. Fold difference of GFP was normalized relative to the co-immunoprecipitated GFP signal in the WT Bcl-2:Nur77 sample.

**LyD9 transduction of WT Bcl-2 or Bcl-2 alanine mutants** –Stably expressing WT Bcl-2 or Bcl-2 alanine mutants in LyD9 cells were obtained by first transfecting Phoenix cells with 3 µg control MSCV-PIG or MSCV-PIG encoding HA-tagged WT Bcl-2 or Bcl-2 alanine mutants along with 0.5 µg VSV-G and 1 µg gag-pol helper plasmid (Nolan lab) with Lipofectamine 2000 (Invitrogen) in 6-well plates. 24 hours post-transfection, the viral supernatant was passed through a 0.2 µM syringe filter, supplemented with 10 µg/mL Polybrene (Santa Cruz Biotechnology), and added to approximately 2-4 x 10^6 LyD9 cells. The LyD9 cells were spun at 2500rpm for 1 hour at room temperature and cultured at 37°C for 24 hours to recover. Selection of successfully transduced cells was obtained by supplementing the culture media with 10 µg/mL Puromycin. Stable transfection was confirmed by GFP expression using flow cytometry and/or by
detecting the presence of the C-terminal HA-tag by Western Blot analysis.

Cell death from chemical apoptotic inducers or IL-3 cytokine deprivation –24 hours post-transfection of WT Bcl-2 or Bcl-2 mutants (in 6 well plates), HeLa cells were transferred to 96 well plates and allowed to settle for another 24 hours. Cell viability was determined by CellTiter-Glo (Promega) at 24, 48, or 72 hours after incubating with cRPMI supplemented with indicated concentrations of staurosporin (STS; Sigma-Aldrich) or cisplatin (CIS; Sigma-Aldrich). Relative Light Units (RLUs) were obtained by normalizing the values to the corresponding WT Bcl-2 treated sample.

To measure cell viability after interleukin-3 (IL-3) deprivation, LyD9 cells were washed three times with 1X PBS (GE HyClone) and seeded into 96 well plates at density of $1 \times 10^5$ cells per well with cRPMI without IL-3. Cell viability was then measured by CellTiter-Glo after indicated number of days following cytokine deprivation. CellTiter-Glo assays were performed in triplicate in 96-well plates according to the manufacturer’s recommendations.

Annexin V+ staining by Flow cytometry – HeLa cells were transfected with the indicated plasmids. After 24 hours, HeLa cells were washed with 1X PBS, resuspended in Annexin V binding buffer (100 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl$_2$), and then incubated with Pacific Blue-conjugated Annexin V antibody (BD Biosciences). Analysis of Annexin V+ was performed on GFP+ and mKate+ cells using FlowJo 10 software (FlowJo).

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FOOTNOTES

The abbreviations used are: Nur77∆DBD, Nur77 lacking a DNA binding domain; Nor-1∆DBD, Nor-1 lacking a DNA binding domain; WT, wild-type; BH, Bcl-2 homology; Bcl, B cell lymphoma; GFP, green fluorescent protein; STS, staurosporin; CIS, cisplatin

FIGURE LEGENDS

FIGURE 1. Bcl-2 Y18 and Y21 within the BH4 domain are essential for a truncated Bcl-2, but not full-length Bcl-2 to interact with the Nur77 family. A, A schematic diagram of GFP fused to wild-type (WT) Bcl-2 or sequential N- or C-terminal deletions of Bcl-2 containing only the BH4 and loop domains with a C-terminal hemagglutinin (HA) tag. B, FLAG immunoprecipitation was performed on HEK293T cell lysates that were previously co-transfected with a FLAG-tagged Nur77 lacking a DNA binding domain (∆DBD) and GFP fused to wild-type (WT) Bcl-2 or the indicated Bcl-2 C-terminal loop deletion mutants. The immunoprecipitates were then run on the gels and blotted with antibodies to the indicated proteins (GFP or FLAG). As controls, the input extracts were also blotted with GFP, FLAG-, or GAPDH-specific antibodies. The fragments are tagged with GFP on the N-terminus and HA on the C-terminus. C-F, FLAG immunoprecipitations were performed similarly as in 1B using a Nur77∆DBD or Nor-1∆DBD construct co-transfected with the control construct containing only the eGFP (peGFP-C1) or the N-terminal deleted Bcl-2 fragments (C), alanine scan mutants within the Bcl-2 BH4-loop fragment (D), single or double alanine amino acid substitutions within the Bcl-2 fragment (E), or Y18A, Y21A mutations within the complete Bcl-2 protein (F). The immunoprecipitates were then run on the gels and blotted with antibodies to the indicated proteins (GFP or FLAG). As controls, the input extracts were also blotted with GFP-, FLAG-, or GAPDH-specific antibodies. Quantification of co-immunoprecipitated GFP bands were performed using Image Studio Lite (Li-CORE) as described in Materials & Methods section. The band intensity of co-immunoprecipitated GFP was normalized to immunoprecipitated FLAG. The normalized GFP signal (NGS) is shown relative to the GFP signal in the WT Bcl-2:Nur77 sample. All experiments have been repeated at least two times with similar results.

FIGURE 2. Bcl-2 Y18 and Y21 mutations or the Bcl-2 loop domain deletion within a complete full-length Bcl-2 protein are not essential for interaction with Nur77. A, FLAG immunoprecipitations were performed as previously described in Figure 1B using Nur77∆DBD or Nor-1∆DBD construct co-transfected with control construct containing only eGFP or Y18A, Y21A mutations within a complete Bcl-2 protein. The immunoprecipitates were run on gels and blotted with antibodies to the indicated proteins (GFP or FLAG). B, FLAG immunoprecipitations were performed using Nur77∆DBD construct co-transfected with control vector pCI or C-terminally MYC-tagged WT Bcl-2 or the indicated Bcl-2 loop deletion mutants. The immunoprecipitates were run on gels and blotted with antibodies to the indicated proteins (MYC or FLAG). Input extracts were blotted with antibodies specific to GFP, MYC, FLAG or GAPDH as a control. All experiments have been repeated at least two times with similar results.

FIGURE 3. Identification of Bcl-2 mutants that abrogate the ability to interact with Nur77 and its family member Nor-1. A, Sequence and structural alignment between Bcl-2 (UniProt P10415) and Bcl-b (UniProt Q9HD36) proteins. Sequences were first aligned using Clustal Omega, followed by manually adjusting the sequence after similar structural positions were identified by Phyre2. Coils above sequences indicate helices and labeled α1-α7. The Bcl-2 homology (BH) domains are shown below the corresponding sequences. Highlighted amino acids are Bcl-B residues involved in Nur77 family interaction as reported in Godoi et al(52). B, A schematic diagram of Bcl-2 mutants used for co-immunoprecipitation assays in C-E. C-E, FLAG-immunoprecipitation were performed as previously described in Figure 1B to identify the potential Nur77 family interacting regions using the indicated alanine mutants (e.g. Bcl-2/Y21A,D102A,D103A) or alanine scanning mutants (e.g. Bcl-2/Ala(95-98)). Quantification of co-immunoprecipitated GFP bands were performed using Image Studio Lite (Li-CORE)
as described in the methods section. The normalized GFP signal (NGS) is shown relative to the GFP signal in the WT Bcl-2:Nur77 sample. All experiments have been repeated at least two times or more where indicated with similar results.

**FIGURE 4.** Bcl-2 mutants that do not interact with Nur77/Nor1 do not affect its anti-apoptotic function. 

A, Immunoblot analysis of transiently transfected HeLa cells with control vector (pEGFP-C1) or constructs containing GFP-fused WT Bcl-2, Bcl-2/Ala(93-96), Bcl-2/Ala(94-97), Bcl-2/Ala(158-161) or Bcl-2/Ala(159-163). Antibodies specific against GFP, and GAPDH were used to assess transfection efficiency and equal loading. B, HeLa cells from 3A with the indicated constructs were treated with 0.25 µM, 0.50 µM or 1 µM staurosporin (STS) for 24 hrs (upper panel) or with 30 µM Cisplatin (CIS) for 48 hrs (lower panel). Cell viability was measured by CellTiter-Glo. Results shown are referenced mean ± SD values to WT Bcl-2 and are representative of three independent experiments performed in triplicate with similar results. Statistics here were calculated by two-way analysis of variance (ANOVA), with Bonferroni’s test compared to control are shown (n.s. not significant, *P < 0.05, **P < 0.01, ***P <0.001). C, Western Blot analysis of LyD9 cells transduced with MSCV-PIG control vector or MSCV-PIG vector encoding hemagglutinin (HA) tagged WT Bcl-2, Bcl-2/Ala(158-161) or Bcl-2/Ala(159-163). 

**FIGURE 5.** Bcl-2 mutants that cannot interact with Nur77 have reduced Nur77-mediated apoptosis. Top, Cell death was measured in HeLa cells by Annexin V+ flow cytometric analysis 24 to 36 hours after transfection with or without Nur77ΔDBD or Nor-1ΔDBD along with a control construct containing only eGFP or GFP-fused WT Bcl-2, Bcl-2/Ala(158-160), or Bcl-2/Ala(159-161) as described in the texts. Results are shown as a mean percentage ± SD of Annexin V+ from GFP+ and mKate+ positive HeLa cells. Background was subtracted. Data are representative of two independent experiments performed in duplicate with similar results. Statistical significance was calculated by Student’s t-test: n.s. not significant, *P < 0.05, **P < 0.01, ***P < 0.001. 

Bottom, Immunoblot analysis of the HeLa cells transiently transfected with the indicated plasmids shown in the top panel. Specific antibodies against FLAG, GFP, or GAPDH were used to assess transfection efficiency and equal loading.
Figure 2
Figure 4
Figure 5
B cell lymphoma 2 (Bcl-2) residues essential for Bcl-2’s apoptosis-inducing interaction with Nur77/Nor-1 orphan steroid receptors
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