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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, under 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 homology (BH) 3 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 removing unwanted and potentially 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 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 members 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)[2] domains (19). The pro-apoptotic BH3-only molecules, such as Bcl-2–interacting mediators of cell death (*e.g.* Bim and Puma), sense and respond to apoptotic signals and activate the effectors molecules Bax and Bak (19, 21–23). Bax and Bak contain the 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 to cells and has been associated with resistance to chemotherapy and a poor prognosis (30). Hence, an ongoing 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 with high affinity for self-antigens induce Nur77 and Nor-1 expression to a level that correlates with apoptosis accompanying negative selection (36, 37, 39). Prior studies have shown that a constitutively active form of either Nur77 or Nor-1 in thymocytes leads to increased apoptosis, whereas 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). Although Bcl-2 is known

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[2] The abbreviations used are: BH, Bcl-2 homology; DBD, DNA-binding domain; IP, immunoprecipitation; eGFP, enhanced GFP; STS, staurosporine; CIS, cisplatin; IL, interleukin; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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 T cell receptor signals induce Nur77 and Nor-1 translocation to the mitochondria, which then associate 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 compared with transgenic wildtype Bcl-2. Most strikingly, unlike wildtype Bcl-2 transgenic mice, these mice exhibited accelerated death because of multiorgan 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 the 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 of 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 the 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. Our study further refines the molecular details of Nur77/Nor-1 and Bcl-2 interaction.

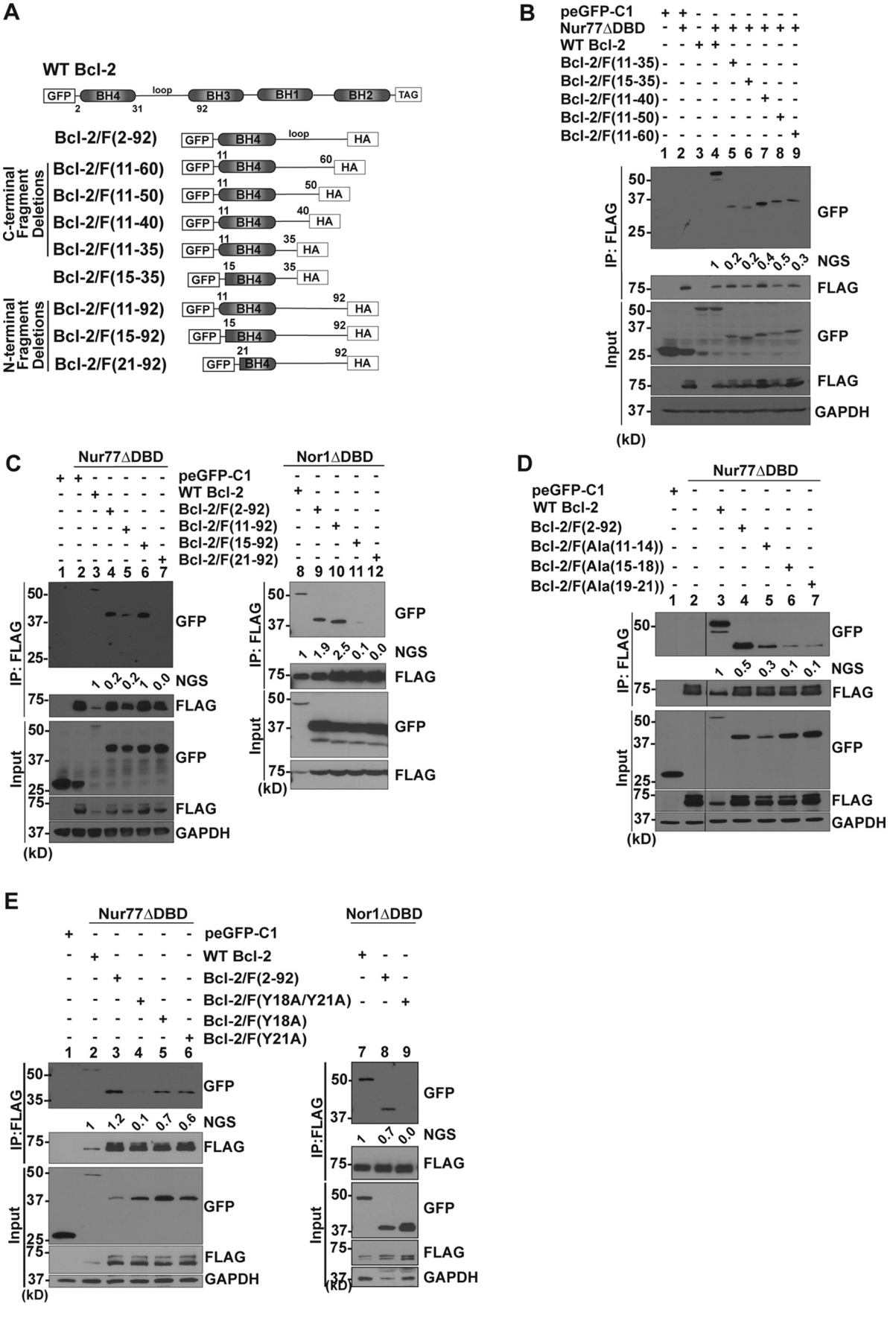
Results

Bcl-2 Tyr-18 and Tyr-21 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 domains. 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 (Fig. 1*A*). 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 nucleus-to-mitochondrion 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 a Western blot using GFP-specific antibodies. Surprisingly, and in contrast to previous reports (44, 47), the Bcl-2/F(11–60) mutant lacking much of the loop was still able to interact with Nur77 (Fig. 1*B*). Several other C-terminal deletions up to 35 amino acids also interacted with Nur77 (Fig. 1*B*).

To further locate the Nur77-interacting site(s) in this Bcl-2 region, we generated progressive N-terminal deletions (Fig. 1*A*). As shown in Fig. 1*C*, 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 (Fig. 1*C*, *lane 7*). Similar results were obtained using Nor-1, with the exception that Bcl-2/F(15–92) consistently exhibited reduced interaction with Nor-1 (Fig. 1*C*, *lane 11*). 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 that 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 Fig. 1*D*, 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)) (Fig. 1*D*, *lanes 6* and *7*). 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 (Fig. 1*E*, *lanes 5* and *6*). A significant loss of interaction with Nur77 was only observed in the Bcl-2 BH4 loop fragment containing both the Y18A and Y21A mutations (Fig. 1*E*, *lane 4*). The Bcl-2/F(Y18A,Y21A) protein completely lost its ability to interact with Nor-1 (Fig. 1*E*, *lane 9*). To see whether Y18A and Y19A within a full-length Bcl-2 protein were sufficient to abrogate Nur77 family binding, a GFP-fused WT Bcl-2 or Bcl-2 containing the double Tyr-18 and Tyr-21 alanine mutation (Bcl-2/Y18A,Y21A) was generated. In contrast to the Bcl-2/F(2–92) Tyr-18/Tyr-21 mutant, however, an interaction with Nur77 or Nor-1 persisted in the context of the full-length protein Bcl-2/Y18A,Y21A (Fig. 2*A*, *lanes 4* and *7*), suggesting that there might be multiple Bcl-2 regions that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WT Bcl-2
GFP
BH4
loop
BH3
BH1
BH2
TAG
2
31
92
Bcl-2/F(2-92)
Bcl-2/F(11-60)
Bcl-2/F(11-50)
Bcl-2/F(11-40)
Bcl-2/F(11-35)
Bcl-2/F(15-35)
Bcl-2/F(11-92)
Bcl-2/F(15-92)
Bcl-2/F(21-92)
HA
C-terminal Fragment Deletions
N-terminal Fragment Deletions
B
peGFP-C1
Nur77ΔDBD
WT Bcl-2
Bcl-2/F(11-35)
Bcl-2/F(15-35)
Bcl-2/F(11-40)
Bcl-2/F(11-50)
Bcl-2/F(11-60)
IP: FLAG
Input
GFP
NGS
FLAG
GAPDH
(kD)
C
Nur77ΔDBD
Nor1ΔDBD
peGFP-C1
WT Bcl-2
Bcl-2/F(2-92)
Bcl-2/F(11-92)
Bcl-2/F(15-92)
Bcl-2/F(21-92)
D
Nur77ΔDBD
peGFP-C1
WT Bcl-2
Bcl-2/F(2-92)
Bcl-2/F(Ala(11-14))
Bcl-2/F(Ala(15-18))
Bcl-2/F(Ala(19-21))
E
Nur77ΔDBD
Nor1ΔDBD
peGFP-C1
WT Bcl-2
Bcl-2/F(2-92)
Bcl-2/F(Y18A/Y21A)
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Bcl-2/F(Y21A)
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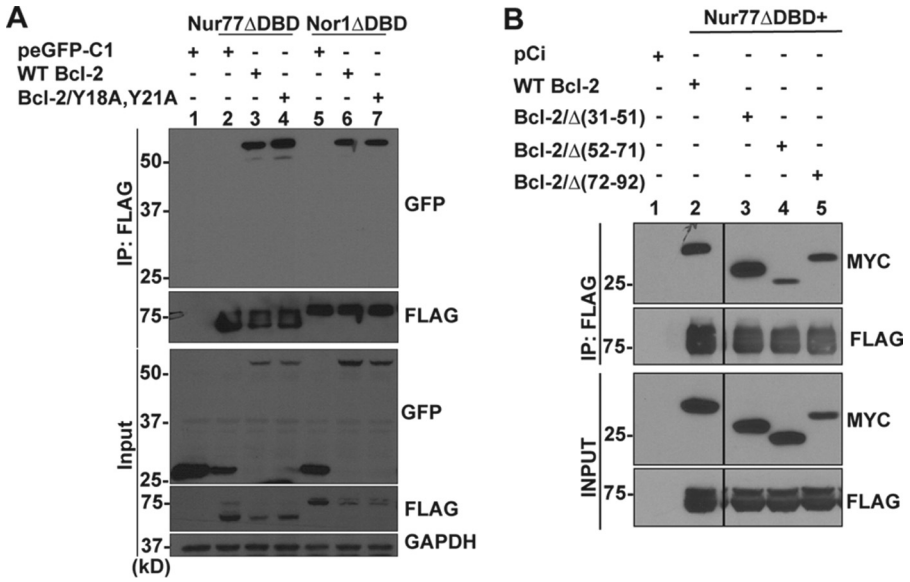


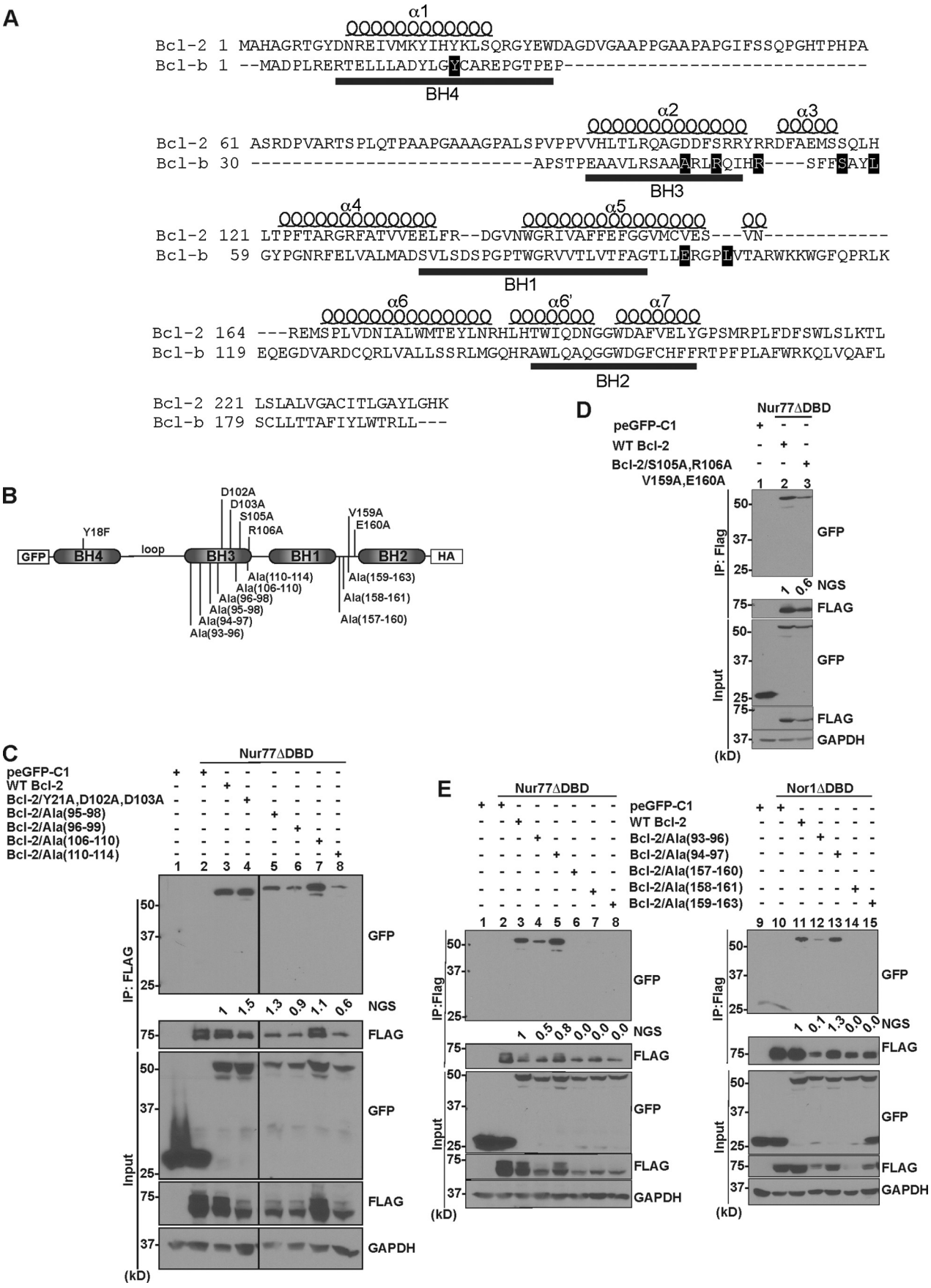
Figure 2. Bcl-2 Tyr-18 and Tyr-21 mutations or the Bcl-2 loop domain deletion within a complete full-length Bcl-2 protein are not essential for interaction with Nur77. *A*, FLAG immunoprecipitations were performed as described previously in Fig. 1*B* using the Nur77ΔDBD or Nor-1ΔDBD construct co-transfected with a control construct containing only eGFP or Y18A and 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 the Nur77ΔDBD 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 were repeated at least twice with similar results.

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 (Fig. 2*B*). Taken together, these data provide evidence that the loop domain is dispensable but that Tyr-18 and Tyr-21 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 Nur77/Nor-1 because combined alanine substitution of Tyr-18 and Tyr-21 in a full-length Bcl-2 protein was unable to abrogate binding. These putative alternative sites could compensate for the loss of Tyr-18 and Tyr-21 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 whether 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 in the Nur77 interaction (Fig. 3*A*). Interestingly, the Bcl-b residue Tyr-19 involved in the Bcl-b–Nur77 interaction aligns with Bcl-2 Tyr-21. 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 with 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 and Bcl-b/R47A,E99A to Bcl-2/S105A,R106A,V159A,E160A; see Fig. 3*B*) 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

Figure 1. Bcl-2 Tyr-18 and Tyr-21 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 of GFP fused to WT Bcl-2 or sequential N- or C-terminal deletions of Bcl-2 containing only the BH4 and loop domains with a C-terminal 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 (Δ*DBD*) and GFP fused to 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 were tagged with GFP on the N terminus and HA on the C terminus. *C–F*, FLAG immunoprecipitations were performed similarly as in *B*, using a Nur77ΔDBD or Nor-1ΔDBD 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 and 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 was performed using Image Studio Lite (LI-COR) as described under "Experimental procedures." 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 were repeated at least twice with similar results.


A
α1
Bcl-2 1 MAHAGRTGYDNREIVMKYIHYKLSQRGYEWDAGDVGAAPPGAAPAPGIFSSQPGHTPHPA
Bcl-b 1 --MADPLRERTELLLADYLGYCAREPGTPEP----------
BH4
α2
α3
Bcl-2 61 ASRDPVARTSPLQTPAAPGAAAGPALSPVPPVVHLTLRQAGDDFSRRYRRDFAEMSSQLH
Bcl-b 30 ----------APSTPEAAVLRSAAARLRQIHR----SFFSAYL
BH3
α4
α5
Bcl-2 121 LTPFTARGRFATVVEELFR--DGVNWGRIVAFFEFGGVMCVES---VN----------
Bcl-b 59 GYPGNRFELVALMADSVLSDSPGPTWGRVVTLVTFAGTLLERGPLVTARWKKWGFQPRLK
BH1
α6
α6'
α7
Bcl-2 164 ---REMSPLVDNIALWMTEYLNRHLHTWIQDNGGWDAFVELYGPSMRPLFDFSWLSLKTL
Bcl-b 119 EQEGDVARDCQRLVALLSSRLMGQHRAWLQAQGGWDGFCHFFRTPFPLAFWRKQLVQAFL
BH2
Bcl-2 221 LSLALVGACITLGAYLGHK
Bcl-b 179 SCLLTTAFIYLWTRLL---
B
GFP
BH4
Y18F
loop
BH3
BH1
BH2
HA
D102A
D103A
S105A
R106A
V159A
E160A
Ala(110-114)
Ala(106-110)
Ala(96-98)
Ala(95-98)
Ala(94-97)
Ala(93-96)
Ala(159-163)
Ala(158-161)
Ala(157-160)
C
Nur77ΔDBD
peGFP-C1
WT Bcl-2
Bcl-2/Y21A,D102A,D103A
Bcl-2/Ala(95-98)
Bcl-2/Ala(96-99)
Bcl-2/Ala(106-110)
Bcl-2/Ala(110-114)
IP: FLAG
GFP
NGS
FLAG
Input
GAPDH
(kD)
D
Nur77ΔDBD
peGFP-C1
WT Bcl-2
Bcl-2/S105A,R106A
V159A,E160A
IP: Flag
GFP
NGS
FLAG
Input
GAPDH
(kD)
E
Nur77ΔDBD
Nor1ΔDBD
peGFP-C1
WT Bcl-2
Bcl-2/Ala(93-96)
Bcl-2/Ala(94-97)
Bcl-2/Ala(157-160)
Bcl-2/Ala(158-161)
Bcl-2/Ala(159-163)
IP:Flag
GFP
NGS
FLAG
Input
GAPDH
(kD)

Nur77 (Fig. 3, *C*, *lane 4*, and *D*, *lane 3*). 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 (Gly-101, Asp-102, and Asp-103) 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) (Fig. 3*B*). 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 WT Bcl-2 (Fig. 3, *C* and *E*). Among these mutants, only Bcl-2/Ala(93–96) was able to consistently reduce, but not fully abolish, Nur77 or Nor-1 interaction (Fig. 3*E*, *lanes 4* and *12*).

Finally, we investigated potential Bcl-2 residues found to align with Bcl-b in the intervening sequence between the 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) (Fig. 3*B*). 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 (Fig. 3*E*). Similar results were also observed with Nor-1 (Fig. 3*E*, *lanes 14* and *15*). Hence, amino acids 158–163 of Bcl-2 within the intervening sequences between its BH1 and BH2 domains contain 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 a 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 staurosporine (STS) or cisplatin (CIS). The cell viability of cells transfected with wildtype Bcl-2 was set at 100%. Immunoblots with the GFP tag confirmed that each Bcl-2 mutant was expressed at similar levels as WT Bcl-2 (Fig. 4*A*). As expected, HeLa cells expressing the control vector resulted in increased cell death with 0.5 μM or 1 μM concentrations of STS (Fig. 4*B*, *top panel*). Interestingly, HeLa cells expressing Bcl-2/Ala(93–96) or Bcl-2/Ala(94–97) proteins resulted in cell death indistinguishable from the empty vector control when challenged with 0.25 μM, 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 staurosporine-induced death at a similar or better level than WT Bcl-2 (Fig. 4*B*, *top panel*). A similar result was obtained when cells were subjected to 30 μM CIS treatment (Fig. 4*B*, *bottom panel*).

We also assessed whether the 158–161 and 159–163 Bcl-2 mutants would affect anti-apoptotic function in a more physiologically relevant setting by testing the Bcl-2 mutants in a classical cytokine deprivation assay. The LyD9 murine hematopoietic progenitor cell line was utilized for this purpose because 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 was confirmed by Western blot analysis (Fig. 4*C*). 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 with the control vector (Fig. 4*D*). A similar protection was also observed for Bcl-2/Ala(158–161) and Bcl-2/Ala(159–163), although the latter exhibited a slightly less protective effect (Fig. 4*D*). Together, these results provide evidence that the Bcl-2/Ala(158–161) and Bcl-2/Ala(159–163) mutants impair the interaction with Nur77 and Nor-1 but have a 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 the 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 (Fig. 5, *top panel*). To ensure equivalent loading and expression, an immunoblot was used to detect the corresponding FLAG or GFP tag (Fig. 5, *bottom panel*). 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/

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 manual adjustment of the sequence after similar structural positions were identified by Phyre[2]. *Coils* above sequences indicate helices and labeled α1-α7. The 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*, schematic of the Bcl-2 mutants used for co-immunoprecipitation assays in *C–E*. *C–E*, FLAG-immunoprecipitation was performed as described previously in Fig. 1*B* 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 was performed using Image Studio Lite (LI-COR) as described under "Experimental procedures." The normalized GFP signal (*NGS*) is shown relative to the GFP signal in the WT Bcl-2:Nur77 sample. All experiments were repeated at least twice or more where indicated with similar results.

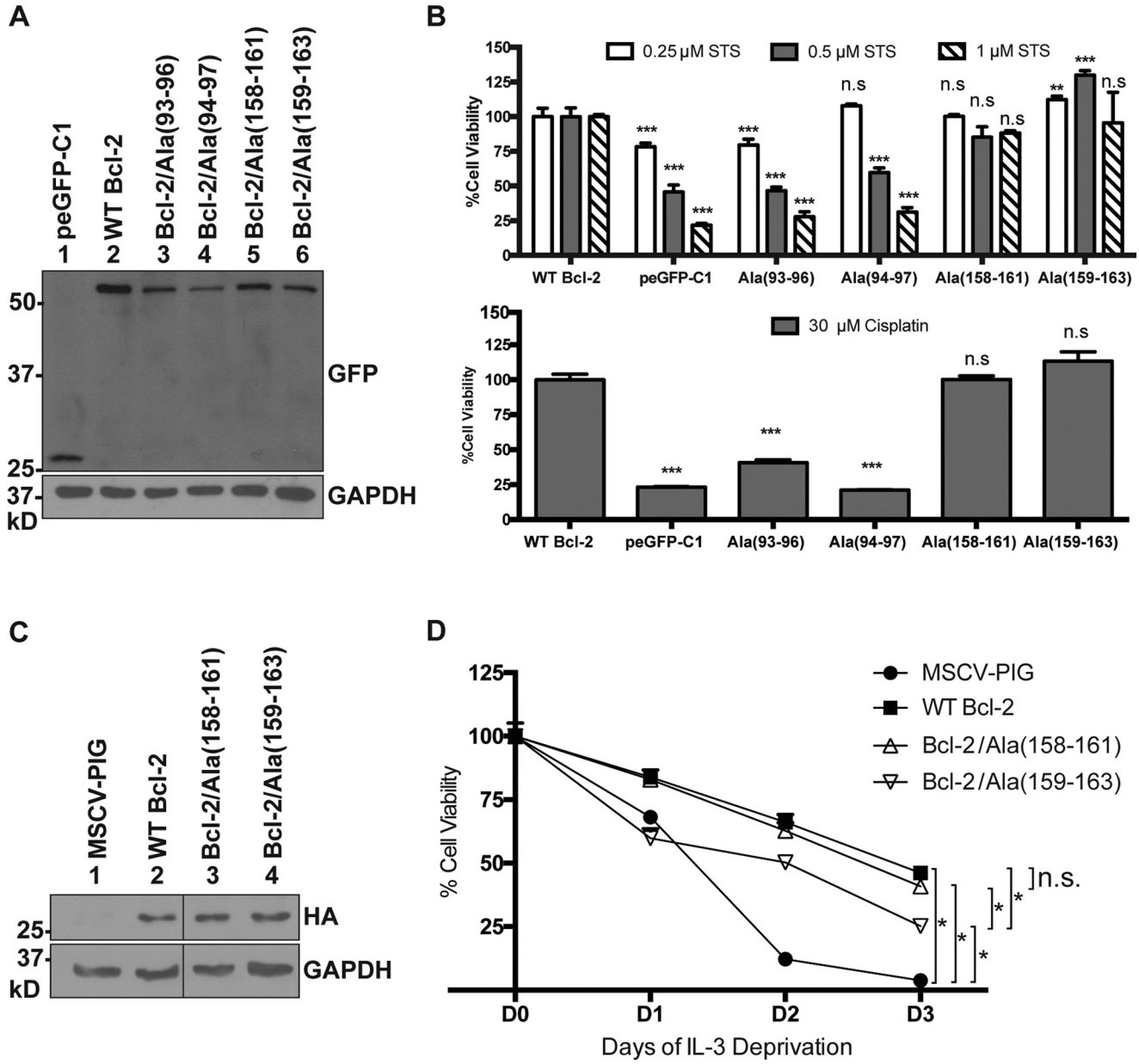


Figure 4. Bcl-2 mutants that do not interact with Nur77/Nor1 do not affect its anti-apoptotic function. *A*, immunoblot analysis of transiently transfected HeLa cells with a control vector (pEGFP-C1) or constructs containing GFP-fused WT Bcl-2, Bcl-2/Ala(93–96), Bcl-2/Ala(94–97), Bcl-2/Ala(158–161), 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 Fig. 3*A* with the indicated constructs were treated with 0.25 μM, 0.50 μM, or 1 μM STS for 24 h (*top panel*) or 30 μM CIS for 48 h (*bottom panel*). Cell viability was measured by CellTiter-Glo. Results shown are referenced mean ± S.D. values to WT Bcl-2 and are representative of three independent experiments performed in triplicate with similar results. Statistics were calculated by two-way analysis of variance with Bonferroni's test compared with control (*n.s.*, not significant; **, $p < 0.01$; ***, $p < 0.001$). *C*, Western blot analysis of LyD9 cells transduced with MSCV-PIG control vector or MSCV-PIG vector encoding HA-tagged WT Bcl-2, Bcl-2/Ala(158–161), or Bcl-2/Ala(159–163). *D*, an IL-3–dependent multipotent stem cell clone, LyD9, transduced with MSCV-PIG vectors encoding WT Bcl-2 or Bcl-2 alanine mutants. Stably transduced cells were selected for resistance against puromycin. The abilities of WT Bcl-2 or Bcl-2 mutants to protect cells from apoptosis were tested by depriving cells of IL-3 for the indicated days (*D0*, *D1*, *D2*, and *D3*). Cell viability was monitored by CellTiter-Glo and normalized to the initial number. All experiments were repeated at least three times with similar results. Statistical significance was calculated by Student's *t* test: *n.s.*, not significant; *, $p < 0.05$.

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 conclude that these Bcl-2 mutants, which are unable to interact with Nur77, also fail to mediate Nur77-induced cell death.

Discussion

The ability of 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, but the molecular details of this interaction are 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 domains has been reported to be the site of Bcl-2/Nur77 interaction (44, 47). However, we found that this Bcl-2 unstructured loop domain is not the primary site for

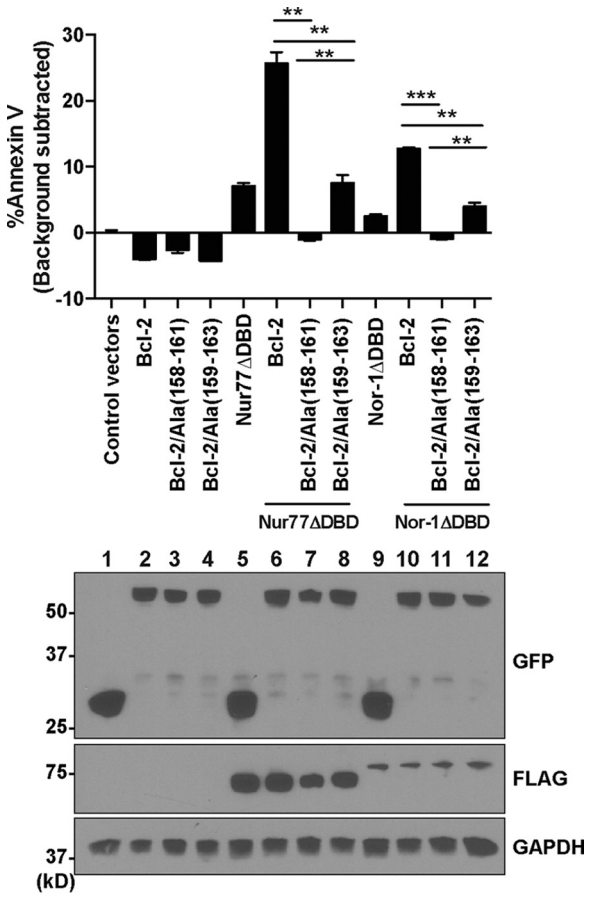


Figure 5. Bcl-2 mutants that cannot interact with Nur77 have reduced Nur77-mediated apoptosis. *Top panel*, cell death was measured in HeLa cells by Annexin V^+ flow cytometric analysis 24 to 36 h after transfection with or without Nur77ΔDBD or Nor-1ΔDBD along with a control construct containing only eGFP or GFP-fused WT Bcl-2, Bcl-2/Ala(158–160), or Bcl-2/Ala(159–161) as described in the text. Results are shown as mean percentage ± S.D. of Annexin V^+ from GFP^+ and $mKate^+$ 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; **, $p < 0.01$; ***, $p < 0.001$. *Bottom panel*, immunoblot analysis of 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.

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, later papers reported that a Bcl-2/Bcl-x(L) chimera without the Bcl-2 loop region was still able to bind to Nur77 (52, 57). Although 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 (Tyr-18 and Tyr-21) 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 Tyr-18 and Tyr-21. 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 of 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 because there is an apparent structural difference between Bcl-b and Bcl-2, with the latter possessing an unstructured "linker" region between the 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 the 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 because 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, Asp-102 from Bcl-2, which 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 of 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 occur over multiple domains (*i.e.* BH4, BH3, and BH1), we identified essential interacting residues within Bcl-2 located in an intervening sequence between the 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 with 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 a 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 immunoblotting. In addition, these Bcl-2 alanine mutants exhibited little to no effect on the anti-apoptotic activities compared with WT Bcl-2. The latter datum also demonstrates that the tagged Bcl-2 behaves normally like that of WT Bcl-2. Interestingly, mutating Val-159 and Glu-160 to alanines together was 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 the 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 intervening sequence between BH1 and BH2. 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 into 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 Bio-Rad iProof high-fidelity DNA polymerase in the 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 the Gibson cloning method (61). All mutations were verified by DNA sequencing (University of California 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 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, non-essential amino acids, 5×10^{-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 medium produced by X61-IL3 cells. Each cell line periodically tested negative for mycoplasma. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer'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 h of transfection, washed with PBS, and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 20 mM EDTA, 1% Nonidet P-40, protease inhibitor mixture (Sigma), and 1 mM DTT). Lysates were clarified by high-speed centrifugation and precleared with protein G–agarose beads (Thermo Scientific) prior to incubation with anti-FLAG or isotyping with protein G–agarose overnight. Beads were washed with lysis buffer prior to being boiled in SDS sample loading buffer, run on a 10% SDS-PAGE gel, and transferred onto nitrocellulose membranes. Membranes were blocked with 5% BSA in Tris-buffered saline 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 was performed using Image Studio Light (LI-COR) as described previously (http://lukemiller.org/index.php/2013/02/analyzing-western-blots-with-image-studio-lite/).[3] 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. The -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 of control MSCV-PIG or MSCV-PIG encoding HA-tagged WT Bcl-2 or Bcl-2 alanine mutants along with 0.5 μg of VSV-G (G glycoprotein of the vesicular stomatitis virus) and 1 μg of gag-pol helper plasmid (Nolan laboratory) with Lipofectamine 2000 (Invitrogen) in 6-well plates. 24 h 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 ~$2–4 \times 10^6$ LyD9 cells. The LyD9 cells were spun at 2500 rpm for 1 h at room temperature and cultured at 37 °C for 24 h to recover. Selection of successfully transduced cells was obtained by supplementing the culture medium 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.

[3] Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party–hosted site.

Cell death from chemical apoptotic inducers or IL-3 cytokine deprivation

24 h 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 h. Cell viability was determined by CellTiter-Glo (Promega) 24, 48, or 72 h after incubation with cRPMI supplemented with the indicated concentrations of STS (Sigma-Aldrich) or CIS (Sigma-Aldrich). Relative light units were obtained by normalizing the values to the corresponding WT Bcl-2–treated sample.

To measure cell viability after IL-3 deprivation, LyD9 cells were washed three times with 1× PBS (GE HyClone) and seeded into 96-well plates at a density of 1×10^5 cells/well with cRPMI without IL-3. Cell viability was then measured by CellTiter-Glo after the 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 h, HeLa cells were washed with 1× PBS, resuspended in Annexin V binding buffer (100 mM HEPES (pH 7.4), 140 mM NaCl, and 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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