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Conversion of Bcl-2 from Protector to Killer by Interaction with Nuclear Orphan Receptor Nur77/TR3

Bingzhen Lin, Siva Kumar Kolluri, Feng Lin, Wen Liu, Young-Hoon Han, Xihua Cao, Marcia I. Dawson, John C. Reed,* and Xiao-kun Zhang*
The Burnham Institute
Cancer Center
10901 North Torrey Pines Road
La Jolla, California 92037

Summary

The Bcl-2 family proteins are key regulators of apoptosis in human diseases and cancers. Though known to block apoptosis, Bcl-2 promotes cell death through an undefined mechanism. Here, we show that Bcl-2 interacts with orphan nuclear receptor Nur77 (also known as TR3), which is required for cancer cell apoptosis induced by many antineoplastic agents. The interaction is mediated by the N-terminal loop region of Bcl-2 and is required for Nur77 mitochondrial localization and apoptosis. Nur77 binding induces a Bcl-2 conformational change that exposes its BH3 domain, resulting in conversion of Bcl-2 from a protector to a killer. These findings establish the coupling of Nur77 nuclear receptor with the Bcl-2 apoptotic machinery and demonstrate that Bcl-2 can manifest opposing phenotypes, induced by interactions with proteins such as Nur77, suggesting novel strategies for regulating apoptosis in cancer and other diseases.

Introduction

Bcl-2-family proteins are evolutionarily conserved regulators of apoptosis (Adams and Cory, 1998; Gross et al., 1999; Reed, 1998; Vander Heiden and Thompson, 1999). All members possess at least one of the four conserved motifs called Bcl-2 homology (BH) domains. Antiapoptotic members, such as Bcl-2 and Bcl-X_L, contain all four BH domains. Some proapoptotic members, such as Bax and Bak, contain BH1, BH2, and BH3 domains, while others, such as Bad and Bid, share sequence homology only at the BH3 domain. The Bcl-2 family proteins primarily act at mitochondria to regulate apoptosis, possibly by forming channels in mitochondrial membranes (Green and Reed, 1998).

One curious and as yet unexplained aspect of some Bcl-2-family proteins is that their phenotypes can be reversed in some cellular contexts. Overexpression of Bcl-2 or Bcl-X_L in some cells promotes rather than prevents apoptosis, whereas Bax and Bak prevent apoptosis under some circumstances (Chen et al., 1996; Fannjiang et al., 2003; Grandgirard et al., 1998; Lewis et al., 1999; Subramanian and Chinnadurai, 2003; Uhlmann et al., 1998). *Drosophila* Bcl-2 homologs exhibit either pro- or antiapoptotic activity (Colussi et al., 2000; Igaki et al., 2000). Similarly, mutants of the Bcl-2-homolog, Ced-9, appear to promote rather than prevent programmed cell death in *C. elegans* (Xue and Horvitz, 1997). Given that the *C. elegans* genome contains no Bax-homologs, Ced-9 may perform the functions of both Bcl-2 and Bax by adopting different conformations to exert opposing effects on cell life and death.

Nur77 (TR3 or NGFI-B), an orphan member of the steroid/thyroid/retinoid nuclear receptor superfamily (Kastner et al., 1995; Mangelsdorf and Evans, 1995; Zhang, 2002), plays roles in regulating growth and apoptosis (Winoto and Littman, 2002; Zamzami and Kroemer, 2001; Zhang, 2002). Nur77 expression is rapidly induced during apoptosis in immature thymocytes and T cell hybridomas (Liu et al., 1994; Woronicz et al., 1994), and cancer cells of lung (Li et al., 1998; Kolluri et al., 2003), prostate (Li et al., 2000; Uemura and Chang, 1998), ovary (Holmes et al., 2002, 2003), colon (Wilson et al., 2003), and stomach (Liu et al., 2002; Wu et al., 2002). High levels of Nor1, a Nur77-family member, are associated with favorable responses to several chemotherapeutic agents in patients with diffuse large B-cell lymphoma (Shipp et al., 2002).

Recently, we discovered a paradigm in cellular apoptosis (Li et al., 2000), wherein Nur77 translocates from the nucleus to the cytoplasm, targeting to mitochondria and inducing cyt *c* release. Nur77 mitochondrial-targeting occurs during apoptosis of different types of cancer cells (Holmes et al., 2003; Kolluri et al., 2003; Liu et al., 2002; Wilson et al., 2003; Wu et al., 2002). Sindbis virus-induced apoptosis also involves Nur77 translocation to mitochondria (Lee et al., 2002). How Nur77 targets mitochondria and induces apoptosis however has been unclear.

In this study, we investigated the mechanism by which Nur77 targets mitochondria and induces apoptosis. Our results demonstrate that Nur77 interacts with Bcl-2 through its ligand binding domain (LBD) and that the interaction is required for Nur77 mitochondrial targeting and Nur77-dependent apoptosis. Interestingly, Nur77 binds to the Bcl-2 N-terminal loop region, located between its BH4 and BH3 domains, resulting in a conformational change in Bcl-2, which converts it from a protector to a killer protein.

Results

Nur77 Interacts with Bcl-2

We investigated whether Nur77 targets to mitochondria by binding Bcl-2. In vitro protein binding assays showed that similar proportions of ^{35}S-labeled Bcl-2 or RXRα, a known Nur77 heterodimerization partner, were selectively pulled-down by GST-Nur77 but not by GST (Figure 1A). Conversely, ^{35}S-labeled Nur77 and Bax, a known heterodimerization partner of Bcl-2, bound equally to GST-Bcl-2 but not to GST (Figure 1A). In mammalian two-hybrid studies, Bcl-2/ΔTM, a Bcl-2 mutant lacking its C-terminal transmembrane domain, strongly inter-

*Correspondence: xzhang@burnham.org (X.-k.Z.), jreed@burnham.org (J.C.R.)

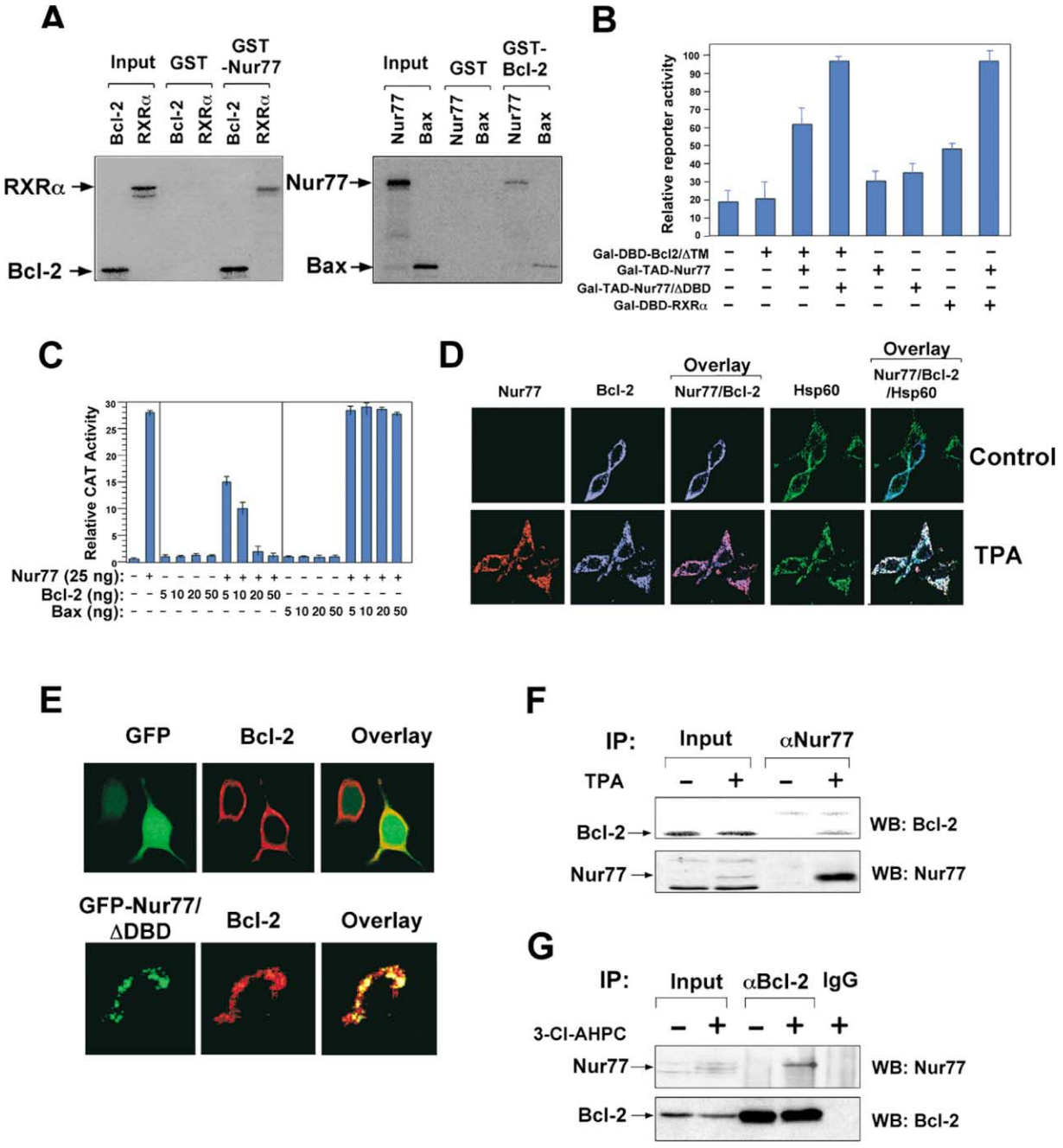

Figure 1. Interaction of Nur77 with Bcl-2

(A) GST-pull down. GST-Nur77, GST-Bcl-2, or GST control protein immobilized on glutathione-Sepharose was incubated with in vitro synthesized ^{35}S-labeled Bcl-2, RXRα, Nur77, or Bax as indicated. Bound proteins were analyzed by SDS-PAGE autoradiography. Input represents 5% of protein used in the pull down assays

(B) Mammalian two-hybrid assay. Gal4 reporter gene $(Gal4)_2$-*tk*-Luc) was transfected into CV-1 cells with the Bcl-2/ΔTM (TM was deleted to prevent Bcl-2 membrane association) or RXRα fused with the Gal-DBD alone or with the Nur77 or Nur77/ΔDBD fused with the Gal-transactivation domain (TAD). Reporter gene activity was determined 48 hr later.

(C) Inhibition of Nur77-dependent transactivation by Bcl-2. CV-1 cells were transfected with the NurRE-*tk*-CAT reporter (Li et al., 2000) with or without Nur77 expression vector together with or without the Bcl-2 or Bax. CAT activity was then determined. The bars in (B) and (C) are means ± SD from three and six experiments, respectively.

(D) Colocalization of endogenous Nur77 and transfected Bcl-2. LNCaP cells were transfected with Bcl-2, treated with or without TPA (100 ng/ml) for 3 hr, then immunostained with polyclonal rabbit anti-Bcl-2, mouse monoclonal anti-Nur77, or anti-Hsp60 antibody. Nur77, Bcl-2, and Hsp60 were visualized using confocal microscopy and images were overlaid (overlay). Approximately 80% of TPA-treated cells demonstrated colocalization.

(E) Colocalization of transfected GFP-Nur77/ΔDBD and Bcl-2. GFP–Nur77/ΔDBD (3 μg) and Bcl-2 (1 μg) were cotransfected into LNCaP cells. After 20 hr, cells were immunostained with anti-Bcl-2 antibody. GFP–fusion and Bcl-2 were visualized as in (D). For control, distribution of transfected GFP empty vector is shown. Approximately 30% of transfected cells exhibited colocalization shown.

(F) In vivo Co-IP assay in LNCaP cells. Lysates from LNCaP cells treated with or without TPA for 3 hr were incubated with mouse monoclonal anti-Nur77 antibody (Abgent, San Diego, CA). For immunoblotting of immunoprecipitates, anti-Bcl-2 or rabbit polyclonal anti-Nur77 antibody (Active Motif, Carlsbad, CA) were used.

(G) In vivo Co-IP in H460 cells. Lysates from H460 cells treated with or without 3-Cl-AHPC (10^{-6} M) for 3 hr were incubated with anti-Bcl-2 antibody. Immunoblotting of immunoprecipitates was conducted as in (F).

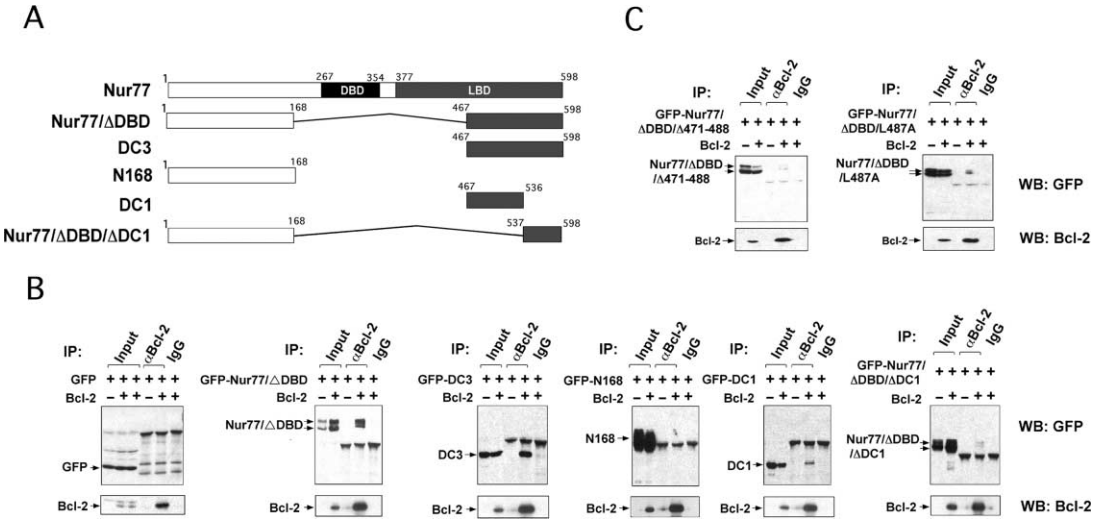

Figure 2. Ligand binding Domain of Nur77 Interacts with Bcl-2

(A) Schematic representation of Nur77 mutants. The Nur77 DBD and LBD are indicated.

(B-C) In vivo Co-IP of Nur77 mutants and Bcl-2. The indicated Nur77 mutant fused with GFP or the empty GFP vector (6 μg) was cotransfected with the empty vector (pRC/CMV) or Bcl-2 expression vector (2 μg) into HEK293T cells. Lysates were immunoprecipitated by using either polyclonal rabbit anti-Bcl-2 antibody (against whole Bcl-2 protein) or control IgG. Cell lysates and immunoprecipitates were examined by immunoblotting using anti-GFP antibody. The same membranes were also blotted with anti-Bcl-2 antibody to determine IP specificity and efficiency. Input represents 5% of cell lysates used in the Co-IP assays.

acted with Nur77 or Nur77/ΔDBD, a Nur77 mutant lacking its DNA binding domain (DBD), comparable to the interaction of Nur77 with RXRα (Figure 1B). Bcl-2 but not Bax potently inhibited Nur77 transactivation (Figure 1C), again suggesting an interaction between Nur77 and Bcl-2.

We next determined whether endogenous Nur77 and transfected Bcl-2 colocalized in cells. The phorbol ester 12-*0*-tetradecanoyl phorbol-13-acetate (TPA) induces the expression of endogenous Nur77 and its mitochondrial localization in LNCaP prostate cancer cells (Li et al., 2000). In TPA-stimulated cells, the distribution patterns of TPA-induced endogenous Nur77 and transfected Bcl-2 overlapped extensively in the cytoplasm and colocalized with Hsp60, a mitochondria-specific protein (Figure 1D). Transfected Nur77/ΔDBD and Bcl-2 also colocalized in cells. The green fluorescent protein (GFP) tagged Nur77/ΔDBD, which constitutively resides on mitochondria in LNCaP cells (Li et al., 2000), displayed a distribution pattern that overlapped extensively with coexpressed Bcl-2, while control GFP protein distributed diffusely in cells (Figure 1E).

The interaction between Nur77 and Bcl-2 was further confirmed by coimmunoprecipitation (Co-IP) assays. As shown in Figure 1F, Bcl-2 was specifically coimmunoprecipitated by anti-Nur77 antibody in TPA-treated cells but not in nontreated cells. Co-IP using lysates from H460 lung cancer cells treated with AHPN analog 3-Cl-AHPC, which potently induces Nur77 expression, mitochondrial targeting and apoptosis (Kolluri et al., 2003), also demonstrated a strong interaction between endogenous Nur77 and Bcl-2 (Figure 1G).

The Nur77 LBD Is Required for Binding Bcl-2

To identify the Nur77 domain responsible for interaction with Bcl-2, we constructed several Nur77 mutants (Figure 2A) as GFP fusions. When GFP-Nur77/ΔDBD and Bcl-2 were cotransfected into HEK293T cells, a significant amount of GFP-Nur77/ΔDBD was coprecipitated with Bcl-2 by anti-Bcl-2 antibody but not by control IgG (Figure 2B). This Co-IP was specific because Bcl-2 coexpression was required and the GFP control protein did not interact with Bcl-2. Analysis of other Nur77 mutants revealed that the C-terminal domain (DC3), but not the N-terminal domain (N168), of Nur77/ΔDBD was responsible for binding Bcl-2. The C-terminal fragment DC1 (467–536 aa) strongly interacted with Bcl-2, while its deletion from Nur77/ΔDBD (Nur77/ΔDBD/ΔDC1) largely abolished the interaction. Furthermore, deletion of a putative amphipathic α-helix (471–488 aa) from Nur77/ΔDBD (Nur77/ΔDBD/Δ471–488) or mutation of Leu487 in the region to Ala (Nur77/ΔDBD/L487A) significantly impaired the interaction between Nur77/ΔDBD and Bcl-2 (Figure 2C). Thus, the DC1 region in the Nur77 LBD is crucial for Bcl-2 interaction.

The Bcl-2 Loop Region, but Not Its Hydrophobic Groove, Is Responsible for Nur77 Binding

Bcl-2 and Bcl-X_L have hydrophobic crevices on their surfaces that bind the BH3 domains of other family members (Sattler et al., 1997). To examine whether Nur77 binds to the Bcl-2 hydrophobic groove, we analyzed the interaction of Nur77/ΔDBD with several deletion or point mutants of Bcl-2 (Figure 3A), which are defective in forming the hydrophobic groove (Petros et al., 2001; Sattler et al., 1997). Deletion (data not shown) or point mutations (Y108K, L137A, G145A, or R146Q) in Bcl-2 abolished or reduced the interaction with Bax (Figure 3B). In contrast, these mutants retained the ability to bind Nur77/ΔDBD (Figure 3C). We also analyzed whether Bax or Bcl-Gs, a BH3-only Bcl-2-family protein (Guo et al., 2001), could compete with Nur77 for binding Bcl-2. Our results

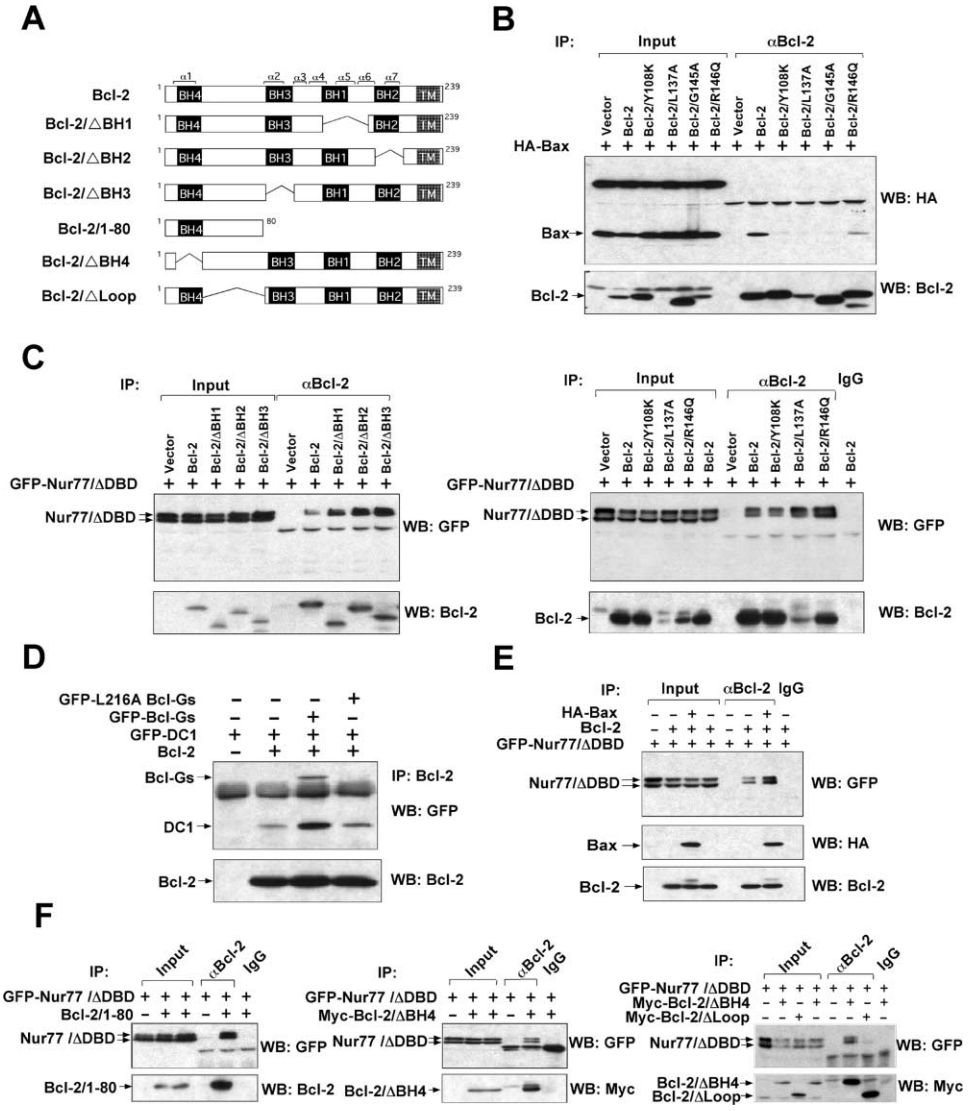

Figure 3. The Bcl-2 Loop Region Interacts with Nur77

(A) Schematic representation of Bcl-2 and its mutants. BH and loop domains and α-helical regions are indicated.

(B) Mutations in the Bcl-2 hydrophobic groove abolish Bcl-2 interaction with Bax.

(C) The Bcl-2 hydrophobic groove is not required for Bcl-2 binding to Nur77/ΔDBD. Co-IP assays (B and C) were performed as in Figure 2B using lysates from HEK293T cells transfected with GFP-Nur77/ΔDBD and the empty vector or the indicated Bcl-2 plasmid. Immunoprecipitates and lysates were examined by immunoblotting using the indicated antibodies.

(D) BH3-only protein Bcl-Gs does not compete with DC1 for binding to Bcl-2. GFP-DC1 (4 μg) was expressed in HEK293T cells with or without Bcl-2 (2 μg) in the presence or absence of GFP-Bcl-Gs or GFP-Bcl-Gs/L216E (4 μg). Lysates were immunoprecipitated by anti-Bcl-2 antibody, followed by immunoblotting with anti-GFP or anti-Bcl-2 antibody.

(E) Bax does not compete with Nur77/ΔDBD for binding to Bcl-2. GFP-Nur77/ΔDBD (6 μg) was expressed in HEK293T cells with or without Bcl-2 (2 μg) in the presence or absence of Bax (2 μg). Lysates were immunoprecipitated by anti-Bcl-2 antibody. Immunoprecipitates and lysates were examined by immunoblotting using anti-GFP, anti-Bcl-2, or anti-HA antibody.

(F) The Bcl-2 N-terminal loop region is essential for Nur77/Bcl-2 interaction. GFP-Nur77/ΔDBD was cotransfected with the indicated Bcl-2 mutant plasmid into HEK293T cells. Lysates were immunoprecipitated by anti-myc or Bcl-2 antibody as indicated. Immunoprecipitates were analyzed by immunoblotting with anti-GFP, anti-Bcl-2, or anti-myc antibody.

showed that neither Bcl-Gs (Figure 3D) nor Bax (Figure 3E) interfered with DC1 or Nur77/ΔDBD binding to Bcl-2. Rather, these proteins consistently enhanced their interaction with Bcl-2. Thus, binding of Bcl-2 to Nur77 is distinct for its binding to Bcl-Gs and Bax and does not require the BH3 binding hydrophobic groove in Bcl-2.

The above observations suggested that the N-terminal portion of Bcl-2 was responsible for binding Nur77. Indeed, the first 80 amino acid residues of Bcl-2 (Bcl-2/1-80), like the full-length Bcl-2, strongly interacted with Nur77/ΔDBD (Figure 3F). The Bcl-2/1-80 encompasses the BH4 domain and an unstructured loop domain. To determine whether the BH4 domain or the loop region was responsible for binding to Nur77/ΔDBD, we investigated Nur77/ΔDBD interaction with Bcl-2 mutants lacking the BH4 domain (Bcl-2/ΔBH4) or the loop region

(Bcl-2/ΔLoop) (Figure 3F). Co-IP assays demonstrated that the Bcl-2/ΔBH4 retained the ability to interact with Nur77/ΔDBD, whereas Bcl-2/ΔLoop did not (Figure 3F). Thus, the loop region of Bcl-2 is required for binding to Nur77.

Interaction with Bcl-2 Mediates Nur77 Mitochondrial Targeting

To determine whether interaction with Bcl-2 mediated Nur77 mitochondrial targeting, we transfected GFP-Nur77/ΔDBD alone or with Bcl-2 into HEK293T cells, which lack detectable levels of endogenous Bcl-2. GFP-Nur77/ΔDBD was diffusely distributed in the cytosol (Figure 4A), indicating that it failed to target mitochondria. However, when coexpressed with Bcl-2, GFP-Nur77/ΔDBD displayed a distribution pattern overlapping with mitochondrial Hsp60. Thus, Bcl-2 expression conferred mitochondrial targeting to Nur77/ΔDBD. The colocalization of Nur77/ΔDBD and Hsp60 was dependent on Nur77/ΔDBD binding to Bcl-2, because mutants of GFP-Nur77/ΔDBD (Nur77/ΔDBD/L487A and Nur77/ΔDBD/Δ471–488) that failed to bind Bcl-2 (Figure 2) did not colocalize with mitochondria.

The role of Bcl-2 in mitochondrial targeting of Nur77 was further studied by examining the accumulation of Bcl-2 binding Nur77/ΔDBD and nonbinding Nur77/ΔDBD/Δ471–488 in mitochondria-enriched heavy membrane (HM) fractions of HEK293T cells (Figure 4B). HM preparation purity was established by assessing levels of mitochondrial Hsp60, nuclear protein PARP, and cytosolic/nuclear protein Jun N-terminal kinase (JNK). Nur77/ΔDBD accumulated in the HM fraction when Bcl-2 was coexpressed, whereas Nur77/ΔDBD/Δ471-488 did not accumulate irrespective of Bcl-2 coexpression.

To complement these gene transfection experiments, small interfering (si)RNA was used to determine whether suppressing endogenous Bcl-2 expression affected Nur77 mitochondrial targeting. In MGC80-3 gastric cancer cells, in which Nur77 was reported to target mitochondria in response to specific apoptotic stimuli (Liu et al., 2002), Bcl-2 expression was almost completely inhibited by Bcl-2-specific siRNA but not by GFP siRNA control (Figure 4C). Both confocal microscopy (Figure 4D) and immunoblotting of HM fractions (Figure 4E) revealed that endogenous Nur77 targeted mitochondria in MGC80-3 cells treated with 3-Cl-AHPC. However, Bcl-2 siRNA, but not control GFP siRNA, largely abolished mitochondrial targeting of Nur77. Similarly, inhibition of endogenous Bcl-2 expression using Bcl-2 antisense oligonucleotides impaired Nur77 mitochondrial targeting in H460 lung cancer cells (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/116/4/527/DC1).

We next studied whether the Bcl-2 loop region could act in a dominant-negative fashion to inhibit Nur77 mitochondrial targeting. In LNCaP cells transfected with GFP-Bcl-2/1-90, a Bcl-2 mutant comprised of the first 90 N-terminal amino acids, TPA-induced Nur77 failed to target mitochondria, displaying a diffuse cytosolic distribution pattern, in contrast to nontransfected cells, which exhibited colocalization of Nur77 and Hsp60 (Figure 4F). Thus, Bcl-2/1-90 inhibits Nur77 mitochondrial targeting, probably by competing with endogenous Bcl-2 for binding to Nur77. Together, these results demonstrate that Bcl-2 acts as a receptor for Nur77 and is responsible for Nur77 mitochondrial targeting.

Nur77 Interaction with Bcl-2 Triggers cyt *c* Release and Apoptosis

Next, we determined the requirement of Nur77 interaction with Bcl-2 for Nur77-induced cyt *c* release and apoptosis. Transient expression of Nur77/ΔDBD or Bcl-2 alone did not cause release of cyt *c* from mitochondria in HEK293T cells, as confocal microscopy analysis showed punctate cyt *c* staining, indicative of mitochondrial cyt *c* (Figure 5A). However, their coexpression resulted in their colocalization and release of cyt *c* from mitochondria (Figure 5A). Cyt *c* release required mitochondrial localization of Nur77/ΔDBD and Bcl-2, because it did not occur upon coexpression of Nur77/ΔDBD with Bcl-2/ΔTM, a Bcl-2 mutant unable to target mitochondria (Figure 5A). Interestingly, coexpression of Nur77/ΔDBD and Bcl-2/Y108K did not induce cyt *c* release, although they colocalized (Figure 5A). These results suggest that the interaction between Nur77/ΔDBD and Bcl-2 is insufficient for inducing cyt *c* release.

Expression of either Nur77/ΔDBD or Bcl-2 alone did not induce apoptosis, as revealed by the absence of nuclear fragmentation and chromatin condensation in HEK293T cells (Figure 5B). However, when coexpressed, Bcl-2 and Nur77/ΔDBD induced striking apoptosis. The proapoptotic effect of Bcl-2 was specific to Nur77, because Bax-induced apoptosis was effectively prevented by Bcl-2 coexpression (see below). Thus, Bcl-2 promotes apoptosis when coexpressed with Nur77 but suppresses apoptosis when coexpressed with Bax.

We next examined the role of endogenous Bcl-2 on Nur77-dependent apoptosis in MGC80-3 cells. Treatment of control GFP-siRNA-transfected cells with 3-Cl-AHPC resulted in apoptosis (Figures 5C and 5D). However, transfection of Bcl-2 siRNA suppressed 3-Cl-AHPC-induced apoptosis by about 60%. Similar results were obtained in H460 cells (Supplemental Figure S1 available on *Cell* website). Moreover, expression of Bcl-2/1-90 protein also suppressed Nur77-dependent apoptosis induced by TPA and 3-Cl-AHPC in LNCaP cells (Figure 5E). Thus, Bcl-2 can manifest a proapoptotic phenotype in settings where Nur77 is expressed and targets to mitochondria.

To extend the above findings to primary cells, we performed experiments using primary cultures of peripheral blood lymphocytes (PBLs). Freshly isolated PBLs were transfected with GFP-Nur77, then treated with TPA plus calcium ionophore ionomycin, which induce Nur77-dependent apoptosis of T-lymphocytes (Woronicz et al., 1994). The treatment caused translocation of GFP-Nur77 from the nucleus to the cytoplasm, colocalizing with cotransfected DsRed2-Mito, a red fluorescent protein (RFP) fused with a mitochondria-targeting sequence (Figure 6A). Subcellular fractionation revealed that the treatment induced accumulation of endogenous Nur77 in HM fractions (Figure 6B). Interestingly, this treatment also altered the migration of Nur77 protein, suggesting a possible posttranslational modification. Thus, both transfected and endogenous Nur77 targets mitochondria in primary lymphocytes.

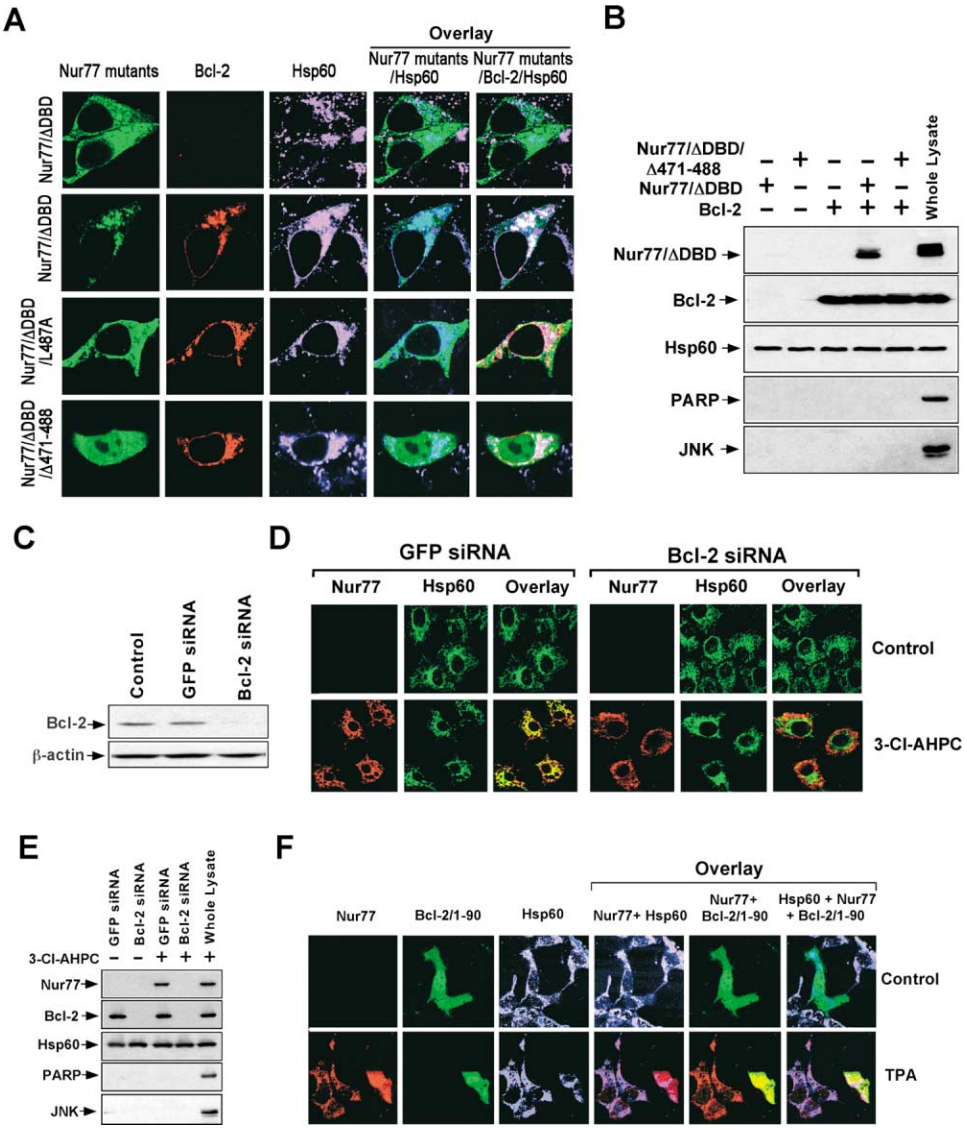

Figure 4. Nur77/Bcl-2 Interaction Mediates Nur77 Mitochondrial Localization

(A) Bcl-2 expression promotes Nur77/ΔDBD mitochondrial localization. The indicated GFP-Nur77 mutant (3 μg) and Bcl-2 (1 μg) were expressed in HEK293T cells alone or together. Cells were immunostained with anti-Bcl-2 or anti-Hsp60 antibody. Bcl-2, Nur77/ΔDBD, its mutants, and mitochondria (Hsp60) were visualized using confocal microscopy and the images were overlaid (overlay). Approximately 30% of cells showed Nur77/ΔDBD colocalization with Bcl-2 and Hsp60, while less than 5% of cells transfected with Nur77/ΔDBD mutants were similarly colocalized. In the absence of Bcl-2, Nur77/ΔDBD did not colocalize with Hsp60.

(B) Immunoblotting analysis of the effect of Bcl-2 expression. GFP-Nur77/ΔDBD or GFP-Nur77/ΔDBD/Δ471-488 (6 μg) and Bcl-2 (2 μg) were transfected into HEK293T cells alone or together. HM fractions were prepared and analyzed for accumulation of Nur77/ΔDBD in mitochondria by immunoblotting using anti-GFP antibody. The same membrane was also blotted with anti-Bcl-2, anti-Hsp60, anti-PARP, or anti-JNK antibody. Whole lysate was prepared from cells transfected with Nur77/ΔDBD and Bcl-2.

(C) Inhibition of Bcl-2 expression by Bcl-2 siRNA. MGC80-3 cells were transfected with Bcl-2 siRNA SMARTpool or control GFP siRNA or left alone. After 48 hr, lysates were prepared and assayed by immunoblotting using anti-Bcl-2 and anti-β-actin antibodies.

(D–E) Inhibition of endogenous Bcl-2 expression abrogates 3-Cl-AHPC-induced Nur77 mitochondrial targeting. MGC80-3 cells transfected with siRNA as described in (C) were treated with 3-Cl-AHPC (10^{-6} M) for 5 hr. Cells were immunostained with anti-Nur77 and anti-Hsp60 antibodies for confocal microscopy analysis (D) or subjected to HM fractionation and analysis (E) as described in (B).

(F) Bcl-2/1-90 inhibits Nur77 mitochondrial targeting. LNCaP cells were transfected with GFP-Bcl-2/1-90 (4 μg). After 24 hr, cells were treated with TPA (100 ng/ml) for 3 hr and immunostained with anti-Nur77 and anti-Hsp60 antibodies, followed by confocal microscopy analysis. Approximately 77% of transfected cells showed the effect presented.

We also studied the role of Bcl-2 in Nur77-dependent apoptosis in PBLs. Treatment with TPA/ionomycin induced extensive apoptosis of PBLs, which was partially inhibited by Bcl-2 antisense oligonucleotides or Nur77 siRNA (Figure 6C). In addition, GFP-Nur77/ΔDBD also colocalized extensively with DsRed2-Mito (Figure 6D) and potently induced PBL apoptosis (Figure 6E), which was almost completely suppressed by Bcl-2 antisense

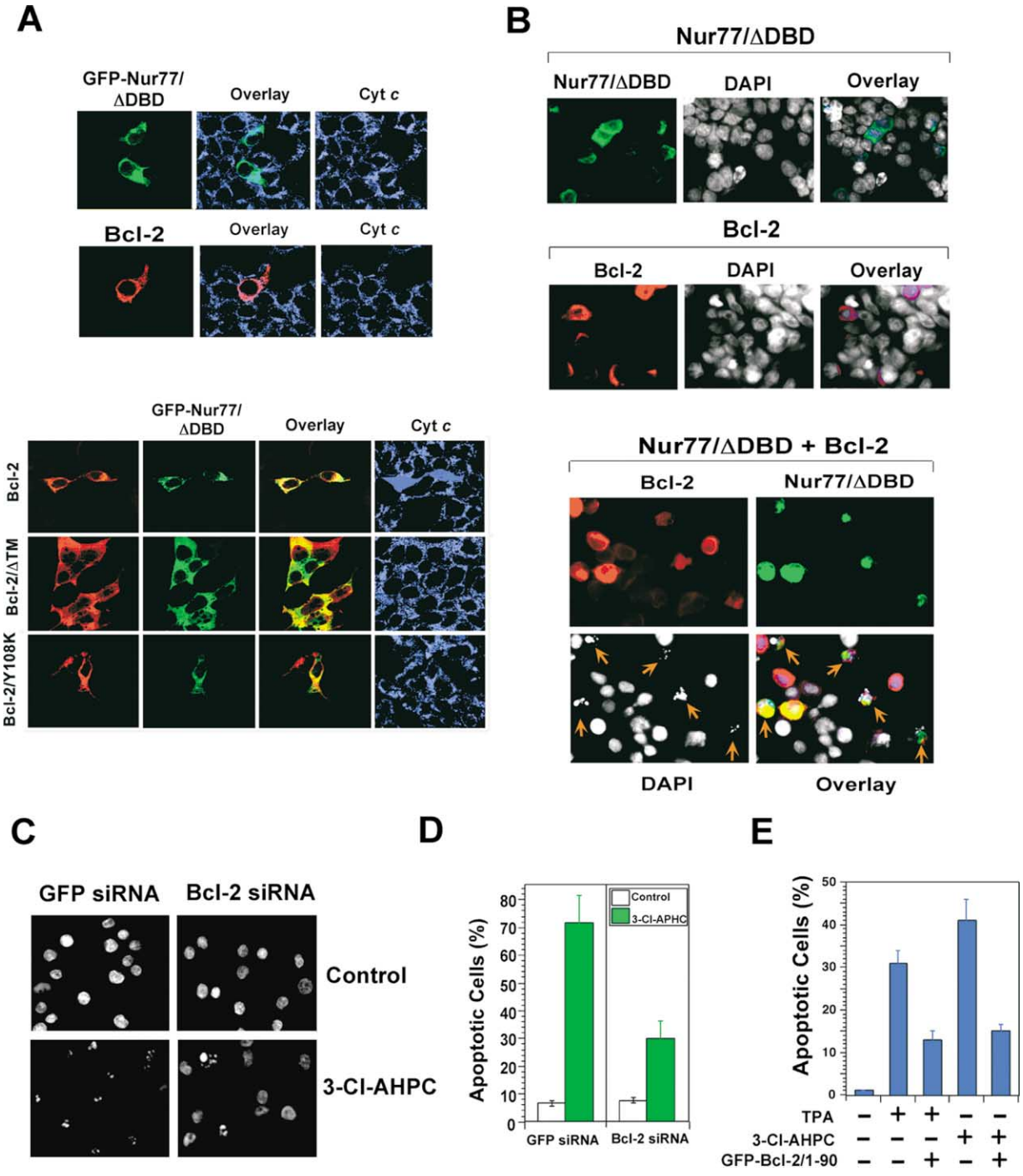

Figure 5. Interaction of Nur77 with Bcl-2 Results in cyt *c* Release and Apoptosis

(A) Induction of cyt *c* release by coexpression of Bcl-2 and Nur77/ΔDBD. GFP-Nur77/ΔDBD (6 μg) and Bcl-2 (2 μg) were expressed in HEK293T cells alone and together. GFP-Nur77/ΔDBD was also coexpressed with Bcl-2/ΔTM or Bcl-2/Y108K (2 μg). Cells were immunostained with anti-Bcl-2 or anti-cyt *c* antibody. Nur77/ΔDBD, Bcl-2 and cyt *c* were visualized using confocal microscopy, and images for Nur77/ΔDBD and Bcl-2 were overlaid (overlay). Approximately 75% of the Nur77/ΔDBD and Bcl-2 colocalized cells displayed various levels of diffuse cyt *c* staining.

(B) Induction of apoptosis by coexpression of Bcl-2 and Nur77/ΔDBD. Bcl-2 and GFP-Nur77/ΔDBD were expressed alone or together in HEK293T cells. After 36 hr, cells were stained by anti-Bcl-2 antibody, followed by TRITC-conjugated secondary antibody (Sigma) and the nucleus was stained by DAPI. Expression of Bcl-2 and GFP-Nur77/ΔDBD, as well as nuclear morphology, were visualized by fluorescence microscopy, and the three images were overlaid. Arrows indicate cells expressing Bcl-2 and GFP-Nur77/ΔDBD. One of four similar experiments is shown.

(C–D) Inhibition of endogenous Bcl-2 expression suppresses 3-Cl-AHPC-induced apoptosis. MGC80-3 cells were transfected with Bcl-2 siRNA SMARTpool or control GFP siRNA. After 36 hr, cells were treated with 3-Cl-AHPC (10^{-6} M) for 48 hr. Apoptosis was determined by DAPI staining as shown in (C) and scored by examining 300 cells for nuclear fragmentation and/or chromatin condensation (D).

(E) Bcl-2/1-90 inhibits TPA and 3-Cl-AHPC-induced apoptosis. LNCaP cells were transfected with GFP or GFP-Bcl-2/1-90. After 24 hr, cells were treated with TPA (100 ng/ml) or 3-Cl-AHPC (10^{-6} M) for 24 hr. Apoptosis was studied as in D.

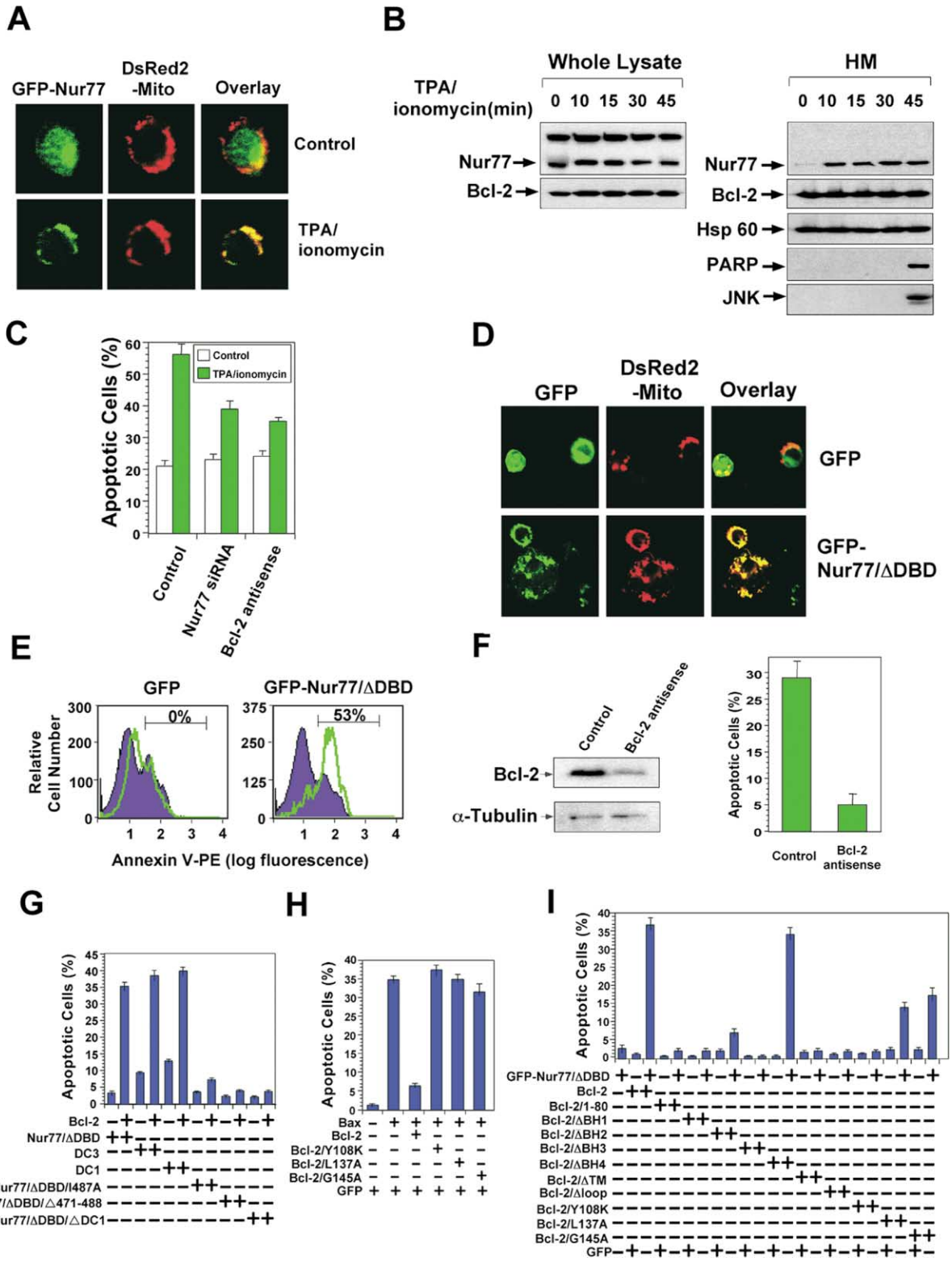

Figure 6. Nur77 Mitochondrial Targeting in Human PBLs and Apoptotic Effects of Nur77 and Bcl-2 Mutants

(A) Mitochondrial targeting of Nur77 in PBLs. GFP-Nur77 (1 μg) and pDsRed2-Mito (1 μg) were transfected into freshly isolated human PBLs. The cells were then treated with TPA (10 ng/ml) and ionomycin (0.5 μM) for 30 min after 10 hr of transfection. GFP-Nur77 and mitochondria (pDsRed2-Mito) were visualized using confocal microscopy. Approximately 20% of the cells showed the pattern presented.

(B) Endogenous Nur77 accumulates in the PBL HM fraction. PBLs were treated with TPA and ionomycin as in (A) for the indicated times and HM fractions were isolated. Total cell lysates and HM fractions were subjected to immunoblotting as described in Figure 4B.

(C) Nur77 and Bcl-2 are required for apoptosis in PBLs. PBLs were transfected with control GFP siRNA, Nur77 siRNA, or Bcl-2 antisense oligonucleotides (2 μg). After 40 hr, cells were treated with TPA and ionomycin for 7 hr and apoptotic cells (Annexin-V positive) were determined by flow cytometry. Bars represent average ± means from two experiments.

oligonucleotides (Figure 6F). Thus, endogenous Bcl-2 contributes to Nur77-dependent apoptosis in primary lymphocytes.

Analysis of Bcl-2 Domain Required for Apoptosis Induction by Nur77

To characterize the proapoptotic mechanism of Bcl-2 in Nur77-induced apoptosis, various Nur77 and Bcl-2 mutants were coexpressed in HEK293T cells. Similar to Nur77/ΔDBD, coexpression of either DC3 or DC1 with Bcl-2 strongly induced apoptosis (Figure 6G). Thus, the minimal C-terminal domain of Nur77, capable of binding Bcl-2, was sufficient to induce apoptosis when coexpressed with Bcl-2. The requirement of Nur77 interaction with Bcl-2 for apoptosis was further illustrated by the failure of Nur77 mutants (Nur77/ΔDBD/ΔDC1, Nur77/ΔDBD/Δ471-488, and Nur77/ΔDBD/L487A) that failed to bind Bcl-2 (Figure 2) to induce apoptosis when coexpressed with Bcl-2 (Figure 6G).

Bcl-2 effectively suppressed apoptosis induced by Bax expression in HEK293T cells (Figure 6H). Bcl-2 mutations (Y108K, L137A, G145A) that impaired its interaction with Bax (Figure 3B) abolished its inhibitory effect on Bax-induced apoptosis (Figure 6H), consistent with previous observations that the Bcl-2 hydrophobic cleft is essential for its antiapoptotic effect.

We then performed experiments to delineate the structure-function relationships for the proapoptotic effect of Bcl-2 in Nur77-induced apoptosis (Figure 6I). Coexpression of Nur77/ΔDBD with Bcl-2/ΔLoop did not induce cell death, consistent with the inability of this Bcl-2 mutant to bind Nur77 (Figure 3). Though capable of binding Nur77/ΔDBD, mutants of Bcl-2 lacking the membrane-anchoring TM domain, the BH1 domain, BH2 domain, or BH3 domain were incapable of inducing apoptosis when coexpressed with Nur77/ΔDBD. Similarly, although binding Nur77, a BH3 domain mutant of Bcl-2 (Y108K) also failed to induce apoptosis when coexpressed with Nur77/ΔDBD. Moreover, mutations of the BH3 binding pocket of Bcl-2, L137A, and G145A, which abrogated the ability of Bcl-2 to suppress Bax-induced apoptosis (Figure 6H), retained the ability to promote apoptosis when coexpressed with Nur77/ΔDBD. Thus, an intact hydrophobic groove in Bcl-2 is required for its antiapoptotic activity but not for its proapoptotic activity, demonstrating a structural distinction between these two opposing phenotypes of Bcl-2.

Bcl-2 Undergoes a Conformational Change upon Nur77 Binding

Bax and Bak undergo conformational changes in association with their conversion from latent to active killer proteins (Griffiths et al., 1999; Nechushtan et al., 1999). We therefore explored whether a conformational change might be involved in converting Bcl-2 function from antiapoptotic to proapoptotic. To this end, we compared the effects of Nur77 on binding of Bcl-2 to various anti-Bcl-2 antibodies that recognize different epitopes. Antibody binding to Bcl-2 was measured by immunofluorescence using flow cytometry or by immunoprecipitation.

First, Bcl-2 was coexpressed with GFP-Nur77/ΔDBD or the control GFP in HEK293T cells, and immunostaining was performed on fixed and permeabilized cells using rabbit polyclonal antibody against the whole Bcl-2 protein (αBcl-2), mouse monoclonal antibody against the Bcl-2 BH3 binding pocket (αBcl-2/BH3-pocket), or polyclonal antibody against the Bcl-2 BH3 domain (αBcl-2/BH3-domain) (Figure 7A). Bcl-2 immunofluorescence was undetectable in control GFP-coexpressing cells stained with the αBcl-2/BH3-domain antibody but dramatically increased in GFP-Nur77/ΔDBD-coexpressing cells, suggesting increased availability of the BH3-domain epitope upon Nur77/ΔDBD coexpression (Figure 7A). In contrast, immunofluorescence obtained by staining with the αBcl-2/BH3-pocket antibody was reduced by coexpression of GFP-Nur77/ΔDBD, suggesting decreased availability of this epitope. Alterations in binding of epitope-specific antibodies to Bcl-2 in response to GFP-Nur77/ΔDBD coexpression were not due to changes in Bcl-2 levels, because GFP-Nur77/ΔDBD coexpression did not alter Bcl-2 immunofluorescence when stained with αBcl-2 antibody. In addition, both immunoblotting analysis (Figure 7A) and BD cytometric bead assays (Supplemental Figure S2 available on *Cell* website) revealed equivalent Bcl-2 levels with GFP or GFP-Nur77/ΔDBD coexpression. Nur77/ΔDBD coexpression also did not modify binding of these epitope-specific antibodies to Bcl-2/ΔLoop (Supplemental Figure S3 available on *Cell* website). The Nur77/ΔDBD-induced change in Bcl-2 conformation was also observed in PBLs (Figure 7B and Supplemental Figure S4 available on *Cell* website).

Second, the effects of Nur77/ΔDBD on Bcl-2 conformation were studied using immunoprecipitation assays. These experiments showed that coexpression of Nur77/ΔDBD reduced binding of Bcl-2 to the αBcl-2/BH3-

(D) Nur77/ΔDBD targets mitochondria in PBLs. pDsRed2-Mito and GFP or GFP-Nur77/ΔDBD (1 μg each) were cotransfected into PBLs. GFP-Nur77/ΔDBD and pDsRed2-Mito were visualized as described in (A). Approximately 30% of the transfected cells showed the pattern presented.
(E) Nur77/ΔDBD induces apoptosis of PBLs. GFP or GFP-Nur77/ΔDBD (2 μg) was transfected into PBLs for 18 hr. The transfected (GFP-positive) cell subpopulation was identified by flow cytometry (Kolluri et al., 1999). The apoptotic cells in the transfected (green histogram) and nontransfected (purple histogram) cells were identified by Annexin-V-PE staining. The numbers represent % of transfected cells showing Annexin-V staining compared to nontransfected cells from the same culture dish.
(F) Bcl-2 is required for Nur77/ΔDBD-induced apoptosis. GFP-Nur77/ΔDBD (1 μg) was cotransfected into PBLs with control oligonucleotides or Bcl-2 antisense oligonucleotides (2 μg). After 48 hr, apoptotic cells were determined as described for (E). Bars represent average ± means from two measurements.
(G) Interaction of Nur77 with Bcl-2 is required for apoptotic effect of Nur77/Bcl-2 coexpression. The indicated Nur77 mutant (6 μg) was transfected with empty or Bcl-2 expression vector (2 μg) into HEK293T cells. After 36 hr, apoptotic cells were determined by DAPI staining.
(H) Effect of Bcl-2 mutations on the apoptotic effect of Bax. HEK293T cells were transfected with the indicated expression vectors and apoptotic cells were determined by DAPI staining.
(I) Effect of Bcl-2 mutations on the apoptotic effect of Nur77/Bcl-2 coexpression. Bcl-2 or a mutant was transfected with GFP or GFP-Nur77/ΔDBD into HEK293T cells. Apoptosis wan then determined by DAPI staining. Bars in (G)–(I) are means ± SD from three independent experiments.

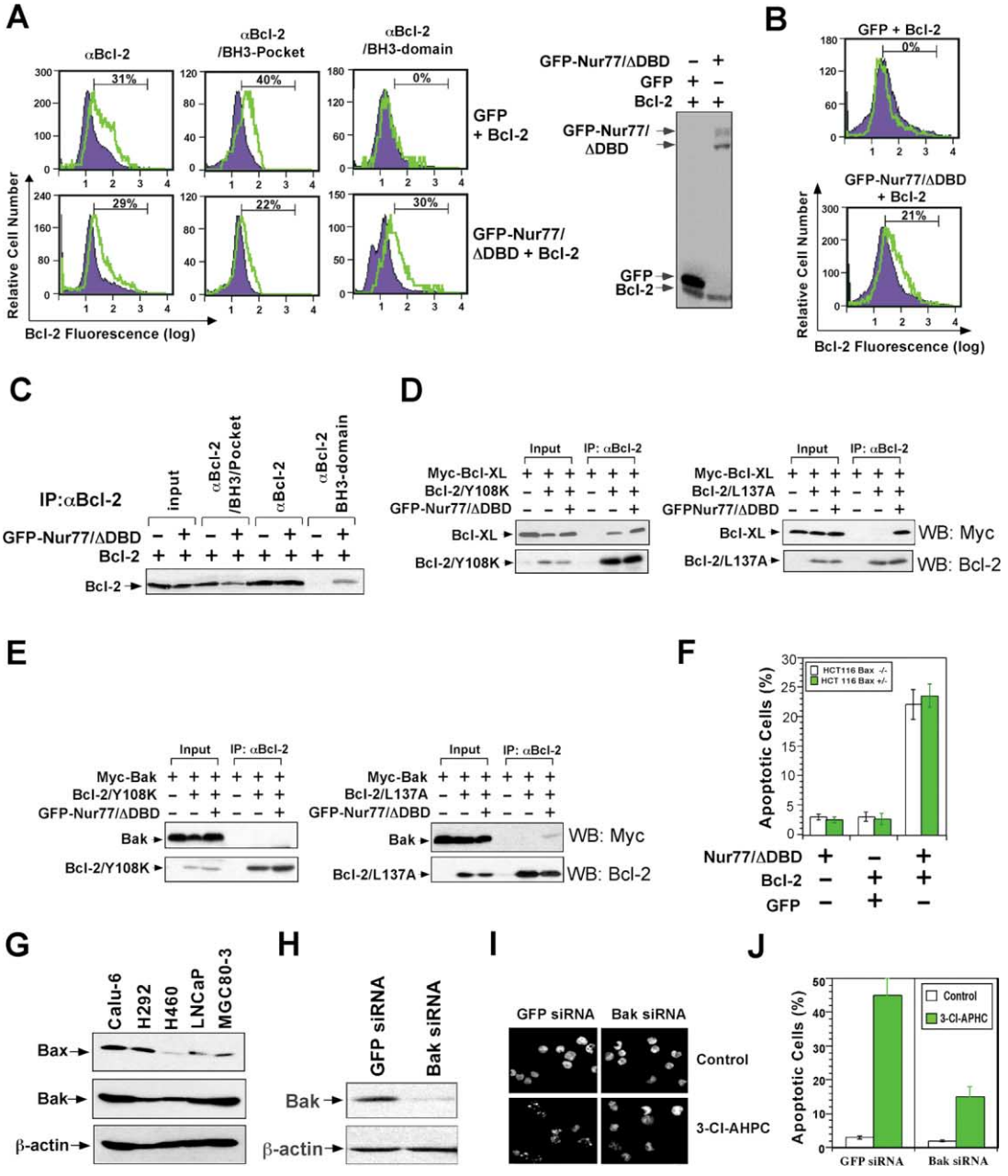

Figure 7. Interaction of Nur77 with Bcl-2 Induces Bcl-2 Conformational Change

(A) Nur77 induces change in Bcl-2 conformation. Bcl-2 (5 μg) was cotransfected into HEK293T cells with GFP or GFP-Nur77/ΔDBD (5 μg) for 14 hr. Portion of the transfected cells were subjected to immunoblotting to confirm similar expression of Bcl-2 in the two samples (right). The remaining cells were divided into three different pools, which were immunostained with three different anti-Bcl-2 antibodies: αBcl-2, αBcl-2/BH3-pocket (BD Transduction Labs), and αBcl-2/BH3 domain (Abgent), followed by SRPD-conjugated secondary antibody (Southern Biotech). Transfected (GFP-positive) cells were identified by flow cytometry. Bcl-2 fluorescence from the transfected cells (green histogram) was compared to that from the nontransfected cells (purple histogram). Similarly fluorescing cells were gated to compare Bcl-2 immunofluorescence after GFP or GFP-Nur77/ΔDBD coexpression. Numbers represent % of transfected cells showing Bcl-2 immunofluorescence compared to the autofluorescence of the nontransfected cells from the same transfection.

(B) Change of Bcl-2 conformation by Nur77/ΔDBD in PBLs. Bcl-2 (1 μg) was cotransfected into PBLs with GFP or GFP-Nur77/ΔDBD (1 μg). A portion of the cells were subjected to immunoblotting (Supplemental Figure S4 available on *Cell* website). The remaining cells were immunostained with αBcl-2/BH3-domain antibody 10 hr after transfection as described in (A).

(C) Nur77/ΔDBD modulates immunoprecipitation of Bcl-2 by epitope-specific anti-Bcl-2 antibodies. HEK293T cells were transfected with the indicated expression vector. After 18 hr, cells lysates were prepared and incubated with the indicated anti-Bcl-2 antibody for immunoprecipitation. Immunoprecipitates were subjected to immunoblotting using anti-Bcl-2 antibody (Santa Cruz).

(D) Alteration of Bcl-2 interaction with Bcl-X_L by Nur77/ΔDBD. The indicated Bcl-2 mutant and Bcl-X_L were coexpressed with or without GFP-Nur77/ΔDBD in HEK293T cells. Lysates were immunoprecipitated by anti-Bcl-2 antibody, and immunoprecipitates examined by immunoblotting using anti-Myc or anti-Bcl-2 antibody.

(E) Alteration of Bcl-2 interaction with Bak by Nur77/ΔDBD. The indicated Bcl-2 mutant and Bak were coexpressed with or without GFP-

pocket antibody, but enhanced binding of Bcl-2 to the αBcl-2/BH3-domain antibody. In contrast, Nur77/ΔDBD did not affect the immunoprecipitation efficiency of the αBcl-2 antibody (Figure 7C). Together, these results demonstrate that Nur77 binding induces a Bcl-2 conformational change that exposes its BH3 domain.

Proapoptotic BH3-only members of the Bcl-2 family induce apoptosis by binding to other Bcl-2 family members through their BH3 domains (Huang and Strasser, 2000; Kelekar and Thompson, 1998). We therefore examined whether Nur77 binding alters the ability of Bcl-2 to bind Bcl-X_L or Bak (Figures 7D-7E). At least when assessed in detergent containing cell lysates by Co-IP, Bcl-2 bound Bcl-X_L and Bak independently of Nur77 (data not shown). To address whether Bcl-2 bound differently to Bcl-X_L and Bak in the presence of Nur77/ΔDBD, two Bcl-2 mutants were analyzed. Bcl-2/L137A, a BH3 binding pocket mutant that retained killing activity in the presence of Nur77/ΔDBD, interacted with Bcl-X_L and Bak only when Nur77/ΔDBD was coexpressed. In contrast, binding of Bcl-X_L and Bak to the Bcl-2/Y108K BH3 domain mutant was unaffected by coexpression of Nur77 (Figures 7D and 7E). Thus, Nur77 binding may result in altered association of Bcl-2 with other Bcl-2 family members. Moreover, the observation that Bcl-2/L137A, but not Bcl-2/Y108K, was capable of killing cells in collaboration with Nur77/ΔDBD (Figure 6I) suggests that exposure of the BH3 domain of Bcl-2 may be responsible for the conversion of Bcl-2 to a proapoptotic molecule.

The above data suggest that Bcl-2, upon Nur77 binding, induces apoptosis through its BH3 domain. BH3-only proteins exert their apoptotic effects through either Bax or Bak. We therefore examined the involvement of Bax and Bak in Bcl-2-dependent apoptosis induced by Nur77. Coexpression of Nur77/ΔDBD and Bcl-2 resulted in a similar degree of apoptosis in HCT116 cells and HCT116 cells lacking Bax (HCT116 $Bax^{-/-}$) (Figure 7F), suggesting that expression of Bax is not crucial. This was also supported by the observation that H460 cells, which underwent extensive apoptosis in response to 3-Cl-AHPC (Supplemental Figure S1 available on *Cell* website), expressed only trace levels of Bax (Figure 7G).

To determine whether Bak, which was highly expressed in H460 cells (Figure 7G), plays a role in Bcl-2-dependent apoptosis induced by Nur77, we examined the effects of suppressing endogenous Bak expression. Significant reductions of Bak protein were observed when H460 cells were transfected with Bak siRNA but not control siRNA (Figure 7H), correlating with significant repression of Nur77-dependent 3-Cl-AHPC-induced apoptosis (Figures 7I–7J). Thus, Bcl-2-mediated apoptosis induced by Nur77 depends on multidomain proapoptotic Bcl-2-family proteins such as Bak.

Discussion

Despite lacking classical mitochondria-targeting sequences, Nur77 translocates from the nucleus to mitochondria, in response to specific cell death stimuli, to trigger cyt *c* release and apoptosis. The results presented here provide evidence that Nur77 targets mitochondria through its interaction with Bcl-2, revealing a crosstalk between Nur77 nuclear receptor and the Bcl-2 signalings. Furthermore, our results demonstrate that the interaction provokes a proapoptotic phenotype of Bcl-2 by inducing a conformational change in Bcl-2 that results in exposure of its BH3 domain. Given that Bcl-2 has been shown to have proapoptotic phenotypes in a variety of contexts, it will be interesting to explore in the future whether Nur77 serves as the mediator of this phenotypic conversion versus other Bcl-2 binding proteins that may await discovery.

Our mutagenesis studies indicate that the loop region located between the BH4 and BH3 domains of Bcl-2 is required for Nur77 binding. Previous studies (Chang et al., 1997) demonstrated that the loop regions of Bcl-2 and Bcl-X_L act as an autoinhibitory domain that reduces the antiapoptotic function of Bcl-2 and Bcl-X_L. Our data suggest that this conserved loop segment found in the vertebrate orthologs of Bcl-2 and Bcl-X_L may also participate in converting the phenotype of Bcl-2 from a protector to a killer of cells. Intriguingly, deletion of the loop region of Bcl-2 blocks paclitaxel-induced apoptosis (Srivastava et al., 1999), thereby suggesting the requirement of the loop region for the apoptotic effect of certain anticancer drugs. Though controversial, paclitaxel may also bind directly to the Bcl-2 loop domain to exert its apoptotic effect (Rodi et al., 1999). For Bcl-X_L, deamidation of residues in its loop region is associated with down regulation of its antiapoptotic activity (Deverman et al., 2002). In another study, insulin receptor substrate (IRS) protein binds to the Bcl-2 loop region, enhancing rather than inhibiting its antiapoptotic function (Ueno et al., 2000). It would be interesting therefore to study whether Nur77 and IRS compete for binding to Bcl-2, exhibiting opposing effects on apoptosis.

The Bcl-2 family members can be divided into two functional subgroups based on whether the BH3 domain is available (Gross et al., 1999). Members with buried BH3 domains are antiapoptotic, while members having an exposed BH3 domain are proapoptotic. Our analysis

Nur77/ΔDBD in HEK293T cells. Lysates were immunoprecipitated by anti-Bcl-2 antibody, and immunoprecipitates examined by immunoblotting using anti-Myc or anti-Bcl-2 antibody.

(F) Absence of Bax does not impair the apoptotic effect of Nur77/ΔDBD. Nur77/ΔDBD and Bcl-2 alone and together were transfected into HCT116 cells (+/−) and HCT116 cells lacking Bax (−/−). After 36 hr, apoptotic cells were determined as in Figure 5D.

(G) Expression of Bax and Bak in cancer cell lines. Cell extracts prepared from the indicated cancer cell lines were analyzed for Bax and Bak expression by immunoblotting.

(H) Inhibition of Bak expression by Bak siRNA in H460 lung cancer cells. H460 cells were transfected with Bak siRNA or control GFP siRNA. After 48 hr, cell lysates were assayed by immunoblotting using anti-Bak antibody.

(I and J) Inhibition of endogenous Bak expression suppresses 3-Cl-AHPC-induced apoptosis. H460 cells were transfected with Bak siRNA or control GFP siRNA. After 36 hr, cells were treated with 3-Cl-AHPC (10^{-6} M) for 24 hr. Apoptosis was determined by DAPI staining (I) and scored (J) as in Figures 5C and 5D.

using epitope-specific anti-Bcl-2 antibodies revealed that Bcl-2 undergoes a conformational change upon Nur77 binding. Given that Nur77/ΔDBD binding reduced epitope availability for an anti-Bcl-2 antibody to the BH3 binding pocket and enhanced the epitope availability for an antibody to the BH3-domain (Figures 7A–7C), it is likely that Nur77 binding induces a rearrangement of the Bcl-2 hydrophobic crevice, resulting in exposure of the otherwise hidden BH3 domain. Such a notion is supported by our findings that the Bcl-2/L137A mutant exhibited enhanced binding to Bak or Bcl-X_L upon Nur77/ΔDBD coexpression, while the Bcl-2 BH3 domain mutant (Bcl-2/Y108K) failed to show such a response (Figures 7D–7E). Based on the observation that Bcl-2 can associate with Bak in Nur77-overexpressing cells, we speculate that Nur77-converted Bcl-2 may similarly function as an agonist of Bak, in addition to an antagonist of Bcl-X_L. Future mutagenesis studies will help to determine whether this hypothesis is correct. However, it is noteworthy that of the 15 known BH3-only proteins in humans and mice, Bid and Bim are the only members that are capable of binding and activating proapoptotic Bcl-2-family proteins Bax and Bak (Korsmeyer et al., 2000; Marani et al., 2002), suggesting a possible role for Bcl-2 when converted to a BH3-displaying killer.

Caspase-mediated cleavage within the loop domain of Bcl-2 converts it into a proapoptotic molecule (Cheng et al., 1997; Grandgirard et al., 1998). However, we do not believe that the Nur77-mediated conversion of Bcl-2 from a protector to a killer involves cleavage of the protein, because we observed no hints of Bcl-2 cleavage in Nur77 overexpressing cells by immunoblotting, and because a mutant of Bcl-2, in which the caspase cleavage site has been mutated (Asp34), remains functional in collaborating with Nur77 to induce apoptosis although with reduced activity (Supplemental Figure S5 available on *Cell* website). Since caspase-mediated or experimental removal of the BH4 domain converts Bcl-2 into a killer, it is conceivable that the BH4 domain of Bcl-2 functions as an inhibitory domain to prevent the exposure of the BH3 domain. Three-dimensional structure of Bcl-2 reveals an extensive interaction between the BH4 domain and the hydrophobic groove (Petros et al., 2001). Therefore, it is tempting to speculate that Nur77, by binding to the loop region in Bcl-2, prevents the inhibitory effect of the BH4 domain, acting as an allosteric regulator to induce a reorganization of the hydrophobic cleft in Bcl-2, leading to exposure of its BH3 domain. This conformational change may be responsible for the conversion of Bcl-2 from an antiapoptotic to a proapoptotic molecule.

Our observations may help explain the paradoxical association of high levels of Bcl-2 protein expression with favorable clinical outcome for patients with several types of cancer, including breast, colon, and nonsmall cell lung cancer (reviewed in Reed, 1996). Possibly, in these tumors, elevated Bcl-2 is a liability, due to conversion of Bcl-2 from an antiapoptotic to a proapoptotic protein through interactions with Nur77 or other proteins. Also, elevated levels of a Nur77-family member are associated with favorable responses to chemotherapeutic agents in patients (Shipp et al., 2002). Interestingly, accumulation of somatic mutations in the region of the *BCL-2* gene encoding the loop domain has also been seen during clinical progression of lymphomas (Tanaka et al., 1992), suggesting the possibility of escape from the conversion mechanism in some types of cancer. Importantly, the discovery of a mechanism for converting Bcl-2 from a protector to a killer might be exploited eventually for developing anticancer drugs that turn overexpression of endogenous Bcl-2, which occurs in approximately half of all human malignancies, into an advantage that promotes tumor cell apoptosis.

Experimental Procedures

(See Supplemental Data available on *Cell* website for detailed procedures)

Bcl-2 siRNAs and Antisense Oligonucleotides

The target siRNA SMARTpools for Bcl-2 and Bak and the siRNA oligonucleotide for Nur77 (5′-CAG UCC AGC CAU GCU CCU dTdT) were purchased from Dharmacon Research Inc. They were transfected into cells according to the manufacturer's recommendations. Bcl-2 antisense oligonucleotide targeting Bcl-2 and negative control oligonucleotides were obtained from Calbiochem.

Nur77/Bcl-2 Interaction Assays

Reporter gene and GST pull-down assays were described previously (Li et al., 2000; Wu et al., 1997). For the mammalian two-hybrid assays, pcDNA-Gal4TAD-Nur77, pcDNA-Gal4TAD-Nur77/ΔDBD, pcDNA-Gal4DBD-Bcl-2/ΔTM, and pcDNA-Gal4DBD-RXRα were cloned and used. For Co-IP assays, HEK293T cells were transiently transfected with various expression plasmids in the presence of caspase inhibitors (zVAD-fmk) to prevent degradation of Nur77 protein due to apoptosis. Antibodies used are: monoclonal mouse anti-GFP (Medical and Biological Laboratories), monoclonal mouse anti-HA (Roche Molecular Biochemicals), monoclonal mouse anti-FLAG (Sigma), monoclonal mouse anti-Myc (Santa Cruz), polyclonal rabbit anti-Nur77 (Active Motif), or monoclonal mouse anti-Bcl-2 (Santa Cruz).

Isolation and Transfection of Human Peripheral Blood Lymphocytes (PBLs)

PBLs were isolated from leukocyte-enriched buffy coats from San Diego Blood Bank by centrifuging on Ficoll-paque Plus (Amersham Pharmacia Biotech). The mononuclear cells were cultured in RPMI containing 10% FBS and 20 mM HEPES. Freshly isolated cells (10^7 cells) were transfected using the human T Cell Nucleofector solution (Amaxa Biosystems) as per the procedure recommended by the manufacturer.

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