Linking of N-Myc to Death Receptor Machinery in Neuroblastoma Cells

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The oncogene MYCN is amplified in aggressive neuroblastomas in which caspase-8, an essential component of death receptor pathways, is frequently inactivated, suggesting a critical role of death receptor-mediated apoptosis in suppression of N-Myc oncogenic activity. Elevated levels of N-Myc sensitize neuroblastoma cells to apoptosis induced by various death ligands. Using tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis as a model, we define the mechanism underlying the sensitization effect. In neuroblastoma cells with increased expression of N-Myc, TRAIL triggers high levels of caspase-8 activation and Bid cleavage, leading to release of cytochrome c and Smac/DIABLO from mitochondria. However, the apoptotic process requires Smac/DIABLO, but not cytochrome c-mediated caspase-9 activation. N-Myc sensitizes neuroblastoma cells to TRAIL by up-regulating TRAIL receptor-2/DR5/KILLER and Bid. Moreover, DR5 mRNA is increased after N-Myc overexpression, and the human DR5 promoter contains two noncanonical E-boxes critical for the transcriptional activation by N-Myc. These findings establish a mechanistic link between N-Myc and death receptor machinery, which may serve as a checkpoint to guard the cell from N-Myc-initiated tumorigenesis.

Apoptosis is executed by caspases, which, based on their roles in apoptotic pathways, can be divided into two general groups. Initiator caspases (such as caspase-2, -8, -9, and -10) function mainly as integrators for upstream apoptotic signals. Once activated, the initiator caspases cleave and activate downstream effector caspases (such as caspase-3, -6, and -7), which are responsible for the proteolytic cleavage of many intracellular proteins, leading to the morphological and biochemical changes associated with apoptosis (1). Two major signaling pathways have been described for activation of initiator caspases in mammalian cells. The intrinsic pathway mediates apoptotic responses to various stress signals such as DNA damage, hypoxia, and growth factor deprivation. It is generally thought that these signals eventually lead to the activation of proapoptotic members of the Bcl-2 family (e.g. Bax and Bak), resulting in mitochondrial release of cytochrome c and other proapoptotic proteins such as Smac/DIABLO (2–4). Cytochrome c activates caspase-9 through Apaf-1 (5, 6), whereas Smac/DIABLO protects active caspases from inactivation by inhibitors of apoptosis (IAPs)³ (7). The extrinsic pathway is initiated by interaction of death ligands with their corresponding death receptors including Fas (CD95/Apo1), TNF receptor-1, TRAIL receptor-1/DR4, and TRAIL receptor-2/DR5/KILLER. The interaction results in oligomerization of the receptors that in turn recruit, via adaptor proteins such as FADD/MORT1, multiple molecules of caspase-8. The high local concentration of caspase-8 molecules allows them to activate each other through their low intrinsic protease activity (8). Activated caspase-8 can process downstream effector caspases directly, leading to apoptosis. In certain cell types, however, death receptor-induced apoptosis requires amplification of death signals via a mitochondrial pathway controlled by Bcl-2 family proteins (9). Within this pathway, caspase-8 cleaves Bid, a member of the proapoptotic Bcl-2 family of proteins. The resulting truncated Bid translocates from the cytoplasm to mitochondria, where it promotes the release of cytochrome c and other apoptotic proteins (10, 11).

Oncogene-induced sensitization of cells to apoptosis, as first noted in early 1990s (12, 13), is an important mechanism for suppression of tumorigenesis (14, 15). Tumor suppressors often play a critical role in linking oncogenes to apoptotic machinery. For example, high level expression of many oncoproteins activates the tumor suppressor p53 (16–18). Activated p53 then targets both intrinsic and extrinsic pathways to promote apoptosis through transcription-dependent and -independent mechanisms (19, 20). Alternatively, oncoproteins may directly activate the intrinsic pathway by up-regulating apoptotic proteins such as Bax and Aapf-1 (21–23). Deregulated activation of oncogenes also renders cells more sensitive to death receptor-mediated apoptosis (24–27), and there is evidence that the extrinsic pathway plays an important role in suppression of tumor development (28–30).

Neuroblastoma, a childhood tumor of the peripheral sympathetic nervous system, provides a particularly useful system for investigation of death receptor-mediated apoptosis as a tumor suppression mechanism. Neuroblastoma cells maintain a functional p53 signaling pathway (31–36), and Aapf-1 and caspase-9, two critical components of the intrinsic pathway, are expressed and active in all neuroblastoma specimens examined (37). By contrast, caspase-8, an essential mediator of death receptor-triggered apoptosis (38–40), is frequently inactivated in neuroblastoma cell lines and specimens, especially in those with MYCN amplification (41–44). Also, overexpression of N-Myc has been shown to sensitize neuroblastoma cells to death

³ The abbreviations used are: IAPs, inhibitors of apoptosis; 7-AAD, 7-aminoactinomycin D; DN, dominant-negative; EGFP, enhanced green fluorescence protein; GFP, green fluorescence protein; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis.

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receptor-induced apoptosis (25). Together, these findings suggest a pivotal role of death receptor-mediated apoptosis in protecting the cell from N-Myc-initiated tumorigenesis. In this study, we address the question of how elevated levels of N-Myc lead to activation of death receptor apoptotic pathways.

**EXPERIMENTAL PROCEDURES**

**Retroviral and Plasmid Constructs**—The following retroviral and plasmid constructs were used to generate SHEP1-derived cell lines for this study: pBabe-hygro/N-Myc, pBabe-EGFP, pBabe-puro/FADD-DN, pBabe-puro/CrmA, pBabe-puro/Bcl-2, pBabe-puro/T7-Casp9(C287A), pBabe-puro/DR5, pRetro-Super/Bcl-2, pRetro-Super/p53si, pRetro-Super/DR5si1 and 2, and pcDNA3.1/Smac, pcDNA3.1/Smac-AS, and pcDNA4/Xpress-XIAP. pRetro-Super/p53si has been described previously (45). To generate pRetro-Super/DR5si1 and 2, and pRetro-Super/Bcl-2, synthesized 64-bp oligonucleotides containing the human DR5 siRNA sequences (5'-GGTTATCCCTAGTGAACTC-3' and 5'-GGCGAGTATTGGAGGACCAC-3'), positions 907–925 and 1158–1176 relative to the start codon, respectively) or the Bid siRNA sequences (5'-GAAGACATCATCCGGAATA-3' and 5'-GAAGAGACGCGTCGGTT-3'), positions 238–256 and 350–360 relative to the start codon, respectively) were cloned into the HindIII and BglII sites in the pRetro-Super vector to form a procedure described previously (36, 47).

The human DR5 promoter luciferase reporter constructs pDR5/−1188, pDR5/−115, and pDR5/−38, as well as the promoter-less luciferase vector pGV2B, have been described previously (46). The promoter region (−1188 to +1) contains seven noncanonical E-box elements, which were individually point mutated using the QuikChange site-directed mutagenesis kit according to the manufacturer’s protocol (Stratagene). The resulting point mutants of DR5 promoter-luciferase reporter constructs are as follows: pDR5/m972 (−972/−967, CAATGG to CAAAGT), pDR5/m930 (−930/−925, CATTGG to TTCCTGG), pDR5/m537 (−537/−532, CAGGTTG to GATATG), pDR5/m428 (−428/−432, CAATGG to AGCTTG), pDR5/m294 (−294/−289, CAGCTG to CAGTAG), pDR5/m221 (−221/−216, CAGTGG to GTAGCT), and pDR5/m177 (CACGCG to CCGCGG). All of the sequences of the constructs were confirmed by DNA sequencing.

**Cell Culture and Cell Lines**—The human neuroblastoma cell line SHEP1 and various cell lines derived from SHEP1 were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma). All cells were cultured at 37 °C in a 5% CO2 humidified incubator. Retrovirus-mediated gene transfer was performed as described previously (36, 47). 24 h after the final round of retroviral infection, cells were cultured in the growth medium containing either 1.0 μg/ml puromycin or 100 μg/ml hygromycin B for 3 days, and drug-resistant cells were pooled. The percentage of retrovirus-infected cells ranged between 80 and 90%, as estimated in parallel cultures using the retrovirus-expressing EGFP. Overexpression or down-regulation of relevant proteins was verified by immunoblotting. For transfection, a transfection of plasmid DNA, 3 × 105 cells were transfected with 2 μg of plasmid DNA using Lipofectamine Plus reagent according to the manufacturer’s protocol (Invitrogen). 48 h after transfection, the cells were selected in growth medium containing 800 μg/ml G418 (Geneticin, Invitrogen) for 6 days. G418-resistant cell clones with overexpression or down-regulation of relevant proteins were pooled and used in apoptosis studies.

**Apoptosis Induction and Analysis**—Exponentially growing cells at 70–80% confluence were either untreated or treated with the indicated concentrations of TRAIL (Calbiochem), 500 ng/ml of an agonistic anti-Fas antibody (clone CH-11, Upstate), 1 ng/ml human TNF-α (Calbiochem) plus 2.5 μg/ml cycloheximide (Sigma), or 0.5 μg/ml doxorubicin (Ben Venue Laboratories). 24 h after treatment, adherent and floating cells were pooled, collected by centrifugation, and washed once with ice-cold phosphate-buffered saline (PBS). Apoptotic cell death was determined by staining of the collected cells with annexin-V and 7-aminocoumarin-3′-yloxyacetic acid (3′,4′-dicarboxyfluorescein diacetate,succinimidyl ester) (Molecular Probes) and 7-AAD. Microscopic images were captured using a Nikon Eclipse E800 microscope. For immunofluorescent staining, cells were fixed with 4% paraformaldehyde in PBS for 5 min. The cells were blocked with 5% milk in PBS for 1 h, incubated with primary antibodies in blocking buffer for 1 h at room temperature, washed in PBS, and then incubated with the appropriate secondary antibody. Primary antibodies were used at 1:400 for a rat monoclonal antibody against Smac/DIABLO (clone 10G7, Calbiochem) and 1:800 for a mouse monoclonal antibody against cytochrome c (clone 6H2.B4, Pharmingen). Texas Red-X goat anti-mouse IgG (1:600) and Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1:600, Molecular Probes) were used as secondary antibodies. 300 nM DAPI in PBS was used for nuclear staining. Cells were visualized using a Nikon Eclipse E800 microscope with Image-Pro Plus software for image analysis.

**Transient Expression and Luciferase Assays**—SHEP1 and SHEP1/N-Myc cells relative to those found in SHEP1/GFP cells were quantified using Kodak Image Station 440CF. For immunofluorescent staining, cells were grown on coverslips and treated with 10 ng/ml TRAIL for 24 h. Cells undergoing apoptosis show characteristics of cell shrinkage. C, SHEP1/GFP and SHEP1/N-Myc cells were either untreated or treated with 100 ng/ml TRAIL for 24 h and analyzed for apoptosis by annexin-V and 7-AAD staining. Percentages of cells undergoing apoptosis (stained positively with both annexin-V and 7-AAD) are indicated. D, SHEP1/GFP and SHEP1/N-Myc cells were either untreated or treated for 24 h with 0.5 μg/ml agonistic anti-Fas antibody CH-11 or 1 ng/ml TNF-α plus 2.5 μg/ml cycloheximide and analyzed for apoptosis as above. Each bar represents the average ± S.D. of three independent experiments.

**RESULTS**

N-Myc Sensitizes SHEP1 Cells to Death Receptor-mediated Apoptosis—The human neuroblastoma cell line SHEP1 is an epitheloid subclone of the neuroblastoma cell line SK-N-SH (48) that contains no MYCN amplification (49). SHEP1 cells

**FIG. 1. N-Myc sensitizes SHEP1 cells to apoptosis induced by death ligands.** A, immunoblot analysis of N-Myc levels in GFP- and N-Myc-overexpressing SHEP1 cell lines, as well as in IMR32 cells, a neuroblastoma cell line with MYCN amplification. Levels of β-tubulin are shown as the loading control. B, micrographs of SHEP1/GFP and SHEP1/N-Myc cells that were either untreated or treated with 100 ng/ml TRAIL for 24 h. Cells undergoing apoptosis show characteristics of cell shrinkage. C, SHEP1/GFP and SHEP1/N-Myc cells were either untreated or treated with 100 ng/ml TRAIL for 24 h and analyzed for apoptosis by annexin-V and 7-AAD staining. Percentages of cells undergoing apoptosis (stained positively with both annexin-V and 7-AAD) are indicated. D, SHEP1/GFP and SHEP1/N-Myc cells were either untreated or treated for 24 h with 0.5 μg/ml agonistic anti-Fas antibody CH-11 or 1 ng/ml TNF-α plus 2.5 μg/ml cycloheximide and analyzed for apoptosis as above. Each bar represents the average ± S.D. of three independent experiments.
have functional death receptor pathways and express very low levels of endogenous N-Myc which are generally undetectable by immunoblotting (Fig. 1A), thus providing a suitable system for examining the effect of elevated levels of N-Myc on death receptor-triggered apoptosis. The human N-Myc oncogene was introduced into SHEP1 cells by retroviral-mediated gene transfer. After 3-day selection in the presence of hygromycin, infected cells were pooled, which expressed N-Myc at the level compatible to that found in IMR32 cells (Fig. 1A), a neuroblastoma cell line with MYCN amplification (49). The control, GFP-expressing SHEP1 (SHEP1/GFP) cells were relatively resistant to TRAIL-induced apoptosis (Fig. 1B). N-Myc expression markedly sensitized SHEP1 cells to TRAIL, and most of the cells exhibited morphological changes characteristic of apoptosis such as cell shrinkage within 24 h of treatment with 100 ng/ml TRAIL (Fig. 1B). The apoptotic cell death was confirmed further by an annexin-V binding assay in which annexin-V binds to externalized phosphatidylserine on the surface of apoptotic cells (Fig. 1C). In addition, SHEP1 cells expressing N-Myc (SHEP1/N-Myc) showed a marked increase in their sensitivity to an agonistic anti-Fas antibody (clone CH-11) and to TNF-α (Fig. 1D). These results confirmed the previous finding that N-Myc is a potent proapoptotic regulator of death receptor-triggered apoptosis (25).

**TRAIL Induces Apoptosis in SHEP1/N-Myc Cells through the Caspase-8-Bid-Mitochondria Pathway**—We next wished to define the signaling pathway through which N-Myc sensitizes SHEP1 cells to death receptor-induced apoptosis, using the TRAIL system as a model. As expected, both FADD and caspase-8 are required for TRAIL-induced apoptosis. Expression of a FADD dominant-negative mutant (FADD-DN) (50) or CrmA, a potent caspase-8 inhibitor, were either untreated or treated with 0.5 μg/ml doxorubicin (Doxo) or 100 ng/ml TRAIL for 24 h and analyzed for apoptosis as above. C, immunoblot analysis of caspase-8 activation and Bid cleavage in SHEP1/GFP and SHEP1/N-Myc cells that were either untreated or treated for 24 h with the indicated concentrations of TRAIL. Caspase-8 activation was indicated by the appearance of the processed products, p43/41 and p18, and Bid cleavage indicated by the appearance of the truncated product tBid. α-Tubulin levels are shown as the loading control. D, vector control and Bcl-2-overexpressing SHEP1/N-Myc cells were either untreated or treated with 100 ng/ml TRAIL for 8 h, fixed, and immunostained with a rat monoclonal antibody against Smac/DIABLO or a mouse monoclonal antibody against cytochrome c (Cyto C). Nuclei were visualized with DAPI. Magnification, ×60. E, SHEP1/N-Myc cells expressing GFP or Bcl-2 were treated with the indicated concentrations of TRAIL for 24 h and analyzed for apoptosis as above. Inset, immunoblot analysis of Bcl-2 overexpression. N-Myc levels are shown as the loading control. Each point in A, E, and F or bar in B represents the average ± S.D. of three independent experiments.
SHEP1/GFP cells (Fig. 2C). Concurrent with the caspase-8 activation, a decrease in the levels of full-length Bid and an increase in the levels of truncated Bid (tBid) were observed (Fig. 2C).

The observed Bid cleavage suggests a possible role of mitochondria in mediating TRAIL-induced apoptosis in SHEP1/N-Myc cells. To test this hypothesis, we examined the location of cytochrome c and Smac/DIABLO in SHEP1/N-Myc cells before and after TRAIL treatment. In untreated cells, immunofluorescent staining revealed a characteristic punctate mitochondrial pattern for both cytochrome c and Smac/DIABLO (Fig. 2D). After TRAIL stimulation, the punctate staining of cytochrome c and Smac/DIABLO became diffused in SHEP1/N-Myc cells (Fig. 2D), indicating the release of these factors from mitochondria. Overexpression of Bcl-2 in SHEP1/N-Myc cells prevented the mitochondrial release of cytochrome c and Smac/DIABLO (Fig. 2D) and protected the cells from TRAIL-induced apoptosis (Fig. 2F). Together, these results suggest that the mitochondrial release of apoptotic factors is an essential step in TRAIL-induced apoptosis in SHEP1/N-Myc cells.

To define further the role of Bid in TRAIL-induced apoptosis in SHEP1/N-Myc cells, we generated two Bid siRNA-expressing retroviral constructs that target different regions of the Bid-coding sequence. Retroviruses produced from both constructs were able to knock down the levels of Bid in SHEP1/N-Myc cells (Fig. 2F, inset) and to decrease their sensitivity to TRAIL-induced apoptosis (Fig. 2F), indicating that Bid plays an essential role in this apoptotic process.

Smac/DIABLO, but Not Cytochrome c-mediated Caspase-9 Activation, Is Required for TRAIL-induced Apoptosis in SHEP1/N-Myc Cells—Release of cytochrome c into the cytoplasm promotes Apaf-1-mediated caspase-9 activation (6). Indeed, caspase-9 became activated in SHEP1/N-Myc cells after TRAIL treatment, as indicated by the appearance of its processed form (Fig. 3C). We next assessed the effect of down-regulation of endogenous Smac/DIABLO on TRAIL-induced apoptosis. Overexpression of Smac/DIABLO increased the sensitivity of SHEP1 cells to TRAIL-induced apoptosis in SHEP1/N-Myc cells through a caspase-9-independent pathway downstream from mitochondria.

Because Smac/DIABLO was also released from mitochondria in SHEP1/N-Myc cells treated with TRAIL (Fig. 2D), we examined its role in TRAIL-induced apoptosis. Overexpression of Smac/DIABLO increased the sensitivity of SHEP1 cells to TRAIL (Fig. 3C). We next assessed the effect of down-regulation of endogenous Smac/DIABLO on TRAIL-induced apoptosis. SHEP1/N-Myc cells were transfected with either pcDNA3.1 or a pcDNA3.1-based plasmid that expresses Smac/DIABLO in an antisense orientation, and G418-resistant clones with significant down-regulation of Smac/DIABLO were pooled. SHEP1/N-Myc cells stably transfected with the control plasmid pcDNA3.1 remained sensitive to TRAIL-induced apoptosis (Fig. 3D). By contrast, the antisense-mediated down-regulation...
of endogenous Smac/DIABLO significantly decreased the sensitivity of SHEP1/N-Myc cells to TRAIL (Fig. 3D), indicating a critical role of Smac/DIABLO in mediating TRAIL-induced apoptosis in these cells.

Smac/DIABLO Targets XIAP for Degradation in SHEP1/N-Myc Cells in Response to TRAIL—A major target of Smac/DIABLO is X-linked inhibitor of apoptosis (XIAP), an inhibitor of caspase-9, -3, and -7 (53). To define the mechanism of Smac/DIABLO action, we investigated the role of XIAP in TRAIL-induced apoptosis in SHEP1/N-Myc cells. Overexpression of XIAP effectively protected SHEP1/N-Myc cells from apoptosis induced by TRAIL (Fig. 4A). Immunoblot analysis revealed that after treatment with TRAIL, the endogenous XIAP levels were decreased markedly in SHEP1/N-Myc cells (Fig. 4B, lanes 4–6 and 13–15). A decrease in XIAP levels was also observed in TRAIL-treated SHEP1 cells expressing both N-Myc and casp9 DN (Fig. 4B, lanes 1–3), which remained sensitive to TRAIL-induced apoptosis (Fig. 3B). By contrast, TRAIL failed to induce XIAP degradation in resistant SHEP1/N-Myc cells expressing FADD-DN (Fig. 4B, lanes 10–12), CrmA (lanes 16–18), or Bel-2 (lanes 7–9). Moreover, down-regulation of endogenous Smac/DIABLO in SHEP1/N-Myc cells blocked TRAIL-induced XIAP degradation (Fig. 4B, lanes 19–21). Together, these observations suggest that Smac/DIABLO promotes XIAP degradation to mediate TRAIL-induced apoptosis in SHEP1/N-Myc cells.

N-Myc Sensitizes SHEP1 Cells to TRAIL by Up-regulating Apoptotic Proteins in the TRAIL Signaling Pathway—Dissection of the TRAIL apoptotic pathway in SHEP1/N-Myc cells allows us to investigate the mechanism by which N-Myc sensitizes cells to TRAIL-induced apoptosis. We first examined the levels of apoptotic proteins and regulators that may function in the TRAIL signaling pathway in GFP- and N-Myc-expressing SHEP1 cell lines. Immunoblot analysis revealed that N-Myc overexpression in SHEP1 cells resulted in a noticeable increase in the levels of DR5, Bid, and p53 (Fig. 5, A and B). We have shown earlier that Bid is essential for TRAIL-induced apoptosis in SHEP1/N-Myc cells (Fig. 2F). Also, given that these cells do not express detectable levels of DR4 (data not shown), TRAIL most likely signals through DR5 to induce apoptosis. Thus, higher levels of DR5 and Bid may account for the TRAIL-sensitive phenotype of SHEP1/N-Myc cells. In support of this hypothesis, overexpression of DR5 was sufficient to increase the sensitivity of SHEP1 cells to TRAIL (Fig. 5C). We examined further the requirement of DR5 induction for the TRAIL-sensitive phenotype of SHEP1/N-Myc cells. Two DR5 siRNA retroviral constructs that target different regions of the DR5-coding sequence were generated. Infection of SHEP1/N-Myc retroviruses produced with either construct resulted in a significant reduction in the levels of endogenous DR5 (Fig. 5D, inset), and these cells became resistant to TRAIL-induced apoptosis (Fig. 5D). By contrast, SHEP1/N-Myc cells infected with control retroviruses remained highly sensitive to TRAIL (Fig. 5D). Thus, induction of DR5 by N-Myc is essential for sensitizing SHEP1 cells to TRAIL-induced apoptosis.

DR5 Is a Transcriptional Target of N-Myc—Northern blot analysis revealed an increase in DR5 mRNA levels in SHEP1/N-Myc cells (Fig. 6A), suggesting that the induction of DR5 occurred at the transcription step. A major transcriptional activator of DR5 expression is p53 (54). SHEP1 cells express wild-type p53 (33), and consistent with a recent study (55), N-Myc expression resulted in a significant increase in the basal levels of p53 in SHEP1 cells (Fig. 5, A and B). A concomitant increase in the expression of p53 target genes such as p21Waf1/Cip1 and HDM2 was also observed (data not shown), indicating a functional p53 signaling pathway in SHEP1/N-Myc cells. Therefore, we examined the possibility that N-Myc may require p53 for induction of DR5. Down-regulation of p53 by a well defined siRNA retroviral construct (45) had no effects on the ability of N-Myc to induce DR5 (Fig. 6B) and to sensitize SHEP1 cells to TRAIL (Fig. 6C). We also obtained similar results with SHEP1/N-Myc cells expressing p53(R175H), which functions as a dominant-negative mutant (36, 56) (data not shown). Thus, N-Myc induces DR5 through a p53-independent mechanism.

Next, we examined the possibility of DR5 being a transcriptional target of N-Myc. Luciferase reporter constructs containing serial 5′-deletions of the human DR5 promoter have been generated previously, and the reporter construct (DR5/−1188) containing the promoter sequence spanning −1188 to +1 (translation start site) was able to induce maximal levels of luciferase activity in transient transfection assays (46). We tested some of these reporter constructs in SHEP1 and SHEP1/N-Myc cells (Fig. 6D). In both cell lines, only the DR5/−1188 reporter construct resulted in significantly higher levels of luciferase activity than the promoterless luciferase vector pGV82 (Fig. 6E). Importantly, SHEP1/N-Myc cells transfected with the DR5/−1188 reporter construct showed a further increase in luciferase activity compared with SHEP1 cells transfected with the same construct (72-fold versus 27-fold, a 2.7-fold induction; Fig. 6E).

Inspection of the DR5 promoter sequence (−1188 to +1) revealed the presence of seven noncanonical E-box elements (six CANNTG sites and one CACCGG site, see “Experimental Procedures”). All of these E-box elements have been shown previously to be able to mediate transcriptional regulation by Myc proteins (for review, see Ref. 57). Like other members of
Identification of signaling pathways that link oncogenes to apoptotic machinery has important implications both for our fundamental understanding of tumorigenesis and for the development of therapeutic approaches that specifically target tumor cells. In the subset of neuroblastoma with MYCN amplification, caspase-8 expression is preferentially suppressed (41). This finding, along with the observation that N-Myc sensitizes neuroblastoma cells to apoptosis induced by various death ligands (25), suggests a coupled relationship between N-Myc and death receptor-triggered apoptosis. To understand the molecular basis of this relationship, we first delineate the transcriptional regulation of N-Myc.

**DISCUSSION**

The Myc family of transcription factors, N-Myc contains a DNA binding/dimerization domain consisting of the basic, the helix-loop-helix, and the leucine zipper regions (bHLHZip). The bHLHZip domain binds to so-called E-box DNA recognition sequences with the core motif 5'-CANNTG (57). To determine the significance of these E-boxes in the regulation of DR5 transcription by N-Myc, SHEP1/N-Myc cells were transfected with DR5/H11032 and 1188 luciferase reporter constructs containing individually mutated E-boxes (Fig. 6D). Mutation of the −221 or the −229 E-box element resulted in a significant reduction in luciferase activity compared with the wild-type DR5/H9252 promoter. pGVB2 is a promoterless luciferase vector. Also shown are mutated E-box elements and their locations within the DR5 promoter.

**FIG. 6.** The DR5 gene is a transcription target of N-Myc. A, Northern blot analysis of DR5 mRNA levels in SHEP1/GFP and SHEP1/N-Myc cells. The levels of 28 S and 18 S ribosomal RNA are shown as the loading control. B, immunoblot analysis of the levels of p53 and DR5 in vector (pRS) control and p53 siRNA-expressing SHEP1/N-Myc cells. α-Tubulin levels are shown as the loading control. C, parental SHEP1 and SHEP1/N-Myc cells infected with the vector (pRS) or p53 siRNA-expressing retroviruses were treated for 24 h with the indicated concentrations of TRAIL and analyzed for apoptosis by annexin-V and 7-AAD staining. Each point represents the average ± S.D. of three independent experiments. D, schematic representation of the luciferase reporter constructs containing serial 5′-deletions of the human DR5 promoter. pGVB2 is a promoterless luciferase vector. Also shown are mutated E-box elements and their locations within the DR5 promoter. E and F, SHEP1 (E) and SHEP1/N-Myc cells (E and F) were transfected with the indicated luciferase reporter constructs along with a β-galactosidase reporter plasmid. 24 h after transfection, the cells were harvested and assayed for luciferase and β-galactosidase activities. Luciferase values were normalized to β-galactosidase activity to account for differences in the transfection efficiency. Fold activation indicates the induction of luciferase activity by transfection with the DR5 promoter-luciferase reporter constructs relative to that induced by transfection with the promoterless luciferase vector pGVB2. Each point in C or bar in E and F represents the average ± S.D. of three independent experiments.

The Myc family of transcription factors plays a dual role in apoptosis by up-regulation of apoptotic proteins in the TRAIL signaling pathway.
Smac/DIABLO in mediating TRAIL-induced apoptosis. In this study, we define further the molecular mechanism by which N-Myc sensitizes neuroblastoma cell to TRAIL-induced apoptosis (Fig. 7). N-Myc induces DR5 and Bid, two essential apoptotic proteins in the TRAIL signaling pathway. The up-regulation of DR5 and Bid is required for N-Myc-induced sensitization of neuroblastoma cells to TRAIL, because abrogation of their induction by siRNA completely reverses the TRAIL-sensitive phenotype of SHEP1/N-Myc cells. Mechanistically, an increase in the levels of DR5 could facilitate the formation of the DR5 death-inducing signaling complex in response to TRAIL, leading to higher levels of caspase-8 activation, as observed in this study (Fig. 2C). Moreover, elevated levels of Bid could further amplify the death signal through mitochondrial release of Smac/DIABLO. The observed induction of Bid also suggests a mechanism for N-Myc to sensitize neuroblastoma cells to other death receptor signals that require Bid for targeting mitochondria.

Several lines of evidence from this study suggest that the DR5 gene is a transcription target of N-Myc. First, DR5 mRNA and protein levels are increased after overexpression of N-Myc. Second, N-Myc is able to activate transcription from the human DR5 promoter in luciferase reporter assays. Finally, the human DR5 promoter region contains two noncanonical E-box elements critical for the transcriptional activation by N-Myc. The mechanism by which N-Myc induces Bid is currently under investigation. Nevertheless, our findings establish a direct connection between N-Myc and death receptor machinery. Conceivably, this connection makes N-Myc-amplified neuroblastoma cells a target of the surveillance network that employs a p53-dependent checkpoint. A molecular understanding of this signaling pathway in a variety of neuroblastoma cell lines examined (31–36). Importantly, loss of p53 has no effects on the rate and incidence of neuroblastomas in MYCN transgenic mice (65).

Together, these observations suggest that the biochemical process that drives neuroblastoma development does not go through a p53-dependent checkpoint. A molecular understanding of this process may shed light on what determines the cell type dependence of p53 as a tumor suppressor and help us identify other tumor suppression mechanisms.

TRAIL is a promising agent for cancer therapy because of its selective cytotoxicity to human cancer cell lines in vitro and to xenografts in immunodeficient mice (66–68). However, the molecular basis for this differential sensitivity is poorly understood. It was initially suggested that the presence of TRAIL decay receptors in normal, but not cancer, cells might account for the selectivity (69–71). But subsequent studies failed to establish a correlation between the expression of the decay receptors and the sensitivity to TRAIL (72, 73). We suggest that activation of oncogenes, a common event in tumorigenesis, may be an important mechanism underlying the selective sensitivity of cancer cells to TRAIL-induced apoptosis. This model is consistent with experimental observations. In addition to N-Myc, a number of oncogenes, including E1A, c-Myc, and Ras, have been shown to sensitize cells to apoptosis induced by TRAIL (74–76). Moreover, in a study to determine how normal human cells acquire TRAIL-sensitive phenotype during a controlled process of malignant transformation, Nesterov et al. (76) showed that both normal and immortalized human embryonic kidney cells and foreskin fibroblasts were resistant to TRAIL, whereas Ras-transformed cells were susceptible, demonstrating that the acquisition of TRAIL-sensitive phenotype depends on activation of oncogenes. Further exploring of the molecular pathways that link oncogenes to the TRAIL death machinery may offer numerous opportunities for targeting TRAIL to cancer cells.

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