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13. ABSTRACT *(Maximum 200 Words)*

The key transcriptional regulators of the cellular hypoxic response, Hypoxia Inducible Factor-1 (HIF-1) and NF-κB, are responsible for induction of genes that regulate anaerobic metabolism, cell survival, and angiogenesis. We hypothesize that cancer cells subvert these normal hypoxia-dependent mechanisms to enable their own deregulated survival, neovasculogenesis, and growth. We find that loss of the p53 tumor suppressor gene promotes the neovascularization and growth of tumor xenografts. Our results indicate that p53 inhibits NF-κB RelA activity via interaction with the p300 transcriptional integrator and promotes ubiquitin-mediated proteasomal degradation of the α subunit of HIF-1. Loss of p53 augments HIF-1- and NF-κB-dependent transcriptional activation of the vascular endothelial growth factor (*VEGF*) gene and contributes to the angiogenic switch during tumorigenesis. In addition, we find that activation of NF-κB by HER-2/neu- and IGF-1 protects breast cancer cells from hypoxia- or death receptor-induced apoptosis. Conversely, repression of NF-κB by inhibition of the IκB kinase-NEMO complex sensitizes breast cancer cells to hypoxia- or TRAIL/Apo2L-induced death. Together, our studies indicate that the constitutive activation of HIF-1 and NF-κB in breast cancers may underlie their angiogenic and apoptosis-resistant phenotype; as such, these transcription factors could provide attractive targets for innovative interventions to treat and prevent human breast cancers.

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INTRODUCTION:

The clonal evolution of tumor cells in hypoxic microenvironments ultimately selects subpopulations that not only resist apoptosis, but also promote angiogenesis. The transcriptional regulators of the normal hypoxic response, Hypoxia Inducible Factor-1 (HIF-1) and NF-κB, are responsible for induction of genes that promote anaerobic metabolism, cell survival, vasodilatation, and angiogenesis. We hypothesize that cancer cells subvert these normal hypoxia-dependent mechanisms to enable their own deregulated survival, neovasculogenesis, and growth. We propose that inhibition of HIF-1 and/or NF-κB can abrogate the angiogenic and apoptosis-resistant phenotype of breast tumors, thereby curtailing their growth and metastases. We aim to elucidate the molecular mechanisms by which the p53 tumor suppressor regulates HIF-1and NF-κB activity and examine the effect of inhibiting HIF-1 and/or NF-κB on the growth, neovascularization, and metastatic potential of breast cancers *in vitro* and *in vivo*. These studies will provide insights into the molecular mechanisms governing the response to hypoxic stress and will determine whether their subversion by breast cancers is responsible for their apoptosis-resistant and angiogenic phenotype. These key transcription factors could provide targets for innovative interventions for the treatment and prevention of breast cancer.

BODY:

09/01/99 – 08/31/00:

The previous annual report (*September 2000*) covered the first year (0-12 months) of the research project and was devoted to the successful completion of Specific Aim 1 (Tasks 1 and 2 of the statement of work).

Specific Aim 1. Investigate the mechanism(s) of p53-mediated repression HIF-1 and its role in regulation of hypoxia-induced angiogenesis.
- A. Elucidate the molecular mechanism(s) responsible for p53-mediated repression of HIF-1 activity.
- B. Define the role of HIF-1 in the angiogenic phenotype conferred by p53-deficiency

Statement of Work (1-12 months)
- Task 1: Elucidate the molecular mechanism(s) responsible for p53-mediated repression of HIF-1 activity.
- Task 2: Define the role of HIF-1 in the angiogenic phenotype conferred by p53-deficiency

We completed the studies proposed in specific aim 1 (Tasks 1, 2a,b) and published the results and conclusions in:

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development* 14:34-44, 2000. (*Reprint of publication enclosed-Appendix 1*).

Abstract: The switch to an angiogenic phenotype is a fundamental determinant of neoplastic growth and tumor progression. We demonstrate that homozygous deletion of the p53 tumor suppressor gene via homologous recombination in a human cancer cell line promotes the neovascularization and growth of tumor xenografts in nude mice. We find that p53 promotes Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α subunit of hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that regulates cellular energy metabolism and angiogenesis in response to oxygen deprivation. Loss of p53 in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activation of the vascular endothelial growth factor (VEGF) gene in response to hypoxia. Forced expression of HIF-1α in p53-expressing tumor cells increases hypoxia-induced VEGF expression and augments neovascularization and growth of tumor xenografts. These results indicate that amplification of normal HIF-1-dependent responses to hypoxia via loss of p53 function contributes to the angiogenic switch during tumorigenesis.

09/01/00 – 08/31/01:

The current report (*September 2001*) covers the second year (12-24 months) of the research project. This period was devoted to the completion of Specific Aim 2 and part of Specific Aim 3 (Tasks 2 and 3 of the statement of work).

Specific Aim 2. Define the role of NF-κB RelA in the angiogenic phenotype conferred by p53 deficiency and the molecular determinants of κB-dependent angiogenesis
- A. Investigate whether repression of RelA by a transdominant mutant IκBα (IκBαM) can inhibit the angiogenic phenotype conferred by p53- deficiency.
- B. Investigate the molecular determinants of NF-κB-mediated angiogenesis.

Specific Aim 3. Examine the effect of inhibiting HIF-1 or RelA on growth, neovascularization, and metastatic potential of breast cancers.

Statement of Work (12-24 months):

Task 2: Define the role of NF-κB in the angiogenic phenotype conferred by p53-deficiency
Task 3: Define the role of NF-κB on growth and neovascularization of breast cancers.

We have completed the studies proposed in specific aim 2 (Task 2) and part of specific aim 3 (Task 3a) and have presented the results and conclusions in the following publication and manuscript:

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* 3:409-416, (2001). (*Reprint of publication enclosed-Appendix 2*)

Ravi, R. and Bedi, A. Sensitization of Breast Cancers to TRAIL/Apo2L-induced Apoptosis by Inhibition of the IκB Kinase-NEMO Complex. Manuscript Submitted, 2001. (*Manuscript enclosed-Appendix 3*)

Research Accomplished:

All the methodology and results (data and figures) detailed in the following summary are included in the publication and manuscript referenced above (Please refer to the publication reprints and manuscript in *Appendices 2, 3, and 4*)

Regulation of Hypoxia-induced Apoptosis by NF-κB

(*Unpublished Data- Please refer to figure 1 in Appendix 4*)

Electrophoretic mobility shift assays demonstrated that hypoxia induces NF-κB DNA-binding activity in 3T3 fibroblasts (Figure 1a). To examine the role of NF-κB in hypoxia-induced expression of VEGF, $RelA^{+/+}$ and $RelA^{-/-}$ 3T3 fibroblasts were analyzed for expression of HIF-1α protein and VEGF mRNA under tissue culture conditions simulating the hypoxic tumor microenvironment. Following exposure to 0.1% O_2, $RelA^{+/+}$ and $RelA^{-/-}$ cells exhibited equivalent induction of HIF-1α protein and VEGF mRNA (Figure 1b and c). Although RelA was not required for hypoxia-induced expression of VEGF, $RelA^{-/-}$ cells exhibited greater levels of hypoxia-induced apoptosis than their $RelA^{+/+}$ counterparts (Figure 1d).

The molecular machinery of cell death comprises an evolutionarily conserved family of cysteine aspartate proteases (caspases) that executes cell disassembly via cleavage of critical substrates that maintain cytoskeletal and DNA integrity. These terminal proteolytic events require activation of the apoptosome, a holoenzyme formed by association of caspase-9 with essential mitochondrial cofactors, such as Apaf-1 and cytochrome c (Cyt c). The release of mitochondrial cofactors is regulated by members of the Bcl-2 family, which comprises pro-survival proteins, such as Bcl-2 and Bcl-x_L, and pro-apoptotic proteins, such as BAX, BAK, and BID. Since heterodimerization of pro- and anti-apoptotic members of the Bcl-2 family can mutually neutralize one another's function, their relative concentrations act as a rheostat that determines the release of mitochondrial cofactors and cell survival. We examined whether hypoxia-induced activation of RelA influences the expression of Bcl-2 family members. The basal and hypoxia-inducible expression of Bcl-x_L was markedly reduced in $RelA^{-/-}$ cells compared to their $RelA^{+/+}$ counterparts (Figure 1e).

Activation of NF-κB requires phosphorylation and ubiquitin-mediated degradation of IκBα via the activity of the IκB-kinase (IKK) complex containing two kinases (IKK-α and IKK-β) and the regulatory protein NEMO (NF-κB essential modifier; IKK-γ). Inhibition of the IKK complex by the non-steroidal anti-inflammatory drug, aspirin (ASA) reduced hypoxia-induced expression of Bcl-x_L and sensitized cells to hypoxia-induced apoptosis (Figure 1d and e).

Together, these results indicate that activation of NF-κB is required for inducing Bcl-x_L expression and protecting cells from hypoxia-induced apoptosis. Hypoxia promotes stabilization and elevation of p53, which in turn, induces expression of TRAIL-R2 (DR5, KILLER), a death receptor belonging to the tumor necrosis factor (TNF) receptor gene superfamily. TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins containing cytoplasmic sequences, termed "death domains", which trigger cell death following engagement by their cognate "death ligand", TRAIL (tumor necrosis factor-related apoptosis-inducing ligand; Apo2L). Therefore, we next examined whether NF-kB-induced expression of Bcl-x_L protects cells from death receptor-transduced apoptotic signals.

Regulation of Death Receptor-induced Apoptosis by NF-κB

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* 3:409-416, (2001). (*Reprint of publication enclosed-Appendix 2*)

Abstract:

While NF-κB promotes expression of death receptors (TRAIL-R1/R2, CD95/Fas), we have demonstrated that HER-2/neu-mediated activation of NF-κB (RelA) also induces expression of Bcl-x_L which protects breast cancer cells from TRAIL/Apo2L. Inhibition of NF-κB by blocking activation of the IκB-kinase complex with either a peptide that disrupts the interaction of IKKβ with NEMO or by acetyl salicylic acid (aspirin;ASA) reduces expression of Bcl-xL and sensitizes breast cancer cells to TRAIL/Apo2L-induced death.

Results:

Exposure of MCF-7 breast cancer cells to recombinant heregulin β1 (HRG β1) increased κB DNA binding activity in EMSA and increased expression of TRAIL-R1 and TRAIL-R2. However, exposure of MCF-7 cells to HRG β1 also promoted expression of Bcl-x_L and rendered them relatively resistant to TRAIL/Apo2L (Appendix 2, Fig. 4). Activation of NF-κB requires phosphorylation and ubiquitin-mediated degradation of IκBα via the activity of the IκB-kinase (IKK) complex containing two kinases (IKK-α and IKK-β) and the regulatory protein NEMO (NF-κB essential modifier; IKK-γ). A cell permeable peptide [NEMO binding domain (NBD) peptide] that blocks the interaction of NEMO with the IKK complex inhibits cytokine-induced NF-κB activation. The sequence of the wild-type peptide indicating the Antennapedia homeodomain (lower case) and the IKKβ (upper case) segments and the mutant peptide with the W→A mutations (underlined) are:

Wild type (WT NBD)- drqikiwfqnrrmkwkkTALDWSWLQTE
Mutant (MU NBD)- drqikiwfqnrrmkwkk TALDA SA LQTE

Acetyl salicyclic acid (aspirin; ASA), also specifically inhibits the activity of IKK-β. Inhibition of the IKK complex by either ASA or WT NBD peptide prevented HRG β1-induced loss of IκBα or activation of NF-κB. Exposure of MCF-7 cells to either ASA or WT NBD (but not MU NBD) prevented HRG β1 from inducing expression of Bcl-x_L and prevented HRG β1 from protecting MCF-7 cells from TRAIL/Apo2L-induced apoptosis (Appendix 2, Fig. 4). Conversely, inhibition of NF-κB sensitizes breast cancer cells to TRAIL/Apo2L.

Ravi, R. and Bedi, A. Sensitization of Breast Cancers to TRAIL/Apo2L-induced Apoptosis by Inhibition of the IκB Kinase-NEMO Complex. Manuscript Submitted, 2001. (*Manuscript enclosed-Appendix 3*)

Abstract:

Breast cancer cells can be induced to undergo apoptosis by engagement of death receptors with TRAIL (Tumour necrosis factor-related apoptosis-inducing ligand)/Apo2L. Here we show that activation of the PI3 Kinase-Akt-NF-κB signaling pathway by HER-2/neu or insulin-like growth factor-1 (IGF-1) renders breast cancer cells relatively resistant to TRAIL/Apo2L-induced apoptosis. Activation of NF-κB requires phosphorylation-dependent degradation of IκB by an IκB kinase (IKK) complex comprising the regulatory protein NEMO (NF-κB essential modifier) in association with two kinases (IKKα and IKKβ). A cell-permeable NEMO-binding domain peptide that blocks the interaction of NEMO with the IKK complex inhibits HER-2/neu- or IGF-1-mediated activation of NF-κB and sensitizes breast cancer cells to TRAIL/Apo2L-induced death. The efficacy of TRAIL/Apo2L in the treatment of breast cancers may be improved by antibody-mediated inhibition of growth factor receptors (HER2/neu or IGF-1R) and/or peptidomimetic drugs that disrupt the IKK-NEMO complex.

Background/ Rationale:

Genetic aberrations that render cells incapable of executing apoptosis not only promote tumorigenesis, but also underlie the observed resistance of human cancers to genotoxic anticancer agents (1, 2). Unraveling mechanisms to unleash the apoptotic program in tumor cells could aid the design of effective therapeutic interventions against resistant breast cancers. One mechanism of triggering tumor cell death involves engagement of death receptors belonging to the tumor necrosis factor (TNF^3) receptor gene superfamily, such as TRAIL-R1 (DR4), and TRAIL-R2

(DR5, TRICK2, KILLER) by their cognate "death ligand", TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)/Apo2L (3-5). TRAIL/Apo2L induces apoptosis of many breast cancer cell lines *in vitro*, and its tumoricidal activity and safety *in vivo* has been confirmed in preclinical animal models of human breast cancer xenografts (5). Although TRAIL/Apo2L inhibits tumor growth when administered immediately after xenotransplantation, delaying application of the same treatment is less effective against more established tumors (5). Moreover, human breast cancer cell lines exhibit a wide heterogeneity in their sensitivity to TRAIL/Apo2L *in vitro*. These data suggest that successful treatment of breast cancers with TRAIL/Apo2L may require its combination with agents that inhibit survival signals responsible for protecting tumor cells from death receptor-induced apoptosis.

Amplification and consequent overexpression c-erbB2 (HER-2/neu), a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, has been observed in a significant proportion of human breast cancers (~30%) and is correlated with increased metastatic potential, resistance to apoptosis, and poor prognosis (6). Akin to EGFR, IGF-1 receptor (IGF-1R) overexpression in breast cancers has been associated with resistance to radiation and chemotherapeutic agents (7). Both HER-2/neu and IGF-1R promote PI3 kinase (PI3-K)-mediated phosphorylation and activation of Akt, a serine-threonine kinase that, in turn, activates the IκB kinase (IKK) complex (8-10). Activation of the IKK complex requires association of the regulatory protein, NEMO (NF-κB essential modifier)/IKKγ/IKKAP1, with the NEMO-binding domains (NBD) of two catalytic subunits, IKKα (IKK-1) and IKKβ (IKK-2)(11). The activated IKK complex induces phosphorylation-mediated degradation of IκB, thereby promoting activation of NF-κB, a family of dimeric transcription factors that protects cells from TRAIL/Apo2L-induced apoptosis (12). Breast cancers frequently exhibit constitutive NF-κB activity associated with aberrant activation of the IKK complex (13).

In this study, we examined whether HER-2/neu or IGF-1 inhibit TRAIL/Apo2L-induced apoptosis of breast cancer cells via activation of the PI3-K-Akt-NF-κB signaling pathway. We investigated whether breast cancer cells can be sensitized to TRAIL/Apo2L by inhibiting NF-κB with a cell-permeable NEMO-binding domain peptide that prevents formation of the IKKβ-NEMO complex.

Results:

Inhibition of TRAIL/Apo2L-induced Apoptosis of Breast Cancer Cells by HER-2/neu or IGF-1. Exposure of MCF-7 breast cancer cells (expressing low levels of HER-2/neu) to TRAIL/Apo2L (100 ng ml^{-1}) resulted in death of 59±3% of the entire population over 72h in culture (Fig. 1, A and B). However, MCF-7 cells stably transduced with an expression vector encoding HER-2/neu (MCF-7-neu) were relatively resistant to the same concentration of TRAIL/Apo2L, with demise of only 27±4% of the cell population over the same time period (Fig. 1, A and B). Treatment of MCF-7-neu cells with trastuzumab (Herceptin™)(1 μM) increased sensitivity to TRAIL/Apo2L, such that the induction of apoptosis over 72h (51±5% of the cell population) approached that of MCF-7 cells treated with the same combination (62±3%) (Fig. 1, A and B). Akin to the effect of HER-2/neu overexpression, exposure to IGF-1 also inhibited TRAIL/Apo2L-induced apoptosis of MCF-7 cells, with death only 30±3 % of the cell population over 72h in culture (Fig. 1, A and B).

HER-2/neu or IGF-1 Protect Cells from TRAIL/Apo2L via PI3-K-dependent Activation of Akt. HER-2/neu and IGF-1R activate phosphoinositide-3-OH kinase [PI3-K], that, in turn, induces phosphorylation-mediated activation of the Akt serine/threonine kinase. MCF-7 cells exhibited low Akt kinase activity in immunoblot assays using a phospho-Akt kinase-specific antibody (Fig. 1C). Compared to MCF-7 cells, MCF-7-neu cells exhibited greater levels of Akt kinase activity which declined in response to treatment with trastuzumab (Fig. 1C). Activation of Akt in both IGF-1-stimulated MCF-7 cells and MCF-7-neu cells was blocked by the PI3-K inhibitor LY294002 (Fig. 1C). Treatment with LY294002 sensitized both MCF-7-neu and IGF-1-exposed MCF-7 cells to TRAIL/Apo2L, with death of more than 80% of either cell population over 48h (Fig. 1D).

To investigate the role of Akt in HER-2/neu- or IGF-1-mediated protection of cells from TRAIL/Apo2L, MCF-7 and MCF-7-neu cells were cotransfected with expression vectors encoding either wild-type Akt (HA-Akt) or a dominant negative catalytically inactive mutant of Akt [HA-Akt(K179M)] (10), together with an expression vector for β-galactosidase (CMV-β-Gal), and then maintained in the presence or absence of IGF-1. Following 48h of treatment with TRAIL/Apo2L, transfected cells (identified by immunostaining cells for β-Gal expression) were scored for apoptosis by analysis of nuclear morphology. MCF-7-neu cells transfected with HA-Akt showed only 19±4% apoptosis in response to TRAIL/Apo2L (Fig. 1D). In contrast, 69±5% of MCF-7-neu cells transfected with HA-Akt(K179M) exhibited apoptotic nuclear morphology following exposure to TRAIL/Apo2L (Fig. 1D). Likewise, IGF-1-stimulated MCF-7 cells transfected with HA-Akt(K179M) exhibited 65±5% apoptosis, while cells transfected with HA-Akt displayed only 23±3% in response to TRAIL/Apo2L (Fig. 1D). Consistent with Akt

functioning downstream of PI3-K, HA-Akt was able to inhibit TRAIL/Apo2L-induced apoptosis even in the presence of LY294002 (Fig. 1D). Taken together, these results indicate that HER-2/neu or IGF-1R inhibit TRAIL/Apo2L-induced death of breast cancer cells by activation of the PI3-K-Akt survival signaling pathway.

HER2/neu and IGF-1 Stimulate NF-κB via Akt-induced Activation of the IKK-NEMO Complex. Electrophoretic mobility shift assays (EMSA) showed greater NF-κB DNA-binding activity in nuclear extracts prepared from MCF-neu cells compared to MCF-7 cells (Fig. 2A). Likewise, NF-κB DNA-binding activity was increased in MCF-7 cells following treatment with IGF-1 (Fig. 2A). To determine whether activation of NF-κB by HER-2/neu or IGF-1 was mediated by Akt, MCF-7 and MCF-7-neu cells were cotransfected with expression vectors encoding either HA-Akt or HA-Akt(K179M) together with CMV-β-Gal, and then maintained in the presence or absence of IGF-1 for 36h before assessment of NF-κB DNA-binding activity. Ectopic expression of HA-Akt(K179M) reduced NF-κB activity in MCF-7-neu cells and prevented IGF-1-induced activation of NF-κB in MCF-7 cells (Fig. 2A). Conversely, MCF-7 cells (without IGF-1) cotransfected with HA-Akt exhibited increased NF-κB activity compared with cells transfected with the empty vector (Fig. 2A).

Signal-dependent activation of NF-κB requires phosphorylation and ubiquitin-mediated degradation of IκBα via the activity of the IκB-kinase (IKK) complex containing two kinases (IKK-α and IKK-β) and the regulatory protein NEMO (NF-κB essential modifier; IKK-γ) (11). *In vitro* phosphorylation assays of immunoprecipitated IKKβ using GST-IκBα as substrate showed greater IKKβ kinase activity in MCF-7-neu and IGF-1-treated MCF-7 cells compared to MCF-7 cells maintained without IGF-1 (Fig. 2B). An amino terminal α-helical region of NEMO interacts with six α_2-region residues in the carboxy terminal of IKKβ and IKKα, termed the NEMO-binding domain (NBD) (14). A cell permeable peptide spanning the IKKβ NBD (consisting of the region T735 to E745) fused with a sequence derived from the Antennapedia homeodomain for membrane translocation (wild-type NBD peptide; WT NBD) blocks the interaction of NEMO with the IKK complex and inhibits TNFα-induced NF-κB activation (14). A corresponding mutant NBD peptide (W739 and W741 mutated to alanines; MU NBD) does not disrupt signal-induced formation of the IKK-NEMO complex (14). To determine whether HER-2/neu- or IGF-1-induced activation of NF-κB requires formation of the IKK-NEMO complex, MCF-7 cells (maintained with or without IGF-1) or MCF-7-neu cells were incubated with either wild-type or mutant NBD peptides for 16h. Immune complex kinase assays using IKKβ immunoprecipitates showed that the wild-type NBD peptide, but not the mutant, reduced IKK activity in MCF-7-neu cells and prevented IGF-1-induced activation of IKK in MCF-7 cells (Fig. 2B). EMSA demonstrated that only the wild-type NBD peptide inhibited NF-κB DNA binding activity in MCF-7-neu or IGF-1-stimulated MCF-7 cells (Fig. 2C). These results indicate that HER-2/neu- or IGF-1-induced Akt-mediated activation of NF-κB can be prevented by disruption of the IKK-NEMO complex.

Blockade of the IKK-NEMO Interaction Sensitizes Breast Cancer Cells to TRAIL/Apo2L-induced Apoptosis. To determine whether inhibition of the interaction of NEMO with the IKK complex can sensitize HER-2/neu- or IGF-1-stimulated breast cancers to TRAIL/Apo2L-induced apoptosis, MCF-7 (in the presence IGF-1) and MCF-7-neu cells were exposed to TRAIL/Apo2L in the presence of either wild-type or mutant NBD peptide. MCF-7-neu cells treated with the wild-type NBD peptide alone resulted in death of 15±4% of the population. Exposure of MCF-7-neu cells to TRAIL/Apo2L in the presence of the wild-type NBD peptide resulted in death of 67±3% of the cell population over 48h (Fig. 3, A and B). In contrast, the mutant NBD peptide had no effect of the viability of MCF-7-neu cells and failed to sensitize cells to TRAIL/Apo2L-induced death (28±5%)(Fig. 3, A and B). Treatment with the wild-type peptide, but not the mutant, also sensitized IGF-1-stimulated MCF-7 cells to TRAIL/Apo2L, with death of 74±4% of the cell population over 48h (Fig. 3, A and B).

To confirm that inhibition of the IKK complex can augment TRAIL/Apo2L-induced death of breast cancer cells that have endogenous amplification and overexpression of the HER-2/neu gene, the SKBr-3 cell line was pretreated with either trastuzumab (Herceptin™) or NBD peptides, and then exposed to TRAIL/Apo2L. Treatment with TRAIL/Apo2L alone resulted in death of 23±5% of SKBr3 cells over 48h (Fig. 3C). Exposure of SKBr3 cells to trastuzumab alone resulted in death of only 13±4% of the cell population over 48h (Fig. 3C). However, pretreatment with trastuzumab sensitized SKBr-3 cells to TRAIL/Apo2L, with death of 46±4% of the cell population over 48h (Fig. 3C). Treatment of SKBr-3 cells with wild-type NBD peptide, but not the mutant peptide, resulted in marked enhancement of sensitivity to TRAIL/Apo2L-induced death. The extent of TRAIL/Apo2L-induced apoptosis in SKBr-3 cells treated with wild-type NBD (73±3%) was not only greater that in cells treated with the mutant peptide (28±4%), but also larger than the level observed in cells pretreated with trastuzumab (Fig. 3, A and C).

While trastuzumab can negate the protective effects of HER-2/neu overexpression, breast cancer cells may also inhibit TRAIL/Apo2L induced apoptosis by activation of NF-κB via diverse other growth factor receptors or genetic alterations (10, 13). The Hs578 breast cancer cell line does not overexpress HER-2/neu, yet exhibits constitutively high IKKβ kinase activity (13). Hs578 cells were extremely resistant to TRAIL/Apo2L, with death of only 7±3% of the population over 48h (Fig. 3C). Unlike SKBr-3 cells, Hs578 cells could not be sensitized to TRAIL/Apo2L-induced death by treatment with trastuzumab (Fig. 3C). To determine whether breast cancer cells with constitutively active IKK can be sensitized to TRAIL/Apo2L by inhibition of the IKK-NEMO complex, Hs578 cells were pretreated with either wild-type or mutant NBD peptide and then exposed to TRAIL/Apo2L. Treatment with the wild-type NBD peptide, but not the mutant peptide, sensitized Hs578 cells to TRAIL/Apo2L-induced apoptosis (Fig. 3, A and C).

Discussion:

TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins containing cytoplasmic sequences, termed "death domains", which recruit and cross-activate the initiator procaspase-8 (FLICE) (3). Caspase-8-mediated proteolysis of BID enables its translocation to the mitochondria, where the truncated protein (tBID) promotes the release of pro-death cofactors (cytochrome c and Apaf-1) via heterodimerization with BAX or BAK (15). The activation of caspase-9 by these mitochondrial cofactors is responsible for the terminal proteolytic events that lead to cell death. Although TRAIL/Apo2L offers a potential mechanism of inducing apoptosis in breast cancers that resist conventional genotoxic therapy, we have demonstrated that TRAIL/Apo2L-induced death signals are inhibited by NF-κB (12). NF-κB protects tumor cells from apoptosis by inducing expression of the caspase-8-inhibitor FLIP, members of the inhibitors-of-apoptosis (IAP) family, and Bcl-x_L, a pro-survival member of the Bcl-2 family that counteracts BAX (12, 16, 17).

Our results indicate that overexpression of HER-2/neu or exposure to IGF-1 protects breast cancer cells from TRAIL/Apo2L-induced death via activation of the PI3K-Akt-NF-κB anti-apoptotic pathway. In accordance with a recent report, we find that inhibition of HER-2/neu signaling by trastuzumab (Herceptin™) sensitizes HER-2/neu-overexpressing breast cancer cells to TRAIL/Apo2L-induced apoptosis (18). However, activation of NF-κB is a final common effect of multiple genetic aberrations [such as amplification of diverse receptor tyrosine kinases (HER-2/neu, IGF-1R, EGFR), mutations of *ras*, or loss of *PTEN*], that operate in human breast cancers (8, 9, 19, 20). Activation of IKK is a converging point for activation of NF-κB by diverse growth factor receptors and genetic alterations (11), and a recent report showed that IKK is aberrantly activated in a high proportion of primary human breast cancers (13). Our results indicate that NBD peptides that interfere with the interaction of NEMO with the IKK complex can block the activation of NF-κB and sensitize breast cancers to TRAIL/Apo2L-induced apoptosis. These findings suggest that either antagonistic antibodies against growth factor receptors (HER-2/neu and IGF-1R) and/or peptidomimetic drugs that disrupt the IKK-NEMO complex may improve the efficacy of TRAIL/Apo2L in the treatment of human breast cancers.

KEY RESEARCH ACCOMPLISHMENTS:

- Our observations indicate that loss of p53 function, via somatic mutations or expression of viral oncoproteins, contributes to activation of the angiogenic switch and promotes tumor growth.
- Our studies define a novel mechanism by which p53 regulates the angiogenic switch; p53 inhibits hypoxia-induced expression of HIF-1α by facilitating its ubiquitination and subsequent degradation.
- Our findings suggest that amplification of HIF-1 activity resulting from loss of p53 function may contribute to the overexpression of VEGF that is observed in a wide variety of human cancers.
- Our results indicate that NF-κB/RelA is required for hypoxia-induced expression of Bcl-x_L and protection of cells from hypoxia- and death receptor-induced apoptosis.
- Our findings indicate that activation of NF-κB by HER-2/neu or insulin-like growth factor-1 (IGF-1) renders breast cancer cells relatively resistant to TRAIL/Apo2L-induced apoptosis. Conversely, breast cancer cells can be sensitized to TRAIL/Apo2L-induced death by antibody-mediated inhibition of growth factor receptors (HER2/neu or IGF-1R) and/or peptidomimetic drugs that disrupt the IKK-NEMO complex.

REPORTABLE OUTCOMES:

Manuscripts/ Abstracts/ Presentations:

We have completed the studies proposed in specific aims 1, 2, and part of 3 (Tasks 1,2, and 3a) and have reported the results and conclusions in:

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development* 14:34-44, 2000. (*Appendix 1-Reprint of publication enclosed*).

These findings were presented (abstract & poster) at the AACR-NCI-EORTC Meeting in Washington, D.C., 1999.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Abstract & Presentation at AACR-NCI-EORTC Meeting*, Washington, D.C., Nov. 1999.

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* 3:409-416, (2001). (*Appendix 2-Reprint of publication enclosed*)

Ravi, R. and Bedi, A. Sensitization of Breast Cancers to TRAIL/Apo2L-induced Apoptosis by Inhibition of the IκB Kinase-NEMO Complex. Manuscript Submitted, 2001. (*Appendix 3-Submitted Manuscript enclosed*)

CONCLUSIONS:

Importance of completed research:

There are two major impediments to the successful treatment of breast cancer. First, surgical extirpation of the primary neoplasm is often followed by the occurrence of metastatic tumors. Second, overt metastases are resistant to conventional chemo- or radio-therapy. Therefore, successful treatment is contingent upon identifying strategies to prevent metastases or eliminate tumor cells that have acquired genetic aberrations that confer resistance to cytotoxic agents. Our results indicate that amplification of HIF-1 activity resulting from loss of p53 function may contribute to the angiogenic phenotype of human cancers. Conversely, the inhibition of HIF-1 may provide a therapeutic strategy to curtail the tumor growth and progression. We have also determined that activation of NF-κB promotes expression of Bcl-x_L protects cells from hypoxia- and death receptor-induced apoptosis. Our findings indicate that activation of NF-κB by HER-2/neu or insulin-like growth factor-1 (IGF-1) renders breast cancer cells relatively resistant to TRAIL/Apo2L-induced apoptosis. Conversely, breast cancer cells can be sensitized to hypoxia- or TRAIL/Apo2L-induced death by peptidomimetic drugs that disrupt the IKK-NEMO complex. Together, our findings provide a scientific foundation for targeting HIF-1 and NF-κB to overcome the hypoxia-resistant angiogenic phenotype of human breast cancers.

Implications and practical applications of completed research:

Strategies to inhibit angiogenesis have hitherto focused upon inhibition of individual angiogenic factors/receptors or suppression of endothelial cell proliferation. Unlike these approaches which target downstream mediators of angiogenesis, strategies that target the proximal transcriptional mediators of angiogenesis and cell survival (HIF-1 or NF-κB) would be expected to inhibit an entire panel of synergizing factors. As such, it may be more potent and less susceptible to evasion by genetically pliable tumor cells that could evolve mechanisms of resistance against any individual factor. By demonstrating that deregulation of HIF-1 contributes to the increased expression of VEGF in p53-deficient cancers, our data provide further support for the hypothesis that inhibition of HIF-1 may abrogate the ability of such tumors to establish an adequate vascular supply and adapt their cellular metabolism to hypoxia, thereby curtailing their growth and metastasis. By identifying NF-κB as a key determinant of tumor cell survival, our studies suggest that inhibition of the IKK complex by NSAIDs or peptidomimetic drugs that target the IKK-NEMO interaction may be used to potentiate hypoxia- or TRAIL/Apo2L-induced death of breast cancer cells.

Future Studies:

As defined in the research proposal and approved statement of work, we have initiated studies devoted to the *in vivo* studies described in specific aim 3 (Tasks 3b,c) of the research project.

REFERENCES:

The references pertinent to the report are listed in the appended publications and manuscript (Appendices 1,2,3).

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development* 14:34-44, 2000. (*Appendix 1-Reprint of publication enclosed*).

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* **3**:409-416, (2001). (*Appendix 2-Reprint of publication enclosed*)

Ravi, R. and Bedi, A. Sensitization of Breast Cancers to TRAIL/Apo2L-induced Apoptosis by Inhibition of the IκB Kinase-NEMO Complex. Manuscript Submitted, 2001. (*Appendix 3-Submitted Manuscript enclosed*)

APPENDICES:

Appendix 1:

Reprint of publication: (Page Numbers 13-23)

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes & Development 14:34-44, 2000.

Appendix 2:

Reprint of publication: (Page Numbers 24-32)

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* **3**:409-416, (2001).

Appendix 3:

Submitted Manuscript: (Page Numbers 33-48)

Ravi, R. and Bedi, A. Sensitization of Breast Cancers to TRAIL/Apo2L-induced Apoptosis by Inhibition of the IκB Kinase-NEMO Complex. Manuscript Submitted, 2001.

Appendix 4:

Unpublished Data: (Page 49-50)

Figure 1

Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α

Rajani Ravi,[1] Bijoyesh Mookerjee,[1] Zaver M. Bhujwalla,[2] Carrie Hayes Sutter,[3] Dmitri Artemov,[2] Qinwen Zeng,[1] Larry E. Dillehay,[1] Ashima Madan,[4] Gregg L. Semenza,[3,5] and Atul Bedi[1]

[1]Johns Hopkins Oncology Center, [2]Department of Radiology, and [3]Institute of Genetic Medicine, Departments of Pediatrics and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland, 21287 USA; [4]Department of Pediatrics, Stanford University School of Medicine, Palo Alto, California, 94305 USA

The switch to an angiogenic phenotype is a fundamental determinant of neoplastic growth and tumor progression. We demonstrate that homozygous deletion of the *p53* tumor suppressor gene via homologous recombination in a human cancer cell line promotes the neovascularization and growth of tumor xenografts in nude mice. We find that p53 promotes Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α subunit of hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that regulates cellular energy metabolism and angiogenesis in response to oxygen deprivation. Loss of *p53* in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activation of the vascular endothelial growth factor (*VEGF*) gene in response to hypoxia. Forced expression of HIF-1α in p53-expressing tumor cells increases hypoxia-induced *VEGF* expression and augments neovascularization and growth of tumor xenografts. These results indicate that amplification of normal HIF-1-dependent responses to hypoxia via loss of p53 function contributes to the angiogenic switch during tumorigenesis.

[*Key Words*: p53; hypoxia-inducible factor-1 (HIF-1); angiogenesis; vascular endothelial growth factor (VEGF); hypoxia; cancer]

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Regions of vascular deficiency or defective microcirculation in growing tumors are deprived of O_2, glucose, and other nutrients. Apoptosis induced by nutrient deficiency counterbalances cell proliferation and limits tumor growth (Holmgren et al. 1995; O'Reilly et al. 1996; Parangi et al. 1996). Clonal evolution of tumor cells in this hypoxic microenvironment results from selection of subpopulations that not only resist apoptosis (Graeber et al. 1996) but also promote the formation of new blood vessels (for review, see Hanahan and Folkman 1996; Folkman 1997). In addition to promoting further growth of the primary tumor, cellular adaptation to hypoxia and tumor neovascularization strongly correlate with the risk of invasion and metastasis (Brown and Giaccia 1998; Dang and Semenza 1999; for review, see Folkman 1997). The switch to an angiogenic phenotype is considered to be a fundamental determinant of neoplastic progression (Gimbrone et al. 1972; Folkman et al. 1989; Bergers et al. 1999). This realization has, in turn, fueled an intense search for the molecular mechanisms by which the angiogenic switch is activated during tumorigenesis.

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Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that regulates O_2 homeostasis and physiologic responses to O_2 deprivation (for review, see Guillemin and Krasnow 1997; Semenza 1999). HIF-1 consists of two subunits, HIF-1α and HIF-1β, that belong to a subfamily of basic helix-loop-helix (bHLH) transcription factors containing a PAS (Per-ARNT-Sim) motif (Wang et al. 1995). A decrease in cellular O_2 tension leads to elevation of HIF-1 activity via stabilization of the HIF-1α protein; conversely, ubiquitin-mediated proteolysis of HIF-1α on reexposure to a normoxic environment results in rapid decay of HIF-1 activity (Semenza and Wang 1992; Wang et al. 1995; Salceda and Caro 1997; Huang et al. 1998; Kallio et al. 1999). The binding of HIF-1α, in conjunction with its dimerization partner HIF-1β, to DNA (consensus binding sequence, 5′-RCGTG-3′) leads to the transcriptional activation of genes that mediate anaerobic metabolism (glucose transporters and glycolytic enzymes), O_2-carrying capacity (erythropoietin, transferrin), and vasodilatation (inducible nitric oxide synthase and heme oxygenase-1) (for review, see Guillemin and Krasnow 1997; Semenza 1999). HIF-1 also binds to the 5′ flanking sequence of the vascular endothelial growth factor (*VEGF*) gene and is required for transactivation of *VEGF* in response to hypoxia (Forsythe

et al. 1996; Carmeliet et al. 1998; Iyer et al. 1998; Ryan et al. 1998). The binding of VEGF to the receptor tyrosine kinases flk1/KDR, flt-1, and flt-4 (VEGFR-1–VEGFR-3) on vascular endothelial cells promotes their proliferation and leads to vessel formation (for review, see Ferrara 1993; Risau and Flamme 1995; Brown et al. 1996). In contrast to wild-type cells, *VEGF* gene expression is not induced by hypoxia in HIF-1α-deficient embryonic stem cells, and dramatic vascular regression occurs in HIF-1α-null mouse embryos (Iyer et al. 1998; Kotch et al. 1999). Therefore, HIF-1 is a key transcriptional mediator of metabolic adaptation and VEGF-mediated angiogenesis in response to hypoxia. Although these responses serve to maintain O_2 homeostasis in normal tissues, they are also co-opted by tumors to facilitate neovascularization and growth. Akin to their role in vascular development and remodeling in normal tissues, HIF-1α (Maxwell et al. 1997; Carmeliet et al. 1998; Ryan et al. 1998) and VEGF (Plate et al. 1992; Shweiki et al. 1992; Kim et al. 1993; Millauer et al. 1994) facilitate tumor angiogenesis, and both HIF-1α (Zhong et al. 1999) and VEGF (for review, see Folkman 1997) are overexpressed in a wide variety of human cancers.

The genetic alterations that are responsible for oncogenesis and tumor progression may also underlie the ability of tumors to switch to an angiogenic phenotype. The human *p53* tumor suppressor gene encodes a multifunctional transcription factor that mediates cellular responses to diverse stimuli, including DNA damage and hypoxia (for review, see Giaccia and Kastan 1998). In addition to being an integral component of the surveillance mechanisms that arrest cell cycle progression under adverse conditions, p53 is also involved in mediating hypoxia-induced apoptosis (Graeber et al. 1996) and inducing inhibitors of angiogenesis such as thrombospondin-1 (Dameron et al. 1994; Van Meir et al. 1994). Evidence also suggests that p53 negatively regulates *VEGF* expression (Mukhopadhyay et al. 1995; Bouvet et al. 1998; Fontanini et al. 1998). Somatic mutations of the *p53* gene represent one of the most common genetic alterations in human cancers, and the acquisition of such defects is strongly associated with tumor progression and metastasis (for review, see Levine 1997).

In this study, we demonstrate that genetic inactivation of p53 in cancer cells provides a potent stimulus for tumor angiogenesis and identify a novel mechanism by which loss of*p53* function contributes to activation of the angiogenic switch in tumors. We find that homozygous deletion of p53 via homologous recombination in human colon cancer cells promotes the neovascularization and growth of tumor xenografts in nude mice. We show that p53 inhibits HIF-1 activity by targeting the HIF-1α subunit for Mdm2-mediated ubiquitination and proteasomal degradation. Conversely, the loss of p53 enhances hypoxia-induced HIF-1α levels and augments HIF-1-dependent expression of VEGF in tumor cells. We further demonstrate that forced expression of HIF-1α in p53-expressing tumor cells promotes *VEGF* expression and neovascularization of tumor xenografts. These findings indicate that inactivation of p53 in tumor cells contributes to activation of the angiogenic switch via amplification of normal HIF-1-dependent responses to hypoxia.

Results

Inhibition of tumor angiogenesis and growth by p53

The effect of p53 on tumor cell growth and angiogenesis was examined by comparing an isogenic set of human colon adenocarcinoma cell lines differing only in their p53 status (Bunz et al. 1998). The parental HCT116 line, containing wild-type p53 ($p53^{+/+}$), and a p53-deficient derivative ($p53^{-/-}$), generated by homologous recombination, demonstrated equivalent growth kinetics in tissue culture, with doubling times of 29 and 32 hr, respectively (Fig. 1A). However, xenografts (2.5×10^4–2.5×10^5 cells) of $p53^{-/-}$ HCT116 cells in athymic BALB/c (nu/nu) mice exhibited a significantly shorter latency and marked increase in tumor growth kinetics compared with their $p53^{+/+}$ counterparts (Fig. 1B,C). Whereas 12/12 animals inoculated with 2.5×10^4 $p53^{-/-}$ cells developed tumors within 3 weeks, only 1/12 mice receiving the same number of $p53^{+/+}$ cells was able to establish a tumor during the entire 8-week observation period. To examine whether the observed differences in growth kinetics in vivo were associated with variation in tumor vascularity, tumors established from $p53^{+/+}$ and $p53^{-/-}$ cells were subjected to histologic analysis and nuclear magnetic resonance (NMR) imaging. Immunohistochemical analyses of tumor sections using an antibody against von Willebrand Factor (vWF) demonstrated significantly increased blood vessel density in $p53^{-/-}$ tumors compared with their $p53^{+/+}$ counterparts (Fig. 1D,E). Analyses of neovascularization by NMR imaging showed that compared with $p53^{+/+}$ tumors, $p53^{-/-}$ tumors had a higher vascular volume (14 ± 2.6 μl/g vs. 8.4 ± 2.4 μl/g in highly permeable regions), as well as a threefold greater vascular permeability (0.4 ± 0.18 μl/g/min vs. 0.13 ± 0.04 μl/g/min in highly vascular zones) (Fig. 1F). Thus, loss of p53 function has a profound effect on the neovascularization and growth of human colorectal cancer xenografts in nude mice.

Effect of p53 genotype on hypoxia-induced VEGF *expression and HIF-1 activity*

Hypoxia-induced, HIF-1-mediated expression of VEGF stimulates angiogenesis and vascular permeability in neoplastic tissues (Plate et al. 1992; Shweiki et al. 1992; Forsythe et al. 1996; Maxwell et al. 1997; Carmeliet et al. 1998). $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells were analyzed for expression of VEGF mRNA and protein under tissue culture conditions simulating the hypoxic tumor microenvironment. Following exposure to 1% O_2, $p53^{-/-}$ cells exhibited a greater induction of VEGF mRNA and protein compared with their $p53^{+/+}$ counterparts (Fig. 2A,B). Transcriptional activation of the *VEGF* gene in response to hypoxia is mediated by binding of HIF-1 to a 47-bp

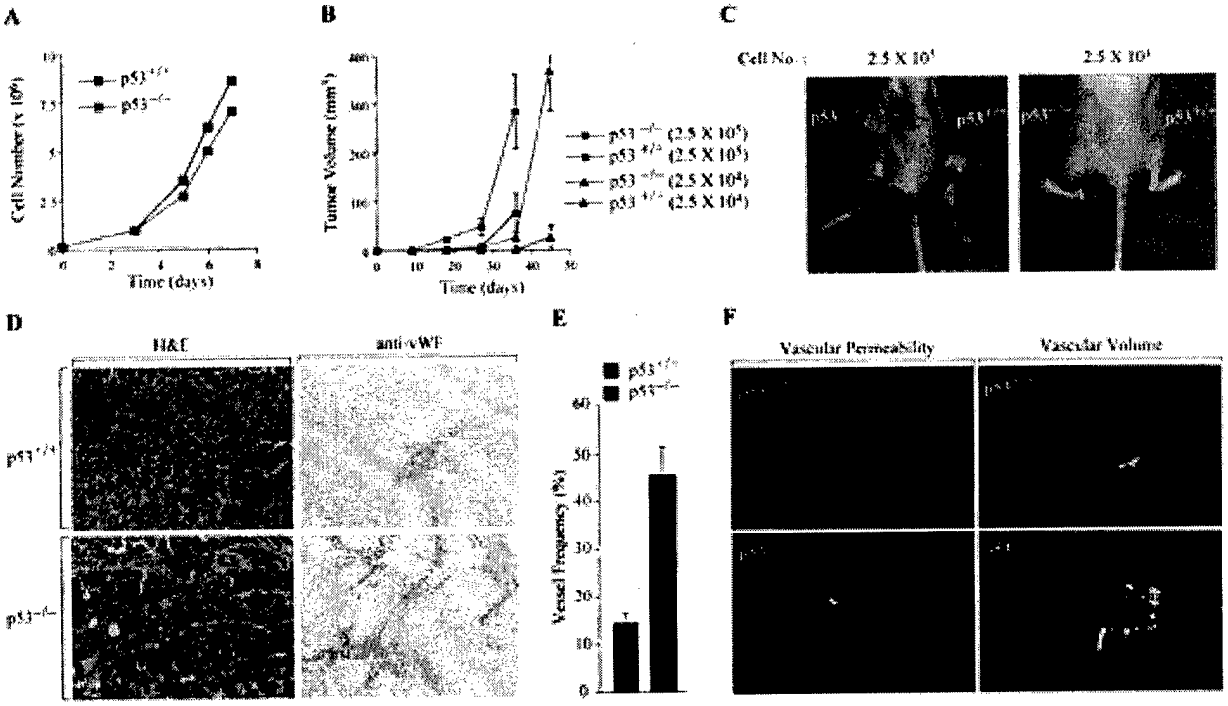

Figure 1. Effect of p53 genotype on tumor growth and angiogenesis. (*A*) Growth of $p53^{+/+}$ (blue) and $p53^{-/-}$ (red) HCT116 cells cultured in DMEM supplemented with 10% fetal calf serum at 37°C and 95%air/5%CO_2. (*B, C*) Growth of $p53^{+/+}$ (blue) and $p53^{-/-}$ (red) HCT116 xenografts [2.5×10^4 (▲) or 2.5×10^5 (■) cells] injected subcutaneously into right ($p53^{+/+}$) or left ($p53^{-/-}$) hind legs of athymic BALB/c (nu/nu) mice. Values expressed represent mean ± S.E. of 12 xenografts of each cell type. (*D*) Histologic analysis of blood vessels in $p53^{+/+}$ and $p53^{-/-}$ HCT116 xenograft tumors by staining with H&E or immunoperoxidase detection of endothelial cells using an anti-vWF antibody (×25). (*E*) Quantification of blood vessel density in $p53^{+/+}$ (blue) and $p53^{-/-}$ (red) xenografts. The data represent the mean ± S.E. of the frequency of vessel hits among 300 random sampling points from each of three tumors of either genotype. (*F*) Representative NMR analysis of in vivo vascular volume (*right*) and permeability (*left*) of $p53^{+/+}$ and $p53^{-/-}$ (*bottom*) HCT116 xenografts.

hypoxia-response element in the 5′ flanking region, and a reporter plasmid containing this sequence (VEGF-p11w) is transactivated by cotransfection of an expression vector encoding HIF-1α (pCEP4/HIF-1α) (Forsythe et al. 1996). To examine whether p53 influences HIF-1-mediated transcriptional activation of VEGF, $p53^{+/+}$ and $p53^{-/-}$ cells were cotransfected with the VEGF-p11w reporter and CMVβgal [encoding β-galactosidase (β-gal)]. Analyses of luciferase and β-gal activity in response to hypoxia (1% O_2) revealed a fourfold greater increase in VEGF-p11w transcription (relative to β-gal) in $p53^{-/-}$ cells compared with $p53^{+/+}$ cells (Fig. 2C). These differences were not seen when the reporter contained a 3-bp substitution in the hypoxia response element that eliminated HIF-1 binding (VEGF-p11m), suggesting that HIF-1 was a target for p53-mediated inhibition. Coexpression of pCEP4/HIF-1α in $p53^{+/+}$ cells increased hypoxia-induced activation of VEGF-p11w to levels that approached the reporter activity exhibited by hypoxic $p53^{-/-}$ cells in the absence of exogenous HIF-1α (Fig. 2C). Conversely, cotransfection of an expression vector encoding wild-type human p53 into $p53^{-/-}$ cells completely repressed hypoxia-induced VEGF-p11w expression (Fig. 2C). Electrophoretic mobility shift assays demonstrated that hypoxia-induced HIF-1 DNA-binding activity was reduced in $p53^{+/+}$ cells compared with $p53^{-/-}$ cells (Fig. 2D). The specificity of binding of HIF-1 to DNA was confirmed by competing hypoxia-induced DNA–protein complexes with excess unlabeled wild-type probe but not with an unlabeled mutant probe containing the same 3-bp substitution in the HIF-1 binding site as in reporter VEGF-p11m. Thus, p53 inhibits HIF-1 activity and *VEGF* expression in response to hypoxia.

Effect of p53 on oxygen-regulated expression and stability of HIF-1α

Hypoxia-induced HIF-1 DNA-binding and transcriptional activity are dependent on increased levels of HIF-1α protein and its heterodimerization with HIF-1β (Wang and Semenza 1993; Wang et al. 1995; Jiang et al. 1996; Huang et al. 1998). To investigate whether p53 influences HIF-1 activity by altering expression of HIF-1α, the levels of HIF-1α protein and mRNA were assessed in $p53^{+/+}$ and $p53^{-/-}$ cells exposed to either 20% or 1% O_2. In response to hypoxia, $p53^{-/-}$ HCT116 cells or mouse embryonic fibroblasts (MEFs) expressed higher levels of HIF-1α protein compared with their $p53^{+/+}$ counterparts (Fig. 3A,B). In contrast to HIF-1α protein levels, HIF-1α mRNA was expressed at equivalent levels

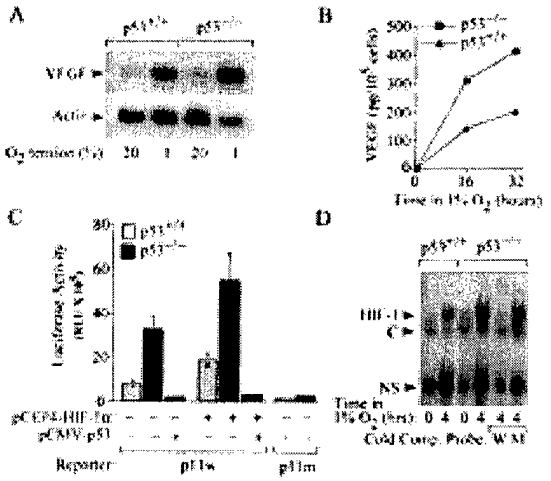

Figure 2. Effect of p53 genotype on hypoxia-induced *VEGF* expression and HIF-1 activity. (*A*) Northern blot analysis of VEGF mRNA expression in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells incubated for 16 hr in either 20%or 1%O_2. (*B*) ELISA of VEGF protein concentration in supernatant medium of $p53^{+/+}$ (blue ▲) or $p53^{-/-}$ (red ■) HCT116 cells incubated for 16–32 hr in 1% O_2. (*C*) Hypoxia-induced and HIF-1-dependent activation of VEGF-reporter activity in $p53^{+/+}$ (shaded bars) and $p53^{-/-}$ (solid bars) HCT116 cells. Wild-type (p11w) and mutant (p11m) copies of the hypoxia response element from the *VEGF* gene were inserted 5′ to a SV40 promoter–luciferase transcription unit. Cells were cotransfected with either VEGF–p11w or VEGF–p11m and CMVβgal, with or without pCEP4/HIF-1α or pCMV–p53, exposed to 1% O_2 for 20 hr, and harvested for luciferase assays. The data represent the mean ± S.E. luciferase activity (normalized for β-gal activity) from three independent experiments. (*D*) Electrophoretic mobility shift assays of HIF-1 DNA-binding activity in nuclear extracts from $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells exposed to 20% (lanes *1* and *3*) or 1% (lanes *2* and *4–6*) O_2. HIF-1 DNA binding was confirmed by competition assays using either unlabeled wild-type oligonucleotide (W) or a mutant oligonucleotide (M) containing the same 3-bp substitution as in p11m. Complexes containing HIF-1, constitutive (C), and nonspecific (NS) DNA-binding activities (Semenza and Wang 1992) are indicated.

in hypoxic $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 3C), suggesting an effect of p53 on HIF-1α protein expression. To confirm this effect, $p53^{-/-}$ cells were cotransfected with pCEP4-HIF-1α and either pCMV-p53 (encoding wild-type human p53) or empty vector (pCMV0) and exposed to 1% O_2 for 8 hr. Immunoblot analysis showed that $p53^{-/-}$ cells cotransfected with pCMV-p53 exhibited reduced levels of HIF-1α compared with cells receiving the control vector (Fig. 3D).

The steady state level of HIF-1α protein is regulated by an oxygen-dependent and iron-sensitive mechanism of ubiquitin-mediated proteasomal degradation (Salceda and Caro 1997; Huang et al. 1998; Kallio et al. 1999). The 20S proteasome is the core catalytic subunit of the 26S proteasome complex that mediates degradation of ubiquitin-tagged proteins (for review, see Hershko and Ciechanover 1998). HIF-1α expression is induced by exposure to hypoxia or treatment with cobalt chloride (Wang et al. 1995). To examine whether p53 influences the stability of HIF-1α protein, HIF-1α expression was analyzed in lysates of cobalt-treated $p53^{+/+}$ and $p53^{-/-}$ cells at serial time intervals following addition of cycloheximide. HIF-1α protein decayed with a half-life of <20 min in $p53^{+/+}$ cells, compared with >40 min in $p53^{-/-}$ cells (Fig. 3E).

HPV-E6 augments HIF-1α stability and VEGF *expression in response to hypoxia*

The human papilloma virus (HPV16) E6 oncoprotein promotes ubiquitin-dependent conjugation and degradation of p53 (Scheffner et al. 1990). To investigate whether E6-induced degradation of endogenous p53 promotes expression of HIF-1α and induction of VEGF, the PA-1 ovarian teratocarcinoma cell line was stably transfected with an expression vector encoding HPV-16 E6 (PA-1 E6) or empty vector (PA-1 Neo) (Ravi et al. 1998). Under hypoxic conditions, PA-1 E6 cells expressed higher levels of HIF-1α protein compared with PA-1 Neo cells (Fig. 4A). Analyses of HIF-1α protein stability in cycloheximide-treated cells showed that HIF-1α protein decayed with a half-life of ~15 min in PA-1 cells, compared with >30 min in PA-1 E6 cells (Fig. 4B). PA-1 Neo or PA-1 E6 cells were cotransfected with either VEGF-p11w or VEGF-p11m reporter and CMVβgal. Analyses of luciferase and β-gal activity in response to hypoxia (1% O_2) revealed a twofold greater increase in VEGF-p11w transcription (relative to β-gal) in PA-1 E6 cells compared

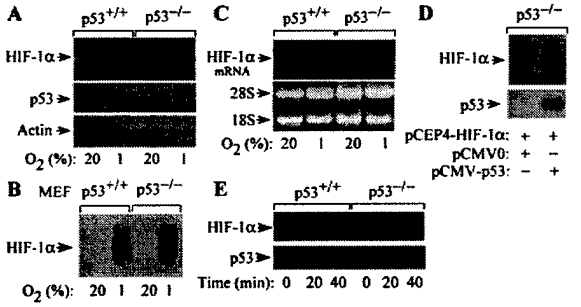

Figure 3. Effect of p53 on oxygen-regulated expression and stability of HIF-1α. (*A*) Immunoblot analysis of HIF-1α expression in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells cultured for 8 hr in 20%or 1%O_2. The blot was analyzed sequentially with monoclonal antibodies against HIF-1α (H1α67), p53 (DO-1), and β-actin. (*B*) Immunoblot analysis of HIF-1α expression in $p53^{+/+}$ and $p53^{-/-}$ MEFs cultured for 8 hr in 20% or 1% O_2. (*C*) Northern blot analysis of HIF-1α mRNA expression in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells cultured as in *A*. (*D*) Immunoblot analysis of HIF-1α protein in $p53^{-/-}$ HCT116 cells cultured in 1% O_2 for 8 hr following cotransfection with pCEP4–HIF-1α and either pCMV–p53 or empty vector. The blot was analyzed sequentially with anti-HIF-1α and anti-p53 monoclonal antibodies. (*E*) Half-life of HIF-1α protein in $p53^{+/+}$ and $p53^{-/-}$ cells exposed to 100 μM cobalt chloride following addition of 100 μM cycloheximide. Lysates of cells harvested at the indicated time intervals were subject to immunoblot analysis of HIF-1α and p53 expression.

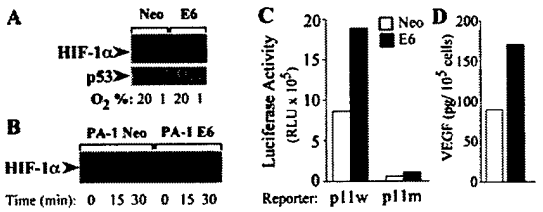

Figure 4. HPV E6 increases expression of HIF-1α and VEGF in response to hypoxia. (*A*) Immunoblot analysis of HIF-1α expression in PA-1 Neo or PA-1 E6 cells cultured for 8 hr in 20% or 1% O_2. (*B*) Half-life of HIF-1α protein in PA-1 Neo or PA-1 E6 cells exposed to 100 μM cobalt chloride following addition of 100 μM cycloheximide. Lysates of cells harvested at the indicated time intervals were subject to immunoblot analysis of HIF-1α expression. (*C*) Hypoxia-induced and HIF-1-dependent activation of VEGF-reporter activity in PA-1 Neo (open bars) and PA-1 E6 (solid bars) cells. Cells were cotransfected with either VEGF–p11w or VEGF–p11m and CMVβgal, exposed to 1% O_2 for 20 hr, and harvested for luciferase assays. The data represent the mean luciferase activity (normalized for β-gal activity) from three independent experiments. (*D*) ELISA of VEGF protein concentration in supernatant medium of PA-1 Neo (open bar) or PA-1 E6 (solid bar) cells incubated for 16 hr in 1% O_2.

with PA-1 Neo cells (Fig. 4C). Neither cell line exhibited significant transcription of the VEGF-p11m reporter. Consistent with the promotion of HIF-1-dependent VEGF transcription by E6 expression, exposure to 1% O_2 resulted in greater induction of VEGF protein expression in PA-1 E6 cells compared with PA-1 Neo cells (Fig. 4D).

p53 promotes ubiquitin-dependent of HIF-1α

To determine whether p53 interacts with HIF-1α in HCT116 cells, as previously demonstrated in MCF-7 cells (An et al. 1998), protein lysates from hypoxic $p53^{+/+}$ and $p53^{-/-}$ cells were immunoprecipitated with an anti-p53 or isotype control antibody, and the resulting immune complexes were subjected to immunoblot assays using an antibody against HIF-1α. HIF-1α was detected in immunoprecipitates derived from $p53^{+/+}$ cells but not $p53^{-/-}$ cells or immune complexes precipitated with the control antibody (Fig. 5A).

To determine whether p53 promotes ubiquitination of HIF-1α, $p53^{+/+}$ and $p53^{-/-}$ cells were cotransfected with an HIF-1α expression vector (pCEP4/HIF-1α) and a vector encoding hexahistidine-tagged ubiquitin (His_6-Ub) or the empty control vectors. Transfected cells were exposed to 1% O_2 for 4 hr in the presence of MG132, a peptide aldehyde inhibitor of the 20S proteasome. Aliquots of whole-cell extracts or His-tagged proteins isolated by affinity purification from cell lysates were subjected to immunoblot assays using an anti-HIF-1α monoclonal antibody (Fig. 5B). Immunoblot analysis of whole cell extracts of $p53^{+/+}$ cells detected a 120-kD protein corresponding to the apparent molecular mass of HIF-1α (Wang et al. 1995), as well as an additional series of slower migrating complexes. The higher molecular weight complexes represented polyubiquitinated forms of HIF-1α as they were also detected by immunoblot analysis of His-tagged proteins with an anti-HIF-1α monoclonal antibody. Compared with $p53^{+/+}$ cells, $p53^{-/-}$ cells transfected with vectors encoding HIF-1α and His_6-Ub demonstrated a higher level of unconjugated HIF-1α and a reciprocal reduction in polyubiquitinated HIF-1α (Fig. 5B). Introduction of a p53 expression vector (pCMV–p53) into $p53^{-/-}$ cells increased the proportion of HIF-1α that was ubiquitinated under hypoxic conditions (Fig. 5B).

Conjugation of Ub to proteins destined for degradation involves conversion of Ub to a high-energy thiol ester by the E1 Ub-activating enzyme followed by the transfer of activated Ub to the substrate via the activity of an E2 Ub-conjugating enzyme and an E3 Ub–protein ligase (for review, see Hershko and Ciechanover 1998). To confirm the requirement of the Ub-proteasome system for p53-mediated degradation of HIF-1α, we examined the effect of p53 on hypoxia-induced HIF-1α expression in the BALB/c 3T3-derived $ts20TG^R$ cell line, which harbors a thermolabile E1, or a derivative cell line (H38-5), in which the temperature-sensitive defect was corrected by introduction of the human E1 cDNA (Chowdary et al. 1994). $ts20TG^R$ and H38-5 cells were transfected with either an expression vector encoding human p53 or a control vector and transferred to hypoxic chambers (1% O_2) at either the permissive temperature (35°C) or the restrictive temperature (39°C). Transfection of p53 into $ts20TG^R$ cells resulted in reduced HIF-1α levels at 35°C but not at 39°C (Fig. 5C). However, E1-expressing H38-5 cells exhibited p53-mediated reduction of HIF-1α levels at both temperatures. Taken together, the data indicate that p53 limits hypoxia-induced expression of HIF-1α by promoting its ubiquitination and proteasomal degradation.

Whereas a single E1 is responsible for activation of ubiquitin, multiple E3 enzymes are responsible for specific selection of proteins destined for degradation. Because p53 induces the Mdm2 E3 Ub–protein ligase and is itself a target for Ub-mediated degradation via its interaction with Mdm2 (Momand et al. 1992; Barak et al. 1993; Wu et al. 1993; Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997), this raised the possibility that HIF-1α is recruited to Mdm2 via its interaction with p53. To test this hypothesis, protein lysates of $p53^{-/-}$ HCT116 cells that were transfected with either pCMV–p53 or empty vector and transferred to 1%O_2 for 6 hr were immunoprecipitated with anti-Mdm2 or isotype control antibody, and the resulting immune complexes were subjected to immunoblot assays using an antibody against HIF-1α. Anti-Mdm2 immunoprecipitates derived from cells transfected with p53 displayed significantly higher levels of coprecipitated HIF-1α protein compared to immune complexes derived from $p53^{-/-}$ HCT116 cells with the empty vector (Fig. 5D).

Amino acid residues Phe-19, Leu-22, and Trp-23 in the amino-terminal transactivation domain of p53 are critical for its interaction with Mdm2 (Lin et al. 1994). A p53 double mutant at residues 22 and 23 (p53 Gln22, Ser23) fails to interact with Mdm2 and is also transactivation

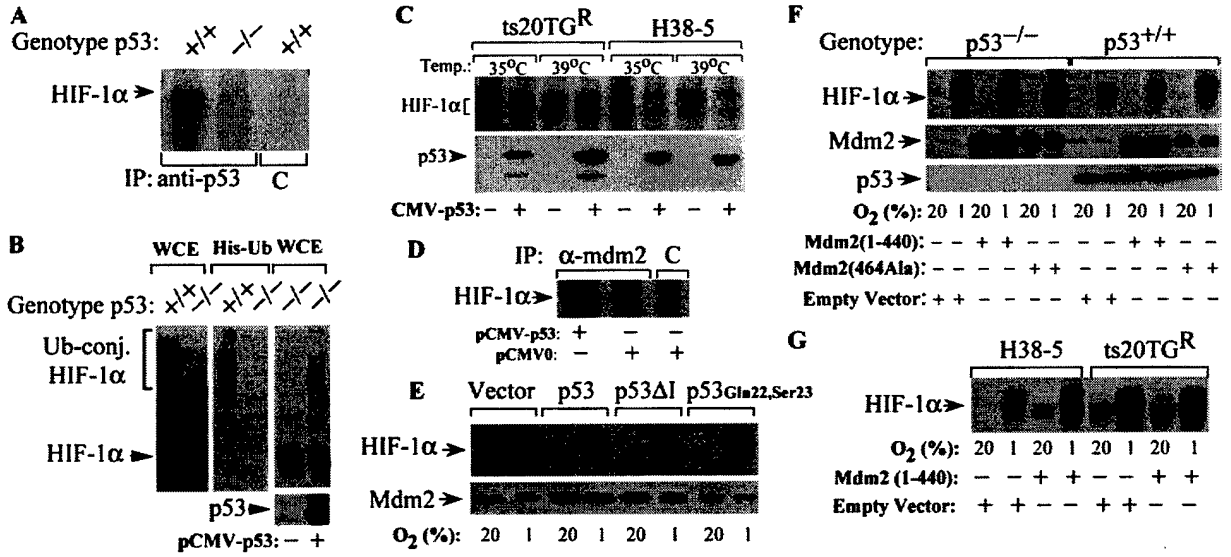

Figure 5. Effect of p53 expression on ubiquitin-mediated degradation of HIF-1α. (*A*) Interaction of p53 with HIF-1α. Lysates of $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells exposed to 1%O_2 for 8 hr were immunoprecipitated with either anti-p53 antibody or isotype control antibody (*C*) and the resultant immune complexes were subjected to immunoblot analysis with anti-HIF-1α monoclonal antibody. (*B*) Differential ubiquitination of HIF-1α in hypoxic $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. Cells were cotransfected with pCMVβgal and pCEP4/HIF-1α with either MT107/His_6-Ub or empty vector (MT107), and cultured in 1%O_2 for 4 hr in the presence of 50 μM MG132. Aliquots of whole-cell extract (WCE) or His-tagged proteins purified from whole-cell lysates (His-Ub) were subjected to immunoblot analysis with anti-HIF-1β antibody. (*C*) Effect of p53 expression on HIF-1α protein levels in hypoxic $ts20TG_R$ and H38-5 cells. Cells transfected with pCMV–p53 or pCMVβgal were maintained at either 35°C or 39°C for 8 hr and exposed to 1%O_2 for an additional 8 hr at their respective temperatures. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α or anti-p53 antibodies. (*D*) Effect of p53 on complex formation between HIF-1α and Mdm2. Lysates of $p53^{-/-}$ HCT116 cells transfected with either pCMV–p53 or empty vector and transferred to 1%O_2 for 6 hr were immunoprecipitated with anti-Mdm2 or isotype control antibody, and the resulting immune complexes were subjected to immunoblot assays using an antibody against HIF-1α. (*E*) Effect of wild-type p53, p53ΔI, or p53Gln22,Ser23 on expression of HIF-1α in response to hypoxia. $p53^{-/-}$ HCT116 cells transfected with pCMVβgal and either pCMV–p53, pCB6 + p53ΔI, pCMV–p53Gln22,Ser23, or empty vector were exposed to 1%O_2 for 8 hr. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α or anti-Mdm2 antibodies. (*F*) Effect of dominant–negative (RING finger) mutants of Mdm2 on hypoxia-induced expression of HIF-1α. $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells transfected with vectors encoding human Mdm2 (1–440) (pCHDM1–440), Mdm2 (464Ala) (pCHDM464Ala), or pCMVβgal were exposed to 1%O_2 for 8 hr. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α, anti-p53, or anti-Mdm2 antibodies. (*G*) Effect of dominant–negative (RING finger deletion mutant) Mdm2 on p53-mediated inhibition of HIF-1α expression in $ts20TG^R$ and H38-5 cells. Cells cotransfected with pCMV–p53 and either pCHDM1–440 or empty vector were maintained at 39°C for 12 hr and then exposed to 20%or 1%O_2 for an additional 8 hr at 39°C. Whole-cell lysates were subjected to anti-HIF-1α immunoblot analysis.

deficient (Lin et al. 1994). A p53 mutant carrying a deletion of residues 13–19 (p53ΔI) is also unable to bind to Mdm2 but retains its transactivation function (Marston et al. 1995). To investigate whether p53 requires interaction with Mdm2 to mediate degradation of HIF-1α, $p53^{-/-}$ HCT116 cells were transfected with encoding either wt p53, p53 22-23, p53ΔI, or control vector and analyzed for HIF-1α expression under hypoxic conditions. In contrast to wild-type p53, the p53 mutants (p53ΔI or p53 Gln22, Ser23) or the control vector were unable to reduce the levels of HIF-1α (Fig. 5E).

The Ub–protein ligase function of Mdm2 is dependent on a RING finger domain (residues 434–490) at the carboxyl terminus (Honda et al. 1997). Mdm2 mutants with a deletion of the RING finger domain [Mdm2 (1–440)] or a substitution of a cysteine residue at position 464 to alanine [Mdm2 (464Ala)] are deficient in Ub–protein ligase function but retain the ability to bind p53, thereby behaving in a dominant negative manner (Kubbutat et al. 1999). Introduction of Mdm2 (1–440) or Mdm2 (464Ala) augmented hypoxia-induced HIF-1α levels in $p53^{+/+}$ HCT116 cells but did not significantly influence HIF-1α expression in hypoxic $p53^{-/-}$ HCT116 cells (Fig. 5F). To determine whether Mdm2 functions as an E3-ligase that mediates p53-induced degradation of HIF-1α, $ts20TG^R$ and H38-5 cells were cotransfected with expression vectors encoding wild-type p53 and either Mdm2 (1–440) or empty control vector and transferred to hypoxic chambers (1%O_2) at 39°C. Cotransfection of Mdm2 (1–440) increased hypoxia-induced HIF-1α expression in E1-proficient H38-5 cells coexpressing p53 to levels observed in E1-deficient $ts20TG^R$ cells (Fig. 5G). Together, the data in Figure 5 are consistent with a model in which p53 acts as a molecular chaperone that facilitates recognition and recruitment of HIF-1α for ubiquitination by Mdm2.

Enhancement of tumor angiogenesis in $p53^{+/+}$ cells by forced expression of HIF-1α

To determine whether p53-mediated degradation of HIF-1α contributes to the suppression of tumor angiogenesis and growth, $p53^{+/+}$ HCT116 cells were stably transfected with pCEP4/HIF-1α (HCT116–HIF-1α) (Fig. 6A). Under hypoxic conditions, stable transfectants overexpressing HIF-1α demonstrated significantly increased VEGF mRNA levels compared with the parental $p53^{+/+}$ cells (Fig. 6B). When inoculated into athymic nude mice, HCT116–HIF-1α cells established tumors with a shorter latency and exhibited a significant increase in tumor growth kinetics compared with the parental cells (Fig. 6C). Histologic evaluation and analyses of NMR maps, as described earlier, revealed a significant increase in blood vessel density, vascular volume (17.4 μl/g) and , (0.8 μl/g/min) in xenografts established from HCT116–HIF-1α cells compared with those derived from the parental $p53^{+/+}$ HCT116 cells (Fig. 6D).

Discussion

Recognition of the importance of angiogenesis for the growth and metastasis of cancers has raised fundamental questions regarding the molecular mechanisms of the angiogenic switch during tumor progression. The genetic alterations involved in tumorigenesis are also responsible for the phenotypic characteristics of cancer cells. The *p53* tumor suppressor gene is one of the most frequently mutated genes in human cancers (for review, see Levine 1997). In addition to p53 mutations, which occur in ~50% of all cancers (involving >50 tissue types), p53 is also inactivated by viral oncoproteins such the E6 protein of cervical cancer-associated HPV 16 and 18, adenovirus E1A, and SV40 large T antigen (for review, see Levine 1997). Our observations indicate that loss of p53 function, via somatic mutations or expression of viral oncoproteins, contributes to activation of the angiogenic switch during tumorigenesis.

In addition to identifying the loss of p53 as a discrete and potentially rate-limiting event in tumor angiogenesis, we define a novel mechanism by which p53 regulates the angiogenic switch. Our observations indicate that p53 inhibits hypoxia-induced expression of HIF-1α by facilitating its ubiquitination and subsequent degradation. This mechanism is distinct from the proposal that p53 inhibits HIF-1-mediated transactivation by competing for the p300 coactivator (Blagosklonny et al. 1998) and is analogous to the proposed role of the von Hippel-Lindau (VHL) tumor suppressor (Maxwell et al. 1999). As in the case of VHL (Maxwell et al. 1999), we demonstrate that p53 interacts with HIF-1α in vivo, as reported previously (An et al. 1998). In addition, we demonstrate for the first time that a tumor suppressor (p53) promotes the ubiquitin-mediated degradation of HIF-1α via recruitment of an E3 ubiquitin–protein ligase (Mdm2). Although ubiquitination is assumed to be the mechanism by which VHL affects HIF-1α degradation, our data provide the first direct evidence for this mechanism of tumor suppressor action. The constitutive stabilization of HIF-1α and the related HIF-2α protein) resulting from VHL loss of function may underlie the predisposition to highly angiogenic tumors in VHL disease, a rare hereditary cancer syndrome. Our findings indicate that deregulation of HIF-1α expression, leading to overexpression of VEGF, may contribute to the angiogenic switch conferred by inactivation of p53 in a broad array of human cancers. In accordance with this hypothesis, HIF-1α is frequently overexpressed in common human cancers and there is a statistically significant correlation between the presence of mutant p53 and HIF-1α overexpression (Zhong et al. 1999). Our findings suggest that increased HIF-1 activity resulting from loss of p53 function may contribute to the overexpression of VEGF that is observed in a wide variety of human cancers (for review, see Brown et al. 1996; Folkman 1997).

The angiogenic switch is regulated by changes in the relative balance between inducers and inhibitors of en-

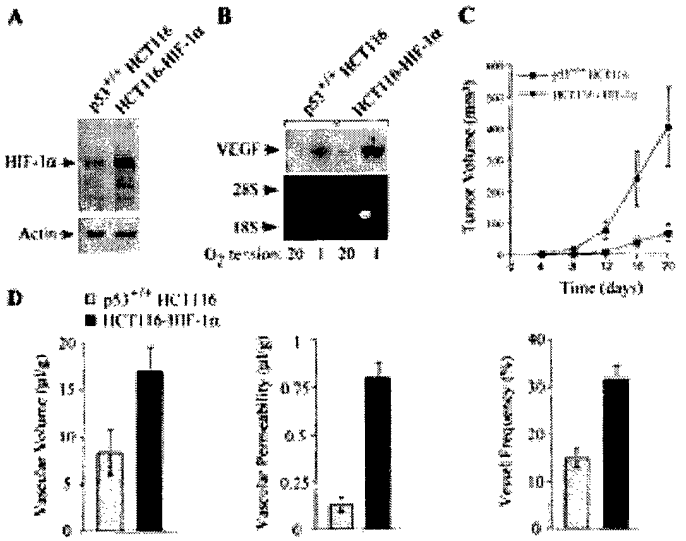

Figure 6. Increased tumor angiogenesis and growth in $p53^{+/+}$ cells with forced overexpression of HIF-1α. (*A*) Immunoblot analyses of HIF-1α protein levels in $p53^{+/+}$ HCT116 cells and $p53^{+/+}$ HCT116 cells stably transfected with a HIF-1α expression vector (HCT116–HIF-1α following exposure to 1% O_2 for 8 hr. (*B*) Northern blot analysis of VEGF mRNA levels in $p53^{+/+}$ HCT116 and HCT116–HIF-1α cells cultured for 16 hr in 20% or 1% O_2. (*C*) Growth of $p53^{+/+}$ HCT116 (blue ■) and HCT116–HIF-1α (red ▲) cells (2.5×10^6) injected subcutaneously into the flanks of athymic BALB/c nude mice. Values expressed represent mean ± S.E. of 12 xenografts of each cell type. (*D*) Quantification of vascular volume, permeability, and blood vessel density in $p53^{+/+}$ HCT116 (shaded bars) and HCT116–HIF-1α (solid bars) xenograft tumors. In vivo vascular volume and permeability of the tumors were determined by NMR analyses, and blood vessel frequency in stained sections of excised tumors was analyzed as described in Fig. 1.

dothelial cell proliferation and migration (for review, see Hanahan and Folkman 1996). The switch can be activated by increasing the levels of inducers, such as VEGF, and/or by reducing the concentration of inhibitors, such as thrombospondin-1 (TSP-1). The p53-mediated inhibition of VEGF expression demonstrated in this study, together with the previously reported ability of p53 to upregulate TSP-1 (Dameron et al. 1994), indicates that p53 provides dual functions that regulate angiogenesis. Thus, the loss of p53 function during tumorigenesis deregulates both arms of the balance, providing a potent stimulus for neovascularization and tumor progression.

In addition to loss-of-function mutations in tumor suppressor genes such as *p53* or *VHL*, oncogene activation is also capable of stimulating HIF-1 activity. Expression of the *v-Src* oncogene induces expression of HIF-1α protein, HIF-1 DNA-binding activity, and transcriptional activation of *VEGF* and enolase 1 (Jiang et al. 1997). A phosphatidylinositol 3-kinase/Akt pathway of HIF-1 activation may induce *VEGF* expression in *Ha-ras*-transformed cells (Mazure et al. 1997). Therefore, increased HIF-1 expression is associated with multiple genetic alterations that promote tumor angiogenesis. Because HIF-1 is also a key transcriptional activator of genes encoding glucose transporters and glycolytic enzymes (Iyer et al. 1998), these genetic alterations also contribute to the metabolic adaptation and enhanced survival of tumor cells in hypoxic microenvironments.

As p53 is an important mediator of DNA damage-induced apoptosis, the angiogenic phenotype conferred by inactivation of p53 in human cancers is frequently associated with resistance to conventional genotoxic anticancer agents (for review, see Lowe 1995). Because p53-deficient tumors remain dependent on angiogenesis for growth and metastasis, inhibition of angiogenesis may represent an effective therapeutic intervention (Boehm et al. 1997; Bergers et al. 1999). Recent studies indicate that inhibition of tumor-derived VEGF expression restricts angiogenesis and promotes vascular regression in experimental tumor models (Kim et al. 1993; Millauer et al. 1994, 1996; Warren et al. 1995; Goldman et al. 1998). Loss of HIF-1 activity is also associated with decreased angiogenesis and growth of tumor xenografts in nude mice (Jiang et al. 1997; Maxwell et al. 1997; Carmeliet et al. 1998; Ryan et al. 1998). By demonstrating that deregulation of HIF-1 underlies the increased expression of VEGF in p53-deficient cancers, our data provide further support for the hypothesis that inhibition of HIF-1 may abrogate the ability of such tumors to establish an adequate vascular supply and adapt their cellular metabolism to hypoxia, thereby curtailing their growth and metastasis.

Materials and methods

Cell lines and culture

The parental HCT116 human colon adenocarcinoma cell line, containing wild-type p53 ($p53^{+/+}$), and a p53-deficient derivative ($p53^{-/-}$) created by homozygous deletion via homologous recombination (Bunz et al. 1998), were a gift from Bert Vogelstein. $p53^{+/+}$ HCT116 cells were transfected with pCEP4/HIF-1α and a pool of stable transformants overexpressing HIF-1α (HCT116–HIF-1α) was selected in the presence of hygromycin (200 μg/ml). $p53^{+/+}$ or $p53^{-/-}$ HCT116 and HCT116–HIF-1α cells were maintained in McCoy's modified medium (Life Technologies, Inc.) supplemented with 10%fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5%CO_2. PA-1 ovarian teratocarcinoma cells stably transfected with pCMV–HPV16 E6 or pCMV–Neo, generated as described (Ravi et al. 1998), were maintained in Basal Eagle medium supplemented with 0.5 mg/ml G418, 10%FCS, and antibiotics (as described above) at 37°C and 5%CO_2. $p53^{+/+}$ and $p53^{-/-}$ MEFs (gift from Tyler Jacks, Massachusetts Institute of Technology, Cambridge, MA), were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10%FCS and antibiotics. The BALB/c 3T3-derived $ts20TG^R$ or H38-5 cell lines (Chowdary et al. 1994) (gift from Harvey L. Ozer) were maintained at 35°C in DMEM supplemented with 10%fetal bovine serum and antibiotics. The permissive and nonpermissive temperatures for the $ts20TG^R$ E1-mutant cell line are 35°C and 39°C, respectively. Cells were plated on 100-mm petri dishes and allowed to approach confluence. For hypoxic conditions, cells were placed in a modular incubator chamber and flushed with a gas mixture containing 1%O_2, 5%CO_2, and balance N_2 (Semenza and Wang 1992).

Growth of tumor xenografts in nude mice

HCT116 cells (2.5×10^4, 2.5×10^5, or 2.5×10^6) suspended in 0.1 ml of PBS were injected subcutaneously into the right ($p53^{+/+}$) or left ($p53^{-/-}$) hind legs or flanks of athymic BALB/c (nu/nu) mice. Tumor volumes were determined by external measurement in three dimensions using the equation V = [L × W × H] × /6, where V = volume, L = length, W = width, and H = height. Care of experimental animals was in accordance with institutional animal care and use committee guidelines.

NMR analyses of in vivo vascular volume and permeability of tumor xenografts

Multislice maps of relaxation rates (T_1^{-1}) were obtained by a saturation recovery T_1 SNAPSHOT-FLASH imaging method (flip angle of 5°, echo time of 2 msec). Images of four slices (slice thickness of 1 mm) acquired with an in-plane spatial resolution of 125 μm (128 × 128 matrix, 16-mm field of view, NS = 8) were obtained for three relaxation delays (100 msec, 500 msec, and 1 sec) for each of the slices; 128 × 128 × 4 T_1 maps were acquired within 7 min. An M_o map with a recovery delay of 7 sec was acquired once at the beginning of the experiment. Images were obtained before intravenous administration of 0.2 ml of 60 mg/ml albumin–GdDTPA in saline (dose of 500 mg/kg) and repeated starting after the injection up to 32 min. Relaxation maps were reconstructed from data sets for three different relaxation times and the M_o data set on a pixel by pixel basis. At the end of the imaging studies, the animal was sacrificed, and 0.5 ml of blood was withdrawn from the inferior vena cava. Vascular volume and permeability product surface area (PS) maps were generated from the ratio of $\Delta(1/T_1)$ values in the images to that of blood. The slope of $\Delta(1/T_1)$ ratios versus time in each pixel was used to compute PS, whereas the intercept of the line at zero time was used to compute vascular volume. Thus, vascular volumes were corrected for permeability of the vessels. Volume and permeability values (mean ± S.E.) were computed for tumor xenografts established with HCT116

$p53^{+/+}$ (n = 4), HCT116 $p53^{-/-}$ (n = 5), and HCT116–HIF-1α (n = 2) cells.

Histologic analyses of blood vessel density in tumor xenografts

Five-micrometer sections prepared from paraffin-embedded tissue were stained with hemotoxin & eosin (H & E) and subjected to immunoperoxidase detection of endothelial cells using an anti-vWF antibody. A circular matrix of 25 random sampling points (per unit area) was superimposed on defined fields, and the points overlying a vessel were scored as a percentage of the total points.

Plasmids

Plasmids encoding human wild-type full-length p53 (pC53-SN; gift from Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD), mutant p53 (pCB6+ p53ΔI) (Marston et al. 1994), p53 double mutants (pCMV–p53Gln22, Ser23) (Lin et al. 1994), human mutant Mdm2 (pCHDM1-440 and pCHD464Ala; provided by Karen Vousden) (Kubbutat et al. 1999), His_6–Ubiquitin (MT107–His_6-Ub; provided by Dirk Bohmann) (Musti et al. 1997), pCMV–HPV16 E6 (provided by Kathy Cho, Johns Hopkins University School of Medicine, Baltimore, MD), and HIF-1α (pCEP4–HIF-1α) (Forsythe et al. 1996; Jiang et al. 1996) have been described previously.

Analysis of VEGF reporter activity

Wild-type (p11w) and mutant (p11m) copies of the hypoxia response element from the *VEGF* gene cloned 5′ to a SV40-promoter–luciferase transcription unit were described previously (Forsythe et al. 1996). $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells and PA-1 Neo or PA-1 E6 cells were cotransfected (using Lipofectin) with either VEGF–p11w or VEGF–p11m and CMVβgal, with or without pCEP4/HIF-1α. Transfected cells were exposed to hypoxia ($1\%O_2$) for 20 hr and harvested for β-gal and luciferase assays (Promega) in fixed protein aliquots. Luciferase activity was normalized for β-gal activity. The data represent the mean ± S.E. from three independent experiments.

Electrophoretic mobility shift assays of HIF-1 DNA-binding activity

Nuclear extracts (5 μg) prepared from $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells exposed to either 20%or $1\%O_2$ were incubated with ^{32}P-labeled double-stranded oligonucleotide probe containing a wild-type HIF-1 binding site and DNA/protein complexes were analyzed by polyacrylamide gel electrophoresis as described previously (Semenza and Wang 1992; Jiang et al. 1996). HIF-1 binding to the probe was confirmed by competition assays using 50 ng of either unlabeled wild-type oligonucleotide or a mutant oligonucleotide containing the same 3-bp substitution as in p11m (Semenza and Wang 1992; Forsythe et al. 1996).

Northern blot

VEGF and HIF-1α mRNA was assessed by Northern blot analyses of total RNA prepared from $p53^{+/+}$ or $p53^{-/-}$ HCT116 and HCT116–HIF-1α cells cultured for 16 hr in either 20%or $1\%O_2$. Total RNA (20-μg aliquots) was fractionated by 1.2%agarose–formaldehyde gel electrophoresis and transferred to nylon membranes. The blots were hybridized to probes for VEGF, HIF-1α, and β-actin mRNA using random primer labeling (Boehringer Mannheim) (Jiang et al. 1997).

ELISA

Quantikine (R & D Systems) was used to measure VEGF protein in supernatant medium of $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells and PA-1 Neo or PA-1 E6 cells cultured as described above for 16–32 hr.

Immunoblot analyses and immunoprecipation

Nuclear extracts or whole-cell lysates were prepared, fractionated by SDS-PAGE, transferred to PVDF membranes (Millipore, Bedford, MA), and immunoblotted with monoclonal antibodies against HIF-1α (H1α67; Novus Biologicals, Inc.) (Zhong et al. 1999), p53 (DO-1, Ab-6; Oncogene Research Products), Mdm2 (Ab-1; Oncogene Research Products), or β-actin (Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham). For analysis of protein interactions, whole-cell lysates were immunoprecipitated with antibodies against either p53 or Mdm2 or isotype control antibody, and the resultant immune complexes were subjected to immunoblot analysis with anti-HIF-1α monoclonal antibody H1α67.

Analysis of HIF-1α protein half-life and ubiquitin-dependent degradation

Cells exposed to 100 μM cobalt chloride for 4 hr were treated with 100 μM cycloheximide. Whole-cell extracts were prepared at intervals of 15–40 min and subjected to immunoblot analyses with anti-HIF-1α antibody. To assess ubiquitination of HIF-1α in hypoxic conditions, $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells were cotransfected (Lipofectin, Life Technologies, Inc.; 100-mm dishes) with 2 μg of pCMVβgal and 6μg each of pCEP4/HIF-1α and either MT107/His_6-Ub or empty vector (MT107) (Musti et al. 1997), and cultured in $1\%O_2$ for 4 hr in the presence of 50 μM MG132 (Peptides International, Inc.). Cells were lysed in buffer supplemented with 5 mM N-ethylmaleimide and 50 mM imidazole, as described (Ravi et al. 1998). Whole-cell extracts or His-tagged proteins (purified from 500 μg of whole cell–protein lysates (normalized to β-gal activity) using Talon Metal Affinity Resin (Clontech)] were subjected to SDS-PAGE and immunoblot analysis with anti-HIF-1α antibody. To analyze whether the effect of p53 on HIF-1α protein levels was dependent on ubiquitination, $ts20TG^R$ and H38-5 cells were transfected (using Lipofectin) with pCMV–p53 and pCMVβgal, maintained at either 35°C or 39°C for 8 hr, and then exposed to $1\%O_2$ for an additional 8 hr at their respective temperatures. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α or anti-p53 antibodies.

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Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB

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TRAIL (tumour-necrosis factor-related apoptosis ligand or Apo2L) triggers apoptosis through engagement of the death receptors TRAIL-R1 (also known as DR4) and TRAIL-R2 (DR5). Here we show that the c-Rel subunit of the transcription factor NF-κB induces expression of TRAIL-R1 and TRAIL-R2; conversely, a transdominant mutant of the inhibitory protein IκBα or a transactivation-deficient mutant of c-Rel reduces expression of either death receptor. Whereas NF-κB promotes death receptor expression, cytokine-mediated activation of the RelA subunit of NF-κB also increases expression of the apoptosis inhibitor, Bcl-x_L, and protects cells from TRAIL. Inhibition of NF-κB by blocking activation of the IκB kinase complex reduces Bcl-x_L expression and sensitizes tumour cells to TRAIL-induced apoptosis. The ability to induce death receptors or Bcl-x_L may explain the dual roles of NF-κB as a mediator or inhibitor of cell death during immune and stress responses.

Apoptosis has an essential role in embryogenesis, adult tissue homeostasis and the cellular response to stressful stimuli, such as DNA damage, hypoxia or aberrations in cell-cycle progression[1]. Increased apoptosis is involved in the pathogenesis of diverse ischaemic, degenerative and immune disorders[2]. Conversely, genetic aberrations that render cells incapable of executing their suicide program promote tumorigenesis and underlie the observed resistance of human cancers to genotoxic anticancer agents[3]. Unravelling mechanisms to unleash the apoptotic program in tumour cells might aid the design of effective therapeutic interventions against resistant human cancers.

The molecular machinery of cell death comprises an evolutionarily conserved family of cysteine aspartate proteases (caspases)[4]. Caspases can be activated by the engagement of death receptors belonging to the tumour-necrosis factor (TNF) receptor gene superfamily[5], such as TNFR1, CD95 (Fas), TRAIL-R1 (DR4)[6] and TRAIL-R2 (DR5, TRICK2, KILLER)[7–13], by their respective cognate 'death ligands', TNF-α, CD95L (Apo1L) and TRAIL (also known as Apo2L)[14,15]. TRAIL induces apoptosis in several tumour cell lines, including those that resist chemotherapeutic agents or ionizing radiation because of inactivating mutations of the p53 tumour suppressor gene[16–20].

TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins containing cytoplasmic sequences, termed 'death domains', that recruit adaptor proteins and activate caspases[16]. Two other TRAIL receptors, TRAIL-R3 (TRID/DcR1) and TRAIL-R4 (TRUNDD/DcR2), have extracellular domains similar to TRAIL-R1 and TRAIL-R2, but lack a functional cytoplasmic death domain[7,8,21–24]. TRAIL-R3 and TRAIL-R4 may serve as 'decoys' that compete with TRAIL-R1/TRAIL-R2 for binding to TRAIL, and overexpression of either protein confers protection against TRAIL-induced death[7,8].

The NF-κB family of dimeric transcription factors is important in modulating cell survival during stress and immune responses[25]. NF-κB protects cells from apoptosis[26–31] by promoting expression of survival factors, such as members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, XIAP)[32] and the Bcl-2 homologues, Bfl-1/A1 (refs 33, 34) and Bcl-x_L (ref. 35). In contrast, much evidence highlights an apparently paradoxical pro-apoptotic role for NF-κB[36–39]. These observations raise the possibility that κB sites in pro- or anti-apoptotic genes may exhibit different preferences for particular subunits comprising the NF-κB dimer, and that NF-κB may have signal-specific effects on cell survival.

Here we show that the RelA and c-Rel subunits of NF-κB are critical determinants of the expression of death receptors and survival genes that modulate TRAIL-induced apoptosis. The signal-specific activation of dimers that induce expression of either death receptors or survival genes might explain how NF-κB adopts either of its dual personalities as a mediator or inhibitor of cell death during immune and cellular stress responses. The identification of NF-κB as a key determinant of cellular susceptibility to TRAIL may have important implications for anticancer therapy.

Results

Subunit-specific effects of NF-κB on death receptor expression and on sensitivity to TRAIL. NF-κB exists in almost all cell types in an inactive cytoplasmic complex with an inhibitory protein, IκB. Signal-dependent phosphorylation and ubiquitin-mediated degradation of IκB by IκB kinases (IKKs) releases the active complex, which functions in transcriptional regulation of target genes after nuclear translocation[25]. Trimerization of TNFR1 by TNF-α leads to degradation of IκB and activation of NF-κB. Mouse embryonic fibroblasts (MEFs) stably transduced with a retrovirus carrying a combined amino- (residues 32 and 36) and carboxy-terminal PEST sequence phosphorylation mutant of IκBα (IκBαM)[28] show reduced basal and TNF-α-inducible κB DNA-binding activity and lower expression of TRAIL-R2 messenger RNA compared with wild-type MEFs carrying a control vector (Fig. 1a, b).

The subunits of NF-κB are known to exhibit different preferences for variations of the 10-base-pair (bp) consensus sequence

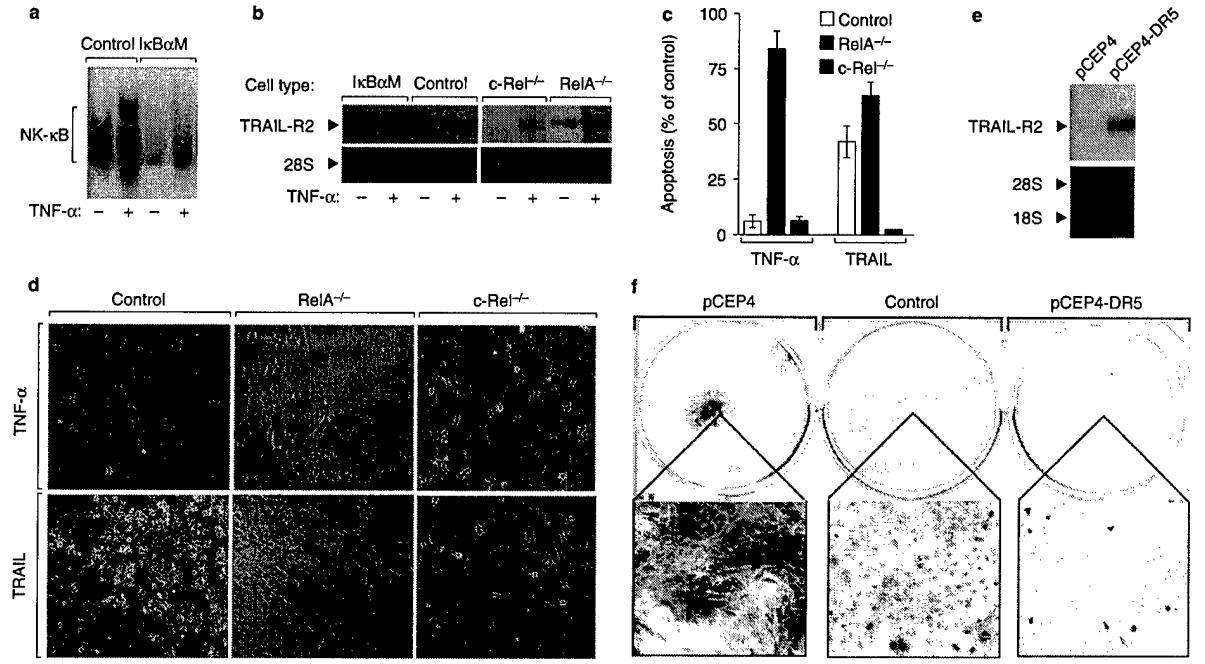

Figure 1 **Subunit-specific effects of NF-κB on death receptor expression and sensitivity to TRAIL. a**, Inhibition of NF-κB by a transdominant mutant IκBα (IκBαM). MEFs stably transduced with a plasmid encoding IκBαM and the empty vector pLXSN[28] (control) were incubated with TNF-α (100 ng ml^{-1}, 1 h) or left untreated. Nuclear extracts were analysed for NF-κB DNA-binding activity by EMSA. **b**, Basal and TNF-α-inducible expression of TRAIL-R2 mRNA in RelA$^{-/-}$, c-Rel$^{-/-}$, IκBαM-expressing, and wild-type mouse fibroblasts carrying an empty vector (control). **c**, **d**, Effect of deficiency of either RelA or c-Rel on TNF-α- or TRAIL-induced cell death. RelA$^{-/-}$, c-Rel$^{-/-}$ and wild-type mouse fibroblasts were exposed to either TNF-α (100 ng ml^{-1}) or recombinant human TRAIL (100 ng ml^{-1}; with enhancer antibody) for 24 h. Data (mean ± s.d.) shown in **c** are the percentage of apoptotic nuclei among total nuclei counted ($n = 3$). Representative photomicrographs illustrating the cytotoxicity of TRAIL are shown in **d**. **e**, Expression of TRAIL-R2 in c-Rel$^{-/-}$ mouse fibroblasts transfected with either pCEP4-DR5 or empty pCEP4 vector. **f**, Susceptibility of c-Rel-deficient cells to TRAIL-R2-induced death. Photomicrographs depict crystal-violet-stained colonies of c-Rel$^{-/-}$ mouse fibroblasts selected for growth in hygromycin B after transfection with either pCEP4-DR5 or empty pCEP4 vector. Cells from an untransfected control population were maintained in hygromycin-free media (control). Similar observations were made in RelA$^{-/-}$ and wild type mouse fibroblasts (data not shown).

(5′-GGGGYNNCCY-3′) in particular target genes[25]. We therefore analysed the role of specific subunit(s) of NF-κB on expression of TRAIL-R2 mRNA levels in RelA-deficient (RelA$^{-/-}$)[26], c-Rel-deficient (c-Rel$^{-/-}$)[40] and wild-type mouse fibroblasts. Whereas TNF-α-inducible expression of TRAIL-R2 mRNA was evident in RelA$^{-/-}$ fibroblasts, this induction was markedly diminished in c-Rel$^{-/-}$ fibroblasts (Fig. 1b).

We distinguished the effects of RelA and c-Rel on cell survival by examining the response of RelA$^{-/-}$, c-Rel$^{-/-}$ or wild-type mouse fibroblasts to either TNF-α or TRAIL. RelA$^{-/-}$ fibroblasts were highly sensitive to TNF-α-mediated cell death, but c-Rel$^{-/-}$ fibroblasts, akin to their wild-type counterparts, remained relatively resistant to such treatment (Fig. 1c, d). Whereas RelA$^{-/-}$ and wild-type fibroblasts were both susceptible to TRAIL-induced apoptosis, c-Rel$^{-/-}$ fibroblasts were almost completely resistant to TRAIL (Fig. 1c, d). c-Rel$^{-/-}$ cells were resistant to TRAIL, but they failed to yield any viable clones after transfection with an expression vector encoding TRAIL-R2 (pCEP4/DR5)[10] (Fig. 1e, f). The resistance of c-Rel$^{-/-}$ cells to TRAIL-induced death seems therefore to result from their deficiency in death receptor expression rather than inhibition of intracellular death signalling. These results suggest that, in contrast to the protection conferred by RelA against TNF-α-induced death, c-Rel mediates the inducible expression of death receptors for TRAIL.

NF-κB c-Rel contains an N-terminal 300-residue conserved region known as the Rel homology domain (RHD), which mediates dimerization and nuclear localization, and a variable C-terminal domain, which is responsible for transactivation. To examine directly the effect of c-Rel or RelA on death receptor expression and sensitivity to TRAIL, c-Rel (CCR), a c-Rel truncation mutant lacking the C-terminal transactivation domain (Δc-Rel; CCR-H) or RelA were conditionally expressed in HeLa cells using a tetracycline-regulated system[33,41] (Fig. 2a). The c-Rel, truncated c-Rel or RelA genes were expressed under control of the tTA fusion activator, comprising the *Escherichia coli* tetracycline repressor and the activation domain of the VP16 protein of herpes simplex virus. Stable cell clones carrying either c-Rel (CCR43) or Δc-Rel (CCR-H5) were subjected to immunoblot analysis using an antibody against the RHD of chicken c-Rel. Removing tetracycline from the culture medium for 48 h resulted in induction of either c-Rel in CCR43 cells or the faster migrating Δc-Rel mutant in CCR-H5 cells (Fig. 2b).

Electrophoretic mobility shift assays with double-stranded oligonucleotides containing a palindromic κB site were performed using nuclear protein derived from CCR43 or CCR-H5 cells maintained in the presence or absence of tetracycline for 48 h. CCR43 cells showed increased κB DNA-binding activity in response to withdrawal of tetracycline, and the DNA-bound complex was supershifted with an anti-c-Rel antibody but not with an antibody against RelA (Fig. 2c). Although the inducible c-Rel is active in binding c-Rel-responsive κB motifs, the transactivation-deficient mutant Δc-Rel competes with endogenous c-Rel for κB binding, thereby behaving in a dominant-negative manner (Fig. 2c)[33,41].

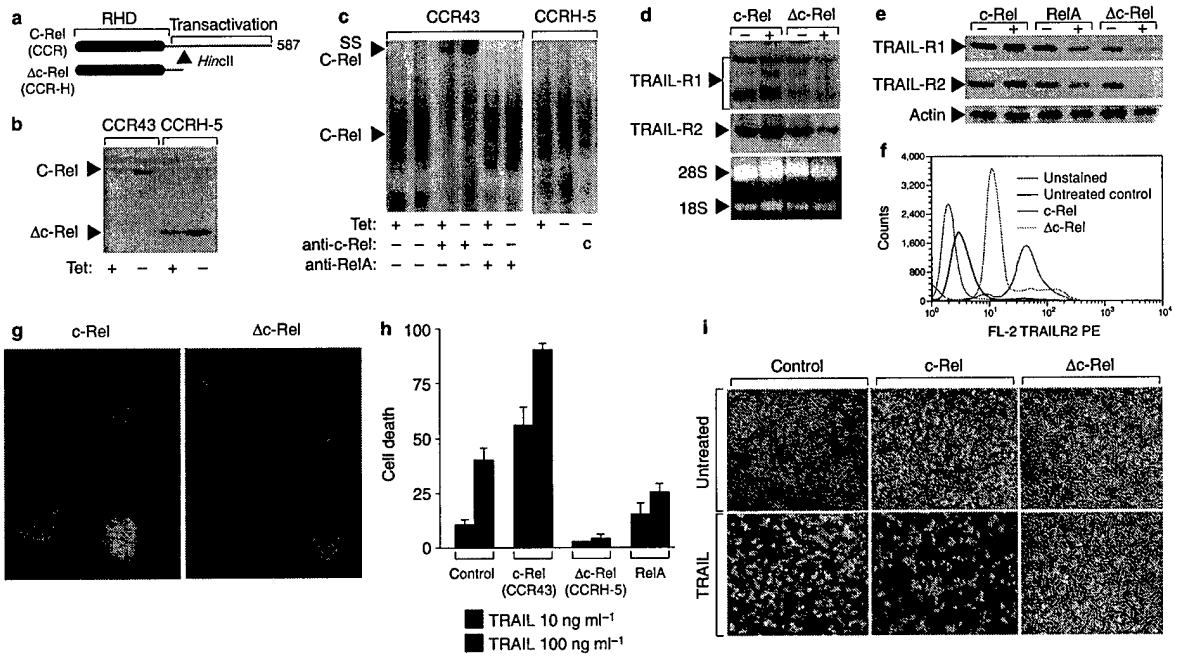

Figure 2 **Effect of inducible expression of c-Rel, ΔRel or RelA on death receptor expression and sensitivity to TRAIL.** **a**, Representation of full-length c-Rel (CCR) and Δc-Rel (CCR-H), a c-Rel mutant that contains a stop codon at the unique *Hin*cII site of c-Rel. **b**, Immunoblot analyses of expression of c-Rel and Δc-Rel in HeLa (HtTA-1) cell clones stably transfected with c-Rel (CCR43) or Δc-Rel (CCR-H5), respectively (in the presence or absence of tetracycline for 48 h). **c**, EMSA of c-Rel-specific DNA-binding activity in nuclear extracts of CCR43 and CCR-H5 cells maintained in the presence or absence of tetracycline (Tet) for 48 h. Supershift (SS) analysis of DNA–protein complexes was performed with anti-c-Rel and anti-RelA antibodies. Competition of the Δc-Rel-induced DNA–protein complex with unlabelled c-Rel-specific oligonucleotides is shown (lane 'c'). **d**, Northern blot analyses of TRAIL-R1 and TRAIL-R2 mRNA in cells maintained in the presence (uninduced, –) or absence (induced, +) of tetracycline for 48 h. **e**, Western blot analyses of the effect of induced expression of c-Rel or Δc-Rel on expression of TRAIL-R1 and TRAIL-R2 protein. **f**, Flow cytometric analysis of the effect of induced expression of c-Rel or Δc-Rel on IR-induced cell surface expression of TRAIL-R2 in HeLa cells. (Unstained controls received secondary antibody alone; untreated controls received no IR.) **g**, Confocal microscopic examination of TRAIL-R2 immunofluorescence in HeLa cells induced to express either c-Rel or Δc-Rel for 48 h. **h**, HeLa (HtTA-1) cell clones stably transfected with either c-Rel (CCR43), Δc-Rel (CCR-H5), or RelA were maintained in the absence of tetracycline for 48 h (to induce gene expression) and then exposed to TRAIL (10–100 ng ml^{-1}; enhancer antibody 2 μg ml^{-1}) or left untreated for another 24 h. Data represent the percentage survival (viable/[apoptotic + viable]) in each cell population (mean ± s.d.) from three independent experiments. **i**, Representative photomicrographs illustrating the cytotoxic effects of TRAIL (100 ng ml^{-1}) on c-Rel (CCR43) cells maintained in the presence of tetracycline (control), or in c-Rel (CCR43) and Δc-Rel (CCR-H5) cells induced to express c-Rel or Δc-Rel, respectively, by culture in tetracycline-free medium for 48 h.

Northern blot analysis showed that c-Rel promotes the expression of death receptors at a transcriptional level, but Δc-Rel interferes with this induction (Fig. 2d). Induction of c-Rel in CCR43 cells resulted in increased protein expression of both TRAIL-R1 (2.2-fold induction relative to an actin control) and TRAIL-R2 (2.6-fold induction) (Fig. 2e). In contrast, induction of the dominant-negative transactivation mutant Δc-Rel in CCR-H5 cells inhibited protein expression of either TRAIL-R1 (2.4-fold repression) or TRAIL-R2 (3.2-fold repression) (Fig. 2e). Flow cytometric analyses confirmed that inducible expression of cell-surface TRAIL-R2 was greater in cells expressing c-Rel compared with cells expressing Δc-Rel (Fig. 2f). Confocal microscopy showed relatively greater immunofluorescent labelling of TRAIL-R2 in the cytoplasm of cells induced to express c-Rel compared with cells forced to express Δc-Rel (Fig. 2g).

Induction of c-Rel by removing tetracycline resulted in a dose-dependent increase in the sensitivity of CCR43 cells to TRAIL-induced death (Fig. 2h, i). By contrast, expression of Δc-Rel by removing tetracycline in CCR-H5 cells rendered these cells relatively resistant to TRAIL (Fig. 2h, i). Consistent with its induction of survival factors, induced expression of RelA reduced sensitivity to TRAIL (Fig. 2h).

NF-kB-induces expression of TRAIL-R2 and TRAIL-mediated tumour cell radiosensitization independently of p53. The cellular response to DNA damage inflicted by genotoxic anticancer agents is modulated by the product of the p53 tumour suppressor gene—a transcription factor that promotes expression of TRAIL-R2/DR5 (ref. 10). As NF-κB has been implicated in p53-mediated cell death[38], we thought that p53 might be required for NF-κB-induced expression of TRAIL-R2. We therefore examined the effect of p53 genotype on the basal, TNF-α- and DNA-damage-induced activation of NF-κB and expression of death receptors in isogenic cell lines that differ only in p53 status.

The effect of TNF-α on expression of TRAIL-R2/DR5 was examined in MEFs of wild-type and $p53^{-/-}$ genotypes. Expression of TRAIL-R2/DR5 was impaired in c-$Rel^{-/-}$ cells (Fig. 1b), but $p53^{-/-}$ cells exhibited normal basal and TNF-α-inducible expression of TRAIL-R2 mRNA (Fig. 3a), indicating that NF-κB mediates TNF-α-induced expression of TRAIL-R2 in a p53-independent fashion. The parental HCT116 line, containing wild-type p53 ($p53^{+/+}$), and a p53-deficient derivative ($p53^{-/-}$), created by homozygous deletion of endogenous *p53* genes through homologous recombination[42], also showed equivalent basal levels of TRAIL-R2 mRNA (Fig. 3d). $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells showed an equivalent reduction in

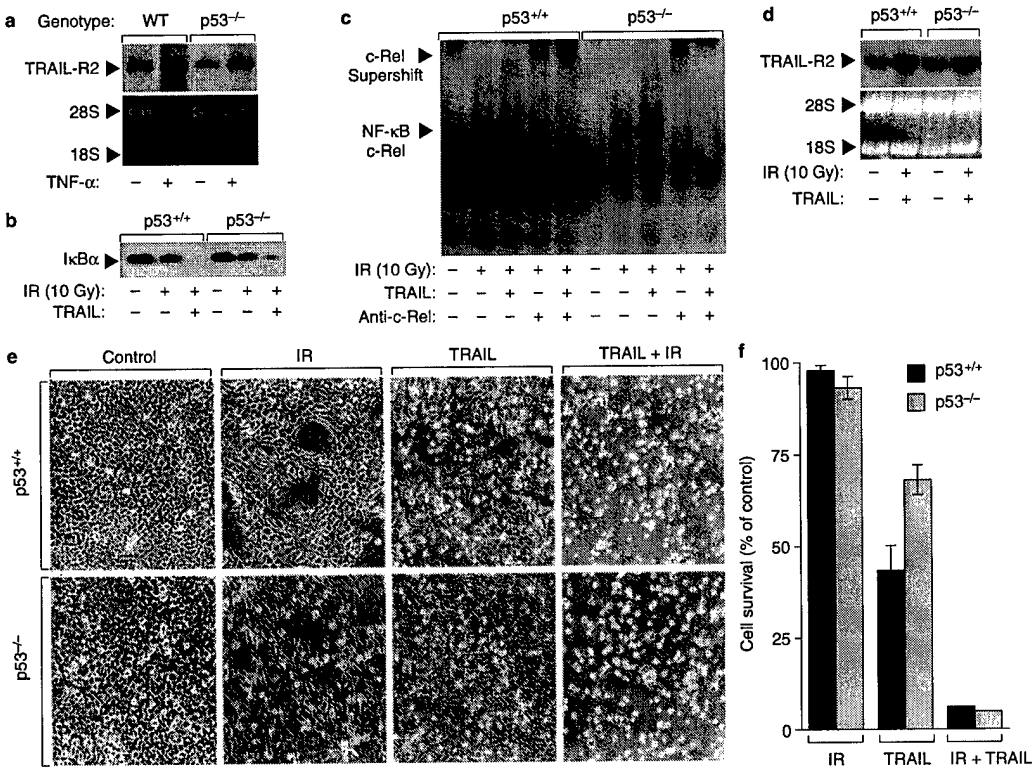

Figure 3 **NF-κB-induced expression of TRAIL-R2 and TRAIL-mediated radiosensitization independent of p53.** **a**, Northern blot analyses of the effect of TNF-α on TRAIL-R2 mRNA levels in wild-type (WT) and p53 $^{-/-}$ MEFs. **b**, Western blot analyses of IκBα expression in p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells exposed to irradiation (10 Gy) in the presence or absence of TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}). **c**, EMSA of κB-specific DNA-binding activity in nuclear extracts of p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells exposed to irradiation (10 Gy) in the presence or absence of TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}). Supershift (SS) analysis of DNA–protein complexes was performed an anti-c-Rel specific antibody. **d**, Western bot analyses of TRAIL-R2 expression in irradiated p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells exposed to TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}). **e**, **f**, Representative photomicrographs illustrating the effects of either IR (10 Gy), TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}), and IR + TRAIL on survival of p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells. Data in **f** represent the percentage survival (viable/[apoptotic + viable]) in each cell population (mean ± s.d.) from three independent experiments

IκBα levels and elevation in κB/c-Rel DNA-binding activity in response to irradiation, and irradiation-induced κB DNA-binding was augmented by exposure to TRAIL in both cell types (Fig. 3b, c). Exposure to ionizing radiation (IR) and TRAIL resulted in an equivalent elevation of TRAIL-R2 mRNA in both *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells (Fig. 3d); therefore, IR-induced expression of TRAIL-R2 in cells exposed to TRAIL was analogous to its p53-independent expression after treatment with TNF-α.

To examine whether the combination of IR with TRAIL can override the radioresistance of HCT116 cells, we exposed *p53*$^{+/+}$ or *p53*$^{-/-}$ HCT116 cells to IR (10 Gy), TRAIL (100 ng ml^{-1}) or both. Although both *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells were resistant to IR-induced apoptosis, exposure to IR resulted in augmentation of TRAIL sensitivity in both cell types, such that either cell population was eliminated within 48 h of treatment (Fig. 3e, f). Together, these data indicate that IR can induce NF-κB-mediated expression of death receptors and augment TRAIL-induced death of both *p53*$^{+/+}$ and *p53*$^{-/-}$ tumour cells. These data have potentially important implications for the treatment of p53-deficient human cancers by TRAIL-mediated radiosensitization.

The RelA subunit of NF-κB induces Bcl-x_L and protects cells from TRAIL/death-receptor-induced apoptosis. We investigated whether the differential activation of c-Rel- and/or RelA-containing dimers of NF-κB in response to physiological signals (immune activation by ligation of CD40) or stressful stimuli (DNA damage) influences the expression of death receptors and sensitivity to TRAIL.

Irradiation of B cells activated κB DNA-binding activity in electrophoretic mobility shift assays (EMSAs), using a c-Rel consensus binding site as an oligonucleotide probe, and the IR-induced DNA–protein complex was supershifted with an anti-c-Rel antibody (Fig. 4a). Although irradiation of wild-type B lymphocytes resulted in induction of TRAIL-R2 mRNA, IR-inducible levels of TRAIL-R2 were diminished in B cells from c-Rel$^{-/-}$ mice (Fig. 4b, c). Ligation of IR-induced TRAIL-R2 with TRAIL resulted in apoptosis of Bcl-2-overexpressing B lymphocytes (from TgN(Bcl-2) mice), which are otherwise relatively resistant to IR[43] (Fig. 4d).

Stimulation of resting mouse B lymphocytes with a monoclonal antibody against CD40 also resulted in activation of κB DNA-binding activity in EMSAs (Fig. 4a). The slower migrating DNA–protein complex was supershifted by an anti-c-Rel antibody that does not recognize RelA (Fig. 4a, lane 4), whereas a faster migrating complex was supershifted with an anti-RelA-specific antibody (Fig. 4, lane 3). CD40-mediated activation of c-Rel also induced TRAIL-R2 expression (Fig. 4b), but (unlike IR) it protected lymphocytes from TRAIL-induced death (Fig. 4d). Either CD40 ligation or IR activated c-Rel, but RelA-induced transcriptional activation of a HIV-CAT reporter (driven by two κB sites contained

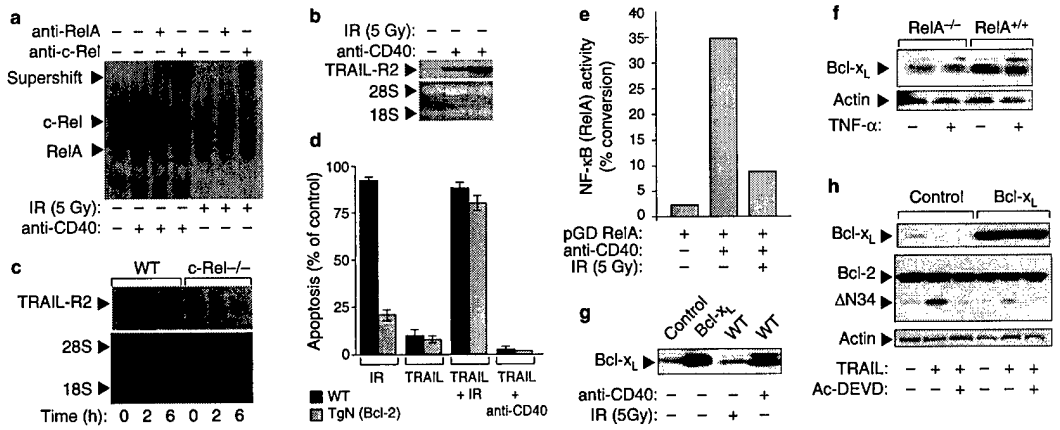

Figure 4 **The RelA subunit of NF-κB induces Bcl-x_L and protects cells from TRAIL/death receptor-induced apoptosis. a**, NF-κB DNA-binding activity in nuclear extracts of primary mouse B lymphocytes exposed to either anti-CD40 antibody (10 μg ml^{-1} for 16 h) or ionizing radiation (IR; 5 Gy). Supershift (SS) analysis of DNA–protein complexes was performed with anti-c-Rel- or anti-RelA-specific antibodies. **b**, Northern blot analysis of TRAIL-R2/DR5 expression in primary mouse B cells exposed to either anti-CD40 antibody or IR (5 Gy). **c**, Northern blot analysis of IR-induced expression of TRAIL-R2 in primary mouse B cells from wild-type (WT) or c-$Rel^{-/-}$ mice. **d**, Effect of IR (5 Gy), TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}), IR + TRAIL, or anti-CD40 antibody + TRAIL on survival of mouse B lymphocytes from WT or TgN(Bcl-2) mice. Data (mean ± s.d.) are the percentage apoptosis relative to untreated controls ($n = 3$). **e**, RelA-mediated HIV-CAT expression in activated B lymphocytes in response to CD40 or IR. **f**, Immunoblot analyses of basal or TNF-α-induced expression of Bcl-x_L in $RelA^{+/+}$ or $RelA^{-/-}$ fibroblasts. **g**, Expression of Bcl-x_L in mouse B cells in response to CD40 ligation or IR. HL-60-Neo (Control) or Bcl-x_L-overexpressing HL-60 (Bcl-x_L) cells were used as controls. **h**, Inhibition of caspase-3-mediated cleavage of Bcl-2 and TRAIL-induced death by expression of Bcl-x_L. HL-60-Neo (Control) or HL-60-Bcl-x_L (Bcl-x_L) cells were exposed to TRAIL (100 ng ml^{-1}) with or without pretreatment with Ac-DEVD-CHO (300 μM) and analysed for expression of Bcl-x_L and Bcl-2 12 h later. The full-length Bcl-2 (26K) and the Bcl-2 cleavage product (23K; ΔN34) are indicated. Percentage of each cell population that underwent apoptosis after 24 h: HL-60-Neo, 80 ± 5%; HL-60-Bcl-x_L cells, 27 ± 3%.

in the long-terminal repeat) was increased by anti-CD40 treatment but not by exposure to IR (Fig. 4e). This suggested that co-activation of RelA by CD40 ligation might inhibit TRAIL-induced apoptosis through RelA-induced expression of survival factor(s).

Compared with $RelA^{+/+}$ cells, $RelA^{-/-}$ cells exhibited reduced basal and TNF-α-inducible expression of the apoptosis inhibitor, Bcl-x_L (Fig. 4f). As Bcl-x_L expression in resting B cells was increased markedly in response to anti-CD40 (Fig. 4g), we investigated whether Bcl-x_L could inhibit TRAIL-induced death. Exposure of HL-60 cells (expressing wild-type Bcl-2; relative molecular mass (M_r) 26,000 (26K)) to TRAIL (100 ng ml^{-1}) resulted in the death of more than 80 ± 5% of the population within 24 h of treatment. This was associated with the appearance of a caspase-3-dependent 23K Bcl-2 cleavage product (Fig. 4h), previously identified as a C-terminal fragment (ΔN34; cleaved at Asp34) that lacks the loop domain and functions as a Bax-like death effector[44]. Stable transfection of a vector encoding Bcl-x_L into HL-60 cells inhibited caspase-3-dependent cleavage of Bcl-2 (Fig. 4h) and reduced TRAIL-induced apoptosis (27 ± 3% death of the total population at 24 h). Therefore, the reduction of TRAIL-induced apoptosis of B cells in the presence of anti-CD40 (despite c-Rel-mediated expression of TRAIL-R2) reflects the dominant protective effect of Bcl-x_L induced through the co-activation of RelA in activated B cells.

Together, these results illustrate the biological significance of NF-κB activity in regulating expression of both the death receptors and survival factors that determine cellular sensitivity to TRAIL. Our observations suggest that IR-induced NF-κB-mediated induction of death receptors can synergize with TRAIL to eliminate B cells overexpressing Bcl-2—a finding that may have implications for the treatment of resistant tumours, such as human follicular lymphomas. Our studies also indicate that RelA-mediated expression of Bcl-x_L may be responsible for the resistance of CD40-activated or transformed B cells to apoptotic signals transduced by death receptors.

Inhibition of NF-κB by blocking activation of the IKK complex sensitizes tumour cells to TRAIL. To determine the physiological significance of NF-κB in both the regulation of death receptor signalling and the sensitivity of tumour cells to TRAIL, we examined the effect of recombinant heregulin β1 (HRG β1), a ligand that induces HER-2/neu (c-erbB2)-mediated activation of NF-κB[45]. Exposure of MCF-7 human breast cancer cells to HRG β1 increased κB DNA-binding activity in EMSAs (Fig. 5a), and increased expression of TRAIL-R1 (4.2-fold induction relative to an actin control) and TRAIL-R2 (3.0-fold induction) (Fig. 5b). However, exposure of MCF-7 cells to HRG β1 also promoted the expression of Bcl-x_L (3.4-fold induction), and rendered them relatively resistant to TRAIL (Fig. 5d, e).

Activation of NF-κB requires the phosphorylation and ubiquitin-mediated degradation of IκBα by the IKK complex, which contains two kinases (IKK-α and IKK-β), and the regulatory protein NEMO (NF-κB essential modifier)[46]. A cell-permeable peptide (NEMO-binding domain (NBD) peptide) that blocks the interaction of NEMO with the IKK complex inhibits cytokine-induced NF-κB activation[46]. The anti-inflammatory agent, acetyl salicylic acid (aspirin; ASA), also specifically inhibits the activity of IKK-β[47].

Inhibiting activation of the IKK complex by either ASA or the wild-type NBD peptide prevented HRG β1-induced loss of IκBα or activation of NF-κB (Fig. 5a, b). Exposure of MCF-7 cells to either ASA or wild-type NBD (but not a mutant NBD peptide) prevented HRG β1 from either inducing expression of TRAIL-R1, TRAIL-R2 or Bcl-x_L (Fig. 5b, c). Exposure to either ASA or wild-type NBD (but not mutant NBD) inhibited HRG β1-mediated protection of MCF-7 cells from TRAIL-induced apoptosis (Fig. 5d, e). These data indicate that NF-κB promotes expression of both death receptors for TRAIL and Bcl-x_L, a protein that blocks death signals transduced by TRAIL. The dominant anti-apoptotic effect of Bcl-x_L allows NF-κB-activating cytokines, such as HRG β1, to confer protection against TRAIL. Conversely, inhibition of NF-κB after death receptor ligation can sensitize tumour cells to TRAIL.

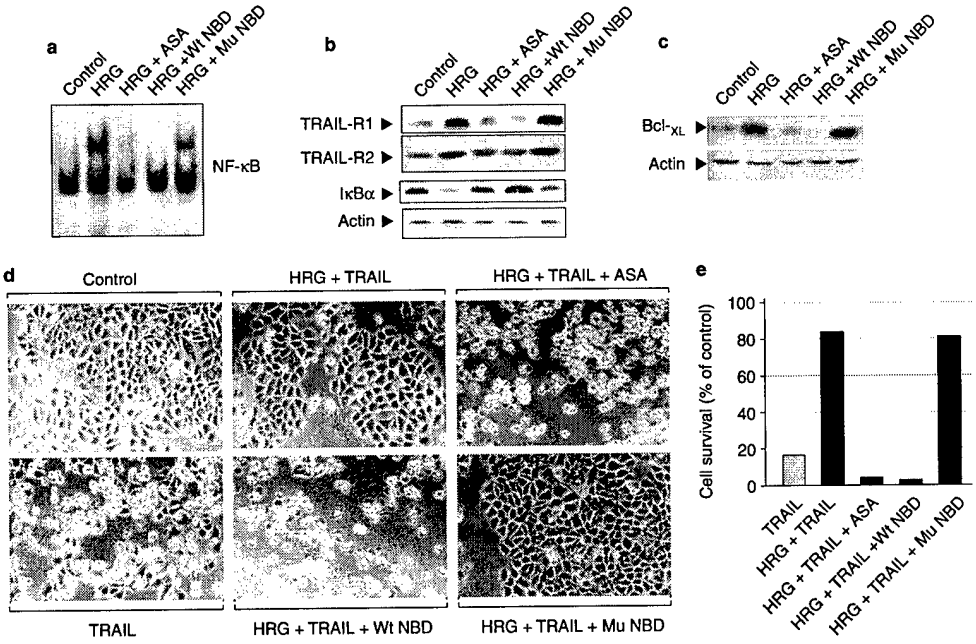

Figure 5 **Inhibition of NF-κB by blocking activation of the IKK complex sensitizes tumour cells to TRAIL. a**, EMSA of NF-κB DNA-binding activity in nuclear extracts of MCF-7 cells exposed to recombinant heregulin β1 (HRG) in the absence or presence of either aspirin (ASA; 3 mM), a cell-permeable peptide spanning the IKKβ NEMO-binding domain (wild-type (Wt NBD) or mutant (Mu NBD); 250 μM). Untreated MCF-7 cells were used as controls (Control). **b**, **c**, Immunoblot analyses of TRAIL-R1, TRAIL-R2, IκBα and Bcl-x_L protein expression in MCF-7 cells after exposure to HRG for 12 h (in the absence or presence of either ASA, Wt NBD or Mu NBD). Untreated MCF-7 cells served as controls. **d**, **e**, Untreated or HRG-treated MCF-7 cells were exposed to TRAIL (100 ng ml^{-1} + enhancer antibody 2 mg ml^{-1}) in the absence or presence of either ASA (3 mM), Wt NBD (250 μM) or Mu NBD (250 μM) for 24 h. Representative photomicrographs illustrating the survival/apoptosis of MCF-7 cells in each group are shown in **d**. Data in **e** represent the percentage survival (viable/[apoptotic + viable]) in each cell population (mean) from three independent experiments.

Discussion

NF-κB has apparently conflicting roles in the regulation of cell survival in several well-defined physiological systems and pathological states[25–39]. Targeted disruption of the RelA subunit of NF-κB results in massive hepatic apoptosis and the embryonic death of mice[26]. RelA deficiency or NF-κB inhibition by phosphorylation mutants of IκBα sensitizes cells to TNF-α-induced death[27–30]. Activation of NF-κB by co-stimulation of lymphocytes mediates cell survival and clonal proliferation, and inhibition of NF-κB by IκB mutants promotes activation-induced apoptosis of T cells, and loss of $CD8^+$ T cells in the thymus[31].

In contrast to its demonstrated protective role in these studies, NF-κB can adopt a pro-apoptotic function in other circumstances. Constitutive activation of NF-κB in mouse embryos through targeted disruption of IκBα results in a lethal phenotype manifesting thymic and splenic atrophy[36], and high levels of the c-Rel subunit of NF-κB are observed during apoptosis in the developing avian embryo[37]. NF-κB has also been reported to be essential in p53-mediated apoptosis[38]. NF-κB exhibits contrasting effects on neuronal cell survival; while it protects neurons from β-amyloid-induced death, it promotes cell death in cerebral ischaemic and neurodegenerative disorders[39]. Activation of NF-κB by ischaemic or stress-induced signals, such as hypoxia or DNA damage, may be protective in some situations and detrimental in others. These observations raise a fundamental issue of how NF-κB can have divergent effects on cell survival depending on the cell type and the specific activating signal.

Here we have shown that NF-κB induces the expression of both death receptors (TRAIL-R1,TRAIL-R2) and survival genes such as Bcl-x_L; however, the κB motifs in pro- or anti-apoptotic genes seem to exhibit selective affinity for activation by dimers composed of specific subunits of NF-κB. The varying phenotypes of knockout mice lacking individual Rel proteins reveal that the different subunits share certain functions, but also perform unique roles that cannot be complemented and may even be opposed by other family members. As κB sites on certain survival or pro-apoptotic genes exhibit specific preferences for RelA and c-Rel, the balance between different NF-κB dimers may determine the susceptibility of cells to diverse stressful stimuli that activate NF-κB.

Although our results suggest that subunit-specific regulation of death-modulating genes provides a mechanism that may underlie the seemingly paradoxical effects of NF-κB on cell survival, it is also conceivable that dimers composed of either subunit could have different effects depending on the cell type and the circumstances or duration of activation. For example, RelA seems able to stimulate expression of Fas/CD95 (ref. 48), and c-Rel can induce expression of genes such as inducible nitric oxide synthase (iNOS), interleukin-2 or Bfl-1/A1 (refs 33, 34), which may serve anti-apoptotic functions. In situations where activity of a particular subunit is deregulated, it may also adopt a promiscuous ability to induce 'death' or 'survival' genes that are not the normal transcriptional targets. As such, the final cellular response to apoptotic signals may be determined by the relative activity of different dimers comprising specific subunits, as well as by the duration and level of activity of the particular dimers involved.

Identifying approaches that sensitize cancer cells to apoptosis while concurrently protecting normal tissues might improve the

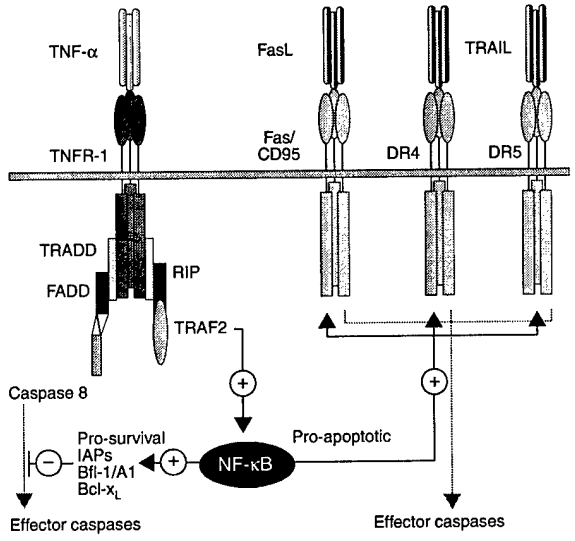

Figure 6 **Representation of the molecular determinants of the contrasting effects of NF-κB on cell survival**. TNF-induced aggregation of the death domains of TNFR1 enables recruitment of the adapter protein TRADD (TNFR1-associated death domain). The death domain of TRADD recruits FADD (Fas-associating protein with death domain)/Mort1 which, in turn, binds and activates caspase-8, the proximal member of a cascade of effector caspases that execute cell death. The TNFR1–TRADD complex also recruits proteins (TRAF2 and RIP) which signal the activation of NF-κB. Activation of NF-κB protects cells against TNF-α- or TRAIL-induced death through induction of pro-survival genes, such as members of the IAP family (c-IAP1, c-IAP2, XIAP) or the Bcl-2 homologues, Bfl-1/A-1 and Bcl-x_L. NF-κB may also function as a pro-death factor by inducing expression of death receptors (CD95/Fas, TRAIL-R1/DR4, TRAIL-R2/DR5) which trigger caspase activation and apoptosis.

therapeutic ratio of anticancer agents. Although the activation of TRAIL-R1/TRAIL-R2 signalling by TRAIL offers a potential mechanism of inducing apoptosis in tumours that resist conventional genotoxic therapy, the therapeutic ratio of this approach depends on the differential basal expression of death or decoy receptors and pro-survival proteins in tumour cells and normal tissues[20].

Our studies indicate that the composition and activity of NF-κB in tumour cells is a key determinant of the expression of TRAIL receptors or survival proteins and their susceptibility to apoptosis after ligation with TRAIL. Our data also indicate that TRAIL can synergize with genotoxic agents to eliminate p53-deficient or Bcl-2-overexpressing tumour cells that are otherwise resistant to DNA-damage-induced apoptosis. However, endogenous or cytokine-induced activation of the RelA subunit induces Bcl-x_L and protects tumour cells from TRAIL. Most significantly, our findings indicate that inhibiting NF-κB after the ligation of death receptors can reduce Bcl-x_L expression and sensitize tumour cells to TRAIL-induced apoptosis. The identified roles of NF-κB in death receptor expression and signalling may aid the rational design of regimens using TRAIL to eliminate tumour cells while sparing normal tissues. □

Methods

Cell lines and cell culture.

RelA$^{+/+}$ and RelA$^{-/-}$ mouse fibroblasts[26] (A. A. Beg, Columbia Univ., USA), c-Rel$^{-/-}$ mouse fibroblasts[40] (S. Gerondakis, WEHI, Australia), p53$^{-/-}$ MEFs (T. Jacks, MIT, USA), and MEFs stably transduced with a plasmid encoding IκBαM (pLIκBαMSN) or the empty control vector (pLXSN)[28] (D. R. Green, La Jolla Institute of Allergy and Immunology, USA) were maintained in high-glucose DMEM (Life technologies, Inc.) supplemented with 10% fetal calf serum (FCS), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C and 5% CO_2.

Genes encoding c-Rel (CCR), Δc-Rel (CCR-H; a c-Rel truncation mutant lacking the C-terminal transactivation domain owing to a stop codon at the unique *Hin*cII site of c-Rel), or RelA were conditionally expressed in HeLa (HtTA-1) cells using a tetracycline-regulated system[33,41]. HtTA-1 cells, which stably express a fusion protein comprising the *E. coli* tetracycline repressor and the activation domain of the herpes simplex virus VP16 protein (tTA), were a gift from H. Bujard (Heidelberg, Germany). We transfected HtTA-1 cells with pUHD10-3–CCR (encoding chicken c-Rel complementary DNA under control of the minimal cytomegalovirus promoter and hepatamerized tetracycline operator sites of pUHD10-3) and pHMR272 (to confer resistance to hygromycin B), pUHD10-3–hygro–CCR-H (encoding Δc-Rel), or pUHD10-3–hygro–RelA using a modified calcium phosphate procedure. Cell clones stably transduced with c-Rel, Δc-Rel or RelA were selected in hygromycin B (225 U ml^{-1}; Calbiochem) and maintained in DMEM supplemented with 10% FCS, 125 μg ml^{-1} G418 (Gibco), 225 U ml^{-1} hygromycin B (Calbiochem), 2 μg ml^{-1} tetracycline (Sigma), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C and 5% CO_2. We induced gene expression by washing the cells and transferring them to tetracycline-free medium for 48–72 h.

The parental HCT116 human colon adenocarcinoma cell line, containing wild-type p53 (*p53*$^{+/+}$), and a p53-deficient derivative (*p53*$^{-/-}$), created by homozygous deletion through homologous recombination (B. Vogelstein, Johns Hopkins Oncology Center, USA), were cultured as described[42]. We isolated primary B lymphocytes from splenocytes obtained from wild-type, Bcl-2-overexpressing (TgN(Bcl-2)36Wehi)[43] (Jackson Laboratories) and c-Rel$^{-/-}$[40] mice. *In vitro* activation of mouse B lymphocytes was performed by anti-CD40 monoclonal antibody (10 μg ml^{-1}, clone HM40-3; Pharmingen) and goat anti-mouse IgG (H+L, 10 μg ml^{-1}; Jackson ImmunoResearch). HL-60 cells transfected with an expression vector encoding Bcl-x_L (HL-60-Bcl-x_L) or control vector (HL-60-Control) (K. Bhalla, Emory University, USA) were cultured as described[49]. We inhibited caspase-3 in HL-60 cells by pre-treating the cells with Ac-DEVD-CHO (300 μM; Bachem) for 30-min before administering TRAIL.

MCF-7 human breast cancer cells were maintained in RPMI medium (Life technologies) supplemented with 10% fetal calf serum (FCS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C and 5% CO_2. MCF-7 cells were cultured in the presence or absence of recombinant heregulin β1 (100 ng ml^{-1}; Neomarkers, Fremont, CA), as indicated.

Exposure to ligands of the TNF family.

We incubated cells in either 10–100 ng ml^{-1} recombinant human TRAIL with enhancer antibody (2 μg ml^{-1}; Alexis Corporation), or 100 ng ml^{-1} rh TNF-α (R & D Systems) for 24 h at 37 °C.

Exposure to inhibitors of the IKK complex.

We inhibited the IKKβ–NEMO interaction and HRG β1-induced NF-κB activation by incubating MCF-7 cells with a 250 μM concentration of a cell-permeable peptide spanning the IKKβ NBD. The sequence of the wild-type peptide indicating the Antennapedia homeodomain (lower case) and the IKKβ (upper case) segments and the mutant peptide with the positions of the W to A mutations (underlined) are as follows[46]: wild type, drqikiwfqnrrmkwkkTALDWSWLQTE; mutant, drqikiwfqnrrmkwkkTALDASALQTE. Both peptides (Genemed Synthesis, San Francisco) were supplied as a 20 mM solution in dimethyl sulphoxide (DMSO). Results for DMSO controls were not different from controls using no peptide. We treated MCF-7 cells with 3 mM acetyl salicyclic acid (aspirin; Sigma), obtained from a 1.0 M stock solution prepared in 0.05 M Tris-HCl.

Exposure to ionizing radiation.

Ionizing radiation (IR) (500 cGy) was delivered with a ^{137}Cs dual source γ-cell irradiator.

Expression vectors and cell transfections.

RelA–/– or c-Rel–/– MEFs were plated at ~50% confluence 16–24 h before serum-free lipofectin-mediated transfection (12–16 h) with either empty pCEP4 vector or pCEP4-KILLER/DR5 (ref. 10; W. El-Deiry, HHMI, Univ. Pennsylvania). Transiently transfected cell populations were assessed for apoptosis 48 h later, and then selected with 0.5 mg ml^{-1} hygromycin B for another 14 d before examination after staining with crystal violet.

Electrophoretic mobility shift assays.

Nuclear extracts were prepared as described[50]. Double-stranded oligonucleotides containing either a consensus binding site for c-Rel (5′-GGG GAC TTT CCC-3′) (Santa Cruz Biotechnology) were 5′ end-labelled using polynucleotide kinase and [γ-^{32}P]dATP. Nuclear extracts (2.5–5 μg) were incubated with ~1 μl of labelled oligonucleotide (20,000 c.p.m.) in 20 μl of incubation buffer (10 mM Tris-HCl, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, 1–2 μg of poly dI-dC) for 20 min at 25 °C. The specificity of NF-κB DNA-binding activity was confirmed by competition with excess cold wild-type or mutant oligonucleotide or supershift with either a rabbit polyclonal antibody against c-Rel (Ab-3)[41] or p65 (RelA) (sc-109, Santa Cruz Biotechnology). DNA–protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and analysed by autoradiography.

Reporter assays.

We co-transfected CD40-activated B cells with HIV1-LTR-CAT reporter, RelA expression vector (pGD RelA) (using reporter and activator DNA in a 1:2 ratio) and a β-galactosidase expression vector (poN260). Transfected cells were irradiated (5 Gy) or left untreated, and assessed 6 h later for HIV-CAT expression using thin layer chromatography and a PhosphorImager (Molecular Dynamics)[50]. HIV-CAT activity was expressed as percentage conversion and normalized to β-galactosidase activity.

Immunoblot assays.

Cell lysates were prepared as described[50], and 50–100 μg of protein were resolved by SDS–PAGE, transferred onto Immobilon-P PVDF membrane (Millipore), and probed with appropriate dilutions of the following primary antibodies: anti-c-Rel (Ab-3)[41]; anti-IκBα (C-12), anti-p65 (RelA)(sc109), anti-Bcl-x_L (S-18), anti-Bcl-2 (100) and anti-Actin (C-11) (Santa Cruz Biotechnology); rabbit polyclonal anti-TRAIL-R1 (AHR5012; Biosource International) or anti-TRAIL-R2 (AHR5022; Biosource International). We visualized immunoreactive protein complexes by enhanced chemiluminescence (Amersham).

RNA extraction and northern blot hybridization.

Total RNA was extracted using Trizol (Life Technologies). RNA samples (20 μg) were analysed in 1.2% agarose–formaldehyde gels, transferred onto Zeta probe membranes (Biorad), and ultraviolet crosslinked with a Stratalinker (Stratagene). The membranes were hybridized to the following ^{32}P-labelled probes: (1) human TRAIL-R1/DR4 cDNA (Alexis Corporation); (2) a partial length cDNA representing a 364-bp *Eco*R1 fragment of mouse KILLER/DR5 (W. El-Deiry, HHMI, Univ. Pennsylvania); (3) human TRAIL-R2/DR5/KILLER cDNA (Alexis); and (4) β-actin. We washed membranes in SSPE/0.1% SDS at 65 °C, and visualized them by autoradiography.

Flow cytometry.

HtTA cells induced to express either c-Rel (CCR43) or Δc-Rel (CCR-H5) for 48 h were irradiated (10 Gy) or left untreated. After 12h, cells were collected with trypsin (0.25% at 37 °C for 5 min), washed twice in PBS at 4 °C and FACS buffer (PBS, 1% BSA, sodium azide 0.05%), and stained with goat anti-human TRAIL-R2 polyclonal antibody (AB1687; Chemicon International) for 30 min at 4 °C. Cells were washed twice with FACS buffer, exposed to PE-conjugated donkey anti-goat IgG for another 30 min at 4 °C (Sigma), and analysed by a FACScan flow cytometer (Becton Dickinson). Cells treated with secondary antibody alone were used as unstained negative controls.

Confocal microscopy.

HtTA cells were seeded onto eight-well chamber slides (Nunc, Naperville, IL) and induced to express either c-Rel (CCR43) or Δc-Rel (CCR-H5) for 48 h before fixation in 2% paraformaldehyde for 5 min. We permeabilized the cells with 0.1% saponin in PBS containing 10% human AB serum for 10 min, and then incubated them with goat anti-human TRAIL-R2 polyclonal antibody (AB1687; Chemicon International)(1:300 in PBS containing 1% human AB serum) at 4 °C for 1 h. After washing with PBS, cells were incubated with FITC-conjugated donkey anti-goat IgG (H+L) secondary antibodies (Molecular Probes, Eugene, OR) at 4 °C for 45 min. Cells were counterstained with propidium iodide and examined using a Zeiss Axiophot microscope with a confocal attachment and a digital camera.

Analysis of cell death.

Cells were assessed for morphological features of apoptosis using phase-contrast microscopy. We quantified cell survival at the indicated intervals by trypan blue dye exclusion. The cell viability was measured by scoring at least 200 cells in each group, and the average per cent viability was calculated from three different experiments.

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Sensitization of Breast Cancers to TRAIL/Apo2L-induced Apoptosis by Inhibition of the IκB Kinase-NEMO Complex[1]

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[3] The abbreviations used are: TRAIL/Apo2L, tumour necrosis factor-related apoptosis-inducing ligand/ Apo2 ligand; TRAIL-R, TRAIL receptor; TNF, tumor necrosis factor; DR, death receptor; IGF-1 – insulin-like growth factor-1; EGFR, epidermal growth factor receptor; IGF-1R, IGF-1 receptor; PI3-K, phosphatidylinositol 3'-kinase; NF-κB - nuclear factor-κB; IκBα - inhibitor of κB; IKK - inhibitor of κB kinase; NEMO – NF-κB essential modifier/modulator; IKKAP1, IκB kinase-associated protein; WT NBD peptide, wild-type NEMO-binding domain, MU NBD, mutant NEMO-binding domain; PNPP, p-nitrophenyl phosphate; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; β-gal, β-galactosidase; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis protein.

ABSTRACT

Breast cancer cells can be induced to undergo apoptosis by engagement of death receptors with TRAIL (Tumour necrosis factor-related apoptosis-inducing ligand)/Apo2L. Here we show that activation of the PI3 Kinase-Akt-NF-κB signaling pathway by HER-2/neu or insulin-like growth factor-1 (IGF-1) renders breast cancer cells relatively resistant to TRAIL/Apo2L-induced apoptosis. Activation of NF-κB requires phosphorylation-dependent degradation of IκB by an IκB kinase (IKK) complex comprising the regulatory protein NEMO (NF-κB essential modifier) in association with two kinases (IKKα and IKKβ). A cell-permeable NEMO-binding domain peptide that blocks the interaction of NEMO with the IKK complex inhibits HER-2/neu- or IGF-1-mediated activation of NF-κB and sensitizes breast cancer cells to TRAIL/Apo2L-induced death. The efficacy of TRAIL/Apo2L in the treatment of breast cancers may be improved by antibody-mediated inhibition of growth factor receptors (HER2/neu or IGF-1R) and/or peptidomimetic drugs that disrupt the IKK-NEMO complex.

INTRODUCTION

Genetic aberrations that render cells incapable of executing apoptosis not only promote tumorigenesis, but also underlie the observed resistance of human cancers to genotoxic anticancer agents (1, 2). Unraveling mechanisms to unleash the apoptotic program in tumor cells could aid the design of effective therapeutic interventions against resistant breast cancers. One mechanism of triggering tumor cell death involves engagement of death receptors belonging to the tumor necrosis factor (TNF^3) receptor gene superfamily, such as TRAIL-R1 (DR4), and TRAIL-R2 (DR5, TRICK2, KILLER) by their cognate "death ligand", TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)/Apo2L (3-5). TRAIL/Apo2L induces apoptosis of many breast cancer cell lines *in vitro*, and its tumoricidal activity and safety *in vivo* has been confirmed in preclinical animal models of human breast cancer xenografts (5). Although TRAIL/Apo2L inhibits tumor growth when administered immediately after xenotransplantation, delaying application of the same treatment is less effective against more established tumors (5). Moreover, human breast cancer cell lines exhibit a wide heterogeneity in their sensitivity to TRAIL/Apo2L *in vitro*. These data suggest that successful treatment of breast cancers with TRAIL/Apo2L may require its combination with agents that inhibit survival signals responsible for protecting tumor cells from death receptor-induced apoptosis.

Amplification and consequent overexpression c-erbB2 (HER-2/neu), a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, has been observed in a significant proportion of human breast cancers (~30%) and is correlated with increased metastatic potential, resistance to apoptosis, and poor prognosis (6). Akin to EGFR, IGF-1 receptor (IGF-1R) overexpression in breast cancers has been associated with resistance to radiation and chemotherapeutic agents (7). Both HER-2/neu and IGF-1R promote PI3 kinase (PI3-K)-mediated phosphorylation and activation of Akt, a serine-threonine kinase that, in turn, activates the IκB kinase (IKK) complex (8-10). Activation of the IKK complex requires association of the regulatory protein, NEMO (NF-κB essential modifier)/IKKγ/IKKAP1, with the NEMO-binding domains (NBD) of two catalytic subunits, IKKα (IKK-1) and IKKβ (IKK-2)(11). The activated IKK complex induces phosphorylation-mediated degradation of IκB, thereby promoting activation of NF-κB, a family of dimeric transcription factors that protects cells from TRAIL/Apo2L-induced apoptosis (12). Breast cancers frequently exhibit constitutive NF-κB activity associated with aberrant activation of the IKK complex (13).

In this study, we examined whether HER-2/neu or IGF-1 inhibit TRAIL/Apo2L-induced apoptosis of breast cancer cells via activation of the PI3-K-Akt-NF-κB signaling pathway. We investigated whether breast cancer cells can be sensitized to TRAIL/Apo2L by inhibiting NF-κB with a cell-permeable NEMO-binding domain peptide that prevents formation of the IKKβ-NEMO complex.

MATERIALS AND METHODS

Cell lines and transfections. The MCF-7, SKBr-3, and Hs578 human breast cancer cell lines were cultured in RPMI 1640, McCoy's 5A, and DMEM medium, respectively, supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). MCF-7 cells overexpressing HER-2/neu (MCF-7-neu) were generated by LipofectAMINE transfection (Invitrogen/Life Technologies, Inc., Gaithersburg, MD) with an expression vector encoding neu and selection of stably transfected clones with 300 μg/ml G418 (Invitrogen/Life Technologies). MCF-7, MCF-7-neu, SKBr-3, and Hs578 cells were transiently cotransfected with either wild-type Akt (HA-Akt) or a catalytically inactive mutant of Akt [HA-Akt(K179M)](provided by Dr. Michael E. Greenberg, Harvard Medical School, Boston, MA) together with an expression vector for β-galactosidase (CMV-β-Gal)(pON260) for 16h, as described (10). All cells were cultured at 37°C and 5% CO_2.

Treatment with recombinant human TRAIL/Apo2L. Exponentially growing cells (2 x 10^5/well) were incubated in 100 ng/ml recombinant human TRAIL with enhancer antibody (2 μg/ml)(Alexis, San Diego, CA).

Stimulation with recombinant human IGF-1. Exponentially growing cells (2 x 10^5/well) were washed twice in serum-free medium and then placed in medium containing recombinant human IGF-1 (50 ng/ml)(R&D Systems, MA) for 24-36h before, and continuing for the duration of, the experiment.

Treatment with trastuzumab (Herceptin™). To inhibit HER-2/neu signaling, exponentially growing cells (2 x 10^5/well) were treated with trastuzumab (Herceptin™)(1 μM)(Genentech, South San Francisco, CA) for 24-36h before, and continuing for the duration of, the experiment.

Inhibition of PI3-Kinase. Inhibition of PI3-K was achieved by treatment of cells with LY294002 (10 μM)(Cell Signaling Inc., Beverly, MA) beginning 1h before, and continuing for the duration of, the experiment. Control cells received dimethyl sulfoxide (vehicle) at the same concentration.

Immunoblot Assays. Cell lysates were prepared as described (12), and 50-100 μg of protein were resolved by SDS-PAGE, transferred onto Immobilon-P PVDF membrane (Millipore, Bedford, MA), and probed with primary antibodies against either Phospho-Akt (Ser473), Akt (Cell Signaling Technology, Beverly, MA), or IKKβ (H-470)(Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive protein complexes were visualized with enhanced chemiluminescence (Amersham, IL).

Inhibition of the IKK complex. Inhibition of the IKKβ-NEMO interaction and NF-κB activation was achieved by incubation of cells with 100-200 μM of a cell-permeable peptide spanning the IKKβ NEMO-binding domain (NBD) (14). The sequence of the wild-type peptide (WT NBD) indicating the Antennapedia homeodomain (lower case) and the IKKβ (upper case) segments and the mutant peptide (MU NBD) with the positions of the W→A mutations (underlined) are indicated below (14):

Wild type: drqikiwfqnrrmkwkkTALDWSWLQTE

Mutant: drqikiwfqnrrmkwkkTALDASALQTE

[Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and W, Trp]. Both peptides were supplied dissolved in dimethyl sulfoxide (DMSO)(Genemed Synthesis Inc., South San Francisco, CA).

IKK Kinase Assays. IKK complexes were immunoprecipitated from whole cell extracts (500 μg) using an antibody against IKKβ (M-280)(Santa Cruz Biotechnology). One half of the immunoprecipitate was subjected to a kinase assay at 30°C for 30 min in kinase buffer [20 mM HEPES (pH 7.6), 3 mM $MgCl_2$, 10 μM ATP, 3 μCi [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT] containing 500 ng of GST-IκBα fusion protein (GST-IκBα)(Santa Cruz Biotechnology, Santa Cruz, CA) as substrate. The kinase reaction was terminated by addition of 2X SDS-PAGE sample buffer, subjected to SDS-PAGE and autoradiography. The remaining half of the immunoprecipitate was subjected to immunoblot analysis, as described above.

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared as described (12). Double-stranded oligonucleotides containing either a consensus binding site for NF-κB (5'-GGG GAC TTT CCC-3') (Santa Cruz Biotechnology) were 5' end-labeled using polynucleotide kinase and [γ-^{32}P] dATP. 2.5-5 μg of

nuclear extracts were incubated with ~1 μl of labeled oligonucleotide (20,000 cpm) in 20 μl incubation buffer (10 mM Tris, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, and 1-2 μg of poly dI-dC) for 20 min at 25°C. The specificity of NF-κB DNA-binding activity was confirmed by competition with excess cold wild-type or mutant oligonucleotide or supershift with an antibody against p65/RelA(Geneka, Montreal, Canada), as described. DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and analyzed by autoradiography and densitometry (Molecular Dynamics).

Analysis of Cell death. Cells were assessed for morphological features of apoptosis using phase-contrast microscopy, and cell death at the indicated intervals was quantified as described (12). In experiments involving transient transfection, transfected cells identified by immunostaining for β-Gal expression were scored in a blinded manner for apoptosis by analysis of nuclear morphology using Hoechst 33258 (10). Average percent viability (mean ± SE) was calculated from triplicate measurements, with each experiment performed three times.

RESULTS

Inhibition of TRAIL/Apo2L-induced Apoptosis of Breast Cancer Cells by HER-2/neu or IGF-1. Exposure of MCF-7 breast cancer cells (expressing low levels of HER-2/neu) to TRAIL/Apo2L (100 ng ml^{-1}) resulted in death of 59±3% of the entire population over 72h in culture (Fig. 1, A and B). However, MCF-7 cells stably transduced with an expression vector encoding HER-2/neu (MCF-7-neu) were relatively resistant to the same concentration of TRAIL/Apo2L, with demise of only 27±4% of the cell population over the same time period (Fig. 1, A and B). Treatment of MCF-7-neu cells with trastuzumab (Herceptin™)(1 μM) increased sensitivity to TRAIL/Apo2L, such that the induction of apoptosis over 72h (51±5% of the cell population) approached that of MCF-7 cells treated with the same combination (62±3%) (Fig. 1, A and B). Akin to the effect of HER-2/neu overexpression, exposure to IGF-1 also inhibited TRAIL/Apo2L-induced apoptosis of MCF-7 cells, with death only 30±3 % of the cell population over 72h in culture (Fig. 1, A and B).

HER-2/neu or IGF-1 Protect Cells from TRAIL/Apo2L via PI3-K-dependent Activation of Akt. HER-2/neu and IGF-1R activate phosphoinositide-3-OH kinase [PI3-K], that, in turn, induces phosphorylation-mediated activation of the Akt serine/threonine kinase. MCF-7 cells exhibited low Akt kinase activity in

immunoblot assays using a phospho-Akt kinase-specific antibody (Fig. 1C). Compared to MCF-7 cells, MCF-7-neu cells exhibited greater levels of Akt kinase activity which declined in response to treatment with trastuzumab (Fig. 1C). Activation of Akt in both IGF-1-stimulated MCF-7 cells and MCF-7-neu cells was blocked by the PI3-K inhibitor LY294002 (Fig. 1C). Treatment with LY294002 sensitized both MCF-7-neu and IGF-1-exposed MCF-7 cells to TRAIL/Apo2L, with death of more than 80% of either cell population over 48h (Fig. 1D).

To investigate the role of Akt in HER-2/neu- or IGF-1-mediated protection of cells from TRAIL/Apo2L, MCF-7 and MCF-7-neu cells were cotransfected with expression vectors encoding either wild-type Akt (HA-Akt) or a dominant negative catalytically inactive mutant of Akt [HA-Akt(K179M)] (10), together with an expression vector for β-galactosidase (CMV-β-Gal), and then maintained in the presence or absence of IGF-1. Following 48h of treatment with TRAIL/Apo2L, transfected cells (identified by immunostaining cells for β-Gal expression) were scored for apoptosis by analysis of nuclear morphology. MCF-7-neu cells transfected with HA-Akt showed only 19±4% apoptosis in response to TRAIL/Apo2L (Fig. 1D). In contrast, 69±5% of MCF-7-neu cells transfected with HA-Akt(K179M) exhibited apoptotic nuclear morphology following exposure to TRAIL/Apo2L (Fig. 1D). Likewise, IGF-1-stimulated MCF-7 cells transfected with HA-Akt(K179M) exhibited 65±5% apoptosis, while cells transfected with HA-Akt displayed only 23±3% in response to TRAIL/Apo2L (Fig. 1D). Consistent with Akt functioning downstream of PI3-K, HA-Akt was able to inhibit TRAIL/Apo2L-induced apoptosis even in the presence of LY294002 (Fig. 1D). Taken together, these results indicate that HER-2/neu or IGF-1R inhibit TRAIL/Apo2L-induced death of breast cancer cells by activation of the PI3-K-Akt survival signaling pathway.

HER2/neu and IGF-1 Stimulate NF-κB via Akt-induced Activation of the IKK-NEMO Complex. Electrophoretic mobility shift assays (EMSA) showed greater NF-κB DNA-binding activity in nuclear extracts prepared from MCF-neu cells compared to MCF-7 cells (Fig. 2A). Likewise, NF-κB DNA-binding activity was increased in MCF-7 cells following treatment with IGF-1 (Fig. 2A). To determine whether activation of NF-κB by HER-2/neu or IGF-1 was mediated by Akt, MCF-7 and MCF-7-neu cells were cotransfected with expression vectors encoding either HA-Akt or HA-Akt(K179M) together with CMV-β-Gal, and then maintained in the presence or absence of IGF-1 for 36h before assessment of NF-κB DNA-binding activity. Ectopic expression of HA-Akt(K179M) reduced NF-κB activity in MCF-7-neu cells and prevented IGF-1-induced activation of NF-κB

in MCF-7 cells (Fig. 2A). Conversely, MCF-7 cells (without IGF-1) cotransfected with HA-Akt exhibited increased NF-κB activity compared with cells transfected with the empty vector (Fig. 2A).

Signal-dependent activation of NF-κB requires phosphorylation and ubiquitin-mediated degradation of IκBα via the activity of the IκB-kinase (IKK) complex containing two kinases (IKK-α and IKK-β) and the regulatory protein NEMO (NF-κB essential modifier; IKK-γ) (11). *In vitro* phosphorylation assays of immunoprecipitated IKKβ using GST-IκBα as substrate showed greater IKKβ kinase activity in MCF-7-neu and IGF-1-treated MCF-7 cells compared to MCF-7 cells maintained without IGF-1 (Fig. 2B). An amino terminal α-helical region of NEMO interacts with six α_2-region residues in the carboxy terminal of IKKβ and IKKα, termed the NEMO-binding domain (NBD) (14). A cell permeable peptide spanning the IKKβ NBD (consisting of the region T735 to E745) fused with a sequence derived from the Antennapedia homeodomain for membrane translocation (wild-type NBD peptide; WT NBD) blocks the interaction of NEMO with the IKK complex and inhibits TNFα-induced NF-κB activation (14). A corresponding mutant NBD peptide (W739 and W741 mutated to alanines; MU NBD) does not disrupt signal-induced formation of the IKK-NEMO complex (14). To determine whether HER-2/neu- or IGF-1-induced activation of NF-κB requires formation of the IKK-NEMO complex, MCF-7 cells (maintained with or without IGF-1) or MCF-7-neu cells were incubated with either wild-type or mutant NBD peptides for 16h. Immune complex kinase assays using IKKβ immunoprecipitates showed that the wild-type NBD peptide, but not the mutant, reduced IKK activity in MCF-7-neu cells and prevented IGF-1-induced activation of IKK in MCF-7 cells (Fig. 2B). EMSA demonstrated that only the wild-type NBD peptide inhibited NF-κB DNA binding activity in MCF-7-neu or IGF-1-stimulated MCF-7 cells (Fig. 2C). These results indicate that HER-2/neu- or IGF-1-induced Akt-mediated activation of NF-κB can be prevented by disruption of the IKK-NEMO complex.

Blockade of the IKK-NEMO Interaction Sensitizes Breast Cancer Cells to TRAIL/Apo2L-induced Apoptosis. To determine whether inhibition of the interaction of NEMO with the IKK complex can sensitize HER-2/neu- or IGF-1-stimulated breast cancers to TRAIL/Apo2L-induced apoptosis, MCF-7 (in the presence IGF-1) and MCF-7-neu cells were exposed to TRAIL/Apo2L in the presence of either wild-type or mutant NBD peptide. MCF-7-neu cells treated with the wild-type NBD peptide alone resulted in death of 15±4% of the

population. Exposure of MCF-7-neu cells to TRAIL/Apo2L in the presence of the wild-type NBD peptide resulted in death of 67±3% of the cell population over 48h (Fig. 3, A and B). In contrast, the mutant NBD peptide had no effect of the viability of MCF-7-neu cells and failed to sensitize cells to TRAIL/Apo2L-induced death (28±5%)(Fig. 3, A and B). Treatment with the wild-type peptide, but not the mutant, also sensitized IGF-1-stimulated MCF-7 cells to TRAIL/Apo2L, with death of 74±4% of the cell population over 48h (Fig. 3, A and B).

To confirm that inhibition of the IKK complex can augment TRAIL/Apo2L-induced death of breast cancer cells that have endogenous amplification and overexpression of the HER-2/neu gene, the SKBr-3 cell line was pretreated with either trastuzumab (Herceptin™) or NBD peptides, and then exposed to TRAIL/Apo2L. Treatment with TRAIL/Apo2L alone resulted in death of 23±5% of SKBr3 cells over 48h (Fig. 3C). Exposure of SKBr3 cells to trastuzumab alone resulted in death of only 13±4% of the cell population over 48h (Fig. 3C). However, pretreatment with trastuzumab sensitized SKBr-3 cells to TRAIL/Apo2L, with death of 46±4% of the cell population over 48h (Fig. 3C). Treatment of SKBr-3 cells with wild-type NBD peptide, but not the mutant peptide, resulted in marked enhancement of sensitivity to TRAIL/Apo2L-induced death. The extent of TRAIL/Apo2L-induced apoptosis in SKBr-3 cells treated with wild-type NBD (73±3%) was not only greater that in cells treated with the mutant peptide (28±4%), but also larger than the level observed in cells pretreated with trastuzumab (Fig. 3, A and C).

While trastuzumab can negate the protective effects of HER-2/neu overexpression, breast cancer cells may also inhibit TRAIL/Apo2L induced apoptosis by activation of NF-κB via diverse other growth factor receptors or genetic alterations (10, 13). The Hs578 breast cancer cell line does not overexpress HER-2/neu, yet exhibits constitutively high IKKβ kinase activity (13). Hs578 cells were extremely resistant to TRAIL/Apo2L, with death of only 7±3% of the population over 48h (Fig. 3C). Unlike SKBr-3 cells, Hs578 cells could not be sensitized to TRAIL/Apo2L-induced death by treatment with trastuzumab (Fig. 3C). To determine whether breast cancer cells with constitutively active IKK can be sensitized to TRAIL/Apo2L by inhibition of the IKK-NEMO complex, Hs578 cells were pretreated with either wild-type or mutant NBD peptide and then exposed to TRAIL/Apo2L. Treatment with the wild-type NBD peptide, but not the mutant peptide, sensitized Hs578 cells to TRAIL/Apo2L-induced apoptosis (Fig. 3, A and C).

DISCUSSION

TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins containing cytoplasmic sequences, termed "death domains", which recruit and cross-activate the initiator procaspase-8 (FLICE) (3). Caspase-8-mediated proteolysis of BID enables its translocation to the mitochondria, where the truncated protein (tBID) promotes the release of pro-death cofactors (cytochrome c and Apaf-1) via heterodimerization with BAX or BAK (15). The activation of caspase-9 by these mitochondrial cofactors is responsible for the terminal proteolytic events that lead to cell death. Although TRAIL/Apo2L offers a potential mechanism of inducing apoptosis in breast cancers that resist conventional genotoxic therapy, we have demonstrated that TRAIL/Apo2L-induced death signals are inhibited by NF-κB (12). NF-κB protects tumor cells from apoptosis by inducing expression of the caspase-8-inhibitor FLIP, members of the inhibitors-of-apoptosis (IAP) family, and Bcl-x_L, a pro-survival member of the Bcl-2 family that counteracts BAX (12, 16, 17).

Our results indicate that overexpression of HER-2/neu or exposure to IGF-1 protects breast cancer cells from TRAIL/Apo2L-induced death via activation of the PI3K-Akt-NF-κB anti-apoptotic pathway. In accordance with a recent report, we find that inhibition of HER-2/neu signaling by trastuzumab (Herceptin™) sensitizes HER-2/neu-overexpressing breast cancer cells to TRAIL/Apo2L-induced apoptosis (18). However, activation of NF-κB is a final common effect of multiple genetic aberrations [such as amplification of diverse receptor tyrosine kinases (HER-2/neu, IGF-1R, EGFR), mutations of *ras*, or loss of *PTEN*], that operate in human breast cancers (8, 9, 19, 20). Activation of IKK is a converging point for activation of NF-κB by diverse growth factor receptors and genetic alterations (11), and a recent report showed that IKK is aberrantly activated in a high proportion of primary human breast cancers (13). Our results indicate that NBD peptides that interfere with the interaction of NEMO with the IKK complex can block the activation of NF-κB and sensitize breast cancers to TRAIL/Apo2L-induced apoptosis. These findings suggest that either antagonistic antibodies against growth factor receptors (HER-2/neu and IGF-1R) and/or peptidomimetic drugs that disrupt the IKK-NEMO complex may improve the efficacy of TRAIL/Apo2L in the treatment of human breast cancers.

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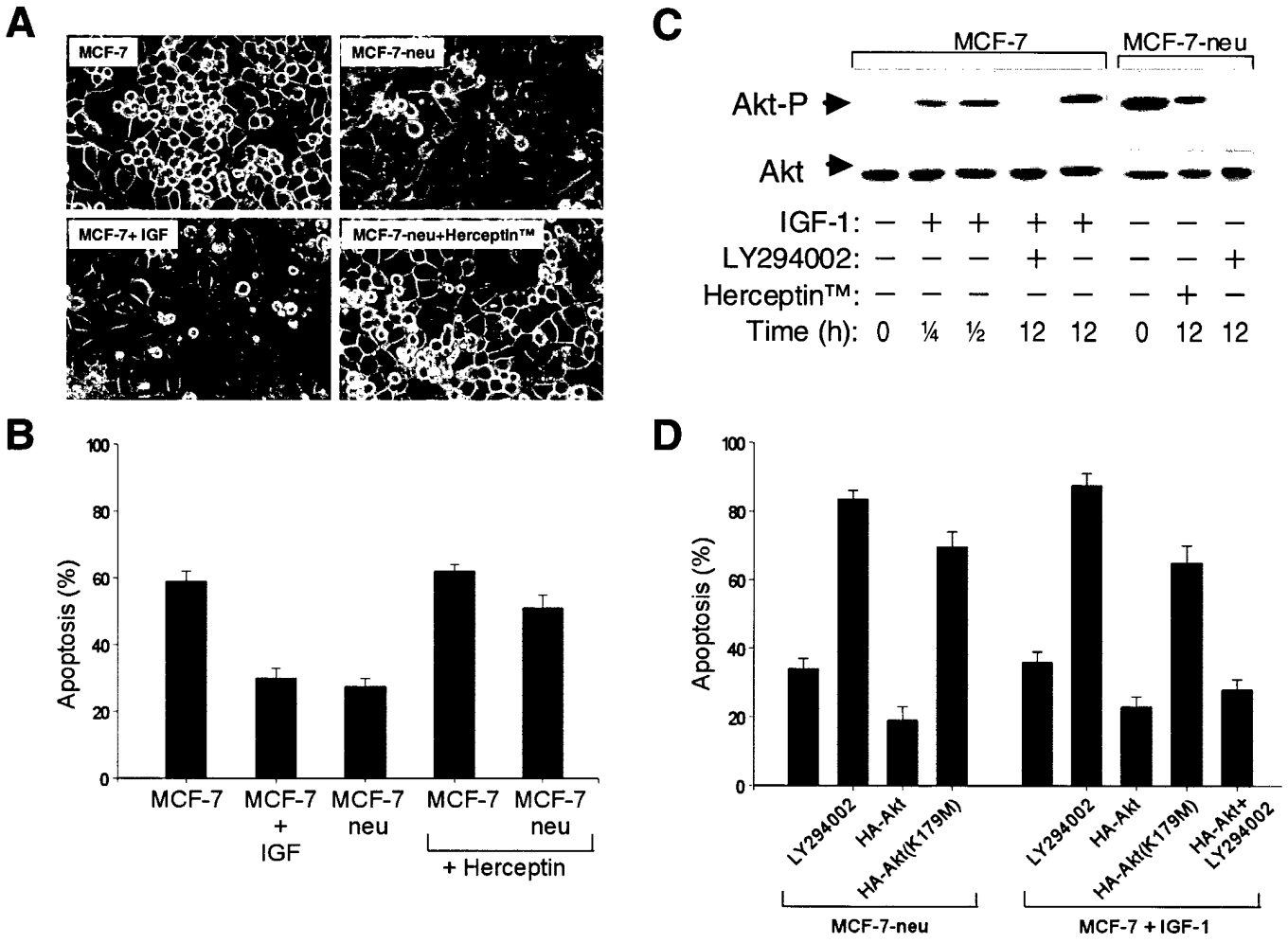
FIGURE LEGENDS

Figure 1. Inhibition of TRAIL/Apo2L-induced apoptosis of breast cancer cells by HER-2/neu or IGF-1 via PI3-K-dependent activation of Akt. ***A, B,*** MCF-7 and MCF-7-neu cells were incubated with or without IGF-1 (50 ng/ml) or trastuzumab (Herceptin™)(1 μM) for 24h and then exposed to TRAIL/Apo2L (100 ng/ml) for another 72h. *A*, Phase contrast photomicrographs of cells after 48h of treatment with TRAIL/Apo2L. *B*, Data represent the percentage of apoptotic cells as a function of total cells at 72h (mean ± SE; n=3). ***C,*** PI3-K-dependent phosphorylation of Akt by either IGF-1 or HER-2/neu. MCF-7 (with or without IGF-1, 50 ng/ml) and MCF-7-neu cells were pretreated with either LY294002 (10 μM) or trastuzumab (Herceptin™, 1 μM), and analyzed for Akt phosphorylation at the indicated time intervals. ***D,*** MCF-7 cells (with IGF-1) and MCF-7-neu cells were cotransfected with expression vectors encoding either HA-Akt or HA-Akt(K179M), together with CMV-β-Gal, and then maintained in the presence or absence of IGF-1. Following 48h of treatment with TRAIL/Apo2L (100 ng/ml), transfected cells were analyzed for apoptotic nuclear morphology. Data represent the percentage of apoptotic cells as a function of total transfectants at 48h (mean ± SE; n=3).

Figure 2. HER2/neu and IGF-1 stimulate NF-κB via Akt-induced activation of the IKK-NEMO complex. ***A,*** HER-2- or IGF-1-induced Akt-dependent activation of NF-κB. MCF-7 and MCF-7-neu cells were cotransfected with expression vectors encoding either HA-Akt or HA-Akt(K179M) together with CMV-β-Gal, and then maintained in the presence or absence of IGF-1 for 36h before assessment of NF-κB DNA-binding activity by EMSA. ***B***, Inhibition of HER-2/neu- or IGF-1-induced activation of IKK by a cell-permeable peptide spanning the IKKβ NBD. MCF-7 (with or without IGF-1) and MCF-7-neu cells were incubated with either wild-type NBD peptide (WT NBD) or mutant NBD peptde (MU NBD)(200 μM) for 16h. The IKK complex was immunoprecipitated using anti-IKKβ and subjected to either a kinase assay (using GST-IκBα as substrate) or an immunoblot assay for IKKβ. ***C,*** Inhibition of HER-2/neu- or IGF-1-induced activation of NF-κB by wild-type NBD peptide. MCF-7 (with IGF-1) and MCF-7-neu cells were incubated with either WT NBD or MU NBD (100, 200 μM) for 16h and analyzed for NF-κB DNA-binding activity by EMSA.

Figure 3. Blockade of the IKK-NEMO interaction sensitizes breast cancer cells to TRAIL/Apo2L-induced apoptosis. MCF-7 (with IGF-1), MCF-7-neu, SKBr-3, and Hs578 cells were pretreated with either trastuzumab (Herceptin™)(1 μM) or NBD peptides (WT NBD or MU NBD; 100 μM), and then exposed to TRAIL/Apo2L (100 ng/ml) for 48h. ***A***, Representative phase contrast photomicrographs of cells in each group after 24h of treatment with TRAIL/Apo2L. ***B,C***, Data represent the percentage of apoptotic cells as a function of total cells at 48h (mean ± SE; n=3).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MCF-7
MCF-7-neu
MCF-7+ IGF
MCF-7-neu+Herceptin™
B
Apoptosis (%)
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80
60
40
20
0
MCF-7
MCF-7 + IGF
MCF-7 neu
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+ Herceptin
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MCF-7
MCF-7-neu
Akt-P
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IGF-1: – + + + + – – –
LY294002: – – – + – – – +
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Time (h): 0 ¼ ½ 12 12 0 12 12
D
Apoptosis (%)
100
80
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40
20
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LY294002
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LY294002
HA-Akt
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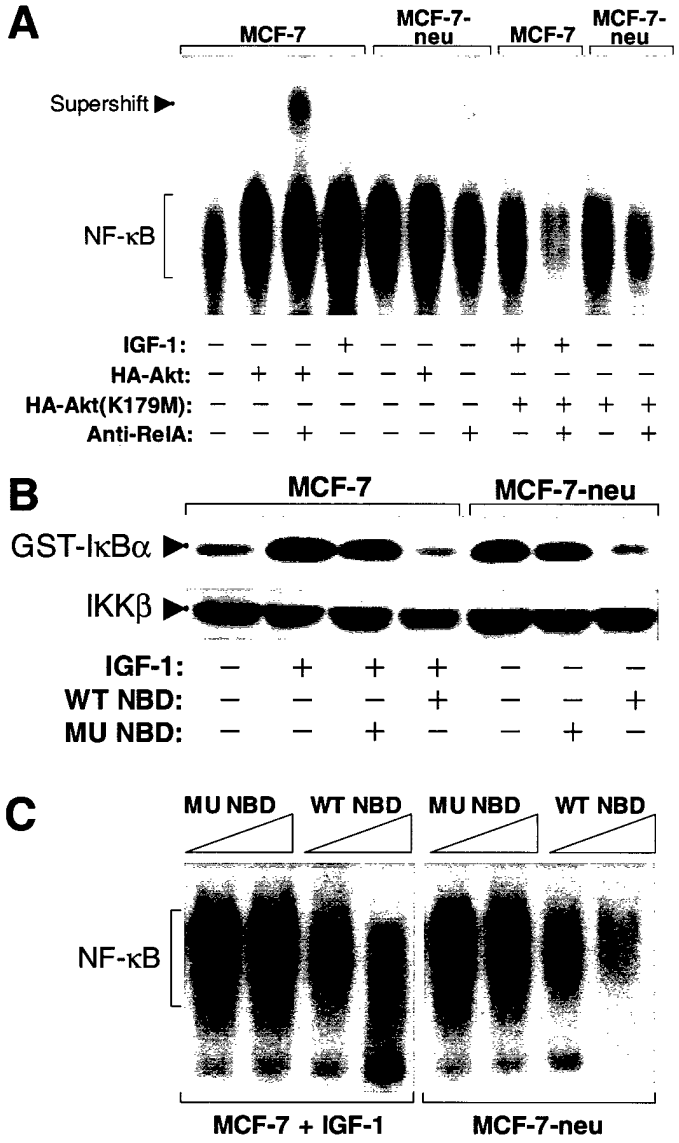
MCF-7-neu
MCF-7 + IGF-1


A
MCF-7
MCF-7-neu
MCF-7
MCF-7-neu
Supershift
NF-κB
IGF-1: − − − + − − − + + − −
HA-Akt: − + + − − + − − − − −
HA-Akt(K179M): − − − − − − − + + + +
Anti-RelA: − − + − − − + − + − +
B
MCF-7
MCF-7-neu
GST-IκBα
IKKβ
IGF-1: − + + + − − −
WT NBD: − − − + − − +
MU NBD: − − + − − + −
C
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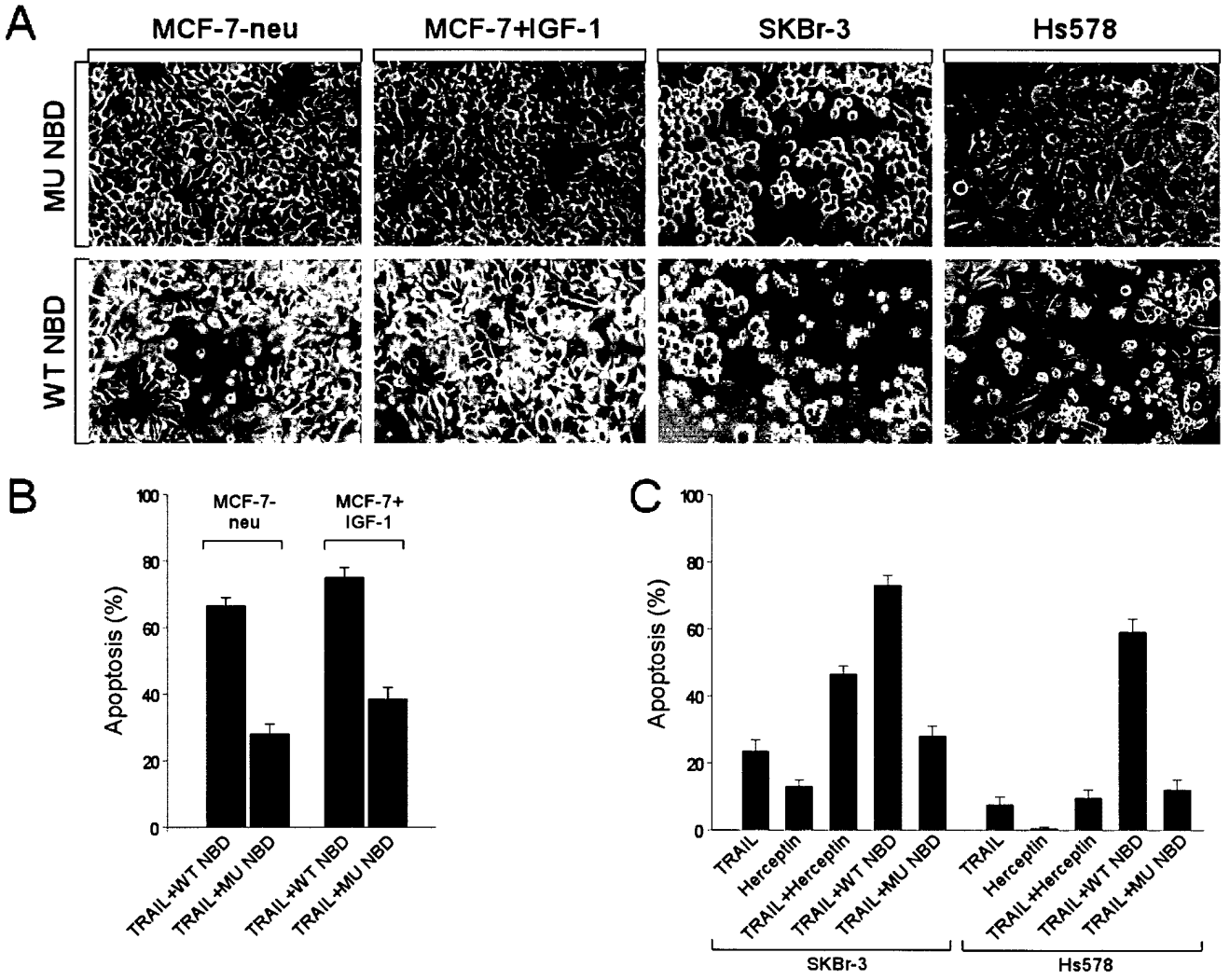
WT NBD
NF-κB
MCF-7 + IGF-1
MCF-7-neu


A
MCF-7-neu
MCF-7+IGF-1
SKBr-3
Hs578
MU NBD
WT NBD
B
MCF-7-neu
MCF-7+IGF-1
Apoptosis (%)
100
80
60
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20
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TRAIL+WT NBD
TRAIL+MU NBD
TRAIL+WT NBD
TRAIL+MU NBD
C
Apoptosis (%)
100
80
60
40
20
0
TRAIL
Herceptin
TRAIL+Herceptin
TRAIL+WT NBD
TRAIL+MU NBD
TRAIL
Herceptin
TRAIL+Herceptin
TRAIL+WT NBD
TRAIL+MU NBD
SKBr-3
Hs578

Appendix 4:

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APPENDIX 4.

Figure 1 Legend

Figure 1. NF-κB/RelA induces expression of Bcl-x_L and protects cells from hypoxia-induced apoptosis. **a.** Electrophoretic mobility shift assay of NF-κB DNA binding activity in 3T3 cells in the presence or absence of aspirin (ASA, 1 mM). **b.** Immunoblot assay of HIF-1a expression in $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts in either normoxia (20% O_2)(N) or hypoxia (0.1% O_2)(H). **c.** Northern blot analysis of VEGF mRNA expression in $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts in either normoxia (20% O_2)(N) or hypoxia (0.1% O_2)(H). **d.** Survival of $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts following exposure to 0.1% O_2 for 48h. **e.** Immunoblot analysis of Bcl-x_L expression in $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts in either normoxia (20% O_2)(N) or hypoxia (0.1% O_2)(H), in the presence or absence of ASA (1 mM).

Appendix 4: Figure 1.