Transforming Growth Factor-\(\alpha\) Inhibits the Intrinsic Pathway of c-Myc-Induced Apoptosis through Activation of Nuclear Factor-\(\kappa\)B in Murine Hepatocellular Carcinomas

Lakita G. Cavin,\(^1\) Fang Wang,\(^1\) Valentina M. Factor,\(^2\) Swayamjot Kaur,\(^1\) Manickam Venkatraman,\(^1\) Snorri S. Thorgeirsson,\(^2\) and Marcello Arsura\(^1\)

\(^1\)Department of Pharmacology, Center for Anticancer Drug Research, College of Medicine, University of Tennessee Cancer Institute, Memphis, Tennessee and \(^2\)Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland

Abstract
Nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) plays an important role during liver neoplastic development through transcriptional regulation of prosurvival genes, which then counteract the death-inducing signals elicited by the host immune response. The c-Myc proto-oncogene is frequently deregulated in liver tumors. Furthermore, enforced expression of c-Myc in the liver promotes the development of hepatocellular carcinomas, a process that is accelerated by coexpression with transforming growth factor-\(\alpha\) (TGF-\(\alpha\)). TGF-\(\alpha\)/c-Myc-derived hepatocellular carcinomas display reduced apoptotic levels compared with those of single c-Myc transgenic hepatocellular carcinomas, suggesting that TGF-\(\alpha\) provides a survival advantage to c-Myc-transformed hepatocytes. Given that TGF-\(\alpha\)/c-Myc hepatocellular carcinomas display constitutive NF-\(\kappa\)B activity, here, we have tested the hypothesis that enforced expression of TGF-\(\alpha\) results in constitutive NF-\(\kappa\)B activation and enhanced cell survival using TGF-\(\alpha\)/c-Myc-derived hepatocellular carcinoma cell lines. We show that TGF-\(\alpha\) induces NF-\(\kappa\)B through the phosphatidylinositol 3-kinase/Akt axis in these bitransgenic hepatocellular carcinomas. Furthermore, we found that adenovirus-mediated inhibition of NF-\(\kappa\)B activity impairs the ability of TGF-\(\alpha\)/c-Myc-derived tumor cells to grow in an anchorage-independent fashion due to sensitization to c-Myc-induced apoptosis. Lastly, we show that NF-\(\kappa\)B inhibits c-Myc-induced activation of caspase-9 and caspase-3 through up-regulation of the antiapoptotic target genes Bcl-X\(_L\) and X-linked inhibitor of apoptosis (XIAP). Overall, these results underscore a crucial role of NF-\(\kappa\)B in disabling apoptotic pathways initiated by oncogenic transformation. (Mol Cancer Res 2005;3(7):403–12)

Introduction
Nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) is a dimeric transcription factor implicated in the regulation of development, regeneration, and neoplastic transformation of the liver (1, 2). In nonstimulated hepatocytes, NF-\(\kappa\)B is sequestered in the cytoplasm through interaction with a family of inhibitory proteins known as I\(\kappa\)B\(\alpha\)s (3). In response to viral infections, DNA damage, and proinflammatory cytokines, the I\(\kappa\)B kinase (IKK) complex promotes NF-\(\kappa\)B activation through phosphorylation-induced ubiquitination of I\(\kappa\)B\(\alpha\)s, which targets these molecules for proteolysis in the 26S proteasome (4). The IKK complex is composed of two catalytic subunits, IKK-\(\alpha\) (IKK-1) and IKK-\(\beta\) (IKK-2), and a scaffolding component termed IKK-\(\gamma\) (4). Mouse embryos lacking either the IKK-2 or the IKK-\(\gamma\) subunit display enhanced liver apoptosis during gestation, which is reminiscent of that observed in RelA-null mice due to sensitization to tumor necrosis factor-\(\alpha\) cell killing (4). Recent evidence indicates that constitutive activation of the IKKs/NF-\(\kappa\)B axis is also involved in liver tumor development through protection from cell death and induction of cell growth (5, 6). For example, we have shown that oncogene/growth factor–mediated transformation of hepatocytes led to altered NF-\(\kappa\)B regulation through constitutive activation of the IKK complex (7, 8). The mechanism by which NF-\(\kappa\)B contributes to liver tumor formation is in part due to transcriptional activation of prosurvival genes, which in turn oppose proapoptotic stimuli elicited by the host immune system and/or by the transforming oncogene. In this regard, we have shown recently that adenovirus-mediated inhibition of NF-\(\kappa\)B activity in murine hepatocellular carcinomas promoted down-regulation of Bcl-X\(_L\), X-linked inhibitor of apoptosis (XIAP), and \(\alpha\)-fetoprotein genes, thereby sensitizing malignant hepatocytes to tumor necrosis factor-\(\alpha\)–mediated cell killing (6). Furthermore, we reported that inhibition of NF-\(\kappa\)B activity potentiated transformed growth factor (TGF)-\(\beta\)–induced apoptosis of oncogenic Ras-transformed rat liver epithelial cells and...
immortalized hepatocytes (7, 9, 10). Lastly, mounting evidence suggests a bridging role of NF-κB between liver tumor initiation and inflammation in hepatitis B virus–infected and hepatitis C virus–infected livers (11-13).

The c-Myc proto-oncogene is a member of the Myc family of b/HLH/LZ proteins that regulate crucial signaling pathways involved in cell proliferation, differentiation, and transformation (14, 15). Deregulation of c-Myc gene expression due to amplification or gene rearrangement is frequently observed in experimentally derived hepatocellular carcinomas (16-18) as well as in primary liver tumors (19, 20). Furthermore, ectopic expression of c-Myc in murine hepatocytes promotes liver neoplastic development (21), and targeted inactivation of c-Myc induces tumor regression of c-Myc-induced hepatocellular carcinomas (22). However, under growth-limiting conditions when c-Myc overexpression is uncoupled from growth signals, c-Myc can sensitize cells to the intrinsic pathway of apoptosis (23, 24). This observation implies that the apoptotic response elicited by c-Myc must be disabled to permit c-Myc-mediated transformation. In this regard, hepatic coexpression of TGF-α in c-Myc–derived hepatocellular carcinoma cell lines to c-Myc–transgenic mice but not from single c-Myc mice, here, only in hepatocellular carcinomas derived from double TGF-α/c-Myc transgenic mice promoted enhanced liver neoplastic development (25) compared with that of single c-Myc or TGF-α transgenic mice (25, 26). Furthermore, acceleration of hepatocarcinogenesis in bitransgenic mice was due to down-modulation of apoptosis (27).

Given that we observed IKK complex and NF-κB activation only in hepatocellular carcinomas derived from double TGF-α/c-Myc transgenic mice but not from single c-Myc mice, here, we have tested the hypothesis that overexpression of TGF-α counteracts c-Myc-induced apoptosis through induction of NF-κB activity, thereby accelerating c-Myc-induced liver neoplastic development. We show that, under growth-limiting conditions, inhibition of NF-κB activity sensitizes TGF-α/c-Myc–derived hepatocellular carcinoma cell lines to c-Myc-induced apoptosis.

Overall, our results indicate that constitutive activation of NF-κB impairs the ability of malignant hepatocytes to commit to engage cell death induced by transforming oncogenes.

Results

The Phosphatidylinositol 3-Kinase/Akt Axis Plays a Role in Constitutive Activation of NF-κB in TGF-α/c-Myc–Derived Hepatocellular Carcinoma Cell Lines

Previously, we showed that hepatocellular carcinomas derived from bitransgenic TGF-α/c-Myc mice displayed constitutive NF-κB activity (8). To determine the mechanism of NF-κB activation and its functional effect on cell survival, we established several cell lines from hepatocellular carcinomas developed in TGF-α/c-Myc mice. Two of these cell lines, named 223ma5 (223) and 263ma2 (263), were found to express high levels of c-Myc and TGF-α mRNA (Fig. 1A). Both cell lines gave rise to tumors on transplantation in nude mice (data not shown) and displayed α-fetoprotein gene expression (6). Similarly to TGF-α/c-Myc tumors (8), we observed constitutive binding of two complexes to a radiolabeled upstream regulatory element-κB probe in nuclear extracts of exponentially growing 223 and 263 cells (Fig. 1B). Supershift analysis revealed that the slower migrating complex contained the p65 (RelA) and p50 (NF-κB1) subunits, whereas the faster migrating complex contained p50 homodimers (Fig. 1B). Furthermore, we found that exponentially growing 223 and 263 cells and two additional TGF-α/c-Myc–derived hepatocellular carcinoma cell lines (ma3 and ma4) expressed enhanced levels of NF-κB-luciferase activity compared with that of a c-Myc-derived hepatocellular carcinoma cell line (604T1; Fig. 1C).

Because our previous data suggested a role of the phosphatidylinositol 3-kinase PI(3)K/Akt axis during constitutive activation of the IKK complex in TGF-α/c-Myc–derived hepatocellular carcinomas (8), we asked whether TGF-α-mediated activation of epidermal growth factor receptor (EGFR) signaling in 223 cells would promote NF-κB activation through Akt. Indeed, 223 and 263 cells displayed constitutive Akt kinase activity that was inhibited by treatment with the PI(3)K inhibitor wortmannin (Fig. 2A). To determine the role of Akt in the regulation of NF-κB activity, we assessed NF-κB DNA-binding...
activity on inhibition of the EGFR/PI(3)K/Akt pathway. We observed down-regulation of NF-κB DNA-binding activity in 223 cells following treatment with the AG1478 compound (data not shown). Likewise, treatment of 223 cells with the PI(3)K inhibitor wortmannin as well as ectopic expression of dominant-negative forms of IKK-2 or Akt resulted in down-regulation of NF-κB DNA-binding activity (data not shown). Moreover, ectopic expression of a kinase-dead Akt, but not its wild-type counterpart, led to reduced kinase-luciferase activities to levels comparable with those seen on transfection of the superrepressor IκB-α (Fig. 2B). Thus, Akt regulates the transcriptional activity of NF-κB in murine hepatocellular carcinomas.

Previously, we reported constitutive IKK complex activity in liver samples of TGF-α/c-myc bitransgenic mice (8). Because Akt-mediated activation of NF-κB in response to certain stimuli requires the IKK complex (7, 28-32), we sought to determine whether inhibition of Akt promotes down-regulation of IKK complex activity. In extracts of exponentially growing 223 cells, we observed constitutive phosphorylation of IKKs (Fig. 2C), which was consistent with the constitutive NF-κB activation observed in these cells (Fig. 1B). In 223 cells treated with the PI(3)K inhibitors LY294002 and wortmannin or with the EGFR inhibitor AG1478, we noticed a significant down-regulation of IKK complex phosphorylation levels (Fig. 2C). Thus, our results support a model in which constitutive activation of EGFR signaling in bitransgenic hepatocellular carcinomas, presumably due to autocrine secretion of TGF-α, induces IKK complex and NF-κB activity through activation of the PI(3)K/Akt axis.

**FIGURE 2.** EGFR mediates constitutive NF-κB activation through a PI(3)K/Akt signaling pathway in bitransgenic TGF-α/c-Myc hepatocellular carcinoma cell lines. A, WCEs were isolated from 223 or 263 cells treated in the presence or absence of 100 nmol/L wortmannin. Samples (80 μg) were immunoprecipitated (IP) using an antibody against phospho-Akt (pAkt). Subsequently, immunoprecipitates were subjected to kinase assay (KA) using purified GSK3β protein as substrate. B, 223 cells were plated in triplicate in 96-well plates and transfected by lipofection with 50 ng α-B-lucifera (α-B-Luc.) construct in the absence or presence of 10 ng vectors directing expression of wild-type Akt (WT), kinase-dead Akt (KD Akt), or the superrepressor 2N3C-IκB-κ and an internal control Renilla luciferase expression plasmid. The final DNA concentration was adjusted to 150 ng with the pCDNA backbone vector. Following 24 hours of transfection, luciferase activity was measured and expressed in arbitrary relative luciferase units. Representative of three independent experiments done in triplicate. Columns, mean; bars, SD. C, 223 cells were either untreated (control) or treated with DMSO, 100 μmol/L AG1478, 100 nmol/L wortmannin, or 10 μmol/L AG1478 for 6 hours. WCEs (30 μg) were subjected to immunoblotting (IB) for pIKK-1/2 and IKK-2.
NF-αB was found to be a potent activator of the active form of IKK-2 (pCMV-IKK-EE; ref. 33) that we have (Fig. 4A-C). Furthermore, ectopic expression of a constitutively active form of IKK-2 accelerates cell death of quiescent c-Myc-derived hepatocellular carcinoma cells (604T1), which express low levels of constitutive NF-αB activity (Fig. 1C). We observed pronounced cell death of serum-deprived 223 cells (604T1), which express low levels of constitutive NF-αB, as determined by the accumulation of reactive oxygen species–mediated activation of the Intrinsic Pathway of Apoptosis (Fig. 6B; data not shown). As predicted by our previous results, the cleavage of caspase-9 into the p37/p41 proteolytically cleaved fragments in serum-starved 223 cells. (Fig. 6A), indicating that the negative cross-talk between the NF-αB and the c-Jun NH2-terminal kinase pathway does not play a role in sensitization to c-Myc-induced apoptosis during serum deprivation.

To further elucidate the antiapoptotic role of NF-αB during neoplastic transformation of murine hepatocytes, we assessed cell death of quiescent c-Myc-derived hepatocellular carcinoma cells (604T1), which express low levels of constitutive NF-αB activity (Fig. 1C). We observed pronounced cell death of serum-deprived 223 cells (Fig. 4D) to an extent comparable with that seen in IKK-2 K>M–expressing cells (control) versus neo/IKK-2 K>M (B). Following 24 hours of incubation in serum-deprived medium, cells were stained with crystal violet and foci were visually counted at the microscope. Values are percentage of foci per well relative to DMSO-treated (DMSO) or exponentially growing cells (control), which was set at 100. Representative of three independent experiments done in triplicate. Columns, mean; bars, SD. **, P < 0.01, significance calculated using the Students’ t test.

Inhibition of NF-αB Activity Causes Reduction of Bcl-XL and XIAP Expression Levels and Sensitizes 223 Cells to the Intrinsic Pathway of Apoptosis

Previously, the ability of c-Myc to sensitize quiescent B cells and fibroblasts to cell death has been associated with destabilization of the mitochondrial integrity (36), presumably through repression of prosurvival Bcl-2 family members and subsequent mitochondrial membrane depolarization (37). On the basis of this observation and because we observed enhanced production of reactive oxygen species in adIKK-2 K>M–infected quiescent 223 cells compared with GFP-expressing cells (data not shown), we sought to determine whether reactive oxygen species–mediated activation of c-Jun NH2-terminal kinase played a role in sensitization to c-Myc-induced cell death. Intriguingly, inhibition of NF-αB activity in serum-deprived 223 cells did not potentiate c-Jun NH2-terminal kinase–mediated phosphorylation of c-Jun (Fig. 6A), indicating that the negative cross-talk between the NF-αB and the c-Jun NH2-terminal kinase pathway does not play a role in sensitization to c-Myc-induced apoptosis during serum deprivation.

To determine whether the rescue from c-Myc-induced apoptosis by NF-αB relied on inhibition of the intrinsic pathway of cell death, we assessed the kinetics of activation of specific caspases as determined by the accumulation of proteolytically cleaved fragments in serum-starved 223 cells. In both GFP-expressing and IKK-2 K>M–expressing 223 cells that had been deprived for 12 hours of serum, we did not notice a significant increase in proteolytic processing of caspase-8 into the p20 subunit (Fig. 6B), implying that the activation of death effector domains containing complexes is not involved in c-Myc-induced apoptosis following serum removal. In contrast, GFP-expressing 223 cells displayed accumulation of both the 37/39-kDa fragment of caspase-9 and the 17/20-kDa subunits of caspase-3 after 12 hours of serum removal (Fig. 6B; data not shown). As predicted by our previous results, the cleavage of caspase-9 into the p37/
p39 subunits and the processing of caspase-3 into the p17/p20 subunits were significantly faster in serum-deprived adIKK-2 K>M–infected cells compared with adGFP-expressing cells (6 versus 12 hours, respectively; Fig. 6B; data not shown). Furthermore, following serum removal, we observed a more robust induction of the enzymatic activity of caspase-3 in IKK-2 K>M–expressing 223 cells than in GFP-infected cells (Fig. 6C). Thus, inhibition of NF-κB activity accelerates the induction of the intrinsic pathway of apoptosis.

To determine the crucial downstream components of the NF-κB signaling pathway involved in suppression of mitochondrial cell death, we measured changes in protein expression levels of two antiapoptotic target genes of NF-κB, Bcl-XL and XIAP. Treatment of 223 cells with either the AG1478 or the LY294002 compound, which we have found previously to inhibit IKK complex activity (Fig. 2C), led to down-regulation of Bcl-XL and XIAP gene products (Fig. 7A). Likewise, we observed a dramatic down-regulation of both Bcl-XL and XIAP protein expression levels in adIKK-2 K>M–infected cells but not in adGFP-infected cells (Fig. 7B). Furthermore, ectopic expression of IKK-EE that protected quiescent 604T1 cells from apoptosis (Fig. 4D) resulted in up-regulation of both Bcl-XL and XIAP protein expression levels (Fig. 7C). Lastly, ectopic expression of XIAP rescued 223 cells expressing IKK-2 K>M from cell death following serum deprivation (Fig. 7D). Thus, down-regulation of XIAP and Bcl-XL gene expression following inhibition of NF-κB activity could play a potential role in sensitizing 223 cells to the intrinsic pathway of c-Myc-induced apoptosis.

Discussion

The apoptotic response elicited by transforming oncoproteins, such as c-Myc, restrains the development of the malignant cell phenotype. Thus, further genetic changes are required to confer a survival advantage to c-Myc-transformed cells. Our results provide evidence that one of these events is represented by activation of NF-κB. We show that hepatocellular carcinomas derived from TGF-α/c-myc bitransgenic mice display constitutive NF-κB activity due in part to TGF-α-mediated activation of the PI(3)K/Akt axis. Constitutive induction of NF-κB activity, in turn, promotes up-regulation of the antiapoptotic target genes Bcl-XL and XIAP, which results in protection against c-Myc-induced apoptosis.

NF-κB is typically found in an inactive state in non-stimulated cells. However, during malignant transformation, NF-κB is constitutively activated due to phosphorylation-mediated degradation of the IκBα gene product by IκB kinases, such as IKKs and CK2 (7, 38-40). In our study, we report a novel autocrine mechanism of NF-κB activation in TGF-α/c-Myc–derived malignant hepatocytes based on the ability of secreted TGF-α, a growth factor that is frequently expressed in primary and experimentally derived tumors, to activate the IKK complex through an EGFR/PI(3)K/Akt signaling pathway. Previously, activation of the PI(3)K/Akt axis in response to cell stimulation with platelet-derived growth factor, tumor necrosis factor-α, and IFNs had been implicated in the induction of NF-κB activity through Akt-mediated activation of IKKs (28-30). Likewise, Ha-Ras-mediated transformation of NIH 3T3 fibroblasts and rat liver epithelial cells resulted in IKK complex activation through multiple pathways involving mitogen-activated protein kinases as well as PI(3)K/Akt (7, 41). Furthermore, in breast cancer cells, the PI(3)K/Akt axis played a role during NF-κB activation in response to EGFR or HER-2/new signaling (31, 42), which could be impeded by the PTEN tumor suppressor (31) or by the pro-peptide domain of lysyl oxidase (43, 44). Consistent with a role of the PI(3)K/Akt axis in IKK activation, we report that TGF-α/c-Myc–derived hepatocellular carcinomas display constitutive phosphorylation...
of Ser\textsuperscript{180}/Ser\textsuperscript{181} in the activation loop of IKKs, which is reduced on treatment with inhibitors of the EGFR/PI(3)K signaling pathway. Although overexpression of Akt has been shown to enhance NF-κB transcriptional activity through IKK-2-mediated phosphorylation of RelA on Ser\textsuperscript{536}/Ser\textsuperscript{529} (32), we were unable to detect increased RelA phosphorylation on Ser\textsuperscript{536} either in tissue sections of TGF-α/c-Myc–derived hepatocellular carcinomas or in 223 cells (data not shown). This observation implies that Akt activity in TGF-α/c-Myc–derived hepatocellular carcinomas promotes IKK-mediated phosphorylation of IκBα rather than RelA. Future experiments will help to clarify whether Akt is activating IKKs through direct phosphorylation of serine residues within their activation loop or whether it mediates additional post-translational modifications of the IKK complex.

Another important finding of our study is the antagonistic effect of NF-κB on c-Myc-induced apoptosis. Overexpression of c-Myc in growth-limiting conditions initiates the intrinsic pathway of apoptosis, which is characterized by mitochondrial depolarization, release of cytochrome c, and formation of the apoptosome (36). The mechanism of c-Myc-induced apoptosis seems to rely on the ability of c-Myc to suppress the activity of antiapoptotic Bcl-2 family members, such as Bcl-X\textsubscript{L} and Bcl-2 (45). Alternatively, c-Myc can induce cell death through up-regulation of the ARF-p53 tumor suppressor pathway, although it remains unclear how c-Myc affects ARF expression levels (46). Our results suggest that one potential mechanism of NF-κB-mediated inhibition of c-Myc-induced apoptosis is through transactivation of the Bcl-\(X_\text{L}\) gene. Indeed, we show that inhibition of NF-κB by the IKK-2
K->M mutant leads to down-regulation of Bcl-X<sub>L</sub> gene product, which correlates with the acceleration of processing of procaspase-9 and caspase-3 and induction of apoptosis. However, we noticed that serum-deprived GFP-expressing cells also displayed caspase-9 activation albeit at later time points than we noticed that serum-deprived GFP-expressing cells also caspase-9 and caspase-3 and induction of apoptosis. However, which correlates with the acceleration of processing of pro-

**FIGURE 6.** Inhibition of NF-κB activity accelerates c-Myc-induced caspase-9 and caspase-3 activation. **A.** The 223 cells were infected adIKK-2 K>M or adGFP as above and treated with or without 0.003% H<sub>2</sub>O<sub>2</sub> for 30 minutes or 100 mol/L butylated hydroxyanisole for 1 hour. Following 6 hours of serum deprivation, WCEs were analyzed by immunoblotting using antibody against IKK-2 K>M, total Jun (tot Jun), or phospho-Jun (pJun). **B.** The 223 cells were infected with adIKK-2 K>M or adGFP as above. Following serum deprivation for the indicated times, WCEs were subjected to immunoblot analysis using antibodies specific for the p20 subunit of caspase-8, the p37/p39 subunits of caspase-9, or an antibody against actin. The ratios of the absorbance of the caspase-9 fragment to that of the actin are expressed in arbitrary units.

**C.** The 223 cells were infected with adIKK-2 K>M or adGFP as above. Following serum deprivation for the indicated times, WCEs were subjected to caspase-3 activity assay using the CaspAce assay system. Caspase-3-specific activity was measured as pmol p-nitroaniline liberated per hour and expressed as arbitrary units. Representative of two independent experiments. Columns, mean; bars, SD.

IKK-2 K->M–expressing cells (12 versus 6 hours) but did not undergo apoptosis. This observation prompted us to examine whether NF-κB was inhibiting apoptosis by targeting an additional prosurvival factor that was acting downstream of the apoptosome. Indeed, we show that inhibition of NF-κB rescues IKK-2 K->M–expressing cells from apoptosis induced by serum deprivation. Interestingly, we detected neither enhanced c-Jun NH<sub>2</sub>-terminal kinase activity or inhibition of antioxidant enzymes, such as manganese superoxide dismutase (data not shown), in response to down-regulation of NF-κB activity, suggesting that c-Jun NH<sub>2</sub>-terminal kinase does not play a role in sensitization to c-Myc-induced apoptosis in hepatocellular carcinoma cell lines, as shown previously in NIH 3T3 fibroblasts (47).

Overall, our data support a model whereby inhibition of NF-κB sensitizes malignant clones to c-Myc-induced cell death through potentiation of the intrinsic pathway of apoptosis. Furthermore, given that we observed reduced apoptotic index in double TGF-α/c-Myc–derived hepatocellular carcinomas versus single c-Myc, our data suggest that enforced expression of TGF-α provides a survival advantage to c-Myc-transformed hepatocytes through up-regulation of NF-κB activity, thus accelerating liver neoplastic development.

**Materials and Methods**

**Cell Culture and Treatment Conditions**

Four hepatocellular carcinoma cell lines (223, 263, ma3, and ma4) were derived from TGF-α/c-myc bitransgenic mice described elsewhere (25). One hepatocellular carcinoma cell line (604T1) was derived from a single c-myc transgenic mouse (25). Cells were cultivated in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin (Sigma Chemical Co., St. Louis, MO). Where indicated, cells were treated with 100 μmol/L AG1478 (Calbiochem, San Diego, CA), an epidermal growth factor inhibitor, 100 μmol/L wortmannin (Calbiochem), a PI(3)K inhibitor, or 100 μmol/L LY294002, another PI(3)K inhibitor (Calbiochem) and 100 μmol/L butylated hydroxyanisole, an antioxidant (Sigma Chemical). For focus-forming assay, cells were plated at 70% confluence in 24-well plates and infected with 10 plaque-forming units (pfu)/mL adenoviral constructs as described below. After 24 hours, cells were either serum starved or grown in the presence of 10% serum for 24 to 48 hours. To visualize foci, cells were stained for 24 hours at room temperature with 0.025% crystal violet dissolved in 1× PBS. Stained foci were counted using a dissecting microscope. Values were given as percentage of stained cells relative to DMSO-treated control cells, which was set at 100. Each experiment was done at least thrice.

**RNA Isolation and Analysis**

Total cellular RNA was isolated by the guanidinium method and samples were subjected to Northern blot analysis as described previously (48). Probes used include the mouse c-Myc cDNA clone pM-c-Myc-54 and the human TGF-α gene (25).
Transfection Conditions and Reporter Assays

For transient transfection, 223 cells were plated at low confluence in 96-well plates and transfected in triplicate with a solution of DNA and LipofectAMINE reagent according to the manufacturer’s instructions (Life Technologies). Cells were harvested after treatment according to the manufacturer’s protocol in the dual-luciferase reporter assay (Promega Corp., Madison, WI). Lysates were analyzed with a Labsystems Luminoskan 96-well plate luminometer (Thermolab System, Needham Heights, MA). Firefly luciferase activity was normalized for Renilla luciferase activity, and results were expressed as percentage of normalized luciferase activities in treated cells versus vehicle-treated cells. Means and SDs are relative to at least three experiments, each done in triplicate.

For adenoviral infection, cells were infected with 10 pfu adIKK-2 K>M, adwtIKK-2, adGFP, and adMAD for 24 hours.

Plasmids and Adenoviruses

The κ-B-luciferase construct has been described previously (49). The adenoviral vectors expressing wild-type IKK-2 or dominant-negative forms of IKK-2 (IKK-2 K>M) were constructed by blunt ligation of the respective IKK cDNA into the replication-deficient vector pAxCA. Virus stocks were amplified to high titer (Quantum Biotechnologies, Montreal, Quebec, Canada). The concentration of viral particles was determined by A260 measurement. Plaque assay to determine infectious virus units gave a viral particles/infectious virus unit ratio of <100:1. Adenoviral preparations were retitrated using human umbilical vein endothelial cells to determine the optimum multiplicities of infection. The adenoviral vector expressing Mad1 (MAD) has been described previously (50). The adenoviral vectors expressing wild-type Akt and kinase-dead Akt have been described previously (51). Adenoviral infections were done using 10 pfu/mL of growth medium.

Whole cell extracts (WCE) were then assayed for p16INK4a protein expression using an anti-p16 antibody (sc-1207).

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from cells as described previously (7). The sequences of the upstream regulatory element-κ-B-containing oligonucleotide from the c-myc gene and the octamer 1-containing oligonucleotide are as follows: upstream regulatory element-κ-B, 5’-AAGTCCGGGTITTTTCC-CAACC-3’, and octamer 1, 5’-TGTCGAATGCAAATCACTAGAA-3’. Electrophoretic mobility shift assay was done as described previously (7).

FIGURE 7. Inhibition of NF-κB activity leads to down-regulation of Bcl-XL and XIAP gene products. A. The 223 cells were either treated with DMSO or treated with 100 μmol/L LY294002 (LY) or 100 μmol/L AG1478 for 6 hours. WCEs (30 μg) were subjected to immunoblotting for Bcl-XL, XIAP, or actin. B. The 223 cells were infected with ad IKK-2 K>M or adGFP for 24 hours and WCEs were subjected to immunoblotting as described above. C. Single c-Myc-derived 604T1 cells were plated in p35 plates and transfected with 1 μg pCMV-IKK-EE or pCMVneo control. After 24 hours, WCEs (30 μg) were subjected to immunoblot assay using antibodies targeted against XIAP, Bcl-XL, IKK-2, and actin. D. The 223 cells were plated in 96-well plates and transfected using LipofectAMINE 2000 with 25 ng pON407 φ-galactosidase expression vector in the presence or absence of 50 ng XIAP expression vector. The final concentration of DNA was adjusted to 150 ng with the XIAP backbone vector. Six hours after transfection, 10 pfu/mL adGFP or adIKK-2 K>M was added to the cultures. After 18 hours of incubation, cells were serum deprived for 24 hours. Cell death was monitored by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining and expressed as the percentage of blue viable cells relative to total cell number. Representative of two independent experiments done in triplicate. Columns, mean; bars, SD.
Protein Isolation and Immunoblot Analysis

To prepare WCEs, cells were washed with cold PBS and resuspended in PD buffer [40 mmol/L Tris (pH 8), 300 mmol/L NaCl, 6 mmol/L EDTA, 6 mmol/L ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′′-tetraacetic acid, 10 mmol/L glycercophosphate, 10 mmol/L NaF, p-nitrophenyl phosphate, 300 μmol/L Na3VO4, 1 mmol/L benzamidine, 2 μmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 1 μg/mL leupeptin, 10 μg/mL aprotinin, 1 μg/mL pepstatin, 0.5% NP40]. Cells were lysed by sonication, and lysates were cleared by centrifugation at 40,000 rpm for 30 minutes at 4°C. Samples (30 μg) were subjected to electrophoresis on 10% SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunoblotting as described previously (49). The antibodies specific for phospho-IKK-1/2 and phospho-EGFR (Tyr1068) were purchased from Cell Signaling Technology (Beverly, MA). The antibodies specific for IKK-1/2 (sc-7607), IκB-α (sc-371), c-Jun (sc-45), phospho-Jun (sc-822), and caspase-8 p20 (sc-7890) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse-specific polyclonal antibody that recognizes the full-length caspase-9 and the 37- to 39-kDa cleaved fragments of caspase-9 was purchased from Cell Signaling Technology. The monoclonal antibody specific for β-actin (CP-01) was purchased from Oncogene Research Products (San Diego, CA). The antibody specific for Bcl-X (-610209) was purchased from BD Biosciences (San Jose, CA). The antibody against XIAP (sc-7607) was purchased from Stressgen (San Diego, CA). We thank Martine Roussel, Frank Mercurio, Ron DePinho, Sanjeev Gupta, David Sisson, Kostantin Kandror, and Kuni Matsumoto for kindly providing retroviral stocks, adenoviral constructs, and cloned DNAs. Dr. Leonard Lothstein and Ludymla Savranskaya for useful suggestions while preparing this article; and Rose Mathew and Marie Guylaine for excellent technical support.

Akt Kinase Assay

To assess the kinase activity of Akt in 223 cells, a kinase assay was done using an Akt kinase assay kit (Cell Signaling Technology). WCEs (80 μg) were immunoprecipitated with phospho-Akt antibody, and kinase assay was done according to the manufacturer’s instructions. The kinase reaction was stopped by addition of SDS-PAGE sample buffer, subjected to SDS-PAGE analysis, and visualized by autoradiography.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done on cultures of live cells using the DeadEnd Colorimetric TUNEL System (Promega) following the manufacturer’s instructions. Cell extracts (100 μg) were subjected to caspase-3 assay using the CaspAce colorimetric assay kit (Promega).

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