p53 stabilization is decreased upon NFκB activation: A role for NFκB in acquisition of resistance to chemotherapy

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Summary

Chemotherapeutic agents simultaneously induce transcription factors p53 and NFκB. p53 induction can activate an apoptotic program, and resistance to chemotherapy correlates with the loss of a functional p53 pathway. By contrast, NFκB prevents apoptosis in response to chemotherapeutic agents. We have analyzed the p53 response in IKK1/2$^{-/-}$ MEFs, which lack detectable NFκB activity. Compared to WT fibroblasts, IKK1/2$^{-/-}$ fibroblasts showed increased cell death and p53 induction in response to the chemotherapeutic agent, doxorubicin. Reconstitution of IKK2, but not IKK1, increased Mdm2 levels and decreased doxorubicin-induced p53 stabilization and cell death. IKK2-mediated effects required its kinase function and were abrogated by coexpression of the dominant negative IκBαM, implying a role for NFκB in blocking chemotherapy-induced p53 and cell death.

Introduction

Cytotoxicity of chemotherapeutic agents has been attributed to apoptosis, a form of programmed cell death (Chao et al., 2000). However, acquisition of resistance to the cytotoxic effects of chemotherapy has emerged as a significant impediment to effective cancer therapy. The tumor suppressor protein p53, a sequence-specific transcription factor, is a key modulator of apoptosis in response to chemotherapeutic agents (Levine, 1997; Lowe et al., 1993a). As p53 plays a central role in sensing and maintaining genetic integrity, its activation and function have been extensively investigated (Levine, 1997; Oren, 1999). Various cellular signals, including activation of oncogenes (Debbas and White, 1993; Serrano et al., 1997), DNA damage (Huang et al., 1996), and hypoxia (Graeber et al., 1994), lead to activation of p53, resulting in either apoptosis or cell cycle arrest. The choice between these outcomes likely depends, among other factors, on the genetic variability (Polyak et al., 1996) and the extent of p53 activation (Chen et al., 1996). Regulation of p53 mainly occurs posttranscriptionally, often via posttranslational modifications that increase its half-life and nuclear concentration (Prives, 1998). In normal cells, p53 protein is rapidly turned over due to ubiquitin-mediated proteolytic degradation (Bottger et al., 1997). Interaction of p53 with Mdm2, a protein that is transcriptionally activated by p53 (Wu et al., 1993), is crucial for regulating this degradation (Haupt et al., 1997; Honda and Yasuda, 1999; Kubbutat et al., 1997). Mutant forms of p53 accumulate in cells because they fail to transcriptionally activate Mdm2. The loss of a wild-type p53 response creates a permissive environment for acquisition of genomic instability (Yin et al., 1992), which can lead to secondary mutations that can engender resistance to the cytotoxicity of chemotherapeutic agents.

NFκB is a family of ubiquitously expressed dimeric sequence specific transcription factors. Initially recognized for controlling gene expression and function in the immune system, the importance of NFκB family members in modulating cellular growth, apoptosis, and development is now well documented. In most unstimulated cells, the NFκB dimers are bound in the cytoplasm by inhibitory IκB molecules (Verma et al., 1995). Since transcriptional activation by NFκB requires its nuclear translocation, signal-induced degradation of IκB molecules is considered a key rate-limiting step in NFκB activation (Karin and Ben-Neriah, 2000). Phosphorylation of IκB molecules at specific serine residues immediately precedes their ubiquitin-mediated degradation (Chen et al., 1995). Two serine threonine IκB kinases (IKK), IKK1 and IKK2, which are 52% identical, were identified to be responsible for phosphorylating and targeting IκB molecules for degradation. Both IKK1 (also called IKKα) and IKK2 (also called IKKβ) normally reside in a large >700 kDa complex in the cyto-

SIGNIFICANCE

Our results demonstrate that IKK2, a central regulator of the antiapoptotic pathway controlled by the oncogenic transcription factor NFκB, can collaterally downregulate the proapoptotic pathway controlled by the tumor suppressor p53. Our data also distinguishes between the functions of the highly homologous kinases, IKK1 and IKK2, in response to chemotherapy. Given that these kinases have distinct in vivo roles, our results suggest that rather than using generic IKK inhibitors, specific inhibitors of IKK2 could be used as adjuvants to existing chemotherapy regimens. Collectively, our results provide a mechanism of acquisition of resistance to chemotherapeutic agents that activate both NFκB and p53 and also suggest a role for the deregulated NFκB activity observed in several leukemias, lymphomas, and breast cancers.

plasm. In addition to these two kinases, the IKK complex also consists of other regulatory proteins (Yamaoka et al., 1998). A genetic dissection of the different roles played by these kinases has been provided by the generation of IKK1 (Li et al., 1999a; Takeda et al., 1999) and IKK2 knockout mice (Li et al., 1999b, 1999c; Tanaka et al., 1999). Mice lacking both IKK1 and IKK2 die embryonically at day 9.5 (Li et al., 2000). Unlike murine embryo fibroblasts (MEFs) derived from IKK1$^{-/-}$ and IKK2$^{-/-}$animals, MEFs derived from IKK1/2$^{-/-}$ double knockout embryos are severely defective in NFκB activation, reiterating that these are the two essential kinases involved in NFκB activation (Li et al., 2000).

Both NFκB and p53 are activated in response to many stimuli, including DNA damage (Devary et al., 1993; Wang et al., 1996) and TNFα (Donato and Perez, 1998). It is conceivable that these transcription factors, which have been documented to have largely opposite effects in response to stress, modulate each other's activities. Indeed, competition for limiting pools of transcriptional coactivators p300 and CBP has been reported to mediate a bidirectional repression between NFκB and p53 (Webster and Perkins, 1999). In addition, transactivation of the p53 promoter by NFκB in response to TNFα (Wu and Lozano, 1994) and anticancer drugs (Hellin et al., 1998; Sun et al., 1995) has been observed.

Although mutational inactivation of p53 is observed in well over 50% of all human cancers (Hollstein et al., 1994), a significant fraction of cancers express wild-type p53, which may be inactivated by other mechanisms, such as increased Mdm2 expression resulting from gene amplification (Oliner et al., 1992), increased Raf (Ries et al., 2000), or Akt signaling (Zhou et al., 2001). One potential additional mechanism might involve the activation of antiapoptotic genes that compete with proapoptotic pathways activated by p53. NFκB is a well-documented antiapoptotic factor, which is turned on simultaneously along with p53 in response to chemotherapeutic agents (Pahl, 1999; Wang et al., 1996). Inhibition of NFκB has been documented to lead to increased efficacy of anticancer drugs (Cusack et al., 1999, 2001). In nude mice, tumors derived from HT1080 fibrosarcoma cells (Wang et al., 1999) or from the colorectal tumor cell line LoVo (Cusack et al., 2000) are more sensitive to chemotherapy when NFκB activity is also inhibited. Since NFκB impinges on multiple aspects of tumor progression, such as the control of apoptosis, cell cycle, and cell migration, it is critical to understand the mechanistic basis of the observation that blocking NFκB is a useful means of blocking acquired resistance to chemotherapy.

In this study, we have used IKK1/2$^{-/-}$ MEFs to decipher the role of IKKs and NFκB in regulating the p53 response induced by the anticancer agent doxorubicin (DoxR). We find that lack of IKK2 activity and the consequent inability to activate NFκB facilitates p53 stabilization and promotes cell death. Our results not only provide evidence of selectivity in NFκB activation by the IκB kinases but also show that IKK2 plays a unique role in activating NFκB and in compromising p53 function in response to chemotherapy.

Results

MEFs lacking IKK1 and IKK2 are more sensitive to DoxR-induced cell death and p53 stabilization

The role of IKK1 and IKK2 in DoxR sensitivity was evaluated using spontaneously immortalized MEFs from IKK1/2$^{-/-}$ and WT embryos. Compared to WT MEFs, IKK1/2$^{-/-}$ MEFs were more sensitive to DoxR-induced cell death (Figure 1A). Further characterization of DoxR mediated cell death in these MEFs indicated that cell death resulted from apoptosis (data not shown). Gel shift analysis to detect the activation of NFκB in response to varying doses of DoxR revealed NFκB DNA binding activity in WT but not in IKK1/2$^{-/-}$ MEFs (data not shown).

Since p53 plays an important role in DoxR-mediated cell death (Lowe et al., 1993a), we analyzed the induction of p53 in WT and IKK1/2$^{-/-}$ MEFs. A titration of DoxR revealed that p53 induction in the IKK1/2$^{-/-}$ MEFs occurred at a lower dose of DoxR (Figure 1B, compare lanes 3–6 and 9–12). Similar results were obtained using two independent sets of WT and IKK1/2$^{-/-}$ MEFs. Densitometric analysis revealed between 2–5 fold higher p53 in IKK1/2$^{-/-}$ cells. The levels of p65, IKK1, and IKK2 remained unchanged upon DoxR titration. RT-PCR analysis revealed no significant differences in p53 expression between WT and IKK1/2$^{-/-}$ cells (data not shown). These results suggest that lack of IKK activity sensitizes cells to p53 stabilization in response to DoxR. More importantly, induction of p53 is not dependent on NFκB activity.

To understand the increased sensitivity of IKK1/2$^{-/-}$ MEFs to DoxR, we evaluated the integrity of the DNA damage-induced p53 response in these cells. Phosphorylation of N-terminal serine 18 in murine p53 (corresponding to serine 15 in human p53) contributes to p53 activation by DNA damage (Chao et al., 2000). Figure 1C demonstrates that DoxR treatment induces comparable levels of S18 phosphorylation in both WT and IKK1/2$^{-/-}$ MEFs (lanes 2 and 4). Total p53 induction was measured by PA421 antibody (top panel, Figure 1C). We evaluated the transactivation potential of the activated p53 by measuring protein levels of the cyclin-dependent kinase inhibitor p21, a key target gene of p53 (el-Deiry et al., 1993). Figure 1D demonstrates that DoxR treatment induced expression of p21 protein in both WT and IKK1/2$^{-/-}$ MEFs. It is worth noting that there are higher endogenous levels of p21 (Figure 1D, compare lane 1 and 3) in the IKK1/2$^{-/-}$ MEFs, which is consistent with the enhanced p53 stabilization in these MEFs (Figure 1B). The subcellular localization of DoxR-induced p53 and p21 in both WT and IKK1/2$^{-/-}$ MEFs is nuclear (data not shown). Retrovirus mediated expression of the dominant negative p53, p53DD (Bowman et al., 1996), partially blocks DoxR-mediated cell death in IKK1/2$^{-/-}$ MEFs (Figure 1E). Similarly, p53DD also partially blocks cell death in WT MEFs (see below; Figure 5B). We conclude that DoxR-induced p53 in WT and IKK1/2$^{-/-}$ MEFs is: (1) modified upon DNA damage; (2) transactivation competent; (3) correctly localized in the cell; and (4) capable of inducing cell death.

Reduction of DoxR-induced cell death and p53 stabilization by IKK2

Since the IKK1/2$^{-/-}$ MEFs were sensitive to DoxR-induced cell death and accumulation of p53, we tested if reconstitution with IKK1 or IKK2 could reverse p53 accumulation and cell death. IKK1/2$^{-/-}$ cells were infected with recombinant adenoviruses (rAD) expressing IKK1WT (rAD-IKK1WT), the kinase inactive IKK1KM (rAD-IKK1KM), IKK2WT (rAD-IKK2WT), and kinase inactive IKK2KM (rAD-IKK2KM). As a control, cells were infected with rAD-GFP, a virus expressing the green fluorescent protein. Recombinant IKK1 and IKK2 proteins were detected using HA and flag tags, respectively. Only transduction with rAD-IKK2WT

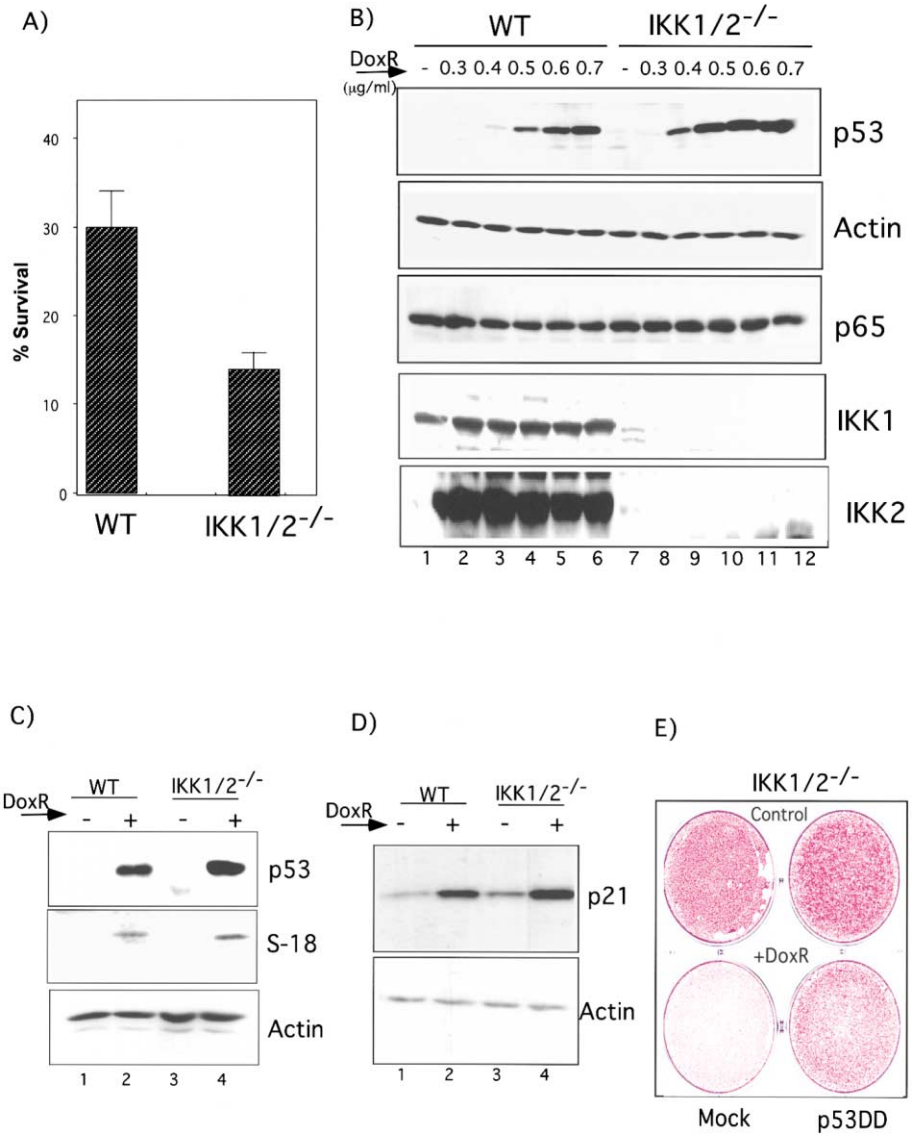

Figure 1. DoxR-induced cell death and p53 stabilization in MEFs

A: WT and IKK1/2$^{-/-}$ MEFs were plated in six-well plates and allowed to reach confluence. Cells were treated with 0.7 μg/ml DoxR and percentage survival was calculated as described. **B:** Confluent cells grown in 6-well dishes were treated with varying does of DoxR. Whole cell lysates were prepared in 2× SDS gel loading buffer and subjected to Western blot analysis with the indicated antibodies. **C:** Confluent WT and IKK1/2$^{-/-}$ MEFs were treated with 0.7 μg/ml DoxR and whole cell extracts prepared. Total and Ser-18 phosphorylated p53 levels were detected using clone PA421 and Phospho-p53 (Ser 15) 16G8 antibody, respectively. **D:** WT and IKK1/2$^{-/-}$ MEFs were treated with 0.5 μg/ml of DoxR, and whole cell lysates were prepared and resolved on a 15% gel. Level of p21 induction was detected using p21 (H-164) antibody. **E:** IKK1/2$^{-/-}$ MEFs were either mock infected or infected with pLXSN-p53DD retrovirus expressing p53DD. Pools of infected cells were treated with DoxR (0.4 μg/ml). Forty-eight hours after DoxR addition, surviving cells were stained. The expression of p53DD protein was confirmed using PA421 antibody (data not shown).

dramatically reduced the accumulation of p53 in response to DoxR in IKK1/2$^{-/-}$ cells (Figure 2A, lane 8). Northern blot analysis (Figure 2B) revealed comparable levels of p53 messenger RNA in rAD-GFP, rAD-IKK2WT, and rAD-IKK2KM transduced cells, indicating that IKK2WT-mediated effects on p53 stabilization operate at the posttranscriptional level. Furthermore, cells expressing IKK2WT but not IKK1WT or the kinase inactive IKKs were also more resistant to DoxR-induced cell death (Figure 2C).

To test the generality of our observations, we determined whether IKK2WT could block p53 stabilization in response to another anticancer agent, etoposide. We found that IKK2WT reduced p53 stabilization (Figure 2D) and cell death (data not shown) in response to etoposide. We conclude that IKK2 significantly antagonizes the ability of anticancer agents like DoxR or etoposide to increase p53 abundance and subsequent apoptosis.

IKK2 but not IKK1 can activate NFκB

NFκB pathway is the only signaling cascade documented to be modulated by IKK1 and IKK2 in vivo. To better understand the molecular distinction between IKK1 and IKK2 in antagonizing DoxR-induced p53 stabilization, we evaluated the potential of these kinases to activate NFκB in IKK1/2$^{-/-}$ cells. Gel shift analysis demonstrated that IKK2 (Figure 3A, lane 3) but not IKK1 was able to induce NFκB DNA binding in these MEFs. The binding to the Oct-1 probe was used as a loading control for the nuclear lysates prepared from IKK1/2$^{-/-}$ cells expressing GFP, IKK1, and IKK2. Treatment of IKK1$^{-/-}$ MEFs with DoxR induced NFκB DNA binding (Figure 3B). Since IKK1$^{-/-}$ cells contain IKK2, activation of NFκB in response to DoxR in these cells supports the notion that this activity is mediated by IKK2.

IKK2 mediated events require activation of NFκB

We next tested if IKK2-mediated activation of NFκB accounts for the observed difference in the abilities of IKK1 and IKK2 to regulate p53 levels. To address this question, we used cell lines expressing IκBαM, a dominant negative form of IκB molecule that blocks NFκB activation (Van Antwerp et al., 1996). The IKK1/2$^{-/-}$ MEFs were transduced with retroviral constructs pLXSH or pLXSH-IκBαM to generate stable cell lines (IKK1/

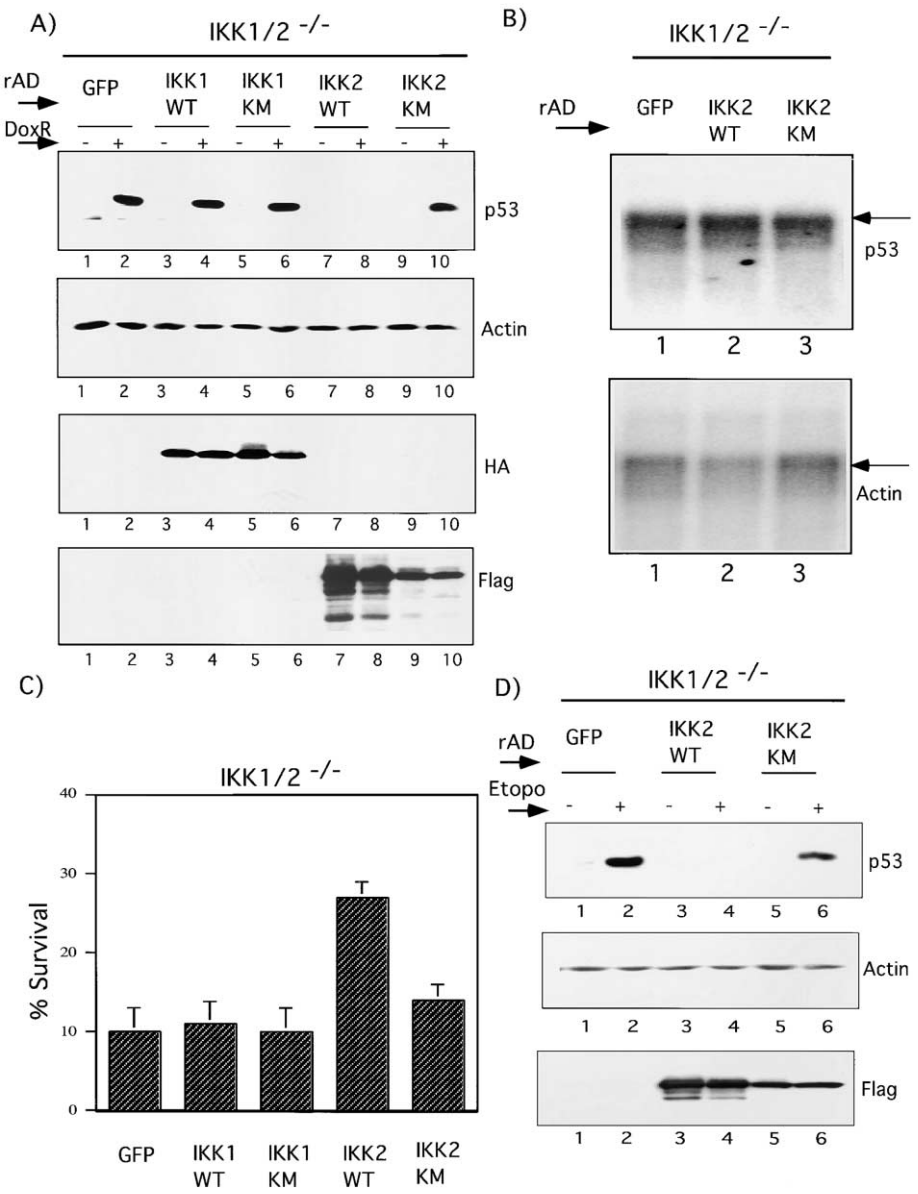

Figure 2. Expression of IKK2WT blocks DoxR and etoposide-induced p53 stabilization and cell death

A: IKK1/2$^{-/-}$ MEFs were cultured in six well dishes. Freshly confluent cells were infected with recombinant adenoviruses (rAD), expressing GFP (rAD-GFP), IKK1WT (rAD-IKK1WT), IKK1KM (rAD-IKK1KM), IKK2WT (rAD-IKK2WT), or IKK2KM (rAD-IKK2KM) in 300 μl DMEM with 5% FCS and antibiotics. The plates were rocked every 10 min and the infection was allowed to proceed for 2 hr, following which cells were replenished with media without removing the virus. This media was changed with fresh media after overnight incubation at 37°C. Forty-eight hours postinfection, cells were treated with DoxR at 0.4 μg/ml, whole cell lysates were prepared, and Western blot analysis with the indicated antibodies was performed. IKK1 and IKK2 proteins were detected using antibodies against HA and Flag tags, respectively. **B:** Northern blot analysis of IKK1/2$^{-/-}$ cells infected with the indicated rADs. Cells were treated with various concentrations of DoxR (shown here is 0.5 μg/ml) for 24 hr. A total of 8–10 μg of total RNA was analyzed by standard Northern blot hybridization. The complete murine p53 cDNA and a 200 bp fragment of actin were used as probes. **C:** IKK1/2$^{-/-}$ MEFs were cultured and infected in six-well dishes as before and treated with 0.7 μg/ml DoxR. Cell survival was estimated between 60 and 72 hr. **D:** IKK2 can block etoposide-mediated p53 stabilization. IKK1/2$^{-/-}$ MEFs were infected with rAD-GFP, rAD-IKK2WT, and rAD-IKK2KM as described before. Forty-eight hours postinfection, cells were treated with 100 μM etoposide. Total lysates were prepared 20 hr later by directly lysing the cells in 2× SDS gel loading buffer. Western blot analysis was performed for the indicated molecules.

2$^{-/-}$ Hygro and IKK1/2$^{-/-}$ IκBαM, Figure 4). Unlike in IKK1/2$^{-/-}$ Hygro cells, expression of IKK2WT in IKK1/2$^{-/-}$ IκBαM cells had significantly reduced effect on both, blocking p53 stabilization (Figure 4A compare lane 4 and lane 10) and inducing cell death (Figure 4B) in response to DoxR. The levels of flag-tagged IKK2WT expressed in IKK1/2$^{-/-}$Hygro and IKK1/2$^{-/-}$IκBαM cells were comparable. The persistence of IκBαM (Figure 4A, lanes 7–12) compared to IκBα (Figure 4A, lanes 1–6) confirms its inability to be modified and degraded. These results support the notion that NFκB activity is required for blocking DoxR-induced p53 and cell death.

Endogenous levels of NFκB inhibit p53 activation

Overexpression of IKK2WT resulted in constitutive levels of nuclear NFκB in both IKK1/2$^{-/-}$ and WT MEFs (data not shown). Although such constitutive NFκB activity is seen in various tumor cells and is important in reducing p53 levels in our assays, we next investigated if blocking the endogenous NFκB activation by dominant negative IκBαM molecule would lower the threshold for DoxR-mediated p53 induction. We made stable lines with WT MEFs expressing either empty pLXSH vector (WT-Hygro) or the IκBαM repressor (WT-IκBαM). We observed lower DoxR threshold and increased stabilization of p53 in WT-IκBαM MEFs (Figure 5A, compare lanes 3 and 4). While expression of IκBαM sensitized WT MEFs to cell death induced by DoxR, expression of p53DD could significantly reduce this death (Figure 5B).

IKK2 but not IKK1 can upregulate Mdm2

Since the ubiquitin ligase Mdm2 is a key determinant of p53 stability, we examined whether IKK2-mediated destabilization of p53 occurred through Mdm2. Reconstitution with IKK2WT and not IKK1WT upregulated the levels of Mdm2 protein (Figure 6A, lane 4). However, unlike in IKK1/2$^{-/-}$ or WT cells, overex-

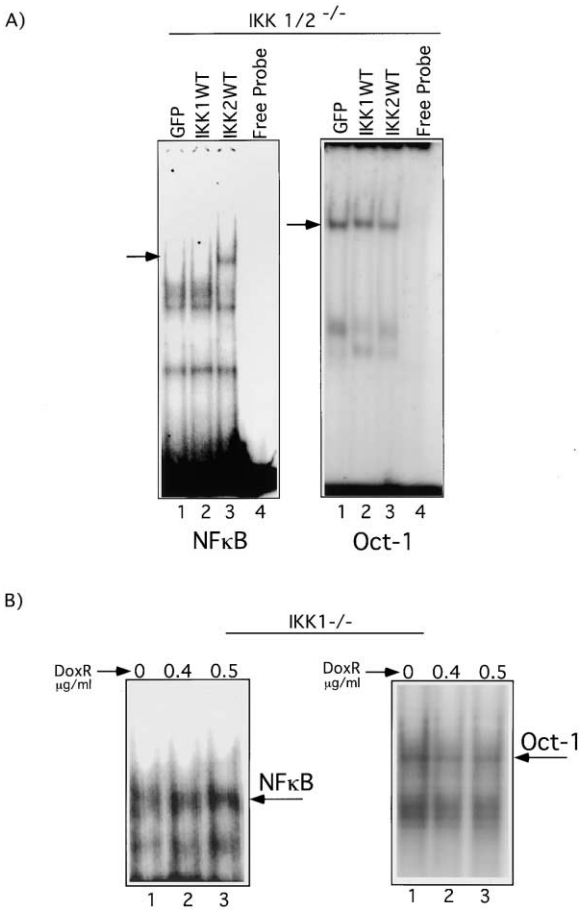

Figure 3. NFκB DNA binding activity in response to DoxR

A: Gel shift analysis of IKK1/2$^{-/-}$ MEFs transduced with the indicated viruses. Forty-eight hours postinfection, cells were treated with various concentrations of DoxR (shown here is 0.5 μg/ml), and nuclear extracts were prepared and analyzed for NFκB DNA binding activity. **B:** IKK1$^{-/-}$ MEFs were treated with indicated doses of DoxR and analyzed for gel shift activity. The oct-1 probe was used as loading control.

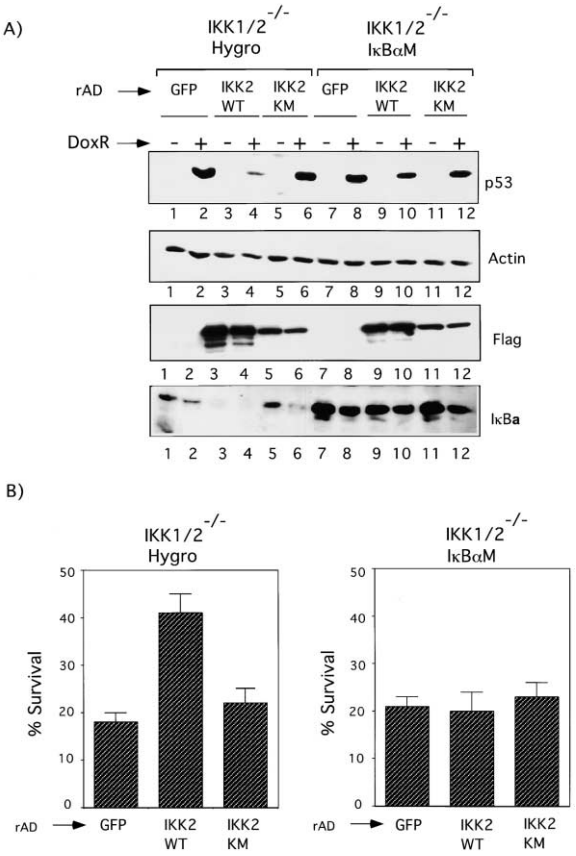

Figure 4. IKK2-mediated block of p53 stabilization and cell death requires activation of NFκB

A: IKK1/2$^{-/-}$ MEFs were infected with either pLXSH or pLXSH-IκBαM retroviruses and stably selected. Mixed populations of IKK1/2$^{-/-}$ Hygro (lane 1–6) and IKK1/2$^{-/-}$ IκBαM cells (lanes 7–12) were infected with rAD-GFP, rAD-IKK2WT, and rAD-IKK2KM as described above. Forty-eight hours postinfection, cells were either left untreated or treated with 0.4 μg/ml DoxR. p53 levels were measured in whole cell lysates using PA421 antibody. Actin levels were used to normalize for protein loading. Expression of IKK2 and IκBα proteins was measured using the Flag M2 and IκBα (C-21) antibodies, respectively. **B:** IKK1/2$^{-/-}$ Hygro and IKK1/2$^{-/-}$ IκBαM were infected with rAD-GFP, rAD-IKK2WT, and rAD-IKK2KM as described and treated with 0.7 μg/ml DoxR 48 hr postinfection. Cell survival was estimated as described before.

pression of IKK2WT in p53$^{-/-}$ did not lead to upregulation of Mdm2 (Figure 6B, compare lanes 2 and 5). Transducing IKK1/2$^{-/-}$Hygro cells with increasing MOI of rAD-IKK2WT caused a concomitant increase in the levels of Mdm2 protein (Figure 6C, lanes 2–6), which was blocked by coexpression of IκBαM (Figure 6C, lanes 8–12), implicating that NFκB activity is essential for IKK2-mediated upregulation of Mdm2. Comparable levels of IKK2 were expressed in both cell types (Figure 6C, lanes 2–6 and 8–12). Notably, levels of p53 at a constant dose of DoxR were much lower in IKK1/2$^{-/-}$Hygro cells (Figure 6C, lanes 2–6) than in the corresponding IKK1/2$^{-/-}$IκBαM cells (Figure 6C, lanes 8–12), once again indicating increased p53 levels in the absence of NFκB activity.

Modulation of Mdm2 messenger RNA levels

Northern blot analysis consistently revealed lower steady-state levels of Mdm2 mRNA in IKK1/2$^{-/-}$, p65$^{-/-}$ (data not shown), and p53$^{-/-}$ MEFs compared to WT MEFs (Figure 7A). Based on densitometric quantification, IKK1/2$^{-/-}$ had 40% less Mdm2 mRNA than WT MEFs (Figure 7B). Reconstitution with IKK2WT but not IKK2KM increased the levels of Mdm2 RNA in IKK1/2$^{-/-}$MEFs (Figure 7C). Unlike in WT and IKK1/2$^{-/-}$ MEFs, overexpression of IKK2WT in p53$^{-/-}$ MEFs did not lead to increase in Mdm2 RNA (data not shown). These results suggest that IKK2WT-mediated increase of Mdm2 mRNA requires p53 activity, and that the observed upregulation of Mdm2 protein by IKK2 most likely results from increased levels of its mRNA.

To test if Mdm2 is a direct target of NFκB, we stimulated WT and IKK1/2$^{-/-}$ cells with TNFα, a potent activator of NFκB. As expected, TNFα treatment increased levels of NFκB target gene IκBα in WT but not in IKK1/2$^{-/-}$ cells (Figure 7D). However, we did not observe an increase in Mdm2 RNA in this time scale (Figure 7D). Treatment of IKK1/2$^{-/-}$ cells with DoxR demonstrated comparable or higher levels of Mdm2 in cells expressing IKK2WT (Figure 7E, compare lanes 2, 4, and 6). We conclude that IKK2-mediated NFκB activation modulates basal levels of Mdm2, and additionally contributes to Mdm2 induction following DNA damage.

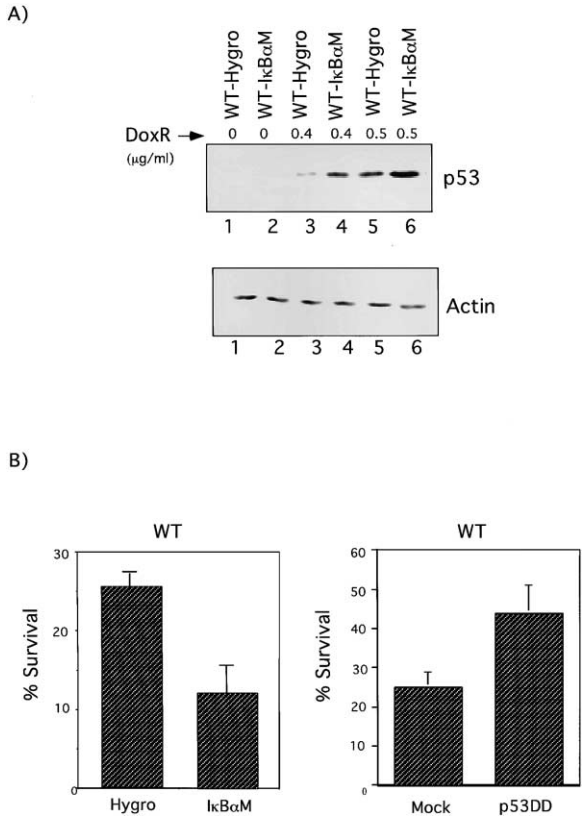

Figure 5. Endogenous levels of NFκB modulate DoxR-mediated induction of p53 and cell death

A: WT MEFs were infected with pLXSH or pLXSH-IκBαM retroviruses to generate stable pools WT-Hygro and WT-IκBαM, respectively. These cells were plated in six-well dishes and confluent cells were treated with indicated doses of DoxR for 18 hr. Cells were washed with PBS and directly lysed in 2× SDS buffer. Western blot analysis with p53 and actin antibodies was performed as described before. **B:** WT MEFs were either mock infected or infected with retroviruses encoding IκBαM and p53DD. Pools of infected cells were treated with 0.7 μg/ml DoxR and cell survival was estimated as described previously.

Discussion

Cell proliferation reflects the interplay of cell survival and cell death pathways. It is becoming apparent that the effectiveness of chemotherapeutic agents may similarly reflect their relative abilities to paradoxically activate cell death and cell survival pathways. p53 has been demonstrated to be important for modulating cell death in response to chemotherapeutic agents (Lowe et al., 1993a). However, a correlation between wild-type status of p53 and response to chemotherapy is not uniformly observed in tumors. In part, this is due to alterations in other factors that regulate p53 abundance or function, such as amplification of Mdm2 (Oliner et al., 1992) and HER-2/neu (Zhou et al., 2001), and mutations/deletions in INK4a/ARF loci (Sherr, 1998). Additional mutations and genetic variability might be other crucial modifiers of a tumor's response to chemotherapy. The findings presented in this paper implicate the NFκB signaling cascade as a potential modulator of p53 in response to chemotherapy.

We demonstrate that IKK1/2$^{-/-}$ cells that are severely defective in NFκB activity are more sensitive to cell death and p53 induction in response to DoxR (Figure 1) and etoposide (Figure 2). Reconstitution of IKK2 but not IKK1 can block this sensitivity to p53 stabilization (Figure 2). Since IKK2 does not downregulate the endogenous p53 promoter (Figure 2B) and upregulates Mdm2 expression (Figure 6A), our results indicate that IKK2 suppresses stabilization of the p53 protein. In addition to a role in cancer treatment, IKK2-mediated regulation of Mdm2 and p53 may have important implications in tumor progression. In tumors with wild-type p53, NFκB-induced Mdm2 might attenuate the p53 response in early phases of tumor development. NFκB-induced Mdm2 overexpression could also counter mutant forms of p53 that retain some p53 functions (Friedlander et al., 1996). Finally, given that p53-independent transformation properties of Mdm2 have been reported (Cordon-Cardo et al., 1994), NFκB-induced Mdm2 might contribute to tumor progression in a p53-independent manner.

NFκB has been shown to protect against a variety of cellular insults (Baichwal and Baeuerle, 1997). Antiapoptotic genes including scavenger enzyme MnSOD, inhibitors of apoptosis c-IAP1 and c-IAP2, Bcl-2 homologs such as Bfl1/A1 and Bcl-x, and the transcription factor A20 have been proposed to mediate NFκB dependent survival signals (Barkett and Gilmore, 1999). Intuitively, the IKK1/2$^{-/-}$ MEFs would fail to activate these NFκB dependent antiapoptotic genes which might be required for protection against both p53 dependent and independent cell death. While reconstitution of IKK2 and thus NFκB activity in IKK1/2$^{-/-}$ MEFs should reconstitute the function of these antiapoptotic genes, the exact contribution of NFκB in specifically repressing p53 dependent cell death in IKK1/2$^{-/-}$ MEFs remains unclear.

Genetic analysis of IKK1$^{-/-}$ and IKK2$^{-/-}$ single knockout mice clearly points to the distinct roles these kinases play in vivo (Li et al., 1999a, 1999b). However, given that these kinases are intimately associated in the IKK signalosome, which is crucial for NFκB activation, individual contributions of IKK1 and IKK2 in NFκB activation have been intensively probed. While IKK2 is crucial for IκBα degradation, IKK1 has recently been postulated to be required for a second NFκB-activating pathway (Senftleben et al., 2001). In this study we have reconstituted IKK1 and IKK2 in cells lacking both these kinases. Our results clearly demonstrate that IKK2 is both necessary and sufficient to activate NFκB (Figure 3) and block p53 stabilization and cell death in a NFκB-dependent manner (Figure 4). Genetic analysis using IKK2$^{-/-}$ MEFs had revealed that IKK1 homodimers can activate NFκB DNA binding in response to TNFα (Li et al., 1999b). The inability of IKK1 to activate NFκB activity in response to DoxR in our assay could indicate that unlike IKK2, IKK1-mediated NFκB activation might occur only in response to selective upstream signals. These results reiterate that apart from activating different signaling cascades in vivo, distinct genetic roles of these kinases could also be reflective of their differential abilities to activate NFκB. Designing IKK1 and IKK2 specific inhibitors could be instrumental in selectively blocking NFκB in response to distinct upstream signals.

Hodgkins lymphoma (Sovak et al., 1997) and breast cancer (Nakshatri et al., 1997) cell lines have constitutively elevated levels of nuclear NFκB. Blocking of NFκB activity has been shown to inhibit *ras* mediated transformation (Mayo et al., 1997). In addition, human T cell leukemia virus type 1 (HTLV-1) (Sun and Ballard, 1999) and Epstein-Barr virus (EBV) (Cahir McFarland et al., 1999) are some of the viruses that induce the activity of

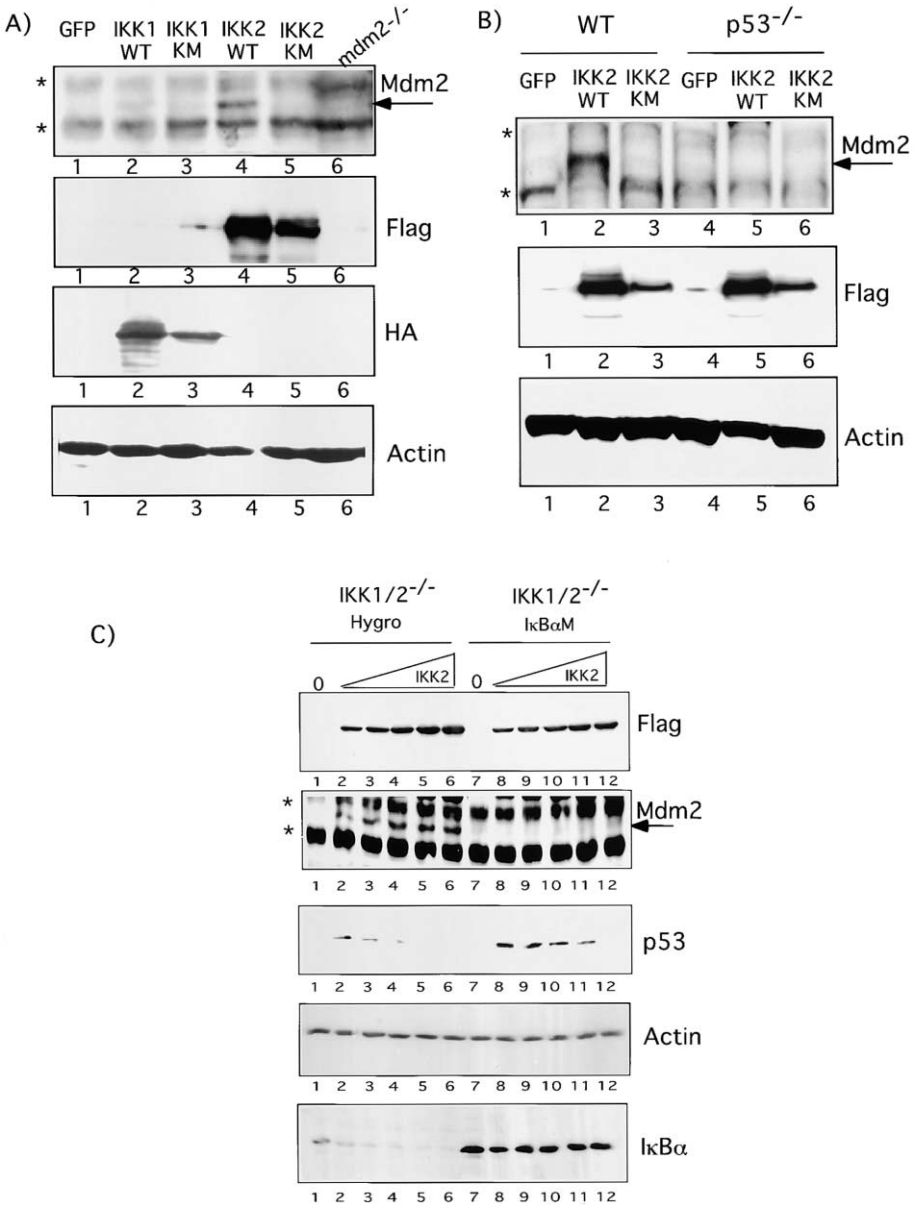

Figure 6. Modulation of Mdm2 protein by IKK2

A: MEFs were infected with the indicated adenoviruses. Western blot analysis with SMP14 antibody was performed 36 hr later to detect Mdm2 protein. The positions of two nonspecific bands have been indicated by * in all the blots. The reactivity of Mdm2 bands was cross-confirmed with another antibody, Ab-2 (Oncogene research products). **B:** WT and p53$^{-/-}$ MEFs were transduced with the indicated viruses and levels of Mdm2 proteins were analyzed 36 to 48 hr postinfection. **C:** IKK1/2$^{-/-}$Hygro (lanes 1–6) and IKK1/2$^{-/-}$IκBαM (lanes 7–12) cells were selected as described in Figure 4. These cells were either uninfected (lanes1 and 7) or infected with increasing titers of rAdIKK2WT (lanes 2–6 and 8–12). Forty-eight hours postinfection, only the infected cells were treated with 0.4 μg/ml of DoxR. Cell lysates were prepared 18 hr after addition of DoxR and analyzed with the indicated antibodies.

NFκB during cellular transformation. Cumulatively, consistent with the role of their viral homolog v-rel (Gilmore, 1999), the NFκB family members have been implicated in malignant transformation of several mammalian cell types. Defective IκBα activity and constitutive IKK activity have been mechanistically linked to the persistent nuclear NFκB seen in many human tumors (Rayet and Gelinas, 1999). In our study, expression of IKK2 alone was sufficient to cause persistently nuclear NFκB activity (data not shown) and significantly reduced cell death and p53 accumulation (Figure 4). It is tempting to speculate that human tumor cells with constitutive NFκB activity might exhibit a compromised p53 response.

To evaluate the role of p53 in NFκB dependent increase in Mdm2, we analyzed whether IKK2 could upregulate Mdm2 in p53$^{-/-}$ MEFs (Figure 6B). Using WT MEFs as matched controls, we showed that IKK2WT overexpression upregulates Mdm2 protein (Figure 6B) and RNA (data not shown) in WT but not in p53$^{-/-}$ cells. Since the p53 promoter has been documented to be upregulated by NFκB (Wu and Lozano, 1994), we addressed the concern that NFκB mediated upregulation of Mdm2 might simply be due to the fact that NFκB upregulates p53, and p53 in turn upregulates Mdm2. However, we do not believe that this is the case since we do not see increased p53 protein levels by overexpression of IKK2WT with or without chemotherapy (Figure 2A, lanes 7 and 8 or Figure 4A, lanes 3 and 4), and titration of increasing amounts of IKK2WT at a constant dose of DoxR showed concomitant increase in Mdm2 protein without increase in p53 (Figure 6C, lanes 1–6). Further, blocking NFκB using IκBαM under similar experimental conditions showed increased p53 (Figure 6C, lanes 7–12), suggesting that this increase in p53 might be a consequence of reduced mdm2 levels. Finally, the demonstration that lack of NFκB activity does not compromise the ability of DoxR to induce p53 (Figure 1B) suggests that NFκB-mediated upregulation of Mdm2 is not merely

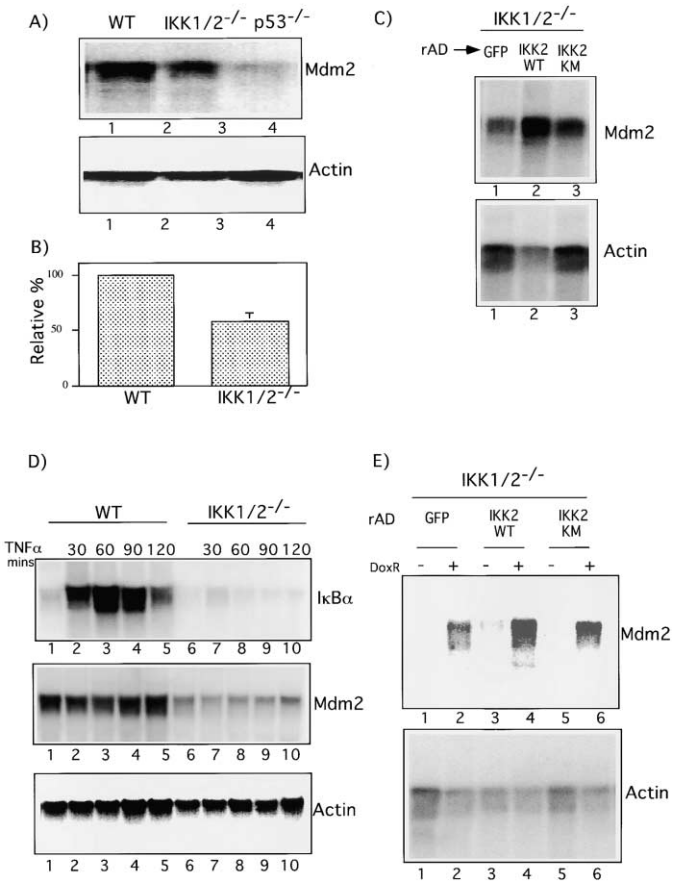

Figure 7. Modulation of Mdm2 mRNA by IKK2

A: Total RNA from indicated MEFs was prepared and analyzed for levels of Mdm2. **B:** Densitometric quantification of Mdm2 mRNA. Relative percent of Mdm2 levels are shown. **C:** IKK1/2$^{-/-}$ MEFs were transduced with the indicated viruses, and levels of Mdm2 were analyzed 48 hr postinfection. **D:** WT and IKK1/2$^{-/-}$ MEFs were treated with 20 ng/ml of TNFα for the indicated timepoints. Complete murine IκBα and Mdm2 cDNAs were used as probes. **E:** IKK1/2$^{-/-}$ MEFs were transduced with the indicated viruses and treated with 0.4 μg/ml of DoxR 48 hr postinfection. 12–16 hr later, total RNA was extracted and analyzed for Mdm2 expression by Northern blot analysis. Actin was used as loading control in all experiments.

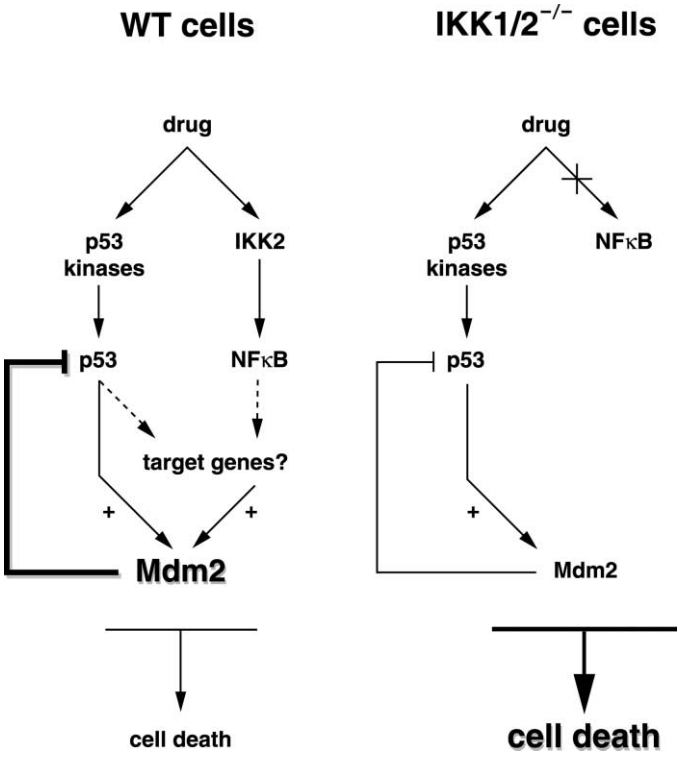

Figure 8. A proposed model for NFκB-mediated modulation of p53

In WT cells, anticancer drugs cause DNA damage and activate p53 via phosphorylation events. These drugs simultaneously activate NFκB via IKK2. NFκB or its target gene(s) cooperates with p53 or its target gene(s) to positively modulate Mdm2 levels. In cells lacking IKK2, NFκB is not activated by DoxR. The lack of this positive modulation results in lower levels of Mdm2 and thus greater sensitivity to p53 stabilization.

reflective of its increased transcription due to enhanced p53 protein levels.

A model based on results obtained in this study is presented in Figure 8. We show that treatment with anticancer drugs activates p53 (Figure 1) and NFκB (Figure 3). Activation of NFκB occurs selectively through IKK2 (Figure 3). NFκB cooperates with p53 and upregulates Mdm2 by increasing its RNA (Figure 7) and protein (Figure 6) levels. There are at least two lines of evidence that suggest that IKK2 positively modulates the expression of Mdm2. First, basal levels of Mdm2 RNA are lower in IKK1/2$^{-/-}$ (Figure 7A) and p65$^{-/-}$ (data not shown) cells, and they can be upregulated by reconstitution of IKK2WT but not IKK2KM (Figure 7C). Second, in response to DoxR, IKK1/2$^{-/-}$ cells expressing IKK2WT have comparable or higher Mdm2 (Figure 7E), despite the fact that they have very low levels of p53 protein (Figure 4, compare lanes 2, 4, and 6). It is conceivable that instead of a direct cooperation between these factors, NFκB or its target gene(s) might cooperate with p53 or its target gene(s) in modulating Mdm2 levels.

Based on transient transfection of IKK2 along with the 1 kb intronic mdm2 promoter (Ouchi et al., 1998) (data not shown) and results presented in Figure 7D, we suggest that Mdm2 might not be a direct transcriptional target of NFκB. Alternatively, the upregulation of Mdm2 mRNA by NFκB might occur by posttranscriptional mechanisms. The only such mechanism that has been reported (Hsing et al., 2000) has been documented to operate in a p53-independent manner. Given that IKK2-mediated upregulation of Mdm2 mRNA requires p53, which is crucial for Mdm2 transcription, we believe that IKK2-mediated effects are more likely to operate at transcriptional level. Recently, p63 and p73 have been shown to modulate p53-dependent transcription (Flores et al., 2002). Similarly, NFκB might be a positive modulator of p53-dependent transcription of Mdm2 and might be required to recruit chromatin remodeling complexes in a p53 dependent manner. The lack of this positive modulation of p53 dependent transcription of Mdm2 in IKK1/2$^{-/-}$ MEFs might result in lower levels of Mdm2 (Figure 7A) and thus greater sensitivity to p53 stabilization and cell death (Figure 1).

In contrast to our study, p53-mediated apoptosis was reported to require NFκB activation (Ryan et al., 2000). In our assays, recombinant adenovirus (rAdp53) mediated overexpression of p53 activated Mdm2, p21, and Bax in both WT and

IKK1/2$^{-/-}$ MEFs (V.T. and I.M.V., unpublished data). However, transduction with rAdp53 did not induce NFκB DNA binding in either of these MEFs. In addition, analysis of γ-irradiation-induced apoptosis of thymocytes, which is p53 dependent (Lowe et al., 1993b), did not reveal any differences between WT and IKK2$^{-/-}$ thymocytes (V.T., I.M.V., E. Dejardin, and D. Green, unpublished observations). These results indicate that activation of NFκB by p53 is a restricted phenomenon and is not essential for p53-induced cell death. Ryan and colleagues also found that compared to WT MEFs, p65$^{-/-}$ MEFs are less sensitive to DoxR-induced cell death. We observe high endogenous p53 levels in unchallenged p65$^{-/-}$ MEFs (data not shown). This is consistent with our observation that NFκB can reduce p53 levels. However, it is possible that the p53 in p65$^{-/-}$ MEFs was mutated, as this occurs frequently during spontaneous immortalization of MEFs (Harvey and Levine, 1991). The existence of mutated p53 in p65$^{-/-}$ MEFs would be consistent with decreased sensitivity of these MEFs to DoxR (Ryan et al., 2000). Additionally, this might explain the extremely low levels of Mdm2 mRNA observed in these cells (data not shown).

In summary, our results support the notion that tumors with persistent activation of NFκB should be targeted with strategies wherein chemotherapeutic agents could be combined with specific inhibitors of IKK2 for more efficient results. Ironically, although inclusion of such NFκB inhibitors could augment chemotherapeutic response against tumors, these strategies might sensitize normal bystander cells to p53-induced cell death.

Experimental procedures

Cell culture

Spontaneously immortalized murine embryo fibroblasts were cultured in DMEM supplemented with 10% FCS (Hyclone), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml of amphotericin B (Gibco). Doxorubicin was purchased from Sigma and dissolved in Milli Q water at a concentration of 10 mg/ml as stock. Etoposide (Calbiochem) was reconstituted in DMSO and stored at –20°C.

Retrovirus generation and stable cell selection

Twenty micrograms of vector plasmid was transfected with 5 μg of an expression vector for vesicular stomatitis virus G protein and 1 μg of a GFP expression plasmid into a cell line 293 gp/bsr (N. Somia and I.M.V., unpublished data). Supernatants containing the viruses were recovered 48 and 72 hr later, filtered through a 0.45 micron filter, and used to infect MEFs on three to six successive days. Freshly infected cells were stably selected on hygromycin (200 μg/ml).

Antibodies

The murine p53 antibody PA421 used to measure total p53 in all the assays shown was obtained from Calbiochem (clone PA421). The specificity of the p53 immunoreactivity of this antibody was cross-confirmed by using the FL-393 antibody (Santacruz Biotechnology). The Phospho-p53 (Ser 15) 16G8 antibody, which recognizes only serine 18 phosphorylated murine p53, was obtained from New England Biolabs. Anti-βactin antibody (Sigma) was used to detect levels of actin, which served as loading controls in all the experiments. The antibodies against IKK1 (M-280), IKK2 (H470), p65 (C-20), IκBα (C-21), IκBβ (C-20), Mdm2 (SMP14), and p21 (H-164) were all from Santacruz Biotechnology. Detection of flag-tagged IKK2 was performed using the Flag-M2 monoclonal antibody (F3165) from Sigma. IKK1WT and IKK1KM proteins were detected using anti HA antibody (12CA5).

Adenovirus production and infections

The rAD expression cassettes carrying the CMV IE enhancer, chicken β-actin promoter, and rabbit β-globin poly A signal were used to express GFP, IKK1WT, IKK1KM, IKK2WT, and IKK2KM proteins. Viruses were generated by homologous recombination in 293 cells (Miyake et al., 1996). rAD vectors do not integrate and replicate in the host cells. Thus, to keep the multiplicity of infection (MOI) constant, only freshly confluent cells were used for infection. A MOI of 200 has been used in all the experiments described.

Western blots

Typically, cells were cultured in six-well dishes, and confluent cells were treated with DoxR or etoposide for the indicated times, washed with PBS, and directly harvested in 2× gel loading buffer containing 100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and β-mercaptoethanol. Whole cell extracts were prepared by boiling the cells collected in the previous step and passing the lysates several times through a 25G needle. Lysates were resolved on Tris-glycine SDS gels (10% for p53, actin, p65, IκBα, IKK1, IKK2, and Mdm2, and 15% for p21). After electrophoresis, proteins were transferred on PVDV membrane (Immobilon P, Millipore) by a overnight transfer, blocked in PBS without Mg^{2+} and Ca^{2+} containing 0.2% tween-20 and 5% nonfat milk, and probed with the indicated antibodies in PBS without Mg^{2+} and Ca^{2+} containing 0.2% tween-20 and 1% milk.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described previously (Li et al., 1999b). Cells were washed in ice-cold PBS, scraped off the dish, and lysed for 10 min on ice using ice-cold hypotonic buffer (buffer A: 10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF). Nuclei were separated from the lysates obtained in the previous step by a brief centrifugation at 12,000 g. Nuclear extracts were prepared by a high salt lysis of the nuclei in buffer containing 20 mM HEPES [pH 7.9], 400 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT, incubated at 4°C for 20 min, supplemented with 5 vol. of storage buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT), and vortexed for 10 s. Lysed nuclei were centrifuged for 10 min at 12,000 × g, and the resulting supernatants were referred to as nuclear extracts. The DNA binding reaction was performed at room temperature in a volume of 20 μl, which contained the binding buffer (25 mM Tris-HCl [pH 7.5], 1.5 mM EDTA, 7.5% glycerol, 75 mM NaCl, 1.5 mM DTT, 0.3% NP-40, and 1 mg/ml BSA), 1 μg of poly (dI-dC), probe, and 7–10 μg of nuclear proteins. After incubation with the radiolabeled probe for 30 min, the samples were fractionated on a 4% polyacrylamide gel and visualized by autoradiography. The NFκB and Oct-1 probes have been described previously (Li et al., 1999b).

Cell survival assays

Cells typically were plated in six-well dishes. Confluent cells were treated with DoxR (0.7 μg/ml) for 60–72 hr. To estimate the percentage of survival, cells were stained with 0.05% crystal violet (w/v in 20% methanol) for 10 min. The excess stain was later washed off with water. The stain retained by surviving cells was leached in methanol and read immediately at 595 nm using a spectrophotometer. The untreated samples in each cell type were considered as 100% survival, and percentage cell survival was quantitated based on survival of untreated samples.

Northern blot analysis

Total RNA was isolated by TRIzol reagent (GibcoBRL) and quantitated by spectrophotometry. For Northern blot analysis, samples were resolved on 1% formaldehyde gels and blotted onto Hybond N+ membranes (Amersham Pharmacia). Following crosslinking, the membranes were hybridized to the probes in QuikHyb hybridization solution using the manufacturers instructions (Stratagene).

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