The Involvement of MAPK Signaling Pathways in Determining the Cellular Response to p53 Activation

CELL CYCLE ARREST OR APOPTOSIS

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The effect of ERK, p38, and JNK signaling on p53-dependent apoptosis and cell cycle arrest was investigated using a Friend murine erythroleukemia virus (FVP)-transformed cell line that expresses a temperature-sensitive p53 allele, DP16.1/p53ts. In response to p53 activation at 32 °C, DP16.1/p53ts cells undergo p53-dependent G1 cell cycle arrest and apoptosis. As a result of viral transformation, these cells express the spleen focus forming env-related glycoprotein gp55, which can bind to the erythropoietin receptor (EPO-R) and mimics many aspects of EPO-induced EPO-R signaling. We demonstrate that ERK, p38 and JNK mitogen-activated protein kinases (MAPKs) are constitutively active in DP16.1/p53ts cells. Constitutive MEK activity contributes to p53-dependent apoptosis and phosphorylation of p53 on serine residue 15. The pro-apoptotic effect of this MAPK kinase signal likely reflects an aberrant Ras proliferative signal arising from FVP-induced viral transformation. Inhibition of MEK alters the p53-dependent cellular response of DP16.1/p53ts from apoptosis to G1 cell cycle arrest, with a concomitant increase in p21WAF1, suggesting that the Ras/MEK pathway may influence the cellular response to p53 activation. p38 and JNK activity in DP16.1/p53ts cells is anti-apoptotic and capable of limiting p53-dependent apoptosis at 32 °C. Moreover, JNK facilitates p53 protein turnover, which could account for the enhanced apoptotic effects of inhibiting this MAPK pathway in DP16.1/p53ts cells. Overall, these data show that intrinsic MAPK signaling pathways, active in transformed cells, can both positively and negatively influence p53-dependent apoptosis, and illustrate their potential to affect cancer therapies aimed at reconstituting or activating p53 function.

The p53 tumor suppressor protein is activated in response to DNA damage and abnormal proliferative signals and can induce apoptosis through the activation of death-promoting gene targets (e.g. Bax, p53AIP1, Pidd/Lrd2, Puma, and Noxa), and cell cycle arrest through the activation of p21WAF1, GADD45, and 14-3-3sr (1). Apoptosis and arrest are believed to be important mechanisms of p53 tumor suppression, and are capable of preventing the expansion of cancer-prone cells (e.g. harboring mutations and/or under the influence of inappropriate growth signals). The factors and mechanisms underlying the decision to undergo apoptosis or growth arrest in response to p53 are not fully understood. This decision is likely complex and governed by multiple factors that depend on extrinsic factors (e.g. the presence of soluble growth factors) and intrinsic factors (e.g. hyperactive survival signaling pathways, defective death-signaling pathways) that are additionally cell type-specific.

We and others have shown that p53-induced apoptosis in hematopoietic cells can be suppressed in the presence of cytokines or mitogenic factors such as phorbol 12-myristate 13-acetate and that cells remain in a viable growth-arrested state (2–7). Survival pathways, commonly activated in transformed cells independently of their normal regulatory signals, can also rescue cells from p53-dependent death. For example, cells with constitutively activated phosphatidylinositol-3′-OH kinase (PI3′K)/protein kinase B (PKB) have a delayed response to apoptosis induced by p53 (8, 9). Ectopic expression of anti-apoptotic Bcl-2 proteins in multiple hematopoietic cell lines blocks p53-dependent apoptosis as well as apoptosis induced by γ-irradiation (6, 10–13). These cells remain in a viable p53-dependent growth-arrested state, reminiscent of that observed when p53 is activated in the presence of growth-promoting cytokines (6, 8, 13).

Extrinsic and intrinsic survival factors that alter the cellular response to p53 activation are likely to impinge upon targets that inhibit the cell death machinery. Anti-apoptotic Bcl-2 proteins comprise a subset of proteins that function in the mitochondrial apoptotic pathway, inhibiting the action of pro-apoptotic family members that trigger the release of cytochrome c and other apoptogenic factors from the mitochondria, leading to caspase activation and apoptosis (14). Under cytokine-stimulated growth and differentiation conditions, the resulting expression of either Bcl-2 or Bcl-XL leads to hematopoietic cell survival (15). Kinases acting in survival pathways like PKB, Rsk, and CAM-dependent protein kinase are all able to inhibit Bad, a pro-apoptotic Bcl-2 family member (16). Once phosphorylated at key residues by these kinases, Bad is bound by 14-3-3 proteins and sequestered in the cytoplasm where it is unable to inactivate Bcl-XL at the mitochondria (17).

Previously, we generated a cell line expressing a temperature-sensitive (ts) p53 transgene from an erythroleukemia cell line null for p53 (DP16.1/p53ts) (18). This cell line was established from a mouse infected with the polycythemia-inducing strain of the Friend murine erythroleukemia virus (FVP), a complex of spleen focus-forming virus (SFFV) and Friend murine leukemia virus. The p53ts mutation consists of an alanine-to-valine amino acid substitution at position 135 (p53Val→Ala135), producing a protein that adopts a mutant conformation at 37 °C and a wild-type conformation at 32 °C, the temperature at which p53 is activated and induces G1 arrest and apoptosis (2, 19). As a result

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of viral transformation, DP16.1/p53ts cells express the SFFV-encoded env-related glycoprotein gp55 (19). The oncogenic potential of gp55 derives from its ability to bind and activate the erythropoietin receptor (EPO-R), mimicking many aspects of EPO/EPO-R-induced signaling (20, 21) including the activation of Ras/Raf-1/MAPK, PI3’K/PKB, and protein kinase C (22–24). The gp55/EPO-R interaction is believed to be responsible for the increase in proliferation of splenic erythroid precursors observed during the initial stages of FVP infection, (25, 26). Recently, an isoform of stem cell-derived tyrosine kinase (SfK (short form)-Stk) was identified as a host factor that renders mice susceptible to FVP-induced disease (21, 27). SfK-Stk can covalently interact with gp55 bound to the EPO-R, resulting in constitutive SfK-Stk kinase activity (28), and SfK-Stk activation of Ras/MAPK and PI3’K/PKB signaling pathways has been implicated in gp55-mediated expansion of primary erythrocytes infected with FVP (29). Other genetic alterations including the inactivation of p53 occur during the late stages of FVP infection leading to erythroleukemia (18, 30).

We have shown previously that the PI3’K/PKB pathway is activated in DP16.1/p53ts cells. PKB is constitutively phosphorylated in these cells, as is its pro-apoptotic target, Bad. Moreover, in the presence of PI3’K inhibitors, phosphorylation of PKB and Bad is inhibited and p53-dependent apoptosis is markedly enhanced (8). Thus, when p53 is activated in the context of an active PI3’K/PKB signaling pathway, its ability to promote apoptosis is limited. It is possible that other EPO-R-dependent signaling pathways are activated in DP16.1/p53ts cells and that these could also influence the cellular response to p53 activation. In this respect, DP16.1/p53ts cells provide a useful model to study the interplay between p53 and growth/survival pathways that are frequently activated in transformed cells and may give insight into how cancer cells evade p53-dependent apoptosis.

Here we have examined the status of three EPO-responsive kinases, ERK, p38, and JNK (31, 32), in DP16.1/p53ts cells and investigated their ability to modulate the cellular response to p53 activation. We show that ERK, p38, and JNK are constitutively active in DP16.1/p53ts cells and that signaling through these MAPK pathways can influence the ability of p53 to promote apoptosis. The constitutive MEK signal is pro-apoptotic and contributes to p53 activation by phosphorylation on serine residue 15. When MEK is inhibited, the response of DP16.1/p53ts cells to p53 activation is redirected from apoptosis toward G1 arrest, with a concomitant increase in p21WAF1 protein expression. Conversely, p38 and JNK MAPKs are anti-apoptotic, and p53-dependent apoptosis increases when signaling through these kinases is inhibited. Thus, p38 and JNK MAPKs represent intrinsically activated survival pathways in DP16.1/p53ts cells. JNK activity in DP16.1/p53ts cells destabilizes p53, which could provide an explanation for the observed increase in p53-dependent apoptosis when JNK signaling is inhibited in these cells. This is consistent with other models that suggest JNK activity in proliferating cells promotes p53 degradation (33). Overall, this work demonstrates that intrinsic MAPK signaling pathways, active in transformed cells, can differentially regulate the cellular response to p53 activation and suggests that the Ras/MEK pathway may be a specific determinant of the decision to undergo p53-dependent apoptosis or G1 arrest.

EXPERIMENTAL PROCEDURES

Cell Culture—The DP16.1/p53ts cell line was derived from the Friend virus-transformed parental murine erythroleukemia cell line (DP16.1) by stable introduction of a temperature-sensitive p53 allele (A135V). Cells were maintained at 37 °C in α-minimal essential medium supplemented with 10% fetal calf serum (Hyclone) and routinely screened for p53 expression by Western blotting. MAPK inhibitors U0126, SB203580, and SP600125 (Calbiochem) were dissolved in dimethyl sulfoxide (Me2SO, 0.025%) and added directly to cell cultures 1 h prior to culturing at 32 °C. No-drug controls contained an equal volume of Me2SO. Cycloheximide (Sigma) was used at a concentration of 2 μg/ml.

Apoptosis and Cell Cycle Analysis—Apoptosis was assessed using annexin V-phycocerythrin (BD Biosciences-Pharmingen) and 7-aminoactinomycin D (7-AAD, Sigma) staining of cells and flow cytometry. Cells staining positive for annexin V and negative for 7-AAD were scored as apoptotic.

For cell cycle analysis, cells were fixed on ice in 70% ethanol, washed with 1% bovine serum albumin-containing phosphate-buffered saline, incubated with 1 mg/ml RNase A for 10 min at 37 °C, and finally resuspended in phosphate-buffered saline containing 0.1 mg/ml propidium iodide. Acquisition and analysis of flow cytometry data were carried out using CellQuest (version 3.3) on a FacScan™ flow cytometer (BD Biosciences). Cell cycle analysis was performed using ModFit LT (version 2.0).

Western Blotting—Buffer consisting of 1% Nonidet P-40, 50 mM HEPES, pH 7.0, 250 mM sodium chloride, and 1 mM EDTA supplemented with protease inhibitor mixture (Roche Applied Science) was used for cell lysis. For the detection of phosphorylated p53, this buffer additionally contained 1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate. The Bradford assay (BioRad) was used to estimate protein concentrations. 80 μg of total protein was routinely used for antigen detection, with the exception of p53, for which 30 μg was sufficient. An equal volume of 2× SDS sample buffer (2% SDS, 10% glycerol, 0.2 M dithiothreitol, bromphenol blue) was added to samples prior to resolution by PAGE. Gels were transferred to polyvinylidene difluoride membranes and incubated with the indicated antibodies according to the manufacturer’s instructions. Panspecific p53 monoclonal antibody (PAb421) and polyclonal p53 antibody (Ab7) from Oncogene were used to detect total p53 protein. Monoclonal phosphospecific p53 (serine 15), ERK1/2 (threonine 202/tyrosine 204), and polyclonal phospho-specific MAPKAPK-2 (threonine 334) antibodies were from Cell Signaling Technology. Polyclonal phosphospecific JunD (serine 73) antibody was from Upstate, and total JunD antibody was from Santa Cruz Biotechnology. Polyclonal p21WAF1 (Santa Cruz) and monoclonal β-actin (Sigma) antibodies were also used in this study.

Transient Expression of Constitutively Active Raf-1 and Dominant Interfering MKK3, MKK6, and SEK1—Constitutively activated pMTSM-Raf-caax is a chimeric protein consisting of full-length human Raf-1 fused to a K-Ras4B membrane-targeting sequence at the carboxyl terminus (34). Dominant interfering MKK3, MKK6, and SEK1 kinase mutants have alanine and leucine amino acid substitutions at critical activating residues: pMT2-MKK3-AL (S189A/T193L), pMT2-MKK6-AL (S207A/T211L), and pcDNA3.1-SEK1-AL (S204A/S207L). (35). These constructs were kindly provided by J. Woodgett (Ontario Cancer Institute). FuGene™ (Roche Applied Science) transfection reagent was used to introduce kinase expression constructs in combination with a pcDNA3.1-CD20 expression plasmid (3 μg 1 μg, respectively) into 2 × 10^6 DP16.1/p53ts cells, according to the manufacturer’s protocol. Cells were cultured at 37 °C for a 24-h recovery period and then diluted to 1 × 10^6 cells/ml and cultured at 32 °C for 12, 16, or 20 h. CD20 expressing cells were identified by flow cytometry with a fluorescein isothiocyanate-conjugated anti-CD20 antibody (BD Biosciences-Pharmingen), and apoptosis was measured in this CD20^+ subpopulation. Raf-caax was additionally transfected into the H1299 human lung carcinoma cell line using FuGene transfection reagent according to the manufacturer’s protocol.
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Antisense Inhibition of ERK Expression—High pressure liquid chromatography-purified, phosphorothioate-modified antisense (AS) and control scrambled (CON) ERK oligonucleotides (Invitrogen) were introduced into DP16.1/p53ts cells using the Amaxa Nucleofector system: AS-ERK: 5′-GCCGCGCAGGCGCCAT-3′; CON-ERK: 5′-CGCGGTCTGCGGACCC-3′ (36). Cells were harvested by washing twice with phosphate-buffered saline and then resuspended in Nucleofector solution to a concentration of 2 × 10^7 cells/ml. 100 μl of this cell suspension was added to 3 μg of AS- or CON-ERK oligonucleotide and exposed to the electrical pulse generated by the Nucleofector device (program U-15). Immediately thereafter, cells were diluted to 2 × 10^5 cells/ml in α-minimal essential medium supplemented with 20% fetal calf serum and cultured at 37 °C for 24 h. Cells were further diluted to 1 × 10^5 cells/ml in α-minimal essential medium supplemented with 10% fetal calf serum prior to culturing at 32 °C. ERK expression was determined by Western blot analysis, and apoptosis was measured by flow cytometry as described.

Detection of ubiquitinated p53—DP16.1 and DP16.1/p53ts cells were cultured in the presence or absence of SP600125 (10 μM) for 15 h at 32 °C. Cells were treated with the proteasome inhibitor MG132 (Sigma) for 5 h prior to harvesting. Cells were lysed into immunoprecipitation buffer consisting of 1% Triton-X-100, 50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 10 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate supplemented with protease inhibitor mixture (Roche Applied Science). 1 mg of total protein was incubated with 3 μg of polyclonal p53 antibody (FL393, Santa Cruz Biotechnology) or rabbit IgG for 12 h at 4 °C. Protein A-Sepharose beads were added to the immunoprecipitates, and following a 1-h incubation at 4 °C, beads were recovered, washed with immunoprecipitation buffer, resuspended in an equal volume of 2× SDS sample buffer, and resolved by SDS-PAGE. Ubiquitinated p53 was detected by Western blot analysis with a mouse anti-ubiquitin monoclonal antibody (6C1.17, BD Biosciences-Pharmingen).

RESULTS

MAPKs Are Intrinsically Active in DP16.1/p53ts Cells—To evaluate how MAPK signaling might modulate the cellular response to p53 activation, we examined the activity of ERK, p38, and JNK kinases in

FIGURE 1. Constitutive activation of ERK, p38, and JNK MAPKs in DP16.1/p53ts cells. Cells were cultured at 32 °C for 8 h in the presence of increasing amounts of MAPK inhibitors as indicated. A, ERK protein expression was determined by Western blot analysis using antibodies for phospho-ERK1/2 (upper panel) and total (tot) ERK1/2 (lower panel). B, phosphorylation of the p38 substrate, MAPKAPK-2 kinase, was detected by Western blot analysis with a phospho-specific MAPKAPK-2 kinase antibody (upper panel) followed by immunoblotting with β-actin (lower panel). C, phosphorylation of the JNK substrate JunD was detected by Western blot analysis with antibodies for phospho-JunD (upper panel) and total JunD (lower panel).
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The expression of a constitutively active Raf-1 kinase (Raf-caax) had the opposite effect and enhanced apoptosis of DP16.1/p53ts cells after p53 activation at 32 °C. Raf-caax-transfected cells showed a modest increase in apoptosis at 32 °C at 12 and 20 h in comparison to untransfected and empty-vector control-transfected cells (Fig. 3A). Importantly, Raf-caax expression did not promote apoptosis of DP16.1/p53ts cells cultured at 37 °C, indicating that the enhanced apoptotic effect was dependent upon p53. The modest effect of Raf-caax likely reflects the high levels of phosphorylated active ERK in DP16.1/p53ts cells and is consistent with the view that MEK may already be fully activated in these cells. The ability of Raf-caax to stimulate MEK/MAPK signaling was demonstrated in transfected H1299 cells in which Raf-caax expressing cells showed increased phospho-MEK1/2 compared with untransfected cells (Fig. 3B). These data suggest that Raf-1 and MEK can act as pro-apoptotic kinases, supporting a model in which constitutive Ras/Raf-1/MEK signaling sensitizes cells to undergo p53-dependent apoptosis.

Phosphorylation of p53 at Serine 15 is Associated with p53 Activation and Apoptosis Induction—A number of reports suggest that ERK phosphorylates p53 on serine 15 in response to various stress-inducing stimuli known to cause apoptosis (37–39). We examined the phosphorylation status of this residue in DP16.1/p53ts cells and found that p53 was not phosphorylated at serine 15 at 37 °C, consistent with p53 inactivation at this temperature. After cells were cultured at 32 °C for 6 h, serine 15-phosphorylated p53 was readily detected (Fig. 3B). Together, these data suggest that Raf-1 and MEK can act as pro-apoptotic kinases, supporting a model in which constitutive Ras/Raf-1/MEK signaling sensitizes cells to undergo p53-dependent apoptosis.

Serine 15 phosphorylation is inhibited by U0126. A, DP16.1/p53ts cells were cultured at 37 or 32 °C for 6 h, and p53 protein expression was determined by Western blot analysis with antibodies that detect p53 phosphorylated on serine 15 (upper panel) or total p53 (lower panel). B, p53 protein expression in DP16.1/p53ts cells cultured at 32 °C for 14 h in the absence (−) or presence (+) of U0126 (10 μM).
phosphorylation decreased upon U0126 treatment (Fig. 4B). The ability of U0126 (10 μM) to block MEK activity and diminish p53 serine 15 phosphorylation and p53-dependent apoptosis raised the possibility that constitutive Ras/MEK sensitizes DP16.1/p53ts cells to undergo p53-dependent apoptosis by contributing to p53 phosphorylation at serine 15. It is likely that other factors also contribute to p53 serine 15 phosphorylation at 32 °C, because serine 15 phosphorylation was not completely suppressed with U0126. To investigate a direct involvement of ERK in mediating p53-dependent apoptosis, we introduced antisense oligonucleotides to inhibit ERK expression in DP16.1/p53ts cells. Despite effective ablation of ERK expression in the antisense-treated cells (Fig. 5A), p53-dependent apoptosis was unaffected (Fig. 5B). Thus, the constitutive Ras/MEK signal that contributes to p53-dependent apoptosis of DP16.1/p53ts cells is likely mediated through MEK independently of ERK.

Inhibition of MEK Alters the Cellular Response to p53 Activation—Upon p53 activation, DP16.1/p53ts cells undergo a transient arrest in G1, which is followed by apoptosis, the latter being the predominant response to p53 activation in these cells. Having shown that MEK inhibition decreases p53-dependent apoptosis, we next investigated the effect of MEK inhibition on p53-dependent cell cycle arrest. Control and U0126-treated DP16.1/p53ts cells were cultured at 32 °C for 16 h, and cell cycle profiles were obtained by propidium iodide staining and flow cytometry (Fig. 6A). As expected the number of DP16.1/p53ts cells containing less than 2N DNA content was decreased in the presence of U0126 at 32 °C, consistent with the suppression of apoptosis observed with annexin V staining. The proportion of cells in each phase of the cell cycle was determined, and the G1/S ratios were calculated to depict the extent of G1 cell cycle arrest. When cells were cultured at 32 °C for 16 and 24 h in the presence of U0126, the G1/S ratio was almost 2-fold greater compared with that of control-treated cells (Fig. 6B). This arrest response can be attributed solely to p53 activation, because the cell cycle profiles of U0126- and control-treated DP16.1 parental cells (null for p53) were unchanged at 32 °C (Fig. 6, A and B). Moreover, U0126 treatment did not affect the cell cycle profile and G1/S ratios of DP16.1/p53ts cells cultured at 37 °C, the temperature at which p53 is inactive (Fig. 6, A and B).

To investigate further how MEK inhibition influences the cellular response to p53 activation, we measured the expression of three p53-responsive genes, p21WAF1, Puma, and Bax. p21WAF1 is a transcriptional target of p53, known to play an important role in mediating G1 arrest, whereas Puma and Bax promote apoptosis. p53-deficient DP16.1 cells do not express p21WAF1 or Puma at 32 °C in the presence or absence of the MEK inhibitor (Fig. 6C). The levels of both proteins were elevated in DP16.1/p53ts cells 16 h after p53 activation at 32 °C. In U0126-treated DP16.1/p53ts cells, we observed a further increase in the level of p21WAF1 and a decrease in the level of Puma compared with untreated cells 16 h after p53 activation at 32 °C. Bax levels remained unchanged under all conditions. Together, these data suggest that the Ras/Raf-1/MEK pathway influences the cellular response to p53 activation by sensitizing cells to undergo apoptosis. Moreover, the increase in p21WAF1 expression and the accompanying decrease in Puma expression seen upon MEK inhibition suggest that MEK activity influences target promotor selection by p53.

Inhibition of p38 and JNK Enhances p53-dependent Apoptosis—To determine the influence of constitutive p38 and JNK MAPK signals on p53-dependent apoptosis, DP16.1/p53ts cells were cultured at 32 °C in the presence of SB203580 and SP600125 inhibitors. With both inhibitors, p53-dependent apoptosis was increased; no effect was observed on inhibitor-treated parental DP16.1 cells cultured at 32 °C or on DP16.1/p53ts cells cultured at 37 °C (Fig. 7A). To confirm these results, DP16.1/p53ts cells were transfected with dominant interfering mutants of MKK3, MKK6, and SEK1; MKK3/6 act upstream of p38, and SEK1/2 act upstream of JNK. Apoptosis was significantly enhanced in cells expressing the dominant interfering MKK6-AL mutant compared with cells transfected with empty vector (p = 0.0013) but not in cells expressing the MKK3-AL mutant (Fig. 7B). Moreover, co-expression of both MKK6-AL and MKK3-AL resulted in a similar increase in apoptosis compared with expression of MKK6-AL alone, suggesting that MKK6 is primarily responsible for activating the anti-apoptotic function of p38 in DP16.1/p53ts cells. A small increase in apoptosis was observed in DP16.1/p53ts cells expressing SEK1-AL (data not shown). It is likely that SEK2, a known JNK MAPK kinase, also contributes to JNK activity in DP16.1/p53ts cells; however, a dominant interfering SEK2 mutant kinase was not available to address this possibility. Overall, these data demonstrate that intrinsic p38- and JNK-mediated signaling pathways suppress p53-dependent apoptosis in DP16.1/p53ts cells.

JNK Regulates p53 Protein Levels and Stability—In proliferating cells under non-stressed conditions, JNK has been reported to target p53 for degradation by the ubiquitin-mediated proteasomal pathway (33). We examined p53 protein levels in SP600125-treated DP16.1/p53ts cells cultured at 32 °C at time points leading up to the appearance of annexin V-positive apoptotic cells and found that p53 was elevated compared with untreated cells (Fig. 8A). To determine whether the elevation in p53 protein level was due to an increase in p53 stability, we performed a cycloheximide-blocking experiment to measure the half-life of p53 pro-
tein. p53 has a half-life of about 1 h in DP16.1/p53ts cells cultured at 32 °C. In the presence of SP600125, the p53 half-life increased to about 3 h (Fig. 8). Moreover, an increase in the level of serine 15-phospho-rylated p53, a post-translational modification associated with increased p53 protein stability, was observed in JNK inhibitor-treated cells; this effect was not observed in cells treated with the p38 inhibitor (Fig. 8). These findings are consistent with the view that JNK mediates p53 degradation in non-stressed cells (33) and suggest that JNK might antagonize the pro-apoptotic function of p53 by promoting its degradation. To determine whether JNK regulates p53 stability at the level of ubiquitination, we treated DP16.1/p53ts cells with the JNK inhibitor SP600125 and measured p53 ubiquitination by immunoprecipitation (p53).

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FIGURE 6. MEK inhibition increases p53-dependent G1 arrest. A, DP16.1/p53ts and parental DP16.1 cells were cultured at 37 °C or 32 °C for 16 h in the absence or presence of 10 μM U0126. DNA content was determined by propidium iodide staining and flow cytometry. B, The G1/S phase ratios, which depict the extent of cell cycle arrest, were determined from the cell cycle profiles of DP16.1/p53ts and DP16.1 cells cultured at 37 or 32 °C for 0, 16 and 24 h, in the absence or presence of 10 μM U0126. For the DP16.1/p53ts cells, each bar represents the average of three independent experiments ± S.E. C, Western blot analysis of p21WAF1, Puma, and Bax protein expression in DP16.1 and DP16.1/p53ts cells cultured at 32 °C for 16 h in the absence (−) or presence (+) of 10 μM U0126. Blots used to examine p21WAF1 and Puma/Bax expression was reprobed individually to determine β-actin protein levels as a protein loading control.

FIGURE 7. Inhibition of p38 and JNK activity increases p53-dependent apoptosis. A, DP16.1/p53ts cells (open bars) were cultured at 32 °C or 37 °C for 16 h in the presence of SB203580 (SB) or SP600125 (SP) as indicated. Parental DP16.1 cells (filled bars) were cultured at 32 °C for 16 h in the presence of inhibitors and served as additional controls. The proportion of cells undergoing apoptosis (annexin V-positive/7-AAD negative) was determined by flow cytometry and is expressed relative to untreated cells (n = 3, ± S.E.). B, DP16.1/p53ts cells were cotransfected with vectors expressing CD20 together with MKK3-AL, MKK6-AL, both MKK3-AL and MKK6-AL, or empty vector (EV) and cultured at 32 °C for 16 h. The percentage of CD20-positive cells undergoing apoptosis (annexin V-positive/7-AAD negative) was determined by flow cytometry (n = 3, ± S.E.). The difference between MKK6-AL and empty vector was significant (t test, p = 0.0031).
Western blotting with an antibody against ubiquitin. The level of ubiquitinated p53 was similar in SP600125-treated and untreated cells (Fig. 8D). This finding was unexpected and suggests that JNK may regulate p53 stability at a step subsequent to ubiquitination.

DISCUSSION

It is unclear why certain cells undergo apoptosis in response to p53 activation, whereas other cells undergo p53-dependent cell cycle arrest. Differences in the cellular response to p53 activation have been attributed to extracellular survival factors and to intrinsic factors that might reflect differences in DNA repair, the activation of intracellular survival pathways, oncogene-mediated sensitization to apoptosis, the magnitude of p53 protein expression, or the selective transactivation/repression of p53 target genes in different cell types. Understanding how intrinsic and extrinsic factors influence p53-dependent cell fate decisions (cell cycle arrest and apoptosis) is of fundamental importance in cancer biology. The common ability of survival-promoting cytokines to suppress p53-induced apoptosis has been widely documented and may reflect a mechanism by which p53-positive tumors gain resistance to apoptosis-inducing anticancer agents (15). The activation of intrinsic survival pathways that rescue cells from p53-dependent death has implications concerning tissue injury and repair, tumor evolution, and response to cancer therapy. Survival pathways are often constitutively activated in cancer cells, thereby bypassing the normal requirement for extracellular factors to protect from apoptosis. Here we show that ERK, p38, and JNK MAPKs are constitutively activated in SFFV-transformed DP16.1/p53ts cells under normal growth conditions. Previously we demonstrated that JAK1 and PI3K were constitutively activated in these cells (8) possibly because of SFFV-encoded gp55 protein-mediated activation of the EPO receptor. Our findings are consistent with observations that gp55 can substitute for EPO in many aspects of EPO-R signaling such as MAPK activation (40). Other SFFV-infected hematopoietic cells show cytokine-independent activation of Raf-1 and MEK (23, 29). Thus, DP16.1/p53ts cells provide a useful model for investigating how intrinsically activated survival signals influence the cellular outcome to p53 activation.

We found that inhibiting MEK activity suppressed p53-dependent apoptosis and that expression of a constitutively activated Raf-1 mutant ( Raf-caax) had the opposite effect, that of enhancing p53-dependent apoptosis. In contrast to the well documented role of the Ras/MEK/ERK pathway in the suppression of apoptosis (41–44), our results indicate that this pathway contributes to p53-dependent apoptosis. One explanation could be that in oncogene-expressing cancer-prone cells (45–47), the Ras/MEK/ERK pathway is deregulated to facilitate tumor cell growth and the resulting hyperproliferation sensitizes cells to apoptotic signals (e.g. mediated by p53 upon activation at 32 °C in DP16.1/p53ts cells). Tremendous selection pressure to lose cellular apoptotic programs during transformation is expected in cells exposed to such an aberrant growth signal (48). It is well established that gp55-mediated activation of the EPO-R promotes polyclonal proliferation of SFFV-infected erythroid cells in the early stage of virus-induced erythroleukemia...
The emergence of malignant clones in the late stage of the disease is accompanied by loss of p53 expression (25).

Several mechanisms of p53 activation by the Ras/MAPK pathway have been described. Ultimately, this pathway leads to Rb phosphorylation and E2F-1 activation, which in addition to activating genes required for entry into the cell cycle also activates p19ARF. p19ARF binds Mdm2 and blocks its ability to target p53 for degradation, leading to p53 protein stabilization and transcriptional activation (49, 50). This mechanism of p53 activation is associated with p53 protein accumulation, an effect that was not observed in DP16.1/p53ts cells when p53 was activated at 32 °C. Other reports suggest that ERK contributes to p53 activation by phosphorylation of p53 on serine 15 (37–39), prompting us to examine the phosphorylation status of this site in DP16.1/p53ts cells. Serine 15-phosphorylated p53 was not detected in DP16.1/p53ts cells cultured at 37 °C, although total p53 was readily detected. Remarkably, phosphoserine 15 was induced upon temperature shift in DP16.1/p53ts cells, suggesting that phosphorylation at this site contributes to p53 activation and the induction of apoptosis at 32 °C. Moreover, the expression of serine 15-phosphorylated p53 correlated with apoptosis, as it was decreased in MEK inhibitor-treated cells. Amino acid substitutions of p53 serine 15 and 20 are known to specifically impair the ability of p53 to induce apoptosis, leaving its transactivating and growth-suppressive functions intact (51). Consistent with our observations, others report a p53-dependent apoptotic response induced by resveratrol, which is associated with MAPK activity and phosphorylation of p53 at serine 15 (52). Other studies, however, demonstrate that unphosphorylated p53, or p53 containing mutations of key amino-terminal serine residues, retains its ability to induce apoptosis (53, 54). The role of specific phosphorylation events in the induction of p53-dependent apoptosis remains unresolved, but these events are likely to be influenced by the nature and magnitude of the activating stimulus (55).

We observed that MEK inhibition suppressed p53-dependent apoptosis and enhanced p53-dependent G1 arrest, with a concomitant increase in the expression of p21WAF1 protein and a decrease in the expression of Puma protein. It is possible that differential promoter activation by p53 could account for this switch from an apoptotic response in favor of growth arrest (56). When p53 is activated in transformed, aberrantly proliferating cells, it may preferentially activate apoptotic target genes; however, when the transforming signal is blocked, p53 may be redirected to promoters like p21WAF1, favoring survival in an arrested state. Our data suggest that serine 15 may play a role in differential promoter activation by p53 in DP16.1/p53ts cells. Alternatively, p53 promoter activation may remain unchanged under apoptotic and growth arrest/survival conditions. In this model, the Ras/MEK pathway may act through a parallel pathway that sensitizes cells to undergo p53-dependent apoptosis and that suppresses p21WAF1 expression.

p21WAF1 is a key modulator of the arrest versus apoptotic response downstream of p53. Cells that typically undergo growth arrest upon ectopic p53 expression or exposure to γ-irradiation undergo apoptosis when rendered p21WAF1-deficient by targeted deletion or antisense inhibition of p21WAF1 protein expression (57–59). Conversely, growth arrest resulting from ectopic p21WAF1 expression rescues p53-induced apoptosis of p21WAF1 null primary mouse embryo fibroblasts and other apoptosis-sensitive cells (59). In vivo tumor models strongly suggest that an intact p21WAF1 arrest response contributes to radioresistance and decreased cure rates in mice treated with ionizing radiation (60–62). The conditions and upstream factors that determine the extent of p53-dependent p21WAF1 induction and whether these results in arrest or apoptosis are not clear. Here we suggest that aberrant proliferative signaling through Ras/MEK may be one such determinant; when attenuated, the cellular response to p53 activation is directed toward growth arrest with an accompanying increase in p21WAF1 protein expression.

Inhibition of p38 and JNK, as well as upstream activating kinases MKK6 and SEK1, resulted in an increase in p53-dependent apoptosis of DP16.1/p53ts cells. These enhanced apoptotic effects suggest that p38 and JNK MAPKs play an anti-apoptotic role in DP16.1/p53ts cells and are capable of limiting p53-dependent death. Although p38 and JNK were originally identified as stress-activated MAPKs involved in apoptosis, mounting evidence supports a role for these kinases in cell survival, particularly in cells of hematopoietic origin (63, 64). Suppression of p53-dependent apoptosis by p38 and JNK in DP16.1/p53ts cells is likely the result of intrinsic kinase activity resulting from p55 activation of the EPO-R. It is possible that other transforming events occurred during the course of FVP-induced transformation to render these kinases constitutively active. Regardless, it is reasonable to postulate that the activity of p38 and JNK in this context differs from that which occurs as a result of exposure to cellular stress. Recently, JNK was shown to target p53 for ubiquitin-mediated proteasomal degradation in proliferating cells under non-stressed conditions, an opposing function to that of JNK activated in response to UV, which leads to p53 phosphorylation and activation (33, 65, 66). Thus, the activity of JNK toward p53 is seemingly stimulus-dependent, acting as an anti-apoptotic kinase under unstressed conditions and a pro-apoptotic kinase under conditions of cellular stress. Supporting this model, we observed an increase in p53 protein levels in JNK inhibitor-treated DP16.1/p53ts cells, which was a result of an increase in p53 protein stability. The ability of JNK to destabilize p53 in DP16.1/p53ts cells could account for the enhanced apoptosis observed upon inhibition of JNK signaling.

Overall, these data suggest that intrinsically activated MAPK pathways exert disparate effects on p53 function and cellular outcome. MEK MAPK kinase activity contributes to p53-dependent apoptosis in DP16.1/p53ts cells and likely reflects an aberrant Ras proliferative signal arising from FVP-induced viral transformation. Inhibition of this signal at the level of MEK alters the p53 cellular response in favor of G1 arrest over apoptosis. Conversely, intrinsic p38 and JNK MAPK activities are capable of restricting p53-dependent apoptosis in DP16.1/p53ts cells. JNK is implicated in facilitating p53 protein turnover, which could account for the enhanced apoptotic effects of inhibiting this MAPK pathway in DP16.1/p53ts cells. Understanding how intrinsic MAPK pathways influence p53 function will benefit the development of cancer therapies aimed at inducing apoptosis either by p53 reconstitution or by chemo- and/or radiotherapy of tumors with intact p53.

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REFERENCES
1. Benchimol, S. (2001) Cell Death Differ. 8, 1049–1051
2. Lin, Y., and Benchimol, S. (1995) Mol. Cell. Biol. 15, 6045–6054
3. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991) Nature 352, 345–347
4. Cannan, C. E., Gilmer, T. M., Couts, S. B., and Kastan, M. B. (1995) Genes Dev. 9, 600–611
5. Abrahamson, J. L., Lee, J. M., and Bernstein, A. (1995) Mol. Cell. Biol. 15, 6953–6960
6. Quelle, F. W., Wang, J., Feng, J., Wang, D., Cleveland, J. L., Ihle, J. N., and Zambetti, G. P. (1998) Genes Dev. 12, 1099–1107
7. Heinrichs, S., and Deppert, W. (2003) Oncogene 22, 555–571
8. Lin, Y., Brown, L., Hedley, D. W., Barber, D. L., and Benchimol, S. (2002) Blood 100, 3990–4000
9. Sabbatini, P., and McCormick, F. (1999) J. Biol. Chem. 274, 24263–24269
10. Chiu, S. K., Tao, L., and White, E. (1994) Mol. Cell. Biol. 14, 2556–2563
11. Schott, A. F., Apel, I. J., Nunez, G., and Clarke, M. F. (1995) Oncogene 11, 1389–1394
