A ROLE FOR AP-1*

Ras Protects Rb Family Null Fibroblasts from Cell Death

Received for publication, October 28, 2003, and in revised form, December 5, 2003
Published, JBC Papers in Press, December 19, 2003, DOI 10.1074/jbc.M311814200

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The retinoblastoma protein (Rb) controls cell proliferation, differentiation, and senescence and provides an essential tumor suppressive function that cells must eliminate to attain unlimited proliferative potential. Elimination of the Rb pathway also results in apoptosis, however, thereby providing an efficient surveillance mechanism to sense the loss of Rb. To become tumorigenic cells must thus overcome not only Rb function but also the apoptotic response caused by the loss of Rb function. We show that oncogenic Ras (RasV12) potently blocks cell death in Rb family member knockout mouse embryo fibroblasts (TKO cells). Activation of phosphatidylinositol 3-kinase and Raf by oncogenic Rb mediated this protection, implying that multiple Ras effector pathways are required, in concert, for this pro-survival signal. Although activation of Raf by selective Ras mutants protected TKO cells from cell death, pharmacologic inhibition of MEK had little effect on RasV12 protection, suggesting that a Raf-dependent, MEK-independent pathway was important for this effect. We show that this Raf-dependent protection occurred through activation of c-Jun and thus AP-1 activation.

These observations could account for the dependence of Ras transformation on c-Jun activity and for the roles of AP-1 in oncogenesis. Our results support the concept of two oncogenic events cooperating to achieve a balance between immortalization and survival.

* This work was supported by National Institutes of Health Grant CA 85839 and American Heart Association Grant 01940116N (to G. D. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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† The abbreviations used are: cdk, cyclin-dependent kinase; TKO, triple knockout; FBS, fetal bovine serum; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI3K, phosphatidylinositol 3-kinase; JNK, c-Jun N-terminal kinase; MEF, mouse embryonic fibroblasts.

The retinoblastoma protein (Rb), a negative regulator of cell proliferation, was the first tumor suppressor discovered. It is thought that the loss of Rb function is a required step for tumorigenesis (1, 2). This theory is supported by the high frequency of oncogenic lesions that directly or indirectly impinge upon the Rb pathway in human cancer (1) and by the demonstration that abrogation of Rb function is necessary for transformation of primary human cells (3). Abrogation of Rb function can occur through deletion of the Rb locus itself, as seen in retinoblastoma, osteosarcoma, lung carcinoma, and some forms of breast cancer (4–8). These instances, however, are relatively rare. More common are genetic lesions in other Rb pathways that result in nonfunctional Rb. Cyclin D/cyclin-dependent kinases (cdk)1 4/6 and cyclin E/cdk2

phosphorylate and inactivate Rb, and cdk inhibitors such as p16Ink4a and p15Ink4b inhibit the cyclin/cdk complexes. Overexpression or amplification of each of the cyclin and cdk subunits, activating point mutations in cdk4, and silencing of p15 or p16 through deletion or methylation of their promoters have all been observed in human cancer (1, 9). Deletion of Rb itself may be rare because upstream lesions affect not only Rb but also its two relatives, p107 and p130, which are not tumor suppressors but share many of the growth inhibitory characteristics of Rb.

However, the loss of Rb function also results in a strong signal for apoptosis in many cell types. This occurs largely through hyperactivity of E2F transcription factors, positive regulators of cell proliferation and apoptosis, which are inhibited by Rb (10). There are also other apoptotic effectors that bind to Rb such as Id2 (11). Viral oncogenes such as adenoviral E1A and HPV E7 induce apoptosis, in part, by binding and inhibiting Rb (12, 13). Overexpression of an E2F mutant that binds DNA (and thus displaces Rb from promoters) but fails to transactivate gene expression can induce apoptosis (14, 15), suggesting that the loss of Rb repression at promoters is sufficient to cause cell death even in the absence of E2F activity. It is thought that this response to loss of Rb is a tumor surveillance mechanism whereby cells in which this essential growth inhibitory pathway is lost are eliminated before they give rise to tumors.

The requirement for the loss of Rb function for tumorigenesis raises questions about certain classic models of tumorigenesis. Growth factor and growth factor receptor signaling, as well as their intracellular effectors Ras and downstream mitogen-activated protein kinases, have long been known to be hyperactivated in many human cancers. These lesions promote cell proliferation, but in the absence of Rb it is unclear how important further enhancement of cell proliferation might be. Loss of Rb function overcomes permanent growth arrest, conferring (potentially) unlimited growth potential, and this may be a more important barrier for would-be tumor cells to overcome rather than simply increasing their growth rate. Several types of human cancers have both mutations in the p16/Rb pathway and activation of Ras (for example, see Refs. 16 and 17). It has been suggested that hyperactivation of Ras pathways may be important for characteristics of advanced neoplasm such as metastatic potential (18). On the other hand, because the loss of Rb induces apoptosis, growth factor/growth factor receptor, Ras, and Ras effector signaling may initially be important for promoting survival, without which a cell in which Rb function was lost might never yield progenitors.

To study the role of activating, oncogenic Ras mutations in

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cell survival and death caused by the loss of the Rb pathway, we
turned to a cell culture system. Mouse embryonic fibroblasts in
which all three Rb family members (Rb, p107, and p130) are
“knocked out” (triple knockouts or TKOs) have been described
(19, 20). These cells undergo accelerated apoptosis when se-
rum/growth factors are removed, making them an ideal model
by which to study the effects of mitogenic signaling on apopto-
sis caused by the loss of Rb function.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Retroviral Infections, and Apoptosis Assays—**Rb family
triple knockout cells (TKOs) were maintained in Dulbecco’s modified
Eagle’s medium plus 10% fetal bovine serum plus sodium pyruvate and
nonessential amino acids. For viability assays, the cells were plated
such that they were ~75% confluent the following day. Serum depriva-
tion was with 0.1% FBS. At harvesting, supernatant, wash, and
trypsinized cells were combined for trypan blue exclusion assay. Ras
effector mutants (RasV12S35, RasV12G37, RasV12E38, and
RasV12C40) were constructed through point mutagenesis using pBa-
beuroRasV12 as the template (QuickChange site-directed mutagenesis
kit; Stratagene). Dominant inhibitory RasN17 was made through point
mutagenesis using pBabe.puro.Ras as template. All of the plasmids
were sequenced to confirm that the appropriate mutations had been
incorporated. MSCV c-Jun was constructed by PCR amplifying c-Jun
cDNA to have ends with BamHI and EcoRI sites and ligating it into the
BglII-EcoRI cloning sites of MSCV.

Retroviruses were produced by co-transfecting 293T cells with the
retroviral vectors and the e2 helper virus. Virus was harvested at five
separate time points spaced 6–10 h apart. Target cells were infected at
three separate time points spaced 3 h apart without removing the previous virus. For pBabe.puro retroviral vector infections, TKOs were
selected in 2.0 μg/ml puromycin. MSCV vectors carried an internal
ribosomal entry site-green fluorescent protein. The efficiency of infec-
tions was generally nearly 100% as assessed by puromycin selection or
green fluorescent protein positivity.

**Western Analysis—**Cells were lysed in ELB bufler (50 mM Hepes, pH
7.2, 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) plus aprotinin,
leupeptin, phenylmethylsulfonyl fluoride, sodium pyrophosphate, and
sodium fluoride. Antibodies used were phospho-Ser 73 c-Jun (Cell Signal-
ing 9271); phospho-Ser 473 Akt (Cell Signaling 9164); phospho-Thr 202/
Ty 204-p44/p42 ERK (Cell Signaling 9910); α-tubulin (Sigma T5168);
phospho-Ser 217/221-MEK1/2 (Cell Signaling 9121); and p21 (Santa Cruz
sc-291).

**RESULTS**

**Oncogenic Ras Protects against Loss-of-Rb-mediated Cell
Death—**Numerous studies have shown that oncogenic Ras can
promote either apoptosis or survival in fibroblasts (for examples,
see Refs. 21–26). Therefore, we asked what effect the
expression of oncogenic Ras would have on the cell death that
results from functional loss of Rb family members, an essential
step in tumorigenesis.

Mouse embryonic fibroblasts triply knocked out for all three Rb
family members, Rb, p107, and p130 (TKOs) underwent cell
dead when serum was removed (0.1% fetal bovine serum) (Fig.
A and Ref. 19), whereas wild type MEFs survived in medium
containing 0.1% FBS without proliferating or undergoing sig-
ificant cell death for at least 2 weeks (data not shown). TKOs were
transduced with either a control retrovirus (pBabe.puro)
or a retrovirus expressing e-H-RasV12 (RasV12) and selected
for stable integration by puromycin treatment. When control
pBabe TKOs were deprived of serum (0.1% FBS), ~65% were
dead 2 days after serum withdrawal (Fig. 1A). By 4 days over
90% of cells were dead (Fig. 1A). In contrast, for RasV12-
expressing TKOs, only ~15% of cells were dead 2 days after
serum withdrawal, and less than 25% were dead after 4 days
(Fig. 1A). RasV12 TKO viability exceeding 70% was present up
to 10 days of continuous culture in 0.1% serum, and after 2
weeks ~50% of cells were still viable (Fig. 1A).

TKO cells transduced with dominant negative Ras (RasN17)
had a higher rate of basal cell death in 10% FBS compared with
control pBabe TKOs (~30% versus 10%, respectively; Fig. 1B).

In addition, when serum was withdrawn, RasN17 expressing
TKOs underwent cell death faster than control TKOs (Fig. 1B).
These results indicated that oncogenic Ras could overcome cell
death resulting from the combination of loss of Rb family mem-
bler function and growth factor deprivation. Furthermore, en-
dogenous Ras appears to provide a basal survival signal to cells
that have lost Rb family member function.

**Multiple, but Not All, Ras Effector Pathways Are Required to
Prevent Cell Death of Rb Family Null MEFs Following Serum
Deprivation—**Next we asked what signaling pathways down-
stream of activated Ras were required to protect TKO cells
cell death following serum deprivation. First, a pharma-
cologic approach was taken. Cells were treated with various
drugs that inhibit specific Ras effector pathways. Two concen-
trations of each drug were used on control pBabe TKOs or
RasV12 TKOs, in the presence or absence of serum, and cell
death was quantified after 2 days of drug treatment.
LY294002, a PI3K inhibitor, significantly blocked the protec-
tive effect of RasV12 at both the low (10 μM) and high (50 μM)
concentrations, accelerated cell death of control pBabe-infected
TKOs when serum-deprived, and caused increased cell death of
control pBabe TKOs grown in the presence of serum (Fig. 2A).
Wortmannin, another PI3K inhibitor, also reversed RasV12
protection against serum deprivation-induced death and increased death in control pBabe TKOs both in the presence and absence of serum (data not shown). SP600125, a JNK inhibitor, also significantly blocked the protective effect of RasV12 at both low (20 μM) and high (50 μM) concentrations, accelerated cell death of serum-deprived control pBabe TKOs, and increased cell death of control pBabe TKOs grown in the presence of serum (Fig. 2A). Two MEK inhibitors were also tested. PD98059 only partially reversed the RasV12 protective effect and only at the high concentration (100 μM; ~2-fold the cell death), but did accelerate the cell death of serum-deprived control pBabe TKOs, and had only a marginal effect on control pBabe TKO survival in the presence of serum (Fig. 2A). U0126 treatment, another MEK inhibitor, gave results similar to that of PD98059 treatment (Fig. 2A). SB203580, a p38 mitogen-activated protein kinase inhibitor, had no significant effect on any of these parameters (Fig. 2A). None of the drugs affected RasV12 TKOs cell survival grown in the presence of serum.

**Fig. 2.** Inhibition of single Ras pathways reverses Ras-mediated protection. A, control pBabe.puro TKOs or RasV12 TKOs were plated at equal confluence, and 24 h later medium was changed to 0.1% (−ser) or 10% (+ser) FBS. At the same time, cells were treated with the following drugs: Me2SO (first columns, white), 10 μM LY294002 (second columns, gray) or 50 μM LY294002 (third columns, black), 20 μM SP600125 (fourth columns, white) or 50 μM SP600125 (fifth columns, gray), 20 μM PD98059 (sixth columns, black) or 100 μM PD98059 (seventh columns, white), 1 μM U0126 (eighth columns, gray) or 5 μM U0126 (ninth columns, black), or 3 μM SB203580 (tenth columns, white) or 10 μM SB203580 (eleventh columns, gray). 48 h later, all cells were collected and cell death was quantified by trypan blue exclusion assay. B, control pBabe.puro TKOs and RasV12 TKOs were plated at equal confluence, and 24 h later the same drugs (D) were added at the same concentrations. 2 or 8 h after addition of drugs total cell lysates were taken, and Western blots were performed as indicated (W). As internal controls, blots were probed for α-tubulin. C, TKOs were transduced with the indicated retroviruses, selected, and plated to be at equal confluence the following day, and then medium was changed to 0.1% FBS. 48 h later, all cells were collected, and cell death was quantified by trypan blue exclusion assay. Shown are the death rates relative to control pBabe.puro TKOs.
except for a small effect of LY294002 but only at the high concentration (Fig. 2A), indicating that changes in cell survival were not due to nonspecific toxicity of the drugs. In summary, these results suggested that activation of PI3K and JNK are both important in Ras-mediated protection against cell death in Rb family TKOs. Because inhibition of either of these pathways also increased death of control TKOs grown in the presence of serum, this suggested that in the absence of Rb function, the base-line activity of the PI3K and JNK pathways contributed to survival.

To determine whether treatment of cells with the pharmacologic inhibitors resulted in the expected inhibition of target signaling proteins, Western blots for activated phosphorylated signaling proteins in the effector pathways were performed. As expected, LY294002 resulted in a decrease in phospho-Akt levels, a direct downstream target of PI3K; SP600125 caused a decrease in phospho-c-Jun levels, a target of JNK; and PD98059 and U0126 both caused a decrease in phospho-ERK (p42/p44) levels, a target of MEK (Fig. 2B). These Western blots also yielded additional information. First, the inhibitory effects of LY294002, SP600125, and U0126, although initially strong (Fig. 2B, 2 h), did not persist for 8 h. In contrast, the effects of PD98059 persisted for 8 h at the high concentration (Fig. 2B). The strong reversal of RasV12-mediated protection of TKO apoptosis despite temporary inhibition of PI3K and JNK suggested that transient inhibition of PI3K or JNK was sufficient to allow initiation of an apoptotic program that continued even after PI3K and JNK function was restored. In support of this possibility, the addition of SP600125 followed by its removal after 8 h still resulted in reversal of RasV12-mediated protection and acceleration of death of control pBabe TKOs (data not shown). Second, RasV12-expressing TKOs had significantly higher basal levels of phospho-Akt and phospho-c-Jun than control TKOs, as expected (Fig. 2B). Unexpectedly, however, control TKOs had clearly higher levels of phospho-ERK than RasV12 TKOs (Fig. 2B), indicating that ERK activation most likely did not contribute to the protective effect of RasV12.

These pharmacologic experiments indicated that multiple Ras effector pathways are required, in concert, for efficient protection against loss-of-Rb-mediated cell death.

As a second approach to determine what signaling pathways downstream of activated Ras contributed to the protection of TKOs from serum-deprived cell death, RasV12 compound mutants that activate only one Ras effector pathway (27) were tested for their capacity to rescue TKOs from cell death following serum deprivation, relative to RasV12 or control pBabe TKOs. RasV12S35 (RasS35) and RasV12E38 (RasE38) activate only Raf, RasV12G37 (RasG37) activates only Raf-GDS, and RasV12C40 (RasC40) activates only PI3K. TKOs were transduced with control pBabe virus, RasV12, or RasV12 effector mutant retroviruses and selected for stable expression. The cells were deprived of serum for 2 days, and cell death was quantified. A significant reduction in cell death upon serum withdrawal was observed for RasS35 and RasE38 TKO cells (Fig. 2C; −50% reduction compared with pBabe control TKO cells) and for RasC40 TKOs (Fig. 2C; 40% reduction compared with pBabe control TKO cells). These reductions were not as strong as observed in RasV12 TKOs, however (Fig. 2C; 80% reduction compared with pBabe control TKO cells). For RasG37 TKOs, only a marginal reduction in cell death was observed (−20% reduction compared with pBabe control TKO cells, Fig. 2C).

Consistent with the results obtained from pharmacologic inhibition of downstream signaling pathways activated by oncogenic RasV12, hyperactivation of a single Ras effector pathway by RasV12 effector mutants was only able to provide partial protection against loss-of-Rb-mediated cell death. These results again indicated that activation of multiple but not all Ras effector pathways were required for RasV12 to protect Rb family TKO cells from cell death following serum deprivation.

Pharmacologic inhibition of PI3K (e.g. LY294002) and selective activation of PI3K (e.g. RasC40) indicated that PI3K-mediated part of the protective effect of oncogenic RasV12. The PI3K/Akt pathway is a well characterized Ras activated anti-apoptotic pathway. PI3K, which is activated by Ras, in turn activates Akt, which phosphorylates and inactivates numerous apoptotic proteins such as Bad and caspase 9, promoting survival in many different systems (28). That LY294002 potently reversed the RasV12 protective effect and that RasC40 was able to partially protect TKOs (Figs. 2A and 3B) from apoptosis were consistent with these results. Interestingly, although activation of Raf by RasS35 or RasE38 was protective, pharmacologic inhibition of MEK (downstream of Raf) had only a minor effect on RasV12 protection. These results suggested the possibility that other Raf-dependent but MEK-independent signaling pathways were important for the protective effect of RasV12 in Rb family TKO cells.

RasV12 Promotes Survival by Signaling to AP-1.—Inhibition of JNK activity appeared to be as potent as inhibition of PI3K activity in the abrogation of oncogenic Ras-mediated survival of Rb family TKO cells deprived of serum (Fig. 2, A and B). This result was unexpected, because JNK is usually pro-apoptotic (29). JNK phosphorylates and activates c-Jun. c-Jun has also been shown to be pro-apoptotic when overexpressed, to transduce the JNK apoptotic signal in neuronal cells, and to mediate UV-induced apoptosis (30). c-Jun is part of the transcription factor complex AP-1, which is comprised of Jun, Fos, Maf, and/or ATF family members (30). Therefore, we asked whether c-Jun activity (i.e. AP-1) contributed to the RasV12-mediated protection from cell death of Rb family null MEFs deprived of serum.

First, control and RasV12 TKO cells (± serum) were treated with curcumin, an AP-1 inhibitor (31, 32). Curcumin reversed RasV12-mediated protection from serum-deprived cell death of TKOs to an extent similar to inhibition of JNK by SP600125 (Fig. 3A versus Fig. 2A, respectively). This suggested that activation of AP-1 could be an important target of RasV12-mediated survival. Because AP-1 autoregulates itself by inducing transcription of c-Jun (33), if AP-1 was activated by RasV12 one would expect the level of c-Jun mRNA to increase in RasV12-expressing TKO cells relative to pBabe control cells. Northern blot analysis demonstrated that c-Jun mRNA levels were indeed elevated in RasV12-expressing TKOs (Fig. 3B), consistent with increased AP-1 activity.

Second, TKO cells were transduced with SupJunD1, a dominant negative inhibitor of AP-1. SupJunD1 expression resulted in an increase in basal (i.e. in the presence of serum) cell death compared with control pBabe TKO cells grown in serum (5-fold; Fig. 3C). This result suggested that endogenous AP-1 was important for viability in cells that have lost Rb family function. Western blot analysis of phospho-c-Jun levels in SupJunD1-transduced TKO cells demonstrated a reduction in phospho-c-Jun (active), confirming that SupJunD1 inhibited AP-1 activity (Fig. 3D, third lane versus first lane).

Third, TKO cells were transduced with a bistrionic retrovirus expressing both RasV12 and SupJunD1. TKOs transduced with RasV12 alone were protected against serum deprivation-induced cell death, as expected (8% for RasV12 versus 64% for control pBabe; Fig. 3E). Co-transduction of SupJunD1 with RasV12 reversed the protective effect of RasV12. In these cells cell death was comparable with control pBabe TKO cells (avg. 58% death; Fig. 3E). Western blot analysis demonstrated that...
phospho-c-Jun levels were increased by RasV12 alone (Fig. 3F, as in Fig. 2B) and that SupJunD1 reduced phospho-c-Jun induction by RasV12 to the levels present in control pBabe TKO cells (Fig. 3F). Fourth, c-Jun was overexpressed in TKO cells. c-Jun-overexpressing TKOs exhibited a ~60% reduction in cell death after 2 days of serum deprivation compared with control TKO cells (Fig. 4A), further supporting a survival role for AP-1 in loss-of-Rb-mediated cell death.

Fifth, v-Jun, the oncogenic viral homolog of c-Jun, is not subject to phosphorylation by JNK but is constitutively active. Although it transactivates an overlapping set of genes as c-Jun, its activity is not identical to c-Jun and can actually antagonize c-Jun-mediated transactivation of certain target genes (33). Therefore, v-Jun expression in Rb family TKO cells could be anticipated to affect cell survival. Indeed, when v-Jun was overexpressed in TKO cells, the cell death induced by serum withdrawal was enhanced (Fig. 3F). Western blot analysis of v-Jun-transduced TKO cells showed that phospho-c-Jun levels were virtually eliminated relative to control TKO cells (Fig. 4B, lane 2 versus lane 1), consistent with the enhancement of cell death by v-Jun. These cumulative results argue that AP-1 activity has a protective effect that counterbalances loss-of-Rb-mediated cell death following serum deprivation.

**Raf Signals to AP-1 to Protect against Loss-of-Rb-mediated Apoptosis**—RasS35 and RasE38 conferred the strongest protection provided by the RasV12 effector mutants, which specifi-
Ras Blocks Loss-of-Rb-mediated Apoptosis

Fig. 5. Ras/Raf signal to AP-1 to protect against cell death. A and B, TKOs transduced with the indicated retroviruses were selected and plated, and 48 h later, total cell lysates were taken and Western blots were performed for phospho-c-Jun (A) or phospho-MEK1/2 (B). α-Tubulin was probed for as an internal control. C, TKOs were transduced with pBabe.puro or ΔRaf1–22W (constitutively active Raf) and selected for stable transduction. The cells were plated to yield equal confluence the next day and 24 h later serum-deprived with 0.1% FBS. 48 h later, cell death was quantified by trypan blue exclusion assay. D, RasS35 and RasE38 TKOs were plated, and 24 h later the medium was replaced with 10% FBS (speckled bars) or 0.1% FBS (all other bars), and at the same time 0 (diagonal lines), 10 μM (wavy lines), or 20 μM (solid gray bars) curcumin was added. 48 h later, cell death was quantified by trypan blue exclusion assay. E, RasS35 and RasE38 TKOs were plated, and 24 h later the medium was replaced with 10% FBS (speckled bars) or 0.1% FBS (all other bars), and at the same time 0 (diagonal lines), 20 μM (wavy lines), or 50 μM (solid gray bars) SP600125 was added. 48 h later, cell death was quantified by trypan blue exclusion assay.

cally activate the Raf pathway. However, pharmacologic inhibition of MEK activity (downstream of Raf) did not appear to significantly affect the protective effect of RasV12 (Fig. 2A). In light of the protective effect of AP-1, we asked whether activation of Raf by oncogenic RasV12 was protecting against cell death through signaling to AP-1. RasV12 TKOs contained significantly elevated levels of phospho-c-Jun compared with control TKO cells (Figs. 2B and 5A, second lane versus first lane). Western blots of the RasS35 and RasE38 effector mutants, which contained elevated levels in phospho-MEK1/2, as expected (Fig. 5B, second and fourth lanes versus first lane), also had elevated levels of phospho-c-Jun relative to control cells (Fig. 5A, fourth and sixth lanes versus first lane). These results suggested that RasV12 could up-regulate phospho-c-Jun, in part, through Raf activation.

To test this directly, TKO cells were transduced with constitutively active Raf (ΔRaf1–22W). Compared with control TKO cells, ΔRaf1–22W TKO cells were protected from cell death by ~40% (Fig. 5C), a level similar to that seen with RasS35 and RasE38 TKO cells (Fig. 2C). Phospho-c-Jun levels also were dramatically increased in Raf (ΔRaf1–22W) TKO cells (Fig. 3D, fourth lane versus first lane). Next, to test whether Raf activation protected TKO cells from death by signaling through AP-1, RasS35 and RasE38 TKO cells were treated with the AP-1 inhibitor curcumin. As seen with RasV12-containing TKO cells, curcumin reversed the protective effect of RasS35 and RasE38 (Fig. 5D), suggesting that Raf was signaling to AP-1 to block loss-of-Rb-mediated cell death. As was the case with RasV12 TKO cells, the JNK inhibitor, SP600125, also reversed the protective effect of RasS35 and RasE38 (Fig. 5D), providing additional evidence that the Raf protective signal required activation of c-Jun/AP-1. The extent of protection from cell death provided by Raf (ΔRaf1–22W) (Fig. 5C) was similar to that achieved following expression of RasS35 (Fig. 2C), RasE38 (Fig. 2C), or overexpression of c-Jun (Fig. 4A), consistent with a role for AP-1 activity in the protection against loss-of-Rb-mediated apoptosis by oncogenic Ras activation of Raf.

p53 Regulates Cell Proliferation but Not Apoptosis in Rb Family TKO Cells—An alternative to up-regulation of an anti-apoptotic pathway in overcoming loss-of-Rb-mediated apoptosis is down-regulation of a pro-apoptotic pathway to which the loss of Rb signals. Overexpression of E2F-1, which mimics the loss of Rb, has been shown to induce apoptosis through both p53-dependent and p53-independent mechanisms (34). That p53 is the most commonly mutated tumor suppressor in human cancers suggests the possibility that abrogation of the p53 pathway could be a means of overcoming loss-of-Rb-mediated apoptosis. Thus, we asked what the effect of inhibiting endogenous p53 would be on cell death of Rb family TKO cells. Two cancer mutants of p53 (dominant negatives), p53R175H and p53R273H (35), were stably transduced into TKO cells. Western blot analysis for p21 levels, a transcriptional target of p53 and a negative regulator of cell proliferation, showed that inhibition of endogenous p53 occurred in both p53R175H and p53R273H TKOs (Fig. 6A; reduction of p21 was moderate for p53R175H and strong for p53R273H TKOs). As another control confirming the loss of p53 function by these dominant negative p53 mutants, the cells were treated with etoposide, a DNA damage-inducing agent (DNA damage induces apoptosis, in part, by a p53-dependent mechanism (36)). Relative to control TKOs (containing wild type p53) p53R175H- and p53R273H-containing TKOs were protected from cell death induced by etoposide (Fig. 6B).

Inhibition of endogenous p53 had a marginal effect on TKO cell death following serum withdrawal (Fig. 6C). However, inhibition of endogenous p53 had a positive effect on cell proliferation, with p53R175H being stronger (Fig. 6D). p53R175H TKOs and p53R273H TKOs showed detectable cell growth even in the absence of serum (0.1%) (data not shown), which may account for the marginal difference in cell death upon serum

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FIG. 6. Inhibition of p53 results in increased cell proliferation but not significant protection against cell death in Rb family TKOs. A, TKOs were transduced with the indicated retroviruses, selected in puromycin, and plated, and 48 h later, total cell lysates were taken, and Western blot was performed for p21. α-Tubulin is shown below as an internal control. B, pBabe, p53R175H, and p53R273H TKOs were treated with 5 μM etoposide, and 15 h later cell death was quantified by trypan blue exclusion assay. C, pBabe, p53R175H, and p53R273H TKOs were plated to give equal confluence the following day, quantified by trypan blue exclusion assay. D, pBabe (circles), p53R175H (squares), and p53R273H (triangles) TKOs were plated at 5.0 × 104/60-mm plate (relative cell number), TKOs were transduced with the indicated retroviruses, selected in puromycin, and plated, and 48 h later, total cell lysates were taken, and Western blot was performed for p21. α-Tubulin is shown below as an internal control. B, pBabe, p53R175H, and p53R273H TKOs were treated with 5 μM etoposide, and 15 h later cell death was quantified by trypan blue exclusion assay. C, pBabe (circles), p53R175H (squares), and p53R273H (triangles) TKOs were plated at 5.0 × 104/60-mm plate (relative cell number) on day 0 in medium with puromycin, and total cell number was counted at the indicated time points. Each count was done with duplicate plates, and shown is an experiment representative of multiple repetitions.

withdrawal. The relative change in p21 levels was also consistent with the higher rate of cell proliferation for p53R175H TKOs than for p53R273H TKOs.

discussion

The retinoblastoma protein plays a critical role in regulation of cell proliferation, and abrogation of its function is thought to be essential for tumorigenesis (1, 2). Deletion of all three members of the Rb family (Rb, p107, and p130) in primary mouse embryo fibroblasts (TKOs) results in immortalization (19, 20), supporting the idea that loss of Rb function is sufficient to confer unlimited proliferative potential. In vivo, these three genes are never deleted, but mutations affecting upstream regulators in the Rb pathway that affect the function of all three family members are common (1). However, loss of this essential tumor suppressor pathway also induces apoptosis. Thus, to give rise to a tumor, a cell not only must eliminate Rb function but must also circumvent the consequent apoptosis. Generally speaking, this can occur in one of two ways: abrogating a pro-apoptotic pathway that is triggered by loss of Rb function or up-regulating an anti-apoptotic pathway that can overcome loss-of-Rb-mediated apoptosis. Here we tested the hypothesis that up-regulation of an anti-apoptotic pathway, e.g. oncogenic Ras, a mutation common in cancer and thought to be important for stimulating cell proliferation, can overcome cell death mediated by loss of Rb family function. Because the loss of Rb already confers unlimited proliferative potential, one of the critical roles for Ras in tumorigenesis may be to promote cell survival in the context of the pro-apoptotic signal resulting from the loss of Rb function. In our studies, Ras potently blocked loss-of-Rb-mediated cell death in response to serum deprivation, signaling through multiple pathways. In contrast, down-regulation of the pro-apoptotic p53 activity, another tumor suppressor frequently mutated in cancer, was not sufficient to prevent loss-of-Rb-mediated cell death but rather enhanced cell proliferation.

Multiple Ras effector pathways (PI3K and Raf) mediated this pro-survival signal. The PI3K/Akt pathway is a well characterized Ras-activated anti-apoptotic pathway. Interestingly, whereas activation of Raf by selective Ras mutants (RasS35 or RasE38) and expression of constitutively active Raf itself were protective, pharmacologic inhibition of MEK (downstream of Raf) had little effect on RasV12 protection, suggesting that a Raf-dependent, MEK-independent pathway was important for the protective effect of RasV12. We found that Raf activation of c-Jun and subsequent AP-1 activation was important for RasV12-mediated protection from cell death in Rb family TKO cells deprived of serum.

AP-1 has been reported to have opposing effects on apoptosis. Overexpression of c-Jun induces apoptosis in mouse fibroblasts, sympathetic neurons, Syrian hamster embryo cells, and a human colorectal carcinoma cell line (RKO) (37). Both c-Jun+/−MEFs and JNK1−/−/JNK2−/−MEFs are resistant to apoptosis induced by UV radiation (38, 39). Evidence for an anti-apoptotic role for AP-1 comes from studies showing increased spontaneous apoptosis in the same c-Jun−/−fibroblasts (38) and protection by c-Jun against both UV-induced and TNFα-induced cell death (40). Interestingly, protection against UV-induced apoptosis was dependent on phosphorylation of c-Jun on Ser63/Ser73, whereas the promotion of cell proliferation by c-Jun was not (40). In vivo, hepatocytes and other liver cells in c-Jun−/−embryos undergo extensive apoptosis in midgestation (41). Like AP-1, JNK, an upstream activator of c-Jun, and thus AP-1, is pro-apoptotic in most systems (29); however, it also has been shown to be anti-apoptotic in some cases (42–44). It is interesting then that in MEFs triply knocked out for all Rb family members (TKOs), c-Jun appears to be anti-apoptotic. Because the loss of Rb function is thought to be an essential step in the development of cancer, the role of AP-1 in regulating apoptosis in this context is likely to be relevant to tumor biology.

AP-1 has been implicated in transformation in a number of ways. c-Jun and c-Fos are both homologs of retroviral oncogenes, and overexpression of either c-Jun or c-Fos transforms immortalized rodent fibroblasts in vitro (33, 37). AP-1 activity is induced by various tumor promoters and activated oncoproteins (37). Jun proteins also cooperate with Ras in transformation of rodent fibroblasts, with c-Jun being the strongest (37). Notably, oncogenic Ras transformation of cells requires c-Jun, as c-Jun−/−cells are not transformed by oncogenic Ras (45), and more specifically Ras transformation requires N-terminal phosphorylation of c-Jun (46).

Transformation is a complex event that involves changes in cell proliferation, survival, and invasiveness. In the context of Rb family function loss, Ras, Raf, and AP-1 are important for preventing cell death, thereby allowing cells in which restraints on proliferative potential have been removed (i.e. loss of Rb function) to progress to tumor formation and malignancy. Indeed, it is well known that oncogenic Ras cooperates with either E1A (an adenoviral gene that inactivates Rb family members) or E2F-1 in cell transformation (13, 47). It is of note that in an in vivo tumor model with conditional Ras expression, Ras was shown to be required for tumor maintenance, with removal of Ras expression causing massive apoptosis (48). A
role for c-Jun in tumor maintenance has also been reported, as inactivation of c-Jun early in liver tumor development results in a decrease in tumor number and size due to an increase in liver cell apoptosis and not a decrease in cell proliferation (49). Specific inactivation of c-Jun in the epidermis also impairs tumor development in SOS transgenic mice (50).

Recently, Raf has been implicated in the promotion of cell survival. Generation of Raf-1−/− mice led to the unexpected result that although Raf-1 is not important for cell proliferation, it is essential for protecting against apoptosis in a variety of embryonic tissues (51, 52). Furthermore, this pro-survival function of Raf is not dependent on MEK/ERK (51, 52). Intriguingly, like c-Jun-deficient embryos, Raf-1-deficient embryos show widespread apoptosis in the fetal liver (52). One putative mechanism for promotion of cell survival by Raf is that mitochondrially localized Raf phosphorylates the pro-apoptotic Bcl-2 family member Bad, which results in inhibition of cytochrome c release (53). Our findings that RasS35, RasE38, and RasE38 protection against loss-of-Rb-mediated cell death require c-Jun phosphorylation and AP-1 activity, together with the striking parallel between constitutively active Raf TKOs and that RasV12, RasS35, and RasE38 protection against loss-of-Rb-mediated cell death require c-Jun phosphorylation and AP-1 activity, together with the striking parallel between constitutively active Raf TKOs and RasV12 TKOs (Fig. 2B), might suggest the possibility that Raf promotes survival at least partially by activating AP-1. The mechanism for the activation of AP-1 by Raf remains uncertain. Raf can activate AP-1 through ERK-mediated induction of c-Fos transcription, which leads to increased c-Jun transcription (37); through phosphorylation of Fra1 and 2 by ERKs, which may enhance DNA binding in conjunction with c-Jun (54); and through direct phosphorylation and AP-1 activity, together with the striking parallel between constitutively active Raf TKOs and RasV12 TKOs (Fig. 2B), might suggest the possibility that Raf promotes survival at least partially by activating AP-1. The mechanism for the activation of AP-1 by Raf remains uncertain. Raf can activate AP-1 through ERK-mediated induction of c-Fos transcription, which leads to increased c-Jun transcription (37); through phosphorylation of Fra1 and 2 by ERKs, which may enhance DNA binding in conjunction with c-Jun (54); and through direct phosphorylation of JunD by ERK (55). However, the anti-apoptotic function of Raf in vivo was shown not to be dependent on MEK/ERK (51, 52). Our results also provide evidence of MEK-independent anti-apoptotic signaling by Raf, in that 1) activated ERK levels were actually lower in RasV12 TKOs (Fig. 2B); 2) MEK inhibitors did not significantly reverse the protective effect of RasV12 (Fig. 2A); and 3) constitutively active MEK did not protect TKOs against apoptosis (data not shown). The mechanism for Raf-dependent, MEK-independent activation of AP-1 is unclear. Furthermore, overexpression of c-Jun did not protect against apoptosis as strongly as RasV12 (compare Fig. 3B with Fig. 2A), suggesting that other signals from Ras synergize with AP-1 in protecting against loss-of-Rb-mediated apoptosis.

Acknowledgments—We thank Tyler Jacks for Bcl-2 family member Bad, which results in inhibition of cytochrome c release (53). Our findings that RasS35, RasE38, and RasE38 protection against loss-of-Rb-mediated cell death require c-Jun phosphorylation and AP-1 activity, together with the striking parallel between constitutively active Raf TKOs and RasV12 TKOs (Fig. 2B); 2) MEK inhibitors did not significantly reverse the protective effect of RasV12 (Fig. 2A); and 3) constitutively active MEK did not protect TKOs against apoptosis (data not shown). The mechanism for Raf-dependent, MEK-independent activation of AP-1 is unclear. Furthermore, overexpression of c-Jun did not protect against apoptosis as strongly as RasV12 (compare Fig. 3B with Fig. 2A), suggesting that other signals from Ras synergize with AP-1 in protecting against loss-of-Rb-mediated apoptosis.

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