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Raf-independent Deregulation of p38 and JNK Mitogen-activated Protein Kinases Are Critical for Ras Transformation*

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Activated Ras, but not Raf, causes transformation of RIE-1 epithelial cells, supporting the importance of Raf-independent pathways in mediating Ras transformation. The p38 and JNK mitogen-activated protein kinase cascades are activated by Ras via Raf-independent effector function. Therefore, we determined whether p38 and JNK activation are involved in Ras transformation of RIE-1 epithelial cells. Rather surprisingly, we found that pharmacologic inhibition of p38, together with Raf activation of ERK, was sufficient to mimic the morphologic and growth transformation caused by oncogenic Ras. p38 inhibition together with ERK activation also caused the same alterations in cyclin D1 and $p21^{CIP1}$ expression caused by Ras and induced an autocrine growth factor loop important for transformation. Finally, in contrast to p38, we found that JNK activation promoted Ras transformation, and that Ras deregulation of p38 and JNK was not mediated by activation of the Rac small GTPase. We conclude that a key action of Raf-independent effector pathways important for Ras transformation may involve inhibition of p38 and activation of JNK.

Ras proteins function as key regulators of cellular signal transduction pathways (1, 2). Diverse extracellular stimuli that promote cell proliferation and differentiation activate cell surface receptors that converge on and promote the transient activation of Ras. Once activated, Ras interacts with and stimulates a spectrum of functionally diverse downstream effectors. These effectors in turn stimulate diverse cytoplasmic signaling cascades that regulate cytoplasmic and nuclear events. Nuclear events include regulation of cell cycle progression and gene expression (3, 4).

The Raf-1 serine/threonine kinase is the best characterized effector of Ras (5). Activated Ras binds to and promotes the activation of Raf, which activates the MEK1/2[1] dual specificity kinases, which in turn phosphorylate and activate the p42/p44 ERK mitogen-activated protein kinases (MAPKs). Although it is clear that Ras activation of the Raf/MEK/ERK protein kinase cascade is important for Ras transformation, it is now well established that Ras transformation also involves its activation of multiple Raf-independent effector pathways (6–8).

Phosphatidylinositol 3-kinases (PI3Ks) represent the second best characterized effectors of Ras, and PI3K activation has been shown to be important for Ras transformation of rodent fibroblasts (9). RalGDS and related proteins have also been implicated as effectors of Ras, and they also promote Ras transformation of rodent fibroblasts by activation of Ral small GTPases (10). Other effectors of Ras include AF-6, Rin1, Nore1, RASSF1, and phospholipase Cε (8, 11–13). However, their contributions to Ras transformation have not been established.

Approximately 30% of all human cancers express mutated and constitutively activated forms of Ras (14, 15). Additionally, the vast majority of these tumors are predominantly of epithelial origin. Because oncogenic Ras has been shown to play an essential role in oncogenesis (16, 17), research efforts have focused on defining the signaling pathways that mediate growth transformation by oncogenic Ras. Although many signaling components have been shown to be regulated by Ras, attempts to define those critically important for Ras transformation have been complicated by several issues (8). First, it is clear that cell type differences exist in the signaling pathways that are regulated by Ras. For example, whereas Ras activation of Raf alone is sufficient to cause transformation of rodent fibroblasts, Ras requires Raf-independent pathways to cause transformation of RIE-1 and a variety of other epithelial cells (18–20).[2] Second, the consequences of transient activation of Ras may differ from those caused by sustained activation of Ras. For example, although transient activation of Ras can activate PI3K, sustained Ras activation did not up-regulate PI3K activity in RIE-1 cells. In contrast to rodent fibroblasts, PI3K was not important for Ras transformation of these epithelial cells (21). Therefore, delineating the importance of Raf-independent effectors in maintenance of the transformed state by sustained expression of oncogenic Ras in epithelial cells will be critical for understanding the mechanism by which oncogenic Ras promotes human oncogenesis.

In addition to the ERK MAPK cascade, oncogenic Ras has been shown to stimulate transient activation of the JNK and p38 MAPK cascades (22–24) by activation of Raf-independent pathways that involve the Rac small GTPase (25). However, whereas ERK MAPKs are activated by stimuli that promote growth and differentiation, p38 and JNK are activated strongly

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[1] The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, Jun NH_2-terminal kinase; PI3K, phosphatidylinositol-4-phosphate kinase; EGF, epidermal growth factor; TGF-α, transforming growth factor-α.

[2] Hamad, N. M., Elconin, J., Karnoub, A. E., Bai, W., Rich, J. N., Der, C. J., Abraham, R. T., and Counter, C. M. (2002) *Genes Dev.*, in press.

by environmental stress and inflammatory cytokines (26). In almost all instances, p38 and JNK are activated coordinately by the same stimuli.

In contrast to ERK activation, the role of p38 and JNK activation in Ras transformation has not been clearly delineated. The function of a key substrate of JNK, the c-Jun transcription factor, has been shown to be required for Ras transformation of rodent fibroblasts (27–29). SEK1 is a kinase activator of JNK, and dominant negative SEK1 was shown to block Ras transformation of NIH 3T3 cells (30). However, a limitation of these studies is that the approaches for inhibition of the JNK pathway were indirect, and may have blocked activities beyond JNK. For example, how the SEK1 dominant negative works is not clear and may block the activities of the ERK and p38, as well as JNK, MAPK pathways (31). The absence of a pharmacologic inhibitor specific for JNK has limited a more precise evaluation of this question. Studies in rodent fibroblasts suggest that p38 acts as a negative regulator of Ras transformation. For example, although transient expression of activated Ras was found to cause activation of p38, further activation of p38 blocked Ras-induced mitogenesis in NIH 3T3 cells (24) or reversed morphologic and growth transformation (32). What is clear from these analyses is that the up-regulation of p38 can block Ras transformation. However, these studies did not determine whether persistent Ras activation causes the up-regulation, or down-regulation, of p38 activity. Finally, the importance of p38 and JNK in Ras transformation of epithelial cells, the cell type from which the vast majority of *ras* mutation-positive human cancers arise, has not been determined.

We have evaluated the possibility that p38 and JNK represent key components of Raf-independent signaling important for Ras transformation of RIE-1 epithelial cells. Previously, we demonstrated that activated Ras, but not Raf, causes transformation of RIE-1 epithelial. Surprisingly, in this study, we found that inhibition of p38, together with Raf activation of ERK, was sufficient to cause the same morphologic and growth transformation caused by activated Ras. Thus, a key action of Raf-independent effector signaling important for Ras transformation involves the down-regulation of p38 activity. Finally, we determined that the regulation and role of the related JNK MAPK was opposite to that seen with p38. Thus, although p38 and JNK are typically activated coordinately, p38 and JNK are regulated differently by oncogenic Ras and have opposing roles in Ras transformation.

MATERIALS AND METHODS

Molecular Constructs and Reagents—Mammalian expression vectors containing cDNA sequences encoding constitutively activated mutants of K-Ras4B(12V) or Raf-1 (the NH_2-terminal truncated and constitutively activated 22W mutant or the constitutively plasma membrane-targeted Raf-C*AAX* mutant) have been described previously (18, 33–36). A constitutively active mutant of the p38 activator MKK6b(EE) (with Ser-207 and Thr-211, the two phosphorylation sites in the activation loop, mutated to Glu) was obtained from Jiahuai Han (Scripps Research Institute, La Jolla, CA) (37). The cDNA sequence was removed from pcDNA3-MKK6b(EE) as an NH_2-terminal hemagglutinin epitope-tagged *Hin*dIII/*Xba*I fragment and subcloned into the pBabe-puro mammalian expression vector. The pcDNA3 expression vectors encoding wild type p38α, p38γ, and the p38γ(AF) dominant negative mutant (with Thr-183 and Tyr-185, the two phosphorylation sites in the activation loop, mutated to Ala and Phe, respectively) also were provided generously by Jiahuai Han (37). Expression vectors encoding hemagglutinin epitope-tagged versions of activated human Rac1(61L) and RhoA(63L) have been characterized previously (38). The SB203580 p38 inhibitor (Calbiochem; catalog no. 559389) and the SP600125 JNK inhibitor were dissolved in dimethyl sulfoxide (Me_2SO), and control cultures were treated with the equivalent final concentration of Me_2SO (vehicle). Anisomycin (A9739) was purchased from Sigma.

SDS-PAGE and Western Blot Analyses—Western blot analyses to assess the expression of proteins in cell lysates were done using monoclonal antibodies against cyclin D1 (17-32G-11 and HD-11; Santa Cruz Biotechnology), monoclonal or rabbit polyclonal antibodies against $p21^{CIP1}$ (Ab4/5; Upstate Biotechnology), a monoclonal antibody against mouse $p27^{Kip1}$ (K25020; Transduction Laboratories), polyclonal antibodies against a synthetic doubly phosphorylated phospho-p38 MAPK (Thr-180/Tyr-182; 9211; Cell Signaling) or a synthetic peptide derived from human p38 (9212; Cell Signaling), and polyclonal antibodies against a synthetic phospho-Thr71 peptide of ATF2 or a synthetic peptide corresponding to residues 65–77 of human ATF2 (9221S; New England Biolabs).

Cell Culture and Retroviral Infection—RIE-1 and Bosc23 cells were grown in Dulbecco's modified Eagle's medium. Production of infectious, replication-incompetent retrovirus was achieved by transfection of pBabe-puro expression constructs into the Bosc23 ecotropic packaging cell line (39). K-Ras(12V)-transformed RIE-1 cells were infected by exposure to 5 ml of retroviral supernatant (with a final concentration of Polybrene at 4 μg/ml) and 5 ml of growth medium. Fresh growth medium was added after 5–10 h, and drug selection was initiated 24 h later in 1–2 μg/ml puromycin.

Transient Expression Reporter Assays—For transient expression reporter assays, parental RIE-1 cells were transfected with Superfect or Effectene (Qiagen) as suggested by the protocols of the manufacturer. Briefly, RIE-1 cells were seeded the day prior to transfection and then incubated with plasmid DNA and cationic lipids for 24 h. Cells were harvested with 300 μl of luciferase lysis buffer (BD PharMingen), and 50 μl lysate was analyzed using enhanced chemiluminescence reagents and a Monolight 2010 luminometer (Analytical Luminescence). The cyclin D1-luciferase construct consists of 963 base pairs of the human cyclin D1 promoter controlling the expression of the luciferase gene (40). Reporter plasmids encoding the Gal4 DNA binding domain fused to the transactivation domains of the Elk-1, Jun, and CHOP (Stratagene) transcription factors have been described elsewhere (38, 41).

Transfections and Focus Formation Assays—RIE-1 cells were transfected with LipofectAMINE PLUS (Invitrogen) according to the protocol of the manufacturer. For each plate, 200 ng of pZIP-H-ras(12V) or 200 ng of pZIP-Neo(SV)x1 empty vector was transfected with 1.8 μg of carrier DNA. Three h after adding DNA mixtures, plates were aspirated and medium was replaced with 4 ml of complete growth medium. Seventeen h after removing the transfection mixtures, growth medium was replaced with 4 ml of complete growth medium supplemented with 0.2% Me_2SO (final concentration), or the indicated compound in a final concentration of 0.2% Me_2SO. Cultures were fed with 4 ml of fresh growth medium containing Me_2SO or the indicated compound every 3 days (four compound additions; total length of compound treatment 11 days). Finally, cultures were fixed with methanol, stained with 0.4% methylene blue (Sigma), and photographed. SB203580 (lot B37347) and PD98059 (lot B38092) were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). The SP6000125 (anthrax[1,9-*cd*]pyrazol-6(2H)-one) JNK inhibitor has been shown to specifically inhibit JNK, and not ERK or p38 kinase activity *in vitro* and *in vivo*. It was synthesized and purified as described (42).

RESULTS

Inhibition of p38 Causes Transformation of Raf-expressing RIE-1 Cells—We showed previously that Ras transformation of RIE-1 rat intestinal epithelial cells required both Raf-dependent and Raf-independent effector activation (18). However, the specific Raf-independent pathway(s) involved in Ras transformation has not been determined. Because Ras causes activation of the p38 and JNK MAPK cascades via Raf-independent signaling (22–24), we assessed the possible involvement of p38 and JNK activation in Ras transformation of RIE-1 cells.

First, we determined the level of p38 activation in untransformed RIE-1 cells stably transfected with empty vector, or RIE-1 cells stably expressing constitutively activated and transforming mutants of Raf-1 or K-Ras4B (Raf-22W and K-Ras(12V); designated RIE(Raf), and RIE(Ras), respectively). Unexpectedly, although transient expression of oncogenic Ras has been shown to activate p38 (22–25), we found that p38 activity was reduced in RIE(Ras) cells, when compared with untransformed vector control and RIE(Raf) cells (Fig. 1). Similarly, we found that the activity of ATF-2, a p38 substrate, was also reduced in RIE(Ras) cells when compared with untrans-

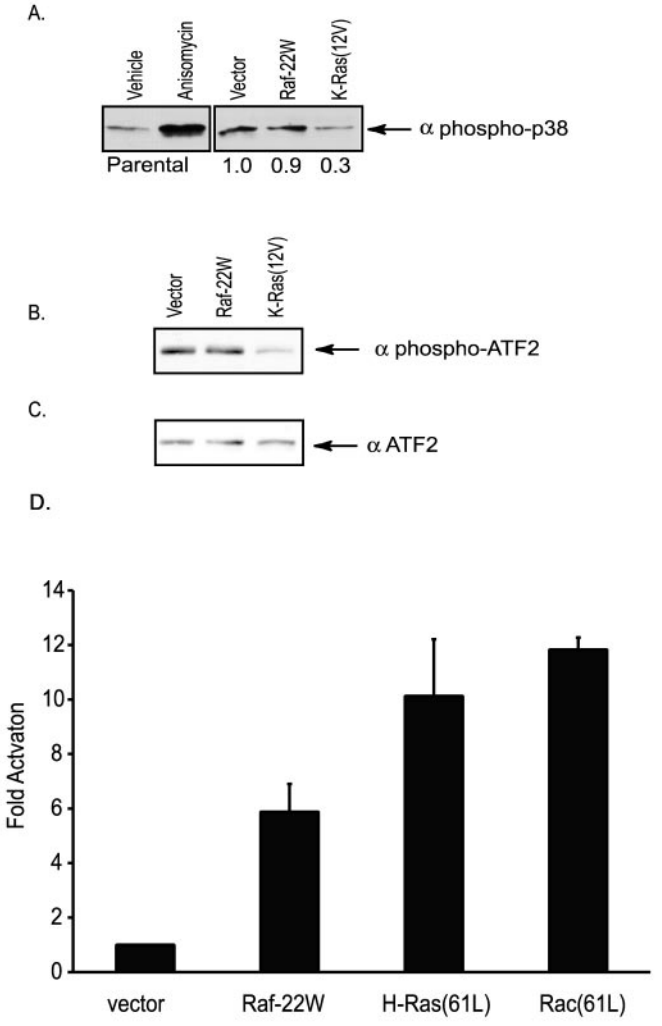

FIG. 1. **Steady-state level of p38 activity is reduced by sustained activation of K-Ras but not Raf in RIE-1 cells.** Equal total protein per cellular lysate of RIE-1 cells expressing the indicated proteins were resolved on SDS/PAGE and transferred to membranes for Western blot analyses to quantitate the levels of activated and dually phosphorylated form of p38 using the Thr-180/Tyr-182 anti-phospho-p38 antiserum (*A*), the total levels of p38 protein using the 9212 p38MAPK antibody against total p38 (data not shown), the levels of transcriptionally active and phosphorylated form of ATF2 using the Thr-71 anti-phospho-ATF2 antiserum (*B*). *C*, the membrane in *panel B* was stripped and re-probed for total ATF2 protein levels using the anti-ATF2 antiserum. Treatment of untransformed RIE-1 cells with 30 μM anisomycin for 20 min was used as a control to evaluate stress-mediated activation of p38. Data shown are representative of three independent experiments. *D*, transient expression of activated Ras causes activation of the p38 target CHOP. RIE-1 cells were transiently transfected with pAX142 (vector), or pAX142 encoding Raf-22W, H-Ras(61L), or Rac(61L), along with PFA-Gal-CHOP (Gal4 DNA binding domain fused to the NH_2-terminal activation domain of CHOP) and the reporter construct PFR-luc (5X-Gal4 binding element). Afterward cells were transfected for 24 h and cultured another 36 h before extract preparation. Luciferase activity was measured and expressed as -fold activation relative to the level of activation seen with the vector control. Data shown are representative of three independent experiments.

formed vector control and RIE(Raf) cells (Fig. 1*B*). Thus, although transient activation of Ras causes an up-regulation of p38 activity (Fig. 1*D*), sustained Ras activation resulted in a down-regulation of p38 activity in RIE-1 cells.

Our finding that steady-state p38 activity was reduced in RIE(Ras) cells suggested that down-regulation of p38 activity in RIE(Raf) cells may promote transformation. To address this possibility, we utilized SB203580, a pharmacologic inhibitor of p38 (43). First, we compared the consequences of p38 inhibition

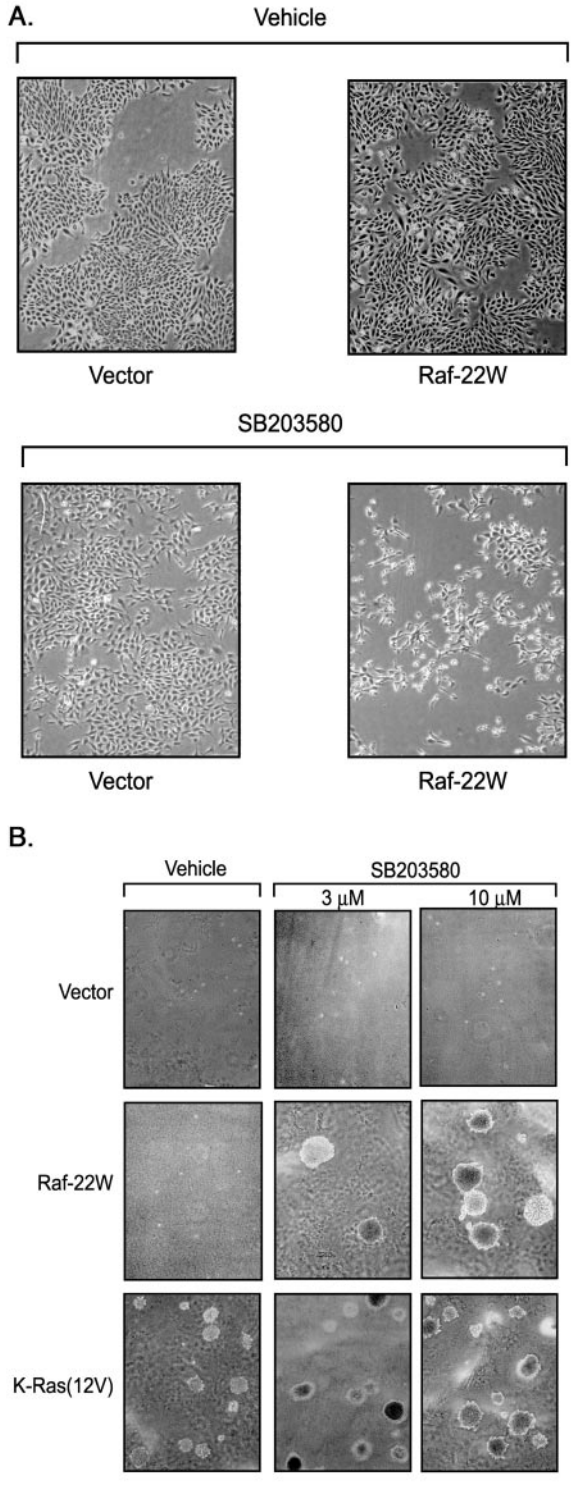

FIG. 2. **Treatment with the SB203580 p38 inhibitor causes morphologic and growth transformation of Raf-expressing, but not untransformed, RIE-1 cells.** *A*, RIE-1 cells stably expressing activated Raf-22W or stably transfected with the empty pZIP-NeoSV(x)1 vector were grown on plastic and incubated in growth medium supplemented with either Me_2SO vehicle control or 3 μM SB203580. Cell morphology was evaluated after 16 h. Data shown are representative of three independent experiments. *B*, treatment with SB203580 promotes the ability of Raf-expressing, but not untransformed, RIE-1 cells to form colonies in soft agar. RIE-1 cells stably expressing the empty pZIP-NeoSV(x)1 vector, or encoding activated Raf-22W or K-Ras(12V), were assayed for their ability to proliferate under anchorage-independent conditions in growth medium containing 0.3% agar and supplemented with Me_2SO, 3 μM, or 10 μM SB203580. The formation of multicellular colonies was visualized after 14 days. Data shown are representative of three independent experiments.

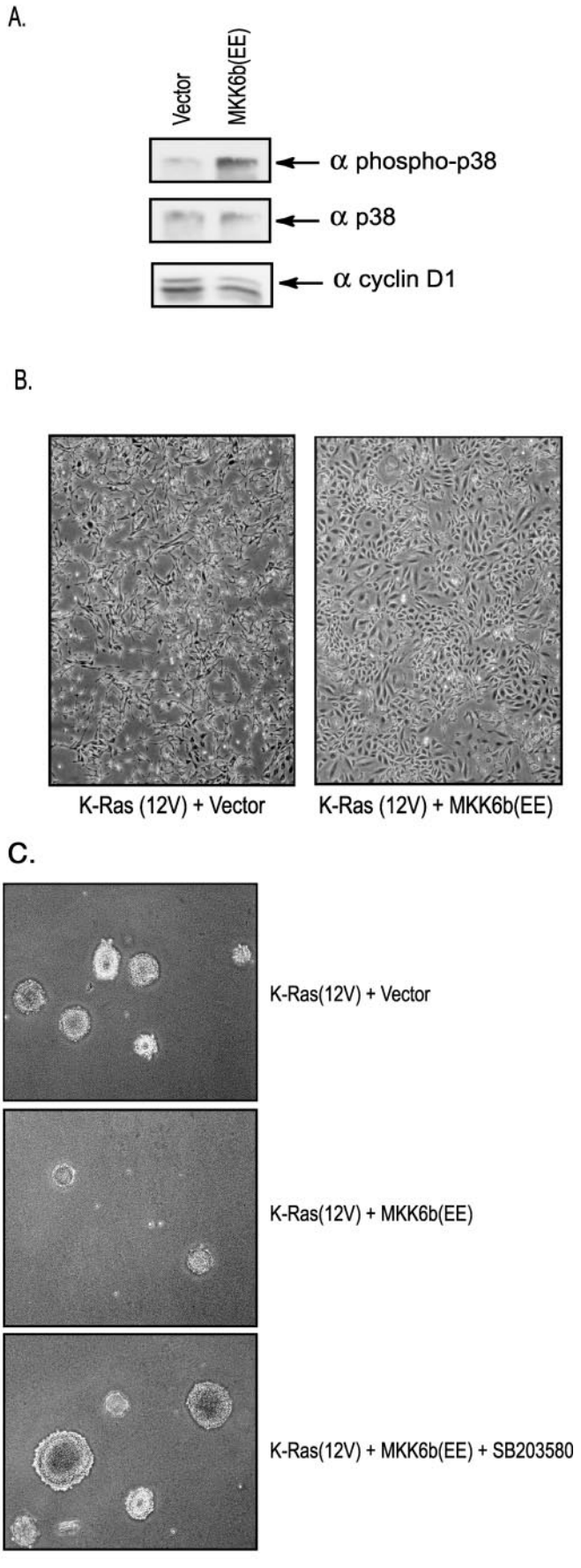

FIG. 3. **Activation of p38 reverses morphologic and growth transformation of Ras-transformed RIE-1 cells.** *A*, the relative levels of phospho-p38, total p38, and cyclin D1 protein expression were determined by Western blot analyses of equal total cellular lysates. The levels of phospho-p38 protein expression were determined using the Thr-180/Tyr-182 anti-phospho-p38 antiserum, and the membrane was stripped and re-probed with the anti-p38 antiserum for total p38 levels. Total cyclin D1 protein expression was determined using the anti-cyclin D1 antiserum. *B*, shown are the morphology of two pooled representative populations of cells harboring the empty vector and expressing MKK6b(EE). Data shown are representative of the analyses of both clonal and pooled populations of empty vector or MKK6b(EE)-expressing cells. *C*, activation of p38 inhibits the ability of Ras to form colonies in soft agar. K-Ras(12V)-transformed RIE-1 cells stably infected with the empty pBabe-puro vector or encoding MKK6b(EE) were established as described in Fig. 4 and were assayed for their ability to proliferate under anchorage-independent conditions. Three $\times 10^4$ cells were seeded in growth medium containing 0.3% agar and supplemented with either Me_2SO or 3 μM SB203580. The formation of multicellular colonies was visualized after 14 days. MKK6b(EE)-expressing cells showed reduced frequency of colony formation and reduced colony size, and treatment with SB203580 enhanced colony formation efficiency and size. Data shown are representative of three independent experiments.

on the morphology of control empty vector-transfected RIE-1 cells or RIE(Raf) cells that display stably up-regulated ERK activity (18). The morphologies of vehicle-treated RIE(Raf) and vector control cells were unchanged and retained the flat, nonrefractile appearance of untransformed RIE-1 cells (Fig. 2*A*). However, 16 h of treatment with the SB203580 p38 inhibitor caused striking morphologic transformation of Raf-expressing, but not vector control, cells. The treated RIE(Raf) cells showed a highly refractile appearance and were indistinguishable in morphology from RIE(Ras) cells (data not shown).

Next, we determined whether p38 inhibition could promote the anchorage-independent growth of RIE(Raf) cells. Like untransformed RIE-1 cells, RIE(Raf) cells fail to grow in soft agar (18). As shown in Fig. 2*B*, treatment with SB203580 promoted the ability of RIE(Raf) cells, but not untransformed vector control RIE-1 cells, to form colonies in soft agar. Overall, we observed that the colony forming efficiency for 10 μM SB203580-treated RIE(Raf) cells was ~80% of that for vehicle-treated RIE(Ras) cells. Thus, blocking p38 activity in Raf-expressing cells mimics the consequences of oncogenic Ras and causes morphologic and growth transformation.

Activation of p38 Antagonizes Ras Transformation in RIE-1 Cells—Because we found that p38 activity was reduced in RIE(Ras) cells, we predicted that stimulation of the p38 pathway would antagonize the transformed phenotype of RIE(Ras) cells. To address this possibility, we established RIE(Ras) cells stably expressing constitutively active MKK6b(EE), a specific upstream kinase activator of p38 (Fig. 3). First, we evaluated these stably expressing MKK6b(EE) for p38 activation and verified that ectopic expression of MKK6b(EE) caused an up-regulation of phospho-p38 levels in RIE(Ras) cells comparable with that observed in RIE(Raf) or untransformed RIE-1 cells (Fig. 3*A*). Second, we observed that expression of MKK6b(EE) caused a marked reversion of the transformed morphology of RIE(Ras) cells, exhibiting the nonrefractile appearance typical of untransformed RIE-1 cells (Fig. 3*B*).

We also found that RIE(Ras) cells expressing MKK6b(EE) were greatly impaired in their ability to grow in soft agar, with a 50% reduction in colony forming activity, as well as a reduction in the size of the colonies that formed (Fig. 3*C*). However, treatment of RIE(Ras)/MKK6b(EE) cells with SB203580 caused an increase in colony forming efficiency and in colony size, supporting the role of MKK6b(EE)-mediated up-regulation of p38 in inhibiting the anchorage-independent growth of RIE(Ras) cells. Finally, because other activities, such as activation of Raf, have been attributed to SB203580 treatment (44), the ability of a specific activator of p38 (MKK6) to reverse Ras transformation argues that the effects of this inhibitor on

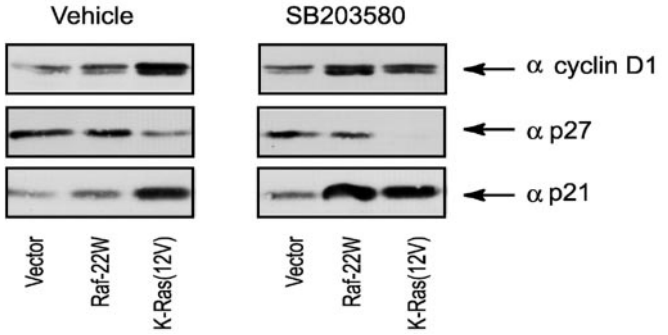

FIG. 4. **SB203580 treatment of Raf-expressing RIE-1 cells causes up-regulation of cyclin D1 and p21 protein expression.** Mass populations of RIE-1 cells stably transfected with the empty pZIP-NeoSV(x)1 vector or encoding activated Raf-22W or K-Ras(12V) were cultured with growth medium supplemented with either 10 μM SB203580 or Me_2SO control (vehicle). After 24–48 h of treatment, equal amounts of total cell lysate protein were resolved by SDS-PAGE and Western blot analyses were performed with anti-cyclin D1, anti-$p27^{KIP1}$, and anti-$p21^{CIP1}$ antisera to determine the steady-state levels of cyclin D1, $p27^{KIP1}$, and $p21^{CIP1}$ protein, respectively. Data shown are representative of at least three independent experiments.

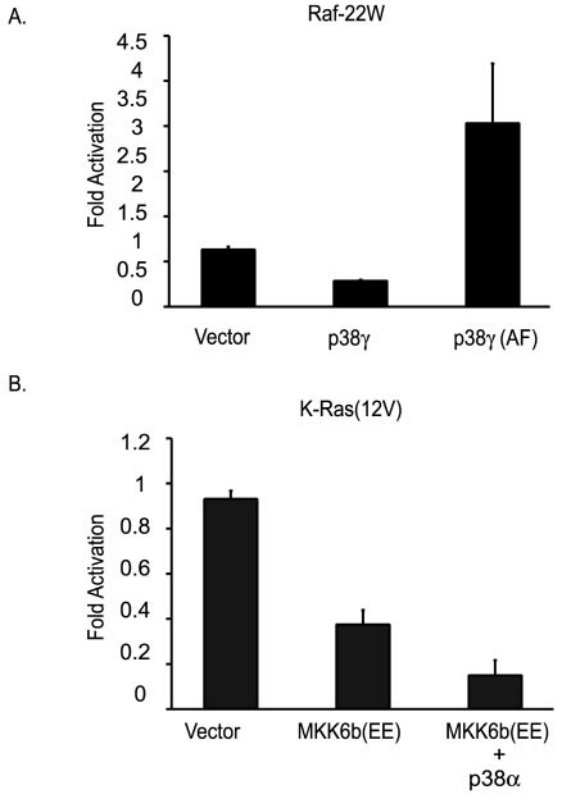

FIG. 5. **p38 is a negative regulator of transcription from the cyclin D1 promoter.** Transient expression analyses were done in RIE-1 cells stably expressing activated Raf-22W or K-Ras(12V) to evaluate the role of p38 in regulating the expression from a reporter plasmid, where the luciferase gene is under the control of the human cyclin D1 promoter. We showed previously that expression of this reporter is stably up-regulated in cells expressing activated Ras but not Raf (46). Whereas expression of dominant-negative (*AF*), but not wild type, p38γ, stimulated up-regulation of transcription from the cyclin D1 promoter in RIE(Raf) cells (*A*), expression of activated MKK6b(EE) causes down-regulation of transcription from the cyclin D1 promoter in RIE(Ras) cells (*B*). Further inhibition was seen when MKK6b(EE) was co-expressed with wild type p38α. Data shown are representative of three independent experiments.

RIE(Raf) cells are caused by inhibition of p38 activity.

Inhibition of p38 Causes Increases in Cyclin D1 and $p21^{CIP1}$—We next wanted to determine a mechanism for p38 regulation of Ras transformation. One possibility was suggested by the previous demonstration that p38 activity antagonizes transcription from the cyclin D1 promoter (45). We observed recently that Ras but not Raf can cause transformation of RIE-1 cells, and that Ras but not Raf could up-regulate cyclin D1 protein expression and function (46). Thus, we asked whether SB203580 treatment of RIE(Raf) cells would mimic this consequence of Ras activation. Strikingly, similar to RIE(Ras) cells, we found that RIE(Raf) cells treated with the p38 inhibitor showed an increase in the steady-state level of cyclin D1 protein (Fig. 4). We also found that MKK6b(EE)-mediated up-regulation of p38 activity in RIE(Ras) cells caused a 2–3-fold reduction in steady-state levels of cyclin D1 (Fig. 3*A*). Finally, we utilized a reporter plasmid where luciferase gene expression is under the control of the human cyclin D1 promoter (40) and determined that p38 negatively regulates cyclin D1 protein expression at the level of gene transcription (Fig. 5). We determined that co-expression of a kinase inactive and dominant negative form of p38, p38γ(AF), augmented cyclin D1 promoter stimulation.

Previously, we observed that the steady-state levels of two cyclin-dependent kinase inhibitors, $p21^{CIP1}$ and $p27^{KIP1}$, were altered by Ras but not Raf in RIE-1 cells (46). Whereas $p27^{KIP1}$ expression was reduced, $p21^{CIP1}$ expression was elevated, in RIE(Ras) but not RIE(Raf) cells (Fig. 4). Treatment of RIE(Raf) cells with SB203580 also caused an up-regulation of $p21^{CIP1}$, but did not cause the down-regulation of $p27^{KIP1}$ protein levels that is seen in RIE(Ras) cells. Thus, p38 inhibition may promote Ras transformation, in part, by deregulating the expression and function of cyclin D1 and $p21^{CIP1}$, whereas $p27^{KIP1}$ down-regulation may not be as critical for transformation of RIE-1 cells.

Inhibition of p38 in Raf-expressing Cells Stimulates an Autocrine Pathway—We also assessed another possible mechanism for p38 regulation of Ras transformation. Because we showed previously that conditioned medium from cultures of RIE(Ras), but not RIE(Raf), contained activity that caused the morphologic transformation of control RIE-1 cells (18), we assessed whether SB203580-treated RIE(Raf) cells now contained a similar activity. Parental RIE-1 cells were treated with conditioned media collected from SB203580-treated vector control, RIE(Raf), or RIE(Ras) cells. We observed that conditioned medium from SB203580-treated RIE(Raf), but not vector-transfected, cells caused morphological transformation of parental cells (Fig. 6). Thus, like RIE(Ras) cells, SB203580-treated RIE(Raf) cells also stimulated production and secretion of a factor(s) that could promote morphologic transformation of RIE-1 cells.

We showed previously that TGF-α was one of the factors secreted into the medium by cultures of RIE(Ras) but not RIE(Raf) cells (47). Therefore, we determined whether up-regulation of TGF-α expression and secretion may be involved in promoting SB203580 treatment-induced transformation of RIE(Raf) cells. First, we determined whether treatment of parental cells with exogenous TGF-α alone would cause up-regulation of cyclin D1 and $p21^{CIP1}$ protein expression. As shown in Fig. 7*A*, treatment with TGF-α caused the up-regulation of both cyclin D1 and $p21^{CIP1}$. Second, we determined whether SB203580 treatment of RIE(Raf) cells caused an up-regulation of the mRNA expression of TGF-α or other EGF family ligands. As we have shown previously, RIE(Ras) cells express TGF-α transcripts, whereas control RIE-1 and RIE(Raf) cells do not. However, we failed to detect an up-regulation of TGF-α transcription in SB203580-treated RIE(Raf) cells (Fig. 7*B*). These data suggest that, although TGF-α production alone can cause the changes in cyclin D1 and $p21^{CIP1}$ expression, up-regulation

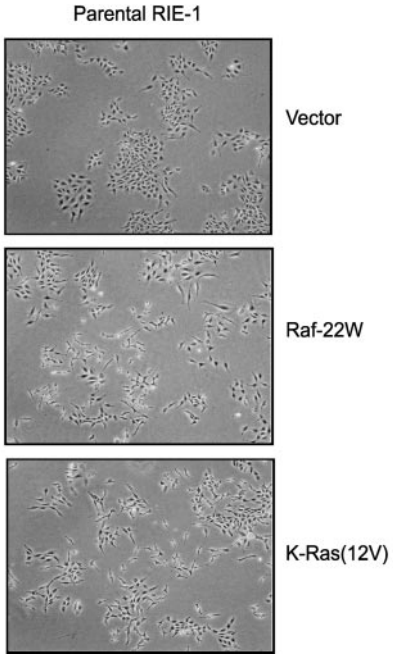

FIG. 6. **Treatment with the conditioned media from SB203580-treated Raf-expressing, but not vector control, RIE-1 cells causes morphologic transformation of parental RIE-1 cells.** To determine whether SB203580-mediated transformation of Raf-expressing RIE-1 cells stimulated the production of these factors, adherent cultures of RIE-1 cells stably expressing the empty pZIP-NeoSV(x)1 vector or expressing activated Raf-22W or K-Ras(12V) were incubated with growth medium supplemented with 5 μM SB203580. After 24 h, conditioned media from each SB203580-treated cell line were collected, passed through a 0.4-μm filter to remove any cells, and added to parental RIE-1 cells plated the previous day at 1.5×10^5 cells/10-cm dish. Changes in cell morphology were then assessed after 24 h.

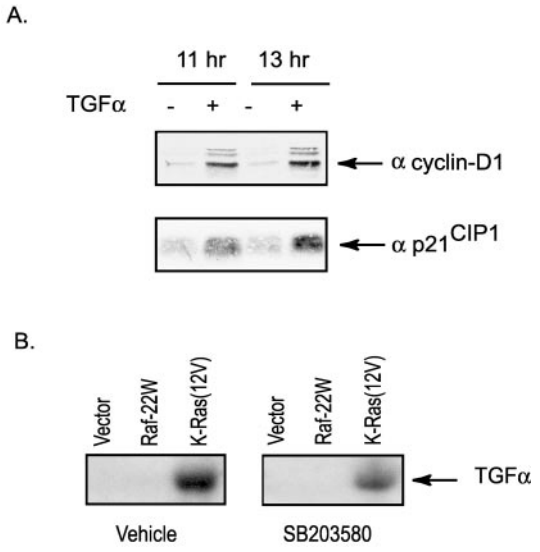

FIG. 7. **TGF-α up-regulates cyclin D1 and p21 in parental RIE-1 cells, but its expression is not stimulated by SB203580 treatment of activated Raf-22W-expressing RIE-1 cells.** *A*, parental RIE-1 cells were treated with 20 ng/ml TGF-α for the indicated times and equal total lysates were analyzed by Western blot analyses to monitor any changes in the levels of cyclin D1 and $p21^{CIP1}$ proteins. Treatment with TGF-α alone was sufficient to cause increased expression of cyclin D1 and $p21^{CIP1}$ in untransformed RIE-1 cells and is similar to what is seen in Ras-transformed RIE-1 cells. *B*, RIE-1 cells stably transfected with the empty pZIP-NeoSV(x)1 or encoding activated Raf-22W or K-Ras(12V) were treated with either Me_2SO vehicle control or 3 μM SB203580. After 48 h the cells were lysed and total RNA was isolated. Northern blot analysis was performed by hybridization with a ^{32}P-radiolabeled rat TGF-α cDNA probe. Data shown are representative of three independent experiments.

of TGF-α is not involved in the transformation of SB203580-treated RIE(Raf) cells. Our Northern blot analyses also failed to find up-regulation of two other EGF family ligands, HB-EGF or amphiregulin, in the SB203580-treated RIE(Raf) cells (data not shown).

Inhibition of JNK Does Not Promote Transformation of RIE(Raf) Cells—Similar to p38, the JNK MAPK cascade is activated by a variety of stress stimuli, and p38 and JNK are often activated coordinately. Additionally, it has been reported that use of SB203580 at higher concentrations (above 3 μM) may result in inhibition of JNK (44). Therefore, we used two approaches to evaluate the possibility that JNK activation may also antagonize Ras transformation of RIE-1 cells. First, we determined whether steady-state JNK, like p38, activity was down-regulated in RIE(Ras) but not RIE(Raf) cells. Second, we determined whether the inhibition of JNK activity would yield the same effects as those observed with inhibition of p38.

We first compared the activity of JNK in RIE-1, RIE(Raf), and RIE(Ras) cells by evaluating the steady-state level of phosphorylated and activated c-Jun, which is a key substrate of JNK. Western blot analyses with an anti-phospho-c-Jun antibody revealed that phosphorylated Jun levels were low in untransformed RIE-1 and RIE(Raf) cells, but elevated in RIE(Ras) cells (Fig. 8*A*). These results contrast with the down-regulation of p38 activity seen in RIE(Ras) cells, suggesting that JNK activation may promote, rather than antagonize, Ras transformation of RIE-1 cells. To evaluate a role for JNK in Ras transformation, we utilized the SP600125 JNK-specific inhibitor (42). SP600125 specifically inhibits all three isoforms of JNK (IC_{50} of 0.11–0.15 μM *in vitro*) but not ERK or p38 (IC_{50} of greater than 30 μM). We first determined the consequences of JNK inhibition on the morphology and growth of control empty vector-transfected, RIE(Raf), and RIE(Ras) cells. In contrast to our observations with SB203580, SP600125-treated RIE(Raf) cells did not undergo any significant change in morphology (Fig. 9*A*). Instead, we found that treatment of RIE(Ras) cells with SP600125 caused a significant impairment in the rate of growth on plastic (Fig. 9*B*) and in the frequency and size of colony formation in soft agar (Fig. 9*C*). The inhibitory activity of SP203580 was not the result of nonspecific inhibition of ERK activity, because we found that this treatment did not reduce the elevated ERK activity present in Ras-transformed RIE-1 cells (data not shown). Furthermore, treatment with 20 μM SP600125 blocked TNFα -induced JNK activation but not EGF-induced ERK activation in untransformed RIE-1 cells (data not shown).

We also compared the ability of pharmacologic inhibitors of ERK, JNK, and p38 to inhibit oncogenic Ras focus formation in RIE-1 cells. Consistent with our previous observations, we found that treatment with the PD98059 MEK1/2 inhibitor caused significant inhibition of oncogenic Ras focus forming activity (greater than 80% inhibition at 20 μM) (Fig. 10). Similarly, treatment with 20 μM SP600125 caused a greater than 90% reduction in focus formation. In contrast, cultures treated with SB203580 showed a slight enhancement of focus forming activity when treated with a concentration that promoted morphologic and growth transformation of RIE(Raf) cells (10 μM). Thus, ERK and JNK activation, but p38 inactivation, promote Ras transformation.

Finally, we also determined the consequence of JNK inhibition on the expression of cyclin D1 and $p21^{CIP1}$. Unlike SB203580 treatment, SP600125 treatment of RIE(Raf) cells did not cause a significant increase in the steady-state level of expression of cyclin D1 or $p21^{CIP1}$ (Fig. 8*B*). Taken together, these results suggest that JNK and p38 have different roles in

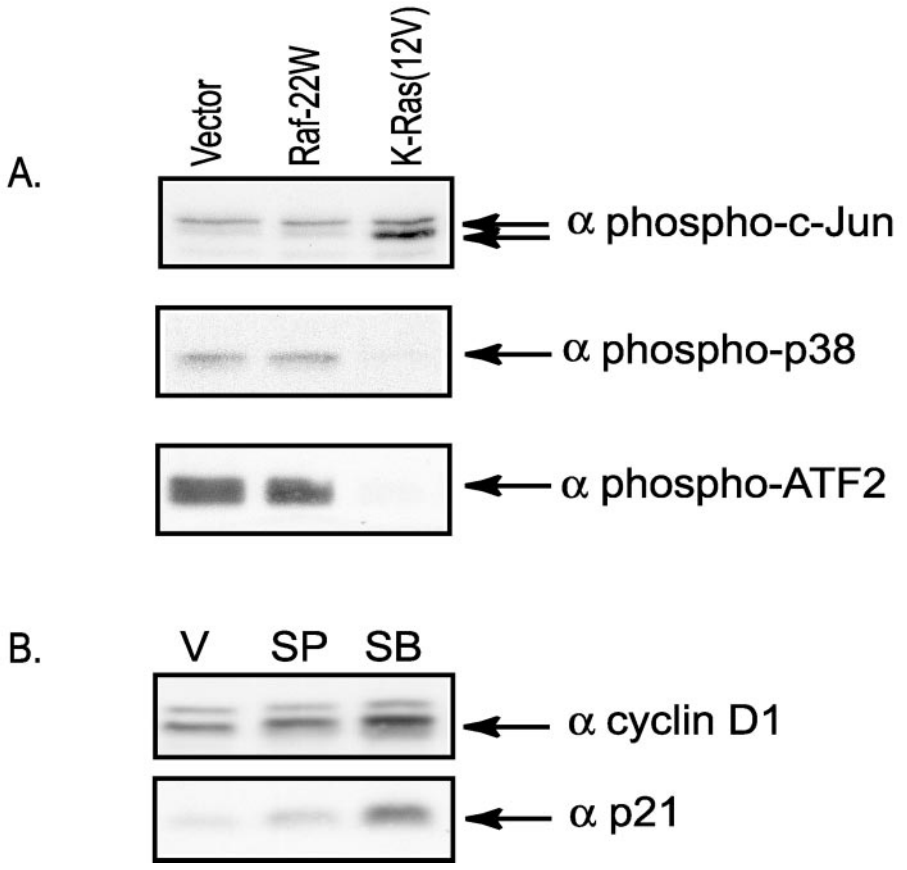

FIG. 8. **JNK is activated in RIE(Ras) but not RIE(Raf) or untransformed RIE-1 cells but is not involved in regulation of cyclin D1 or p21^{CIP1} expression.** *A*, JNK is activated in RIE(Ras) cells. Western blot analyses were done on control RIE-1 (*Vector*) or RIE-1 cells stably expressing Raf-22W or K-Ras(12V) to determine the levels of transcriptionally active phosphorylated c-Jun (06-659; Upstate Biotechnology, Inc.). In parallel, the levels of activated and dually phosphorylated p38 using the Thr-180/Tyr-182 anti-phospho-p38 antiserum and transcriptionally active and phosphorylated form of ATF2 using the Thr-71 anti-phospho-ATF2 antiserum were determined. *B*, equal total cell lysates from RIE(Raf) cells treated with vehicle control (*V*), 20 μM SP600125 (*SP*), or 5 μM SB203580 (*SB*) were analyzed by Western blot analyses to monitor any changes in the levels of cyclin D1 and p21^{CIP1} proteins.

mediating Ras transformation of RIE-1 cells and that our observations with SB203580 are not a consequence of nonspecific inhibition of JNK activity.

DISCUSSION

Activated Ras and Raf show equivalent abilities to cause transformation of NIH 3T3 and other rodent fibroblast cell lines. In contrast, activated Ras but not Raf causes transformation of RIE-1 rat intestinal and other epithelial cells (18–20).[2] Thus, Raf-independent effector activation is critical for Ras transformation of epithelial cells. Although most commonly activated by stress and inflammatory stimuli (26), the p38 and JNK MAPK cascades are also activated by transient expression of oncogenic Ras via Raf-independent pathways (22–25). Therefore, we assessed their contribution to Ras transformation of RIE-1 cells. We found that pharmacologic inhibition of p38 alone was sufficient to mimic the contribution of Raf-independent effector pathways to cooperate with the Raf/ERK pathway to cause morphologic and growth transformation. The fact that p38 inhibition alone caused the same changes in cyclin D1 and p21^{CIP1} expression, and induction of an autocrine growth mechanism, as caused by activated Ras but not Raf, supports the possibility that the down-regulation of p38 is a key action of Raf-independent effector pathways involved in Ras transformation. In contrast, although p38 and JNK are commonly activated by the same stimuli, we found that JNK activation contributed to, rather than antagonized, Ras transformation. To date, we have been unable to clearly identify the Raf-independent effector function required for Ras transformation of RIE-1 cells. Although logical candidates include the PI3K or RalGDS effector pathways shown to be important in Ras transformation of rodent fibroblasts, we recently eliminated these two effectors as being important for Ras transformation of RIE-1 cells (21). In this study, we found surprisingly that inhibition of p38, in cooperation with Raf activation of ERK, caused the same morphologic and growth transformation observed with activated Ras. Thus, p38 inhibition strongly mimicked the contribution of Raf-independent effector signaling in promoting Ras transformation. Other activities have been described for SB203580, such as inhibition of JNK, and may account for the consequences seen (44). However, our observations that restoration of p38 activity in Ras-transformed cells reversed morphologic and growth transformation, and that an inhibitor of JNK did not mediate the same consequences, argue that the ability of SB203580 treatment to cooperate with Raf activation to promote transformation is the result of inhibition of p38.

Two previous studies also showed that p38 activation was antagonistic for Ras-induced mitogenesis or growth transformation of NIH 3T3 cells. Siebenlist and colleagues (32) showed that MEK3-mediated activation of p38 caused the reversion of morphologic and growth transformation of Ras-transformed NIH 3T3 cells. Stacey and colleagues (24) found that, although transient expression of oncogenic Ras did cause activation of p38, further activation of p38 blocked Ras-induced DNA synthesis in NIH 3T3 cells. However, although these studies showed that p38 activation is antagonistic for Ras growth promotion and because Ras causes activation of p38 in NIH 3T3 cells, whether Ras caused alteration in p38 function to promote transformation was not evaluated in these studies. Thus, these studies did not directly address how oncogenic Ras regulates p38 activity to promote transformation. In contrast, our analyses showed that the down-regulation of p38 was a mechanism by which oncogenic Ras causes transformation. Finally, because we found that transient expression of oncogenic Ras activated p38, the down-regulation of p38 seen in RIE(Ras) cells is the result in part of the different consequences of

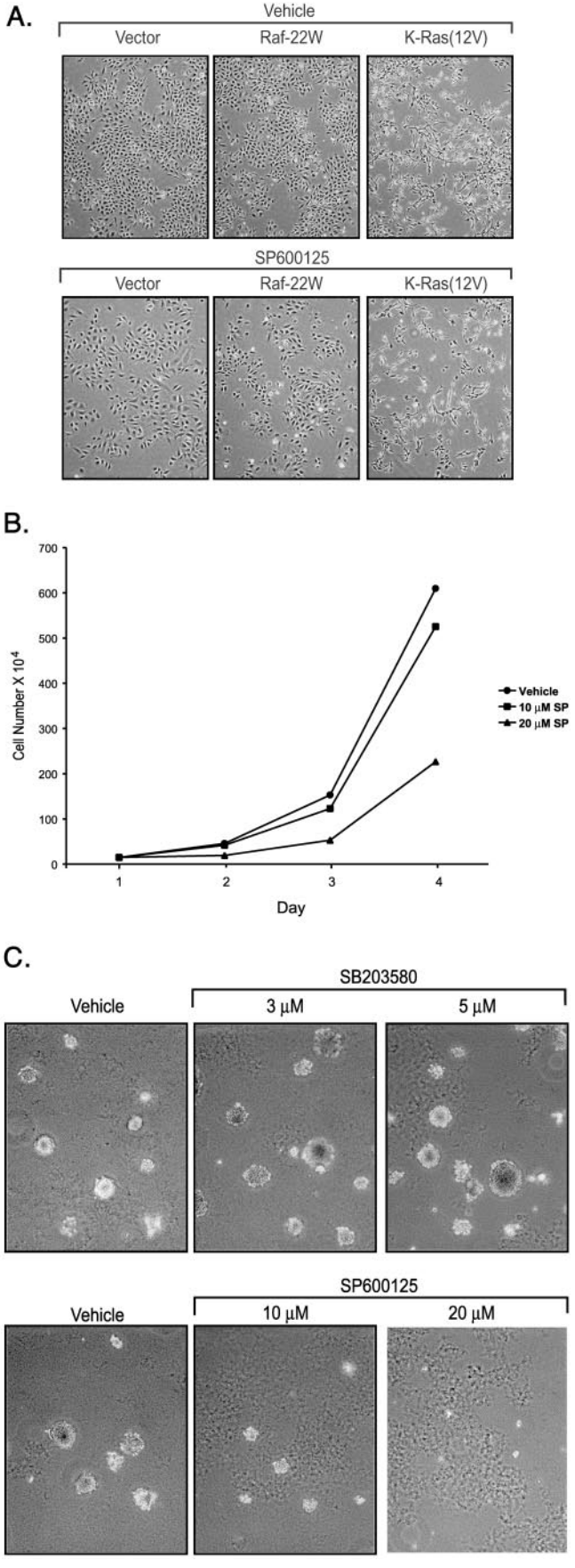

FIG. 9. **Treatment with the SP600125 JNK inhibitor does not cause morphologic transformation of RIE(Raf) cells, but inhibits the growth of RIE(Ras) cells.** RIE-1 cells stably expressing activated K-Ras(12V), Raf-22W, or stably transfected with the empty pZIP-NeoSV(x)1 vector were grown on plastic and incubated in growth medium supplemented with either Me_2SO vehicle control or 20 μM SP600125. *A*, SP600125 treatment does not alter the morphology of RIE(Raf) or RIE(Ras) cells. The morphology of the cells was evaluated after 24 h. *B*, SP600125 treatment reduces the rate of growth of RIE(Ras) cells when grown on plastic. Cultures of RIE(Ras) cells were plated in six-well plates at 1.5×10^5 cells/well in growth medium on day 1, and the growth medium was then replaced with growth medium supplemented with Me_2SO only (*Vehicle*) or with 10 μM or 20 μM SP600125. Triplicate wells were analyzed on the indicated days, and the number of cells per well was determined. *C*, SP600125 treatment reduces the anchorage-independent growth of RIE(Ras) cells in soft agar. RIE-1 cells stably expressing K-Ras(12V) were assayed for their ability to proliferate under anchorage-independent conditions in growth medium containing 0.3% agar and supplemented with Me_2SO, 3 μM SB203580, and 5 μM SB203580 or Me_2SO, 10 μM SP600125, and 20 μM SP600125. The formation of multicellular colonies was visualized after 14 days. Data shown are representative on three independent experiments.

transient *versus* sustained activation of Ras.

Does p38 play an antagonistic role in transformation in other situations? In addition to RIE-1 cells, we have determined that Ras but not Raf causes transformation of ROSE rat ovarian epithelial cells, and SB203580 treatment of Raf-expressing ROSE cells also mimics Ras transformation of these cells (data not shown). Inhibition of p38 enhanced the proliferation of the Panc-1 human pancreatic carcinoma cell line, which harbors a *ras* mutation (49). In contrast, p38 activity was constitutively up-regulated in Src-transformed NIH 3T3 cells, and inhibition of p38 abolished the ability of these cells to grow in soft agar (50). Thus, p38 activity may be anti-proliferative in some, but not all, situations. Further analyses of the role of p38 in human cancers will be needed to determine whether inhibitors of p38 will be useful, or detrimental, for cancer treatment.

Does p38 inhibition accurately mimic the actions of Raf-independent effector signaling to promote Ras transformation? Our studies have identified two mechanisms by which p38 inhibition might cooperate with Raf to promote cellular transformation, and both are events also caused by oncogenic Ras. First, one involves p38 deregulation of cell cycle progression through G_1. We showed previously that sustained activation of Ras but not Raf caused an up-regulation of cyclin D1 and $p21^{CIP1}$, but a down-regulation of $p27^{KIP1}$ protein expression (46). In the present study, we found that p38 inhibition also promoted the up-regulation of cyclin D1 and $p21^{CIP1}$ protein expression, but no significant change in $p27^{KIP1}$ expression was observed. The ability of p38 inhibition to cause both transformation as well as alterations in these regulators provides further support for a role for cyclin D1 and $p21^{CIP1}$ in Ras transformation, but indicates that down-regulation of $p27^{KIP1}$ may not be as critical for transformation. Ellenger-Ziegelbauer *et al.* (32) also found that p38 activation blocked mitogen-driven expression of cyclin D1 in NIH 3T3 cells. We showed previously that conditioned media from cultures of RIE(Ras), but not RIE(Raf), contained activity that caused the morphologic and growth transformation of control RIE-1 cells (18). We also determined that SB203580 treatment of Raf-expressing RIE-1 cells involved activation of an autocrine pathway. In the present study, we observed that conditioned medium from SB203580-treated RIE(Raf), but not vector-transfected, cells caused morphological transformation of parental cells. Thus, like activated Ras, p38 inhibition in RIE(Raf) cells also stimulated production and secretion of a factor(s) that could promote morphologic transformation of RIE-1 cells. However, we failed to detect an up-regulation of TGF-α or two other EGF family ligands, HB-EGF or amphiregulin, in SB203580-treated RIE(Raf) cells. Whether the autocrine activity represents yet other EGF family ligands (EGF, betacellulin, epiregulin) or

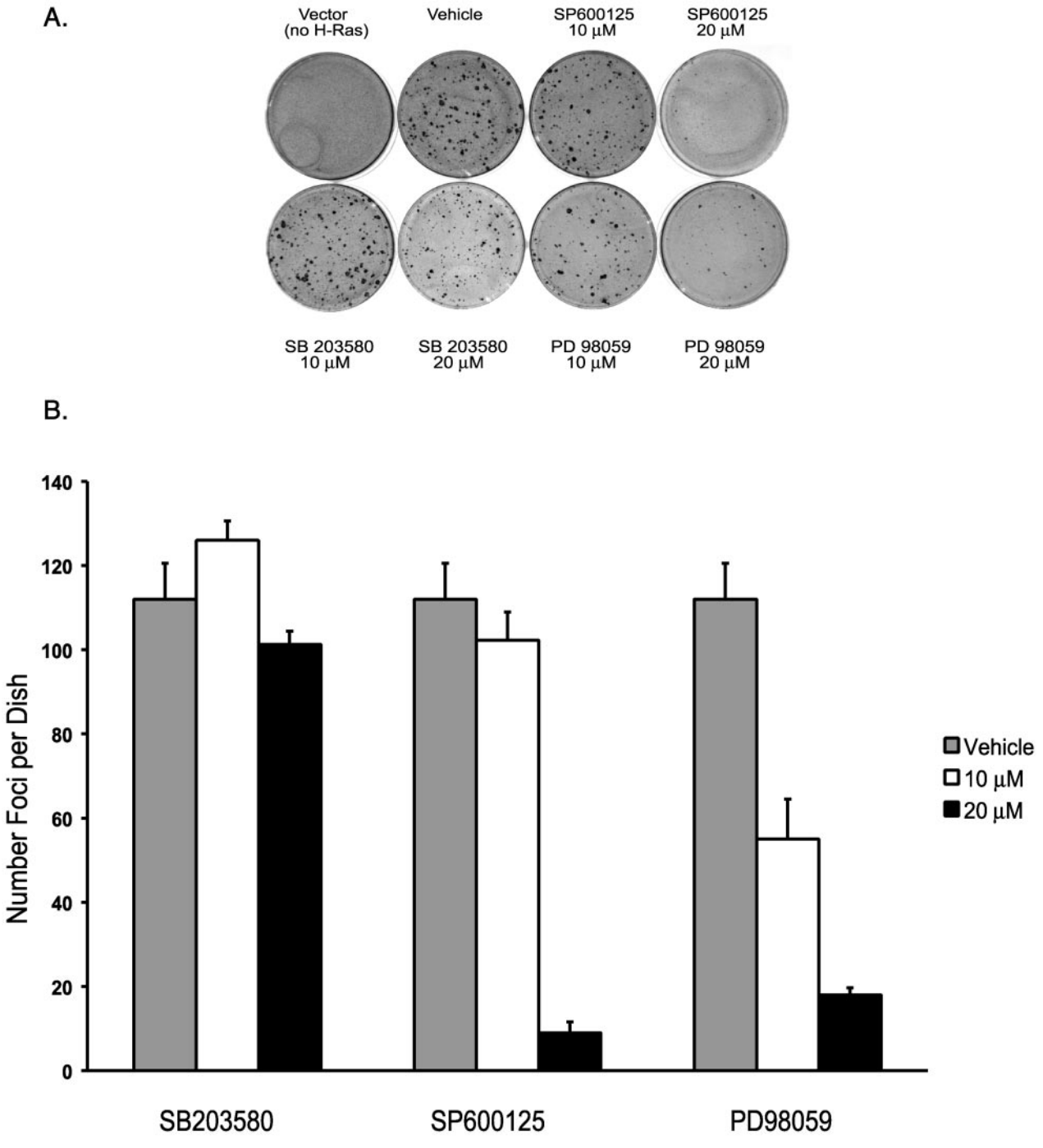

FIG. 10. **Treatment with the SP600125 JNK inhibitor and PD98059 MEK inhibitor, but not SB203580 p38 inhibitor, inhibits the Ras-mediated focus formation.** RIE-1 cells were transfected with empty pZIP-NeoSV(x)1 vector (no H-Ras) or with 200 ng of pZIP-H-*ras*(12V) (all other plates) and subsequently treated with vehicle (Me_2SO) or the indicated compounds. After 11 days of treatment, cells were fixed and stained, and transformed foci were visualized on a Kodak Image Station 400CF. Shown are representative plates from each condition assayed (*A*) and quantitation of total foci from triplicate plates (*B*). Data are expressed as mean foci per plate ± S.D.

other peptide growth factors remains to be determined. Concerning the mechanism of Ras deregulation of p38 and JNK, one logical mechanism for the Raf-independent regulation of p38 and transformation involves activation of Rac small GTPase (22). Therefore, we determined whether Rac activation may be involved in Ras transformation of RIE-1 cells using RIE-Raf cells and established cell lines stably co-expressing activated Rac1(61L) (data not shown). These cells retained the morphology of untransformed RIE-1 cells and failed to form colonies when suspended in soft agar (data not shown). Furthermore, Rac-expressing RIE-1 cells showed up-regulated, rather than down-regulated, p38 activity. Thus, in contrast to rodent fibroblasts, activated Rac1 or RhoA fails to cooperate with Raf to cause transformation on RIE-1 cells. Therefore, it appears that Rac activation is not the key Raf-independent event required for Ras down-regulation of p38.

In summary, we have determined that one critical component of Raf-independent signaling important for Ras transformation of RIE-1 cells involves down-regulation of p38 activity. Consequently, delineating the molecular consequences of p38 inhibition may lead to the identification of the critical mechanisms of Ras transformation of these and other epithelial cells.

In particular, it appears that p38 inhibition promotes changes in gene expression also caused by activated Ras but not Raf. For example, we recently showed that activated Raf, but not Ras, caused a down-regulation of tropomyosin gene expression in RIE-1 cells (48). However, SB203580-treated RIE(Raf) cells showed the same down-regulation of tropomyosin seen in Ras-transformed cells. Therefore, our future studies will concentrate on defining the gene expression changes caused by p38 inhibition that are also caused by activated Ras but not Raf. Finally, because Ras transformation of other epithelial cells also requires Raf-independent signaling, whether p38 inhibition will also play a role in Ras transformation of other epithelial cells will be important to establish. Further identification of key distinctions in the roles of MAPK family members in epithelial cells may provide a good assessment of their potential as therapeutic targets in oncology.

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