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Ras Inactivation of the Retinoblastoma Pathway by Distinct Mechanisms in NIH 3T3 Fibroblast and RIE-1 Epithelial Cells*

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Although Ras and Raf cause transformation of NIH 3T3 fibroblasts, only Ras causes transformation of RIE-1 intestinal epithelial cells. To determine if the inability of Raf to transform RIE-1 cells is due to a failure to deregulate cell cycle progression, we evaluated the consequences of sustained Ras and Raf activation on steady state levels of cyclin D1, $p21^{CIP/WAF}$, and $p27^{KIP1}$. Both Ras- and Raf-transformed NIH 3T3 cells showed up-regulated expression of cyclin D1, p21, and p27 protein, increased retinoblastoma (Rb) hyperphosphorylation, and increased activation of E2F-mediated transcription. Similarly, Ras-transformed RIE-1 cells also showed up-regulation of cyclin D1, p21, and hyperphosphorylated Rb. In contrast, Ras-mediated down-regulation of p27 was seen in RIE-1 cells. Conversely, stable expression of activated Raf alone caused only a partial up-regulation of p21 and Rb hyperphosphorylation but no activation of E2F-responsive transcription or down-regulation of p27 in RIE-1 cells. Moreover, we found that the AP-1 site was dispensable for Ras-mediated stimulation of the cyclin-D1 promoter in NIH 3T3 cells but was essential for Ras-mediated stimulation in RIE-1 cells. Thus, up-regulation of p21, rather than the down-regulation seen in previous transient expression studies, is seen with sustained Ras activation. Additionally, p27 may serve a positive (NIH 3T3) or negative (RIE-1) regulatory role in Ras transformation that is cell type-dependent. The involvement of Raf and phosphatidylinositol 3-kinase in mediating Ras changes in cyclin D1, p21, and p27 was also very distinct in NIH 3T3 and RIE-1 cells. Taken together, these results demonstrate the importance of Raf-independent pathways in mediating oncogenic Ras deregulation of cell cycle progression in epithelial cells.

Mutated and constitutively activated forms of Ras are found in 30% of human cancers (1, 2). Therefore, the signaling pathways that mediate oncogenic Ras growth transformation have been studied intensively. Although it is clear that Ras activation of the Raf/MEK[1]/ERK mitogen-activated protein kinase cascade is important for Ras transformation, it has also become clear that Ras transformation also requires the activation of multiple Raf-independent effector pathways (3). Furthermore, the pathways important for Ras transformation also exhibit cell type differences.

In addition to Raf, Ras also associates with a spectrum of other downstream effector targets. Among these, the phosphoinositide 3-phosphate lipid kinases (PI3Ks) represent the second best characterized effectors of Ras (4). Activated PI3K, a lipid kinase, facilitates the conversion of phosphatidylinositol 4,5-phosphate to phosphatidylinositol 3,4,5-phosphate. Phosphatidylinositol 3,4,5-phosphate levels are elevated in Ras-transformed cells and promote the activation of the Akt/protein kinase B serine/threonine kinase (5, 6). A third class of Ras effectors is a family of guanine nucleotide exchange factors (RalGDS, RGL, and Rlf/RGL2) that serve as activators of the Ral small GTPases (7, 8). The contribution of PI3K and RalGDS to Ras transformation has been supported by studies with effector domain mutants (E37G and Y40C) of activated Ha-Ras^{Val-12} that cause the loss of Raf binding and consequently a failure to activate ERKs (6, 9–11). $Ras^{Val-12/Gly-37}$ is also impaired in interaction with PI3K but retains interaction with RalGDS, whereas the $Ras^{Val-12/Cys-40}$ mutant is impaired in interaction with RalGDS but retains interaction with PI3K. The ability of both mutants to cause growth transformation of NIH 3T3 cells demonstrates that Ras can also cause transformation by Raf-independent pathways.

One aspect of Ras signaling clearly important for growth transformation involves regulation of components of the cell cycle machinery that is critical for progression through the G_1 phase (12–14). A significant component of Ras regulation of cell cycle progression involves Ras stimulation of events that lead to the hyperphosphorylation and inactivation of the Rb tumor suppressor protein (15). Rb hyperphosphorylation then results in the release of bound E2F transcription factors, thus allowing E2F to stimulate the transcription of genes whose products promote G_1 progression (16). Although how Ras causes inactivation of the Rb pathway has been studied extensively, a clear understanding of this process remains to be determined.

The prevailing evidence implicates Ras regulation of both positive (cyclin D1) and negative ($p21^{CIP1/WAF1}$ and $p27^{KIP1}$) factors in causing Rb inactivation to promote G_1 progression. However, which Ras effector pathway regulates these cell cycle components and how their functions are then altered are not clearly understood. The lack of a coherent picture of Ras and cell cycle regulation is due, in part, to differences in experimen-

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[1] The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-4-phosphate kinase; CDK, cyclin-dependent kinase; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; Rb, retinoblastoma.

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tal analyses (transient *versus* stable Ras activation) and to cell type differences (fibroblasts *versus* epithelial cells) in how Ras functions. Additionally, although p21 and p27 can act as inhibitors of cyclin D-CDK complexes, they may also serve a positive role in G_1 progression by promoting cyclin D-CDK assembly and nuclear localization. Much of our knowledge has come from the study of fibroblast models. Thus, whether the links between Ras and the cell cycle in epithelial cells, the cell type from which the majority of *ras* mutation positive cancers arise, will show cell type differences is not clear.

The best characterized cell cycle target of Ras is cyclin D1. Cyclin D1, a major positive regulator of cell cycle progression, is up-regulated in response to both transient and sustained Ras activation in a variety of cell types (17–22). Increased cyclin D1 facilitates increased formation of active cyclin D1-cyclin-dependent kinase (CDK4 and CDK6) complexes that phosphorylate and inactivate Rb function. Ras up-regulation of cyclin D1 protein expression is mediated, in large part, by stimulation of transcription from the cyclin D1 promoter (19). To date, this up-regulation has been attributed mainly to Ras activation of the Raf/ERK pathway. However, a contribution of PI3K and RalGDS effector pathways has also been shown (23, 24).

The second most studied link between Ras and cell cycle regulation involves the regulation of expression of the $p21^{CIP1}$ CDK inhibitor. The levels of p21 are low during quiescence and tend to increase in response to mitogenic stimuli, particularly those that activate the Raf/MEK/ERK pathway (25, 26). However, the majority of observations suggest that p21 antagonizes Ras growth stimulation. Transient activation of low levels of Ras or Raf in NIH 3T3 cells did not induce p21 expression and caused a mitogenic response (27, 28). In contrast, high Ras or Raf induction caused an up-regulation of p21 and was growth inhibitory. Similarly, it was observed that microinjection of activated Ras alone into Swiss 3T3 mouse fibroblasts induced p21 and did not stimulate proliferation, whereas co-expression of activated RhoA prevented p21 induction and thereby allowed Ras to induce a proliferative response (29). Consistent with a role for p21 in antagonizing Ras function, loss of p21 but not p27 function in primary mouse keratinocytes caused increased susceptibility to transformation by oncogenic Ras (30). Finally, ectopic expression of p21 inhibited Ras transformation of NIH 3T3 cells (31). Thus, it is currently believed that oncogenic Ras down-regulation of p21 expression, via the Raf/ERK pathway, is required for Ras to cause growth transformation.

A link between Ras and a second CDK inhibitor $p27^{KIP1}$, where Ras causes down-regulation of p27 expression via the Raf/ERK pathway, has also been observed in a variety of cell types. p27 protein levels are regulated post-translationally and exhibit a pattern of expression that is opposite that of p21. p27 levels are elevated in quiescent cells and down-regulated in response to mitogenic stimuli via a Ras-dependent mechanism (32, 33). Microinjection of activated Ras caused transient down-regulation of p27 in Swiss 3T3 cells (29). Transient activation of Raf-1 or MEK1 activation caused down-regulation of p27 protein levels in NIH 3T3 cells (21, 34). Sustained expression of oncogenic Ras caused a down-regulation of p27 protein levels in CCL39 fibroblasts and IEC-6 intestinal epithelial cells that was dependent on the Raf/ERK pathway (35, 36). In contrast to these studies, p27 levels were unchanged in Ras-transformed NIH 3T3 cells (37), and induction of activated MEK did not cause down-regulation of p27 in NIH 3T3 cells (38). Thus, whether a down-regulation of p27 is required for Ras transformation and how Ras might regulate p27 expression are issues that are currently not clearly understood.

Previously, we reported that the Raf/ERK cascade alone, although necessary, was not sufficient for Ras to cause transformation of RIE-1 rat intestinal epithelial cells (39). Based on the importance of the Raf/ERK pathway in the deregulation of cell cycle components in rodent fibroblasts, the failure of Raf to cause transformation of RIE-1 cells suggests that activation of the Raf/ERK pathway alone may not be sufficient to inactivate the Rb pathway in RIE-1 cells. Alternatively, both Ras and Raf may cause Rb inactivation, but this alone is not sufficient, and Ras causes additional changes required to deregulate cell cycle progression. In the present study, we evaluated whether the contribution of the Raf/ERK pathway to Ras deregulation of cell cycle progression differs in NIH 3T3 fibroblasts and RIE-1 epithelial cells. Our results demonstrate cell type differences in Ras regulation of cell cycle progression and the importance of Raf-independent signaling pathways in Ras transformation of epithelial cells.

EXPERIMENTAL PROCEDURES

Molecular Constructs and Reagents—-Mammalian expression vectors containing cDNA sequences encoding constitutively activated mutants of Ras (Ha-Ras^{Leu-61}, K-$Ras4B^{Val-12}$, and N-Ras^{Asp-12}) or Raf-1 (the NH_2-terminal truncated and constitutively 22W mutant or the constitutively membrane-targeted Raf-C*AAX* mutant) were generated using pZIP-NeoSV(x)1 retrovirus vector with a neomycin resistance marker where expression of the inserted gene is regulated from the Moloney retrovirus long terminal repeat promoter and have been described previously (39–43). pCGN-hygro mammalian expression vectors encoding the highly transforming Raf-C*AAX* and Ha-Ras^{Leu-61} proteins were generated by subcloning each cDNA sequence into pCGN in frame with an NH_2-terminal hemagglutinin epitope tag where expression is under the control of the cytomegalovirus (CMV) promoter (39). The pBSTR1 mammalian expression vector encoding human cyclin D1 cDNA expression vector, as described previously (44), is under the control of a tetracycline-repressible CMV promoter and also contains a puromycin-selectable marker. Western blot analyses to verify expression were done using monoclonal antibodies against cyclin D1 (17–32G-11 and HD-11; Santa Cruz Biotechnology), polyclonal antibodies against an amino-terminal peptide of rat cyclin E (sc-481; Santa Cruz Biotechnology), rabbit polyclonal anti-Raf-1 (C-12; Santa Cruz Biotechnology), monoclonal or rabbit polyclonal antibodies against $p21^{WAF}$ (Ab4/5; Upstate Biotechnology, Inc.), and anti-Rb monoclonal antibody (14001A; PharMingen) which detects both active hypophosphorylated Rb and inactive hyperphosphorylated Rb.

Cell Culture and Transformation Assays—RIE-1 rat intestinal epithelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. NIH 3T3 cells were maintained in DMEM supplemented with 10% calf serum. The introduction of plasmid DNA constructs into NIH 3T3 cells was done by calcium phosphate precipitation (45), whereas RIE-1 cells were transfected using Superfect (Qiagen) according to the manufacturer's suggestions. To establish cell lines of NIH 3T3 or RIE-1 cells stably expressing activated Ras (Ha-Ras^{Leu-61}, N-Ras^{Asp-12}, or K-$Ras4B^{Val-12}$) or Raf-22W, transfected cultures were maintained in growth medium supplemented with 400 μg per ml of G418 (Geneticin; Life Technologies, Inc.), and multiple G418-resistant colonies (>50) were pooled together and used for analyses. Some analyses were also performed on RIE-1 cells stably infected with pZIP-NeoSV(x)1 retrovirus vector constructs expressing activated Ha-Ras^{Leu-61}, K-Ras^{Val-12},, N-Ras^{Asp-12}, or Raf-22W. The resulting cell lines were designated NIH(Ras) and NIH(Raf) or RIE(Ras) and RIE(Raf). The U0126 MEK1/2 (provided by J. Trzaskos; DuPont) and LY294002 PI3K (Sigma) inhibitors were dissolved in dimethyl sulfoxide (Me_2SO), and control cultures were treated with the equivalent final concentrations of Me_2SO (vehicle).

To determine if cyclin D1 overexpression, either alone or in cooperation with Raf-22W, could transform RIE-1 cells, co-transfection focus-formation assays were performed. Cells were co-transfected with 0.5 μg of empty pZIP-NeoSV(x)1, pZIP-K-*ras*(12V), or pZIP-*raf*-22W, either alone or together with 0.5 μg of pCMV-cyclin D1. The appearance of transformed foci was quantitated after 21 days.

Western Blot Analyses of Protein Expression—Asynchronous, exponentially growing subconfluent cultures of cells were harvested with lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 25 μg/ml leupeptin. Protein concentrations were determined using BCA protein assay kit (Pierce). After separation by SDS polyacrylamide gel electrophoresis

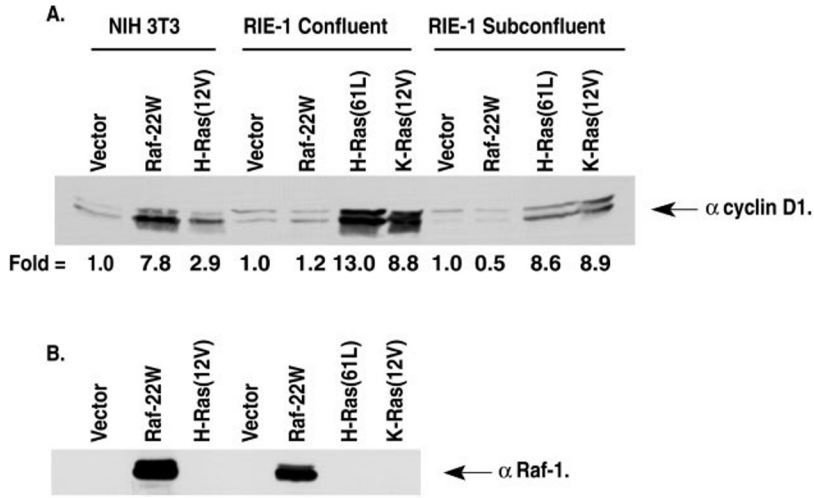

FIG. 1. **Ras, but not Raf, up-regulates steady state cyclin D1 protein levels.** *A*, equal numbers of RIE-1 cells stably transfected with pZIP-NeoSV(x)1 empty vector or encoding Raf-22W, Ha-Ras^{Leu-61}, or K-Ras^{Val-12} were plated and cultured 1–2 days before harvesting for analyses. Exponentially growing and confluent cells were harvested, and total protein concentrations were determined by BCA protein assay. The relative levels of cyclin D1 protein expression were determined by a Western blot of equal total cellular lysates using an anti-cyclin D1 antiserum. Lysates from both NIH 3T3 and RIE-1 stably expressing the indicated constructs were analyzed for comparison. *B,* protein expression of Raf-22W was analyzed in both NIH 3T3 and RIE-1 cells as stated in *A*.

(SDS-PAGE), the resolved proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h in 5% nonfat dry milk, probed with the appropriate antibody for 1–2 h at room temperature or overnight at 4 °C, washed, probed with the appropriate secondary antibody (Amersham Pharmacia Biotech) for 1 h, washed, and exposed to x-ray film.

Transient Expression Reporter Assays—For transient expression reporter assays, parental RIE-1 cells were transfected with Superfect or Effectene (Qiagen) as suggested by the manufacturer's protocols. Briefly, RIE-1 cells were seeded the day before transfection and transfected with cationic lipids for 24 h. The growth medium was then replaced with DMEM supplemented with 0.2% fetal bovine serum. Cells were serum-starved for 24 h and harvested with 300 μl of luciferase lysis buffer (PharMingen), and 50 μl of lysate was analyzed by using enhanced chemiluminescence reagents and a Monolight 2010 luminometer (Analytical Luminescence). To normalize fold activation of the reporter construct to protein levels, protein concentrations were determined by BCA protein assay (Pierce). The cyclin D1-luciferase construct consists of 963 base pairs of wild type sequences or sequences with point mutation altering the AP1 site of the human cyclin D1 promoter controlling the expression of the luciferase gene (19). The E2F-1-responsive luciferase promoter was provided by P. Farnham and has been described previously (46)

Immunoprecipitation and Kinase Assays—Cyclin D1-associated kinase or CDK4 assays were performed as described previously (47). Briefly, RIE-1 cells stably expressing the indicated DNAs were seeded 1–2 days before harvesting. Cyclin D1 or CDK4 were immunoprecipitated (17–32G and anti-mouse CDK4, gift from Y. Xiong, University of North Carolina, Chapel Hill), and the activity of the complex was determined using 0.5–2 μg of GST-Rb substrate. Reactions were stopped with 2× Laemmli sample buffer and were subjected to SDS-PAGE. The gel was then stained with Coomassie Blue to visualize protein substrates, destained, and subsequently dried for 1 h. Hyperfilm (Amersham Pharmacia Biotech) or a PhosphorImager screen was exposed for an appropriate amount of time, with radioactivity then measured using a PhosphorImager (Molecular Dynamics). For immunoblotting, cells were harvested as described above, and equal protein was subjected to SDS-PAGE. Cyclin E-associated kinase assays were performed as described previous (47). Briefly, cyclin E was immunoprecipitated (sc-481; Santa Cruz Biotechnology), and the activity of the complex was determined using 2 μg of histone H1 as substrate.

RESULTS

Oncogenic Ras Utilizes Raf-independent Pathways to Cause Up-regulation of Cyclin D1 in RIE-1 Epithelial Cells—It is well documented that oncogenic Ras causes up-regulation of cyclin D1, in large part, by activation of the Raf/MEK/ERK pathway (12–14). Considering these observations, we sought to determine the importance of Raf-dependent and Raf-independent pathways in the up-regulation of cyclin D1 in RIE-1 epithelial cells. Since we demonstrated previously that Ras, but not Raf, could transform RIE-1 epithelial cells (39), we postulated that the failure of activated Raf to transform RIE-1 cells may be due to its inability to deregulate cyclin D1 or other key components of the cell cycle machinery.

First, we compared the consequences of sustained Ras or Raf activation on cyclin D1 expression in NIH 3T3 and RIE-1 cells. Consistent with previous studies, we observed that NIH 3T3 cells stably expressing activated and transforming mutants of Raf-1 or Ras (designated NIH(Raf) or NIH(Ras), respectively) showed elevated steady state levels of cyclin D1 protein (Fig. 1*A*). In contrast, only RIE-1 cells stably expressing activated Ras (RIE(Ras)), and not Raf (RIE(Raf)), showed up-regulated levels of cyclin D1 protein when assayed in either exponentially growing or confluent cultures. Since RIE(Raf) cells are morphologically indistinguishable from those expressing the empty vector, we verified that the activated Raf-22W protein was stably expressed in RIE(Raf) cells and that it caused stable up-regulation of ERK activity (Fig. 6*C*). These observations show that activation of a Raf-independent pathway(s) is required for Ras to cause cyclin D1 up-regulation in RIE-1 epithelial cells.

Next, we determined whether this increase in cyclin D1 protein levels corresponded to differences in the ability of Raf and Ras to stimulate transcription from the cyclin D1 promoter. For these analyses, transient expression analyses were performed on parental RIE-1 cells transiently transfected with expression plasmids encoding two different activated versions of Raf-1 (Raf-22W or Raf-C*AAX*) or Ras (Ha-Ras^{Leu-61} or K-Ras^{Val-12}) together with a reporter plasmid where the luciferase gene is under the control of the human cyclin D1 promoter. When analyzed in NIH 3T3 cells, we observed that both activated Raf and Ras caused comparable activation of the promoter (data not shown). However, Ras but not Raf, stimulated cyclin D1 transcription when assayed in RIE-1 cells (Fig. 2). Additionally, Northern blot analyses determined that the steady state levels of endogenous cyclin D1 mRNA were elevated in RIE(Ras) but not RIE(Raf) cells (data not shown). These results suggest that the Ras-mediated increase in cyclin D1 protein is due, in part, to increased transcription from the cyclin D1 promoter via stimulation of Raf-independent pathways.

Oncogenic Ras Causes Increases in Cyclin D1-associated Kinase Activity and Rb Hyperphosphorylation—Next, we determined if the elevated levels of cyclin D1 protein observed in Ras-transformed RIE-1 cells corresponded to increased cyclin D1-associated kinase activity. *In vitro* kinase assays were performed on immunoprecipitated cyclin D1 and showed that there was an increase in cyclin D1-associated kinase activity in RIE(Ras) but not RIE(Raf) cells (Fig. 3*A*). We also determined

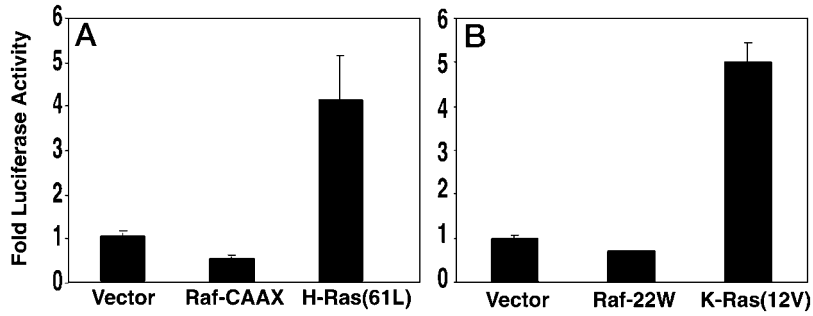

FIG. 2. **Ras, but not Raf, up-regulates transcription from the cyclin D1 promoter.** RIE-1 cells were transiently co-transfected with the cyclin D1-Luciferase reporter plasmid, together with either the empty pCGN-hygro expression vector (*A*) or pCGN-hygro encoding activated Raf-C*AAX* or Ha-Ras^{Leu-61}, or the empty pZIP-Neo(x)1 expression vector or pZIP-NeoSV(x)1 encoding activated Raf-22W or K-Ras^{Val-12} (*B*). DNA was transfected via incubation with cationic lipids for 24 h followed by incubation in complete medium for 24 h and a subsequent starvation for 12–16 h. Fold luciferase activity was normalized by total protein analyzed.

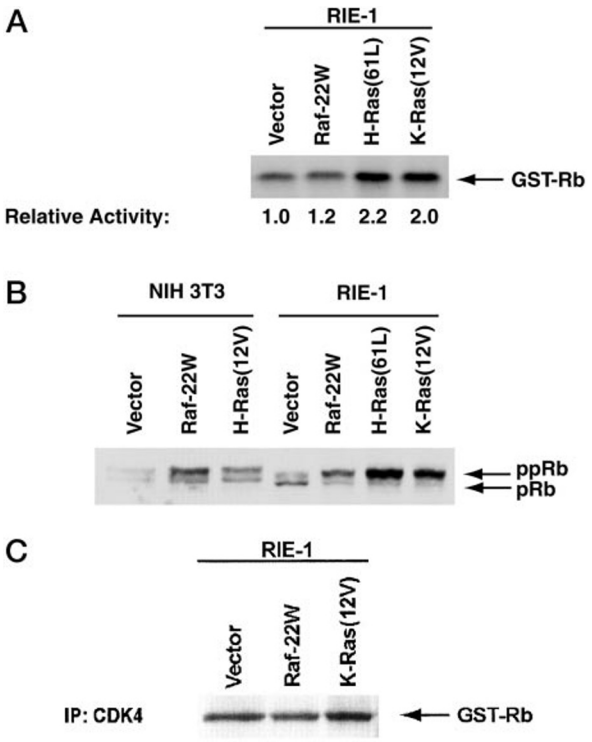

FIG. 3. **Ras utilizes Raf-independent pathways to up-regulate cyclin-associated kinase activity in RIE-1 cells.** *A,* exponentially growing cells stably expressing each DNA were harvested, and cyclin D1-associated kinase assays were performed. Cyclin D1 was immunoprecipitated, and phosphorylation of recombinant GST-Rb fusion protein was measured by an *in vitro* kinase assay, and phosphorylation was quantitated using PhosphorImager analyses. *B,* Ras utilizes Raf-independent pathways to up-regulate cyclin E-associated kinase activity. Exponentially growing RIE-1 cells stably expressing the indicated protein were harvested, and cyclin E-associated kinase activity was determined by immunoprecipitation (*IP*) of cyclin E, and phosphorylation of histone H1 was determined in an *in vitro* kinase assay. Recombinant Sf9 insect cell-expressed cyclin-E/CDK2 was assayed in parallel as an internal control for the *in vitro* kinase assay. Ras utilizes Raf-independent pathways to up-regulate cyclin E-associated kinase activity. *C,* Ras, but not Raf, activation causes an increase in CDK4 kinase activity. Exponentially growing RIE-1 cells stably expressing the indicated protein were harvested, and CDK4 was immunoprecipitated, and phosphorylation of recombinant GST-Rb fusion protein was determined in an *in vitro* assay. Data shown are representative of at least three independent experiments.

that cyclin E-associated kinase activity (Fig. 3*B*), as well as CDK4 activity (due to cyclin D1 and D2 association; Fig. 3*C*) were elevated in RIE(Ras) but not RIE(Raf) cells. Thus, Ras activation of CDK activity important for G_1 progression was critically dependent on activation of Raf-independent signaling.

We then determined if this corresponded to an increased hyperphosphorylation and inactivation of Rb. By using an antibody that recognizes both hypo- and hyperphosphorylated Rb, Western blot analyses showed that both NIH(Ras) and NIH(Raf) cells showed elevated levels of hyperphosphorylated Rb protein. Although RIE(Raf) cells showed elevated Rb hyperphosphorylation over empty vector transfected cells, it was lower than that observed for Rb in RIE(Ras) cells (Fig. 4*A*). This suggested that Raf may cause a partial inhibition of Rb function and, consequently, lead to activation of E2F-mediated transcription. To address this possibility, we evaluated the ability of Raf and Ras to activate E2F-responsive gene expression. Whereas both Ras and Raf caused activation of transcription from an E2F-responsive reporter plasmid when assayed in NIH 3T3 cells, only Ras activated transcription when assayed in RIE-1 cells (Fig. 4*B*). Thus, the limited ability of Raf to cause Rb hyperphosphorylation in RIE-1 cells was not sufficient to lead to activation of E2F-mediated transcription.

The inability to cause up-regulation of cyclin D1 may contribute to the inability of Raf to cause transformation of RIE-1 cells. To address this possibility we forced overexpression of cyclin D1, together with activated Raf-22W, to determine if this could cause transformation of RIE-1 cells. Two approaches were employed to accomplish this. First, we performed focus formation assays in RIE-1 cells to determine whether co-expression of exogenous cyclin D1 together with Raf-22W was sufficient to cause focus formation in RIE-1 cells. We found that forced expression of cyclin D1 alone or together with activated Raf-22W did not result in focus-forming activity in transfected RIE-1 cells (Fig. 5*A*). Second, we established RIE-1 cells that stably overexpressed both cyclin D1 and Raf-22W to determine if cyclin D1 and Raf-22W overexpression could cooperate and cause morphologic transformation. Western blot analyses of the resulting cells confirmed the expression of activated Raf and the overexpression of cyclin D1 at levels comparable to that seen in RIE(Ras) cells (Fig. 5*B*). However, simultaneous expression of both cyclin D1 and Raf was not sufficient to cause morphologic or growth transformation of RIE-1 cells. We conclude that the inability of Raf to up-regulate cyclin D1 alone does not account for its inability to cause transformation of RIE-1 cells.

Both Raf and PI3K Effector Pathways Are Important for Cyclin D1 Up-regulation—In contrast to NIH 3T3 cells, our analyses showed that Ras up-regulation of cyclin D1 is dependent on the activity of Raf-independent pathways. To evaluate the nature of these pathways, we employed three experimental approaches. First, we utilized pharmacologic inhibitors of MEK and PI3K to assess the importance of the Raf and PI3K effector pathways in causing up-regulation of cyclin D1. Strikingly, we found that the treatment of RIE(Ras) cells with the MEK inhibitor (U01026), but not the PI3K inhibitor (LY294002), caused a reversion of the transformed morphology of RIE(Ras) cells (Fig. 6*A*). However, treatment with either U01026 or

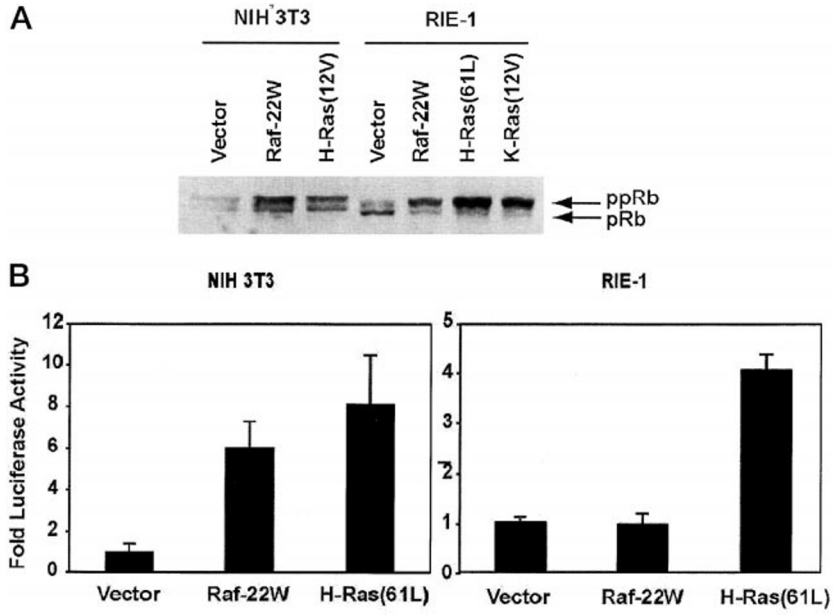

FIG. 4. **Ras and Raf differentially regulated Rb hyperphosphorylation and activation of E2F-responsive gene expression.** *A,* exponentially growing cells stably expressing each DNA were harvested, and equal total protein was analyzed by Western blotting to detect changes in Rb phosphorylation. *B,* Ras, but not Raf, causes up-regulation of transcription from the E2F-responsive reporter plasmid in RIE-1 cells. NIH 3T3 or RIE-1 cells were transiently transfected with pAX142 expression vectors encoding activated Ha-Ras^{Leu-61} or Raf-22W, together with a reporter plasmid where the luciferase gene is under control of the mouse E2F1 (−176 to +36) promoter. Whereas both Ras and Raf stimulated expression in NIH 3T3 cells, only Ras stimulated expression in RIE-1 cells. Data shown are representative of at least three independent assays.

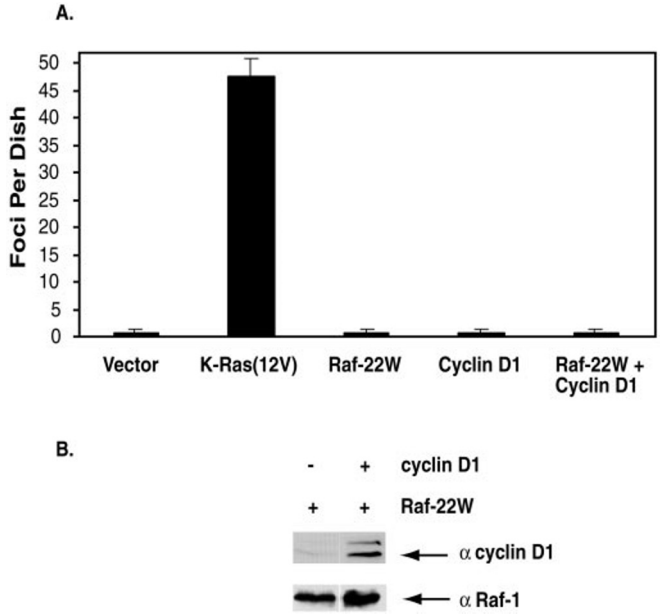

FIG. 5. **Cyclin D1 alone or in combination with oncogenic Raf is not sufficient for transformation of RIE-1 cells.** *A,* 500 ng of Raf-22W, cyclin D1 or K-Ras^{Val-12} (or 500 ng of each for cooperation assays) was transfected into RIE-1 cells, and the total number of foci was scored after 21 days. The number of foci of transformed cells represents the average of three dishes. Data shown are representative of three independent experiments and are the average of triplicate plates for each condition. *B,* exponentially growing RIE-1 cells stably expressing Raf-22W and cyclin D1 were harvested, and total protein concentrations were determined by BCA protein assay. The relative levels of cyclin D1 protein and Raf-22W expression were determined by a Western blot of equal amounts of total cellular lysates.

LY294002 inhibitor treatment caused a reduction in cyclin D1 protein expression to levels that were less than, or similar to, the low level seen in untransformed RIE-1 cells (Fig. 6*B*). Moreover, measurement of phospho-ERK levels indicated that the reduction in cyclin D1 levels associated with the inhibition of PI3K was not due to nonspecific LY294002 inhibition of ERK activation (Fig. 6*C*). These results suggest that both Raf and PI3K effector pathways are important for up-regulation of cyclin D1 in RIE-1 cells by oncogenic Ras and are consistent with recent studies implicating PI3K in stimulation of cyclin D1 levels in rodent fibroblasts (23, 24, 48). Additionally, since LY294002 treatment reduced cyclin D1 levels and reduced rates of proliferation (data not shown), but did not cause a reversion of morphologic transformation, up-regulation of cyclin D1 appears to be required to maintain only some, but not all, aspects of the transformed state. This observation is consistent with studies with Ras-transformed IEC-18 rat intestinal epithelial cells, where antisense down-regulation of cyclin D1 did cause a reduction in cell proliferation (17).

We next utilized effector domain mutants of Ha-Ras^{Val-12} to assess the contribution of the Raf and PI3K effector pathways to Ras up-regulation of cyclin D1 expression (6, 9–11). The Ha-Ras$^{Val-12/Ser-35}$ effector domain mutant retains the ability to interact with Raf, but not PI3K or RalGDS. The Ha-Ras$^{Val-12/Cys-40}$ effector domain mutant retains the ability to interact with PI3K but not Raf or RalGDS. We showed previously that both effector domain mutants could cause limited growth transformation of RIE-1 cells, although at reduced potencies when compared with Ha-Ras^{Val-12} (49). We established RIE-1 cells stably expressing the parental and mutant Ha-Ras^{Val-12} proteins and verified that each protein was expressed at comparable levels by Western blot analysis (data not shown). Each mutant caused partial increases in cyclin D1 and Rb hyperphosphorylation that were less than the increases caused by Ha-Ras^{Val-12} (Figs. 7, *A* and *B*). These results, when taken together with the inhibitor analyses described above, suggest that Ras up-regulation of cyclin D1 expression is dependent on the coordinate activation of Raf and PI3K pathways. Finally, the ability of Ha-Ras$^{Val-12/Ser-35}$, but not activated Raf-22W, to cause a limited up-regulation of cyclin D1 may be due to its ability to bind to other Ras effectors, including AF-6 (10).

We also used transient expression analyses of cyclin D1 promoter reporter plasmids as a third approach to evaluate the Ras signaling pathways important for up-regulation of cyclin D1. Cyclin D1 promoter constructs containing either the wild type sequence or with a point mutation in an AP-1 site were used for these analyses (19). We found that the AP-1 site was dispensable for Ras-mediated stimulation of the cyclin D1 expression in NIH 3T3 cells but was essential for Ras-mediated stimulation in RIE-1 cells (Fig. 8). These results suggest that Ras activation of AP-1, via a Raf-independent pathway, is important for up-regulation of cyclin D1 expression in RIE-1 cells.

Up-regulation of p21 Is Associated with Ras Transformation of Fibroblasts and Epithelial Cells—A number of studies sug-

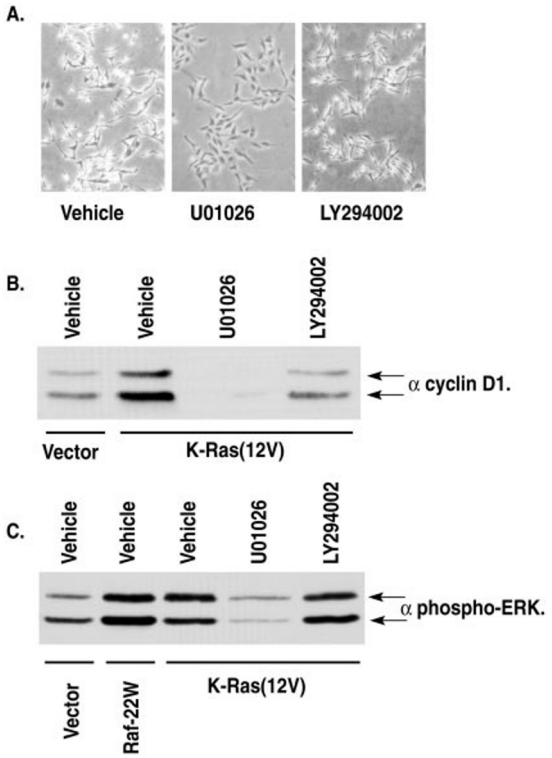

FIG. 6. **Inhibition of ERK activation causes morphological reversion of RIE(Ras) cells and decreases cyclin D1 levels.** *A,* cells stably expressing the K-Ras^{Val-12} were seeded, allowed to adhere, and each inhibitor or Me_2SO control was added to the growth medium. Concentrations of pharmacological inhibitors used were 10 μM for MEK inhibitor (*U0126*) and 20 μM for the PI3K inhibitor (*LY294002*). Photos were taken after 48-h treatments. *B* and *C,* RIE-1 cells treated with vehicle or drugs were harvested, and total protein concentrations were determined by BCA protein assay. The relative levels of cyclin D1 protein (*B*) and phospho-ERK (*C*) expression were determined by a Western blot of equal total cellular lysates.

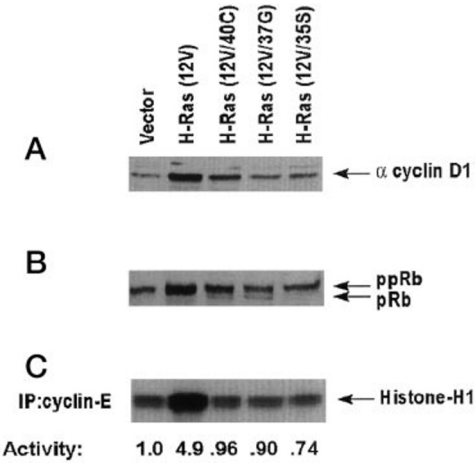

FIG. 7. **Individual Ras effector mutants do not fully up-regulate cyclin D1 protein levels and Rb hyperphosphorylation.** Mass populations of RIE-1 cells stably expressing Ha-Ras^{Val-12}, or the Ser-35, Gly-37, or Cys-40 effector domain mutants of Ha-Ras^{Val-12}, were harvested, and equivalent amounts of total protein were analyzed by Western blotting to detect cyclin D1 protein levels (*A*), Rb hyperphosphorylation (*B*), and cyclin E-associated kinase activity (*C*), by *in vitro* kinase analyses using recombinant histone H1 as a substrate. *IP*, immunoprecipitation.

gested that p21 antagonizes the ability of Ras or Raf to stimulate cellular proliferation (12–14). However, since these studies evaluated the relationship between p21 expression and transient Ras or Raf activation, the role of p21 has in the maintenance of Ras-induced transformation remained unresolved. Additionally, we wanted to determine if Ras and Raf regulated p21 expression similarly in NIH 3T3 and RIE-1 cells.

We used Western blot analyses to evaluate the p21 levels in NIH 3T3 and RIE-1 cells stably expressing activated Ras or Raf. Unexpectedly, we found that NIH(Ras), and to a lesser degree NIH(Raf), cells showed greatly up-regulated levels of p21 protein (Fig. 9*A*). This result contrasts with transient expression studies that indicated that high p21 expression antagonized Ras and Raf induction of cellular proliferation (27, 28). We also found that activated versions of all three Ras proteins caused up-regulation of steady state p21 protein levels in RIE-1 cells, whereas only a very limited increase was seen in RIE(Raf) cells. These observations suggest that up-regulation, rather than down-regulation, of p21 may contribute to Ras transformation of NIH 3T3 and RIE-1 cells.

The limited elevation of p21 seen in RIE(Raf) cells suggested that Ras requires activation of multiple effector pathways to cause up-regulation of p21 expression. We found that treatment with the U0126 or PD98059 MEK inhibitors completely abolished p21 up-regulation in RIE(Ras) cells (Fig. 9*B*; data not shown), suggesting a necessary role of the Raf/ERK pathway in Ras regulation of p21. In contrast, the limited ability of treatment with LY294002 to reduce p21 levels suggests a minor role of the PI3K effector pathway in regulating p21 expression. Consistent with these observations, we found that RIE-1 cells stably expressing the Ha-Ras$^{Val-12/Ser-35}$ effector domain mutant showed a greater increase in p21 than was seen in cells expressing the Ha-Ras$^{Vas-12/Cys-40}$ and Ha-Ras$^{Val-12/Gly-37}$ mutants (Fig. 9*C*; and data not shown). Thus, Ras activation of Raf is important for up-regulation of p21 in both NIH 3T3 and RIE-1 cells. However, additional effector pathways are important for Ras to up-regulate p21 in RIE-1 cells.

Ras Causes Opposing Changes in p27 Expression in NIH 3T3 and RIE-1 Cells—A clear understanding of Ras regulation of p27 expression and the role of p27 in mediating Ras transformation have not been established. However, several lines of evidence suggest that Ras down-regulation of p27 protein levels, via activation of the Raf/ERK pathways, may contribute to Ras stimulation of G_1 progression (12–14). Since many of these studies assessed the consequences of transient activation of Ras, we compared the consequences of sustained Ras and Raf activation on NIH 3T3 and RIE-1 cells on p27 protein levels.

As expected, p27 protein levels were low in subconfluent and proliferating cultures of untransformed NIH 3T3 cells (Fig. 10*A*). Surprisingly, we found that p27 protein levels were greatly elevated in both NIH(Raf) and NIH(Ras) cells, suggesting that p27 up-regulation may facilitate both Ras and Raf transformation of NIH 3T3 cells. In contrast, we found that p27 levels were high in untransformed RIE-1 cells, unchanged in RIE(Raf) cells, and reduced in RIE(Ras) cells. Thus, Ras down-regulation of p27 protein expression, via Raf-independent pathways, may contribute to Ras transformation of RIE-1 cells. Since treatment of RIE(Ras) cells with either U0126 or LY294002 did not restore p27 levels to that seen in control, vector-transfected cells, it appears that Ras down-regulation of p27 expression occurs via activation of Raf- and PI3K-independent pathways (Fig. 10*B*).

DISCUSSION

A clear understanding of what Ras-regulated cell cycle events cause growth transformation remains to be achieved, in part, due to possible cell type differences and to different consequences of transient *versus* sustained activation of Ras (12–14). In the present study we have addressed cell type differences and compared the consequences of sustained Ras activation in NIH 3T3 mouse fibroblasts and RIE-1 rat intes-

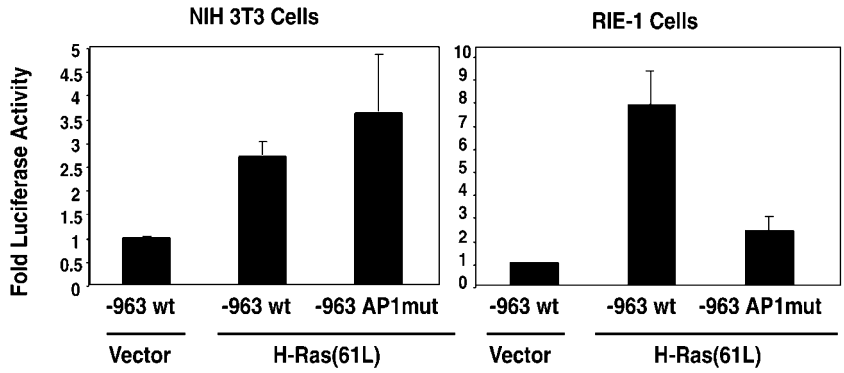

FIG. 8. **AP1 site is important for Ras-mediated stimulation of transcription from the cyclin D1 promoter in RIE-1 but not NIH 3T3 cells.** RIE-1 cells were transiently transfected with the wild type (*wt*) cyclin D1-Luciferase reporter plasmid or the −963 promoter with a point mutation in the AP1 site and activating constructs consisting of pAX142 empty vector or pAX142 encoding Ha-Ras^{Leu-61}. DNA was transfected via incubation with cationic lipids for 24 h followed by incubation in complete medium for 24 h and a subsequent starvation for 12–16 h. Luciferase activity was determined and converted to fold activation relative to the activity detected with the empty vector transfected control.

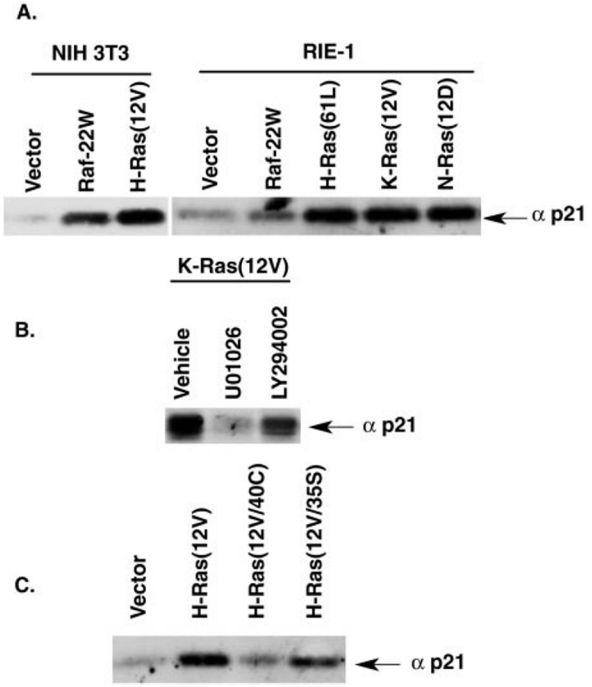

FIG. 9. **Oncogenic Ras-mediated up-regulation of p21 is Raf-dependent.** *A,* exponentially growing cells stably expressing the indicated constructs were harvested, and equal total protein was analyzed by Western blotting to detect changes in p21 levels. *B,* concentrations of pharmacological inhibitors used were 10 μM for MEK inhibitor (*U0126*) and 20 μM for the PI3K inhibitor (*LY294002*). *C,* exponentially growing cells stably expressing either pDCR Ha-Ras^{Val-12}, Ha-Ras$^{Val-12/Cys-40}$, or Ha-Ras$^{Val-12/Ser-35}$ were harvested, and equal total protein was analyzed by Western blotting to detect changes in p21.

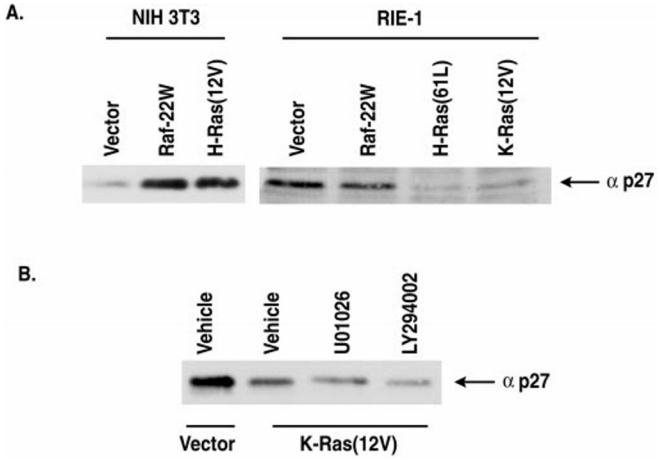

FIG. 10. **Activation of the Raf/ERK pathway does not down-regulate p27 expression.** *A,* exponentially growing cells stably expressing each DNA were harvested, and equal total protein was analyzed by Western blot analyses to detect changes in p21 or p27 levels. *B,* RIE-1 cells stably expressing K-Ras^{Val-12} were seeded and allowed to adhere, and each inhibitor or Me_2SO control was added to the growth medium. Concentrations of inhibitors used were 10 μM for MEK inhibitor (*U0126*) and 20 μM for the PI3K inhibitor (*LY294002*).

tinal epithelial cells. Ras causes transformation of NIH 3T3 cells by activation of either Raf-dependent or Raf-independent pathways, whereas Raf-dependent pathways are necessary but not sufficient to cause transformation of RIE-1 cells. Therefore, we also assessed the contribution of Raf-dependent and Raf-independent effector pathways in regulating the expression of cyclin D1, p21, and p27. Several novel and unexpected conclusions were reached. First, the up-regulation of p21 seen in Ras-transformed NIH 3T3 and RIE-1 cells contrasts with previous transient expression analyses and instead supports a positive role for p21 in mediating Ras transformation of NIH 3T3 and RIE-1 cells. Second, whereas up-regulation of p27 may promote Ras transformation of NIH 3T3 cells, down-regulation may promote Ras transformation of RIE-1 cells. Finally, whereas Raf activation is sufficient to facilitate Ras regulation of cyclin D1, p21, and p27 in NIH 3T3 cells, Raf-independent pathways, including PI3K-dependent activity, are critical for Ras regulation of these cell cycle components in RIE-1 cells. When taken together, our observations emphasize the importance of cell type differences, and different consequences of transient *versus* sustained Ras activation, in how Ras causes deregulation of cell cycle progression.

We first assessed the ability of Ras to cause up-regulation of cyclin D1 in NIH 3T3 and RIE-1 cells. In agreement with previous reports (27, 28, 50), we found that activation of Raf alone was sufficient to increase steady state levels of cyclin D1 protein levels in NIH 3T3 cells. In contrast, our analyses implicated both Raf and PI3K as important effectors for Ras up-regulation of cyclin D1 in RIE-1 cells. Transient expression reporter analyses, as well as Northern blot analyses of stably transfected cells, determined that Ras up-regulation of cyclin D1 in RIE-1 cells occurred at the level of gene transcription. Recent studies have implicated PI3K as an important regulator of cyclin D1 protein expression, by promoting increased gene transcription or by decreasing protein degradation (23, 24). Finally, we found that forced overexpression of cyclin D1 in Raf-expressing RIE-1 cells was not sufficient to allow Raf to cause transformation of RIE-1 cells. Thus, Ras must cause other changes, in addition to the up-regulation of cyclin D1 expression, to cause inactivation of Rb and to promote growth transformation of RIE-1 cells.

We next evaluated the consequences of Ras transformation on p21 expression in NIH 3T3 and RIE-1 cells. Although a previous study found increased p21 levels in Ras-transformed NIH 3T3 cells (37), the majority of studies suggested that p21

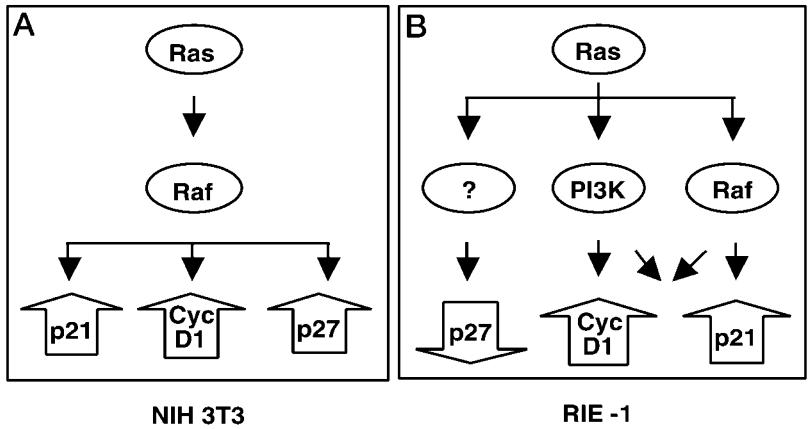

FIG. 11. **Ras regulation of cyclin D1, p21, and p27 by distinct signaling pathways in NIH 3T3 and RIE-1 cells.** *A,* NIH 3T3 cells stably expressing activated Ras and Raf show increased steady state levels of cyclin D1 (*Cyc D1*), p21, and p27. Consistent with previous observations, we found that Ras activation of the Raf/ERK pathways alone was sufficient to regulate the expression of cyclin D1, p21, and p27. However, in contrast to other studies, we found that sustained up-regulation of p21 and p27 was associated with Ras transformation of NIH 3T3 cells. *B,* RIE-1 cells stably expressing activated Ras showed increased steady state levels of cyclin D1 and p21, but down-regulation of p27, protein levels. RIE-1 cells stably expressing activated Raf showed a limited increase in steady state levels of p21 protein but no change in cyclin D1 or p27 protein levels. However, since U0126 inhibition of ERKs reduced cyclin D1 and p21 protein levels in Ras-transformed RIE-1 cells, the Raf/ERK pathway is necessary, but not sufficient, to regulate cyclin D1 and p21 expression. The partial down-regulation of cyclin D1 and p21 seen when Ras-transformed RIE-1 cells are treated with LY294002 supports a role for the PI3K effector pathway in cyclin D1 and p21 regulation. Finally, since neither U0126 nor LY294002 treatment restored p27 expression levels in Ras-transformed RIE-1 cells, Ras must down-regulate p27 expression by Raf- and PI3K-independent effector pathways.

expression antagonized the proliferative activity of Ras (12–14). Therefore, we were surprised to find that the steady state levels of p21 were up-regulated in both Ras-transformed NIH 3T3 and RIE-1 cells. These opposing observations may reflect the different consequences of transient *versus* stable and sustained Ras activity. Since our goal was to determine how p21 may support the transformed growth state caused by sustained Ras activation, our results argue that up-regulation of p21 may facilitate the inactivation of Rb to promote Ras transformation. The stoichiometry of p21 and other components can influence whether p21 serves a negative or positive regulatory role in G_1 progression. The up-regulation of p21 seen in Ras-transformed NIH 3T3 and RIE-1 cells may serve a positive regulatory role and facilitate activation of cyclin D1-CDK complexes. Such a role has been observed in other studies where p21 is found complexed with active cyclin D-CDK complexes (38, 51–53). In contrast to our observations, it was found that the loss of p21 function allowed Ras to cause transformation of mouse keratinocytes (30). Therefore, p21 may exhibit cell type distinct roles in either facilitating or antagonizing Ras transformation.

The significant up-regulation of p21 in NIH(Raf) cells suggests that Ras causes up-regulation of p21 primarily by activation of Raf in NIH 3T3 cells. This is consistent with previous studies that showed that Raf activation alone can cause p21 up-regulation in fibroblasts. In contrast, RIE(Raf) cells showed only limited up-regulation of p21 levels. Treatment of RIE(Ras) cells with LY294002 caused a modest reduction in p21, whereas U0126 treatment reduced the level of p21 to that seen in untransformed RIE-1 cells. Thus, Ras activation of Raf is necessary, but not sufficient, to cause up-regulation of p21. Instead, the coordinate activation Raf and PI3K (and other) effector pathways may be required for Ras up-regulation of p21 protein expression in RIE-1 cells. Finally, it remains possible that the up-regulation of p21 in Ras-transformed cells may not be a direct consequence of Ras activation and, instead, may reflect a compensatory response of cells to Ras-induced growth transformation.

We also evaluated the consequences of Ras transformation on a second cell cycle inhibitor p27. Although p27 expression is correlated strongly with growth inhibition, a clear relationship between Ras transformation and p27 expression has not been established. Ras transformation of some cell lines resulted in down-regulation of p27 expression (*e.g.* Rat 6 fibroblasts, CCL39 hamster fibroblasts, and IEC-6 rat intestinal epithelial cells) but no change in others (NIH 3T3 cells) (37). In our analyses we found that p27 protein levels were stably up-regulated in Ras-transformed NIH 3T3 cells but down-regulated in Ras-transformed RIE-1 cells. The opposing consequences of sustained Ras activity on p27 expression suggest different roles of p27 in Ras transformation of these two cell types. The increased expression of p27 in NIH(Ras) cells suggests that p27 may serve a positive regulatory role, perhaps by promoting the assembly and nuclear translocation of active cyclin D1-CDK complexes (38, 54, 55). In contrast, a reduction of p27 function appears to be important for Ras transformation of RIE-1 cells. The down-regulation of p27 in intestinal epithelial cell transformation is consistent with the decreased levels of p27 seen in a variety of epithelial cell-derived human cancers (16). p27 can act as an inhibitor of cyclin E-dependent kinase activity. Consistent with this role, we found that both cyclin D- and cyclin E-dependent kinase activities were increased in Ras-transformed RIE-1 cells.

p27 protein levels were elevated in both Ras- and Raf-transformed NIH 3T3 cells, suggesting that Ras up-regulation of p27 in fibroblasts is mediated primarily through the Raf effector signaling pathway (21, 27, 28). However, how Ras causes down-regulation of p27 levels in RIE-1 cells is not clear. RIE(Raf) cells showed levels of p27 that were similar to the high levels seen in untransformed RIE-1 cells, and neither U0126 nor LY294002 treatment of RIE(Ras) cells was able to restore p27 protein levels to that seen in untransformed RIE-1 cells. Thus, this decrease in p27 levels does not appear to be due to Ras activation of Raf or PI3K. Other possible mechanisms for Ras-mediated down-regulation of p27 may involve an increase in activation of cyclin E-CDK2 complexes, which have been shown to phosphorylate p27 and initiate its targeting for degradation. RhoA has also been implicated in plate-derived growth factor-induced p27 degradation through regulation of cyclin E/CDK2 (56, 57). Thus, like cyclin D1 and p21, Ras regulation of p27 expression is mediated by distinct signaling pathways in NIH 3T3 and RIE-1 cells.

In summary, we have found that Raf-dependent and Raf-

independent signaling have distinct contributions to Ras regulation of cyclin D1, p21, and p27 expression in NIH 3T3 *versus* RIE-1 cells (Fig. 11). Furthermore, p27 may have opposing roles in the promotion of cell cycle progression in NIH 3T3 and RIE-1 cells. Our observations also emphasize that transient and sustained Ras activation may have different consequences on the expression and function of cell cycle inhibitors. An important strength of transient expression analyses is the ability to make functional connections between Ras activation and specific components of cell cycle regulation. In contrast, the importance of defining the consequences due to sustained Ras activation is that this determines the changes that may be involved in maintenance of the transformed state. These changes will reflect the net consequences of Ras activation, together with subsequent actions in response to these changes, and finally, changes that occur in response to altered cell growth. Thus, our analyses of the consequences of sustained Ras activation in RIE-1 cells may provide a more accurate view of what changes in cell cycle regulation are important for aberrant Ras transformation of human carcinoma cells, and hence, how specific cell cycle regulators may be targeted for cancer treatment.

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