TC21 (R-Ras2), a Ras-related GTPase with transforming potential similar to H-, K- and N-Ras, is implicated in the pathogenesis of human cancers. Transforming growth factor β (TGF-β), a cytokine that plays a significant role in modulating tumorigenesis, normally prevents uncontrolled cell proliferation but paradoxically induces proliferation in H-Ras-transformed cancer cells. Although TC21 activates some pathways that mediate cellular transformation by the classical Ras proteins, the mechanisms through which TC21 induces tumor formation and how TGF-β regulates TC21 transformed cells is not known. To better understand the role of TC21 in cancer progression, we overexpressed an activated G23V mutant of TC21 in a nontumorigenic murine mammary epithelial (EpH4) cell line. Mutant TC21-expressing cells were significantly more oncogenic than cells expressing activated G12V H-Ras both in vivo and in vitro. TC21-induced transformation and proliferation required activation of p38 MAPK, mTOR (the mammalian target of rapamycin), and phosphoinositide 3-kinase but not Akt/PKB. Transformation by TC21 rendered EpH4 cells insensitive to the growth inhibitory effects of TGF-β, and the soft agar growth of these cells was increased upon TGF-β stimulation. Despite losing responsiveness to TGF-β-mediated growth inhibition, both Smad-dependent and independent pathways remained intact in TC21-transformed cells. Thus, overexpression of active TC21 in EpH4 cells induces tumorigenicity through the phosphoinositide 3-kinase, p38 MAPK, and mTOR pathways, and these cells lose their sensitivity to the normal growth inhibitory role of TGF-β.

Ras proteins are signal switch molecules that regulate cell function by cycling between active GTP-bound and inactive GDP-bound conformations. The R-Ras family of Ras-related proteins, including R-Ras, TC21(R-Ras2), and M-Ras(R-Ras3), shares 55% amino acid identity with H-Ras including identical core effector regions (1). Aside from H-, K-, and N-Ras, TC21 is the only Ras superfamily member known to transform epithelial and fibroblast cell lines (2) and induce tumor formation in vivo (3). Increased TC21 expression is observed in breast cancer cells (4), and TC21 mutations are present in cells derived from uterine sarcoma, ovarian, and mammary tumors (5, 6). TC21 is also up-regulated in oral and esophageal carcinomas, suggesting a correlation between TC21 expression and the early stages of tumorigenesis (7, 8).

Downstream effectors of TC21 include three members of the mitogen-activated protein kinas (MAPKs), namely Erk1/2, c-Jun N-terminal kinase, and p38 MAPK as well as phosphoinositide 3-kinase (PI3K) (2, 9). Of these, only PI3K, which phosphorylates phosphoinositides to generate the second messenger lipid phosphatidylinositol 1,4,5-trisphosphate, is required for TC21-induced tumorigenesis. The serine/threonine kinase, Akt, a key target of phosphatidylinositol 1,4,5-trisphosphate, is activated by TC21 (2), resulting in increased cell proliferation, transformation, and survival through numerous effectors, including Bad, GSK-3β, and mTOR. Additional targets of phosphatidylinositol 1,4,5-trisphosphate include protein kinase C, phospholipase Cγ, and exchange factors for Rac, Rho, and Ras GTPases (10).

Transforming growth factor-β (TGF-β) regulates numerous processes including cell proliferation, differentiation, and apoptosis (11). Normal epithelial and differentiated carcinoma cells are generally growth-inhibited by TGF-β, but dedifferentiated or Ras-transformed cells often grow increasingly malignant following TGF-β treatment. TGF-β signals via a heterotetrameric signaling complex comprising the type I and type II TGF-β receptors. Smad2/3 are phosphorylated by the type I receptor and translocate along with Smad4 to the nucleus to activate gene transcription leading to G1/S cell cycle arrest. TGF-β can also activate a number of Smad-independent pathways (12). Whereas H-Ras can cooperate with TGF-β to induce proliferation, invasion, and metastasis (13, 14), it is unknown whether similar interactions occur between TC21 and TGF-β signaling.

To examine how TC21 induces tumorigenesis, we transformed a nonmalignant murine breast line (EpH4) with activated H-Ras(G12V) or TC21(G23V) mutants. We demonstrate that G23V TC21 is significantly more oncogenic than G12V H-Ras both in vivo and in vitro and that TC21-induced proliferation and tumorigenesis was due to activation of p38 MAPK, mTOR, and PI3K, but independent of Akt. In contrast to H-Ras, TC21 alters the cellular response to TGF-β from growth arrest...
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to increased proliferation via a Smad-independent pathway. Thus, expression of TC21 is sufficient for cells to lose their responsiveness to the growth inhibitory effects of TGF-β.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Phoenix 293 cells were provided by Dr. Gary Nolan (Stanford University, Stanford, CA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Murine EpH4 cells were obtained from Dr. Carlos Arteaga (Vanderbilt University, Nashville, TN) and maintained in DMEM with 10% FBS. PAI/L cells were obtained from Dr. Dan Rifkin (New York University, New York, NY) and maintained in 10% FBS.

**Plasmids and Cell Lines**—(G23V)TC21 and (G12V)H-Ras were subcloned into the LZRS-GFP vector (15) modified for bicistronic expression of GFP and the protein of interest. Vectors were transfected into Phoenix 293 packaging cells using Lipofectamine (Invitrogen), and EpH4 cells were subsequently infected with retrovirus daily for 10 days. Stable populations of cells expressing mutant TC21, H-Ras or empty vector were isolated by GFP using a FACStar Plus cell sorter (BD Biosciences, Franklin Lakes, NJ). The dominant-negative construct pCMV6- AKT-K179M (16) was transfected using Lipofectamine. Pooled siRNA for Akt was obtained from Ambion (Austin, TX) and AKT-K179M (16) was transfected using Lipofectamine. Pooled siRNA for p38 MAPK and mTOR were transfected into Phoenix 293 packaging cells using DharmaFECT reagent 2 (Dharmacon, Lafayette, CO). Pooled siRNA for Akt was obtained from Ambion (Austin, TX) and transfected using DharmaFECT reagent 2 (Dharmacon, Lafayette, CO). Pooled siRNA for p38 MAPK and mTOR (FRAP), specific siRNA for PI3K(p110β), and control siRNA were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and transfected using the manufacturer’s reagents and protocol.

**Antibodies and Other Reagents**—TGF-β1 was from R & D Systems (Minneapolis, MN). Antibodies to TC21, H-Ras, TGF-β type II receptor, and actin were from Santa Cruz Biotechnology Inc. Antibodies to phosphorylated and total Erk1/2, p38 MAPK, Akt, mTOR, p70S6K, and Smad2 were from Cell Signaling Technology (Beverly, MA). Total Smad3 antibodies were from Zymed Laboratories (San Francisco, CA), and antibodies to phosphorylated Smad3 were kindly provided by Dr. Ed Leof (Mayo Clinic, Rochester, MN). LY294002, SB203580, U0126, PD98059, Akt inhibitor II, Akt inhibitor III, and rapamycin were from Calbiochem (EMD Biosciences, La Jolla, CA).

**Tumor Formation**—5-week-old female BALB/c athymic mice were obtained from Harlan Laboratories (Indianapolis, IN). The cells were trypsinized, resuspended in phosphate-buffered saline, and then injected subcutaneously on either side of the back (1.0 × 10⁶ cells/100 μl of phosphate-buffered saline/injection). Tumor size was measured after 3 weeks using a digital caliper, and the volumes were calculated as length × width × height.

**Colony Formation**—1 × 10⁴ cells in suspension (DMEM, 10% FBS, 0.3% agar) with TGF-β (5 ng/ml) or inhibitors (0.1 μM) were overlaid onto a solidified layer of agar (DMEM, 10% FBS, 0.7% agar) in 35-mm dishes. The cells were incubated at 37 °C for 9 days. The colonies were scored counting multiple fields using an inverted microscope.

**Cell Proliferation**—3 × 10⁵ cells were plated per well in 24-well plates and maintained in DMEM (2% FBS) for 70 h and then pulsed for 2 h with 4 μCi/well [³H]thymidine (PerkinElmer Life Sciences). The cells were washed with 10% trichloroacetic acid and solubilized with 0.2 N NaOH, and radioactivity was measured using a scintillation counter. Cell counting assays were performed by plating 2.5 × 10⁵ cells (subconfluent) or 3 × 10³ cells (confluent) per 35-mm dish and counting the cell number over 5 days using a hemocytometer.

**Immunoblotting**—The cells were serum-starved overnight and stimulated with either TGF-β in some experiments or 10% FBS in others for the times indicated. The cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) supplemented with protease and phosphatase inhibitors. Total cell lysates were run onto 10% SDS gels, then transferred to nitrocellulose membranes, and blocked with 5% milk in Tris-buffered saline with Tween 20 (150 mM NaCl, 100 mM Tris, pH 7.5, 0.1% Tween 20). Immunoblotting was performed with primary (1:1000) and secondary (1:5000) antibodies in Tris-buffered saline with Tween 20 with 5% milk and visualized using the ECL Western blotting detection system (PerkinElmer Life Sciences).

**Reporter Assays**—The cells were transiently transfected using Lipofectamine with the 3TP-Lux luciferase or CAGA reporter construct in conjunction with a cytomegalovirus-driven Renilla luciferase plasmid. Subsequently the cells were either untreated or treated with TGF-β (5 ng/ml) for 24 h and lysed, and dual luciferase assays were performed as indicated by the manufacturer (Promega, Madison, WI) and measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Ratios of firefly and Renilla luciferase were calculated by normalizing data to relative luminescent units. The PAI/L assay was performed as previously described (17). Briefly, PAI/L cells were incubated for 24 h in serum-free medium with or without TGF-β (5 ng/ml) or in conditioned serum-free medium collected from TC21/EpH4, H-Ras/EpH4, or LZRS/EpH4 cells. The cells were then lysed, and luciferase assays were performed as described above.

**Statistical Analysis**—Student’s t test was used to compare two groups. The values with p ≤ 0.05 were considered significant. The results from colony formation, proliferation, and reporter assays are representative of three independent experiments.

**RESULTS**

(G23V) TC21-transformed EpH4 Cells Are Highly Tumorigenic—Nontumorigenic EpH4 mouse mammary cells were used in this study because they have been well characterized for their response to both H-Ras(G12V) overexpression and TGF-β stimulation (18, 19). These cells express endogenous H-Ras and TC21 (Fig. 1A) and undergo G1/S cell cycle arrest in response to TGF-β (20). EpH4 cells were infected with either empty LZRS-GFP retroviral vector (LZRS/EpH4), active (G23V) TC21(TC21/EpH4), or active (G12V) H-Ras/H-Ras/EpH4. Cell populations with equal expression of GFP were sorted by fluorescence-activated cell sorting (data not shown), and mutant Ras expression levels were verified by Western blot analysis (Fig. 1A). To test tumorigenicity in vivo, the cells were injected subcutaneously into nude mice. TC21/EpH4 cells formed large tumors within 14 days post-injection; H-Ras/
Eph4 cells formed very small tumors, whereas LZRS/Eph4 cells were nontumorigenic (Fig. 1B).

The differences in tumorigenicity between cell populations in vivo were correlated in vitro by evaluating soft agar colony formation. Within 9 days in culture, TC21/Eph4 cells formed numerous large-sized colonies, whereas H-Ras/Eph4 cells formed very few colonies, and LZRS/Eph4 cells failed to grow (Fig. 1C). Relative cell proliferation rates using [3H]thymidine incorporation, and cell counting assays were performed on the different cell populations. TC21/Eph4 cells proliferated approximately three times faster than H-Ras/Eph4 or LZRS/Eph4 cells (Fig. 1, D and E). Furthermore, when plated at high density, H-Ras/Eph4 and LZRS/Eph4 cells were contact-inhibited, whereas TC21/Eph4 cells continued to grow (Fig. 1F). Thus, TC21/Eph4 cells are significantly more tumorigenic than H-Ras/Eph4 cells both in vivo and in vitro.

TC21-induced Soft Agar Growth Requires p38 MAPK and PI3K but Not Akt Activation—Because TC21 is known to activate Erk1/2, p38 MAPK, and PI3K (14, 21), we investigated the roles of these pathways in TC21-induced transformation of Eph4 cells. Serum-starved TC21/Eph4 cells showed markedly elevated basal levels of phosphorylated Akt and p38 MAPK, but not Erk1/2 (Fig. 2A). In contrast, H-Ras/Eph4 cells showed a slight increase in Akt activity relative to LZRS/Eph4 cells (Fig. 2A). Serum stimulation induced a similar transient increase in p38 MAPK and Erk1/2 activity in LZRS and H-Ras/Eph4 cells, whereas Akt activation was slightly increased in H-Ras/Eph4-expressing cells (Fig. 2A). In contrast, marked and sustained activation of Akt and p38 MAPK was evident in TC21/Eph4 cells. To determine whether these pathways were required for TC21-induced transformation and proliferation, soft agar and cell proliferation assays were performed in the presence of specific inhibitors for these pathways as well as following gene silencing with siRNA. As shown in Fig. 2B, significant decreases in PI3K(110β), Akt, or p38 MAPK expression was obtained following gene silencing. Inhibition of the Erk1/2 pathway with the MAPK/Erk kinase inhibitors U0126 (Fig. 2C) or PD98059 (data not shown) had no effect on colony formation of either the TC21 or H-Ras/Eph4 cells. In contrast TC21/Eph4 colony formation was decreased 50–60% with PI3K inhibition (LY294002, PI3K siRNA) or p38 MAPK inhibition (SB203580, p38 MAPK siRNA) (Fig. 2C). Combined inhibition of PI3K and p38 MAPK had no additional effect (data not shown). Surprisingly, inhibition of Akt activity by siRNA (Fig. 2C), specific inhibitors (Akt inhibitor II, Akt inhibitor III) (data not shown), or transfection of a dominant-negative Akt construct (pcMV6-AKT-K179M) (data not shown) did not affect TC21/Eph4 colony formation. In H-Ras/Eph4 cells, PI3K inhibition blocked ~80% of colony formation, and Akt inhibition reduced colony formation by 50% (Fig. 2C), whereas inhibiting p38 MAPK had little effect.

The same strategies described above were utilized to determine which pathways played a role in cell proliferation as determined by [3H]thymidine incorporation assays. As shown in Fig. 2D, inhibition of PI3K or p38 MAPK reduced TC21/Eph4 cell proliferation by roughly 90%, whereas blocking Akt activity had no effect. The proliferation rates of both H-Ras/Eph4 and LZRS/Eph4 cells were decreased by ~80% with inhibition of PI3K, Akt, or p38 MAPK activity (Fig. 2D). Inhibition of Erk1/2 activity did not affect the growth of any of the cell populations. Thus, transformation and increased growth of TC21/Eph4 cells is mediated by p38 MAPK and PI3K, but not Akt.

TC21 Activates mTOR Signaling—Because TC21-mediated transformation of Eph4 cells was PI3K-dependent but Akt-independent, we investigated whether mTOR played a role in this process, because mTOR is known to act downstream of PI3K/
Akt and mediate Ras transformation (22). TC21/EpH4 cells demonstrated a marked increase in phosphorylation of mTOR and its effector p70S6K, both basally and following serum stimulation compared with H-Ras/EpH4 and LZRS/EpH4 cells (Fig. 3A). To determine whether mTOR activation was required for transformation and growth, colony formation and proliferation assays were performed using the mTOR inhibitor rapamycin as well as siRNA directed against mTOR. mTOR expression levels were significantly decreased in all the cell populations following gene silencing with siRNA (Fig. 3B). Inhibition of mTOR activity decreased TC21/EpH4 and H-Ras/EpH4 colony formation by 60 and 80%, respectively (Fig. 3B). Interestingly, the basal growth of H-Ras/EpH4 and LZRS/EpH4 cells on plastic was not significantly reduced by rapamycin or siRNA directed against mTOR, whereas TC21/EpH4 cell proliferation was decreased by 50–60% (Fig. 3C). These observations suggest a role for mTOR in TC21-induced tumorigenesis.

**PI3K/mTOR Signaling in TC21/EpH4 Cells**—Based on the finding that transformation of EpH4 cells depended on PI3K and mTOR, but not Akt, we investigated whether PI3K and mTOR were signaling through a common pathway. As seen in Fig. 4A, inhibition of either PI3K or mTOR reduced TC21/EpH4 colony formation by ~50%, whereas inhibition of both PI3K and mTOR abolished colony formation completely. Combined inhibition of PI3K and mTOR also had an additive effect on TC21/EpH4 cell proliferation (Fig. 4B). Because rapamycin virtually abolished all H-Ras/EpH4 colony formation and proliferation, it was not possible to determine an additive effect of these inhibitors. To determine whether mTOR activation was dependent on PI3K, we tested whether the inhibitors indicated above could block activation of Akt or mTOR by serum stimulation (Fig. 4C). Akt phosphorylation was unaffected by rapamycin treatment in all cell lines, suggesting that PI3K is not dependent or downstream of mTOR activity. Phosphorylation of mTOR was slightly decreased by LY294002 in H-Ras/EpH4 and LZRS/EpH4 cells, but not in TC21/EpH4 cells, suggesting that PI3K-independent pathways are predominantly responsible for mTOR activation.

**mTOR Activation Is Downstream of p38 MAPK**—Because p38 MAPK mediates TC21-induced transformation independent of PI3K activity (data not shown), we investigated whether p38 and mTOR were signaling through a common pathway. As shown in Fig. 5 (A and B), combined inhibition of p38 MAPK and mTOR was no more effective than inhibiting either one alone in reducing TC21/EpH4 colony formation or cell proliferation. The combinatorial effect on H-Ras/EpH4 colony formation could not be tested because rapamycin alone completely blocked growth in soft agar; however, combined inhibition did not decrease H-Ras/EpH4 or LZRS/EpH4 cell proliferation any further. We then examined the effect of these
inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 5C). The serum-induced phosphorylation of mTOR in TC21/Eph4 cells was blocked by p38 MAPK inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 5C). The serum-induced phosphorylation of mTOR in TC21/Eph4 cells was blocked by p38 MAPK inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 5C). The serum-induced phosphorylation of mTOR in TC21/Eph4 cells was blocked by p38 MAPK inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 5C). The serum-induced phosphorylation of mTOR in TC21/Eph4 cells was blocked by p38 MAPK inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 5C). The serum-induced phosphorylation of mTOR in TC21/Eph4 cells was blocked by p38 MAPK inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 5C). The serum-induced phosphorylation of mTOR in TC21/Eph4 cells was blocked by p38 MAPK inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 5C).
inhibition, whereas treatment with rapamycin blocked serum-induced mTOR phosphorylation and reduced mTOR activation below basal levels. Combined inhibition of p38 MAPK and mTOR had no additive effect on mTOR phosphorylation, and rapamycin treatment alone did not affect the activation of p38 MAPK. Activation of mTOR was also partially blocked by p38 MAPK inhibition in H-Ras/EpH4 and LZRS/EpH4 cells, but rapamycin alone did not block activation of p38 MAPK. These data suggest that TC21-induced transformation and proliferation is mediated in part by a p38/mTOR-dependent pathway.

**TGF-β Signaling Is Intact in TC21/EpH4 Cells**—Because TGF-β is known to cooperate with H-Ras in promoting colony formation, cell proliferation, migration, and invasion (18), we examined whether TGF-β signaling could modulate the proliferation and transformation of TC21/EpH4 cells. Interestingly, although TC21/EpH4 cells have a much higher basal level of colony formation than H-Ras/EpH4 and LZRS/EpH4 cells (Fig. 1C), TGF-β stimulation resulted in a bigger percentage increase in colony size (data not shown) and number in the H-Ras/EpH4 cells when compared with TC21/EpH4 cells (Fig. 6A). In contrast, proliferation of H-Ras/EpH4 and LZRS/EpH4 cells on plastic was 30–40% growth-inhibited by TGF-β, whereas TC21/EpH4 cells showed significantly increased proliferation (Fig. 6B).

Because TC21/EpH4 cells were unresponsive to TGF-β-mediated growth inhibition, the integrity of TGF-β-mediated signaling pathways was determined by reporter assays using the 3TP-Lux reporter containing a TGF-β-inducible promoter and the CAGA reporter of Smad-induced transcriptional activation. As shown in Fig. 6 (C and D), all cell lines showed similar increases in 3TP-Lux and CAGA reporter activity following 24 h of stimulation with TGF-β. In addition, Smad2 and Smad3 phosphorylation was the same in TC21/EpH4, H-Ras/EpH4, and LZRS/EpH4 cells (Fig. 6E). To determine whether TC21/EpH4 cells produced less active TGF-β than H-Ras/EpH4 or LZRS/EpH4 cells, PAI/L reporter cells were incubated with conditioned serum-free medium from all cell populations, and luciferase activity was measured. As shown in Fig. 6F, luciferase activity induced by conditioned medium from each cell population was similar. Finally we demonstrate there is no difference in expression of the TGF-β type II receptor in the different cell populations (Fig. 6G). Together, these data suggest that the proliferative effects of TGF-β on TC21/EpH4 cells are mediated through activation of Smad-independent pathways and are not due to alterations in the endogenous production of active TGF-β or changes in expression of the TGF-β type II receptor.
Akt, p38 MAPK, and mTOR in response to TGF-β. We examined the effects of TGF-β on multiple common pathways, including Erk1/2, p38 MAPK, Akt, or a scrambled control, and in cells treated in the presence or absence of 10 μM U0126, 10 μM LY294002 (LY), 10 μM SB203580 (SB), or 2 ng/ml rapamycin (Rapa) and treated with or without TGF-β (5 ng/ml). Colony formation was scored after 9 days. Differences in colony formation of cells treated with inhibitors compared with basal levels (**, p < 0.05) or to TGF-β stimulated colony formation (*, p < 0.05) were significant. C, 72-h cell proliferation was evaluated in cells grown on plastic transfected with siRNA for PI3K, p38 MAPK, Akt, or a scrambled control, and in cells treated in the presence or absence of 10 μM U0126, 10 μM LY294002 (LY), 10 μM SB203580 (SB) or 2 ng/ml rapamycin (Rapa) and treated with or without TGF-β (5 ng/ml). Differences in proliferation of TC21/EpH4, H-Ras/EpH4, and LZRS/EpH4 cells treated with inhibitors. *, p < 0.01. The values from transformation and proliferation assays are the means ± S.D. from triplicate wells of a representative experiment. NT, nontreated.

**FIGURE 7. Signaling pathways activated by TGF-β in TC21/EpH4.** A, cell populations were analyzed for levels of phosphorylated or total Akt, p38 MAPK, Erk1/2, and mTOR expression following stimulation with TGF-β (5 ng/ml). Serum-starved cells were treated with TGF-β for the times indicated, and immunoblots were performed on 20 μg of total cell lysate using the antibodies indicated. A representative of three experiments performed is shown. B, cell populations were seeded in soft agar in the presence or absence of 10 μM U0126, 10 μM LY294002 (LY), 10 μM SB203580 (SB), or 2 ng/ml rapamycin (Rapa) and treated with or without TGF-β (5 ng/ml). Colony formation was scored after 9 days. Differences in colony formation of cells treated with inhibitors compared with basal levels (**, p < 0.05) or to TGF-β stimulated colony formation (*, p < 0.05) were significant. C, 72-h cell proliferation was evaluated in cells grown on plastic transfected with siRNA for PI3K, p38 MAPK, Akt, or a scrambled control, and in cells treated in the presence or absence of 10 μM U0126, 10 μM LY294002 (LY), 10 μM SB203580 (SB) or 2 ng/ml rapamycin (Rapa) and treated with or without TGF-β (5 ng/ml). Differences in proliferation of TC21/EpH4, H-Ras/EpH4, and LZRS/EpH4 cells treated with inhibitors. *, p < 0.01. The values from transformation and proliferation assays are the means ± S.D. from triplicate wells of a representative experiment. NT, nontreated.

**DISCUSSION**

During tumor progression, cells commonly acquire mutations in Ras proteins that result in constitutive activation of proliferative pathways, allowing these cells to override normal growth control mechanisms (23). TC21 is known to be a powerful oncopogene, yet it is not clear how constitutive TC21 activity induces cell proliferation and transformation. We show that TC21/EpH4 cells are significantly more oncogenic than H-Ras/EpH4 cells both in vitro and in vivo and that TC21-induced proliferation and transformation requires PI3K, p38 MAPK, and mTOR activity. Although Smad-dependent and -independent TGF-β signaling pathways remained intact, TC21/EpH4 cells lost their susceptibility to growth inhibition by TGF-β, suggesting that TC21 transformation is sufficient to inhibit the role of TGF-β as a growth suppressor.

TC21/EpH4 cells were highly tumorigenic both in vitro and in vivo compared with H-Ras/EpH4 cells. These results contrast with earlier observations where TC21-transformed NIH3T3 fibroblasts formed the same number of soft agar colonies as H-Ras-transformed cells, despite forming more aggressive tumors in nude mice (3), and where TC21-transformed MCF10A human epithelial cells formed significantly more colonies than H-Ras-expressing cells, but neither formed tumors in vivo (4). The low level of tumorigenicity in H-Ras/EpH4 cells was surprising because others report EpH4 cells transformed with H-Ras(EpRas) form tumors by 5–7 days in BALB/c mice.
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(18) and 4 weeks in nude mice (14). Despite these differences in vivo, both EpRas cells (18) and H-Ras/EpH4 cells were 30–40% growth-inhibited by TGF-β.

The pathways that mediate TC21-induced tumorigenesis are not well established. Our data show that TC21-mediated transformation of EpH4 cells is independent of Erk1/2, which contrasts with data showing that TC21 overexpression in NIH3T3 cells increases Erk1/2 (24) and Raf activity, which is required for transformation (25). These data in turn contrast with other studies suggesting that TC21 does not activate Erk1/2 directly (2), and TC21 can transform NIH3T3 cells independent of Raf (26). A marked increase in basal levels of phosphorylated p38 MAPK was noted in TC21/EpH4 cells, which was important in promoting colony formation and cell proliferation. These data are consistent with findings that TC21 can activate p38 MAPK in Cos7 cells (2), and p38 MAPK activation is important for TC21-induced ureteric bud cell proliferation (27). The requirement of p38 MAPK but not Erk1/2 for EpH4 cell transformation once again demonstrates the heterogeneity by which TC21 induces its effects in different cell types.

Like others, we demonstrate that TC21 activates Akt, and TC21 transformation is PI3K-dependent (24); however, transformation of EpH4 cells was independent of Akt. Although this finding was surprising, TC21-induced migration of murine Schwann cells is dependent on Erk1/2 and PI3K but not Akt activation (6). Treatment of TC21/EpH4 cells with rapamycin reduced cell proliferation and transformation by 50%, suggesting a role for mTOR in these processes. Although mTOR has not previously been associated with TC21 transformation, it does mediate K-Ras-induced alveolar epithelial neoplasia in mice (28). Our finding that mTOR mediates TC21 transformation downstream of p38 MAPK and not PI3K/Akt was unexpected. Although current models suggest that mTOR signals both downstream and in parallel with PI3K to converge on common downstream targets (29), it is not known whether mTOR is directly activated by p38 MAPK.

The molecular mechanisms through which the TGF-β and Ras signaling pathways interact are not well defined. Recent studies suggest constitutive H-Ras activity switches the effect of TGF-β from tumor suppressor to promoter by blocking phosphorylation of the C terminus of Smad3 and inducing phosphorylation of residues in the Smad3 linker region (30). A role for Akt and mTOR in suppressing TGF-β-mediated activation of Smad3 has also been suggested (22). Smad2/3 phosphorylation and Smad-induced transcriptional activation was not changed in TC21/EpH4 cells, suggesting that the proliferative effect of TGF-β is mediated by activation of Smad-independent pathways.

In conclusion we show that activated TC21 causes marked transformation of nontumorigenic mammary cells by activating PI3K and p38 MAPK/mTOR signaling, and these cells are rendered insensitive to the growth inhibitory effects of TGF-β. Although the prevalence of TC21-induced tumorigenesis in humans is unclear, aberrant TC21 activity has been found in breast, oral, and esophageal carcinomas (4, 7, 8), and there is genetic evidence that nonmutated TC21 is a frequent target for tumorigenic activation by retroviruses (31). Thus, identifying the effectors and pathways activated by this Ras superfamily member may provide insights into the pathogenesis of cancer.

REFERENCES

1. Ehhardt, A., Ehrhardt, G. R., Guo, X., and Schrader, J. W. (2002) Exp. Hematol. 30, 1089–1106
2. Graham, S. M., Oldham, S. M., Martin, C. B., Drugan, J. K., Zohn, I. E., Campbell, S., and Der, C. J. (1999) Oncogene 18, 2107–2116
3. Chan, A. M., Miki, T., Meyers, K. A., and Aaronson, S. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 16, 7558–7562
4. Clark, G. J., Kinch, M. S., Gilmer, T. M., Burridge, K., and Der, C. J. (1996) Oncogene 12, 169–176
5. Barker, K. T., and Crompton, M. R. (1998) Br. J. Cancer 79, 296–300
6. Huang, Y., Saez, R., Chao, L., Santos, E., Aaronson, S. A., and Chan, A. M. (1995) Oncogene 11, 1255–1260
7. Arora, S., Matta, A., Shukla, N. K., Deo, S. V., and Ralhan, R. (2005) Mol. Carcinog. 42, 97–108
8. Sharma, R., Sud, N., Chattopadhyay, T. K., and Ralhan, R. (2005) Oncology 69, 10–18
9. Rosario, M., Paterson, H. F., and Marshall, C. J. (2001) Mol. Cell. Biol. 21, 3750–3762
10. Toker, A. (2002) Cell Mol. Life Sci. 59, 761–779
11. Bierie, B., and Moses, H. L. (2006) Cytokine Growth Factor Rev. 17, 29–40
12. Moustakas, A., and Heldin, C. H. (2005) J. Cell Sci. 118, 3573–3584
13. Oft, M., Heider, K. H., and Beug, H. (1998) Curr. Biol. 8, 1243–1252
14. Janda, E., Lehmann, K., Killisch, I., Jechlinger, M., Herzig, M., Downward, J., Beug, H., and Grunert, S. (2002) J. Cell Biol. 156, 299–313
15. Kinsella, T. M., and Nolan, G. P. (1996) Hum. Gene Ther. 7, 1405–1413
16. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Cell 81, 727–736
17. Abe, M., Harpel, J. G., Metz, C. N., Nunes, I., Loskutoff, D. J., and Rifkin, D. B. (1994) Anal. Biochem. 216, 276–284
18. Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., and Reichmann, E. (1996) Genes Dev. 10, 2462–2477
19. Reichmann, E., Ball, R., Groner, B., and Friis, R. R. (1989) J. Cell Biol. 108, 1127–1138
20. Petritsch, C., Beug, H., Balmain, A., and Oft, M. (2000) Genes Dev. 14, 3093–3101
21. Kim, E. S., Kim, M. S., and Moon, A. (2005) Cytokine 29, 84–91
22. Song, K., Wang, H., Krebs, T. L., and Danielpour, D. (2006) EMBO J. 25, 58–69
23. Akhurst, R. J., and Derynck, R. (2001) Trends Cell Biol. 11, 544–551
24. Rong, R., He, Q., Liu, Y., Sheikh, M. S., and Huang, Y. (2002) Oncogene 21, 1062–1070
25. Rosario, M., Paterson, H. F., and Marshall, C. J. (1999) EMBO J. 18, 1270–1279
26. Graham, S. M., Cox, A. D., Drivas, G., Rush, M. G., D’Eustachio, P., and Der, C. J. (1994) Mol. Cell. Biol. 14, 4108–4115
27. Pozzi, A., Coffa, S., Bulus, N., Zhu, W., Chen, D., Chen, X., Mernaugh, G., Su, Y., Cai, S., Singh, A., Brissova, M., and Zent, R. (2006) Mol. Cell Biol. 26, 2046–2056
28. Wislez, M., Spencer, M. L., Izzo, J. G., Juroske, D. M., Balhara, K., Cody, D. D., Price, R. E., Hittelman, W. N., Wistuba, I., and Kurie, J. M. (2005) Cancer Res. 65, 3226–3235
29. Finger, D. C., and Blenis, J. (2004) Oncogene 23, 3151–3171
30. Sekimoto, G., Matsuzaki, K., Yoshiida, K., Mori, S., Murata, M., Seki, T., Matsu, H., Fujisawa, J., and Okazaki, K. (2007) Cancer Res. 67, 5090–5096
31. Kim, R., Trubetskoy, A., Suzuki, T., Jenkins, N. A., Copeland, N. G., and Lenz, J. (2003) J. Virol. 77, 2056–2062