Raf and RhoA Cooperate to Transform Intestinal Epithelial Cells and Induce Growth Resistance to Transforming Growth Factor β

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Abstract

Although unregulated activation of the Ras/Raf/mitogen-activated protein kinase kinase/Erk signaling pathway is believed to be a central mechanism by which many cell types undergo oncogenic transformation, recent studies indicate that activation of Raf kinase by oncogenic Ras is not sufficient to cause tumorigenic transformation in intestinal epithelial cells. Thus, identification of signaling proteins and pathways that interact with Raf to transform intestinal epithelial cells may be critical for understanding aberrant growth control in the intestinal epithelium. Functional interactions between Raf and the small GTPase RhoA were studied in RIE-1 cells overexpressing both activated Raf(22W) and activated RhoA(63L). Double transfectants were morphologically transformed, formed colonies in soft agar, grew in nude mice, overexpressed cyclin D1 and cyclooxygenase-2 (COX-2), and were resistant to growth inhibition by transforming growth factor (TGF) β. RIE-Raf and RIE-RhoA single transfectants showed none of these characteristics. Expression of a dominant-negative RhoA(N19) construct in RIE-Ras(12V) cells was associated with markedly reduced COX-2 mRNA, COX-2 protein, and prostaglandin E_2 levels when compared with RIE-Ras(12V) cells transfected with vector alone. However, no change in transformed morphology, growth in soft agar, cyclin D1 expression, TGFα expression, or TGFβ sensitivity was observed. In summary, coexpression of activated Raf and RhoA induces transformation and TGFβ resistance in intestinal epithelial cells. Although blockade of RhoA signaling reverses certain well-described characteristics of RIE-Ras cells, it is insufficient to reverse the transformed phenotype and restore TGFβ sensitivity. Blockade of additional Rho family members or alternate Ras effector pathways may be necessary to fully reverse the Ras phenotype. (Mol Cancer Res 2004; 2(4):233–41)

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Introduction

Ras proto-oncogenes are functionally complex cytoplasmic and membrane-bound GTPases that function as key points of signaling convergence for diverse extracellular stimuli. Following activation of Ras, signaling diverges to include activation of the well-characterized Raf/mitogen-activated protein kinase kinase (MEK)/Erk kinase cascade as well as an increasingly recognized diversity of alternate Ras effector pathways. Hyperactivation of Ras is a critical event in carcinogenesis. Activating Ras gene mutations have been identified in about 30% of all human tumors (1), including ~50% of colorectal carcinomas (1, 2). In addition, constitutive activation of Ras may occur in a subset of mutation negative colon tumors (3).

Recent studies have begun to characterize Ras signaling pathways responsible for transformation of intestinal epithelial cells. Results thus far indicate that there are critical differences in the signaling pathways by which Ras transforms intestinal epithelial cells *versus* fibroblastic cells, which are far more extensively characterized (4). As expected from studies in fibroblasts, nontransformed intestinal epithelial cells transfected with activated H-Ras or K-Ras oncogenes become morphologically transformed. These transformed cells also become resistant to negative regulation by growth factors such as transforming growth factor (TGF) β (5–8). However, while activation of Raf kinase is sufficient to cause transformation of fibroblasts, multiple lines of evidence indicate that activation of Raf-independent pathways are necessary for transformation of intestinal epithelial cells. For example, mutationally activated Ras transforms RIE-1 intestinal epithelial cells while constitutively active mutant Raf(22W) or membrane-associated Raf(CAAX), both of which are transforming in fibroblasts, fail to transform RIE-1 cells (7). One proposed explanation for this difference is the finding of an activated epidermal growth factor receptor (EGFR) autocrine loop in cells transformed by mutant-activated Ras but no such activation in Raf overexpressing cells (9). Ras-transformed RIE-1 cells are also resistant to growth inhibition by TGFβ while overexpression of activated Raf does not result in TGFβ resistance (10). Collectively, these observations indicate that activation of a critical Raf-independent effector pathway(s) is necessary for transformation of intestinal epithelial cells. This critical pathway may involve p38 signaling because blockade of p38 kinase activity in Raf expressing RIE-1 cells results in morphological transformation and a profile of gene expression closely resembling Ras-transformed cells (11). In addition, inhibition of the c-Jun NH_2-terminal kinase (JNK) kinase activity reverses certain features

of the Ras-transformed phenotype (11). Phosphatidylinositol 3-kinase and Ral-GEF signaling do not appear to be involved at least with respect to resistance to anoikis in Ras-transformed RIE-1 cells (12).

Published work supports involvement of Rho proteins in Ras-mediated cellular transformation of fibroblasts. For example, dominant-negative Rho mutants reduce Ras transforming activity while overexpression of activated Rho proteins interact with oncogenic Ras to cooperatively transform fibroblastic cells (13–16). It has been hypothesized that one mechanism by which Rho may cooperate with Raf is activation of $p42^{MAPK}$ and $p44^{MAPK}$ (17). In contrast to this evidence in fibroblasts, involvement of Rho proteins in epithelial cell transformation is limited to a few recent reports. For example, RhoC has recently been shown to be a transforming oncogene in immortalized human mammary epithelial cells (18) and p21 activated protein kinase 4 (PAK4), a Rho effector, is required for anchorage-independent growth of human cancer cells lines, including colon cancer lines (19).

Herein, we describe in intestinal epithelial cells cooperativity between activated RhoA and activated Raf, the result being cellular transformation and acquisition of other growth characteristics reminiscent of Ras-transformed cells. Accumulating data implicate Rho proteins as key growth regulators in epithelial cells, including those of the gastrointestinal tract (20). Our data show that consideration of Rho signaling, perhaps in addition to other Raf-independent signaling pathways, is important in the analysis of growth signaling networks activated in gastrointestinal neoplasia.

Results

Activated RhoA and Raf(22W) Cooperate to Transform RIE-1 Cells

Ras is a transforming oncogene in fibroblasts and epithelial cells, but constitutive overexpression of the immediate downstream kinase, Raf(22W) or Raf(CAAX), is transforming only in fibroblasts (7, 15). Thus, activation of Raf-independent signaling must occur for mutant-activated Ras to transform epithelial cells. In fibroblasts, activation of Rho family proteins such as RhoA (15) and Rac (16) is necessary for oncogenic transformation by Ras. To understand whether Rho family GTPases might interact with activated Raf to transform RIE-1 cells, RIE-Raf(22W) cells were stably transfected with activated RhoA(63L). The anchorage-dependent morphology and anchorage-independent growth were compared with parental RIE-1, RIE-Ras(12V), RIE-RhoA(63L), and RIE-Raf(22W) overexpressing cells. Results are shown in Fig. 1A. The parental RIE-1 as well as Raf(22W) and RhoA(63L) overexpressing cells have a typical nontransformed appearance, including a flat, epithelial morphology with abundant cytoplasm. RIE-Ras(12V) cells have the expected transformed morphology, characterized by spindle cell shape, refractile nuclei, and limited cytoplasm (7, 10). All cells cloned as a result of cotransfection with RIE-Raf(22W) with RhoA(63L) were transformed and were morphologically indistinguishable from Ras-transformed RIE-1 cells. Assays of anchorage-independent growth in soft agar were also conducted (Fig. 1A, insets). RIE-Ras(12V) and RIE-Raf(22W)/RhoA(63L) cells formed colonies in soft agar whereas RIE-1, RIE-Raf(22W), RIE-RhoA(63L), and control cells did not. When 10,000 cells were plated in soft agar assays, mean colony numbers were 368 and 1624 colonies/plate for RIE-Raf(22W)/RhoA(63L) and RIE-Ras(12V) cells, respectively. We also determined whether RIE-Raf(22W)/RhoA(63L) cells grow as tumors in athymic nude mice. Randomly selected RIE-Ras(12V) and RIE-Raf (22W)/RhoA(63L) clones formed rapidly growing tumors within 1 week of inoculation whereas parental RIE-1, RIE-Raf(22W), and RIE-RhoA(63L) formed no tumors or slowly growing tumors detectable more than 3 weeks after inoculation (data not shown). Singly and dually transfected cells were also analyzed for relative expression of Raf and RhoA (Fig. 1B, top panel) and for relative activity of RhoA determined by binding of RhoA GTP to immobilized rhotekin (Fig. 1B, bottom panel). These experiments confirm expression and activity of RhoA in the double transfectants used in these experiments. Collectively, these results show that constitutively active RhoA(63L) cooperates with Raf(22W) to effect transformed morphology and transformed behavior while overexpression of each activated signaling protein alone does not. Data also show that coexpression of dominant-negative RhoA in RIE-Ras(12V) cells does not fully reverse the transformed phenotype (Fig. 1), although colony numbers were reduced by 4.8-fold.

One mechanism by which Raf(22W) and RhoA(63L) might cooperate to transform RIE-1 cells is the induction of a soluble factor that is transforming in the context of Raf or RhoA activation. RIE-RhoA(63L) and RIE-Raf(22W) cells were treated with cell culture medium conditioned for 48 h by RIE-Raf(22W) or RIE-RhoA(63L), respectively. This condition medium failed to cause cellular transformation, suggesting that transformation is not due to production and secretion of a soluble factor(s).

Cooperative Interaction Between Activated RhoA and Raf Does Not Precisely Recapitulate Characteristics of Ras-Transformed Cells

Prior studies in Ras-transformed intestinal epithelial cells transformed by oncogenic Ras have identified distinctive patterns of gene expression that are believed to contribute to the transformed phenotype and that differentiate Ras(12V) from Raf(22W) overexpressing cells. Among others, these include increased expression of cyclin D1 (the key cyclin driving G_1 traverse in intestinal epithelial cells; Refs. 10, 21), increased expression of epidermal growth factor (EGF)-related peptides such as TGFα (9), activation of cyclooxygenase-2 (COX-2) expression resulting in elevated prostaglandin synthesis (22, 23), and resistance to growth inhibition by TGFβ (10, 24). Specific pharmacological blockade of EGFR signaling and COX-2 activity reverts transformed intestinal epithelial cell behavior, indicating pivotal roles in the process of Ras-mediated transformation (9, 25, 26). We therefore examined expression of cyclin D1, TGFα, and COX-2 levels in RIE-Raf(22W)/RhoA(63L) cells. TGFβ sensitivity is described below. Cyclin D1 expression in parental RIE-1 cells was not detected under the specific experimental conditions shown in Fig. 2A while levels in RIE-Raf(22W) and RhoA(63L) were

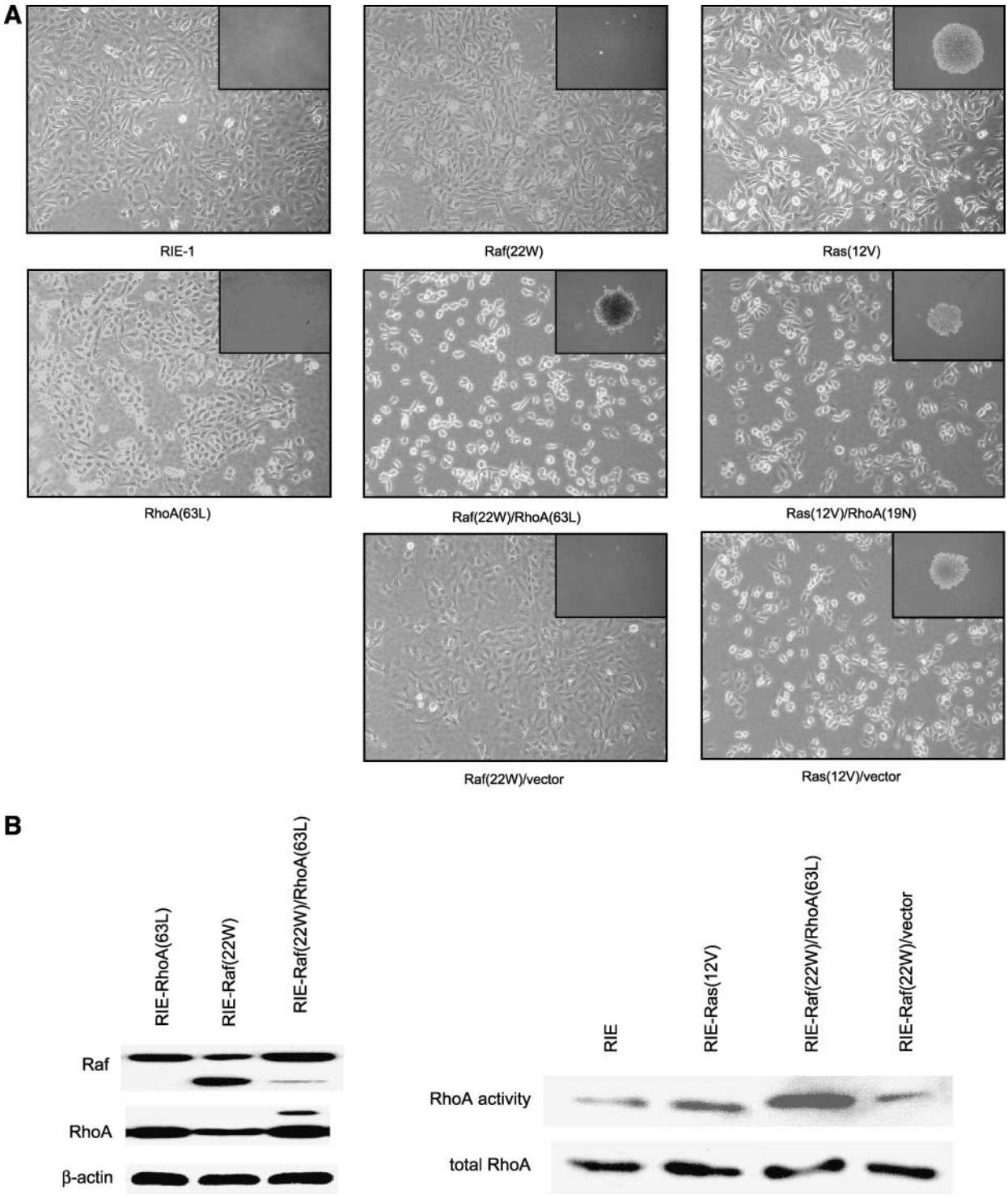

FIGURE 1. Morphology and soft agar growth of stable RIE-1 cell transfectants. RIE-1 cells were singly and dually transfected with the indicated signaling constructs as described in Materials and Methods. Morphology was assessed by light microscopy and anchorage independence was determined by growth in soft agar (*insets*; **A**). RIE-Raf cells stably express mutant active Raf(22W); RIE-Ras express mutant active Ras(12V); RIE-RhoA express mutant active RhoA(63L); RIE-Raf/RhoA express both mutant Raf(22W) and RhoA(63L); RIE-Ras/RhoA(DN) express Ras(12V) and RhoA(19N); RIE-Raf/vector express Raf(22W) and an empty pcDNA3.1 vector; and RIE-Ras express Ras(12V) and an empty pcDNA3.1 vector. Multiple double transfectant clones were analyzed and representative results are shown. **B.** Relative levels of RhoA and Raf in singly and dually transfected cells. Raf(22W) is a NH_2-terminal truncated, mutant, activated construct that migrates faster than the native protein. The RhoA(63L) construct transfected into RIE-Raf(22W) cells is HA tagged to facilitate differentiation from endogenous RhoA. RhoA(63L) construct used to transfect the parental line is not HA tagged. *Right panel,* increased RhoA activity in RIE-Raf(22W)/RhoA(63L) cells. Activity was measured by affinity precipitation of Rho GTP with immobilized rhotekin as described in Materials and Methods.

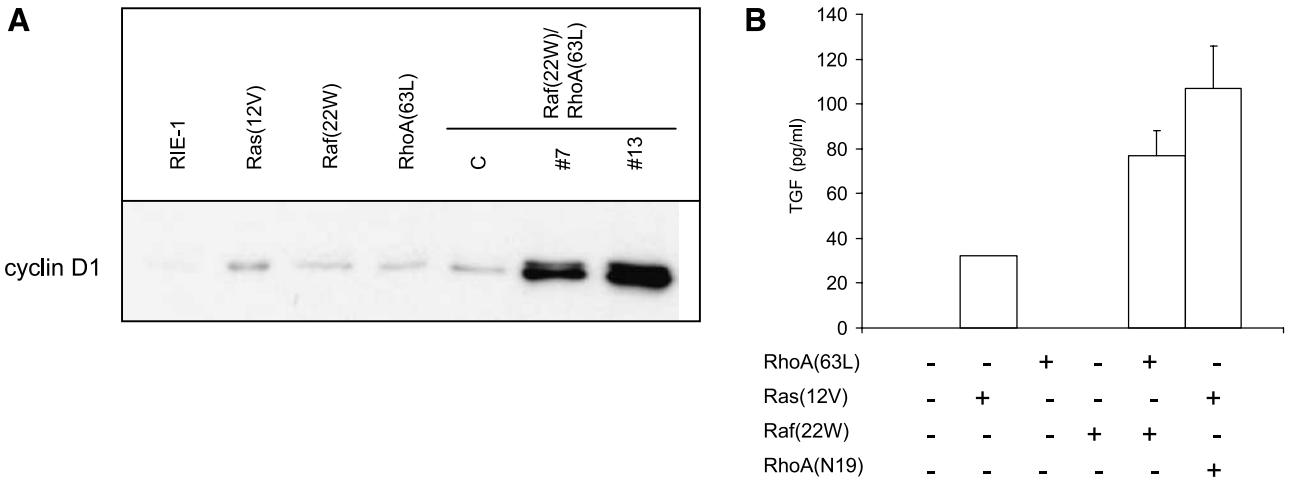

FIGURE 2. Cyclin D1 and TGFα levels in stable RIE-1 cell transfectants. **A.** Basal levels of cyclin D1 were determined using Western analysis of total cell lysates isolated from the indicated cell lines. *C,* a control for transfection of Raf(22W) cells with an empty pcDNA3.1 vector. *#7* and *#13,* representative clones of dually transfected cells. **B.** TGFα levels were determined by RIA in lysates prepared from cells grown in serum-free medium for 48 h as described in Materials and Methods. TGFα levels from two different clones of Raf(22W)/RhoA(63L) are shown.

lower than Ras(12V)-transformed cells as described previously (10). RIE-Raf(22W)/RhoA(63L) dually transfected cells markedly overexpressed cyclin D1 when compared with RIE-Raf(22W) cells transfected with vector alone (Fig. 2A). We also measured TGFα protein levels by RIA ELISA in cell lysates (Fig. 2B). As expected from prior work, we did not detect TGFα in RIE and RIE-Raf(22W) cell lysates while levels were significantly greater in RIE-Ras(12V) cells (9). TGFα was not detectable in RIE-RhoA(63L) while levels in RIE-Raf(22W)/RhoA(63L) were equal to or greater than RIE-Ras(12V) cells.

Figure 3A shows that although the expected increase in COX-2 was seen in RIE-Ras cells, markedly diminished levels of COX-2 protein by Western analysis were seen in doubly transfected Raf(22W)/RhoA(63L) clones. These are the same cell lysates shown in Fig. 2A, indicating a reciprocal effect on cyclin D1 and COX-2 in the doubly transfected line. This finding was supplemented with mRNA (Fig. 3B) and prostaglandin E_2 (PGE_2; Fig. 3C) levels, which confirmed a low level of expression and activity of COX-2 in RIE-Raf(22W)/RhoA(63L) cells when compared with control cell

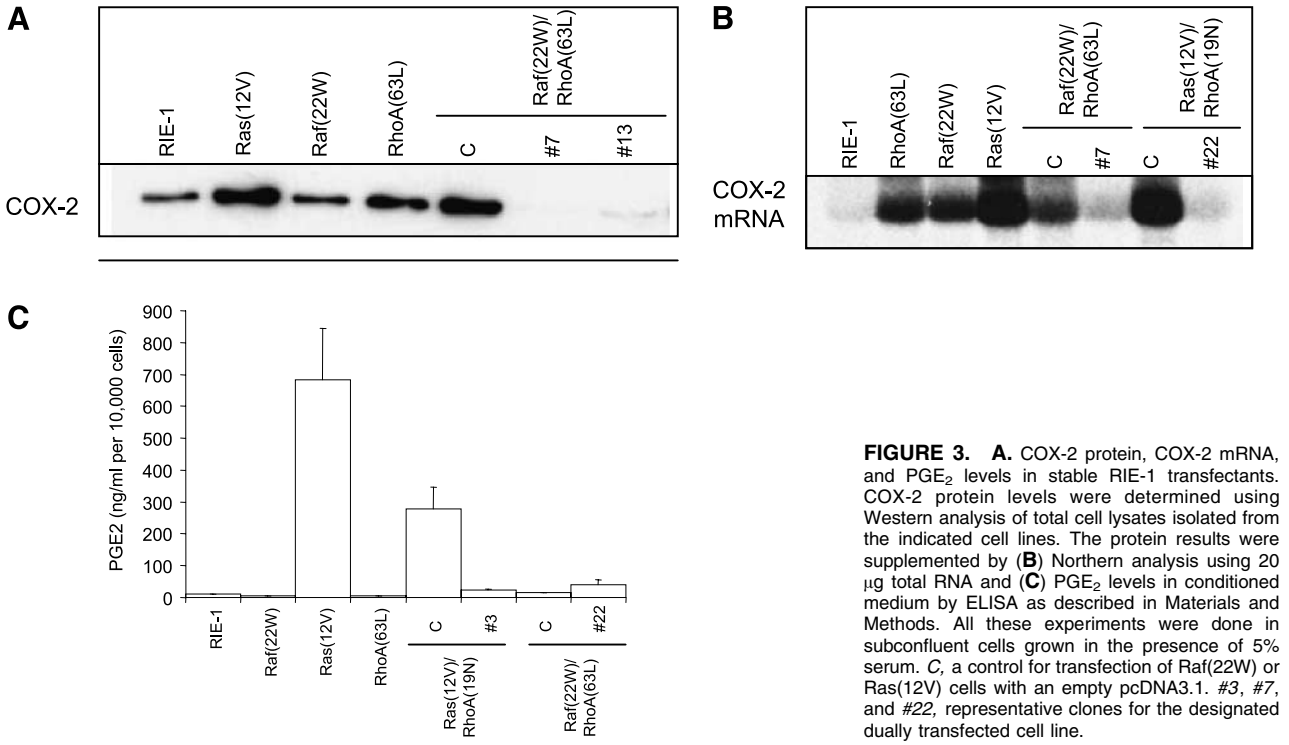

FIGURE 3. **A.** COX-2 protein, COX-2 mRNA, and PGE_2 levels in stable RIE-1 transfectants. COX-2 protein levels were determined using Western analysis of total cell lysates isolated from the indicated cell lines. The protein results were supplemented by (**B**) Northern analysis using 20 μg total RNA and (**C**) PGE_2 levels in conditioned medium by ELISA as described in Materials and Methods. All these experiments were done in subconfluent cells grown in the presence of 5% serum. *C,* a control for transfection of Raf(22W) or Ras(12V) cells with an empty pcDNA3.1. *#3*, *#7*, and *#22,* representative clones for the designated dually transfected cell line.

lines. Collectively, these results indicate that RIE cells transformed by dual overexpression of RhoA(63L) and Raf(22W) express a gene profile that is similar but not identical to Ras(12V)-transformed cells. These data also point out that regulation of COX-2 expression is complex and merits examination independently from the current presentation.

RIE Cells Coexpressing Activated RhoA and Raf Are Resistant to Growth Inhibition by TGFβ

Intestinal epithelial cells overexpressing oncogenic Ras, including the RIE-Ras(12V) cells used herein, are resistant to growth inhibition to TGFβ (10, 27). In previous work, we have shown that RIE-Raf(22W) cells are sensitive to TGFβ, as are parental RIE-1 cells (10), indicating that Raf-independent signaling pathways contribute to TGFβ resistance as well as transformed cell behavior. In the present study, we examined the sensitivity of singly and dually transfected RIE cells to inhibition by TGFβ (Fig. 4A). Thymidine incorporation assays showed that the dually transfected cells are relatively resistant to inhibition by TGFβ when compared with parental RIE-1, RIE-RhoA(63L), and RIE-Raf(22W) cells transfected with a control vector. These findings were confirmed using a luciferase reporter assay composed of a TGFβ-responsive Smad binding element (SBE4) linked to luciferase as shown in Fig. 4B. This mechanism of TGFβ resistance in doubly transfected cells is uncertain. Although TGFβRII levels are decreased in RIE-Raf(22W)/RhoA(63L) cells, Smad2 phosphorylation is not disrupted (Fig. 4C), indicating preserved proximal TGFβ signaling, analogous to findings reported previously in RIE-Ras cells (28).

Farnsyltransferase Inhibition Blocks Soft Agar Growth of RIE-Raf/RhoA Cells

Our results show that activated RhoA and activated Raf, each of which is nontransforming when individually expressed in RIE cells, sufficiently activate cytoplasmic signal transduction pathways to cause transformation when expressed together. We attempted to identify candidate activated pathways based on the ability of selected signaling pathway inhibitors to block morphological transformation in RIE-Raf(22W)/RhoA(63L) cells. Figure 5A shows colony formation in soft agar assays containing inhibitors of Rho-associated coiled coil-forming protein kinase (p160 ROCK; Y27632), EGFR tyrosine kinase (PD153035), MEK (PD98059), and farnesyltransferase (FTI-227). Inhibitor concentrations were selected based on inhibition of the respective enzyme activity in RIE-1 cells (PD153035 and PD98059; data not shown) or based on product literature (FTI-227 and p160 ROCK). Only FTI-227 is able to completely block soft agar growth of the double transfectants, not an unexpected observation because of the well-known inhibition of both Ras and Rho activity by farnesyltransferase inhibition (29). Thus, activation of signaling pathways such as the traditional Raf/MEK/Erk pathway and the EGFR tyrosine kinase (9), both of which might be expected candidates based on observations in Ras-transformed RIE cells, does not appear to be primarily responsible for transformation in RIE-Raf(22W)/RhoA(63L) cells. To further investigate the Erk pathway in RIE-Raf(22W)/RhoA(63L) cells, the highly specific MEK inhibitor U-0126 was added to cell cultures for 24 h. Results shown in Fig. 5A show that MEK is clearly inhibited by U-0126 in Raf, RhoA, and Raf(22W)/RhoA(63L) double transfectants while levels of total Erk were unchanged. Notwithstanding, the high levels of cyclin D1 in Raf(22W)/RhoA(63L) cells, as shown in Fig. 2A, are not decreased by MEK inhibition. In aggregate, data in Fig. 5, A and B, indicate that signaling pathways leading to transformed cell behavior are preserved in the setting of MEK inhibition.

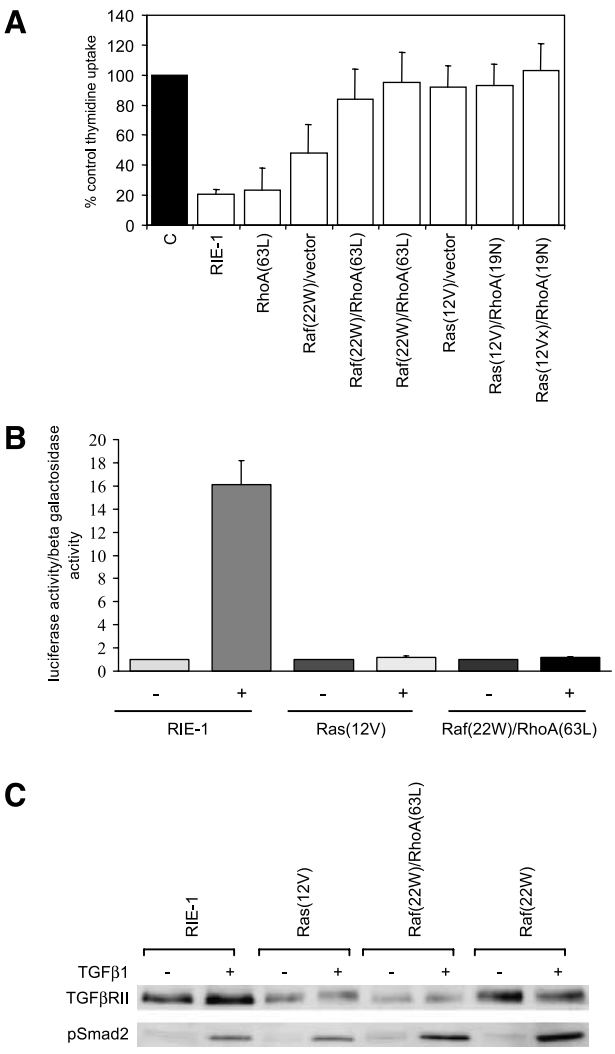

FIGURE 4. Sensitivity of RIE-1 transfectants to growth inhibition by TGFβ. **A.** [^{3}H]thymidine incorporation assays were performed as described in Materials and Methods. Rapidly growing, subconfluent cells were treated with 5 ng/ml TGFβ1 or vehicle for 18 h prior to a 3 h [^{3}H]thymidine pulse. [^{3}H]thymidine incorporation into trichloroacetic acid insoluble material was counted in a scintillation counter and expressed as the mean of quadruplicate experiments done in triplicate assays. Results of two different clones of Raf(22W)/RhoA(63L) and Ras(12V)/RhoA(19N) are shown. **B.** The designated cell line was transiently cotransfected with SBE4-LUC and a pCMV control plasmid as described in Materials and Methods. Cells were treated with TGFβ1 (+) or vehicle (-) for 24 h followed by determination of luciferase activity. Transfection efficiency was low in the parental RIE-1 line; therefore, results were normalized for β-gal activity and expressed relative to treatment with vehicle alone. **C.** Selected components of the TGFβ signaling pathway were also examined in the indicated RIE-1 transfectants. TGFβRII and pSmad2 levels were determined in total cell lysates by Western analysis in the absence (-) or presence (+) of 2 ng/ml TGFβ1 for 30 min.

Dominant-Negative RhoA Does Not Reverse Most Effects of Ras Transformation

In experiments described above, the critical dependence of Ras transformation on RhoA activation was tested by overexpression of a dominant-negative form of RhoA(N19). The transformed morphology, growth in soft agar, and tumor formation in nude mice observed in RIE-Ras cells were not reversed by coexpression of Ras(12V) and RhoA(N19), nor was TGFβ sensitivity restored (Figs. 1 and 4). Similarly, increased expression of TGFα (Fig. 2B) and cyclin D1 (Fig. 6) was not reversed. COX-2 mRNA (Fig. 3B), COX-2 protein (Fig. 5), and PGE_2 levels (Fig. 3C) were all reduced in Ras(12V)/RhoA(N19) cells. This observation is perhaps not unanticipated because RhoA response elements have been identified in the COX-2 promoter (30).

Discussion

In fibroblasts, activation of the Raf/MEK/Erk cascade is the central signaling event leading to transformation by oncogenic Ras. Accumulating data indicate that this is not the case in intestinal epithelial cells (4, 7, 11). Although oncogenic Ras transformation of RIE-1 cells is dependent on activation of Erk, Ras effector domain mutants such as Ras(12V,35S), which do not bind to Raf, cause transformation of RIE-1 cells albeit less efficiently than oncogenic Ras(12V) (31). In addition, overexpression of activated Raf constructs, which are transforming in fibroblasts, are not transforming in intestinal epithelial cells (7). More recently, specific Raf-independent signaling pathways have been identified that, when activated or repressed, cooperate with Raf to effect transformation. For example, inhibition of p38α and p38β kinase activities in RIE-1 cells overexpressing activated Raf results in morphological transformation; conversely, stimulation of p38 activity by overexpression of an upstream kinase antagonizes Ras transformation (11). Activation of JNK occurs in the context of Ras transformation in RIE-1 cells and inhibition of JNK activity using pharmacological inhibitors blocks transformation. These observations suggest that repression of p38 kinase signaling in the context of Raf activation may be sufficient to transform intestinal epithelial cells and that activation of JNK may be important as well. In summary, increasingly specific evidence is now emerging to support the importance of Raf-independent signaling in intestinal epithelial transformation by oncogenic Ras.

In the present study, the potential cooperation between RhoA and activated Raf in intestinal epithelial transformation was examined. RhoA belongs to the large Rho GTPase family, now numbering more than 60 members. Rho proteins function as molecular switches that regulate a diversity of cellular processes, the most extensively studied of which are induction of actin stress fiber assembly and formation of actin-rich cell surface protrusions called lamellipodia and actin-rich membrane extensions called filopodia (32).

Rho proteins also play a key role in regulation of cell proliferation and cell cycle traverse, although these actions are less well studied (33). Early studies in fibroblasts found that expression of activated Rho proteins stimulates G_1 traverse (34)

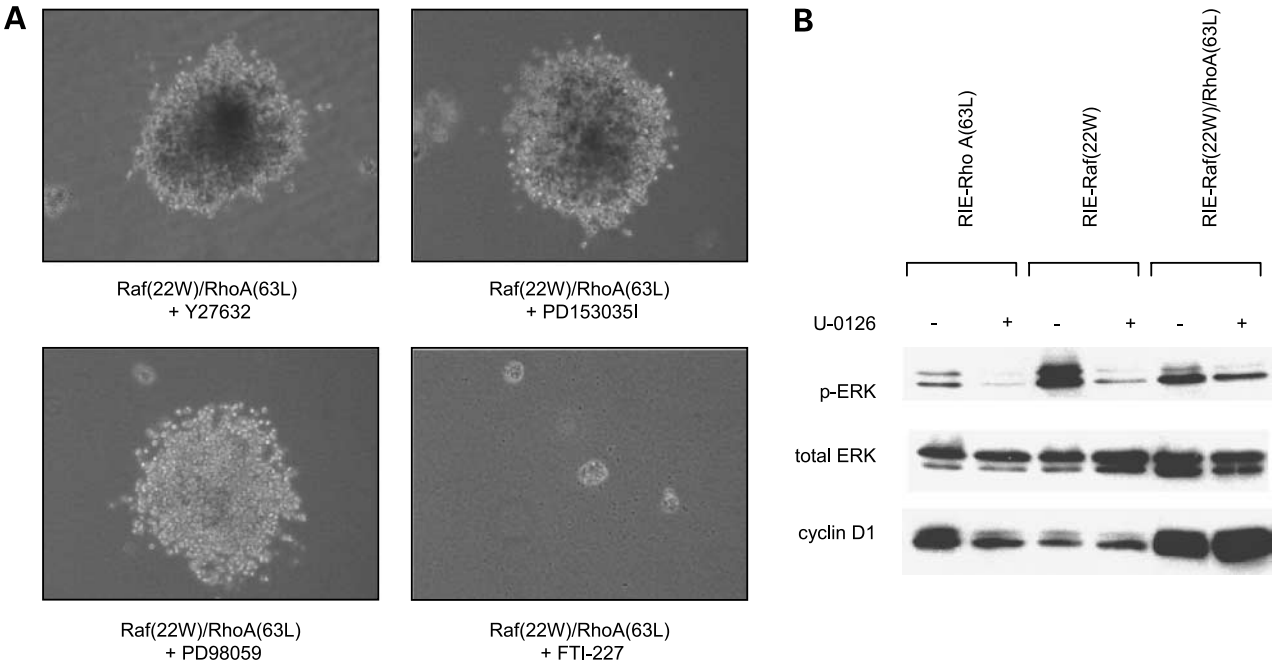

FIGURE 5. Inhibition of anchorage-independent growth of RIE-Raf(22W)/RhoA(63L) by signaling inhibitors. **A.** Cells were grown in soft agar as described in Materials and Methods. Inhibitors of p160 ROCK (Y27632; 20 μg/ml), EGFR tyrosine kinase (PD153035; 10 μg/ml), MEK (PD98059; 25 μg/ml), and farnesyltransferase (FTI-227; 20 μg/ml) were included in the soft agar. The appearance of representative colonies is shown. **B.** Log-phase RIE-RhoA(63L), RIE-Raf(22W), and RIE-Raf(22W)/RhoA(63L) cells were treated with the highly specific MEK inhibitor U-0126 (5 μg/ml) for 24 h. Western blotting was used to assess activated Erk (*p-Erk*), total Erk, and cyclin D1 levels in each cell line in the presence of U-0126 (+) or DMSO (-).

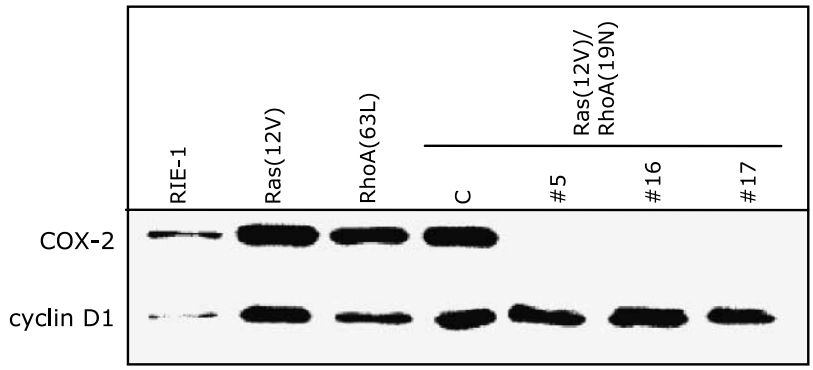

FIGURE 6. Effect of dominant-negative RhoA expression on growth-related protein levels in RIE cells transformed by Ras(12V). COX-2 and cyclin D1 levels were determined by Western analysis using total cell lysates prepared from the indicated cell lines growing logarithmically. *C,* a control for Ras(12V) cells transfected with an empty pcDNA3.1 vector. *#5*, *#16*, and *#17,* representative clones expressing both Ras(12V) and RhoA(19N).

and that Rho is transforming in selected cell types (35). Furthermore, Rho activity is essential for Ras transformation of fibroblasts (14, 36). More recent mechanistic studies implicate Rho-mediated repression of $p21^{waf1/cip1}$ and stimulation of cyclin D1 expression as central events leading to cellular proliferation, including proliferation stimulated by oncogenic Ras in epithelial cells (37–39). Overexpression of the PAK4 serine/threonine kinases, which are effectors of Rho GTPases, maybe a key Rho downstream event in driving transformation in Ras-transformed cells, including fibroblasts and RIE-1 cells (19). In fact, the activity of PAK4, a Rho effector, is increased in a large percentage of human cancer cell lines (19). In contrast to a growing body of literature in cell lines, studies in human cancers are relatively lacking. Overexpression of various Rho GTPases is detectable in human breast tumors (40), but this does not occur as a result of mutational activation or gene amplification. Point mutations in RhoA and other Rho GTPases are also not observed in human colorectal neoplasia (41), although it is certainly possible that activation of Rho activity occurs by a growth factor-mediated autocrine or paracrine mechanism (33).

In the present study, we examined cooperation of Raf and RhoA in regulation of growth-related events in the RIE-1 intestinal epithelial cell line. RhoA was selected for study because it is the Rho protein most characterized as a growth regulator, and the relationship of Rho proteins and cellular transformation is being increasingly recognized (33). We found that stable coexpression of activated Raf and RhoA mutants cooperate to transform RIE-1 cells whereas neither was transforming when expressed alone. We then examined expression of selected genes and proteins that are believed based on prior studies to contribute to the transformed phenotype and that differentiate Ras(12V) from Raf(22W) overexpressing cells (9, 10, 21, 26). Transformation was accompanied by a marked elevation of cyclin D1 expression in all clones isolated, even more so than in Ras-transformed cells. This observation is congruous with prior work in mammary epithelial cells suggesting that RhoA induces cyclin D1 promoter activity and cooperates with activated Ras to further enhance promoter activity (37). Similarly, in Swiss 3T3 fibroblasts, Rho activity is required for sustained Erk activity and induction of cyclin D1 in mid-G_1 (38). Cyclin D1 is emerging as a key oncogene in many cell types, including gastrointestinal epithelial cells (42, 43). Thus, the hyperinduction of cyclin D1 that we observed in RIE-Raf(22W)/RhoA(63L) cells may be a critical cell cycle disturbance resulting in transformation. RIE cells that dually express activated RhoA and Raf also have increased expression of TGFα. This too is a characteristic of Ras-transformed cells. TGFα is an EGF-related peptide that binds to the EGFR and prior studies suggest that its up-regulation may functionally contribute to the Ras-transformed phenotype (9, 25). Notwithstanding, it does not seem likely that elevated levels of EGFR ligands and activation of the EGFR is a central mechanism by which RIE-Raf(22W)/RhoA(63L) are transformed because treatment with an EGFR tyrosine kinase inhibitor does not revert the transformed phenotype (Fig. 4) or reduce elevated cyclin D1 levels (data not shown). Finally, overexpression of COX-2, believed to be an important feature of intestinal epithelial transformation, was not observed in our transformed, dually transfected cells, emphasizing that coexpression of activated RhoA and Raf does not precisely mimic all the complexities of the gene expression profile characteristic of Ras-transformed cells.

Another feature of the transformed intestinal epithelial cells described in this report is resistance to growth inhibition by TGFβ. Resistance to TGFβ is a central feature of transformation of various epithelial cell types transformed by oncogenic Ras, including intestinal epithelial cells. Various mechanisms have been proposed to account for the resistance of Ras-transformed cells to TGFβ, including reduced levels of TGFβRII (10, 24); disturbed translocation of Smad2 and Smad3 into the nucleus following TGFβ treatment (44); accentuated proteasome-mediated degradation of Smad4 (8); stabilization of TGIF, a Smad transcriptional corepressor protein (45); and disruption of TIEG2, a TGFβ-inducible corepressor protein (28). In the present study, we found that transformed RIE-Raf(22W)/RhoA(63L) cells are resistant to growth inhibition by TGFβ and have reduced levels of TGFβRII but normally phosphorylate Smad2 in response to TGFβ treatment as described previously for Ras-transformed cells (28). It is plausible that loss of TGFβ growth inhibitory signaling is also a central component in the transformed behavior of the dually transfected cells.

In summary, dual overexpression of activated RhoA and activated Raf in the RIE intestinal epithelial cell line results in cellular transformation and acquisition of characteristics similar but not identical to Ras-transformed cells. Taken together with other published work, there apparently are several signaling pathways, including the p38 and RhoA pathways that may antagonize or cooperate with activated Raf to cause

transformation in intestinal epithelial cells. Full delineation of these pathways will permit a more complete understanding of the complexities of Raf-dependent and Raf-independent signaling via oncogenic Ras. The recent identification of mutations in RafB in a variety human cancers, including colon cancers, further underscores the importance of understanding the specific signaling pathways that cooperate and interact with the Raf proteins to affect cell proliferation (46). Ultimately, this information will provide a basis for rational design of strategies to interrupt aberrant growth signaling that accommodates the complexity of Ras downstream signaling (47).

Materials and Methods

Cell Lines and Reagents

RIE-1 rat intestinal epithelial cells were obtained from Ken Brown (Cambridge, United Kingdom) and maintained in DMEM supplemented with 5% FCS. These cells are a diploid, nontransformed, EGF- and TGFβ-sensitive, crypt-like cell line derived from rat small intestine. RIE cells stably transfected with mutant-activated Ras, Raf, and RhoA cells have been characterized elsewhere (9). In brief, RIE-Ras cells were established by stable transfection of the parental cells with pSV2-H-Ras(12V) containing human sequences encoding the constitutively active H-Ras(12V) protein. RIE-Raf(22W) cells were obtained by transfection with a constitutively active NH_2-terminal truncated Raf(22W) mutant [pZIP-ΔRaf(22W)] and RIE-RhoA(63L) were obtained by transfection with mutant active RhoA(63L) [pZIP-RhoA(63L)]. In these cells, multiple G418-resistant clones (>50) were pooled for use in subsequent studies.

RIE-Ras were transfected with dominant-negative mutant RhoA(N19) (kindly supplied by Dr. Gary Bokoch) and RIE-Raf cells with hemagglutinin (HA)-tagged RhoA(63L) (kindly supplied by Dr. Melanie Cobb). These constructs were subcloned into pcDNA3.1 and transfected into RIE-Raf(22W) or RIE-Ras(12V) cells using the LipofectAMINE2000 protocol. Stable clones were selected in Zeocin. Expression of HA-tagged RhoA(63L) was verified by Western blot using both anti-HA and anti-Rho(A) antibodies. Successful RhoA activation was determined by affinity precipitation of Rho GTP with immobilized rhotekin (Upstate Cell Signaling Solutions, Waltham, MA).

COX-2 and cyclin D1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD153035 (an EGF tyrosine kinase inhibitor), Y27632 (a p160 ROCK inhibitor), and PD98059 (a MEK inhibitor) were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Secreted PGE_2 was measured by enzyme immunoassay using a kit from Cayman Chemical Co. (Ann Arbor, MI).

Cell Proliferation Assays

[^{3}H]thymidine incorporation assays were carried out in 24-well tissue culture plates. Cells were seeded at a density of 20,000 cells/well, allowed to attach for at least 24 h, and treated as described in the figure legend. A [^{3}H]thymidine (Amersham, Piscataway, NJ) pulse (1 μCi/well) was provided between the 18th and the 21st h of treatment. Radioactivity incorporated into trichloroacetic acid insoluble material was determined by scintillation counting and results are presented as means ± SE for triplicate or quadruplicate measurements. Each experiment was repeated at least thrice.

Soft Agar Assays and Growth in Nude Mice

Cells (1×10^4) were suspended in 1 ml growth medium containing 0.4% low melting temperature agarose with appropriate antibiotics and signaling inhibitors as indicated in figure legends. The suspension was overlaid onto a bottom laye of medium containing 0.8% low melting point agarose in six-well plates. At least three assays were performed. The cultures were incubated at 37°C and 5% CO_2. Colonies were photographed 2 weeks after plating and photographs were taken using a Spot digital imaging system. Colonies were counted manually using an inverted microscope. The tumorigenic potential of each transfected RIE-1 cell line was determined by s.c. injection of 2×10^6 cells into athymic nude mice. Tumor formation was monitored for up to 3 weeks.

Immunoprecipitation and Western Blotting

Cells in 12-well plates or 100 mm culture dishes were solubilized in lysis buffer [20 mM Tris-HCl (pH 7.4), 120 mM NaCl, 100 mM NaF, 200 μM Na_3VO_4, 4 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5% NP40, 2 mM benzamidine] for 30 min at 4°C. The lysate was clarified by centrifugation and the supernatant was incubated overnight with the antibody of interest. Immunoprecipitates were incubated with protein A agarose for 1.5 h and then washed repeatedly in PBS containing 0.05% NP40. The immune complexes were eluted in SDS sample buffer and transferred onto polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine, and 20% methanol buffer at 30 V overnight. Membranes were then blocked, incubated with the diluted antibody of interest, and incubated with horseradish peroxidase-conjugated IgG. The Enhanced Chemiluminescence Plus Detection System (Amersham) was used to detect the antigen-antibody complexes.

Reporter Assays

Cell lines were transiently transfected with 0.65 μg/well SBE4-LUC (kindly provided by Dr. Bert Vogelstein) and 0.35 μg pCMV-β-gal control plasmid using the LipofectAMINE2000 protocol supplied by Invitrogen (Carlsbad, CA). Transfected cells were cultured in DMEM containing 10% FCS for 24 h to permit recovery. TGFβ1 (10 ng/ml) was then added for 24 h. Cell lysates were prepared in reporter lysis buffer and luciferase activities were measured using the Promega Luciferase Assay System (Promega, Madison WI) using a model TD-20/20 luminometer from Turner Designs (Sunnyvale, CA). Values were normalized to β-galactosidase activity to control and adjust for transfection efficiency. Separate assays were performed at least thrice.

TGFα RIA

Confluent parental RIE-1 and each transfected cell line were placed in serum-free medium for 48 h. Cells were lysed at room

temperature in 1 ml or 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% sodium deoxycholate, and 0.5% NP40 for 1 h. The RIA has been described in detail previously (48). Duplicate wells were trypsinized and cells were counted to normalize the data.

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