

A Raf-independent Epidermal Growth Factor Receptor Autocrine Loop Is Necessary for Ras Transformation of Rat Intestinal Epithelial Cells*

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Lisa M. Gangarosa^{‡§}, Nywana Sizemore^{‡§}, Ramona Graves-Deal[§], Sean M. Oldham[¶],
Channing J. Der[¶], and Robert J. Coffey^{§||**}

From the [§]Departments of Medicine and Cell Biology, Vanderbilt University and Veterans Affairs Medical Center, Nashville, Tennessee 37232 and the [¶]Department of Pharmacology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

We recently have shown that activated Ras, but not Raf, causes transformation of intestinal (RIE-1, IEC-6) epithelial cells, whereas both activated Ras and Raf transform NIH 3T3 fibroblasts (Oldham, S. M., Clark, G. J., Gangarosa, L. M., Coffey, R. J., and Der, C. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6924–6928). The observations that conditioned medium from Ras-, but not Raf-, transfected RIE-1 cells, as well as exogenous transforming growth factor α (TGF α), promoted morphological transformation of parental RIE-1 cells prompted us to identify epidermal growth factor (EGF) receptor (EGFR) ligands produced by Ras-transformed RIE-1 cells responsible for this autocrine effect. Since studies in fibroblasts have shown that v-Src is transforming, we also determined if v-Src could transform RIE-1 cells. H- or K-Ras-transformed cells secreted significant amounts of TGF α protein, and mRNA transcripts for TGF α , amphiregulin (AR), and heparin-binding EGF-like growth factor (HB-EGF) were induced. Like Ras, v-Src caused morphological and growth transformation of parental RIE-1 cells. However, TGF α protein was not secreted by RIE-1 cells stably expressing v-Src or activated Raf, and only minor increases in EGFR ligand mRNA expression were detected in these cells. A selective EGFR tyrosine kinase inhibitor PD153035 attenuated the Ras-, but not Src-, transformed phenotype. Taken together, these observations provide a mechanistic and biochemical basis for the ability of activated Ras, but not activated Raf, to cause transformation of RIE-1 cells. Finally, we suggest that an EGFR-dependent mechanism is necessary for Ras, but not Src, transformation of these intestinal epithelial cells.

A remarkable convergence of biological, biochemical and genetic evidence has established that Ras proteins mediate many of their actions via recruitment of Raf-1 to the cell surface, which allows this serine/threonine kinase then to become acti-

ated. Raf-1 causes activation of a mitogen-activated protein kinase (MAPK)¹ kinase (MEK), which in turn phosphorylates p42 and p44 MAPKs. These two MAPKs then translocate to the nucleus where they activate the Elk-1 transcription factor. The ability of dominant negative mutants of Raf-1, MEK, and MAPKs to block oncogenic Ras transformation of rodent fibroblasts demonstrates the essential role of this kinase cascade in Ras function (1–4). However, recent studies also support the importance of Raf-independent effector pathways in mediating Ras transformation (4–6).

Although mutated *ras* genes are most frequently associated with human tumors of epithelial origin (7, 8), most of our knowledge on the signaling pathways that mediate oncogenic Ras function is based on studies of rodent fibroblasts. Therefore, we have been interested in examining transformation by oncogenes such as *ras*, *raf*, and *src* in epithelial cell systems. We have observed that activated Ras, but not Raf, causes transformation of the rat intestinal epithelial cell line RIE-1, as determined by altered morphology, growth in soft agar, and rapid appearance of tumors in nude mice (9). Thus, activation of the Raf/MEK/MAPK cascade alone was not sufficient to cause transformation. Furthermore, our observation that Ras-, but not Raf-, expressing cells secreted factors that promoted RIE-1 morphological transformation suggested that Ras transformation of these epithelial cells was mediated, at least in part, via an autocrine mechanism. Previous studies in other systems have found increased expression of transforming growth factor- α (TGF α) associated with Ras transformation (10–12).

In the present study, we have evaluated the role of EGF receptor (EGFR) ligands in mediating Ras transformation of RIE-1 cells. We observed that Ras-, but not Raf-, expressing cells exhibited increased expression and secretion of TGF α protein. In contrast, Src transformation did not cause up-regulation of TGF α production. Furthermore, inhibition of EGFR function impaired the morphological and growth characteristics of Ras-, but not Src-, transformed cells. These observations distinguish the activities of Ras *versus* Raf and suggest that Ras transformation is mediated by a Raf-independent, EGFR-dependent mechanism. Transformation by v-Src, however, is not dependent on an EGFR autocrine mechanism.

¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; TGF α , transforming growth factor- α ; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding EGF-like growth factor; AR, amphiregulin; BTC, betacellulin; DMEM, Dulbecco's modified Eagle's medium; RIA, radioimmunoassay.

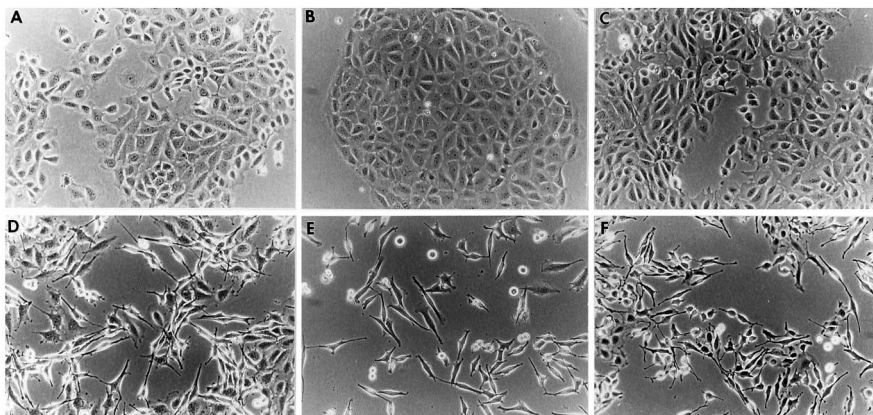
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[‡] Contributed equally to this work.

[¶] Veterans Administration Clinical Investigator.

** To whom correspondence should be addressed: CC-2218 Medical Center North, Vanderbilt University, Nashville, TN 37232-2583. Tel.: 615-343-6228; Fax: 615-343-1591; E-mail: coffeyrj@ctrvax.vanderbilt.edu.

FIG. 1. Morphology of parental RIE-1 cells and oncogene-transfected RIE-1 cells. Cell lines were generated and maintained as discussed under "Materials and Methods." *A*, RIE-1 cells; *B*, RIE-1 pSV2neo cells; *C*, RIE-1 pZIP-*raf* 22W cells; *D*, RIE-1 pSV2-H-*ras* (12V) cells; *E*, RIE-1 pZIP-K-*ras* (12V) cells; and *F*, RIE-1 pZIP/*v-src* cells. Magnification $\times 200$.



MATERIALS AND METHODS

Cell Lines and Reagents

RIE-1 cells were obtained from Dr. Kenneth Brown (Cambridge, UK) and are a diploid, nontransformed, EGF-responsive cell line derived from rat small intestine (13, 14). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen). All assays were done before passage 30. The EGFR kinase inhibitor PD153035 was obtained from Parke-Davis (15).

Constructs and Transfections

The pZIP-K-*ras*(12V) and pZIP- Δ raf22W retrovirus expression vector constructs, which encode transforming mutants of human K-Ras 4B and Raf, respectively, have been described (9, 16). The pSV2-H-*ras* (12V) expression vector construct contains the genomic human sequences encoding the transforming H-Ras(12V) protein and was provided by Dr. Jorge Filmus (Sunnybrook Health/Science Ctr., Toronto, Canada) (17). The *psrc* construct encodes viral Src and was a gift from Dr. Mark Kamps (VCSA, San Diego, CA) (18). The constructs pZIP-K-*ras*(12V), pZIP- Δ raf22W, and *psrc* with pZIP-NeoSV(x)1, as well as the pZIP-NeoSV(x)1 vector control, were each transfected into the RIE-1 cells (1–3 μ g of plasmid DNA/60-mm dish). Transfections were done using 5 μ l of LipofectAMINE (Life Technologies, Inc.) for 16–20 h on cells seeded at $1\text{--}5 \times 10^5$ /60-mm dish. Transfected cells were selected and maintained in medium containing 400 μ g/ml G418 (Life Technologies, Inc.). Multiple G418-resistant colonies (>50) were pooled together for further studies. The pSV2-H-*ras* (12V) as well as the pSV2neo vector control were each transfected into the RIE-1 cells by calcium phosphate precipitation as described previously (19). Transfected cells were selected in medium containing 500 μ g/ml of G418 (Life Technologies, Inc.) and subcloned by limiting dilution.

TGF α Radioimmunoassay

Parental RIE-1 cells and each of the transfected RIE-1 lines were grown to confluence in 24-well dishes, washed in isotonic buffer twice, and switched to serum-free DMEM (1 ml/well) for 48 h, at which time media and lysates were harvested. The cells were washed twice with isotonic buffer and then lysed at room temperature in 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40 (1 ml/well) on a rocker for 1 h. The rat TGF α antibody used for the RIA was developed in collaboration with East Acres Biologicals (Southbridge, MA). The RIA has been described previously in detail (20) and was used to measure TGF α in both the conditioned media and lysates of each transfected cell line. Representative wells were trypsinized, and the cells were counted with a hemacytometer to normalize the data. These experiments were performed on cell lines prior to passage 11.

Isolation of Poly(A)⁺ RNA and Northern Blot Analysis

Cells were grown to near confluence, washed twice with isotonic buffer, and then switched to serum-free DMEM for 72 h. Then total cellular RNA was extracted by the method of Schwab *et al.* (21). Oligo(dT)-selected RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels, and Northern blotting was performed as described previously (22, 23). Hybridizations with species-specific probes labeled by RNA polymerase-directed reverse transcription (EGF, TGF α , amphiregulin (AR), betacellulin (BTC), and 1B15) or random primer extension (heparin-binding EGF-like growth factor (HB-EGF)) were performed in hybridization ovens as described previously (24, 25). 1B15 is a constitutively expressed sequence previously described (26). PhosphorImager

analysis (Molecular Dynamics) was performed to quantify band intensities.

Growth Assays

Anchorage-dependent—Parental, H-Ras, K-Ras, and v-Src RIE-1 cells were plated in 6-well cluster plates at a density of 1×10^6 cells/well. Cells were counted 24 h later to determine plating efficiency. Cells were then treated with the indicated concentrations of PD153035 (10 nM, 25 nM, and 50 nM) or Me₂SO (1 μ l/ml). Cells were treated every other day (days 2 and 4), and triplicate wells were counted by hemacytometer on days 3 and 6.

Anchorage-independent— $1\text{--}20 \times 10^3$ cells/ml of each cell line (H-*ras*-, K-*ras*-, or v-*src*-transfected RIE-1 cells) were plated in 0.4% SeaPlaque agarose (FMC Corp. BioProducts) over a hardened layer of 0.8% agarose. Growth medium containing Me₂SO alone or PD153035 (dissolved in Me₂SO) was added prior to plating. After 7–10 days, colonies in triplicate (>50 μ m) were counted with a colony counter (Bausch & Lomb). Paired *t* test was used to test for significant differences compared with control.

Morphology Experiments

Morphological reversion experiments were performed by adding 1 μ M PD153035 or Me₂SO in growth medium to $\sim 50\%$ confluent *ras*- and *v-src*-transfected RIE-1 cells growing in 6-well dishes. Morphological changes were monitored over the next 72 h and photographs were taken. Conditioned media experiments were performed by harvesting 48-h serum-free conditioned medium from the Ras-transformed and control cell lines. The conditioned media were then filtered through a 0.2 micron filter and added to 50% confluent parental RIE-1 cells at a 3:1 ratio with fresh serum-free medium alone or with 1 μ M PD153035 or 3 μ l/ml anti-TGF α antibody S-574. The cells were examined for morphological changes over the next 48 h and photographed.

EGFR Analysis

Parental and H-Ras RIE-1 cells were plated in 24-well cluster dishes at a density of 1×10^5 cells/well and allowed to grow to 70–80% confluence. Cells were then treated for 24 h with 1 μ M PD153035 or Me₂SO followed by a 5-min pulse with TGF α (10 ng/ml) where indicated. Cells were then lysed with buffer containing 20 mM Hepes, pH 7.4, 1% (w/v) Triton X-100, 2 mM EGTA, 2 mM EDTA, 500 μ M Na₃VO₄, 50 μ M PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Samples of equal protein concentrations were run on SDS-polyacrylamide gel electrophoresis and Western blotted onto polyvinylidene difluoride membranes. The Western blot was probed with anti-human EGFR sheep polyclonal (Upstate Biotechnology) and detected with a donkey anti-sheep horseradish peroxidase-conjugated IgG secondary antibody (Jackson Laboratories) followed by ECL chemiluminescence (Amersham Life Science Inc.) and autoradiography. The phosphorylated tyrosine content of the receptor was determined by subsequently probing the Western blot with an horseradish peroxidase-conjugated anti-phosphotyrosine, clone 4G10 (Upstate Biotechnology), followed by ECL chemiluminescence detection (Amersham Life Science Inc.) and autoradiography.

RESULTS

v-Src, Like Activated Ras, Transforms RIE-1 Cells—We recently have reported that constitutively activated Ras, but not Raf, caused transformation of RIE-1 cells and that Ras-condi-

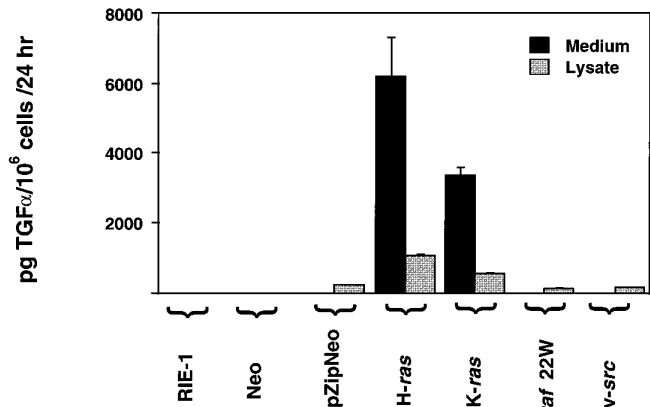


FIG. 2. TGF α protein production in conditioned media and cell lysates of parental and oncogene-transfected RIE-1 cells. TGF α protein was measured by RIA in 48-h serum-free conditioned medium and cell lysates as described under "Materials and Methods."

tioned medium morphologically transformed parental RIE-1 cells (9). Since studies in fibroblasts have demonstrated that v-Src causes transformation, we examined whether RIE-1 cells were also transformed by v-Src. A line of RIE-1 cells was generated with a v-src construct. We initially compared the morphological appearance of the parental RIE-1 cells (Fig. 1A) with that of those transfected with pSV2-H-ras(12V) (Fig. 1D), pZIP-K-ras(12V) (Fig. 1E), and psrc (Fig. 1F). RIE-1 cells stably expressing oncogenic H-Ras, K-Ras, and v-Src showed morphological alterations from a flat monolayer of cuboidal cells to spindle-shaped cells with a poorly adherent, highly refractile subpopulation. The H-Ras- and v-Src-expressing RIE-1 cells, like the recently described K-Ras-expressing RIE-1 cells (9), also grew in soft agar and formed tumors rapidly in nude mice (data not shown). The RIE-1 cells transfected with pZIP- Δ raf22W (Fig. 1C), the pSV2neo vector (Fig. 1B), and the pZIP-Neo SV(x)1 vector (data not shown) appeared identical in morphology to parental cells. Thus, in contrast to activated Raf, v-Src caused a fully transformed phenotype that was similar to that caused by activated Ras.

TGF α Production by Activated Ras-, but Not Raf- or Src-, Expressing RIE-1 Cells—Studies then were directed toward identification of factors that contribute to the morphological changes induced by Ras-conditioned medium. Since administration of TGF α to parental RIE-1 cells led to morphological alterations similar to Ras-conditioned medium (9), we initially examined TGF α protein production by Ras-transformed cells and compared the results with raf- and src-transfected cells. RIE-1 cells transfected with activated H-ras and K-ras secreted ~3–6 ng of TGF α /10⁶ cells/48 h into conditioned media, and ~500–1000 pg of TGF α /10⁶ cells were measured in cell lysates (Fig. 2). Neither the parental RIE-1 cells nor the cells transfected with activated raf, v-src, or control vectors had detectable TGF α in their conditioned media (Fig. 2). Less than 100 pg of TGF α /10⁶ cells was detected in cell lysates of pZIPneo-, raf-, and v-src-transfected RIE-1 cells. Thus, production of TGF α clearly distinguishes Ras- from Raf-expressing RIE-1 cells. Furthermore, v-Src-mediated transformation was not associated with increased TGF α protein levels, and thus, production of TGF α is not required for RIE-1 cellular transformation. Administration of a neutralizing TGF α antibody to Ras-conditioned medium was able to partially revert morphological transformation of parental RIE-1 cells (Fig. 6C) although it was able to completely block the effects of exogenous TGF α (data not shown), suggesting that additional factors participate in Ras morphological transformation.

Ras, Src, and Raf Exhibit Differential Expression of EGFR

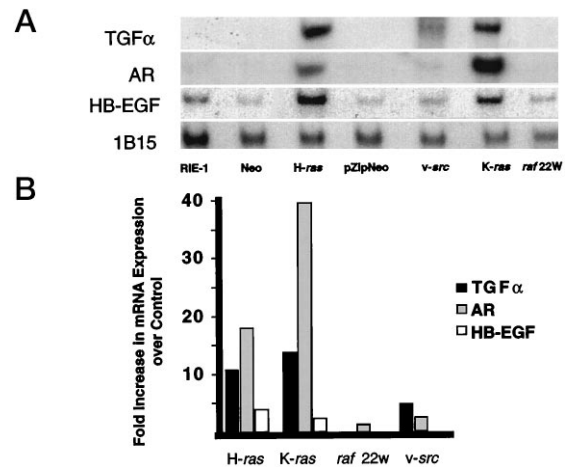


FIG. 3. mRNA expression of EGFR ligands in parental and oncogene-transfected RIE-1 cells. Poly(A)⁺ RNA was isolated from near confluent cultures incubated in serum-free medium for 72 h. Two μ g of RNA were loaded per lane. Northern blots were prepared and probed with ³²P-labeled complementary sequences as described in "Materials and Methods." Blots were scanned with a phosphorimager to obtain quantification of band intensities. The transcript signals of each lane were normalized to the 1B15 loading control signal in that lane. A, northern blot; B, relative EGFR ligand expression represented as -fold increase over neo control.

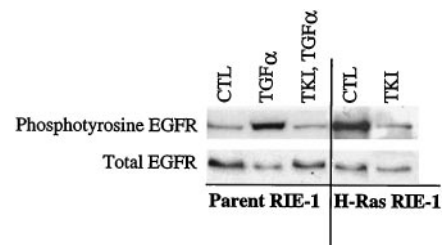
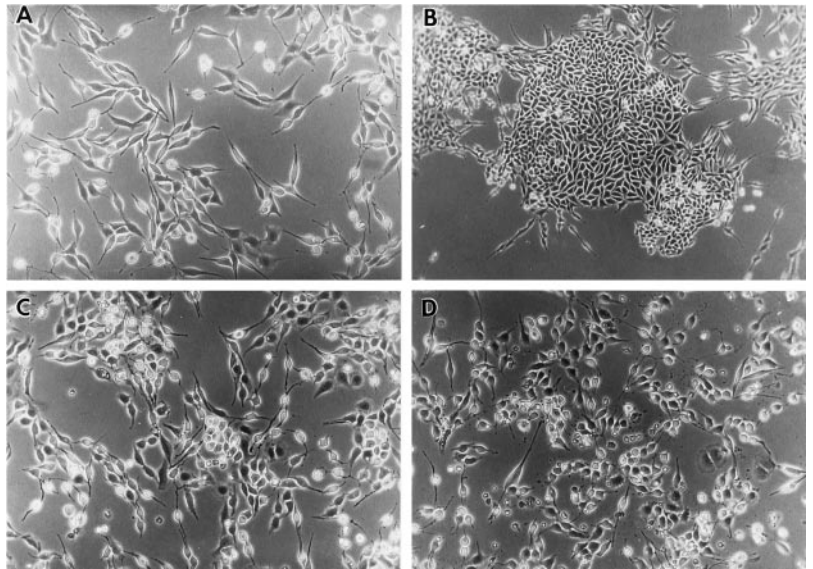


FIG. 4. Effects of EGFR tyrosine kinase inhibition on TGF α -induced and basal EGFR tyrosine phosphorylation in parental and Ras-transformed RIE-1 cells. Parental and H-Ras RIE-1 cells were treated for 24 h with 1 μ M PD153035 or Me₂SO (control, CTL) and then pulsed with TGF α (10 ng/ml). Cells were lysed, and samples of equal protein content were electrophoresed on SDS-polyacrylamide gels. Total EGFR and tyrosine-phosphorylated EGFR content were determined separately by Western blot analyses using specific antibodies as described under "Materials and Methods."

Ligands—We have reported previously that EGFR ligand expression is coordinantly regulated in parental RIE-1 cells (27), and up-regulation of several EGFR ligands has been observed in keratinocytes infected with v-H-ras (12). Inasmuch as reliable protein assays are not available for AR and HB-EGF, we examined expression of mammalian EGFR ligands by Northern blot analysis. Poly(A)⁺ RNA was isolated from near confluent cultures of each cell line maintained serum-free for 72 h. Primary data are shown in Fig. 3A and phosphorimager quantification of band intensities are depicted in Fig. 3B. Results are expressed as -fold increase over appropriate neo control, after normalization to the constitutively expressed 1B15 mRNA of each lane. The only transcript detected in parental RIE-1 cells and RIE-1 cells transfected with vector controls was HB-EGF. RIE-1 cells transfected with H-ras and K-ras constructs exhibited a greater than 10-fold induction of signals for AR and TGF α and a 2–3-fold increase in HB-EGF mRNA expression. In contrast to Ras-transformed cells, raf- and v-src-transfected cells had less than a 2-fold increase in AR and HB-EGF expression. A TGF α transcript was observed in v-Src-transformed cells although no TGF α protein was detected as noted above. Transcripts for EGF and BTC were not seen in any of the cell lines. Thus, selective overexpression of TGF α ,

FIG. 5. Effects of EGFR tyrosine kinase inhibition on morphological appearance of K-Ras- and v-Src-transformed RIE-1 cells. Me₂SO (control) or 1 μ M PD153035 was added to the growth media as described under "Materials and Methods," and photographs of the cells were taken 72 h later. *A*, K-Ras-transformed RIE-1 cells plus control; *B*, K-Ras-transformed RIE-1 cells plus PD153035; *C*, v-Src-transformed RIE-1 cells plus control; *D*, v-Src-transformed RIE-1 cells plus PD153035. Magnification $\times 200$.



AR, and HB-EGF distinguishes Ras RIE-1 cells from both nontransformed Raf and transformed v-Src RIE-1 cells.

EGFR Kinase Inhibition Selectively Alters the Morphology and Growth of Ras-transformed RIE-1 Cells—PD153035 has been demonstrated to be a highly specific EGFR tyrosine kinase inhibitor (15). The ability of this compound to block EGFR function in RIE-1 cells is shown in Fig. 4. Pretreatment with PD153035 (1 μ M) blocked TGF α -induced EGFR tyrosine phosphorylation in serum-starved parental RIE-1 cells (Fig. 4). This agent was then used to determine the contribution of up-regulated EGFR ligands to the Ras-transformed phenotype under four experimental conditions. First, PD153035 (1 μ M) reduced basal EGFR tyrosine phosphorylation in H-Ras-transformed RIE-1 cells (Fig. 4). Based on the previous data, we suggest that this enhanced basal EGFR phosphorylation is likely due to increased levels of endogenous EGF-like ligands activating the EGFR in Ras-transformed cells. Second, addition of 1 μ M PD153035 to RIE-1 cells expressing activated K-Ras caused morphological reversion to a more normal appearance within 72 h (Fig. 5). Third, addition of the compound to conditioned medium from K-Ras-transformed cells prevented morphological transformation of parental RIE-1 cells caused by K-Ras-conditioned medium alone (Fig. 6). Finally, PD153035 caused a dose-dependent reduction in the ability of H- and K-Ras-transformed RIE-1 cells to proliferate on plastic (Fig. 7). We observed, however, that PD153035 treatment of Src-transformed cells failed to cause any significant morphological reversion (Fig. 5) and did not inhibit the monolayer growth of these Src-transformed cells or parental RIE-1 cells (Fig. 7). This agent also significantly reduced soft agar growth of H- and K-Ras-, but not v-Src-, transformed RIE-1 cells (data not shown). Thus signaling through the EGFR contributes significantly to Ras transformation of RIE-1 cells, but Src transformation does not appear dependent on this pathway.

DISCUSSION

These results provide a mechanism to explain why activated Ras, but not activated Raf, transforms RIE-1 cells (9). Herein, we have shown that there is up-regulation of EGFR ligand expression in activated *ras*-, but not *raf*-, transfected RIE-1 cells and that autocrine growth factor signaling through the EGFR contributes significantly to the Ras-transformed phenotype. Additional recent support for the importance of EGFR signaling in mediating the Ras-transformed phenotype comes from studies using EGFR "knock-out" keratinocytes infected

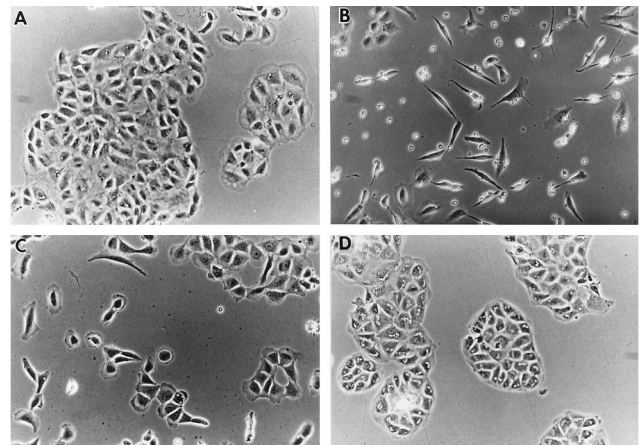


FIG. 6. Effects of TGF α antibody neutralization and EGFR tyrosine kinase inhibition on morphological alterations of parental RIE-1 cells induced by Ras-conditioned medium. Conditioned medium from vector control or K-Ras-transfected RIE-1 cells alone or together with either anti-TGF α antibody (1:250) or PD153035 (1 μ M) was added to parental RIE-1 cells as described under "Materials and Methods." Photographs were taken 48 h later. *A*, control conditioned medium; *B*, K-Ras-conditioned medium; *C*, K-Ras-conditioned medium plus anti-TGF α antibody; *D*, K-Ras-conditioned medium plus PD153035. Magnification $\times 200$.

with H-*ras* retroviral constructs. As compared with H-*ras*-infected, wild-type keratinocytes, H-*ras*-infected EGFR $-/-$ cells form smaller tumors in a primary engrafted papilloma model.² The failure of Raf-expressing cells to cause induction of EGFR ligand expression suggests that Ras activates a Raf-independent pathway that promotes EGFR ligand expression. Studies are underway to identify the Ras signaling pathway that is responsible for EGFR ligand overexpression.

These studies do not delineate the role of individual EGFR ligands in contributing to the Ras-transformed phenotype. Filmus *et al.* (28) studied H-*ras* transformation of an immature rat epithelial crypt cell line (IEC-18) and also found TGF α mRNA induction and increased protein production. Soft agar growth of the H-*ras*-transfected IEC-18 cells was attenuated with both TGF α neutralizing antibodies and anti-sense TGF α construct transfection although transfection of IEC-18 cells

² A. A. Dlugosz, R. J. Coffey, and S. H. Yuspa, unpublished observations.

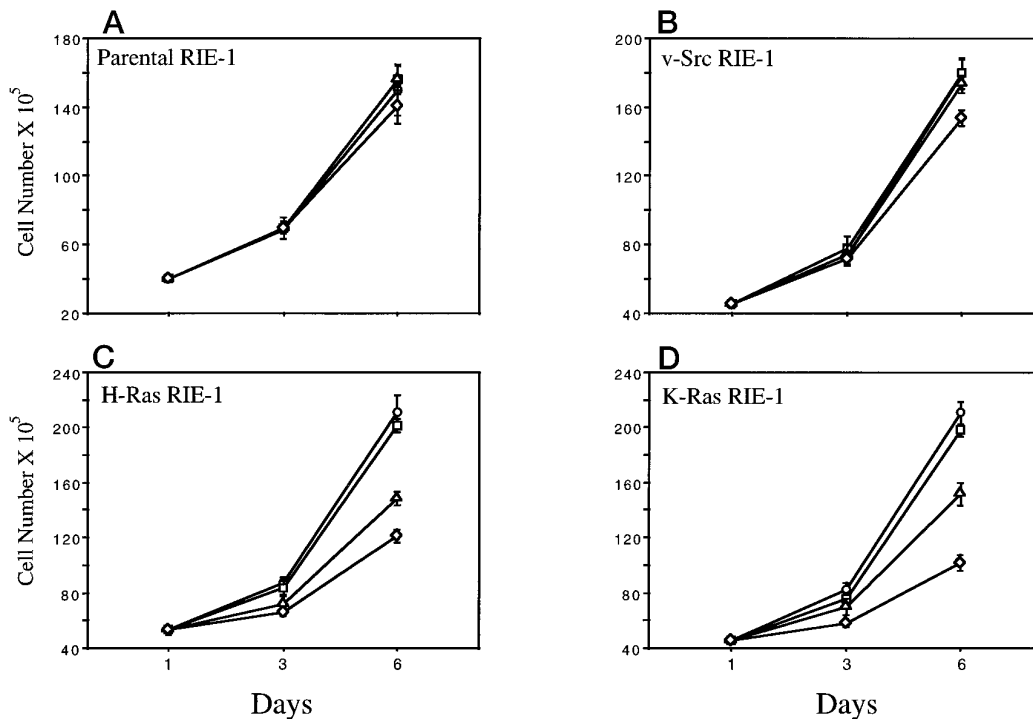


FIG. 7. Effects of EGFR tyrosine kinase inhibition on anchorage-dependent growth of parental RIE-1 cells and its transformants. Cells were plated on plastic as described under "Materials and Methods" either in the presence of increasing doses of PD153035 or Me₂SO (control). Cell number was determined by hemacytometer in triplicate wells.

with TGF α was not sufficient to transform these cells. Expression of other EGFR ligands was not examined in this study. The observation that EGFR blockade (via pharmacological inactivation of its tyrosine kinase (Fig. 6D)), but not neutralization of TGF α (via antibody neutralization (Fig. 6C)), is able to fully revert morphological alterations induced by Ras-conditioned medium suggests that secreted AR and HB-EGF contribute to this effect. It is possible that TGF α along with AR and/or HB-EGF act in concert to mediate morphological transformation of RIE-1 cells. Future studies will address whether overexpression of AR or HB-EGF may be sufficient to transform RIE-1 cells or whether a combination of EGFR ligands is necessary.

v-Src transformation of RIE-1 cells appears to be mediated by a mechanism distinct from that of Ras. In contrast to events related to Ras transformation of RIE-1 cells, v-Src cells do not highly express EGFR ligand transcripts nor produce TGF α protein, and administration of a specific EGFR tyrosine kinase inhibitor did not lead to morphological reversion or a decrease in growth of these Src-transformed cells. Collectively, these data indicate that Src transformation of RIE-1 cells occurs independently of EGFR-mediated events. Thus, whereas Src transformation of rodent fibroblasts is dependent on Ras function, a similar requirement may not be involved in Src transformation of RIE-1 cells. Ras-independent Src signaling events have been observed recently (29, 30). Whether Ras activation is required for Src transformation awaits future analyses.

These results in intestinal epithelial cells underscore the potentially important distinct pathways and mechanisms underlying oncogenic transformation of epithelial cells and fibroblasts. This is not unanticipated in view of marked differences in the growth regulation of these two cell types. For example, platelet-derived growth factor is a mitogen for fibroblasts, but epithelial cells are nonresponsive due to their lack of platelet-derived growth factor receptors. TGF β stimulates growth of fibroblasts, whereas it is a potent epithelial cell growth inhibitor. Moreover, half-lives of farnesylated Ras proteins markedly

differ between fibroblasts and epithelial cells in the presence of farnesyltransferase inhibitors.³ Finally, activated *ras* and *raf* constructs transform NIH 3T3 fibroblasts, whereas only the former is able to transform intestinal and mammary epithelial cells (6). An important lesson from these studies is that one cannot necessarily extrapolate results from growth regulation and oncogene transformation of fibroblasts to epithelial cell systems.

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