Activation of the Raf-1/MAP kinase cascade is not sufficient for Ras transformation of RIE-1 epithelial cells

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ABSTRACT The potent transforming activity of membrane-targeted Raf-1 (Raf-CAAX) suggests that Ras transformation is triggered primarily by a Ras-mediated translocation of Raf-1 to the plasma membrane. However, whereas constitutively activated mutants of Ras [H-Ras(61L) and K-Ras4B(12V)] and Raf-1 (RARaf-22W and Raf-CAAX) caused indistinguishable morphologic and growth (in soft agar and nude mice) transformation of NIH 3T3 fibroblasts, only mutant Ras caused morphologic transformation of RIE-1 rat intestinal cells. Furthermore, only mutant Ras-expressing RIE-1 cells formed colonies in soft agar and developed rapid and progressive tumors in nude mice. We also observed that activated Ras, but not Raf-1, caused transformation of IEC-6 rat intestinal and MCF-10A human mammary epithelial cells. Although both Ras- and RARaf-22W-expressing RIE-1 cells showed elevated Raf-1 and mitogen-activated protein (MAP) kinase activities, only Ras-transformed cells produced secreted factors that promoted RIE-1 transformation. Incubation of untransformed RIE-1 cells in the presence of conditioned medium from Ras-expressing, but not RARaf-22W-expressing, cells caused a rapid and stable morphologic transformation that was indistinguishable from the morphologic of Ras-transformed RIE-1 cells. Thus, induction of an autocrine growth mechanism may distinguish the transforming actions of Ras and Raf. In summary, our observations demonstrate that oncogenic Ras activation of the Raf/MAP kinase pathway alone is not sufficient for full tumorigenic transformation of RIE-1 epithelial cells. Therefore, Raf-independent signaling events are essential for oncogenic Ras transformation of epithelial cells, but not fibroblasts.

Ras proteins are GDP/GTP-regulated switches that function downstream of receptor tyrosine kinases and upstream of a cascade of serine/threonine kinases that include the mitogen-activated protein (MAP) kinases (1–3). Upon activation by ligand-stimulated receptors, activated Ras complexes with and promotes the activation of the Ras-1 serine/threonine kinase. Raf-1 then activates MAP kinase kinases (MEK1 and MEK2), which in turn activate p42 and p44 MAP kinases also referred to as extracellular signal regulated kinases (ERKs). The central role of the Raf-1/MAP kinase pathway in Ras-mediated transformation of fibroblasts is supported by the observations that kinase-deficient mutants of Raf-1, MEK, and MAP kinases are potent inhibitors of Ras signal transduction and transformation (4–9). Furthermore, since constitutively activated mutants of Raf-1 or MEK cause tumorigenic transformation of NIH 3T3 cells (4, 5, 10), activation of the Raf-1/MAP kinase pathway alone is believed to be sufficient to mediate Ras transforming activity.

The precise mechanism by which Ras triggers Raf-1 activation remains to be determined. However, the recent demonstration that addition of the Ras COOH-terminal plasma membrane targeting sequence to Raf-1 converted it to a potent transforming protein suggested that Ras transformation is mediated, in large part, by promoting the translocation of Raf-1 to the plasma membrane (11, 12). Once at the membrane, additional Ras-independent events occur to complete the activation of Raf-1 kinase activity (13, 14). These observations, taken together with the comparable transforming potencies and properties of activated Ras and Raf-1 in rodent fibroblast transformation assays, support the possibility that Ras transformation is mediated solely through activation of the Raf-1/MAP kinase cascade in these cells.

Despite evidence that Raf-1 is a critical downstream target for Ras, there is increasing evidence that Ras may mediate its actions by stimulating multiple downstream targets, of which Raf-1 is only one. First, the recent identification of a mutant Ras protein that failed to bind Raf-1 yet retained signaling activities that contribute to Ras transformation suggested that Raf-1-independent pathways are also important for promoting full Ras transformation (15). Second, genetic studies of S. pombe Ras (ras1) function have identified two distinct ras1 effector-mediated activities (16). One involves ras1 interaction with byr2 (a MEK kinase homolog), and the other is triggered by ras1 interaction with scd1 (a putative Rho guanine nucleotide exchange factor). scd1 in turn may regulate the function of the cdc42p Rho family protein. Evidence that Ras transformation is also mediated by Rho family proteins in mamalian cells includes recent observations that the function of three Rho family proteins (RhoA, RhoB, and Rac1) are necessary for full Ras transformation activity (17–19). Finally, the increasing number of candidate Ras effectors provides additional support for the existence of Raf-independent Ras signaling pathways (20). Included in this expanding roster of functionally diverse proteins are the two Ras GTPase activating proteins (p120 and NF1 GTPase activating proteins), two guanine nucleotide exchange factors of the Ras-related proteins RaIa and RaIb (Rag1DS and RGL) (21–23) and phosphatidylinositol-3-OH kinase (24). Like Raf-1, these proteins show preferential binding to active Ras-GTP and require an intact Ras effector domain (residues 32–40) for this interaction. Presently, the contribution of these candidate effectors to Ras signal transduction and transformation has not been determined.

Although mutant Ras is most frequently associated with epithelial cell-derived tumors (25), the majority of Ras signal transduction and transformation studies have been performed in rodent fibroblast cells (1–3). Therefore, we were interested in addressing the possibility that the signaling pathways in

Abbreviations: MAP, mitogen-activated protein; TGF-α; transforming growth factor type α; ERK, extracellular signal regulated kinase.

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volved in oncogenic Ras transformation of epithelial cells may differ from those required for transformation of NIH 3T3 cells. Unexpectedly, whereas constitutively activated mutants of Ras and Raf-1 showed comparable transformation of NIH 3T3 cells, only mutant Ras could cause potent tumorigenic transformation of RIE-1 cells. Furthermore, we determined that constitutively activated Ras, but not Raf-1, caused activation of a potent autocrine mechanism that contributed significantly to RIE-1 transformation.

MATERIALS AND METHODS

Molecular Constructs. Mammalian expression vectors containing cDNA sequences for human H-ras, K-ras4B, and c-ras-1 were generated using the pZIP-NeoSV(x) retrovirus vector (neomycin resistant), where expression of the inserted gene is regulated from the Moloney long terminal repeat promoter. The pZIP-rasH(61L) and pZIP-rasK(12V) retrovirus expression vector constructs, which encode transforming mutants of human H-Ras(61L) and K-Ras(12V), respectively, have been described (26, 27). pZIP-Δraf22W and pZIP-raf-CAAX encode transforming mutants of human c-Raf-1. ΔRaf-22W is activated by NH2-terminal truncation (28), whereas Raf-CAAX is a chimeric protein that contains the COOH-terminal 18-aa plasma membrane-targeting sequence from K-Ras4B at the COOH terminus of full-length human Raf-1. Recent studies have shown that membrane-targeted Raf-1 shows potent transforming activity in NIH 3T3 cells (11, 12).

Cell Culture and Transformation Assays. RIE-1 rat intestinal epithelial cells were maintained in DMEM supplemented with 5% fetal calf serum. DNA transfections (0.1–10 μg of plasmid DNA per 60-mm dish) were done using 5 μl of Lipofectamine (GIBCO/BRL) for 16–20 hr on cells seeded at 1–5 × 105 per 60-mm dish. NIH 3T3 cells were grown in DMEM supplemented with 10% calf serum. DNA transfections (10–25 ng plasmid DNA per 60-mm dish) were done using calcium phosphate precipitation (29). Transformed foci were quantitated 21 (RIE-1) or 14–16 (NIH 3T3) days after transfection. Representative dishes were stained with crystal violet to visualize transformed foci.

To isolate cell lines stably expressing mutant Ras or Raf-1 proteins, NIH 3T3 and RIE-1 cultures were transfected with the neo-resistant pZIP expression plasmids and were maintained in growth medium supplemented with 400 μg/ml G418 (GIBCO/BRL). Multiple G418-resistant colonies were then pooled together (>50 colonies) and used for growth transformation assays. To assess colony formation in soft agar, each transfected cell line was seeded at 103 to 106 cells per 60-mm dish in growth medium containing 0.3% agar over a base layer of 0.6%. Tumorigenic growth potential of the transfected RIE-1 cells was determined by subcutaneous inoculation into athymic nude mice (1 × 106 cells per site) using procedures that we have described previously (29).

Raf-1 and MAP Kinase Assays. Laemmli protein sample buffer lysates of each transfected cell line were resolved by SDS/PAGE and transferred onto Immobilon filters for Western blot analyses with the C-12 anti-Raf-1 or K-23 anti-MAP kinase (p42 and p44) antisera (Santa Cruz Biotechnology). Detection of secondary antibody was done by enhanced chemiluminescence (Amersham). MAP kinase activation was determined as described previously in serum-starved cells by Western blot analysis to detect the phosphorylated, active and nonphosphorylated, inactive forms of p42MAPK/ERK2 and p44MAPK/ERK1 (7). The MAP kinase immunocomplex assay was carried out by incubating the immunoprecipitated MAP kinase with myelin basic protein in a kinase assay for 30 min at room temperature. The reactions were then stopped using 2× SDS sample buffer. The proteins were then separated on an SDS/15% polyacrylamide gel and visualized by autoradiography (30). Raf-1 kinase activity was determined by immunoprecipitation of Raf-1 using the C12 anti-Raf-1 antiserum from detergent lysates [modified RIPA buffer: 150 mM NaCl/1% (vol/vol) Nonidet P-40/50% (wt/vol) sodium deoxycholate/5 mM EDTA/50 mM Hepes, pH 7.5/1 mM Na3VO4/50 mM NaF/1 μM okadaic acid/1 mM phenylmethylsulfonyl fluoride/5 mM benzamidine/0.1% (vol/vol) aprotinin] derived from each cell line. The immune-complexed Raf-1 was then combined with 26 μl of kinase mix, which consisted of 4 μl of 10× universal kinase buffer (0.1 M Tris·HCl, pH 7.5/0.1 M MgCl2/10 mM DTT), 1 mM ATP, and 5 μCi of 32P-ATP, and 0.5 μg of recombinant human wild-type MEK1 for 15 min, and then 2.0 μg of recombinant kinase-deficient MAP kinase was added for an additional 15 min. The reaction was terminated by the addition of Laemmli protein sample buffer.

Conditioned Media Assay. After 2 days, 5 ml of medium was collected from confluent cultures of RIE-1 cells stably transfected with either the empty pZIP-NeoSV(x) vector or pZIP constructs encoding mutant Ras or ΔRaf-22W proteins. After filtration through a 0.22 μm filter, the different conditioned media, or fresh growth medium supplemented with 20 ng/ml transforming growth factor type α (TGF-α), were added onto subconfluent cultures (103 to 105 cells per 60-mm dish) of untransformed RIE-1 cells. Cells were monitored for 24–48 hr for morphological changes, and photographs were taken after 18 hr.

RESULTS

RIE-1 is an established rat intestinal cell line that displays properties of normal epithelial cells (31, 32). We first deter-
determined the sensitivity of RIE-1 cells to transformation by constitutively activated mutants of Ras and Raf-1. For these analyses, we used expression vectors that encoded oncogenic H-Ras(61L) or K-Ras4B(12V) and two different activated mutants of Raf-1 (ΔRaf-22W and Raf-CAAX). The Ras and Raf expression vectors were previously shown to cause comparable focus-forming activities in NIH 3T3 assays (3–4 × 10^5 foci/µg or 1–2 × 10^6 foci/µg of transfected plasmid DNA, respectively) (26, 28). Surprisingly, whereas oncogenic mutants of Ras readily induced transformed foci in RIE-1 cultures that were transfected with as little as 100 ng of plasmid DNA (~50 foci/µg of DNA) (Fig. 1A), no focus-forming activity was observed in cultures transfected with up to 10 µg of plasmid DNA encoding the two Raf-1 mutants (Fig. 1B) (11, 12, 28). Thus, activated Raf-1, but not Ras, showed differential abilities to cause focus-formation in RIE-1 and NIH 3T3 cells.

We next evaluated the biological properties of RIE-1 cells stably transfected with constructs encoding the different transforming mutants of Ras and Raf-1. Whereas untransformed RIE-1 cells displayed a very flat, well-adherent and nonretractile appearance, Ras-transformed RIE-1 cells were very retractile and poorly adherent (Fig. 2A). In contrast, the morphology of ΔRaf-22W- or Raf-CAAX-transfected cells was indistinguishable from the control RIE-1 cells, which were transfected with the empty pZIP-NeoSV(x)1 retrovirus expression vector. Furthermore, Raf-transfected, but not Ras-transfected, cells showed the ability to form colonies in soft agar (Fig. 2B). Finally, we determined whether mutant Ras or Raf expression caused tumorigenic transformation of RIE-1 cells. Inoculation of Ras-transformed cells into athymic nude mice caused rapidly growing tumors that were greater than 1 cm in diameter within 6 days. In contrast, ΔRaf-22W-transfected cells were negative for tumor formation until day 27, at which time slow-growing tumors became detectable. Data from a representative nude mouse assay are summarized in Table 1. These results contrast with the analyses of NIH 3T3 cells, where both Raf- and Ras-transformed NIH 3T3 cells are highly tumorigenic in nude mice.

The failure of both Raf-1 expression constructs to cause transformation of RIE-1 cells may simply be due to the absence of Raf-1 protein expression from these exogenously introduced naf expression constructs. To address this possibility, we performed Western blot analysis using the C-12 anti-Raf-1 antisera on cell lysates from stably transfected RIE-1 cells. Whereas RIE-1 cells stably transfected with the pZIP-Δraf22W construct showed high levels of the NH2-terminal truncated ΔRaf-22W (~37 kDa) protein (Fig. 3A), we could not readily detect Raf-CAAX expression in the stably transfected RIE-1 cells. This may be due to the apparent growth inhibitory activity that we have observed with Raf-CAAX in

![Fig. 2](image-url)
we measured Raf-1 kinase in cells, activated in both vector-transfected and transfected with the indicated plasmid DNA.

Table 1. Tumorigenicity analysis of Ras- and Raf-expressing RIE-1 cells

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Day of appearance†</th>
<th>Day of death</th>
<th>Mean volume, mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>pZIP-rasK(12V)</td>
<td>6 (4/4)</td>
<td>19</td>
<td>1436.3</td>
</tr>
<tr>
<td>pZIP-rf22W</td>
<td>27 (4/4)</td>
<td>36</td>
<td>510.5</td>
</tr>
<tr>
<td>pZIP-NeoSV(x)1</td>
<td>— (0/4)</td>
<td>42</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A, not applicable.

*Pooled populations of G418-resistant colonies transfected with either
vector transfection.
†Number of cells positive for tumor formation/number of animals injected.

RIE-1 (and NIH 3T3) cells and suggests that the biological properties of these two Raf-1 mutants are not identical. Whereas RIE-1 cultures transfected with either empty vector, pZIP-ras, or pZIP-Araf22W, followed by selection in G418-containing growth medium, resulted in the efficient appearance of drug-resistant colonies, a considerably reduced frequency of colonies was observed with cultures transfected with pZIP-rf-CAAX (data not shown). However, the high levels of Araf-22W expression indicate that the failure of this mutant to cause transformation is not due to lack of expression.

Neither NH₂-terminal truncation (ΔRaf-22W) nor the addition of a plasma membrane targeting sequence (Raf-CAAX) alone is sufficient to activate Raf-1 kinase activity (13, 14). Thus, it is possible that other events required to trigger the activation of ΔRaf-22W or Raf-CAAX transforming activity may not occur in RIE-1 cells (14). To address this possibility, we measured Raf-1 kinase activity in Ras- and Raf-expressing cells using an in vitro MEK-dependent MAP kinase phosphorylation assay. Whereas Raf-1 kinase activity was low in control, vector-transfected cells, Raf-1 kinase activity was greatly elevated in both oncogenic Ras- and ΔRaf-22W-expressing cells (Fig. 3B). Additionally, we detected low levels of constitutively activated MAP kinase activity in both Ras- and Raf-expressing cells by the appearance of the slower migrating, phosphorylated and activated forms of p42 and p44 (Fig. 3C) and by using a MAP kinase immune-complex kinase assay (data not shown). Thus, constitutive Raf and MAP kinase activity alone is not sufficient for transformation of RIE-1 cells, and Ras may trigger the stimulation of Raf/MAP kinase-independent events to promote tumorigenic transformation of RIE-1 cells.

Since up-regulation of TGF-α has been observed in Ras-transformed IEC-18 rat intestinal epithelial cells (33), we evaluated the possibility that the induction of an autocrine growth mechanism may distinguish the transforming activities of Ras and Raf-1 in RIE-1 cells. Whereas conditioned medium from vector-transfected cells showed no activity when added on to untransformed RIE-1 cells, conditioned medium from Ras-transformed cells caused a very dramatic morphologic transformation that was indistinguishable from the highly refractile and rounded morphology of Ras-transformed RIE-1 cells (Fig. 4). In contrast, conditioned medium from ΔRaf-22W-expressing cells did not cause morphologic transformation of RIE-1 cells. Finally, since we have observed that TGF-α expression is enhanced 50-fold in oncogenic Ras-expressing, but not ΔRaf-22W-expressing, RIE-1 cells (unpublished data), we determined whether TGF-α alone could cause the same changes as conditioned medium from Ras-transformed cells. Although growth of untransformed cells in the presence of 20 ng/ml TGF-α resulted in morphologic transformation, the effect was only transient (<24 hr) and distinct from the persistent changes that were seen with the conditioned medium from Ras-transformed cells. Therefore, although we have observed that TGF-α alone is sufficient to promote the growth of untransformed RIE-1 cells in soft agar (data not shown), TGF-α is not likely to be the only component present in the conditioned medium that contributes to RIE-1 transformation. We conclude that constitutive activation of Ras, but not Raf-1, causes induction of an autocrine mechanism that may contribute significantly to transformation of RIE-1 cells.

DISCUSSION

Since constitutive activation of either Ras or Raf-1 causes full tumorigenic transformation of NIH 3T3 fibroblasts, it has been suggested that oncogenic Ras causes transformation solely by promoting activation of the Raf/MAP kinase cascade (11, 12). However, in this study we observed that activated Raf-1, despite triggering constitutively elevated Raf-1 and MAP kinase activity, failed to cause morphologic and growth transformation of RIE-1 epithelial cells in vitro. Thus, oncogenic Ras-mediated up-regulation of the Raf/MAP cascade alone is not sufficient to cause potent transformation of RIE-1 epithelial cells. Since we have also observed that activated Ras, but not Raf-1, causes transformation of the IEC-6 rat intestinal and the MCF-10A human breast epithelial cell lines (data not shown), we suggest that oncogenic Ras requires activation of additional, Raf-independent pathways to cause potent morphologic and growth transformation of epithelial cells.

Although the Raf-expressing RIE-1 cells lacked the rapid tumorigenic growth properties seen with Ras-transformed RIE-1 cells, a delayed onset of tumor formation that was not seen for the vector-transfected RIE-1 cells was observed. One possible explanation for the latent tumorigenic capability of Raf-expressing cells may be that a subpopulation of cells with greatly enhanced Raf-1 expression was responsible for tumorigenesis. However, these tumor-derived cells did not show increased Raf-1 protein or kinase expression when compared with the cells that were injected (data not shown). Instead, it is likely that secondary genetic events that complement activated Raf-1 to promote full tumorigenic transformation have occurred in vivo. Consistent with this possibility, the tumor-
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**FIG. 4.** Conditioned medium from Ras-expressing, but not Raf-expressing, RIE-1 cells causes morphologic transformation of RIE-1 cells. Media collected from confluent cultures of RIE-1 cells stably transfected with either the empty pZIP-NeoSV(a) vector or the pZIP constructs encoding transforming Ras or Raf-1 proteins, or fresh growth medium supplemented with 20 ng/ml TGF-α were added onto subconfluent cultures of parental RIE-1 cells and the cultures were photographed after 18 hr.