Distinct requirements for Ras oncogenesis in human versus mouse cells

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**The spectrum of tumors associated with oncogenic Ras in humans often differs from those in mice either
treated with carcinogens or engineered to sporadically express oncogenic Ras, suggesting that the mechanism
of Ras transformation may be different in humans. Ras stimulates primarily three main classes of effector
proteins, Rafs, PI3-kinase, and RalGEFs, with Raf generally being the most potent at transforming murine
cells. Using oncogenic Ras mutants that activate single effectors as well as constitutively active effectors, we
find that the RalGEF, and not the Raf or PI3-kinase pathway, is sufficient for Ras transformation in human
cells. Thus, oncogenic Ras may transform murine and human cells by distinct mechanisms, and the RalGEF
pathway—previously deemed to play a secondary role in Ras transformation—could represent a new target for
anti-cancer therapy.**

Extracellular signals detected by cell-surface receptors can be transmitted to the cell by stimulating the conversion of the Ras oncoprotein from an inactive GDP-bound to an active GTP-bound state. In the GTP-bound state, Ras stimulates downstream targets, or effectors, which, in turn, affect numerous activities of the cell, such as proliferation, apoptosis, and differentiation (Shields et al. 2000). Mutated and constitutively activated forms of Ras are found in 30% of all human cancers (Bos 1989). Significant experimental and epidemiological evidence supports that such aberrant Ras activation plays a critical role in human oncogenesis. Consequently, there is considerable effort to develop inhibitors of the Ras-signaling pathway for use as novel anticancer drugs (Kloog and Cox 2000; Shields et al. 2000).

Ras transformation is mediated by interactions with multiple downstream effectors whose contribution to Ras transformation has been evaluated primarily in rodent fibroblast model cell systems. Substantial evidence from these studies supports the role of Raf serine/threonine kinases (c-Raf-1, A-Raf, and B-Raf) as key effectors and mediators of Ras-transforming activity. Raf kinases phosphorylate and activate the MEK1 and MEK2 dual

specificity kinases, which, in turn, phosphorylate and activate the ERK1/p44 and ERK2/p42 mitogen-activated protein kinases (MAPKs) (Kyriakis et al. 1992). The critical contribution of the Raf/MEK/ERK pathway to Ras transformation is shown by the ability of constitutively activated mutants of Raf or MEK to cause tumorigenic transformation of NIH 3T3 cells (Bonner et al. 1985; Stanton et al. 1989; Leevers et al. 1994; Stokoe et al. 1994). Additionally, dominant-negative mutants of Raf-1, MEK, and ERK, as well as pharmacologic inhibitors of MEK, have been shown to effectively block Ras transformation in vitro or suppress tumorigenic growth of cancer cell lines in vivo (Kolch et al. 1991; Schaap et al. 1993; Cowley et al. 1994; Westwick et al. 1994; Khosravi-Far et al. 1995; Qiu et al. 1995; Monia et al. 1996; Sebolt-Leopold et al. 1999).

Perhaps the second best-characterized effector important for Ras transformation is phosphatidylinositol 3-kinase (PI3-kinase). Activated PI3-kinase, a lipid kinase, facilitates the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 levels are elevated in Ras-transformed cells. Dominant-negative mutants of PI3-kinase can effectively block Ras transformation of NIH 3T3 cells. Finally, whereas activated PI3-kinase alone cannot cause transformation of NIH 3T3 cells, activated variants of PI3-kinase can cooperate with activated Raf to cause synergistic transforming activity (Rodriguez-Viciana et al. 1997).

The third class of effectors with a role in Ras transformation are guanine nucleotide exchange factors (GEFs)

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for the Ral small GTPases, RalGDS, RGL, RGL2/Rlf, and RGL3 (Wolthuis and Bos 1999). Inhibition of these RalGEFs by expression of a dominant-negative Ral protein blocks Ras focus formation or hematogenous metastatic growth of specific cell lines (Urano et al. 1996; White et al. 1996; Lu et al. 2000; Ward et al. 2001). Furthermore, whereas constitutively activated variants of RalGEFs are not transforming in NIH 3T3 cells, their coexpression with activated Raf induced synergistic focus formation (Urano et al. 1996; White et al. 1996). Thus, although current evidence does not implicate RalGEFs as key players in Ras transformation, they may contribute to the process.

Activating mutations of Ras are found in humans in nearly all pancreatic cancers, one-half of colon and thyroid tumors, and one-third of lung tumors (Bos 1989). However, transgenic mice that model these sporadic Ras mutations do not give rise to the same tumors linked to Ras mutations of human cancer patients. In particular, these mice fail to form pancreatic, thyroid, or colon tumors (Johnson et al. 2001). Additionally, a divergent association of Ras mutations with distinct neoplasms is also seen when compared in humans and rodents. For example, whereas Ras mutations are strongly associated with the carcinogen-induced mammary carcinomas in mice and rats (Zarbl et al. 1985; Miyamoto et al. 1990), such mutations are rare in human breast cancers (Bos 1989). One interpretation of these results is that Ras-mediated tumorigenic growth may display subtle, but important differences between mice and humans. We therefore wished to determine whether the mechanisms of Ras-induced transformation and tumorigenesis operate in a similar fashion in mice and humans.

One drawback of studying Ras-induced transformation in human cells is that ectopic expression of Ras causes cellular senescence in primary human cells (Serrano et al. 1997). However, we recently described that normal primary human cells can be converted to a tumorigenic state by the enforced expression of oncogenic (12V) H-Ras and two other genes, *hTERT* and the early region of *SV40*. *hTERT* is the catalytic subunit of telomerase (Nakamura and Cech 1998), which is essential to immortalize human cells (Bodnar et al. 1998; Counter et al. 1998; Vaziri and Benchimol 1998) and is illegitimately activated to restore telomerase activity in 85% of human cancers (Shay and Bacchetti 1997). The SV40 early region encodes the oncoproteins T and t-Ag, which transform mammalian cells through, in part, disrupting the functions of the tumor suppressor proteins p53 and Rb (Livingston 1992; Ludlow 1993) and the phosphatase PP2A (Sleigh et al. 1978; Rubin et al. 1982; Pallas et al. 1990; Hahn et al. 2002). SV40 DNA, virus, or the T-Ag oncoprotein have also been detected specifically in tumor, but not adjacent tissues, from a wide spectrum of cancers, potentially implicating this virus in the etiology of certain human tumors (Bergsagel et al. 1992; Carbone et al. 1994; Lednicky et al. 1995; Martini et al. 1996). Expression of the T-Ag gene, *hTERT*, and H-Ras12V in primary cultures of human fibroblasts, embryonic kidney, mammary epithelial, and astrocyte cells leads to the cre-

ation of sarcomas (Hahn et al. 1999), carcinomas (Hahn et al. 1999; Elenbaas et al. 2001), and glioblastomas (Rich et al. 2001), respectively, suggesting that ectopic expression of these genes comprise a core set of changes required for tumor growth of human cells. Moreover, because oncogenic Ras is absolutely required for these cells to become tumorigenic, primary cultures of human cells expressing large and small T-Ag and hTERT proteins provide a genetically defined system to circumvent the above limitation, allowing for the study of Ras-mediated oncogenesis in human cells. Although this system does not mimic the gradual selection of spontaneous mutations over time, it does allow one to delineate the contribution of specific genetic changes to the tumorigenic process, starting with primary human cells. Therefore, in this study, we utilized these human cell model systems to evaluate the role of specific effectors in mediating Ras transformation. Our results reveal a striking difference in the contribution of distinct effector pathways to Ras transformation in rodent and human cells, and suggest that RalGEF may play a key role in Ras-mediated human oncogenesis.

Results

Ras12V37G, but not Ras12V35S, transforms human cells

As a first step to elucidate the signaling pathways through which Ras transforms human cells, we sought to determine which effectors of Ras are essential for the transformation of primary human cells. Activated Ras binds its downstream effectors through the core effector domain (residues 32–40). Specific missense mutations in this domain of H-Ras12V that selectively impair the binding and activation of Raf, PI3-kinase, and RalGEFs have been utilized widely to delineate the role of the specific effector pathways in Ras function. Oncogenic H-Ras12V with the mutations T35S, Y40C, or E37G bind to and activate primarily Raf, PI3-kinase, or RalGEFs, respectively. In NIH 3T3 cells, the H-Ras12V35S mutant shows much greater potency than either the 37G or 40C mutants, providing important evidence that Ras transformation of rodent fibroblasts is mediated primarily through Raf (White et al. 1995; Khosravi-Far et al. 1996; Shields et al. 2000). Therefore, we utilized H-Ras12V effector domain mutants to evaluate the importance of Raf, PI3-kinase, and RalGEFs to Ras transformation of human cells.

Prior to evaluating the Ras effector domain mutants in human cells, we verified that our expression vectors for each effector mutant exhibited the differential ability to transform NIH 3T3 cells, as has been described in previous studies (White et al. 1995; Khosravi-Far et al. 1996; Shields et al. 2000). The three described effector domain mutants of H-Ras12V, oncogenic H-Ras12V as a positive control, the null vector, or nontransforming H-Ras as dual negative controls were stably introduced into NIH 3T3 mouse fibroblasts. Immunoblot analysis for ectopic Ras in extracts isolated from the resultant polyclonal

populations revealed a similar amount of this protein in each of the Ras-infected cells (Fig. 1A), presumably at levels higher than typically observed in cancer cells. Cells were next assayed for growth in soft agar. This assay is one of the most rigorous tests of cellular transformation in vitro and provides the best in vitro correlate to tumorigenic growth potential. Additionally, polyclonal populations were assayed immediately after infection to limit the possibility of cells with sporadic mutations skewing the transformation properties of the culture. As expected, NIH 3T3 cells failed to grow in soft agar unless oncogenic, but not wild-type, Ras was expressed. Moreover, as reported by others, Ras12V35S, which activates Raf but not PI3-kinase or RalGEFs (White et al. 1995; Khosravi-Far et al. 1996; Rodriguez-Viciana et al. 1996), was the most highly transforming mutant when expressed in these cells (Fig. 1B).

Having shown that the Ras effector domain mutants function as expected on the basis of the most stringent criteria – biological transformation – we next introduced by retroviral infection the expression vectors encoding the same effector domain mutants and appropriate positive and negative control vectors into human cells. We chose to first test primary human embryonic kidney (HEK) epithelial cells that ectopically express *hTERT* and the *T-Ag* gene (encoding large and small T-Ag). These HEK–HT cells are ideal for assaying Ras-induced oncogenesis, because the addition of oncogenic Ras renders them transformed, as assessed by growth in semisolid medium, and tumor formation when injected into immunocompromised mice (Hahn et al. 1999). After infection, the cells were assayed for H-Ras12V expression by immunoblot analysis and for transformation by the soft agar assay. We found that ectopic Ras was expressed

at similar levels in each of the Ras-infected cells (Fig. 1A). Surprisingly, the transformation profile of human cells by use of the identical effector domain mutants was quite different from that observed in NIH 3T3 cells. Ras12V35S, which activates Raf but not PI3-kinase or RalGEFs, did not support anchorage-independent growth of the human HEK–HT cells (Fig. 1B), despite the fact that this protein is the most potent effector mutant of Ras12V in the murine NIH 3T3 cell-transformation assay. Rather, Ras12V37G, which activates RalGEFs but not Raf or PI3-kinase (White et al. 1996; Rodriguez-Viciana et al. 1997; Wolthuis and Bos 1999), was the only effector domain mutant that yielded transformed growth with human cells (Fig. 1B). These results indicate that oncogenic Ras utilizes different signaling pathways in the transformation of murine NIH 3T3 versus human HEK–HT cells.

Species variation underlies the differences in Ras-induced transformation between murine and human fibroblasts

We envisioned three differences between NIH 3T3 and HEK–HT cells that could account for the dramatic discrepancies in Ras-induced transformation between these cells. First, the difference may stem from the fact that these cells are of different lineage (mesoderm vs. ectoderm). Second, the expression of the *T-Ag* gene in HEK–HT cells, but not NIH 3T3 cells, may be a factor, especially in light of the fact that the alternatively spliced product small t-Ag can indirectly affect MAP-kinase function (Rubin et al. 1982). Although the HEK–HT cells were also infected with an hTERT-encoding retrovirus to restore telomerase activity, this gene was not considered
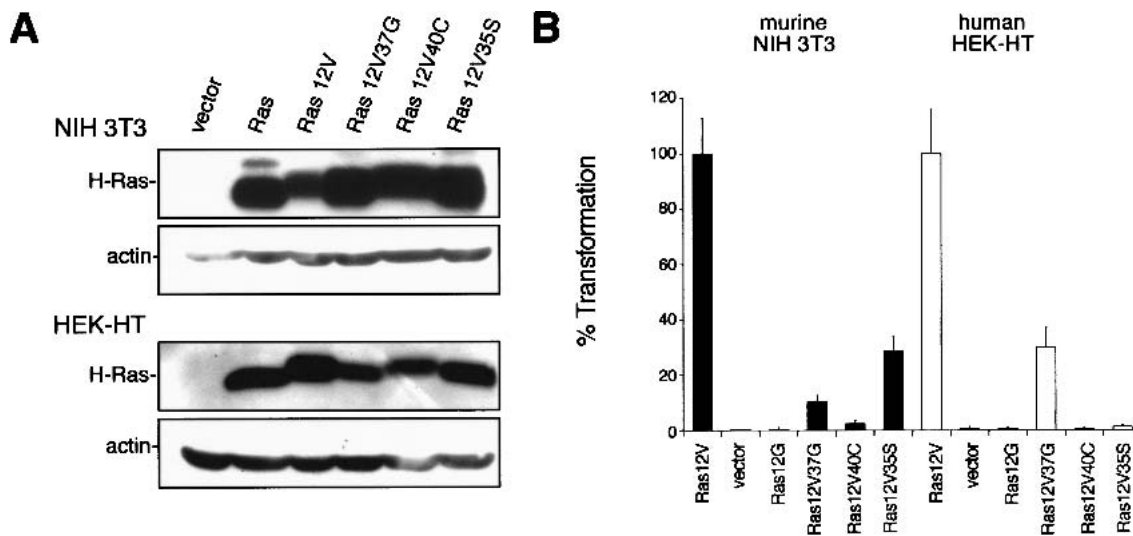

Figure 1. Ras12V37G transforms human cells. (*A*) Expression of Ras mutants was confirmed in NIH 3T3 or HEK-HT cells stably infected with retroviruses encoding the described FLAG-tagged H-Ras cDNAs or with a vector or H-Ras control, by immunoblotting with an anti-FLAG antibody. Actin levels serve as a loading control. (*B*) Anchorage-independent growth of NIH 3T3 (black bars) or human HEK–HT (white bars) cells expressing the described constructs, calculated from the average number of colonies observed from three plates and expressed as the percent of colonies observed in Ras12V-transformed cells. A total of 50,000 Ras12V-transformed NIH 3T3 or HEK–HT cells yielded 380 ± 50 or 289 ± 47 colonies in soft agar, respectively.

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a confounding factor, as NIH 3T3 cells retain expression of the endogenous gene and are telomerase positive (Greenberg et al. 1998). Third, species differences may be important, because NIH 3T3 cells are murine, whereas HEK–HT cells are human.

To directly test whether species variation underlies the difference in transformation, we created identically matched pairs of transformed human and murine cells from the same cell type. Specifically, we isolated normal primary fibroblasts from mice and humans and stably introduced the exact same *T-Ag* gene into both. Equivalent T-Ag expression was confirmed in both the human and murine fibroblasts by immunoblotting with an anti-T-Ag antibody (Fig. 2A). Additionally, because cultured murine fibroblasts are telomerase positive (Chadeneau et al. 1995; Greenberg et al. 1998), hTERT was stably introduced into the human cells to restore telomerase activity (data not shown; Hahn et al. 1999). The cells were next infected with a vector control retrovirus or one encoding oncogenic H-Ras, or the three effector domain mutants. Immunoblot analysis for ectopic H-Ras expression verified that the different H-Ras12V proteins were expressed at similar levels (Fig. 2B). As before, the polyclonal populations were immediately assayed for anchorage-independent growth. We found that T-Ag expressing primary murine fibroblasts behaved as NIH 3T3 cells, in which Ras12V35S was still the most potent effector domain mutant at promoting transformation (Fig. 2C). We

also repeated the experiment with fibroblasts isolated from a different mouse. Specifically, primary cultures of murine fibroblasts were stably infected with retroviruses encoding T-Ag, and Ras12V or the aforementioned effector domain mutants and assayed for anchorage-independent growth. Although the differences between the various effects of the mutants on anchorage-independent growth was less pronounced, Ras12V35S was the most potent effector domain mutant in this transformation assay (Fig. 2D). On the other hand, the human fibroblasts expressing T-Ag and hTERT resembled HEK–HT cells, in which Ras12V37G was again the only effector domain mutant capable of supporting growth in soft agar (Fig. 2C). Thus, the transforming potential of Ras12V37G in human cells cannot be ascribed to either cell type or expression of large or small T-antigens. The simplest interpretation of these data is that the observed discordance in Ras transformation is due to a fundamental difference between mouse and human cells.

Transformation profiles of Ras effector mutants are not cell-type specific in human cells

The transformation potential of Ras effectors has been reported to vary depending on cell type in rodents. For example, an activated version of Raf1 promotes tumorigenic growth of NIH 3T3 fibroblasts (Cowley et al. 1994; Mansour et al. 1994), whereas RIE-1 epithelial cells are
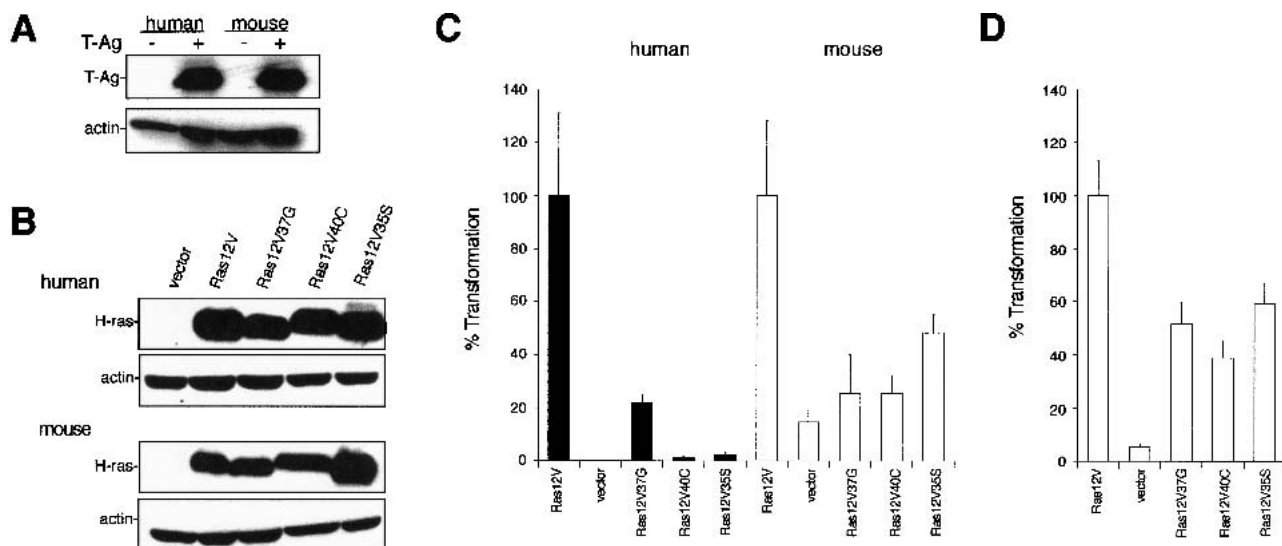

Figure 2. Ras-induced transformation is different in human vs. murine cells. (*A*) Expression of T-Ag was confirmed in human and murine fibroblasts stably infected with a retrovirus encoding the *T-Ag* gene by immunoblotting with an anti-T-Ag antibody. Actin serves as a loading control. (*B*) Expression of Ras mutants was confirmed in T-Ag expressing murine and human fibroblasts (the latter of which also ectopically express hTERT) stably infected with retroviruses encoding the described FLAG-tagged H-Ras cDNAs or with a vector control, by immunoblotting with an anti-FLAG antibody. Actin levels serve as a loading control. (*C*) Anchorage-independent growth of the aforementioned T-Ag-expressing human (black bars) and murine (white bars) fibroblasts, calculated from the average number of colonies observed from three plates and expressed as the percent of colonies observed in Ras12V-transformed cells. A total of 50,000 Ras12V-transformed human or mouse fibroblasts yielded 320 ± 100 or 481 ± 135 colonies in soft agar, respectively. (*D*) Anchorage-independent growth of a different independently derived mouse fibroblast cell strain stably infected with constructs encoding T-Ag and the described H-Ras mutants, calculated from the average number of colonies observed from three plates and expressed as the percent of colonies observed in Ras12V-transformed cells. A total of 50,000 Ras12V-transformed mouse fibroblasts yielded 497 ± 64 colonies in soft agar.

resistant to transformation by this protein (Oldham et al. 1996). Thus, it is possible that the observed transformation of human cells by only the Ras effector mutant that activates RalGEFs may be restricted to specific cell types. However, we showed that both human fibroblasts and HEK cells expressing T-Ag and hTERT behaved identically with respect to Ras transformation. Both cell types grew in soft agar only in the presence of the 37G effector domain mutant of H-Ras12V (Figs. 1B and 2C). Moreover, we found identical results with a third, completely different cell type. Human astrocytes expressing *hTERT* and the *T-Ag* gene (Rich et al. 2001) were stably infected with a control retrovirus or one encoding H-Ras12V or the effector domain mutants (Fig. 3A) and assayed for growth in soft agar. Again, the 37G effector domain mutant was the only variant of Ras12V that retained the ability to transform astrocytes (Fig. 3B). Given that HEK cells are derived from ectoderm, fibroblast from mesoderm, and astrocytes from neuroectoderm, we suggest that the 37G mutant of H-Ras12V in the presence of the *T-Ag* gene and hTERT may promote the

Figure 3. Ras12V37G transforms human astrocytes. (*A*) Expression of Ras mutants was confirmed in hTERT, T-Ag-expressing human astrocytes stably infected with retroviruses encoding the described FLAG-tagged H-Ras cDNAs or with a vector control, by immunoblotting with an anti-FLAG antibody. Actin levels serve as a loading control. (*B*) Anchorage-independent growth of the aforementioned hTERT, T-Ag human astrocytes expressing the described constructs, calculated from the average number of colonies observed from three plates and expressed as the percent of colonies observed in Ras12V-transformed cells. A total of 50,000 Ras12V-transformed human astrocytes yielded 120 ± 40 colonies in soft agar.

growth transformation of a diverse variety of human cell types. The signaling pathway emanating from Ras12V37G may therefore be a general requirement for Ras transformation of human cells.

Activation of neither the MAP-kinase nor the PI3-kinase pathway is sufficient to transform human cells

The observation that expression of Ras12V35S failed to transform a wide spectrum of human cells argues that, unlike murine fibroblasts, activation of Raf and subsequently the MAP-kinase pathway is alone insufficient to transform human cells. To directly evaluate this possibility, an amino-terminally truncated and constitutively active version of Raf1, ΔRaf1-22W (Stanton et al. 1989), was stably expressed in HEK–HT cells (Fig. 4A). We determined that HEK–HT cells stably overexpressing ΔRaf1-22W possessed elevated levels of phosphorylated and activated ERK that were comparable with the elevated level seen in Ras-transformed HEK–HT cells. In contrast, there was no increase in Akt phosphorylation, a downstream target of activated PI3-kinase, indicating the specific activation of the ERK pathway by ΔRaf1-22W (Fig. 4B). Having shown that ΔRaf1-22W caused sustained up-regulation of ERK in HEK–HT cells, we next assayed the ability of these cells to grow in soft agar. In accordance with our observations with Ras12V35S, the resultant cells failed to form colonies in semi-solid medium (Fig. 4C).

We similarly confirmed that activation of the PI3-kinase pathway is not transforming. Specifically, HEK–HT cells were infected with a retrovirus encoding p110-CAAX, a membrane-targeted and constitutively activated version of the p110 α catalytic subunit of PI3-kinase (Rodriguez-Viciana et al. 1997). We verified that the HEK–HT cells stably expressed p110-CAAX and showed up-regulated levels of activated Akt, but not ERK (Fig. 4A,B). Similar to our results with Ras12V40C, activation of the PI3-kinase pathway by p110-CAAX also failed to promote growth of the HEK–HT cells in soft agar (Fig. 4C). Thus, by two different criteria, we show that selective activation of either the Raf or PI3-kinase limbs of the Ras-signaling machinery does not promote the transformed phenotype of anchorage-independent growth in human epithelial cells.

Raf and PI3-kinase pathways fail to cooperate to transform human cells

Although we determined that activation of Raf or PI3-kinase alone was not sufficient to promote the transformation of HEK–HT cells, perhaps the coordinate activation of both effector pathways is required to cause growth transformation. Evidence for this possibility is provided by the observation that PI3-kinase, activated by H-Ras12V40C, can cooperate with Raf activated by H-Ras12V35S to cause synergistic transformation of NIH 3T3 cells (Khosravi-Far et al. 1996; Rodriguez-Vici-
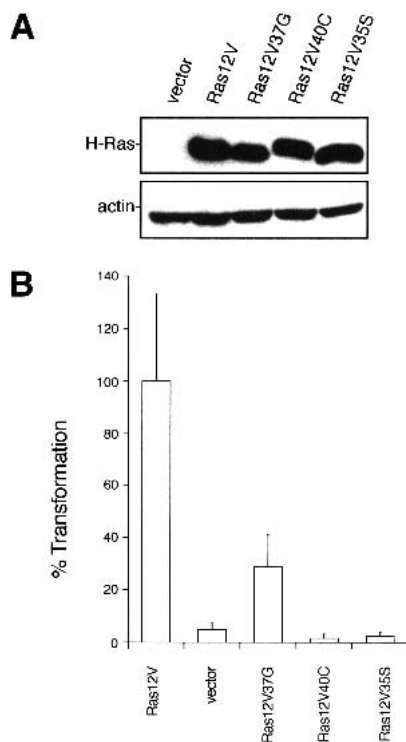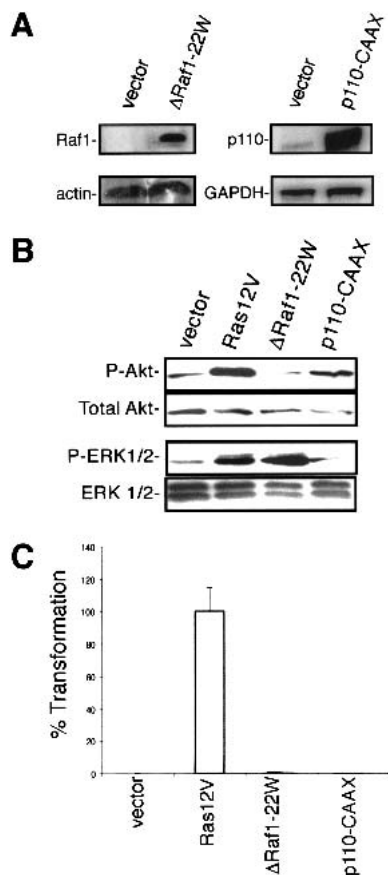
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Figure 4. Specific activation of Raf or PI3-kinase is not sufficient to promote anchorage-independent growth of human cells. (*A*) Expression of ΔRaf1-22W or p110-CAAX was confirmed in the described HEK–HT cells by immunoblotting with an anti-Raf1 antibody or by RT–PCR with primers specific for p110. Actin and GAPDH levels serve as loading controls. These cells also were infected with an empty vector. (*B*) Specific activation of either the MAP-kinase or PI3-kinase pathway by ΔRaf1-22W or p110-CAAX, respectively, was confirmed in the described HEK–HT by immunoblotting with antibodies specific for the phosphorylated forms of ERK1 and ERK2 or Akt. Total ERK 1/2 and Akt levels serve as a loading control. (*C*) Anchorage-independent growth of HEK–HT cells expressing ΔRaf1-22W or p110-CAAX, as calculated from the average number of colonies observed from three plates, and expressed as the percent of colonies observed in Ras12V-transformed cells. A total of 50,000 HEK–HT cells expressing H-Ras12V seeded in soft agar yielded 311 ± 46 colonies.

ana et al. 1997). Therefore, we sought to determine whether the concomitant activation by effector mutants of both these effector pathways could, as has been observed in murine cells, transform human cells.

To test whether the combination of effector domain mutants Ras12V35S and Ras12V40C, which respectively stimulate Raf-1 and PI3-kinase, can promote transformation, we first confirmed that introduction of a second (null) vector had no effect on the transforming potential of any of the effector domain mutants in HEK–HT cells (Fig. 5A,B). Subsequently, HEK–HT cells stably expressing H-Ras12V35S (Fig. 1A) were stably infected with a retrovirus encoding H-Ras12V40C. Because these effector mutants differ by only a single nucleotide, equivalent expression of both transgenes was verified by direct sequencing of cDNA derived from these cells (Fig. 5A). We then evaluated the cells for growth transformation using the soft agar assay. We found that the combined expression of Ras12V35S and Ras12V40C could only lead to an extremely low amount of anchorage-independent growth when compared with the Ras-transformed control cells (Fig. 5B). Thus, these effector domain mutants failed to cooperate and support the transformation of human cells. Moreover, HEK–HT cells stably infected with retroviruses encoding ΔRaf1-22W and p110-CAAX completely failed to grow in semi-solid medium (Fig. 5C). Thus, we conclude that stimulation of both the MAP-kinase and PI3-kinase arms of oncogenic Ras together by two different approaches is insufficient to promote the transformed cell growth of HEK–HT cells.

Raf and PI3-kinase enhance Ras12V37G-induced transformation

We found it surprising that the combined activation of both the PI3-kinase and the MAP-kinase pathways failed to transform the human cells, especially because the combination of these two pathways in murine cells is known to increase transformation above that observed with activation of either effector alone (Khosravi-Far et al. 1996; Rodriguez-Viciana et al. 1997). However, as Ras12V37G alone can transform human cells, albeit to a lesser extent than oncogenic Ras, we next determined whether the coactivation of Raf and PI3-kinase could cooperate with 37G and cause enhanced transforming activity. To directly test this hypothesis, we utilized Ras effector domain mutants or activated effector proteins to cause activation of PI3-kinase or Raf in cells coexpressing H-Ras12V37G.

The expression vector encoding H-Ras12V37G was stably introduced (Fig. 5A; data not shown) into HEK–HT cells that had already been confirmed to express H-Ras12V35S (Fig. 1A) or ΔRaf1-22W (Fig. 4A). The resultant cells from both experiments were then assayed for anchorage-independent growth; we found that both now showed colony forming efficiencies that exceeded that seen with cells transformed by oncogenic H-Ras12V (Fig. 5B). Thus, activation of the MAP-kinase pathway by two independent approaches significantly enhanced the transforming potency of Ras12V37G in human cells.

Similarly, the expression vector encoding H-Ras12V37G was stably introduced (Fig. 5A; data not shown) into HEK–HT cells that expressed either H-Ras12V40C (Fig. 1A) or p110-CAAX (Fig. 4A), and the resultant cells were assayed for growth in semi-solid medium. Again, both methods to activate the PI3-kinase pathway potentiated the transformed growth of Ras12V37G expressing HEK–HT cells to levels greater than that observed in Ras12V-transformed control cells (Fig. 5B). Thus, consistent with the known roles of the MAP-kinase and PI3-kinase pathways in human cancer,
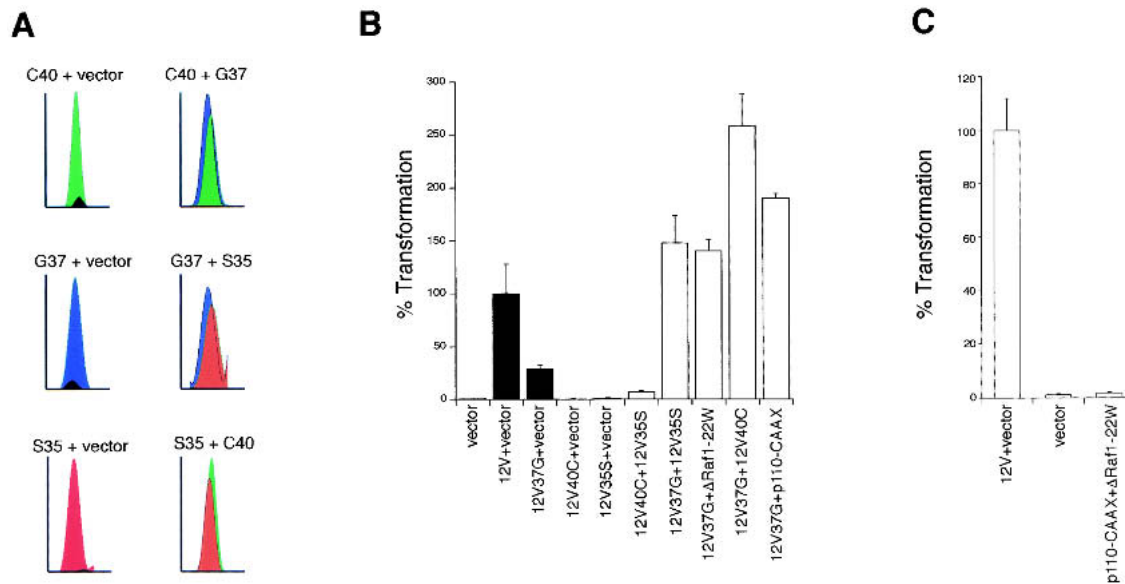
Figure 5. The MAP-kinase and PI3-kinase pathways together are not transforming, but greatly enhance Ras12V37G-mediated transformation. (*A*) Coexpression of Ras12V effector mutants was confirmed from direct sequencing of H-Ras cDNA to identify the point mutations giving rise to 40C (green), 37G (blue), or 35S (red) effector mutations or the corresponding nucleotide of endogenous H-Ras (black in vector control) in the described double-infected HEK–HT cells. (*B*) Anchorage-independent growth of HEK–HT cells expressing the described combinations of Ras12V mutants with control vector (black bars) or the described effector mutants, ΔRaf1-22W or p110-CAAX (white bars), as calculated from the average number of colonies observed from three plates, and expressed as the percent of colonies observed in Ras12V+vector-transformed cells. A total of 50,000 Ras12V+vector-transformed HEK–HT yielded 295 ± 83 colonies in soft agar. (*C*) Anchorage-independent growth of HEK–HT cells expressing Ras12V and control vector, vector alone, or ΔRaf1-22W and p110-CAAX, as calculated from the average number of colonies observed from three plates, and expressed as the percent of colonies observed in Ras12V+vector-transformed cells. A total of 50,000 Ras12V+vector-transformed HEK–HT yielded 463 ± 55 colonies in soft agar.

activation of these pathways does enhance transformation in human cells. However, because anchorage-independent growth was not observed unless Ras12V37G was expressed, these results suggest that the effector-signaling activity of 37G plays an essential role in Ras-induced transformation.

### The Ras12V37G effectors RalGEFs transform human cells

Because Ras12V37G is critical for the Ras oncogenesis in human cells, we sought to identify the underlying signaling pathway responsible for transformation by this effector mutant. Although the transforming activity of Ras12V37G is generally ascribed to its ability to activate RalGEFs (Wolthuis and Bos 1999), this effector domain mutant does retain the ability to interact with other proteins (Khosravi-Far et al. 1996; Han et al. 1997; Linnemann et al. 1999; Cullen 2001). To test whether Ras12V37G transformation of HEK–HT cells involves the activation of RalGEF function, we first determined whether inhibition of RalGEF activity could impair Ras-transforming activity. The S28N dominant-negative mutant of RalA forms a nonproductive complex with RalGEFs (Urano et al. 1996), impeding RalGEF activation of endogenous Ral small GTPases. We found that expression of RalA-28N in H-Ras12V-transformed HEK–

HT cells (Fig. 6A) did curb the ability of Ras-transformed cells to grow in soft agar (Fig. 6D) without decreasing Akt or ERK phosphorylation (Fig. 6B). Similarly, there was a growth reduction in Ras12V37G-expressing cells infected with the RalA-28N-expressing retrovirus (Fig. 6D). Thus, RalGEFs are necessary for Ras-mediated transformation.

Whereas RalA-28N attenuated Ras-induced transformation, it did not completely block it. Such a result could suggest the presence of other effectors downstream of Ras12V37G that could contribute to the oncogenic signal. Therefore, to directly test whether activation of RalGEFs alone is sufficient for transformation, we evaluated whether a constitutively activated RalGEF could cause the transformation of HEK–HT cells. As described above, there are four known RalGEF proteins that can interact with Ras (Wolthuis and Bos 1999; de Bruyn et al. 2000; Rebhun et al. 2000; Shao and Andres 2000), with the Ras-binding domain of Rlf having the highest affinity for GTP–Ras (Esser et al. 1998). Therefore, we established HEK–HT cells stably expressing Rlf-CAAX, a plasma membrane-targeted and constitutively activated version of Rlf (Wolthuis et al. 1997; Ramocki et al. 1998). We verified expression of this protein by immunoblot analysis (Fig. 6A), and, importantly, confirmed that Rlf-CAAX did not activate either ERK or Akt (Fig. 6B) but did activate Ral, as assessed by an increase in Ral–GTP

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Figure 6. RalGEFs are essential for Ras transformation of human cells. (*A*) RalA-28N and Rlf-CAAX expression was confirmed in HEK–HT or Ras12V-transformed HEK–HT cells, respectively, by immunoblotting with either an anti-Ral antibody (*top*) or an anti-HA antibody specific for the HA epitope cloned into Rlf (*bottom*). Actin levels serve as loading controls. (*B*) Phosphorylated Akt (P-AKT) or ERK 1 and 2 (P-ERK 1/2) levels were determined in the described cells by immunoblotting with antibodies specific for the phosphorylated forms of these proteins. The total amount of Akt, ERK 1/2 or actin detected by anti-Akt, ERK, or actin antibodies serve as loading controls. (*C*) Ral–GTP levels were determined in the described cells by binding cellular Ral–GTP to the recombinant Ral-binding domain of the RalBP protein, followed by immunoblotting with a RalA-specific antibody. The total amount of Ral detected by the same anti-RalA antibody serve as loading controls. (*D*) Anchorage-independent growth of Ras12V or Ras12V37G-transformed HEK–HT cells in the absence (vector) or presence of RalA28N, or HEK–HT cells in the absence (vector) or presence of Rlf-CAAX, as calculated from the average number of colonies observed from three plates, and expressed as the percent of colonies observed in Ras12V (black bars) or Ras12V37G (white bars) transformed cells. A total of 50,000 Ras12V-transformed HEK–HT cells yielded 295 ± 83 colonies in soft agar.
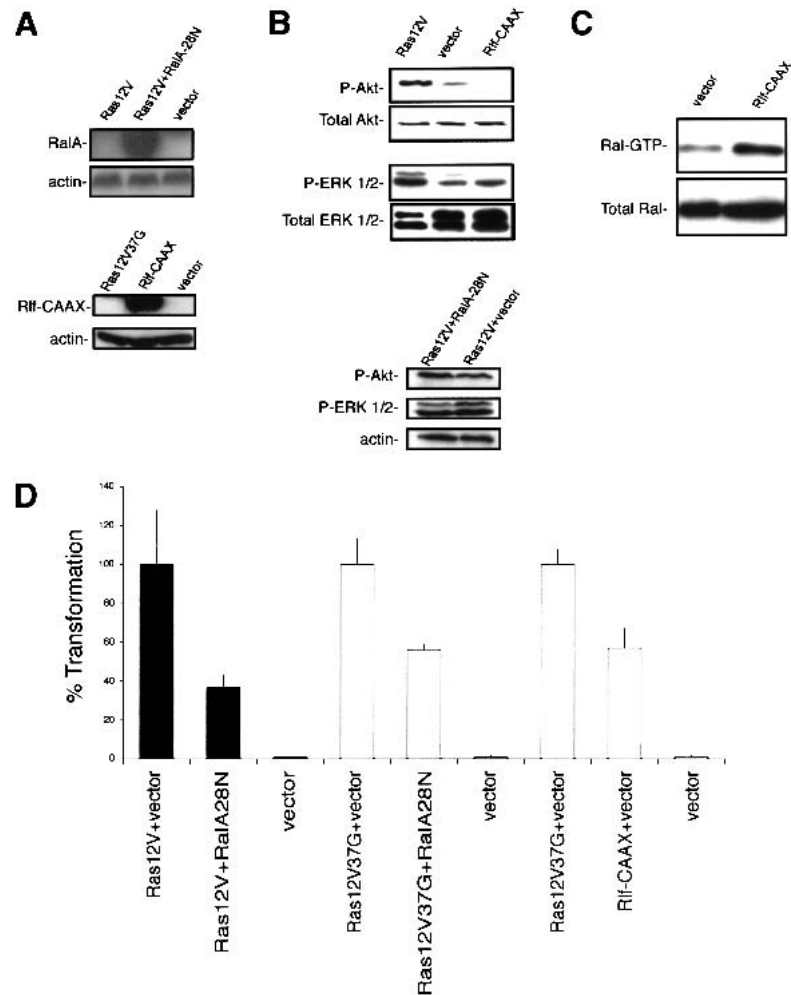
levels (Fig. 6C). The cells were next tested for anchorage-independent growth. Expression of Rlf-CAAX was sufficient to support the anchorage-independent growth of HEK–HT cells up to 60% of the level seen in the same cells transformed by Ras12V37G (Fig. 6D). Although it remains formally possible that this Rlf-CAAX-mediated transformation is unique to HEK cells, the observation that H-Ras12V37G transformed T-Ag and hTERT-expressing HEK cells, astrocytes and fibroblasts suggest that activation of a single RalGEF may be transforming in other human cell types. When taken together with the ability of the Ral dominant-negative to impair Ras12V transformation, these results suggest that oncogenic transformation of human cells by Ras12V37G is mediated, at least in part, by the activation of RalGEFs.

Ras12V37G is essential for Ras-induced tumorigenesis of human cells

Ras12V37G expression alone is sufficient to promote anchorage-independent growth of HEK cells. We next sought to determine whether signaling by this protein is required for the prior observations that primary human cells expressing hTERT and T-Ag require oncogenic Ras

to form tumors when injected in immuno-compromised mice (Hahn et al. 1999). HEK–HT cells expressing H-Ras12V35S and H-Ras12V40C (Fig. 5A) were stably infected with either an empty vector control or an H-Ras12V37G-expressing construct. The resultant cells were then each injected into four immuno-compromised mice to assay for tumor growth. HEK–HT cells that coordinately expressed all three effector mutants readily formed tumors (Fig. 7) at a rate indistinguishable from that caused by oncogenic H-Ras12V (data not shown; Hahn et al. 1999). However, cells lacking Ras12V37G expression completely failed to form detectable masses in vivo, even after ~50 days, that is, twice as long as Ras12V37G-expressing cells required to form tumors (Fig. 7). Effectors of Ras12V37G are therefore necessary for Ras-dependent tumorigenesis in vivo. We next addressed whether Ras12V37G alone is sufficient for tumorigenic growth. In this case, HEK–HT cells expressing Ras12V37G failed to form tumors in vivo (data not shown). Such a result is consistent with our observations that Ras12V37G is not as potent as Ras12V at promoting anchorage-independent growth (Fig. 1), and the known roles of the PI3-kinase and MAP-kinase pathways (Shields et al. 2000) in the more complex process of tu-
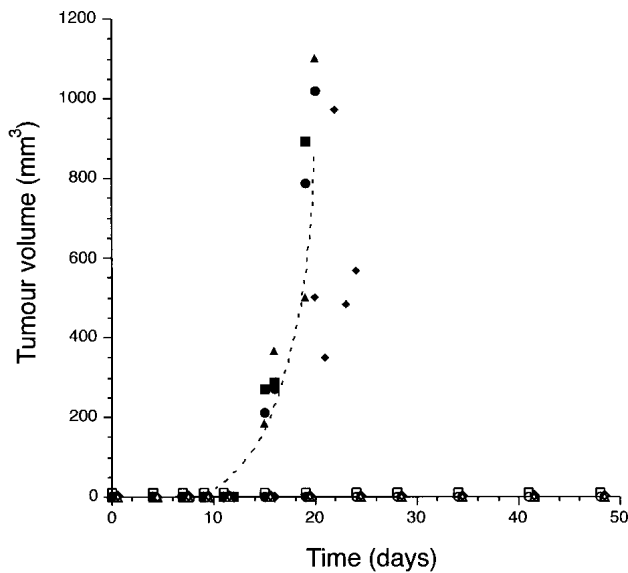
Figure 7 Ras12V37G is essential for tumor growth. Tumor volume (mm^3) of four mice injected with HEK–HT cells expressing H-Ras12V40C and H-Ras12V35S either in the absence (vector: ○□△◇) or presence (●■▲◆) of H-Ras12V37G, plotted against time (days).

morigenesis (Stanbridge and Wilkinson 1980). Thus, whereas effectors of H-Ras12V37G are not alone sufficient for tumorigenic growth they are, unlike in murine cells, essential for this process.

Discussion

Studies done primarily in NIH 3T3 and other rodent fibroblast model cell systems have established that the transforming activity of Ras is mediated largely by activating the Raf>Mek>Erk signal cascade. In NIH 3T3 cells, activated mutants of Raf-1 (Bonner et al. 1985; Stanton et al. 1989; Leevers et al. 1994; Stokoe et al. 1994) or its target MEK (Alessi et al. 1994; Mansour et al. 1994) are completely sufficient to replace oncogenic Ras in the tumorigenic assays. This prompted earlier suggestions that Raf is the key effector of Ras transformation. Although studies of Ras transformation using other cell types now indicate that Raf-independent effector function also contributes to Ras transformation (Shields et al. 2000), Raf is still generally considered to form the backbone of Ras oncogenesis. The importance of this pathway in Ras transformation is reflected by the current evaluation of anti-Raf and anti-MEK strategies in preclinical and clinical studies (Monia et al. 1996; Sebolt-Leopold et al. 1999; Stevenson et al. 1999; Cunningham et al. 2000; Kloog and Cox 2000; Sebolt-Leopold 2000; Stein and Waterfield 2000). There is also strong evidence for an important supportive role for PI3-kinase in Ras transformation (Rodriguez-Viciana et al. 1997; Tolkacheva and Chan 2000). Aside from limited evidence for the importance of RalGEFs in Ras transformation, the role of other effectors in Ras transformation remains largely unresolved. In the present study, we determined

unexpectedly that activation of the Raf and PI3-kinase effector pathways, either alone or together, was not sufficient to cause transformation of human cells. Instead, we found that activation of the RalGEF effector signaling alone, although found previously to not be sufficient to transform rodent fibroblasts, was sufficient to cause growth transformation of a variety of human cells. Moreover, because expression of an activated RalGEF did not lead to an increase in ERK or Akt phosphorylation, we rule out indirect activation of the MAP-kinase and PI3-kinase pathways by RalGEFs as a mechanism accounting for the transforming capacity of RalGEFs. Whereas we did find that Raf and PI3-kinase could cooperate with H-Ras12V37G to promote transformation, our results suggest that it is the activation of effectors of this Ras mutant, such as RalGEFs, that is critical for Ras transformation in human cells, at least in this model system.

As such, we propose an effector-usage model, whereby multiple effectors of Ras contribute to the oncogenic signal, but that there exists significant differences between humans and mice in the relative potency of each pathway. Such species-specific differences in carcinogenesis are not restricted to Ras-related neoplasms. Transgenic mice lacking functional alleles of the tumor suppressors p53 (Donehower et al. 1992), Rb (Jacks et al. 1992), and NF-1 (Jacks et al. 1994), to name a few, also fail to give rise to the same types of tumors observed in familial human diseases harboring the same genetic changes (Cavenee et al. 1985; Legius et al. 1993; Colman et al. 1995; Kleihues et al. 1997). The recurrent discrepancies in the tumor phenotypes between humans and mice emphasize the value of dissecting oncogenesis in human cells.

We have shown that RalGEFs are transforming, supporting the premise that these proteins mediate, at least in part, Ras12V37G transformation. An interesting parallel to these findings is the observation that RalGEFs, and not other Ras effectors, are critical for proliferation of PC12 cells, although, in this case, RalGEFs oppose the growth-inhibitory action of Raf and PI3-kinase (Goi et al. 1999). With regards to the potency of RalGEFs for transformation, we note that whereas RalA-28N inhibited Ras-mediated transformation, it did not abolish it. Similarly, Rlf-CAAX promoted anchorage-independent growth, but not to the same level as Ras12V37G. It therefore remains possible that Ras12V37G may activate other effectors, in addition to RalGEFs, to promote the transformed phenotype. For example, H-Ras12V37G has been shown recently to retain the ability to bind and activate phospholipase C ε and stimulate production of two second messengers, inositol trisphosphate and diacylglycerol (Cullen 2001). These second messengers in turn promote an increase in intracellular calcium and activation of protein kinase C, events that may contribute to growth transformation. Alternatively, the mutants utilized to implicate a RalGEF pathway may be only partially effective in mimicking the consequences of Ras activation of RalGEFs. For example, at least four distinct RalGEFs have been identified as Ras effectors, and, hence, expression of only one activated form may

not be sufficient. Consistent with this possibility, we found that coexpression of two constitutively activated RalGEF members caused a more robust transformation than one alone (data not shown).

The observation that Ras12V37G was required for tumorigenic growth supports the notion that RalGEFs are important for tumorigenesis. Because RalGEFs are bona fide effectors of Ras (Wolthuis and Bos 1999), and Ras is commonly activated in human cancers, either through an activating mutation (Bos 1989) or by inappropriate activation of growth factor receptors (Shields et al. 2000), it stands to reason that the RalGEF pathway is activated in at least some human cancers. We also point out that a RalGEF has been identified that is oncogenic (D'Adamo et al. 1997), opening up the possibility that this pathway could even be activated selectively during tumorigenesis. We can, however, only speculate on the mechanism whereby RalGEFs transform human cells. The only known substrates for these proteins are the monomeric G-proteins RalA and RalB. RalGEFs bind to the GDP-bound state of Ral and facilitate the exchange of GDP for GTP, thereby activating these proteins (Wolthuis and Bos 1999; de Bruyn et al. 2000; Rebhun et al. 2000; Shao and Andres 2000). However, no oncogenic potential has been shown for activated Ral proteins alone, although activated Ral can modestly potentiate the transforming activity of activated Raf in rodent fibroblast transformation assays (Urano et al. 1996; White et al. 1996). Consistent with these data, an activated form of Ral failed to promote significant growth of the human cells in soft agar (data not shown). Thus, it is possible that activation mutations in Ral may not recapitulate the activation by Rlf-CAAX or H-Ras. Alternatively, Rlf-CAAX may activate other signaling proteins, either because they are natural substrates or by virtue of Rlf-CAAX overexpression or constitutive membrane targeting. Future studies will be needed to determine how RalGEFs transform human cells and whether RalGEFs are up-regulated in human tumors that harbor mutated Ras.

In murine cells, the MAP-kinase pathway is clearly critical for tumorigenesis and is also commonly activated in human tumors (Hoshino et al. 1999). Consequently, this pathway has been the target of aggressive efforts to develop inhibitors to treat human cancers. A variety of pharmacologic and antisense approaches have been developed to block the activities of Raf and MEK and are now under clinical evaluation (Stevenson et al. 1999; Cunningham et al. 2000; Kloog and Cox 2000; Sebolt-Leopold 2000). Similarly, PI3-kinase has been implicated in human cancer, particularly for its role in cell survival and proliferation. In certain cancer types, the PI3-kinase gene is amplified, the protein overexpressed (Phillips et al. 1998; Ma et al. 2000) or expression of the PTEN gene, a negative regulator of the PI3-kinase pathway, is lost (Cantley and Neel 1999). Targeted inhibition of specific PI3-kinases has also been proposed as an avenue of cancer treatment (Stein and Waterfield 2000). Given that inhibition of these Ras effectors has profound effects on tumor growth and that Ras12V37G was found to be essential for transformation of human cells, we

speculate that inhibition of effectors of Ras12V37G, such as RalGEFs, may also hold promise as a cancer treatment strategy.

In summary, there is considerable debate regarding the accuracy of mouse models for human cancer. In this study of both human and murine cells, we now show that the signaling pathway engaged by H-Ras12V37G is sufficient for Ras-dependant transformation and necessary for tumor formation in human cells, and that RalGEFs, known targets of this effector mutant, are critical components of this process. A surprising observation from our analyses was that the two most critical effectors of Ras transformation of rodent fibroblasts were not sufficient, either alone, or in combination to cause transformation of the human cells. These observations highlight the value of dissecting the signaling pathways of oncoproteins in human cells and suggest that the effectors of H-Ras12V37G, such as RalGEF, could represent potential targets for the development of anti-Ras drugs for the treatment of human cancers.

Materials and methods

Cell lines

Retrovirus constructs were created by subcloning FLAG–H-Ras and FLAG–H-Ras12V (an amino-terminal FLAG tag was inserted in frame into H-Ras or H-Ras12V by PCR) in the absence or presence of effector domain mutants S35, C40 or G37 (White et al. 1995; Rodriguez-Viciana et al. 1997) generated by site-directed mutagenesis, RalA-28N [the 28N mutation (Urano et al. 1996) was introduced by site-directed mutagenesis into RalA (Frech et al. 1990), a kind gift of R.A. Weinberg (Massachusetts Institute of Technology, Cambridge, MA)], HA-Rlf-CAAX (Wolthuis et al. 1997), p110α-CAAX (Rodriguez-Viciana et al. 1997), ΔRaf1-22W (Stanton et al. 1989), and T-Ag [generated by PCR amplifying the early region of SV40 of plasmid pSVneo (Southern and Berg 1982) with primers that incorporate BamHI and EcoRI sites] into pBabe vectors (Morgenstern and Land 1990) creating the following plasmids pBabepuro: FLAG–H-Ras, FLAG–H-Ras12V (and 35S, 37G, or 40C), HA-Rlf-CAAX, p110α-CAAX, and ΔRaf1-22W; pBabebleo: FLAG–H-Ras12V (and 35S or C40), RalA-28N; and pBabeneo: T/t-Ag. FLAG–H-Ras12V35S was also cloned into the vector pWZL-Blast [a kind gift of J.P. Morgenstern (Millennium Pharmaceuticals Inc., Cambridge, MA)].

At the earliest passage, the appropriate derived amphotrophic retroviruses encoding H-Ras or effector mutants thereof or Ras effectors were used to infect T-Ag + hTERT expressing primary HEK (Armbruster et al. 2001), human astrocyte (Rich et al. 2001), human BJ fibroblasts (Hahn et al. 1999), or primary murine fibroblasts from two independent strains of C57/Bl6;129Sv mice stably infected with pBabeneoT/t-Ag [a kind gift of X.-F. Wang and A.M. Pendergast (Duke University Medical Center, Durham, NC)] or NIH 3T3 (Khosravi-Far et al. 1996) cells, similar to approaches described previously (Hahn et al. 1999; Rich et al. 2001). Stable polyclonal populations resistant to the appropriate drug selection markers were assayed for anchorage-independent growth by the third confluent plate. The same procedure was followed to add additional transgenes.

Immunoblotting

Exponentially growing cells from the described cell lines were lysed, protein concentrations were measured by Lowry assay

(Bio-Rad), and 30 µg of soluble lysate separated by SDS-PAGE and immunoblotted similar to methods described previously (Armbruster et al. 2001) by use of the M2 anti-FLAG (Sigma), anti-RalA (Signal Transduction Laboratories), E-10 anti-Raf1, C-2 or I-19 anti-actin (Santa Cruz Biotechnology), or 12CA5 anti-HA (Roche) antibodies to detect FLAG–H-Ras, RalA28N, ΔRaf1-22W, actin, and HA-Rlf-CAAX. To monitor activation of the MAP-kinase and PI3-kinase pathways, the described cell lines were serum starved in medium supplemented with 0.5% fetal bovine serum for 48 h, lysed, resolved by SDS-PAGE, and immunoblotted similar to that described above. The K-23 anti-ERK1/2 (Santa Cruz), E10 anti-phospho(Thr 202/Tyr 204)-p42/44 MAPK, anti-Akt (Cell Signaling Technology), and S472 anti-phospho(Ser 473)-Akt (New England Biolabs) antibodies were used to detect ERK 1 and ERK 2, phosphorylated ERK 1 and ERK 2, Akt, and phosphorylated Akt, respectively.

Ral–GTP assay

Levels of endogenous Ral–GTP were assayed as described previously (Murphy et al. 2002) by incubating cell lysates with glutathione-agarose bound recombinant GST–RalDB (produced and purified from bacteria), which adheres specifically to Ral in the GTP-bound state. Beads were washed and bound proteins resolved by SDS-PAGE and Ral detected by immunoblotting with the aforementioned anti-RalA antibody. Total Ral levels were determined by immunoblotting lysates with the same antibody.

RT–PCR

Total RNA was isolated and RT–PCR amplified using methodologies described previously (Hahn et al. 1999; Rich et al. 2001) with primers specific for H-Ras (5′-ACGACGATGACAAGACGGAA-3′ and 5′-ATGGCGCTGTACTCCTCCT-3′), p110 (5′-GACAATGTGAACACTCAAAGAG-3′; and 5′-CACCAATAGGGTTCAG CAGA-3′) and GAPDH (Hahn et al. 1999). To identify specific effector mutants, H-Ras RT–PCR amplified products were sequenced to determine the nucleotide at positions +105, +111, and +121 (corresponding to the 35S, G37, and 40C mutations, respectively).

Soft agar

Cells were suspended in soft agar and colonies >30 cells scored after 3 wk, as described previously (Cifone and Fidler 1980). Assays were done in triplicate, and in most cases independently, at least twice.

Tumors

Under a protocol approved by the Duke University Institutional Animal Care and Use Committee, 2×10^6 cells were injected subcutaneously into the flanks of four SCID/Beige mice, after which tumor volumes were determined at regular intervals, as described previously (Hahn et al. 1999; Gou et al. 2001).

Acknowledgments

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