A Direct Interaction between Oncogenic Ha-Ras and Phosphatidylinositol 3-Kinase Is Not Required for Ha-Ras-dependent Transformation of Epithelial Cells

Maria Karasarides‡§, Bela Anand-Apte¶, and Alan Wolfman§**

From the ‡Department of Biological, Geological and Environmental Science, Cleveland State University, Cleveland, Ohio 44115, the §Department of Cell Biology, Cleveland Clinic Foundation, Lerner Research Institute, Cleveland, Ohio 44195, and the ¶Department of Ophthalmic Research, Cleveland Clinic Foundation, Cole Eye Institute, Cleveland, Ohio 44195

Cells expressing oncogenic Ras proteins transmit a complex set of signals that ultimately result in constitutive activation of signaling molecules, culminating in unregulated cellular function. Although the role of oncogenic Ras in a variety of cellular responses including transformation, cell survival, differentiation, and migration is well documented, the direct Ras/effector interactions that contribute to the different Ras biological end points have not been as clearly defined. Observations by other groups in which Ras-dependent transformation can be blocked by expression of either dominant negative forms of Phosphatidylinositol (PI) 3-kinase or PTEN, a 3-phosphoinositide-specific phosphatase, support an essential role for PI 3-kinase and its lipid products in the transformation process. These observations coupled with the in vitro observations that the catalytic subunits of PI 3-kinase, the p110 isoforms, bind directly to Ras-GTP foster the implication that a direct interaction between an oncogenic Ras protein and PI 3-kinase are causal in the oncogenicity of mutant Ras proteins. Using an activated Ha-Ras protein (Y64G/Y71G/F156L) that fails to interact with PI 3-kinase, we demonstrate that oncogenic Ha-Ras does not require a direct interaction with PI 3-kinase to support anchorage-independent growth of IEC-6 epithelial cells. We do find, however, that IEC-6 cells expressing an oncogenic Ha-Ras protein that no longer binds PI 3-kinase are greatly impaired in their ability to migrate toward fibronectin.

The Ras proteins exert their effects on cells by directly associating with downstream effector molecules, thereby initiating the activation of the signaling networks responsible for Ras-mediated biological events. A number of molecules have been identified as potential Ras effectors, including Raf-1, B-Raf, RafGDS, PI3-kinase, Rin 1, AF6, and Noret 1 (1, 2). Among these putative effector molecules, three major candidates, Raf-1, RafGDS, and PI 3-kinase, have been implicated as direct downstream Ras effectors that may contribute to Ras-dependent transformation (3–9). Raf-1 is the only known effector, however, that has been detected in stable complexes with Ras in vivo, at physiological levels of expression (10–12). The consequences of such Ras/Raf interactions on cellular function have been extensively studied and found to result in the activation of the Raf/MAPK cascade. Ras-stimulated MAPK activity is not only required for cellular proliferation, but elevated MAPK activity is commonly associated with Ras transformation of both fibroblasts and epithelial cells. Although elevated MAPK is absolutely required to maintain the transformed phenotype, the current model for Ras function implicates the requirement of additional signaling networks to produce the full spectrum of Ras-dependent biological end points. Several lines of evidence, in support of this model, indicate that oncogenic Ras facilitates the activation of multiple Ras effector pathways that cooperate to establish cellular transformation.
The most direct evidence comes from Ras effector domain mutants that have been characterized to bind only a single Ras target (13). Rodriguez-Viciana et al. (9) demonstrated that Ras effector domain mutants, which interact solely with Raf (G12V/T35S/E37G) or RafGDS (G12V/G37) or PI 3-kinase (G12V/Y40C) are not by themselves sufficient to transform fibroblasts. Co-expression, however, of any two of these Ras effector domain mutants induced the formation of foci, suggesting that Ras must activate more than one effector pathway to generate a transformed phenotype (9).

Transient expression of G12V Ha-Ras results in elevated levels of 3-phosphorylated phosphatidylinositides that are potentiated by the co-transfection of the p110 catalytic subunit of PI 3-kinase (8, 14). The role of PI 3-kinase lipid products in the transformation process is further highlighted by observations that PTEN, a phosphatase that specifically dephosphorylates 3-phosphorylated phosphatidylinositides, has significantly reduced activity in a number of human tumors (15–17). Ectopic expression of PTEN in Ras transformed cells reduces the number of foci in a focus formation assay. It is not clear, however, whether the role of PI 3-kinase in Ras-dependent transformation arises from a direct association with Ras-GTP or through other unidentified indirect mechanisms. This uncertainty is amplified by the failure to detect a stable complex between Ras and PI 3-kinase in vivo, even when both are transiently overexpressed (14).

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Our laboratory has previously published data showing that specific Ras isoforms have different affinities for Raf-1 in vivo. Using 10T1/2 fibroblasts transformed by minimal levels of G12V Ha-Ras, we observe that Raf-1 preferentially associates with c-N-Ras and not the ectopically expressed oncogenic Ha-Ras (12). We have extended these observations by demonstrating that G12V Ha-Ras induces the secretion of EGF receptor (EGFR) ligands that promote the activation of c-N-Ras-Raf-1 complexes and, hence, up-regulate MAPK activity (18). Together these observations suggest that G12V Ha-Ras does not interact with Raf-1 and requires the co-operative function of other signaling modules to generate transformation and elevate basal MAPK. Observations that Raf-CAAX does not substitute for oncogenic G12V Ha-Ras in the transformation of RIE-1 cells provide further evidence that Ha-Ras transformation is mediated by additional Raf-independent mechanisms (19). These observations prompted us to investigate whether oncogenic Ha-Ras requires a direct interaction with PI 3-kinase to mediate the transformation of epithelial cells. To address this issue we constructed a constitutively active Ha-Ras protein (Y64G/Y71G/F156L) that binds all known downstream Ras effectors except PI 3-kinase. Our results indicate that oncogenic Ha-Ras does not require a direct association with PI 3-kinase to transform rat intestinal epithelial (IEC-6) cells. Although a direct Ha-Ras/PI 3-kinase interaction is dispensable for the transforming activities of oncogenic Ha-Ras, we do find that loss of this direct interaction between Ha-Ras and PI 3-kinase greatly impairs migration of epithelial cells toward fibronectin.

MATERIALS AND METHODS

Construction of Ha-Ras Mutants—The F156L point mutation was introduced into Ha-Ras cDNA sequence using Altered Sites II in vitro Mutagenesis System (Promega). Wild-type Ha-Ras cDNA and Y64G/Y71G/Y171G Ha-Ras cDNA were subcloned into the pAL-EX2 vector. Mutagenesis reactions were carried out using the following mutagenesis oligonucleotide: 5′-gaggagtctttatacagg (Life Technologies, Inc.). The integrity of the Ha-Ras mutants was verified by sequence analysis. The cDNAs for the mutant Ha-Ras proteins were subcloned into pQE-9 vector for bacterial expression and into the pcDNA3 vector for mammalian expression.

Transfections and Cell Lines—IEC-6 (normal rat epithelial; small intestine) cells were maintained in DMEM supplemented with 10% FBS and 0.1 unit/ml bovine insulin. Stable cell lines were generated by transfecting IEC-6 cells with the indicated constructs using LipofectAMINE Plus (Life Technologies, Inc.). The cells were plated at 105 in 100-mm dishes and transfected the next day with 4 g of DNA, as specified by manufacturer. After 48 h the transfected cultures were split and placed in selection medium containing G418 (400 μg/ml). Control cell lines were transfected with empty vector (pcDNA3). After 14 days of drug selection individual resistant colonies were isolated and propagated for further analysis.

Protein Expression and Western Blot Analysis—Protein expression was assessed by Western blotting using anti-Ha-Ras monoclonal antibody (Santa Cruz). Stable clones that expressed the various Ha-Ras proteins at levels consistent with levels expressed in T24 bladder carcinoma cells were selected for further analysis. Partially purified membrane fractions were prepared by first lysing cells in 1× p21 buffer (20 mM MOPS, pH 7.4, 200 mM sucrose, 5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol) containing 0.1% saponin, protease inhibitors (aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (saponin lysis buffer). The cell ghosts were isolated by centrifugation at 13,000 rpm for 10 min at 4 °C. The pellet was then solubilized in 1× p21 buffer containing 1% (w/v) CHAPS, protease, and phosphatase inhibitors (CHAPS lysis buffer), the samples were clarified by centrifugation at 13,000 rpm for 10 min at 4 °C, and the supernatant was saved as the enriched membrane fraction. Fifty micrograms of the CHAPS fraction was resolved on SDS-PAGE (13% gel) and transferred to polyvinylidene difluoride membrane (Amerham Pharmacia Biotech). Membranes were blocked with TBS (20 mM Tris, pH 7.4, 140 mM NaCl), containing 0.1% Nonidet P-40, 5% dry milk, and 0.1% calf serum for 1 h at room temperature. After washing with TBS/Nonidet P-40, the membranes were incubated with the primary antibody overnight at 4 °C. The membranes were washed for 1 h (four changes) with TBS/ Nonidet P-40 and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody. The membranes were then washed for 2 h, and the immunoreactive bands were detected with ECL Plus Reagent (Amerham Pharmacia Biotech) and visualized on a Molecular Dynamics StormImager.

Nucleotide Exchange Assay—Recombinant His-tagged wild-type Ha-Ras, F156L Ha-Ras, and Y64G/Y71G/F156L Ha-Ras were purified from Escherichia coli using a standard Ni2+ column and elution with EDTA. The purified proteins were concentrated, and the buffer was exchanged to p21 buffer using a Centricon 10 filtration unit. Ras proteins were then incubated with GTP (final concentration, 10 μM) and 30 mM MgCl2 at 30 °C. At the indicated time points aliquots were collected on 0.2-μm nitrocellulose filters, and the amount of bound [α-32P]GTP was determined by scintillation counting. The total amount of Ras-[α-32P]GTP for each sample was determined by the addition of excess (35 mM) EDTA in identical reactions.

Affinity Precipitation of Ras Effector Proteins with Ha-Ras Mutants—Recombinant Ha-Ras proteins were purified and assayed for activity using a standard filter binding assay. 300-μg aliquots of recombinant Ha-Ras proteins (wild type, F156L, Y64G/Y71G/F156L, and G12V/Y40C) were covalently immobilized on silica resin as described previously (3, 20). Following the immobilization protocol, the covalently linked Ras proteins were then assayed for GTP binding activity. Equal amounts of each Ras protein were then loaded on p21 buffer or GMP-PNP (a nonhydrolyzable GTP analog) (3, 21). IEC-6 cell lysate (250 μg) was incubated with the immobilized Ras proteins (25 μg) for 60 min at 4 °C. Similar experiments were performed using a rat brain lysate as a source of Ras effectors (20). Following the 1-h incubation, the immobilized Ras proteins were washed three times with 1× p21 buffer containing 0.2% Nonidet P-40 and 1 mM dithiothreitol and 3 times with 1× p21 buffer alone. Lysosomal sample buffer was then added to the washed silica beads, and the samples were resolved by SDS-PAGE and transfflipped to polyvinylidene difluoride membranes. The membranes were then immunoblotted for the known Ras effectors using the following antibodies: Raf-1 (Transduction Labs), B-Raf antisemur (Walter Kolch), PI 3-kinase, anti-p85 (Upstate Biotechnology), Raf1 antisemur (Steve Martin, Franz Hofer), Rin 1 (Transduction Labs), and AF6 (K. Kaibuchi). Antibodies for the p110 isoforms were obtained from Santa Cruz and used at the recommended 1:200 dilution.

Phosphorylated ERK1/2 was detected by Western analysis using a monoclonal phosphospecific antibody against the TEY phosphorylation sites of ERK1/2 (Santa Cruz). Total ERK2 was detected using anti-MAPK/ERK2 antibody (Upstate Biotechnology). Phosphorylated Akt was detected by Western analysis using a polyclonal phosphospecific antibody against Ser473 of Akt, and total Akt using a polyclonal Akt antibody (New England Biolabs).

Transformation Assays—Subconfluent cells stably expressing the various Ha-Ras mutants were plated at 1× 105/100-mm dish in medium containing 10% serum and 0.3% agar over a previously poured layer of 0.6% agar. The cells were photographed after 21 days.

Protein Expression and Western Blot Analysis—Protein expression was assessed by Western blotting using anti-Ha-Ras monoclonal antibody (Santa Cruz). Stable clones that expressed the various Ha-Ras proteins at levels consistent with levels expressed in T24 bladder carcinoma cells were selected for further analysis. Partially purified membrane fractions were prepared by first lysing cells in 1× p21 buffer (20 mM MOPS, pH 7.4, 200 mM sucrose, 5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol) containing 0.1% saponin, protease inhibitors (aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (saponin lysis buffer). The cell ghosts were isolated by centrifugation at 13,000 rpm for 10 min at 4 °C. The pellet was then solubilized in 1× p21 buffer containing 1% (w/v) CHAPS, protease, and phosphatase inhibitors (CHAPS lysis buffer), the samples were clarified by centrifugation at 13,000 rpm for 10 min at 4 °C, and the supernatant was saved as the enriched membrane fraction. Fifty micrograms of the CHAPS fraction was resolved on SDS-PAGE (13% gel) and transferred to polyvinylidene difluoride membrane (Amerham Pharmacia Biotech). Membranes were blocked with TBS (20 mM Tris, pH 7.4, 140 mM NaCl), containing 0.1% Nonidet P-40, 5% dry milk, and 0.1% calf serum for 1 h at room temperature. After washing with TBS/Nonidet P-40, the membranes were incubated with the primary antibody overnight at 4 °C. The membranes were washed for 1 h (four changes) with TBS/ Nonidet P-40 and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody. The membranes were then washed for 2 h, and the immunoreactive bands were detected with ECL Plus Reagent (Amerham Pharmacia Biotech) and visualized on a Molecular Dynamics StormImager.
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A

B

C

FIG. 1. Biochemical characterization of the PI 3-kinase Ras interaction. A, nucleotide exchange rate of Ha-Ras proteins. Purified recombinant Ha-Ras proteins were incubated in p21 buffer containing 10 μM GTP, 30 mM MgCl₂, and 10 μCi of [α-³²P]GTP at 30 °C. At the indicated time points, the aliquots were filter-bound, and protein-associated [α-³²P]GTP was measured by scintillation counting. The values are expressed as percentages of total bound. The total amount of bound [α-³²P]GTP was determined from incubating identical reactions in the presence of excess EDTA (35 mM). B, effector binding specificity of Ha-Ras effector mutants. Fifty micrograms of the indicated immobilized recombinant Ras proteins were loaded with either GDP or GMP-P NP and then incubated with 0.5 ml of brain lysate (panels i and ii, 11 mg/ml) or IEC cell lysate (panels iii-vi, 5 mg/ml) for 1 h at 4 °C. The immobilized Ras beads were washed, and the associated proteins were resolved on SDS-PAGE and immunoblotted with antibodies against either the p85 subunit of PI 3-kinase, Raf-1, or antibodies for the specific p110 subunits of PI 3-kinase. Immunoreactive bands were detected by standard ECL techniques. C, cytotoxic extracts (1 ml at 20 mg/ml) were incubated with 100 μg of immobilized Ha-Ras-GDP or GMPPNP. The samples were washed and separated on SDS-PAGE along with 20 μl of untreated cytosol and a 20-μl aliquot from the Ha-Ras-GMPPNP supernatant (post-Ras incubation). The membranes were immunoblotted for the presence of Rin 1 and RalGDS. 64:156 or 64G:17G:156L, Y64H:Y71H:F156L; 156 or 156L, F156L, wt, wild type; C40, Y40C Ha-Ras.
Y11G Ha-Ras forms stable complexes with Raf-1 but fails to physically associate with PI 3-kinase and neurofibromin. The structural changes arising from these point mutations also diminish its ability to form nucleotide-free stable complexes with guanine nucleotide exchange factors (21). It is very likely, therefore, that when expressed in cells these Ha-Ras proteins would not reach an adequate level of bound GTP to be active in signaling. To make Y64G/Y11G Ha-Ras exchange factor-independent we inserted an additional F156L point mutation. The insertion of this point mutation into wild-type Ha-Ras results in a protein with an accelerated exchange rate, making the Ras protein exchange factor-independent (26).

We first examined the in vitro biochemical properties of Y64G/Y11G/F156L Ha-Ras. To verify that the addition of the F156L mutation yielded a Ras protein with constitutive activity, we tested its in vitro guanine nucleotide exchange rate. In the presence of millimolar MgCl₂, wild-type Ha-Ras has a very slow guanine nucleotide exchange rate, with the half-life of Ras-GDP being greater than 20 min (27). As shown in Fig. 1A, wild-type Ha-Ras exchanges nucleotides very slowly, whereas both F156L and Y64G/Y71G/F156L Ha-Ras proteins exchange guanine nucleotides at a greatly accelerated rate.

We next verified that the F156L mutation did not alter the effector selectivity of the Y64G/Y11G mutant. Covalently immobilized recombinant Ha-Ras proteins (wild type, F156L, or Y64G/Y71G/F156L) were loaded with GDP or GMP-PNP and incubated with whole cell lysates from IEC-6 cells. We also compared the in vitro binding properties of our mutant proteins to the previously characterized G12V/Y40C Ha-Ras effector mutant, which binds only PI 3-kinase. Wild-type Ha-Ras, F156L, and Y64G/Y71G/F156L Ha-Ras-GMP-PNP proteins formed stable complexes with Raf-1, whereas the G12V/Y40C mutant did not stably associate with Raf-1 (Fig. 1B, panel i). All of the Ha-Ras proteins tested, except the variant carrying the Y64G mutation, formed stable complexes with the p85 subunit of dimeric PI 3-kinase (Fig. 1B, panel ii). The binding site for Ras-GTP and PI 3-kinase has been mapped to the p110 subunit. Work by other groups has also shown that Ha-Ras-GTP can associate with multiple isoforms of the p110 family in an in vitro binding assay (6, 8, 14, 28). We tested, therefore, whether the mutation at position 64 rendered the Ha-Ras protein unable to associate with all of the different p110 subunits expressed in IEC-6 cells. All of the Ras proteins tested except the Y64G/Y71G/F156L Ha-Ras formed stable complexes with p110α and p110β (Fig. 1B, panels iii and iv). Although IEC-6 cells express easily detectable levels of both p110β and p110γ, we failed to detect a stable interaction between these two p110 isoforms and wild-type F156L, or G12V/Y40C Ha-Ras (Fig. 1B, panels iv and v). These data were confirmed using saturation experiments. Lysate exposed to a second round of naïve Ha-Ras-GMPPNP did not possess any bound PI 3-kinase (either p85 or p110α or δ; data not shown). The remaining lysate possessed easily detectable levels of both p110β and γ, although there was a significantly reduced amount of both p110α and δ (data not shown). This supports our previous observation suggesting that Ha-Ras forms stable complexes with only p110α and p110β from whole cell lysates.

Wild-type Ha-Ras, F156L, and Y64G/Y71G/F156L Ha-Ras-GMP-PNP proteins formed stable complexes with Raf-1, whereas the G12V/Y40C mutant did not stably associate with Raf-1 (Fig. 1B, panel i). Both the F156L and Y64G/Y71G/F156L Ha-Ras proteins formed stable complexes with B-Raf (data not shown), RalGDS (Fig. 1C, panel ii), Rin 1 (Fig. 1C, panel i), and AF6 (data not shown) to a similar extent as those observed with wild-type Ha-Ras. The detection of an interaction between these Ha-Ras proteins and RalGDS is particularly instructive.

The interaction between c-Ha-Ras-GTP and RalGDS has been reported to be in the low micromolar range (1–3 μM), a significantly weaker interaction than that of Ras-GTP with either Raf-1 (100 nM) or the p110 subunit of PI 3-kinase (150 nM) (8, 29–31). The ability of this assay to detect the interaction between Ha-Ras-GTP and RalGDS suggests that the Y64G mutation results in a Ha-Ras protein that has a significantly lower affinity for the p110α and p110β catalytic subunits than that of RalGDS.

The Ras effector molecules clearly preferred wild-type Ha-Ras bound with GMPPNP rather than GDP. Our data, however, appear to indicate that Ha-Ras containing a F156L mutation does not possess guanine nucleotide specificity for target proteins. It is likely that because of its accelerated, factor-independent exchange rate that there is probably a substantial level of conversion of Ha-Ras-GDP to Ha-Ras-GTP during the incubation of the immobilized Ras with the lysates. These data, however, do indicate that the point mutations Y64G/Y71G/F156L result in a Ha-Ras protein that has accelerated nucleotide exchange activity and interacts with all effectors tested except PI 3-kinase p110 subunits. This provides us with a tool to test whether the direct interaction between activated Ha-Ras and PI 3-kinase is required for Ras-dependent biological end points.

Minimal Expression of Constitutively Active Ha-Ras Proteins Results in the Transformation of IEC-6 Cells—We chose rat intestinal epithelial cells (IEC-6) to test the oncogenic potential of the Y64G/Y71G/F156L Ha-Ras protein. We established stable cell lines expressing F156L Ha-Ras, Y64G/Y71G/F156L Ha-Ras, or the well characterized G12V Ha-Ras as a positive control for anchorage-independent growth. We specifically selected clones that expressed low levels of the various Ha-Ras proteins. The rationale for this approach stems from our previous observations demonstrating that overexpression of oncogenic Ha-Ras can result in promiscuous interactions that may not mimic physiologically relevant signaling events (12). The T24 human bladder carcinoma cell line provides an example of cellular transformation by the spontaneous expression of an oncogenic Ha-Ras protein (32). Normally bladder epithelia do not express detectable levels of Ha-Ras, as is the case with the J82 cell line, whose transformation is not Ras-dependent (33). The T24 human bladder carcinoma expresses an oncogenic Ha-Ras protein carrying a G12V point mutation (32). We determined that the level of G12V Ha-Ras expressed in the T24 bladder carcinoma is ~20% of the amount of the endogenous c-N-Ras. We used this as our model for minimal protein expression, because it is sufficient for cellular transformation. As shown in Fig. 2A, the expression levels of the various mutant Ras proteins are comparable with the level of G12V Ha-Ras detected in the naturally occurring T24 human bladder carcinoma. We documented that this minimal level of Ha-Ras expression was maintained throughout the entire course of all experiments (data not shown).

The consequence of oncogenic Ha-Ras expression on the morphology of IEC-6 epithelial cells is shown in Fig. 2B. We first established a critical role for PI 3-kinase activity in the Ras-dependent transformation of IEC-6 cells. Cells expressing the oncogenic G12V Ha-Ras (clones V12(8) and V12(11)) possessed a refractile morphology and proliferate in soft agar (Fig. 2B). IEC-6 cells transfected with empty vector retained a flat, cuboidal morphology to that resembling the parental IEC-6 cells (Fig. 2B). We chose rat intestinal epithelial cells (IEC-6) to test the oncogenic potential of the Y64G/Y71G/F156L Ha-Ras protein. We established stable cell lines expressing F156L Ha-Ras, Y64G/Y71G/F156L Ha-Ras, or the well characterized G12V Ha-Ras as a positive control for anchorage-independent growth. We specifically selected clones that expressed low levels of the various Ha-Ras proteins. The rationale for this approach stems from our previous observations demonstrating that overexpression of oncogenic Ha-Ras can result in promiscuous interactions that may not mimic physiologically relevant signaling events (12). The T24 human bladder carcinoma cell line provides an example of cellular transformation by the spontaneous expression of an oncogenic Ha-Ras protein (32). Normally bladder epithelia do not express detectable levels of Ha-Ras, as is the case with the J82 cell line, whose transformation is not Ras-dependent (33). The T24 human bladder carcinoma expresses an oncogenic Ha-Ras protein carrying a G12V point mutation (32). We determined that the level of G12V Ha-Ras expressed in the T24 bladder carcinoma is ~20% of the amount of the endogenous c-N-Ras. We used this as our model for minimal protein expression, because it is sufficient for cellular transformation. As shown in Fig. 2A, the expression levels of the various mutant Ras proteins are comparable with the level of G12V Ha-Ras detected in the naturally occurring T24 human bladder carcinoma. We documented that this minimal level of Ha-Ras expression was maintained throughout the entire course of all experiments (data not shown).

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This is consistent with recent reports supporting a requirement for PI 3-kinase activity in the transformation of intestinal epithelial cells (34, 35). Those stably expressing F156L, Y64G/Y71G/F156L, or G12V Ha-Ras were spindle-shaped and displayed a refractile morphology consistent with a transformed phenotype. Cells expressing F156L, Y64G/Y71G/F156L, or G12V Ha-Ras proliferated without attachment to an extracellular matrix in a standard soft agar assay (Fig. 2C and Table I). These observations suggest that a direct interaction between oncogenic Ha-Ras and PI 3-kinase is not required for transformation.

Steady-state Changes in PI 3-Kinase-dependent Lipid Products in Ha-Ras Transformed IEC-6 Cells—Our data suggest that a direct interaction between PI 3-kinase and oncogenic Ha-Ras is not required to generate a transformed morphology. Because we observed no differences in the ability to transform IEC-6 cells, which was dependent on the direct interaction between PI 3-kinase and oncogenic Ha-Ras, we next designed experiments to analyze whether IEC-6 cells transformed by the different Ras constructs displayed any differences in PI 3-kinase-dependent lipid metabolism. We first examined whether Ha-Ras transformed cells displayed elevated PI 3-kinase activity above control cells. We were unable to detect significant differences in PI 3-kinase activity between control and Ha-Ras transformed IEC-6 cells in whole cell lysates (data not shown). We also did not observe significant changes in PI

FIG. 2. Minimal expression of constitutively activated Ha-Ras proteins results in the cellular transformation of IEC-6 cells. A, levels of ectopic Ha-Ras expressed in the indicated IEC-6 stable cell lines relative to the naturally occurring GV12 Ha-Ras expressed in T24 human bladder carcinoma cell line. The J82 human bladder carcinoma, whose transformation is Ras-independent, is shown to provide a reference point for the level of Ha-Ras expression in bladder epithelia. Fifty micrograms of membrane-enriched cell lysates from the indicated cell lines was resolved on SDS-PAGE (13%) and immunoblotted for Ha-Ras expression using a Ha-Ras-specific monoclonal antibody. B, morphology of subconfluent IEC-6 cells stably expressing either empty vector or the indicated mutant Ha-Ras proteins. The cells were plated at equal densities in DMEM containing 10% FBS and photographed the next day. C, anchorage-independent growth of the indicated IEC-6 cell lines was assayed by growth in soft agar. 1 × 10^5 IEC-6 cells expressing the indicated Ha-Ras mutants were plated in DMEM containing 10% FBS and 0.3% agar over a previously poured layer of 0.6% agar. The cells were photographed 21 days later. A single representative clone is shown for each cell line. 64:156, Y64G/Y71G/F156L; 156, F156L; V12, G12V Ha-Ras.
3-kinase activity from serum-starved cells stimulated with EGF (data not shown).

In an effort to maximize the putative Ras-dependent activated PI 3-kinase pool, plasma membranes were partially purified from serum-starved IEC-6 cells expressing the indicated Ha-Ras proteins. PI 3-kinase was immunoprecipitated from CHAPS-solubilized plasma membranes, and its activity was determined by an in vitro kinase assay. All of the cell lines possessed similar levels of plasma membrane-associated PI 3-kinase activity (Fig. 3A) and protein levels (data not shown).

To monitor more subtle changes in PI 3-kinase activity, as reflected by changes in 3-phosphorylated phosphoinositide levels, we examined the phosphorylation and activity of Akt/PKB (36). Changes in AKT were monitored by immunoprecipitation followed by an in vitro kinase assay using histone H2B as a substrate and Western analysis for changes in the level of serine 473 phosphorylation. We did not detect elevated levels of Ser473 phosphorylation of Akt from IEC-6 cells transformed by the indicated mutant Ha-Ras proteins as compared with control cells (Fig. 3B). Similar results were reflected in the in vitro kinase assay (data not shown). In all cases, however, significant changes in the level of phosphorylated Akt (Fig. 3B) and phosphorylated ERK (data not shown) were observed when serum-starved cultures were challenged with EGF.

Oncogenic Ras proteins are thought to be transforming by virtue of their ability to maintain higher steady-state levels of Ras-GTP. The naturally occurring oncogenic proteins maintain higher steady-state GTP-bound forms than their reduced intrinsic GTPase activity coupled with their reduced sensitivity to GAP-like molecules (37). The F156L Ha-Ras proteins achieves higher steady-state GTP levels through their accelerated guanine nucleotide exchange rates. In both cases, however, elevated steady-state Ras-GTP levels should result in constitutive complexes between the activated Ha-Ras and its physiological downstream targets. Downward and co-workers (38) have also reported that activated Ha-Ras proteins can increase the catalytic activity of the p110 PI 3-kinase subunits. This group has demonstrated, in transient assays, that expression of G12V Ha-Ras does slightly elevate lipid products of PI 3-kinase. This effect was potentiated by the co-transfection of the p110 catalytic subunit of PI 3-kinase (8, 14). Although these observations were unexpected, they are consistent with the elevated levels of PI (3,4,5)P3 in all of the Ras transformed cell lines (Fig. 3C, panel i). Inclusion of the PI 3-kinase inhibitor LY294002 resulted in a complete disappearance of the glycerol phosphoinositide 1,3,4,5 tetrasphosphate lipid product but only about a 50% reduction in the glycerol phosphoinositide 1,3,4,5 tetrasphosphate lipid product (although the background for this particular sample is significantly higher than for other samples).

We were surprised to find that there was a significant decrease (or absence) of the gPPI (1,3,4)P2 in all of the Ras transformed cell lines (Fig. 3C, panel ii, top panel). In contrast to this, however, there was a modest (2-fold) increase, consistent with those observed in transient assays by Downward and co-workers, of the glycerophosphoinositide 1,3,4,5 tetrasphosphate product of PI 3-kinase (8, 14). Although we currently do not have an explanation for the apparent opposing effects of the oncogenic Ras proteins on the steady-state levels of PI (3,4)P2 and PI (3,4,5)P3, the important issue is that the PI 3-kinase-defective Ras protein produced identical changes in PI 3-kinase-dependent lipid products as both the F156L Ha-Ras and G12V Ha-Ras. This suggests that the elevated levels of PI (3,4,5)P3 in Ha-Ras transformed cells are not generated through a direct interaction between the oncogenic Ras protein and PI 3-kinase itself.

Elevated MAPK Activity Is Not Required for Ha-Ras Transformation of IEC-6 Cells—Elevated MAPK activity, above basal, is often detected in Ras transformed cells. It has also been reported that elevated MAPK activity is required to maintain the transformed phenotype of RIE-1 epithelial cells induced by the expression of Ras but not Src (38). Fig. 4A shows the in vitro kinase activity of immunoprecipitated, endogenous MAPK from serum-starved cells expressing the indicated mutant Ras proteins. Initially, we expected to see elevations in MAPK activity consistent with the transformed morphology, especially in cells expressing G12V Ha-Ras. We did not observe elevated MAPK activity in any of the cell lines transformed with F156L, Y64G/Y71G/F156L, or even the well characterized oncogenic G12V Ha-Ras. The same trend is reflected in the phosphorylation state of MAPK using a phospho-specific antibody to activated ERK1/2 (Fig. 4B).

Although we did not detect elevated MAPK activity, control IEC-6 cells have slightly elevated basal levels of MAPK activity that can be further activated by the addition of EGF (Fig. 4B). The marginal MAPK activity in serum-starved IEC-6 cells is not significantly reduced with prolonged starvation (72 h) (data not shown). Treatment of cells with the MEK1 inhibitors U0126 (Fig. 4B) or PD98059 (data not shown) reverts their transformed morphology (data not shown) and abrogates the basal level of MAPK in control or Ras transformed IEC-6 cells. Although these observations were unexpected, they are consistent with the elevations in MAPK activity we observe with other cell types transformed with minimal levels of G12V Ha-Ras. Both 10T1/2 fibroblasts and 23A2 myoblasts transformed by G12V Ha-Ras have elevated MAPK activity that is 2–3-fold over the basal level. The basal level of MAPK activity in the untransformed 10T1/2 fibroblasts and 23A2 myoblasts, however, can be significantly reduced by serum starvation (12, 39). This suggests that the basal level of MAPK activity, detected in either control or cells transformed by an oncogenic Ha-Ras, is both required and sufficient to support the transformed phenotype. These observations are consistent with recent reports.
that also failed to detect elevated levels of MAPK activity in Ras transformed cells (40, 41).

The Role of PI 3-Kinase in Cell Migration—The data presented in the previous sections support the hypothesis that a direct interaction between oncogenic Ha-Ras and PI 3-kinase is not required for generation of a transformed phenotype. We
have yet to distinguish, either biologically or biochemically, any significant differences between cells expressing a fully functional oncogenic Ras protein or one that is defective in PI 3-kinase association. There have been a few reports suggesting that PI 3-kinase might play a role in cell migration (42–44).

Migration of cells toward growth factors or extracellular matrix proteins requires signaling from cell surface receptors to the motility machinery. Ras plays a critical role in regulating the migration of fibroblasts toward platelet-derived growth factor and fibronectin (24, 45). IEC-6 cells expressing F156L Ha-Ras, Y64G/Y71G/F156L Ha-Ras, or G12V Ha-Ras were tested for the ability to migrate toward soluble fibronectin using a multiwell Boyden chamber assay (25). This assay measures the directional movement of cells across a porous membrane in response to a chemoattractant concentration gradient. Cells expressing either F156L Ha-Ras or G12V Ha-Ras displayed slightly increased (25–30%) motility toward fibronectin. IEC-6 cells expressing Y64G/Y71G/F156L Ha-Ras were greatly impaired in their ability to migrate toward soluble fibronectin, even compared with the IEC-6 control cell line (Fig. 5). These results suggest that a direct interaction between Ha-Ras and PI 3-kinase may play an important role in the migratory response of epithelial cells to fibronectin. More importantly, however, this observation demonstrates that the expression of Y64G/Y71G/F156L Ha-Ras protein does alter some Ras-dependent biological end points compared with the expression of either G12V Ha-Ras or F156L Ha-Ras transforming proteins. This observation strengthens our conclusion that a direct interaction between oncogenic Ha-Ras and PI 3-kinase is not required for the transformation of epithelial cells.

**DISCUSSION**

The significance of constitutive Ras signaling through the Raf kinases, resulting in the up-regulation of the Raf/MEK/MAPK pathway, and its role in cellular transformation is well established (46). Although overexpression of activated Raf is sufficient to transform fibroblasts, several lines of evidence suggest that Ras-dependent transformation results from the constitutive signaling through at least two separate effector pathways. The most compelling data supporting this hypothesis were generated using partial loss of function Ras mutants that bind only Raf (G12V/T35S), only RalGDS (G12V/E37G), or PI 3-kinase (G12V/Y40C) (9, 13). Studies using these mutants lead to the conclusion that expression of an oncogenic Ras protein that interacts with a single effector is not sufficient to transform cells. Further support for additional Ras/effector mechanisms that contribute to Ras-dependent transformation arise from reports demonstrating that constitutive Raf activation, by the overexpression of either ΔRaf-22W or Raf-CAA, do not substitute for oncogenic Ras to establish the transformed phenotype of RIE-1 cells (19). These observations are quite different from those in fibroblasts where overexpression of activated Raf is sufficient for transformation (47), suggesting that Ras signaling involves different mechanisms in epithelial cells.
Although activated versions of all four Ras isoforms transform cells, we have previously published data supporting the hypothesis that they do so by utilizing distinct downstream effector molecules. Raf-1 preferentially associates with c-N-Ras in mouse fibroblasts minimally expressing an oncogenic Ha-Ras protein (12). Using IEC-6 cells transformed by G12V Ha-Ras, Raf-1 co-immunoprecipitated with c-N-Ras and not with the oncogenic G12V Ha-Ras (data not shown). These data suggest that Raf-1 may not act as a direct downstream effector for oncogenic Ha-Ras. The functional consequences of these observations are supported by reports that the transformed phenotype of RIE-1 epithelial cells expressing G12V Ha-Ras can be reverted by blocking EGFR function, indicating that oncogenic Ha-Ras mediates transformation in part by stimulating the production of EGFR ligands (48). We have extended those observations by demonstrating that blocking the EGFR in Ha-Ras transformed cells down-regulates MAPK activity, failing to activate the endogenous c-N-Ras-Raf-1 complex (18).

Taken together these findings point out the relevance of additional Ras effector interactions that may contribute to Ras transformation. PI 3-kinase is a likely effector molecule that has the potential to function directly downstream of the Ras proteins and participate in the transformation process. Cells transformed by oncogenic Ras proteins can be partially reverted by blocking PI 3-kinase activity using dominant negative p85 constructs or by the overexpression of PTEN, a phosphatase that specifically dephosphorylates the 3 position on the inositol ring (49, 50). There is some ambiguity, however, as to whether this increase in PI 3-kinase-dependent lipid products results from a direct interaction with oncogenic Ras-GTP. In this report, we focus on the specific question concerning the requirement of a direct interaction between oncogenic Ha-Ras and PI 3-kinase for cellular transformation.

We use an activated Ha-Ras mutant (Y64G/Y71G/F156L Ha-Ras) that interacts with known Ras effectors (B-Raf, AF6, Rin 1, RalGDS, and Raf-1) similarly to wild-type Ha-Ras but does not interact with PI 3-kinase. We demonstrate that minimal protein expression of Y64G/Y71G/F156L Ha-Ras supports both a transformed phenotype- and anchorage-independent growth of IEC-6 epithelial cells. Interestingly, all of the Ras expressing IEC cell lines exhibited identical changes in steady-state PI 3-kinase-dependent lipid products. All of the Ras-transformed cell lines possessed a 2-fold increase in steady-state PI (3,4,5)P3 and a rather striking absence of PI (3,4)P2 compared with the easily detectable levels in the control cultures. These observations suggest that the two predominant lipid products of PI 3-kinase might have opposing effects on the transformation process. Because we did not detect increased basal phosphorylation of AKT, this might also suggest that it is the PI (3,4,5)P3, PI 3-kinase-dependent lipid product that is responsible for the activation of AKT through its phosphorylation by PDK1 (51). Our data also imply, because the PI 3-kinase-defective mutant (Y64G/Y71G/F156L Ha-Ras) showed changes in PI 3-kinase-dependent lipids identical to those of the control transforming Ras proteins, that the observed changes in steady-state lipids do not arise from the direct interaction between oncogenic Ha-Ras and PI 3-kinase.

We did not detect significant changes in cellular (or plasma membrane-associated) PI 3-kinase activity in Ras transformed IEC-6 cells, even though we documented a 2-fold increase in the steady-state level of the PI 3-kinase product, PI (3,4,5)P3. We did find, however, that inhibition of the basal PI 3-kinase activity was sufficient to revert the transformed morphology. As with the basal MAPK activity in this cell type, the basal PI 3-kinase activity is both sufficient and required to generate the transformed phenotype. These observations are in agreement with work by another group suggesting that the basal PI 3-kinase activity is required for low, mitogenic doses of EGF to activate both Ras and the MAPK cascade (52).

The use of selective Ras mutants that fail to interact with a single putative downstream effector molecule have distinct advantages over the previously well used selective effectors that were characterized to interact with only a single target. The original “single” interacting Ras mutants (the T35S/E37G, 37G, and Y40C) were only characterized relative to “known” Ras binding partners. Identification of additional Ras binding partners and the possibility that each or all of these single interacting Ras mutants might interact with an unidentified target does leave data obtained with these mutants somewhat in question. We have found that the G12V/Y40C Ha-Ras protein interacts with Nore 1 in a manner identical to that of wild-type Ha-Ras (data not shown). This might suggest that previously identified PI 3-kinase-specific biological outcomes might also be attributed to the interactions between oncogenic Ha-Ras and Nore 1 or a co-operative effect arising from the interaction between both PI 3-kinase and Nore 1. Data obtained using the Y64G/Y71G/F156L Ha-Ras mutant are not subject to this limited interpretation. If we were to find that the Y64G/Y71G/F156L Ha-Ras protein fails to interact with a novel Ras binding partner, this would only add to our general conclusions that the putative novel binding partner and PI 3-kinase need not directly interact with oncogenic Ha-Ras to generate a transformed phenotype. Although it is attractive to speculate that a direct interaction between Ha-Ras and PI 3-kinase is important in cell migration, it is also possible that
another Ras effector that fails to interact with the Y64G/Y71G/F156L Ras-Ras protein might also play a role in cell migration. The recently identified phospholipase Cε (53–55), whose binding properties to the Y64G/Y71G/F156L Ras-Ras protein have not been characterized, is certainly a candidate for this Ras-dependent biological end point.

The results presented in this report raise two important issues concerning the direct interactions of oncopgenic Ha-Ras and putative effector proteins. First, if Ha-Ras does not require a physical interaction with either Raf-1 or PI 3-kinase to transform cells, which direct downstream effector(s) of oncopgenic Ha-Ras is responsible for the initial events in the transformation process? The most obvious candidate is RalGDS. Although the expression of activated RalGDS in NIH3T3 cells can induce anchorage-independent growth and proliferation in low serum, its immediate target, Ral, does not behave as an oncogene when expressed in NIH3T3 cells (7, 56, 57). Several studies, however, suggest that the role of RalGDS in Ras-dependent transformation is cooperative with other Ras effectors (9, 13). Even if RalGDS is a direct and specific effector of Ha-Ras, our data implicate at least one other Ras target that might cooperate with RalGDS or function independently of RalGDS as the critical element mediating Ha-Ras-dependent transformation.

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