Human cells contain four homologous Ras proteins, but it is unknown whether each of these Ras proteins participates in distinct signal transduction cascades or has different biological functions. To directly address these issues, we assessed the relative abilities of constitutively active (G12V) versions of each of the four Ras homologs to activate the effector protein Raf-1 in vivo. In addition, we compared their relative abilities to induce transformed foci, enable anchorage-independent growth, and stimulate cell migration. We found a distinct hierarchy between the four Ras homologs in each of the parameters studied. The hierarchies were as follows: for Raf-1 activation, Ki-Ras 4B > Ki-Ras 4A >> N-Ras > Ha-Ras; for focus formation, Ha-Ras ≈ Ki-Ras 4A >> N-Ras = Ki-Ras 4B; for anchorage-independent growth, Ki-Ras 4A ≈ N-Ras >> Ki-Ras 4B = Ha-Ras = no growth; and for cell migration, Ki-Ras 4B >> Ha-Ras > N-Ras = Ki-Ras 4A = no migration. Our results indicate that the four Ras homologs significantly differ in their abilities to activate Raf-1 and induce distinctly different biological responses. These studies, in conjunction with our previous report that demonstrated that the Ras homologs can be differentially activated by upstream guanine nucleotide exchange factors (Jones, M. K., and Jackson, J. H. (1998) J. Biol. Chem. 273, 1782–1787), indicate that each of the four Ras proteins may qualitatively or quantitatively participate in distinct signaling cascades and have significantly different biological roles in vivo. Importantly, these studies also suggest for the first time that the distinct and likely cooperative biological functions of the Ki-ras-encoded Ki-Ras 4A and Ki-Ras 4B proteins may help explain why constitutively activating mutations of Ki-ras, but not N-ras or Ha-ras, are frequently detected in human carcinomas.

Ras proteins function as molecular relay switches in signal transduction cascades that regulate cell proliferation, differentiation, and apoptosis. Extracellular ligands activate cell surface receptors and thereby induce the activation and/or membrane recruitment of guanine nucleotide exchange factors (GEFs),1 which convert inactive GDP-bound Ras into active GTP-bound Ras. To date, the known GEFs for Ras proteins include Sos 1, Sos 2, Ras-GRF/CDC25K, Ras-GRF 2, and Ras GRP (reviewed in Ref. 1). Following activation, GTP-bound Ras binds to/activates one or more effector proteins that subsequently initiate downstream signaling pathways that ultimately induce a programmed cellular response. Although members of the Raf serine/threonine kinase family (consisting of Raf-1, A-Raf, and B-Raf) are the best characterized effectors of Ras, numerous other putative effectors have been identified, including phosphatidylinositol 3-kinase, GEFs for the small GTPase Ral (Ral GDS, RGL, RLF/RGL2), AF6, RIN1, MEK kinase 1, protein kinase Cζ, and Nore1 (reviewed in 1–4). In addition, the GTPase-activating proteins, p120 GTPase-activating protein and neurofibromin/NF1-GTPase-activating protein, which inactivate Ras proteins by catalyzing GTP hydrolysis, have also been implicated as Ras effectors (1–4).

Human cells contain four Ras proteins, Ha-Ras, N-Ras, Ki-Ras 4A and Ki-Ras 4B. (The Ki-ras gene encodes two proteins, 4A and 4B, via alternative splicing of two fourth exons). These proteins are 85% homologous (5, 6) and, with the exception of Ki-Ras 4A, appear to be ubiquitously expressed (7–9). Although no studies have directly compared the biological functions of each of the four Ras homologs, several observations suggest that these proteins likely have different biological roles. For instance, recent studies have shown that Ki-ras, but not N-ras or Ha-ras, is essential for mouse embryogenesis (reviewed in Ref. 1). Mouse embryos lacking a functional Ki-ras gene develop cardiac, liver, neurologic, and hematologic defects and die before term, whereas mice lacking a functional Ha-ras or N-ras gene are born and grow normally. In addition, although mutations at codons 12, 13, or 61 constitutively activate each of the ras genes in vitro, there is clear selectivity with regard to which ras homolog is mutationally activated in a given type of human cancer. Whereas ~90% of pancreatic, 50% of colon, and 30% of lung adenocarcinomas harbor mutant Ki-ras genes, mutant Ha-ras or N-ras genes are rarely detected in these cancers (10). Likewise, whereas ~25% of acute myelogenous leukemias and myelodysplastic syndromes contain mutant N-ras genes, mutant Ha-ras and Ki-ras genes are not frequently observed in these malignancies (11). Moreover, mutant Ha-ras genes are rarely detected in any type of human cancer (10).

Given the likelihood that the four Ras homologs have different biological roles in vivo, we hypothesized that each of the four Ras homologs participates in distinct signal transduction pathways. Recent studies from our laboratory have shown that the GEF Ras-GRF activates Ha-Ras but does not activate N-Ras or Ki-Ras 4B protein in vivo (12). In addition, previous studies have shown that extracellular ligands can selectively trigger a specific GEF to activate Ras proteins. For instance, ligands that activate tyrosine kinase receptors induce Sos, but not Ras-GRF, to activate Ras, whereas ligands that activate G protein-coupled muscarinic receptors induce Ras-GRF, but not Sos, to activate Ras proteins (13–15). These combined studies...
suggest that each of the Ras homologs could potentially be selectively activated by a distinct upstream extracellular ligand/receptor/GEF(s) in vivo. If, therefore, it could be demonstrated that each of the four Ras homologs selectively activates a distinct downstream effector protein(s) in vivo, these studies would support the premise that the four Ras homologs participate in distinct signaling cascades. Accordingly, in this report, we assessed whether the four Ras homologs could differentially activate the best characterized effector protein, Raf-1, in vivo. In addition, to directly test the notion that the four Ras homologs have different biological roles in vivo, we compared the ability of each of the Ras homologs to induce transformed foci, enable anchorage-independent growth, and stimulate cell migration.

**EXPERIMENTAL PROCEDURES**

**Molecular Constructs—**To distinguish exogenous from endogenous Ras proteins, a Glu-Glu (EE) epitope tag (EEREYMPME) (16) was added to the N termini of constitutively active (G12V) human Ha-ras, N-ras, Ki-ras 4A, and Ki-ras 4B cDNAs. EE-tagged G12V ras cDNAs and wild type ras cDNA were cloned into the mammalian transfection vectors pZIP-Neo-SVIII (17). pRK5 Myc, which contains an SV40 origin of replication and adds a 10 amino acid N-terminal Myc tag to expressed proteins (18), and/or pEGFP-C1, which fuses green fluorescent protein (GFP) to the N termini of expressed proteins (CLONTECH).

**Raf-1 Kinase Assays—**Raf-1 kinase activity was assessed by coupled MEK/ERK2 2 kinase assays, according to previously described methods (19, 20) with minor modifications. Briefly, COS-1 cells (8  10^5 cells/100-mm dish) were co-transfected with 2 µg of wild type ras-p190Ras or Ki-ras 4A or Ki-ras 4B/pRK5 Myc (empty pRK5 Myc) plasmid DNA using 60 µl of SuperFect (Qiagen). 24 h following transfection, cells were serum-starved for 16 h in serum-free medium containing 4 µM mevalonolactone (COS-1 cells do not contain sufficient amounts of endogenous isoprenoid precursors to efficiently isoprenylate Ras proteins, so medium was supplemented with mevalonolactone to augment available precursors). Cells were subsequently lysed in 100 µl of lysis buffer (20 mM Tris-HCl, pH 7.5; 1% Triton X-100; 1 mM EDTA; 1.5 mM KCl; 0.1 mM phenylmethylsulfonfluryl fluoride; 5 µg/ml leupeptin; 1 mM benzamidine; 5% glycerol; 0.3% β-mercaptoethanol; 5 mM NaF; 0.2 mM Na_2VO_4; 0.5 µM okadacid acid) and diluted 1:10 in lysis buffer containing 10% glycerol and no KCl. Cleaved Ras proteins (25 µg) were subsequently incubated in 100 µl of lysis buffer containing 6.5 µg/ml of Raf-1 C12 antibody (Santa Cruz) for 1 h on ice, followed by 50 µl of Pansorbin Staph A cells (Calbiochem) for 1 h at 4°C. Immunoprecipitates were washed successively one time each in wash buffer 1 (30 mM Tris-HCl, pH 7.5; 0.1% Triton X-100; 0.2 mM EDTA; 0.3% β-mercaptoethanol; 1 mM KCl; 5 µg/ml leupeptin; 1 mM benzamidine; 1 mM phenylmethylsulfonfluryl fluoride; 5 µg/ml leupeptin; 0.2 mM Na_2VO_4; 0.2 µM Na_3VO_4; 0.5 µM okadacid acid), wash buffer 2 (same as wash buffer 1 except KCl is reduced to 0.1 M), and wash buffer 3 (same as wash buffer 1 except KCl is excluded); resuspended in 20 µl of reaction buffer containing 30 mM Tris-HCl, pH 7.5; 0.1% Triton X-100; 0.05% Brij 35; 10 mM MgCl_2; 0.3% β-mercaptoethanol; 5 mM NaF; 0.2 mM Na_2VO_4; 0.5 µM okadacid acid) containing 6.5 µg/ml MEK-1 (Santa Cruz), 100 µg/ml ERK2 (Santa Cruz), and 0.8 mM ATP; and incubated/vortexed for 15 min at 37°C. Reactions were terminated by the addition of 20 µl of reaction buffer containing 20 mM EDTA and no MgCl_2. Supernatants (10 µl) of terminated reactions were added to 40 µl of ice-cold reaction buffer (50 mM Tris, pH 7.5; 0.1 M EGTA; 12.5 mM MgCl_2) containing 16 µg of myelin basic protein (MBP) and 0.125 mM [γ-^32P]ATP (10  10^6 cpm/mmol), and incubated/vortexed for 30 min at 37°C. Reactions were terminated by the addition of 50 µl of 2X SDS-polyacrylamide gel electrophoresis sample buffer, boiled for 3 min, and resolved on 15% SDS-polyacrylamide gels. The radioactivity incorporated into MBP was visualized by autoradiography and quantitated on a PhosphorImager (Molecular Dynamics). PhosphoImager counts due to Raf-1 (and the empty Ras vector) were subtracted from counts due to Raf-1 and each of the four Ras homologs.

**Transformation Assays—**Cell transformation was assessed by focus-forming assays and soft agar colony formation assays. For focus-forming assays, NIH 3T3 mouse fibroblasts (5  10^5 cells/60-mm dish), Rat-1 fibroblasts (1  10^5 cells/60-mm dish), and RIE-1 rat intestinal epithelial (5  10^5 cells/60-mm dish) cells were transfected by calcium phosphate precipitation (NIH 3T3 and Rat-1) or LipofectAMINE (RIE-1) with 50 ng, 2 µg, and 2.5 µg, respectively, of G12V Ha-ras, N-ras, Ki-ras 4A, or Ki-ras 4B/pZIP-Neo-SVIII plasmid DNA; after 14 (NIH 3T3) or 21 (Rat-1, RIE-1) days, dishes were stained with crystal violet, and the number of transformed foci was counted (21, 22).

For soft agar colony formation assays, Rat-1 and RIE-1 cells, transfected as described above, were selected in G418, suspended in reduced-serum (2%) medium containing 0.2% agar, and overlaid onto a 0.6% agar base at a density of 2  10^4 cells/90-mm dish. Colony formation was monitored for up to 1 month (22, 23).

**Cell Migration Assays—**Cell migration was assessed by quantitating the number of cells that directionally migrate through membranes to a collagen undercoating (24). Briefly, COS-7 cells (5  10^5/100-mm dish) were transfected with 0.1 µg of pCMV-N-ras or 0.2 µg of N-ras, Ki-ras 4A, or Ki-ras 4B/pEGFP-C1 plasmid DNA using 50 µl of LipofectAMINE. (Amounts of transfected DNA represent amounts required to achieve comparable expression levels). 24 h following transfection, cells were incubated in serum-free medium containing 4 µM mevalonolactone for 16 h and loaded (1  10^6 cells) into modified Boyden chambers containing collagen type I-undercoated membranes. Cells were allowed to migrate through membranes for 3 h at 37°C, and the number of GFP-Ras-expressing cells that migrated was quantitated on a fluorescence microscope. At least five fields (magnification, 10) were counted for each membrane.

**Immunodetection of Raf-1 and/or Ras Homologs—**Expression levels of Raf-1 and/or Ras homologs in COS-1 and COS-7 cells were assessed by Western blot, and expression levels of Ras homologs in RIE-1 cells were assessed by immunoprecipitation. Briefly, for Western blots, lysates of COS-1 and COS-7 cells (5 µg) were resolved on 15% SDS-polyacrylamide gels and transferred to nitrocellulose. Raf-1 and Ras proteins contained in COS-1 cell lysates were probed with Raf-1 C12 (Santa Cruz) and Myc 9E10.2 antibodies, respectively. GFP-Ras fusion proteins contained in COS-7 cell lysates were probed with monoclonal GFP antibody (CLONTECH). Immunoblots were developed using enhanced chemiluminescence (Pierce). For immunoprecipitations, G418-selected RIE-1 cells were labeled overnight with 100 µCi/ml [35S]methionine/cysteine (ICN) and lysed in high SDS/Tris/radioimmunoprecipitation assay buffer. Ras proteins were immunoprecipitated from cleared cell lysates with EE antibodies, resolved on 15% SDS-polyacrylamide gels, and visualized by fluorography (21, 23).

**RESULTS**

**Differential Activation of Raf-1 by Ras Homologs—**To determine whether the four Ras homologs might differentially activate Raf-1 in vivo, we transiently co-transfected COS-1 cells with wild type ras-p190Ras and constitutively active (G12V) Ha-ras, N-ras, Ki-ras 4A, or Ki-ras 4B expression plasmids and assessed the activity of Raf-1 immunoprecipitated from these cells by quantitating MBP substrate phosphorylation in coupled MEK/ERK2 in vitro kinase assays. As shown in Fig. 1, A and B, Ki-ras 4B activated Raf-1 8.4-, 4.4-, and 2.3-fold more efficiently than Ha-ras, N-ras, or Ki-ras 4A protein, respectively (p < 0.005). Importantly, the differential abilities of the Ras homologs to activate Raf-1 were not due to variations in Ras homolog or Raf-1 expression levels (Fig. 1C). (Levels of expression of exogenous Ras and Raf-1 proteins were approximately 5–6-fold and 6–8-fold higher, respectively, than endogenous Ras and Raf-1 proteins). For instance, although Ki-ras 4B had an enhanced ability and Ha-ras had a reduced ability to activate Raf-1, Raf-1 expression levels were comparable and exogenous Ha-ras expression was significantly higher than exogenous Ki-ras 4B protein expression. (Note that the small, slower-migrating Ha-ras band in Fig. 1C represents non-post-translationally modified Ha-ras protein). Likewise, although Raf-1 levels were comparable and exogenous Ki-ras 4A was expressed at significantly higher levels than exogenous Ki-ras 4A protein, Ki-ras 4A activated Raf-1 significantly better than N-ras protein. Furthermore, although Ki-ras 4A expression was slightly lower than Ki-ras 4B expression, the relative ability of Ki-ras 4A versus Ki-ras 4B protein to activate Raf-1 did not significantly change when the amount of transfected Ki-ras 4A plasmid DNA was titrated to achieve comparable Ki-ras 4A and Ki-ras 4B expression levels (data not shown). Our results indicate, therefore, that the four Ras homologs
significantly differ in their abilities to activate Raf-1 in COS-1 cells. Ki-Ras 4B efficiently activates Raf-1, and Ki-Ras 4A, N-Ras, and Ha-Ras proteins show progressively decreasing abilities to activate Raf-1.

**Differential Biological Properties of Ras Homologs**—To determine whether the four Ras homologs might have different biological functions in *vivo*, we assessed their relative abilities to induce transformed foci, enable anchorage-independent growth, or stimulate cell migration.

For focus-forming assays, NIH 3T3 mouse fibroblast, Rat-1 fibroblast and RIE-1 rat intestinal epithelial cells were transfected with constitutively active (G12V) Ha-ras, N-ras, Ki-ras 4A, Ki-ras 4B, or N-ras expression plasmids. The activity of immunoprecipitated Raf-1 was assessed by quantitating MBP substrate phosphorylation in coupled MEK/ERK2 in *vitro* kinase assays. Data shown are representative of four independent experiments done in triplicate. A, autoradiogram of MBP phosphorylation. B, PhosphorImager quantitation of MBP phosphorylation. PhosphorImager counts shown are arbitrary and represent the relative intensity of photon emissions. Counts due to Raf-1 alone, in the absence of exogenous Ras homologs (282 × 10^3), have been subtracted. Data shown represent the mean ± S.E. C, immunoblot of COS-1 cell lysates showing expression levels of Raf-1 and exogenous Ras homolog proteins.

![Fig. 1](image1.png)

**Fig. 1.** Ki-Ras 4B activates Raf-1 more efficiently than Ha-Ras, Ki-Ras 4A, or N-Ras. Raf-1 was immunoprecipitated from COS-1 cells transiently transfected with wild typeraf-1 and constitutively active (G12V) Ha-ras, Ki-ras 4A, Ki-ras 4B, or N-ras expression plasmids. The activity of immunoprecipitated Raf-1 was assessed by quantitating MBP substrate phosphorylation in coupled MEK/ERK2 in *vitro* kinase assays. Data shown are representative of four independent experiments done in triplicate.

For cell migration haptotaxis assays, COS-7 cells were transiently transfected with G12V GFP-ras fusion constructs, serum-starved, and loaded into Boyden chambers. Numbers of GFP-Ras-expressing cells that migrated through polycarbonate membranes to a collagen-undercoating during a 3 h incubation selected in G418 and suspended in reduced-serum soft agar medium. Colony formation was monitored for up to 1 month. As shown in Figs. 2 and 3A, the ability of Ki-Ras 4A or Ki-Ras 4B to enable RIE-1 cells to grow in soft agar paralleled their ability to induce transformed foci in these cells. Ki-Ras 4A efficiently induced foci and enabled soft agar growth, whereas Ki-Ras 4B neither efficiently induced foci nor enabled soft agar growth. In contrast, although Ha-Ras efficiently induced foci in RIE-1 cells, Ha-Ras-expressing RIE-1 cells were completely unable to grow in soft agar. Moreover, although N-Ras demonstrated little ability to induce foci in RIE-1 cells, N-Ras efficiently enabled RIE-1 cells to grow in soft agar. As shown in Fig. 3B, in RIE-1 cells, expression levels of exogenous Ha-Ras, Ki-Ras 4B, and N-Ras proteins were comparable and slightly higher than exogenous Ki-Ras 4A protein. It is unlikely, therefore, that differences in protein expression accounted for the ability of Ki-Ras 4A and N-Ras or inability of Ha-Ras and Ki-Ras 4B proteins to enable soft agar growth. However, we are unable to definitively conclude whether there may be differences in the relative ability of Ki-Ras 4A or N-Ras protein to enable RIE-1 cells to grow in soft agar, due to the slight differences in expression between these homologs. Interestingly, although only Ki-Ras 4A and N-Ras enabled RIE-1 epithelial cells to grow in soft agar, each of the four Ras homologs enabled Rat-1 fibroblast cells to grow in soft agar (data not shown).

For cell migration haptotaxis assays, COS-7 cells were transiently transfected with G12V GFP-ras fusion constructs, serum-starved, and loaded into Boyden chambers. Numbers of GFP-Ras-expressing cells that migrated through polycarbonate membranes to a collagen-undercoating during a 3 h incubation selected in G418 and suspended in reduced-serum soft agar medium. Colony formation was monitored for up to 1 month. As shown in Figs. 2 and 3A, the ability of Ki-Ras 4A or Ki-Ras 4B to enable RIE-1 cells to grow in soft agar paralleled their ability to induce transformed foci in these cells. Ki-Ras 4A efficiently induced foci and enabled soft agar growth, whereas Ki-Ras 4B neither efficiently induced foci nor enabled soft agar growth. In contrast, although Ha-Ras efficiently induced foci in RIE-1 cells, Ha-Ras-expressing RIE-1 cells were completely unable to grow in soft agar. Moreover, although N-Ras demonstrated little ability to induce foci in RIE-1 cells, N-Ras efficiently enabled RIE-1 cells to grow in soft agar. As shown in Fig. 3B, in RIE-1 cells, expression levels of exogenous Ha-Ras, Ki-Ras 4B, and N-Ras proteins were comparable and slightly higher than exogenous Ki-Ras 4A protein. It is unlikely, therefore, that differences in protein expression accounted for the ability of Ki-Ras 4A and N-Ras or inability of Ha-Ras and Ki-Ras 4B proteins to enable soft agar growth. However, we are unable to definitively conclude whether there may be differences in the relative ability of Ki-Ras 4A or N-Ras protein to enable RIE-1 cells to grow in soft agar, due to the slight differences in expression between these homologs. Interestingly, although only Ki-Ras 4A and N-Ras enabled RIE-1 epithelial cells to grow in soft agar, each of the four Ras homologs enabled Rat-1 fibroblast cells to grow in soft agar (data not shown).
Ras Isoforms Have Distinct Biochemical/Biological Properties

Fig. 3. Ki-Ras 4A and N-Ras, but not Ha-Ras or Ki-Ras 4B, enable anchorage-independent growth in soft agar. RIE-1 cells were transfected with constitutively active (G12V) Ha-ras, Ki-ras 4A, Ki-ras 4B, N-ras, or empty (vector alone) expression plasmids; selected in G418 and suspended in reduced-serum soft agar medium. Colony formation was monitored for up to 1 month. Data shown are representative of four independent experiments done in duplicate. A, ×10 magnification of soft agar colonies. B, immunoblot of RIE-1 cell lysates showing expression levels of exogenous Ras homolog proteins.

were counted on a fluorescence microscope. As demonstrated in Fig. 4A, in comparison to GFP alone, Ki-Ras 4B efficiently and Ha-Ras minimally induced cell migration (p < 0.005), but Ki-Ras 4A and N-Ras did not significantly induce cell migration (p > 0.3). As shown in Fig. 4B, the relative inability of Ha-Ras, Ki-Ras 4A, or N-Ras to stimulate cell migration was not due to differences in protein expression. (Levels of expression of exogenous GFP-Ras proteins were approximately 1–2-fold higher than endogenous Ras proteins). Furthermore, even when increasing amounts of GFP-ras constructs were transfected into COS-7 cells, Ki-Ras 4A and N-Ras continued to show little or no ability to induce cell migration in comparison to Ki-Ras 4B protein (data not shown). Importantly, similar migration results were observed with native Ras proteins that were not fused to GFP (data not shown).

These combined studies clearly indicate that the four Ras homologs have different biological properties.

DISCUSSION

Our results indicate that the four human Ras homologs significantly differ in their abilities to activate the effector protein, Raf-1, in vitro. Ki-Ras 4B efficiently activates Raf-1, Ki-Ras 4A is intermediate, and N-Ras and Ha-Ras have progressively decreasing and inefficient abilities to activate Raf-1. Our studies also demonstrate that the four Ras homologs can induce significantly different biological responses in vitro. Ha-Ras and Ki-Ras 4A induce 6–8 times more transformed foci than Ki-Ras 4B or N-Ras in RIE-1 cells, but only Ki-Ras 4A and N-Ras enable RIE-1 cells to undergo anchorage-independent growth in soft agar. Moreover, Ki-Ras 4B efficiently stimulates and Ha-Ras inefficiently stimulates COS-7 cell migration, but N-Ras and Ki-Ras 4A proteins do not.

Although differences in the abilities of the four Ras homologs to activate Raf-1 were quantitative rather than qualitative, it is likely that the quantitative differences observed under our “ideal” experimental conditions significantly underestimate actual differences between the Ras homologs. For instance, our studies utilized mutant (G12V) Ras homologs that are constitutively active, and this constitutive activity might have enabled Ha-Ras and N-Ras proteins to promiscuously, albeit inefficiently, activate Raf-1. Likewise, because the exogenous Ras homologs were expressed at significantly higher levels than their endogenous counterparts, this overexpression might also have enabled Ha-Ras and N-Ras to promiscuously activate Raf-1. Furthermore, because each of the exogenous Ras homologs was expressed in cells individually, the exogenous Ras homologs did not have to “compete” with each other for available effector proteins. This lack of competition could also have artifactually enabled some Ras homologs to activate Raf-1. It is possible, therefore, that under normal cellular conditions, wild type endogenous Ha-Ras or N-Ras proteins may have little or no ability to activate Raf-1. We would like to emphasize, however, that even if each of the four wild type endogenous Ras proteins can activate Raf-1 in vivo, differences in their relative abilities to activate Raf-1 could still enable each of the Ras homologs to induce different biological responses. For instance, recent studies have shown that low levels of Raf kinase activity induce proliferation, whereas high levels of Raf kinase activity induce cell cycle arrest and/or differentiation (reviewed in Ref. 1). Furthermore, although our studies did not address the question of whether the homologs might activate Raf-1 with different time courses, previous studies have shown that differences in the duration of Raf kinase activation can also influence whether a cell undergoes proliferation or differentiation (25, 26).

We do not currently know the mechanism(s) underlying the differential abilities of the four Ras homologs to activate Raf-1. Raf-1 contains a C-terminal catalytic domain and an N-terminal regulatory domain that inhibits its catalytic activity (1, 4, 27). Ras binds to two distinct regions within the N-terminal regulatory domain. Specifically, the switch 1 region of Ras (amino acids 30–37) binds to the minimal Ras binding domain of Raf-1 (amino acids 55–131), and the switch 2 region of Ras (amino acids 59–76) binds to the cysteine-rich domain (CRD) of Raf-1 (amino acids 139–184) (1, 4, 27). In current models, Ras is thought to recruit Raf-1 (and the acidic protein, 14-3-3, which...
is bound to the CRD and other sites of Raf-1) to plasma membranes by binding to the Ras binding domain of Raf-1. Following membrane recruitment, negatively charged membrane phospholipids, in particular phosphatidylserine, are believed to competitively displace 14-3-3 from the CRD of Raf-1. This enables Ras to bind the CRD and ultimately permits full Raf-1 activation by other factors (such as membrane-associated tyrosine kinases, serine/threonine kinases, hsp90 and p50 molecular chaperones, and/or the kinase suppressor of Ras, etc.) (1, 4, 27). Because the four Ras homologs are identical in their switch 1 and switch 2 regions, it is unlikely that differential binding to the Ras binding domain or CRD of Raf-1 accounts for the discrepant abilities of the Ras homologs to activate Raf-1. The hypervariable domain, consisting of the 25 C-terminal amino acids of Ras proteins, is the only region where the highly homologous Ras proteins substantially differ from each other. It seems likely, therefore, that residues within this domain might account for differential Raf-1 activation. This domain contains a CAAX motif (where C indicates cysteine, A indicates an aliphatic amino acid, and X indicates serine or methionine) that signals the Ras proteins to undergo a series of posttranslational modifications. Each of the Ras proteins becomes palmitoylated, truncated, and carboxymethylated at its CAAX residues, and these modifications are required for Ras proteins to become associated with plasma membranes (6). In addition, Ha-Ras, N-Ras, and Ki-Ras 4A proteins become palmitoylated on one or two non-CAAX cysteine residues within their hypervariable domains (see Fig. 5) (6). Ki-Ras 4B lacks these cysteine residues and does not become palmitoylated. Rather, Ki-Ras 4B contains a polybasic domain, consisting of six contiguous lysines, within its hypervariable domain (see Fig. 5). Previous studies have shown that palmitoylation (Ha-Ras, N-Ras, and Ki-Ras 4A) or a polybasic domain (Ki-Ras 4B) is required for Ras proteins to become fully membrane associated (21, 28). Palmitoyl moieties likely facilitate Ha-Ras, N-Ras, or Ki-Ras 4A membrane association through hydrophobic or van der Waals interactions, whereas the polybasic domain likely facilitates Ki-Ras 4B membrane association through ionic interactions with negatively charged membrane phospholipid head groups. 

These hypervariable domain differences (and perhaps other hypervariable domain differences) could, therefore, enable each of the four Ras homologs to bind to distinct regions of the plasma membrane and/or to distinct membrane target proteins. It is possible, therefore, that Ki-Ras 4B activates Raf-1 more efficiently than the other Ras homologs because Ki-Ras 4B recruits Raf-1 to a membrane site where other components required for Raf-1 activation (such as negatively charged membrane phospholipids) are co-localized. Similarly, the two contiguous basic lysines upstream of the CAAX of Ki-Ras 4A (see Fig. 5) might partially enable Ki-Ras 4A to bind to membrane phospholipids and account for its intermediate ability to activate Raf-1. Alternatively, it is possible that hypervariable domain differences enable Ki-Ras 4B (and to a lesser extent Ki-Ras 4A) to recruit Raf-1 to the membrane and/or activate Raf-1 more efficiently than the other Ras homologs. Recent studies have shown that Raf-1 must be complexed with 14-3-3 for efficient membrane recruitment by Ras (29), and it has been demonstrated that two basic contiguous residues within the CRD domain of Raf-1 (Arg-143 and Lys-144) are required for 14-3-3 binding (30). It is possible, therefore, that Ki-Ras 4B and Ki-Ras 4A activate Raf-1 more efficiently than Ha-Ras or N-Ras because their polybasic residues bind to the acidic 14-3-3 protein and thereby facilitate Raf-1 membrane recruitment, and/or their polybasic residues competitively displace 14-3-3 from the CRD of Raf-1 and thus allow their switch 2 regions to bind the CRD and facilitate Raf-1 activation. The recent demonstration that Ki-Ras 4B membrane recruits and activates Raf-1 more efficiently than Ha-Ras is consistent with this possibility (31). Moreover, our finding that a Ki-Ras 4B mutant that contains six neutral glutamates substituted for the six contiguous lysines of the polybasic domain of Ki-Ras 4B is unable to activate Raf-1 also supports this notion.2

There was often little or no correlation between the abilities of each of the four Ras homologs to activate Raf-1 in vivo and their relative activities in the three biological assays studied. For instance, although Ki-Ras 4B had the greatest and Ha-Ras had the least ability to activate Raf-1 in COS-1 cells, Ki-Ras 4B had the least and Ha-Ras had the greatest ability to induce transformed foci in NIH 3T3, Rat-1, and RIE-1 cells. Our results with Ki-Ras 4B suggest that Raf-1 activation may not be sufficient for efficient focus formation, and our findings with Ha-Ras suggest that little or no Raf-1 activation may be required for efficient focus formation in the cell lines we employed. Our results are consistent with previous studies that demonstrated that Raf-1 activation is not sufficient or necessary for the induction of transformed foci in some cells (reviewed in Refs. 1–4 and 32). In addition, our studies support the notion that activation of multiple effectors is likely required for full Ras transformation (1–4, 32). Although Ki-Ras 4B activates Raf-1 efficiently, it may inefficiently activate other effectors required for focus formation. Likewise, although Ha-Ras activates Raf-1 inefficiently, it may efficiently activate other effectors capable of inducing focus formation. Similarly, although Ki-Ras 4A moderately activates Raf-1, Ki-Ras 4A likely also activates other effectors required for focus formation.

Interestingly, differences observed between the four Ras homologs depended on the cell line utilized. For instance, although we demonstrated that only two of the four Ras homologs, Ki-Ras 4A and N-Ras, can enable RIE-1 epithelial cells to grow in soft agar, we found that each of the four Ras homologs enables Rat-1 fibroblast cells to grow in soft agar, and a previous report (33) showed that chimeric Ha-Ras, N-Ras, and Ki-Ras 4B proteins enable Rat-2 and NIH 3T3 fibroblast cells to grow in soft agar. These results suggest, therefore, that the effector(s) activated by a given Ras homolog may vary in different cell types and/or the effector(s) required to induce a

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1. Jackson and S. Wong, unpublished results.
given biologic response may vary in different cell types. We also showed that differences between the focus-forming activities of each of the four Ras homologs were much more marked in RIE-1 epithelial cells than in NIH 3T3 or Rat-1 fibroblast cells. We found, however, that the relative abilities of the four Ras homologs to induce transformed foci (Ha-Ras ≥ Ki-Ras 4A >> N-Ras = Ki-Ras 4B) were consistent among each of the cell lines we employed. Similarly, although their nonstandard methodology made their results inconclusive, a previous study (33), utilizing G418-selected Rat-2 cells infected with ras retroviruses, suggested that chimeric Ha-Ras proteins might have greater focus-forming activity than chimeric N-Ras or Ki-Ras 4B proteins. Because the vast majority of human cancers are of epithelial cell origin, our combined results underscore the crucial importance of using epithelial cells as model systems for carcinogenesis studies.

Notably, there was often no correlation between the abilities of the Ras homologs to induce focus formation and their abilities to enable anchorage-independent growth. For instance, whereas Ha-Ras efficiently and N-Ras inefficiently induced focus formation in RIE-1 cells, N-Ras, but not Ha-Ras, enabled RIE-1 cells to grow in soft agar. Our results suggest, therefore, that in epithelial cells, the effector(s) required for efficient focus formation may differ from the effector(s) required for anchorage-independent growth. Consistent with this premise, previous studies have shown that Ras effector domain mutants that have lost their ability to bind to some, but not all, of their effectors fail to induce transformed foci, but still enable growth in soft agar (34).

Multiple signaling pathways are believed to play a role in directed cell migration. For instance, mitogen-activated protein kinase has been shown to promote cell migration by phosphorylating myosin light chain kinase (35); Rac, Rho, and Cdc42 facilitate cell motility through their abilities to reorganize the actin cytoskeleton (36); and phosphatidylinositol 3-kinase, Ras, and phospholipase Cγ have also been implicated as potential mediators of cell migration (37, 38). Because Ki-Ras 4B activates Raf-1 more efficiently than Ha-Ras, N-Ras, or Ki-Ras 4A, it is possible that Raf-1-induced mitogen-activated protein kinase activation accounts for the enhanced ability of Ki-Ras 4B to induce cell migration. It should be noted, however, that although Ki-Ras 4A activates Raf-1 more potently than Ha-Ras, Ha-Ras induces cell migration significantly better than Ki-Ras 4A. Our results support the notion, therefore, that multiple signaling pathways contribute to cell migration. Future studies will be required to delineate which signaling component(s) accounts for the enhanced ability of Ki-Ras 4B to induce cell migration.

Interestingly, although the Ki-Ras 4A and Ki-Ras 4B proteins are alternative splice variants of the same gene, their biological activities are significantly different. Ki-Ras 4A, but not Ki-Ras 4B, efficiently induces transformed foci and enables anchorage-independent growth, whereas Ki-Ras 4B, but not Ki-Ras 4A, induces cell migration. These different biological properties suggest that in addition to Raf-1, Ki-Ras 4A and Ki-Ras 4B likely differ in their abilities to qualitatively or quantitatively activate other effector proteins. As noted previously, Ki-ras gene mutations are detected in human malignancies much more frequently than N-ras or Ha-ras gene mutations. Because Ki-ras gene mutations occur at codons 12, 13, or 61, Ki-ras gene-encoded Ki-Ras 4A and Ki-Ras 4B proteins are both constitutively active. If, therefore, Ki-Ras 4A and Ki-Ras 4B proteins activate different, but cooperative, effector pathways, the combination of these constitutively active signaling pathways could account, at least in part, for the increased frequency of Ki-ras gene mutations in human cancers. For instance, Ki-Ras 4A could activate effector pathways that transform cells and enable anchorage-independent growth, whereas Ki-Ras 4B could activate effector pathways that enable cell motility and thereby facilitate angiogenesis, invasion, and ultimately metastasis. Consistent with this premise, a recent study, utilizing Ras effector domain mutants, has shown that the effector(s) required for metastasis is distinct from the effector(s) required for tumorigenesis (39). Thus, whereas the constitutive activity of only one of the two Ki-Ras proteins might not be sufficient to efficiently enable a malignant tumor to develop, cooperativity of the two Ki-Ras proteins (in conjunction with other oncogene/tumor suppressor gene abnormalities) could efficiently enable the development of a clinically detectable malignancy. Importantly, although Ki-ras 4B was previously reported to be transcribed 10–20-fold more than Ki-ras 4A (40, 41), a recent study (9) demonstrated that in some tissues, such as the colon, transcription of Ki-ras 4A is almost comparable to Ki-ras 4B.

In summary, our results demonstrate that the four human Ras proteins significantly differ in their abilities to activate Raf-1 and also vary in their abilities to induce transformed foci, enable anchorage-independent growth, and stimulate cell motility. It will be important, in future studies, to assess the relative abilities of each of the four Ras homologs to activate other Raf kinase family members (A-Raf and B-Raf), as well as other putative effector proteins. In addition, it will be important to further define the biological differences between the four Ras homologs and determine their biological roles in vivo.

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