Ras proteins are key regulators of cell growth and differentiation. Mammalian cells express three closely related Ras proteins: Ha-Ras, K-Ras, and N-Ras. We have compared the abilities of the Ha-Ras and K-Ras isoforms to activate the Rac effector pathway, using three Rac-dependent readouts: induction of membrane ruffling and pinocytosis, stimulation of cell motility, and Pak binding. The total surface area of membrane ruffles induced by K-RasV12 was 2-fold greater than that induced by Ha-RasV12. Likewise, the number of K-RasV12-induced pinocytic vesicles per cell was 2-fold greater than that induced by Ha-RasV12. In a wound healing assay, K-RasV12-injected cells migrated twice as fast as Ha-RasV12-injected cells. Moreover, the Pak binding activity of Rac, which is indicative of the amount of GTP-bound Rac, was higher in K-RasV12-expressing cells than Ha-RasV12-expressing cells. These results suggest that K-Ras activates Rac more efficiently than Ha-Ras. The preferential activation of Rac by K-Ras is dependent on the mode of membrane anchoring and impacts on the ability of K-Ras to regulate cell survival.

Ras proteins are small molecular weight GTPases that link cell surface receptors to intracellular effector pathways that regulate cell growth, differentiation, and survival. The mammalian genome contains three ras genes that encode highly related proteins of either 188 or 189 amino acids: Ha-Ras, N-Ras, K4A-Ras, and K4B-Ras (1). The first 86 amino acids of Ras proteins are 100% identical, and the next 80 amino acids exhibit 85% homology between any pair of Ras proteins. The remaining C-terminal sequence consisting of residues 166–185, known as the hypervariable domain, is highly divergent with the exception of the last four amino acids (the CAAX motif), which are required for post-translational processing and plasma membrane association (2). Although ubiquitously expressed, the three Ras isoforms have been postulated to perform distinct biological functions. This notion is supported by the finding that K-Ras but not Ha-Ras or N-Ras is essential for normal mouse development (3, 4). Additionally, mutagenically activated forms of the three ras genes are selectively expressed in different human tumors. For example, K-Ras mutations occur in 90% of pancreatic adenocarcinomas, whereas in acute myeloid leukemia over 25% of mutations detected involve N-Ras (5).

The biological effects of Ras proteins are exerted through the activation of several downstream effectors including the Ser/Thr kinase Raf, the p110 catalytic subunit of phosphoinositide 3-kinase (PI3K), and Ral GDS, an exchange factor for Ral GTPases (6). The interaction of Ras with its effectors is mediated by the effector binding loop, which spans residues 32–40. Despite the fact that the amino acid sequence corresponding to the effector binding loop is identical among Ras proteins, recent studies have demonstrated that the three Ras isoforms can differentially activate Raf-1 and PI3K. K-Ras activates Raf-1 more efficiently than Ha-Ras and N-Ras (7, 8). On the other hand, Ha-Ras is a more potent activator of PI3K than K-Ras (8). These hierarchies appear to result, at least in part, from differences in the mechanisms of membrane attachment of the three Ras isoforms (8). However, their physiological significance remains to be determined.

Through genetic and biochemical studies it has been established that the growth promoting activity of Ras proteins is dependent on the synergistic activation of multiple effector pathways (9, 10). Of particular relevance to the present work are the observations that the activation of a pathway that engages the small GTPase Rac is critical for both the mitogenic and oncogenic effects of Ras (11, 12). Rac itself controls at least three effector pathways leading to transcriptional activation, rearrangement of the actin cytoskeleton, and the generation of superoxide (13). Of these, the effector functions involved in regulating the actin cytoskeleton and production of superoxide are necessary components of the mitogenic response (14–16). Additionally, Rac plays an essential role in providing protection against apoptosis induced by high intensity Ras signaling (17).

In the present study we sought to determine whether the Ha-Ras and K-Ras isoforms differ with respect to their ability to activate the Rac pathway. We demonstrate that K-Ras is a more potent activator of Rac relative to Ha-Ras. Our findings indicate that the efficiency of Rac activation is dictated by the mode of membrane anchoring of Ras and impacts on the ability of Ras to regulate cell survival.

EXPERIMENTAL PROCEDURES

Expression Plasmids—All Ras constructs were generated by subcloning the human cDNAs into the mammalian expression vector pCGT, which is derived from pCGN with a replacement of the HA epitope with the T7 epitope (18). The Ha-RasV12–184/K-RasV12 cDNA fragments were amplified by polymerase chain reaction using Pfu DNA polymerase. The primers used are as follows: Ha-RasV12, 5′-CAGGCTCAGGACTTAGC-3′ and 5′-GATGGATCCTTATATAGGATC-3′; K-Ras V12, 5′-CAGGCTCAGGACTTAGC-3′ and 5′-GATGGATCCTTATATAGGATC-3′; K-Ras WT, 5′-CAGGCTCAGGACTTAGC-3′ and 5′-GATGGATCCTTATATAGGATC-3′. The final polymerase chain reaction product was digested by the restriction enzymes BamHI and XhoI and ligated into pCGT. The expression plasmid encoding T7 epitope-tagged RacV12 and RacWT were kindly provided by Linda VanAelst (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The expression plasmid encodes 2 The abbreviations used are: PI3K, phosphoinositide 3-kinase; HA, hemagglutinin; DME, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; PH, pleckstrin homology.
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odig HA epitope-tagged ERK2 has been previously described (11). Myr-
HrasV12S186 expression plasmid was kindly provided by J. Gibbs (Merck).

Cell Culture and Transient Transfections—REF-52 cells were cul-
tured in DMEM supplemented with 10% fetal calf serum in a humidi-
fied incubator with 6% CO2 at 37 °C. The cells were grown in DMEM supplemented with 5% DMEM fetal calf serum in a humidified incubator with 5% CO2 at 37 °C. Transient transfections using a calcium phosphate method were performed as described previously (19). Briefly, DNA was diluted in Tris/EDTA buffer (pH 7) containing 2 mM CaCl2 and added to HEPES/Na2HPO4 buffer. The solution was incubated at room temperature for 15 min prior to the addition to the cells. After 12–16 h the cells were washed with DMEM supplemented with 2% FBS and transfected with SDS sample buffer. The products were resolved by SDS-

The reactions were incubated for 30 min at 30 °C and terminated by the addition of [γ-32P]ATP (PerkinElmer Life Sciences; 6000 Ci/mmol) in kinase reaction buffer. The lysates were clarified by centrification for 20 min at 14,000 × g and then incubated with anti-HA antibody (12CA5) for 2 h. The immune complexes were captured by incubation with protein A beads for 45 min. The immune complexes were washed twice in cold lysis buffer and subsequently washed with kinase reaction buffer. The lysates were verified by indirect immunofluorescence.

Mitogen-activated Protein Kinase Assay—REF-52 cells were washed with cold PBS and lysed in (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 1 mM sodium vanadate, 10 μg/ml leupeptin, 1% aprotinin, 10 μg/ml pepstatin, 1 mM benzamidine, 10 μM okadaic acid, 10 mM benzamidine). The lysates were clarified by centrifugation for 20 min at 14,000 × g and then incubated with anti-HA antibody (12CA5) for 2 h. The immune complexes were captured by incubation with protein A beads for 45 min. The immune complexes were washed twice in cold lysis buffer and subsequently washed with kinase reaction buffer. The lysates were clarified by centrification for 20 min at 14,000 × g. Aliquots from the supernatant were taken to compare protein amounts. The lysates were incubated with either GST alone or GST-PBD (p21-binding domain) fusion protein bound to glutathione-coupled to Sepharose beads at 4 °C for 30 min. The beads and the proteins bound to the fusion protein were washed three times with cold lysis buffer, and the eluted bound proteins were analyzed by Western blotting using a monoclonal mouse antibody against Rac1.

Immunoblot Analysis—Samples were resolved on SDS-polyacryl-

Molecular differences, the membrane ruffles induced by Ha-RasV12 occurred predominantly along the cell periphery and had a long wavelike appearance. K-RasV12 induced elaborate and highly convoluted membrane ruffles at multiple sites both on the dorsal surface and the periphery of the cell. In addition to the distinct morphological differences, the membrane ruffles induced by the two Ras isoforms displayed different kinetic behavior. The K-RasV12-induced membrane ruffles were highly dynamic as indicated by the pronounced changes in ruffle appearance over a 120-s interval (Fig. 1A). In comparison, the Ha-RasV12-induced membrane ruffles remained mostly unchanged over a 120-s interval. Furthermore, based on the sampling of 10 cells, the average surface area of the K-RasV12-induced membrane ruffles was 2-fold greater than the Ha-RasV12 membrane ruffle surface area (Fig. 1B). The Ras isoforms were expressed to similar levels and displayed an identical cellular distribution as determined by indirect immunofluorescence (Fig. 1C). Moreover, the distinct ruffling patterns displayed by Ha-RasV12 and K-RasV12 were retained at higher concentrations of expression plasmid and occurred irrespective of cell density (data not shown). Because Ras-induced membrane ruffling is mediated by Rac (21), the differences in the membrane ruffling activity of the Ras isoforms suggest that Ha-Ras and K-Ras are differentially coupled to the Rac pathway.

As Figure 1D shows, the Ha-RasV12 and K-RasV12 differentially stimulate fluid phase pinocytosis and cell motility. The K-RasV12-induced membrane ruffles were highly dynamic as indicated by the pronounced changes in ruffle appearance over a 120-s interval (Fig. 1A). In comparison, the Ha-RasV12-induced membrane ruffles remained mostly unchanged over a 120-s interval. Furthermore, based on the sampling of 10 cells, the average surface area of the K-RasV12-induced membrane ruffles was 2-fold greater than the Ha-RasV12 membrane ruffle surface area (Fig. 1B). The Ras isoforms were expressed to similar levels and displayed an identical cellular distribution as determined by indirect immunofluorescence (Fig. 1C). Moreover, the distinct ruffling patterns displayed by Ha-RasV12 and K-RasV12 were retained at higher concentrations of expression plasmid and occurred irrespective of cell density (data not shown). Because Ras-induced membrane ruffling is mediated by Rac (21), the differences in the membrane ruffling activity of the Ras isoforms suggest that Ha-Ras and K-Ras are differentially coupled to the Rac pathway.
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K-RasV12 is a more potent activator of Rac than Ha-RasV12—The enhanced motility induced by K-RasV12 together with the more potent effects of this isoform on membrane ruffling and pinocytosis suggests that K-Ras might be a more effective activator of the Rac pathway compared with Ha-Ras. To directly compare the abilities of Ha-RasV12 and K-RasV12 to activate Rac, we have used a Pak binding assay (23). COS-1 cells were transiently transfected with T7 epitope-tagged RacWT and Ha-RasV12 or K-RasV12 expression plasmids. Following 24 h of serum starvation, cells were lysed, and the lysates were incubated with the GTPase binding domain (CRIB domain) of the kinase Pak, a downstream effector of Rac, fused to GST. This domain has been found to specifically bind to the activated GTP-bound form of Rac (24). The eluted proteins bound to the fusion protein GST-PBD were analyzed by Western blotting. As shown in Fig. 4 (A and B), the binding of Rac-GTP to GST-PBD was ~2-fold higher in the presence of K-RasV12 than Ha-RasV12, which is consistent with K-Ras being a stronger activator of Rac. It is important to note that the differential effects of Ha-RasV12 and K-RasV12 on membrane ruffling displayed by REF-52 cells were also observed in COS-1 cells (Fig. 4C).

Ras Membrane Attachment Influences the Activation of the Rac Pathway—The attachment of Ras to the membrane is dependent on specific modifications of the C terminus. The CAAX motifs of Ha-Ras and K-Ras undergo the same post-translational modifications involving farnesylation, proteolytic cleavage, and carboxyl methylation (25). In contrast, the hypervariable region preceding the CAAX motif is processed differently between Ha-Ras and K-Ras. Ha-Ras becomes palmitoylated on cysteine residues within the hypervariable region, whereas K-Ras lacks these cysteine residues but contains a polybasic domain that is required for effective membrane association (2). To test whether these differences could account for the differential activation of the Rac pathway, a chimeric Ras protein was constructed containing the first 164 amino acids of Ha-RasV12 and the last 25 amino acids of K-RasV12 (Ha-Ras1–164/K-Ras). The chimeric protein showed the same subcellular distribution as Ha-RasV12 following injection into quiescent REF-52 cells (Fig. 5A). Significantly, the membrane ruffling phenotype of the Ha-Ras1–164/K-Ras protein resembled closely the phenotype induced by K-RasV12 including an enhanced accumulation of pinocytic vesicles (Fig. 5A). These results suggest that the membrane attachment mechanisms can impact the efficiency of Ras-dependent activation of the Rac pathway.

To further test this idea, we compared the signaling activities of Ha-RasV12 and Myr-HRasV12S186, which contains a mutation in the farnesylation site and a myristoylation signal. The CAAX motifs of Ha-Ras and K-Ras undergo the same post-translational modifications involving farnesylation, proteolytic cleavage, and carboxyl methylation (25). In contrast, the hypervariable region preceding the CAAX motif is processed differently between Ha-Ras and K-Ras. Ha-Ras becomes palmitoylated on cysteine residues within the hypervariable region, whereas K-Ras lacks these cysteine residues but contains a polybasic domain that is required for effective membrane association (2). To test whether these differences could account for the differential activation of the Rac pathway, a chimeric Ras protein was constructed containing the first 164 amino acids of Ha-RasV12 and the last 25 amino acids of K-RasV12 (Ha-Ras1–164/K-Ras). The chimeric protein showed the same subcellular distribution as Ha-RasV12 following injection into quiescent REF-52 cells (Fig. 5A). Significantly, the membrane ruffling phenotype of the Ha-Ras1–164/K-Ras protein resembled closely the phenotype induced by K-RasV12 including an enhanced accumulation of pinocytic vesicles (Fig. 5A). These results suggest that the membrane attachment mechanisms can impact the efficiency of Ras-dependent activation of the Rac pathway.

To further test this idea, we compared the signaling activities of Ha-RasV12 and Myr-HRasV12S186, which contains a mutation in the farnesylation site and a myristoylation signal on the N terminus, enabling the protein to be anchored to the membrane via the N terminus. Myr-HRasV12S186 and Ha-RasV12 had a similar subcellular distribution and activated the ERK kinase cascade to a similar level (Fig. 5B). However, Myr-HRasV12S186 failed to induce membrane ruffling when

![Image](http://www.jbc.org/)

**Fig. 1.** Ha-RasV12 and K-RasV12 induce distinct membrane ruffling patterns and exhibit similar subcellular localizations. A, REF-52 cells were serum-starved for 24 h prior to the microinjection of T7-tagged Ha-RasV12 and K-RasV12 expression vectors (50 ng/μl). 4 h after injection, the cells were imaged at 60-s intervals by time lapse video microscopy. Bar, 20 μm. B, examples of membrane ruffle traces of three cells injected with Ha-RasV12 (top panels) and three cells injected with K-RasV12 (bottom panels). The phase images corresponding to the first cell from the left are shown. The traces were generated and analyzed as described under “Experimental Procedures.” The average surface area per injected cell is denoted in pixels ± standard deviations. The results represent the average of at least five experiments with two injected cells analyzed per experiment (p = 0.001). C, REF-52 cells were immunostained for the expression and subcellular localization of Ha-RasV12 and K-RasV12 with anti-T7 monoclonal antibody followed by rhodamine-conjugated goat anti-mouse secondary antibody. Bar, 20 μm.

each of the Ras isoforms had a unique pattern, we hypothesized that there would be isoform-dependent differences in the stimulation of fluid phase pinocytosis. To test this hypothesis, quiescent REF-52 cells were microinjected with T7-tagged expression plasmids and analyzed by phase contrast microscopy at 6 h after injection. The pinocytic vesicles can be readily identified under these conditions by their refractile appearance (Fig. 2B), and we have established previously that they correspond to pinosomes (20). The number of K-RasV12-induced pinocytic vesicles per cell was ~2-fold greater than that induced by Ha-RasV12 (Fig. 2, A and B). Moreover, the average size of pinocytic vesicles formed in the K-RasV12-injected cells was twice as large when compared with Ha-RasV12-injected cells (Fig. 2C). As has been shown for Ha-RasV12 (21), the stimulation of membrane ruffling and pinocytosis by K-RasV12 was blocked by the co-injection of dominant negative Rac, RacN17 (data not shown).

Membrane ruffling is a critical event both in cell spreading and migration (22). Therefore, we compared the effects of Ha-RasV12 and K-RasV12 on cell motility using a wound healing assay. A 140–180-μm scratch was introduced into a monolayer of quiescent REF-52 cells, and the cells aligning the edges of the wound were injected with T7-tagged Ha-RasV12 and K-RasV12. The K-RasV12 injected cells migrated at a rate of 18 μm/h (± 1.5), whereas the Ha-RasV12 injected cells migrated at a rate of 12 μm/h (± 1.2) (Fig. 3).

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microinjected into quiescent REF-52 cells, indicating that activation of the Rac pathway is uniquely sensitive to the positioning of Ras in the membrane.

Ha-RasV12 but Not K-RasV12 Can Induce Apoptosis—We have recently shown that Rac-mediated signals are essential for protection against Ha-RasV12-induced apoptosis (17). Overexpression of Ha-RasV12 promotes apoptosis, and this response is blocked by the concurrent expression of dominant active RacV12 and potentiated by the co-expression of dominant negative RacN17. If Ha-Ras and K-Ras differentially activate the Rac pathway, then they might be expected to differ in their ability to induce apoptosis. To test this prediction, quiescent REF-52 cells were microinjected with 50 ng/ml Ha-RasV12, K-RasV12, and Ha-Ras1–164/K-Ras expression plasmids, and apoptosis was visualized by the appearance of rounded cells after 16 h (Fig. 6A). To quantitate the apoptotic effect, the number of cells stained with FITC-annexin V, an apoptosis marker, was determined in each injected area. In agreement with our earlier observations, microinjection of Ha-RasV12 induced apoptosis in 38% (± 4.5) of the cells (Fig. 6B). In contrast, microinjection of K-RasV12 had no apparent effect on cell viability (Fig. 6). Thus, the differential effects of Ha-RasV12 and K-RasV12 on apoptosis cor-
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FIG. 5. The interaction of Ras with the plasma membrane influences the activation of the Rac pathway. A, REF-52 cells were serum-starved for 24 h prior to the microinjection of Ha-RasV12 and Ha-RasV121–164/K-Ras expression vectors (50 ng/μl). Left panels, the cells were immunostained for the expression and localization of Ha-RasV12 and Ha-RasV121–164/K-Ras with anti-T7 monoclonal antibody followed by rhodamine-conjugated goat anti-mouse secondary antibody. Bar, 20 μm. Right panels, phase contrast micrographs of cells expressing Ha-RasV12 and Ha-RasV121–164/K-Ras, 4 h after microinjection. Bar, 20 μm. B, REF-52 cells were serum-starved 24 h prior to the microinjection of Ha-RasV12 and Myr-HRasV12S186 expression plasmids (50 ng/μl). Top panels, the cells were immunostained for the expression and localization of Ha-RasV12 and Myr-HRasV12S186 with Y13–259 antibody followed by rhodamine-conjugated goat anti-rat secondary antibody. Bar, 20 μm. Middle panels, phase contrast micrographs of cells expressing Ha-RasV12 and Myr-HRasV12S186 4 h after microinjection. Bar, 20 μm. Bottom panel, REF-52 cells were transiently transfected with HA-tagged ERK2 and Ha-RasV12, Ha-RasV12S186, or Myr-HRasV12S186. ERK2 was immunoprecipitated with anti-HA monoclonal antibody (12CA5) and incubated with myelin basic protein (MBP) in the presence of [γ-32P]-ATP for 30 min at 30 °C. The myelin basic protein phosphorylation was visualized by autoradiography (bottom panel).

FIG. 6. Effects of Ha-RasV12, K-RasV12 and Ha-Ras1–164/K-Ras on apoptosis. REF-52 cells were serum-starved for 24 h prior to the microinjection of Ha-RasV12, K-RasV12, and Ha-Ras1–164/K-Ras expression plasmids (50 ng/μl). A, phase contrast micrographs of Ha-RasV12 and K-RasV12 injected cells. Apoptotic cells appear rounded and refractile (arrowheads). Images were captured 16 h after injection. Bar, 80 μm. B, quantitation of Ras-induced apoptosis. The percentage of apoptotic cells was determined by counting the number of FITC-annexin V-positive cells as a proportion of the total number of cells present in the injected field 16 h after injection. Results represent the averages of at least three experiments ± standard deviations.

FIG. 7. The interaction of Ras with the plasma membrane influences the activation of the Rac pathway. A, REF-52 cells were serum-starved for 24 h prior to the microinjection of Ha-RasV12 and Ha-RasV121–164/K-Ras with anti-T7 monoclonal antibody followed by rhodamine-conjugated goat anti-mouse secondary antibody. Bar, 20 μm. Right panels, phase contrast micrographs of cells expressing Ha-RasV12 and Ha-RasV121–164/K-Ras, 4 h after microinjection. Bar, 20 μm. B, REF-52 cells were serum-starved 24 h prior to the microinjection of Ha-RasV12 and Myr-HRasV12S186 expression plasmids (50 ng/μl). Top panels, the cells were immunostained for the expression and localization of Ha-RasV12 and Myr-HRasV12S186 with Y13–259 antibody followed by rhodamine-conjugated goat anti-rat secondary antibody. Bar, 20 μm. Middle panels, phase contrast micrographs of cells expressing Ha-RasV12 and Myr-HRasV12S186 4 h after microinjection. Bar, 20 μm. Bottom panel, REF-52 cells were transiently transfected with HA-tagged ERK2 and Ha-RasV12, Ha-RasV12S186, or Myr-HRasV12S186. ERK2 was immunoprecipitated with anti-HA monoclonal antibody (12CA5) and incubated with myelin basic protein (MBP) in the presence of [γ-32P]-ATP for 30 min at 30 °C. The myelin basic protein phosphorylation was visualized by autoradiography (bottom panel).

DISCUSSION

It is well established that the signaling activities of Ras are mediated by multiple effector pathways. In the present study we have demonstrated that the activation of the Rac effector pathway is differentially regulated by Ha-Ras and K-Ras. A primary determinant in conferring this difference is the hyper-variable region located in the C-terminal portion of Ras proteins. Because this region specifies the membrane targeting of Ras, the distinct properties of Ha-Ras and K-Ras with respect to Rac activation most likely reflect variations in their membrane anchoring mechanisms.

Several lines of evidence support the notion that Ha-Ras and K-Ras are localized to distinct membrane domains. First, Ha-Ras traffics to the plasma membrane via the endomembrane system, whereas K-Ras appears to reach the plasma membrane through a different transport pathway (26, 27). Second, interfering with the function of caveolin, an integral membrane protein that binds cholesterol (28) impairs Ha-Ras but not K-Ras-dependent signaling, indicating that Ha-Ras is selectively localized to cholesterol-rich membrane domains (29). Third, K-Ras but not Ha-Ras binds to tubulin, and this association is critical for the plasma membrane targeting of K-Ras (30).

It is possible to envisage at least two mechanisms by which the site of anchoring of Ras to the membrane might influence effector interactions. Ras effectors or their co-activators could themselves be localized to distinct subdomains within the plasma membrane. Indeed, a number of signaling molecules involved in Ras signaling, including the Ras target Raf, have been shown to be preferentially localized to specialized cholesterol-rich microdomains also known as caveola (31). Thus, differences in the spatial availability of effector molecules could account for the unique signaling properties of Ha-Ras and K-Ras. Alternatively, the efficiency of interaction with effector molecules might be influenced by the mode of anchoring to the plasma membrane. The association of Ha-Ras with the membrane is mediated by a farnesyl/palmitoyl anchor, whereas K-Ras uses farnesylation and a polybasic domain to bind to the

A RasV12 and Ha-RasV121–164/K-Ras with anti-T7 monoclonal antibody followed by rhodamine-conjugated goat anti-mouse secondary antibody. Bar, 20 μm. Right panels, phase contrast micrographs of cells expressing Ha-RasV12 and Ha-RasV121–164/K-Ras, 4 h after microinjection. Bar, 20 μm. B, REF-52 cells were serum-starved 24 h prior to the microinjection of Ha-RasV12 and Myr-HRasV12S186 expression plasmids (50 ng/μl). Top panels, the cells were immunostained for the expression and localization of Ha-RasV12 and Myr-HRasV12S186 with Y13–259 antibody followed by rhodamine-conjugated goat anti-rat secondary antibody. Bar, 20 μm. Middle panels, phase contrast micrographs of cells expressing Ha-RasV12 and Myr-HRasV12S186 4 h after microinjection. Bar, 20 μm. Bottom panel, REF-52 cells were transiently transfected with HA-tagged ERK2 and Ha-RasV12, Ha-RasV12S186, or Myr-HRasV12S186. ERK2 was immunoprecipitated with anti-HA monoclonal antibody (12CA5) and incubated with myelin basic protein (MBP) in the presence of [γ-32P]-ATP for 30 min at 30 °C. The myelin basic protein phosphorylation was visualized by autoradiography (bottom panel).

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plasma membrane (32). Palmitate contributes to membrane interactions by virtue of its ability to insert deep into the lipid bilayer, whereas the polybasic domain facilitates membrane association through ionic interactions with negatively charged phospholipid head groups (33, 34). These differences could influence the positioning of each Ras isoform at the inner face of the plasma membrane, thereby affecting accessibility to effector molecules.

The lack of a detailed understanding of the effector pathways that couple Ras to Rac makes it difficult to investigate the mechanistic basis for the differential activation of Rac by Ha-Ras and K-Ras. Like all GTP-binding proteins, the activation of Rac is mediated by guanine nucleotide exchange factors that promote the exchange of GTP for GDP. Guanine nucleotide exchange factors for Rac belong to the Dbl family of proteins, all members of which contain the tandem arrangement of a PH domain adjacent to the catalytic Dbl homology domain (35). In certain cases, the PH domains have been shown to regulate the targeting of Dbl homology domains to the appropriate subcellular location (36, 37). Although the determinants that specify the targeting properties of PH domains are not well defined, it has been proposed that, depending on their lipid binding properties, different PH domains could be recruited to different membrane domains (38–40). Hence, activators of Rac might be differentially accessible to Ha-Ras and K-Ras.

P13K has been implicated in the regulation of Rac activation by Ras (41, 42). At least one mechanism by which this might occur involves the generation of lipid mediators that modulate the guanine nucleotide exchange activity of the Dbl homology domain containing proteins Vav and Sos (41, 43). It has been shown that Ha-Ras is a more potent activator of PI3K than K-RasV12 (8). However, we have shown K-Ras to be a more effective activator of Rac than Ha-RasV12, suggesting the existence of other coupling mechanisms linking Ras to the Rac cascade. In addition, it is important to note that mammalian cells express three isoforms of the catalytic subunit of P13K, p110α, p110β, or p110δ (44). Moreover, it has been recently shown that each isoform has distinct signaling properties in the context of receptor-mediated regulation of the actin cytoskeleton (45). Thus, differential interactions of Ha-Ras and K-Ras with p110 isoforms could contribute to functional specificity. A biochemical assay for isoform-specific activation of P13K by Ras will be required to test this hypothesis.

Ras proteins play a role in regulating the turnover of focal adhesions and are essential for cell movement (22). In agreement with an earlier report (7), we have found that K-Ras is more effective in stimulating cell motility compared with Ha-Ras. This difference is most likely a consequence of the preferential activation of Rac by K-Ras because the movement of cells requires the coordinated assembly and disassembly of stress fibers (46). In colon epithelial cells K-Ras but not Ha-Ras disrupts basolateral polarity by altering the expression of several intercellular adhesion proteins including β1 integrin and N-cadherin (47). The preferential ability of K-Ras to induce loss of cell-to-cell and cell-to-substratum adherence and to stimulate cell motility could account for the highly invasive and metastatic phenotype of K-Ras-derived tumor cells (48, 49).

The frequency of K-Ras mutations in human malignancies is considerably higher with Ha-Ras (5). We have found that K-Ras displays a reduced capacity to induce apoptosis presumably because of its ability to activate more efficiently the anti-apoptotic cascade. The capacity of oncoproteins to induce apoptosis or cell cycle arrest is thought to represent safeguard mechanisms to limit the growth of tumor cells. Thus, the compromised ability of K-Ras to induce apoptosis might explain, at least in part, the selective growth advantage of tumor cells harboring K-Ras mutations.

All Ras effectors interact with a common and highly conserved region within Ras known as the effector binding loop. Our observation that myristoylated Ha-Ras retains the ability to activate mitogen-activated protein kinase but has lost the ability to activate Rac raises the possibility that each effector interaction might be uniquely regulated by the mode of attachment of Ras to the membrane. This is supported by a recent study suggesting that the C-terminal domain of Ras influences effector interaction (50). This adds another level of complexity to the regulation of Ras signaling, which along with the multiplicity of effector pathways and their cross-interactions enable the finely tuned control of cellular events that mediate cell growth and differentiation.

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