Ras Isoforms Vary in Their Ability to Activate Raf-1 and Phosphoinositide 3-Kinase*

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Ha-, N-, and Ki-Ras are ubiquitously expressed in mammalian cells and can interact with the same set of effector proteins. We show here, however, that in vivo there are marked quantitative differences in the ability of Ki- and Ha-Ras to activate Raf-1 and phosphoinositide 3-kinase. Thus, Ki-Ras both recruits Raf-1 to the plasma membrane more efficiently than Ha-Ras and is a more potent activator of membrane-recruited Raf-1 than Ha-Ras. In contrast, Ha-Ras is a more potent activator of phosphoinositide 3-kinase than Ki-Ras. Interestingly, the ability of Ha-Ras to recruit Raf-1 to the plasma membrane is significantly increased when the Ha-Ras hyper-variable region is shortened so that the spacing of the Ha-Ras GTPase domains from the inner surface of the plasma membrane mimicks that of Ki-Ras. Importantly, these data show for the first time that the activation of different Ras isoforms can have distinct biochemical consequences for the cell. The mutation of specific Ras isoforms in different human tumors can, therefore, also be rationalized.

Ras proteins operate as molecular switches in signal transduction pathways downstream of tyrosine kinases. An interesting yet unresolved issue is whether the Ras isoforms that are ubiquitously expressed in mammalian cells serve distinct functions. Several lines of evidence suggest that they may. First, recent studies have shown that Ki-Ras but not Ha- or N-Ras has an essential function in mouse development (1, 2). Secondly, specific Ras isoforms are mutated in different tumors: Ki-ras mutations occur in 50% of colon cancers and 90% of pancreatic cancers, whereas N- and Ha-ras mutations are extremely uncommon. Conversely, N-ras mutations occur in 25% of acute leukemias, whereas Ha-ras and Ki-ras mutations are much less common (3). The simplest interpretation of these observations is that oncogenic activation of different Ras proteins has distinct biological consequences for the cell.

The N-terminal 165 amino acids of Ras contain all of the critical domains for GTPase function. The N-terminal 85 residues of all Ras isoforms are identical and contain the two switch regions that undergo conformational changes on GTP binding; in addition the next 80 amino acids are 95% conserved. All Ras proteins in the activated GTP bound state interact with the same set of effectors: Raf kinases, phosphoinositide 3-kinase (PI3-K), RapGDS, and AF6 (4–10). Mutational and structural studies have demonstrated that the Ras effector domain (residues 32–40) is a critical binding site for all these Ras effectors. Moreover, where measured using recombinant proteins, no marked differences in binding affinities of Ha-, N-, and Ki-Ras for these various effector proteins are apparent (11). Thus, there are no differences in effector domains or flanking sequences that could account for distinct signaling outcomes downstream of each Ras isoform.

The major differences between Ras proteins are confined to the hypervariable region (HVR) between residues 166 and 185. Here the Ras sequences diverge significantly with fewer than 15% conserved residues (12). C-terminal to the HVR all Ras proteins terminate in a conserved CAAX motif (C, cysteine; A, aliphatic amino acid; X, methionine or serine) that directs post-translational processing. In previous studies we have shown that one role of the HVR is to cooperate with the processed C-terminal CAAX motif and provide a second signal for Ras plasma membrane localization (13, 14). In Ki-Ras this second signal comprises a polylsine domain (lysine residues 175–180), whereas in Ha-Ras and N-Ras the second signal comprises palmitoylation sites at cysteines 181 and/or 184 (15).

Ras must be localized to the inner surface of the plasma membrane to be biologically active. In addition, Raf-1 and PI3-K are constitutively activated when targeted to the plasma membrane using Ras localization motifs (16–18), indicating that the recruitment of these effectors to the plasma membrane by Ras is a critical step for activation. Taken together, these data suggested that the ability of Ras proteins to activate specific effectors may be influenced by their different mechanisms of attachment to the plasma membrane. To test this hypothesis we investigated whether Ki-Ras and Ha-Ras, which use markedly different plasma membrane localization signals, are equipotent activators of Raf-1 and PI-3K.

MATERIALS AND METHODS

Cell Fractionation and Immunoblotting—COS cells were electroporated as described previously (19) using 1–30 μg of EVX-Ki-RasG12V, EVX-Ha-RasG12V, or EVX-Ha-RasG12V expression plasmids. 72 h later, after an 18-h serum-free incubation, cells were washed, scraped on ice into 0.5 ml of Buffer A (10 mM Tris-Cl, pH 7.5, 25 mM NaF, 5 mM MgCl2, 1 mM EGTA, 1 mM DTT, and 100 μM NaVO4), and homogenized in a tight fitting Dounce homogenizer. The postnuclear supernatants were spun at 100,000 × g for 1 h, the supernatants (S100) were removed, and the sedimented fractions (P100) were rinsed and sonicated for 5 min in 100 μl of ice-cold Buffer A. Protein content was measured by the Bradford reaction. Expression of Ras was determined by quantitative immunoblotting. 20 μg of each P100 fraction was resolved on 15%
SDS-PAGE gels and transferred to polyvinylidene difluoropolyvinylidene difluoride membranes using semidy transfer. Aliquots of GST-Ras, ranging from 5–400 ng (containing therefore 2.1–171 ng of Ras protein) were also loaded onto gels and transferred onto the same polyvinylidene difluoride membrane as the P100 fractions. Westerns were done with anti-Ras (Y13–259) and then anti-FLAG horseradish peroxidase (Pierce) antibodies, developed using enhanced chemiluminescence (SuperSignal, Pierce) and quantitated by phosphorimaging with a CH screen (Bio-Rad). The signals from GST-Ras were used to construct a dose response curve from which the actual Ras content of each P100 fraction was determined. For co-transfections 10 μg of ExV-FLAG Ras plasmid (20) was electroporated with 1–30 μg of Ras plasmid. The cells were harvested and washed with 20 μg of P100 was determined by immunoblotting as described. 20 μg of each S100 and P100 fraction were immunoblotted for FLAGRaf with M2 anti-FLAG monoclonal (Kodak) and anti-mouse horseradish peroxidase (Pierce). FLAG immunoblots were quantitated by phosphorimaging and the percentage of Raf-1 recruited to the P100 fraction was calculated as P100-Raf/P100-Raf + S100-Raf.

**Raf-1 Membrane Kinase Assays**—This assay is discussed in detail elsewhere (20). Briefly, P100 aliquots (20 μg) were adjusted to 20 μl with Buffer A. 2.2 μl of 10% Nonidet P-40 was added, and the membranes were sonicated in a sonicating waterbath for 2 min at 4 °C. A 10-μl aliquot of sonicated P100 fraction was incubated with 6 μl of Buffer B. A second 10-μl aliquot of sonicated P100 fraction was incubated with 6 μl of Buffer A containing 4 μl of 0.5 mM ATP/40 mM MgCl₂ and 1 μl of 0.5 mM EGTA, 100 μM NaNO₃, and 1 μM DTT. 10 μl of these diluted samples were taken into a second incubation with 5 μl of MBP (16 μg) and 10 μl of an ATP mix containing 0.5 mM ATP, 50 mM MgCl₂, [γ-32P]ATP (2,400 cpm/pmol). The MBP kinase reaction was performed in duplicate. After 10 min the reaction was stopped by adding 6 μl of 5x SDS-PAGE sample buffer, and the reaction products were resolved on 15% SDS-PAGE gels. The radioactivity incorporated into MBP was measured by phosphorimaging after spotting the gels with a known amount of radioactive [γ-32P]ATP. Background counts due to any P100-associated MEK and ERK were estimated from the control tubes and subtracted from the assay counts (<5% total activity).

**Raf-1 Immunoprecipitation Kinase Assays**—To measure the specific activity of FLAGRaf, P100 fractions were normalized for FLAGRaf content, adjusted to 1% Nonidet P-40, sonicated for 90 s at 4 °C, incubated on ice for 10 min, and microcentrifuged for 5 min, and the soluble extract was diluted to 400 μl with Buffer B (50 mM Tris-Cl, pH 7.5, 75 mM NaCl, 5 mM MgCl₂, 25 mM NaF, 5 mM EGTA, 100 μM NaNO₃, and 1 mM DTT). The samples were incubated with 10 μl of anti-FLAG-Sepharose beads (Kodak) for 2 h at 4 °C and washed six times in Buffer B. The immunoprecipitates were split into two, and incubated with both MEK and ERK alone, and the kinase assay was completed as described above. The beads were then collected, washed, and immunoblotted to verify the amount of Raf-1 present in the assay.

**Immunofluorescent Assays**—BHK cells were cultured at 37 °C (5% CO₂) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) bovine calf serum and 100 units/ml of penicillin and streptomycin. Cells were plated onto glass coverslips at 60% confluence and 4 h later were transfected using lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions, with 1.6 μg of ExV expression plasmid for Ha-RasG12V and Ha-RasG12V. After an overnight incubation, cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 3% bovine serum albumin in PBS. Cells were then incubated in undiluted anti-p85 (Upstate Biotechnology Inc.) prebound to protein A-Sepharose beads. The immunoprecipitates were washed three times with lysis buffer, twice in 0.5 μl LiCl, 100 mM Tris-Cl, pH 7.6, twice with 10 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1 mM EDTA, and twice with 20 mM HEPEs, pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40, 30 μM NaNO₃, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml leupeptin. Kinase reactions were carried out for 20 min at 25 °C in a vortexing heat block. Kinase buffer contained 20 mM Tris-Cl, pH 7.6, 75 mM NaCl, 10 mM MgCl₂, phosphatidylinositol (200 μg/ml) sonicated in 20 mM HEPEs, pH 7.5, 20 μM ATP, 20 μM adenosine, 10 μg of [γ-32P]ATP (6,000 Ci/mmol). Reactions were stopped with 100 μl of 1 n HCl and phospholipids extracted once with 200 μl of CHCl₃/MeOH (1:1) and once with 160 μl of 1 n HCl/MeOH (1:1). The organic phase was dried under N₂ and resuspended in 10 μl of CHCl₃/MeOH (1:1) containing PIP standard (1 μg/ml). Phosphorylated products were resolved on oxalate impregnated silica60 plates (Merck) using CHCl₃/MeOH:4 μNH₄OH (9:7:2) as solvent. Standards were visualized in iodine vapor, and radioactive products were visualized and quantitated by phosphorimaging. To determine the amount of post-translationally processed, membrane localized Ras expressed, 10% of the harvested cells were lysed in 1% Triton X114. The detergent-partitioning protein fraction was isolated as described (25, 26), concentrated by acetone precipitation, normalized for protein content, and quantitatively immunoblotted.

**RESULTS AND DISCUSSION**

**Ki-Ras Is a More Potent Activator of Raf-1 Than Ha-Ras**—We first investigated whether Ha-Ras and Ki-Ras are equipotent activators of Raf-1. The two Ras isoforms were chosen for this study because of their very different membrane localization signals. COS cells were transiently transfected with varying amounts of Ha-RasG12V or Ki-RasG12V expression plasmids. These fractions were normalized for protein content, immunoblotted for Ras (upper panel) and Raf-1 activity measured in a coupled MEK/ERK activation assay (lower panel). B, MBP phosphorylation, which is the readout from the coupled Raf-1 activation assay, was measured by phosphorimaging and plotted against Ras expression (study using P100 protein measured by quantitative Western blotting). The graph shows data pooled from three independent experiments; the images shown in A are from one of these experiments.

**Fig. 1**. Ki-Ras is a more potent activator of Raf-1 than Ha-Ras. A, membrane fractions were prepared from COS cells transfected with increasing amounts of Ha-RasG12V or Ki-RasG12V expression plasmids. These fractions were normalized for protein content, immunoblotted for Ras (upper panel) and Raf-1 activity measured in a coupled MEK/ERK activation assay (lower panel). B, MBP phosphorylation, which is the readout from the coupled Raf-1 activation assay, was measured by phosphorimaging and plotted against Ras expression (study using P100 protein measured by quantitative Western blotting). The graph shows data pooled from three independent experiments; the images shown in A are from one of these experiments.
against known amounts of recombinant Ras. The Raf-1 activity present in 20 μg of cell membrane was measured in a coupled MEK/ERK activation assay using MBP phosphorylation as readout (Fig. 1A). The results from three such experiments are summarized in Fig. 1B. The data show that Ki-Ras is a significantly more potent activator of Raf-1 than Ha-Ras because minimal overexpression of Ki-Ras to 5 ng/20 μg P100 (which equals 0.025% total membrane protein) resulted in a 5-fold greater activation of endogenous Raf-1 than the same minimal overexpression of Ha-Ras (Fig. 1B). The mechanism of Raf-1 activation is complex, but it is clear that one important role of Ras is to recruit Raf-1 to the plasma membrane (16, 17, 27, 28) where a series of events is initiated that ultimately leads to full Raf-1 activation. These events include tyrosine, serine, and threonine phosphorylation (29–33) plus interactions with Ras (20, 34, 35), phospholipids (36, 37), 14-3-3 proteins and their associated proteins (38–44), and possibly dimerization (45, 46). We reasoned, therefore, that the more potent activation of Raf-1 by Ki-Ras could reflect differences in the relative ability of Ki-Ras and Ha-Ras to recruit Raf-1 to the plasma membrane, and/or differences in Ki-Ras and Ha-Ras catalyzed membrane activation events.

To discriminate between these two possibilities COS cells were co-transfected with epitope-tagged FLAGRaf and varying amounts of Ha-RasG12V or Ki-RasG12V plasmids. S100 and P100 fractions were prepared from these transfectants and immunoblotted for Ras and FLAGRaf. The immunoblots were quantitated by phosphorimaging, and the data were used to calculate the fraction of FLAGRaf recruited to the membrane by increasing amounts of co-expressed Ras. The analysis in Fig. 2A shows that Ki-Ras recruits Raf-1 more efficiently than Ha-Ras, because recruitment of 50% of FLAG-Raf to the plasma membrane required expression of 20 ng (per 20 μg of membrane) of Ki-Ras compared with 130 ng (per 20 μg of membrane) of Ha-Ras (Fig. 2A). We next determined whether Raf-1 recruited to the membrane fraction by Ki- and Ha-Ras differed in specific activity. Membrane fractions from the COS cells coexpressing FLAGRaf and Ki- or Ha-Ras were normalized for FLAGRaf content and immunoprecipitated using anti-FLAG-Sepharose. Raf-1 activity was then measured in the anti-FLAG immunoprecipitates. Significantly, Raf-1 recruited to the membrane by Ki-Ras was found to have a 4-fold higher specific activity than Raf-1 recruited to the membrane by Ha-Ras (Fig. 2B). We conclude that Ki-Ras is a more potent in vivo activator of Raf-1 because Ki-Ras recruits Raf-1 to the plasma membrane more efficiently than Ha-Ras and also because Ki-Ras more efficiently catalyzes Raf-1 activation at the cell membrane.

**Influence of the Ras HVR on Raf-1 Membrane Recruitment—**

The polylysine domain of Ki-Ras (residues 175–180) interacts with negatively charged phospholipid head groups on the inner surface of the plasma membrane (15, 47), residues C-terminal of residue 175 in Ki-Ras will therefore be tightly associated with the membrane. In contrast, the N-terminal limit of membrane tethering of Ha-Ras is the palmitoylation site at cysteine 181 (Fig. 3A). In consequence, the N-terminal conserved domains of Ras (1–166) are likely positioned closer to the plasma membrane in Ki-Ras than in Ha-Ras, i.e. by 7 Ki-Ras HVR residues (167–174) versus 13 Ha-Ras HVR residues (167–180). We therefore examined whether shortening the HVR of Ha-Ras by 6 amino acids would improve the ability of Ha-Ras to recruit and activate Raf. Ha-RasΔ was constructed by deleting amino acids 167–173, leaving intact all of the sequences required for plasma membrane localization (Fig. 3A). As expected, when expressed in BHK cells Ha-RasΔ was found to localize to the plasma membrane to the same extent as full-length Ha-Ras (Fig. 3B). The ability of Ha-RasΔ to recruit and activate Raf-1 was then compared with Ki-Ras and Ha-Ras. COS cells were co-transfected with FLAGRaf and varying amounts of Ha-RasG12V or Ki-RasG12V plasmids. Fig. 2A shows that shortening the HVR of Ha-Ras by 6 amino acids significantly improved its ability to recruit Raf-1 because the recruitment of 50% of expressed FLAGRaf required 35 ng (per 20 μg of membrane) of Ha-RasΔ compared with 135 ng (per 20 μg of membrane) of full-length Ha-Ras. The recruitment of Raf-1 to the plasma membrane by Ha-RasΔ therefore compares favorably with Ki-Ras (Fig. 2A). However, the activation of membrane-recruited Raf-1 was not improved by shortening the Ha-Ras HVR because the specific activities of FLAGRaf recruited by Ha-Ras and Ha-RasΔ were not significantly different (Fig. 2B). Finally, we compared the membrane recruitment of endogenous Raf-1 by Ha-RasΔ, Ha-
The membrane attachment sequences of Ki-Ras comprise the polybasic domain K175–180 plus the CVIM (CAAX motif). The Ha-Ras membrane attachment sequences comprise cysteine residues Cys181 and Cys184 plus the CVLS (CAAX motif). Thus there are 15 HVR residues in Ha-Ras compared with 9 HVR residues in Ki-Ras between the conserved Ras sequence (1–165) and the N-terminal limit of membrane attachment. The Ha-Ras cDNA sequences encoding residues His166 though Leu171 were deleted using oligonucleotide directed mutagenesis to generate Ha-RasΔ, which therefore has 9 HVR residues between the conserved Ras sequence (1–165) and the N-terminal limit of membrane attachment. B, BHK cells were lipofected with expression plasmids for Ha-RasΔ and Ha-Ras (control) and Ras protein visualized by indirect immunofluorescence in a confocal microscope. Because the membrane targeting motifs are intact, Ha-RasΔ localizes normally to the plasma membrane. C, cytosol and membrane fractions of COS cells expressing increasing amounts of Ha-RasG12V, Ki-RasG12V, or Ha-RasG12V were normalized for protein content and immunoblotted for Ras and endogenous Raf-1. The figure shows that even a very low level of Ki-Ras expression is sufficient to fully recruit all endogenous Raf-1 to the membrane. Thus, when taken together with the data in Fig. 2, the increase in endogenous Raf-1 activation, shown in Fig. 1B, that is associated with the immunoprecipitates was measured in an in vitro lipid kinase assay using phosphatidylinositol as substrate. The phosphorylated products of the reaction were resolved by TLC and visualized by phosphorimaging (upper panel). The arrow indicates radioactive PIP product identified on the basis of disappearance in cells treated with wortmannin (data not shown) and by co-migration with an unlabeled PIP standard, in turn visualized by iodine staining. The last lane on the TLC plate represents the PI3-K activity present in serum starved cells transfected with empty vector (control). Immunoprecipitates that are duplicates of those used in the kinase assay were immunoblotted for p85 to confirm that equivalent amounts of PI3-K were being captured from the cell lysates (middle panel). An aliquot of cells from each transfection was lysed in Triton-X114. The detergent partitioning protein fraction, containing processed, membrane localized Ras, was concentrated by acetone precipitation and immunoblotted for Ras (lower panel). B, after phosphorimaging of the TLC plates, Ras-stimulated PI3-K activity was calculated by subtracting the activity present in the control from the activity present in the Ras-transfected samples. The amount of biologically active Ras present in each lysate was determined by quantitative Western blotting of the Triton-X114 detergent partitioning fraction. Data are from four independent experiments, including that shown in A.

**Raf-1 and PI3-K Activation by Ras**

**FIG. 3.** The length of the Ras HVR influences the efficiency with which Ras recruits Raf-1 to the plasma membrane. A, the membrane attachment sequences of Ki-Ras comprise the polybasic domain K175–180 plus the CVIM (CAAX motif). The Ha-Ras membrane attachment sequences comprise cysteine residues Cys181 and Cys184 plus the CVLS (CAAX motif). Thus there are 15 HVR residues in Ha-Ras compared with 9 HVR residues in Ki-Ras between the conserved Ras sequence (1–165) and the N-terminal limit of membrane attachment. The Ha-Ras cDNA sequences encoding residues His166 though Leu171 were deleted using oligonucleotide directed mutagenesis to generate Ha-RasΔ, which therefore has 9 HVR residues between the conserved Ras sequence (1–165) and the N-terminal limit of membrane attachment. B, BHK cells were lipofected with expression plasmids for Ha-RasΔ and Ha-Ras (control) and Ras protein visualized by indirect immunofluorescence in a confocal microscope. Because the membrane targeting motifs are intact, Ha-RasΔ localizes normally to the plasma membrane. C, cytosol and membrane fractions of COS cells expressing increasing amounts of Ha-RasG12V, Ki-RasG12V, or Ha-RasG12V were normalized for protein content and immunoblotted for Ras and endogenous Raf-1. The figure shows that even a very low level of Ki-Ras expression is sufficient to fully recruit all endogenous Raf-1 to the membrane. Thus, when taken together with the data in Fig. 2, the increase in endogenous Raf-1 activation, shown in Fig. 1B, that is associated with the immunoprecipitates was measured in an in vitro lipid kinase assay using phosphatidylinositol as substrate. The phosphorylated products of the reaction were resolved by TLC and visualized by phosphorimaging (upper panel). The arrow indicates radioactive PIP product identified on the basis of disappearance in cells treated with wortmannin (data not shown) and by co-migration with an unlabeled PIP standard, in turn visualized by iodine staining. The last lane on the TLC plate represents the PI3-K activity present in serum starved cells transfected with empty vector (control). Immunoprecipitates that are duplicates of those used in the kinase assay were immunoblotted for p85 to confirm that equivalent amounts of PI3-K were being captured from the cell lysates (middle panel). An aliquot of cells from each transfection was lysed in Triton-X114. The detergent partitioning protein fraction, containing processed, membrane localized Ras, was concentrated by acetone precipitation and immunoblotted for Ras (lower panel). B, after phosphorimaging of the TLC plates, Ras-stimulated PI3-K activity was calculated by subtracting the activity present in the control from the activity present in the Ras-transfected samples. The amount of biologically active Ras present in each lysate was determined by quantitative Western blotting of the Triton-X114 detergent partitioning fraction. Data are from four independent experiments, including that shown in A.

**FIG. 4.** Ha-Ras is a more potent activator of PI3-K than Ki-Ras. A, whole cell lysates (1 mg of total protein) prepared from cells expressing increasing amounts of Ha-RasG12V, Ki-RasG12V, or Ha-RasG12V were immunoprecipitated using anti-p85 antibodies. PI3-K activity associated with the immunoprecipitates was measured in an in vitro lipid kinase assay using phosphatidylinositol as substrate. The phosphorylated products of the reaction were resolved by TLC and visualized by phosphorimaging (upper panel). The arrow indicates radioactive PIP product identified on the basis of disappearance in cells treated with wortmannin (data not shown) and by co-migration with an unlabeled PIP standard, in turn visualized by iodine staining. The last lane on the TLC plate represents the PI3-K activity present in serum starved cells transfected with empty vector (control). Immunoprecipitates that are duplicates of those used in the kinase assay were immunoblotted for p85 to confirm that equivalent amounts of PI3-K were being captured from the cell lysates (middle panel). An aliquot of cells from each transfection was lysed in Triton-X114. The detergent partitioning protein fraction, containing processed, membrane localized Ras, was concentrated by acetone precipitation and immunoblotted for Ras (lower panel). B, after phosphorimaging of the TLC plates, Ras-stimulated PI3-K activity was calculated by subtracting the activity present in the control from the activity present in the Ras-transfected samples. The amount of biologically active Ras present in each lysate was determined by quantitative Western blotting of the Triton-X114 detergent partitioning fraction. Data are from four independent experiments, including that shown in A.

**Ha-Ras Is a More Potent Activator of Phosphoinositide 3-Kinase Than Ki-Ras**—We next examined the ability of Ki-Ras and Ha-Ras to activate a second Ras effector, PI3-K. Anti-p85 antibodies were used to immunoprecipitate endogenous PI3-K from COS cells expressing increasing amounts of Ki-RasG12V and Ha-RasG12V. PI3-K activity was then measured in an in vitro lipid kinase assay using phosphatidylinositol as substrate. Fig. 4A shows the result of a representative experiment and Fig. 4B shows data pooled from four independent experiments. These data clearly show that Ha-Ras is a considerably more potent activator of PI3-K than Ki-Ras. Fig. 4 also shows that shortening the Ha-Ras HVR does not significantly affect the ability of Ha-Ras to activate PI3-K, because the dose response curves of Ha-Ras and Ha-RasΔ are similar. The more potent activation of PI3-K by Ha-Ras can be explained in two
ways: efficient activation may require Ha-Ras specific sequences, located in the HVR or elsewhere, or Ha-Ras may recruit PI-3K to a distinct subdomain of the plasma membrane where activation proceeds more efficiently than in the subdomain to which PI-3K is recruited by Ki-Ras.

**Concluding Remarks**—Although all Ras isoforms qualitatively activate the same effector pathways, we have shown here that in *vivo* there are marked quantitative differences in the activation of c-Raf-1 and PI3-K by Ha-Ras and Ki-Ras. It seems probable that quantitative differences will extend to the activation of other Ras effector pathways, and therefore, activation of each Ras isoform can have distinct biochemical consequences for the cell. These different effector activation profiles may also account for the selective activation of different Ras isoforms in specific human tumors. We propose that the selection pressure for which Ras isoform is activated is determined by the background level of activity in all signaling pathways and that given the nature of multistep oncogenesis, this background activity will vary between target cells. In which case, activation of Ha-Ras may be favored in tumor cells that already have increased MAPK activity but low levels of PI3-K activity, whereas in tumor cells with constitutively elevated PI3-K activity, activation of Ki-Ras may be favored because it will result in coincident robust activation of the Raf/MAPK pathway. A recent study of human colorectal tumors adds significant weight to this hypothesis. Some 86% of human colorectal tumors tested were found to have significantly higher levels of PI3-K activity than normal colonic mucosa sampled from the same patient (48). Moreover, the level of PI3-K activity in these tumors was not further elevated in the presence of a Ki-ras mutation (48). This study is consistent with our finding that Ki-Ras is not a potent *in vivo* activator of PI3-K and, importantly, when taken together with the data presented here, rationalizes the preferential activation of Ki-Ras in colon cancer. In summary, our study emphasizes the importance of the RasHVR in influencing the interaction of Ras with cer. In summary, our study emphasizes the importance of the RasHVR in influencing the interaction of Ras with cer.

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