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Activated K-Ras and H-Ras display different interactions with saturable nonraft sites at the surface of live cells

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Ras–membrane interactions play important roles in signaling and oncogenesis. H-Ras and K-Ras have nonidentical membrane anchoring moieties that can direct them to different membrane compartments. Ras–lipid raft interactions were reported, but recent studies suggest that activated K-Ras and H-Ras are not raft resident. However, specific interactions of activated Ras proteins with nonraft sites, which may underlie functional differences and phenotypic variation between different Ras isoforms, are unexplored. Here we used lateral mobility studies by FRAP to investigate the membrane interactions of green fluorescent protein–tagged H- and K-Ras in live cells. All Ras isoforms displayed stable membrane association, moving by lateral diffusion and not by exchange with a cytoplasmic pool. The lateral diffusion rates of constitutively active K- and H-Ras increased with their expression levels in a saturable manner, suggesting dynamic association with saturable sites or domains. These sites are distinct from lipid rafts, as the activated Ras mutants are not raft resident. Moreover, they appear to be different for H- and K-Ras. However, wild-type H-Ras, the only isoform preferentially localized in rafts, displayed cholesterol-sensitive interactions with rafts that were independent of its expression level. Our findings provide a mechanism for selective signaling by different Ras isoforms.

Introduction

Ras proteins are small GTPases that regulate signaling pathways controlling cell growth, differentiation, and survival (Campbell et al., 1998; Downward, 1998; Bar-Sagi and Hall, 2000; Reuther and Der, 2000). The *ras* gene is the most frequently mutated gene in human tumors, where mutations at positions 12, 13, or 61 lead to constitutive activation (Bos, 1989). The prototypic mammalian Ras proteins (H-Ras, K-Ras 4B, K-Ras 4A, and N-Ras) share over 90% sequence homology and have similar, although not overlapping, activities (Jones and Jackson, 1998; Yan et al., 1998; Booden et al., 2000; Clyde-Smith et al., 2000; Walsh and Bar-Sagi, 2001).

Ras proteins' function and oncogenic potential require their association with the inner plasma membrane leaflet (Willumsen et al., 1984; Marshall, 1996). This most likely reflects the need to recruit Ras effectors to the plasma membrane for their activation (Campbell et al., 1998). Ras–membrane anchorage is promoted by two signals, both localized in the "hypervariable" COOH-terminal region (Casey et al., 1989; Hancock et al., 1989; Kato et al., 1992; Prior et al., 2001). One signal shared by all Ras proteins is a COOH-terminal S-farnesylcysteine carboxy methylester. The second signal differs among the Ras isoforms, consisting of a six-lysine stretch in K-Ras4B (hereafter designated K-Ras), two adjacent S-palmitoyl moieties in H-Ras and one palmitoyl in N-Ras (Hancock et al., 1989; Kato et al., 1992). These moieties and possibly the entire hypervariable region sequence not only confer membrane-tethering capacity on Ras, but are also involved in the trafficking of Ras proteins to the plasma membrane (Choy et al., 1999).

The interactions of Ras proteins with the plasma membrane can differ from one isoform to the other due to their different membrane anchoring moieties. At least some of the differences may arise from different degrees of association with lipid rafts, which are cholesterol/sphingolipid-enriched microdomains that dynamically organize specific membrane proteins (Anderson, 1998; Jacobson and Dietrich, 1999; Kurzchalia and Parton, 1999; Brown and London, 2000; Simons and Toomre, 2000). Originally, both H-Ras and K-Ras were reported to cofractionate with the caveolar fraction (Mineo et al., 1996; Song et al., 1996; Furuchi and Anderson,

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1998), which is considered to be a specialized form of rafts (Jacobson and Dietrich, 1999; Simons and Toomre, 2000). However, recent studies suggest that H-Ras, but not K-Ras, is predominantly localized in lipid rafts. The controversy regarding the domain localization of K-Ras could reflect the operational nature of determining raft association by membrane extraction and cofractionation, which depend on the extraction conditions and flotation gradients used (Janes et al., 1999; Simons and Toomre, 2000). Therefore, it is important to measure the interactions of differently anchored Ras proteins with lipid rafts (and possibly with other structures) in the membranes of live cells (Jacobson and Dietrich, 1999; Simons and Toomre, 2000). The need to explore interactions of activated Ras isoforms with membrane sites or domains distinct from lipid rafts is also important. The potential importance of such interactions is highlighted by the report that activated H-Ras is largely released from raft domains, resulting in nonraft distribution resembling activated K-Ras (Prior et al., 2001). Thus, selective activation of specific signaling pathways by different Ras isoforms (Yan et al., 1998; Coats et al., 1999; Booden et al., 2000; Walsh and Bar-Sagi, 2001; Jaumot et al., 2002) would require differential interactions of activated Ras subtypes with nonraft sites. To date, such interactions were not investigated thoroughly.

Earlier studies on Ras–membrane association used cell-free systems or fixed cells. To investigate this issue in live cells, we used FRAP to study the lateral mobility of green fluorescent protein (GFP)*–tagged Ras isoforms expressed in Rat-1 cells. We compared H-Ras and K-Ras because their membrane anchors differ significantly. Our studies demonstrate that both H-Ras and K-Ras, either wild type (wt) or the constitutively active 12V mutants, undergo fast lateral diffusion at the plasma membrane rather than exchange between membrane and cytoplasmic pools. A novel phenomenon revealed by our studies is that K-Ras(12V) and H-Ras(12V), but not H-Ras(wt), interact with saturable nonraft sites or domains that retard their lateral mobility, and that these interactions differ between the two isoforms of activated Ras. We also demonstrate for the first time in live cells that H-Ras(wt), but not K-Ras, is significantly concentrated in cholesterol-dependent rafts. These observations have important implications for the regulation of Ras functions and phenotypic variation by specific interactions with the plasma membrane.

Results

The GFP-tagged Ras isoforms are biologically active

To investigate the interactions of H-Ras and K-Ras with the plasma membrane, we prepared GFP-tagged constructs of the cDNAs encoding these proteins (GFP-H-Ras, GFP-H-Ras(12V), GFP-K-Ras, and GFP-K-Ras(12V)). To validate that GFP-H-Ras(12V) and GFP-K-Ras(12V) are constitutively active, we used an assay in intact cells, based on glutathione *S*-transferase (GST) fused to the Ras binding domain of Raf-1 (RBD), that binds Ras-GTP but not Ras-GDP (Herrmann et al., 1995). Fig. 1 A demonstrates that the constitutively active Ras mutants bound GTP to a much higher extent than their wt counterparts, either when stably expressed in Rat-1 cells (H-Ras isoforms) or transiently in Cos-7 cells (K-Ras isoforms). Stable expression of GFP-K-Ras(12V) (Niv et al., 1999) or GFP-H-Ras(12V) induced cell transformation. The GFP-tagged wt isoforms respond to upstream signals, as demonstrated by their strong activation in cells exposed to EGF; the level of activated Ras peaked after a 1–5-min incubation with EGF, whereas the total Ras level was not altered (Fig. 1 B). This was accompanied by enhanced EGF-mediated formation of phospho-Erk (unpublished data). Taken together, these data indicate that the GFP-Ras fusion proteins are biologically active.

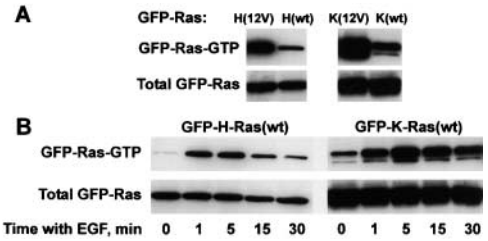

Figure 1. **Activation of GFP-Ras isoforms strongly enhances GTP binding.** The amounts of GFP-Ras-GTP were determined by the RBD assay (Herrmann et al., 1995). Total GFP-Ras levels were determined in parallel (Materials and methods). The data are representative of three independent experiments. (A) Constitutively active GFP-Ras isoforms preferentially bind GTP. GFP-H-Ras(12V) binds a high amount of GTP as compared with GFP-H-Ras(wt) in stably expressing Rat-1 cells. The total GFP-Ras levels are similar. Analogous results were obtained for GFP-K-Ras(12V) versus GFP-K-Ras(wt) transiently expressed in Cos-7 cells. A high level of GTP loading was also found for GFP-K-Ras(12V) stably expressed in Rat-1 cells (unpublished data). (B) Wild-type GFP-Ras isoforms receive upstream signals. GFP-H-Ras(wt) (in Rat-1 cells) or GFP-K-Ras(wt) (transiently expressed in Cos-7 cells) were stimulated by EGF (100 ng/ml, 37°C) for the times indicated and assayed for the levels of GFP-Ras-GTP.

All H-Ras and K-Ras isoforms stably associate with the plasma membrane

Ras proteins do not span the membrane and are tethered only to its cytoplasmic face. Thus, they can either diffuse laterally in the plasma membrane (stable association) or undergo dynamic exchange between membrane-bound and cytoplasmic pools (transient interactions with the membrane). We have demonstrated previously (Niv et al., 1999) that K-Ras(12V) diffuses laterally in the plasma membrane of Rat-1 cells without appreciable exchange. In view of the different membrane anchoring moieties of H-Ras and K-Ras and the possible dissimilarities between the membrane interactions of GDP- and GTP-bound Ras proteins, it was important to investigate the mode of interactions of the various Ras isoforms with the plasma membrane (stable vs. transient association). Lateral diffusion studies using FRAP can distinguish between these two modes of membrane interaction. The distinction is based on a "beam-size" test (Elson, 1985; Niv et al., 1999), where the area illuminated by the laser beam in the FRAP experiment is increased, and the effect of changing the beam size on the characteristic fluorescence recovery time τ (the time required to attain half of the recoverable

*Abbreviations used in this paper: GFP, green fluorescent protein; GST, glutathione *S*-transferase; RBD, Ras binding domain of Raf-1; wt, wild type.

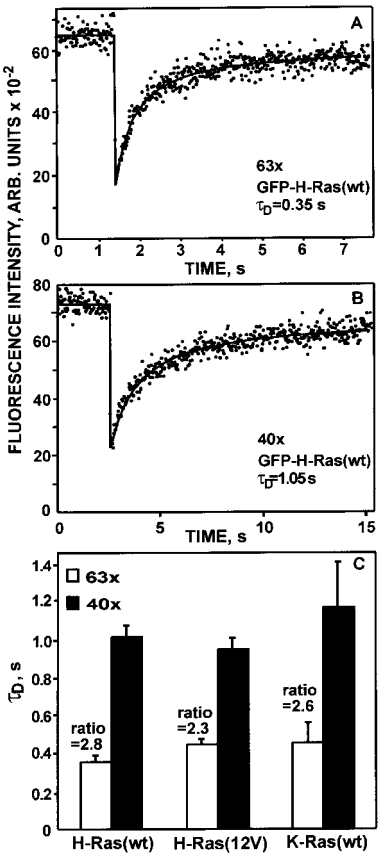

Figure 2. **The fluorescence recovery times of GFP-Ras isoforms are directly proportional to the laser beam size.** Two beam sizes were generated using a 63× microscope objective (Gaussian radius $\omega = 0.85$ μm, $\omega^2 = 0.72$ μm^2) or a 40× objective ($\omega = 1.36$ μm, $\omega^2 = 1.85$ μm^2). Typical FRAP curves obtained on Rat-1 cells stably expressing GFP-H-Ras(wt) are shown in A (63× objective) and B (40× objective); solid lines are the best fit to the lateral diffusion equation using nonlinear regression (Petersen et al., 1986). Similar curves were obtained for stably expressed GFP-H-Ras(12V) and for transiently expressed GFP-K-Ras in Rat-1 cells. (C) Average τ_D values obtained in these experiments. The experiments were conducted on cells expressing comparable levels of GFP-Ras isoforms (400–800 fluorescence intensity units). The ratios of the τ_D values between the 40 and 63× objectives were very close to the theoretical value expected for pure diffusion according to the ratio of the areas illuminated by the beam (proportional to the ratio of the ω^2 values, 1.85/0.72 = 2.56). Each bar is the mean ± SEM of 30–50 measurements. The mobile fractions were high throughout for all Ras proteins: 87–90% for GFP-H-Ras(wt) and GFP-H-Ras(12V), and 92–95% for GFP-K-Ras(12V).

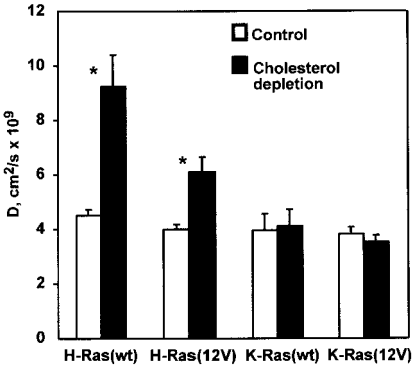

Figure 3. **Cholesterol depletion selectively increases the lateral diffusion rate of GFP-H-Ras(wt).** Rat-1 cells stably expressing GFP-tagged H-Ras(wt), H-Ras(12V), K-Ras(12V), or transiently expressing GFP-K-Ras(wt) were subjected to cholesterol depletion as described in Materials and methods or left untreated (control). FRAP experiments were conducted as in Fig. 2 (63× objective) on cells expressing comparable levels of GFP-Ras proteins (400–800 fluorescence intensity units). Because the fluorescence recovery of all the GFP-Ras isoforms measured here occurred by lateral diffusion (Fig. 2), the results were expressed in terms of the lateral diffusion coefficient *D*, which is a direct measure of the lateral diffusion rate. The bars are mean ± SEM of 40–80 measurements in each case. Asterisks indicate significant differences from control ($P < 0.001$, *t* test). The effects of cholesterol depletion on *D* of GFP-K-Ras(wt) and GFP-K-Ras(12V) were not significant ($P > 0.25$ and $P > 0.1$, respectively). Note that the small differences between the *D* values of the different GFP-Ras proteins before cholesterol depletion in this experiment become significantly higher at specific expression levels of the Ras proteins (see Fig. 5).

fluorescence intensity for a Gaussian bleach profile; Axelrod et al., 1976) is determined. The two modes of interaction predict highly different effects. For lateral diffusion, τ is the characteristic diffusion time τ_D, directly proportional to the illuminated area ($\tau_D = \omega^2/4D$, where ω is the Gaussian laser beam radius, and *D* is the lateral diffusion coefficient). For dynamic exchange with a cytoplasmic pool, τ reflects the chemical relaxation time, which is independent of the beam size (Elson, 1985; Niv et al., 1999). The results of these experiments (Fig. 2) clearly demonstrate that all the Ras isoforms examined (GFP-H-Ras(wt), GFP-H-Ras(12V), and GFP-K-Ras(wt)) diffuse laterally in the plasma membrane without a significant contribution of exchange to the fluorescence recovery. This is demonstrated by the fact that the τ values of the various Ras isoforms change along with the laser beam size by the same factor as the area illuminated by the beam.

Cholesterol depletion increases the lateral mobility of GFP-H-Ras(wt) but has little effect on the other Ras isoforms

Former studies based on cell-free systems or fixed cells reported localization of various Ras isoforms both to raft and nonraft domains (Mineo et al., 1996; Song et al., 1996; Furuchi and Anderson, 1998; Roy et al., 1999; Prior et al., 2001). Clearly, it is important to obtain evidence for differential interactions of Ras isoforms with raft domains in live cells. Therefore, we studied the effects of cholesterol depletion on the lateral mobility of the various GFP-tagged H- and K-Ras proteins in the plasma membrane. Cells were subjected to cholesterol depletion by incubation with compactin and mevalonate in the presence of lipoprotein-deficient serum (see Materials and methods), which reduced the membrane cholesterol content of the various Rat-1 cell lines by 32–35%. This treatment resulted in a twofold elevation in the lateral diffusion rate (directly proportional to *D*) of GFP-H-Ras(wt) (Fig. 3), which became equal to that of the freely diffusing lipid probe 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{16}$) on the same cell line ($D = (8.8 \pm 0.8) \times 10^{-9}$ cm^2/s, mobile fraction =

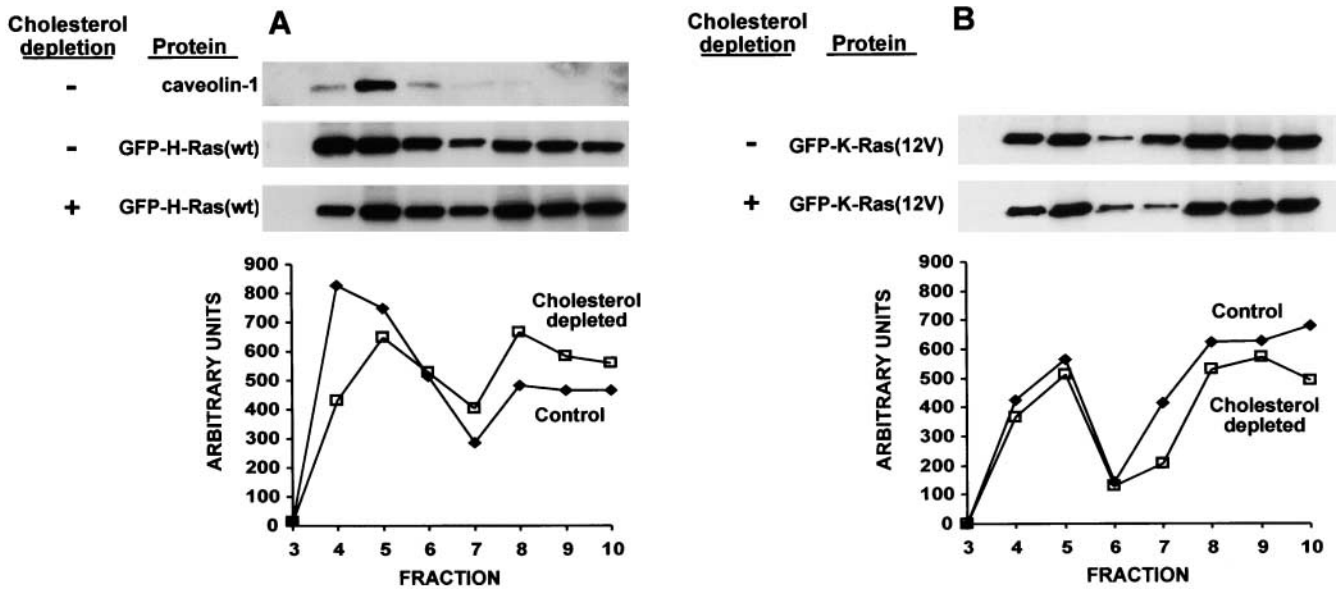

Figure 4. **Cholesterol depletion shifts GFP-H-Ras(wt) toward nonraft membrane fractions.** Rat-1 cells stably expressing GFP-H-Ras(wt) (A) or GFP-K-Ras(12V) (B) were depleted of cholesterol as in Fig. 3 or left untreated (control). The membrane pellet of the cells was fractionated using a nondetergent sucrose gradient flotation procedure (Song et al., 1996; Materials and methods). 1.2-ml fractions were collected from top (fraction 1) to bottom (fraction 10). In this protocol, lipid raft components are typically enriched along with caveolin-1 in fractions 4 and 5 (light membranes floating above the 35% sucrose and into the 5% sucrose region). After precipitation of the proteins by TCA, one-tenth of each fraction was taken for immunoblotting with anti-Ras (1:2,000) or with anti–caveolin-1 (1:1,000), and the GFP-Ras bands were quantified by densitometry.

88 ± 3%, $n = 35$). This probe distributes equally into both the external and internal leaflets of the plasma membrane (Schootemeijer et al., 1994). However, D of GFP-H-Ras(12V) exhibited a much smaller increase, and the D values of either GFP-K-Ras or GFP-K-Ras(12V) were essentially unaffected (Fig. 3). These results are in full agreement with those of previous biochemical studies that demonstrated predominant raft localization of H-Ras but not H-Ras(12V) or K-Ras (Roy et al., 1999; Prior et al., 2001). To obtain a biochemical correlate in our cells, we fractionated the membranes of Rat-1 cells stably expressing GFP-H-Ras(wt) or GFP-K-Ras(12V) using a nondetergent sucrose gradient flotation procedure (Song et al., 1996). This experiment (Fig. 4) demonstrated that cholesterol depletion shifted a significant portion of GFP-H-Ras(wt), but not GFP-K-Ras(12V), from the light membrane fractions (rafts) to nonraft fractions (Fig. 4). H-Ras(12V) yielded results similar to those obtained with K-Ras(12V) (unpublished data). We conclude that interactions with cholesterol-dependent lipid rafts specifically restrict the lateral diffusion of GDP-bound H-Ras in the plasma membrane of live cells.

The lateral mobilities of K-Ras(12V) and H-Ras(12V), but not H-Ras(wt), depend on their expression levels

The above experiments (Fig. 3) demonstrate that after cholesterol depletion, the lateral diffusion rate of GFP-H-Ras(wt) is significantly higher than that of GFP-H-Ras(12V) or the GFP-tagged K-Ras isoforms. This raises the possibility that the lateral diffusion of the latter proteins (H-Ras(12V) and the K-Ras isoforms) is restricted by interactions with raft-independent entities. This possibility is supported by the small differences between the lateral diffusion rates of GFP-H-Ras(wt) and the other nonraft GFP-Ras isoforms at the expression levels used in Fig. 3, suggesting that the lateral diffusion of the latter isoforms is also slowed down, albeit by interactions with other sites or domains. Such interactions can be with nonraft domains or protein sites. Because the pool of such sites is likely limited, we hypothesized that they may become limiting at high expression levels of these Ras isoforms. Thus, differences in the lateral diffusion rates of distinct Ras isoforms due to interactions with such sites would be evident at lower expression levels, but fade at higher expression levels where the great majority of the Ras protein is outside of these sites or domains. To test this hypothesis, we measured the lateral diffusion of stably expressed GFP-K-Ras(12V), GFP-H-Ras(12V), and GFP-H-Ras(wt) as a function of their surface expression levels. These levels are directly proportional to the fluorescence intensity before the bleach point in the FRAP experiment, thus enabling us to correlate the lateral diffusion rate (D) with the level of the GFP-tagged protein in the plasma membrane. The results of these experiments (Fig. 5) demonstrate that the D values of either GFP-K-Ras(12V) or GFP-H-Ras(12V) increase along with the expression level, until a saturation value is reached. In contrast, the D value of GFP-H-Ras(wt) was independent of its surface expression level (Fig. 5 A). The finding of a saturable dependence of the diffusion rate on the surface expression level for Ras isoforms that do not partition preferentially into rafts, but not for the raft-enriched H-Ras(wt) (Figs. 3 and 4), suggests that they arise due to interactions with saturable nonraft sites.

H-Ras–membrane interactions depend on its activation state

Fig. 5 shows that at relatively low surface expression levels (200–600 fluorescence intensity units), the lateral diffusion rate of GFP-H-Ras(wt) is faster than that of GFP-

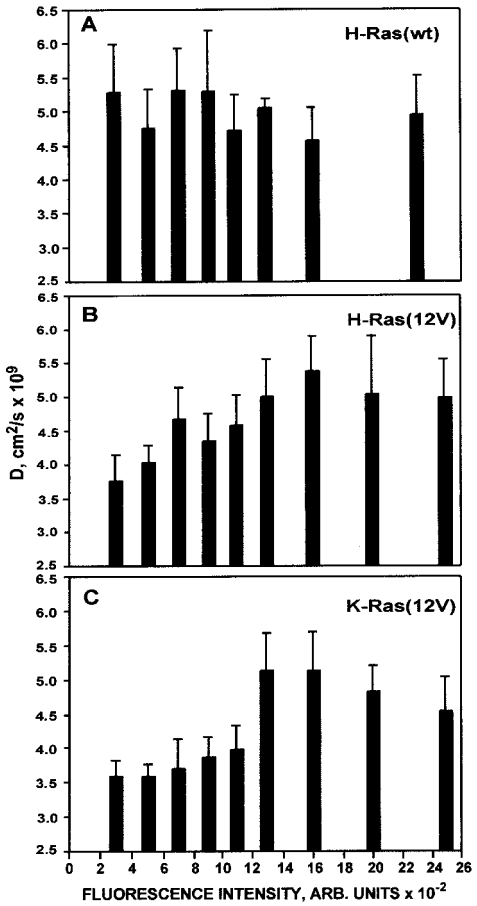

Figure 5. **The lateral diffusion rates of GFP-H-Ras(12V) and GFP-K-Ras(12V) increase with their expression levels in a saturable manner.** FRAP experiments were conducted on Rat-1 cells stably expressing GFP-tagged H-Ras(wt), H-Ras(12V), or K-Ras(12V) as described in Fig. 2 using the 63× objective. The surface expression levels of the GFP-tagged proteins are proportional to the fluorescence intensities given by the prebleach fluorescence levels (Fig. 2). The data were grouped according to the fluorescence intensities into specific intensity ranges (indicated value ± 100 units). Each bar is the mean ± SEM of 30–50 measurements. The mobile fractions were high throughout (87–95%). (A) GFP-H-Ras(wt); (B) GFP-H-Ras(12V); (C) GFP-K-Ras(12V).

H-Ras(12V). This difference could reflect different membrane interactions that depend on the activation state of H-Ras. To ensure that the different lateral mobility of GFP-H-Ras(12V) is not due to secondary alterations in the cells after long-term expression of the transforming H-Ras(12V) isoform, we performed transient expression studies. Rat-1 cells were transfected with GFP-H-Ras(12V) or GFP-H-Ras(wt), and their lateral mobilities were measured shortly after transfection (18–20 h after transfection) on cells showing similar expression levels. Fig. 6 shows that the difference between the activated (GTP-loaded) and nonactivated (GDP-loaded) H-Ras isoforms was retained, closely resembling the results obtained on the stably expressing Rat-1 cell lines. This experiment also circumvents possible effects due to down-regulation of caveolin-1 in cells stably expressing constitutively active Ras (Koleske et al., 1995; Gana-Weisz et al., 2002). It was recently shown (Prior et al., 2001) that at such short posttransfection periods, H-Ras(12V) does not significantly

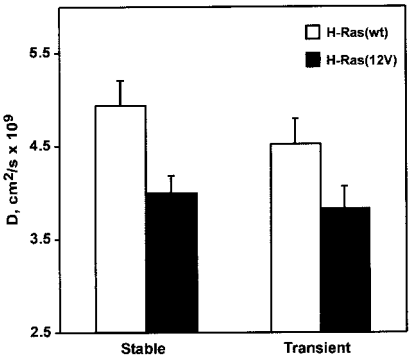

Figure 6. **Similar differences between the lateral mobilities of GFP-H-Ras(wt) and GFP-H-Ras(12V) in stably and transiently expressing cells.** FRAP experiments were conducted as in Fig. 2 using a 63× objective on cells with relatively low surface expression levels of GFP-H-Ras isoforms (200–600 fluorescence units; Fig. 5). Experiments were performed in parallel on stably expressing Rat-1 cell lines and on Rat-1 cells transiently expressing the GFP-H-Ras proteins (18–20 h after transfection). Bars are the mean ± SEM of 30–60 measurements. The mobile fractions were similar in all cases (87–90%). The differences between the D values of the two H-Ras isoforms were significant in both stably ($P < 0.001$) and transiently ($P < 0.005$) expressing cells.

alter the level of caveolin-1 or caveolae. To further validate this issue, we measured the effect of the MAP kinase kinase inhibitor PD 98059 on the lateral mobility of GFP-H-Ras(12V) stably expressed in Rat-1 cells. Treatment with this inhibitor was shown to counteract the down-regulation of caveolin-1 in H-Ras(12V)–transformed fibroblasts (Engelman et al., 1999). Fig. 7 demonstrates that although this treatment elevated the expression of caveolin-1, the GFP-H-Ras(12V) lateral diffusion rate was not affected. The lack of dependence of the GFP-H-Ras(12V) D value on the level of caveolin-1 is in line with its much lower sensitivity to cholesterol depletion as compared with GFP-H-Ras(wt) (Fig. 3). We conclude that the different lateral mobilities of GFP-H-Ras(wt) and GFP-H-Ras(12V) arise due to differences in their membrane interactions; although GFP-H-Ras(wt) interacts mainly with lipid rafts, the lateral mobility of GFP-H-Ras(12V) is restricted by saturable interactions with nonraft sites.

Discussion

The different membrane anchoring moieties of H-Ras and K-Ras may lead to different interactions with the plasma membrane, and such interactions can also depend on the activation state of specific Ras isoforms (Mineo et al., 1996; Song et al., 1996; Furuchi and Anderson, 1998; Prior et al., 2001). As signaling is mediated by activated Ras, whose localization for both H-Ras and K-Ras is outside lipid raft domains, the interactions of these activated Ras isoforms with nonraft sites is highly important. However, the data on such interactions are lacking, and they were not hitherto characterized in the membranes of live, intact cells. In the current study, we used FRAP to investigate these issues in live cells expressing GFP-tagged Ras isoforms. Our studies demonstrate that the membrane interactions of H- and K-Ras iso-

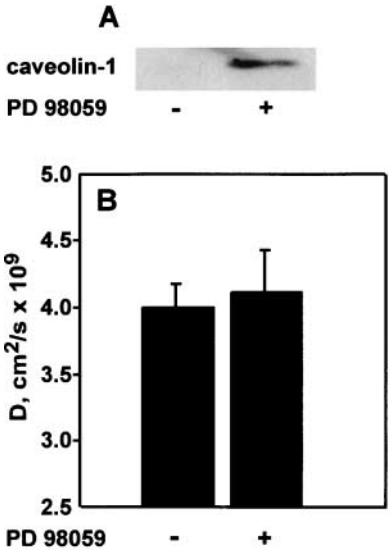

Figure 7. **Treatment with PD 98059 increases the level of caveolin-1 but does not affect the lateral mobility of GFP-H-Ras(12V).** Rat-1 cells stably expressing GFP-H-Ras(12V) were treated with 50 μM PD 98059 for 48 h as described previously (Engelman et al., 1999). They were subjected either to Western blotting (A) to determine caveolin-1, or to FRAP experiments to measure the lateral mobility of GFP-H-Ras(12V) (B). (A) Data are representative of three experiments. (B) Cells with GFP-Ras levels yielding 200–600 fluorescence units were selected for FRAP measurements that were conducted using the 63× objective as in Fig. 2. Bars are mean ± SEM of 30–40 measurements. The differences between the lateral diffusion rates of GFP-H-Ras(12V) before and after treatment with PD 98059 were not significant ($P > 0.25$).

forms vary, depending on both nonraft and raft interactions. Constitutively active GFP-H-Ras(12V) and GFP-K-Ras(12V) exhibit isoform-specific, concentration-dependent interactions with saturable nonraft sites or domains, whereas only GFP-H-Ras(wt) interacts significantly with cholesterol-dependent domains. The importance of these differences for Ras–membrane interactions is highlighted by their potential relevance to the selective activation of specific signaling pathways by different Ras isoforms (Yan et al., 1998; Coats et al., 1999; Booden et al., 2000; Walsh and Bar-Sagi, 2001; Jaumot et al., 2002).

After establishing the biological activities of the GFP-Ras fusion proteins (Fig. 1), we conducted FRAP studies on Rat-1 cells stably expressing these proteins to determine the mode of their interactions with the plasma membrane. FRAP experiments using different laser beam sizes provide a sensitive way to distinguish between lateral diffusion within the membrane and dynamic exchange between membrane-bound and cytoplasmic pools (Elson, 1985; Niv et al., 1999). Our results (Fig. 2) clearly demonstrate that the fluorescence recovery of GFP-tagged H-Ras(wt), H-Ras(12V), and K-Ras(wt) occurs by lateral diffusion and not by exchange, as evidenced by the similar change in their fluorescence recovery rates along with the change in the membrane area illuminated by the beam. Together with our former demonstration that GFP-K-Ras(12V) fluorescence recovery occurs by lateral diffusion (Niv et al., 1999), these findings indicate that all the Ras isoforms studied here stably associate with the plasma membrane. Therefore, their fluorescence recovery rates are determined by the properties of the membrane domains to which they localize and their interactions with membrane-associated sites or structures. Such interactions can occur with either raft or nonraft sites.

Thus far, experimental evidence was presented only for Ras interactions with raft microdomains or caveolae in which H-Ras(wt) (Song et al., 1996; Furuchi and Anderson, 1998; Prior et al., 2001) and in some reports also K-Ras (Furuchi and Anderson, 1998), was found to be preferentially localized. The current study not only provides the first evidence for H-Ras(wt) association with cholesterol-sensitive microdomains in live cells, but also demonstrates novel isoform- and activation-dependent interactions of Ras proteins with nonraft sites. Clearly, among the Ras isoforms studied here, only GFP-H-Ras(wt) experiences strong mobility-restricting interactions with cholesterol-dependent domains (Figs. 3 and 4). These conclusions agree with a recent report (Prior et al., 2001) that H-Ras(wt), but not H-Ras(12V) or K-Ras, is preferentially associated with rafts. The ability of interactions with rafts to retard the lateral mobility of GFP-H-Ras(wt) is also suggested by our finding that disruption of rafts by cholesterol depletion elevates its lateral diffusion rate to that of a freely diffusing lipid probe (Fig. 3). In view of the controversy regarding the identification of lipid rafts in live cells (Kenworthy and Edidin, 1998; Varma and Mayor, 1998), an alternative explanation to the results with GFP-H-Ras(wt) is that it interacts with nonraft sites or domains. However, these interactions must be cholesterol dependent, as they are disrupted after cholesterol depletion.

Our findings are in line with a study (Pralle et al., 2000) that used single particle tracking to show a cholesterol-dependent elevation in viscous drag (equivalent to reduced diffusion coefficient) for some glycosylphosphatidylinositol-anchored and transmembrane raft proteins. The extent of retardation was somewhat higher than that for GFP-H-Ras(wt) (2.5–5-fold vs. 2-fold). This may be because not all the H-Ras(wt) molecules are raft resident. Alternatively, the association of H-Ras(wt) with rafts may be transient, in which case the lateral diffusion of an H-Ras(wt) molecule would be retarded only during the raft association cycle. Under such conditions, all the GFP-H-Ras(wt) molecules would be retarded to the same degree because each would spend a fraction of the time bound to the slower-diffusing entity, undergoing free diffusion during the dissociation cycle. This predicts that the FRAP curves would fit a single component fluorescence recovery (single D), as is the case (Fig. 2). Interestingly, the interactions of GFP-H-Ras(wt) with rafts as reflected by its lateral diffusion are independent of its concentration (Fig. 5), which is in accordance with earlier studies on glycosylphosphatidylinositol-anchored folate receptors (Varma and Mayor, 1998), suggesting that the raft-resident proteins themselves are involved in creating the domains in which they organize.

In contrast to GFP-H-Ras(wt), the constitutively active GFP-H-Ras(12V) and GFP-K-Ras(12V) exhibit concentration-dependent interactions with saturable nonraft sites (Fig. 5). The detection of saturable nonraft interactions for GFP-H-Ras(12V), but not for GFP-H-Ras(wt), is in full agreement with a report (Prior et al., 2001) that GTP loading redistributes H-Ras from rafts, and that this release is required for effi-

cient activation of Raf. For the constitutively activated isoforms of H- and K-Ras, the lateral diffusion rate (*D*) increased with the expression level until a saturation value was reached (Fig. 5). This indicates that these proteins behave differently from transmembrane receptors such as the EGF receptors, whose *D* values were reduced at elevated surface densities (Benveniste et al., 1988). The elevation in *D* of GFP-H-Ras(12V) and GFP-K-Ras(12V) at high expression levels is in line with the notion that their lateral diffusion is restricted by interactions with a limiting population of saturable sites. At low expression levels, a significant portion of the activated Ras isoforms can be bound to the mobility-restricting sites. However, as their expression levels increase, they would saturate these sites, additional Ras molecules would not find unoccupied sites available for binding, and their fraction accommodated within these sites would become negligible. Interestingly, at saturating expression levels the *D* values of GFP-H-Ras(12V) reach the value measured for GFP-H-Ras(wt), which interacts with rafts. It can be argued that this may indicate that at these high levels they leak into raft domains. However, similar *D* values do not necessarily reflect presence in mutual complexes or domains, and may be coincidental. It should be noted that although the interacting sites for either H-Ras(12V) and K-Ras(12V) are distinct from rafts, they are most likely nonidentical, as suggested by the differences between the concentration dependencies of their *D* values (Fig. 5, B and C). This suggestion is in accord with the recent demonstration (Paz et al., 2001) that galectin-1 interacts preferentially with H-Ras(12V), enhancing its membrane association and facilitating signaling, but does not affect K-Ras(12V) membrane association. The different interactions of Ras isoforms can be due to and/or lead to association with different sets of signaling molecules, thus providing a mechanism for selective activation of certain signaling pathways by one Ras isoform but not by the other (Yan et al., 1998; Coats et al., 1999; Booden et al., 2000; Walsh and Bar-Sagi, 2001; Jaumot et al., 2002).

Materials and methods

Plasmids

GFP-K-Ras(12V) in pEGFP-C3 was described previously (Niv et al., 1999). GFP-K-Ras, GFP-H-Ras, and GFP-H-Ras(12V) were prepared similarly, by inserting the respective full-length cDNAs of the human Ras isoforms into the PstI-BamHI sites of pEGFP-C3 (CLONTECH Laboratories, Inc.), after the addition of suitable restriction sites by PCR. The constructs encode EGFP fused in frame to the 5′ end of the Ras isoforms. The sequence of all constructs was verified by sequencing.

Cell culture, antibodies, and reagents

All cell lines were maintained in DME with 10% FCS as described previously (Niv et al., 1999). Cell lines stably expressing GFP-H-Ras or GFP-H-Ras(12V) were generated by transfecting Rat-1 cells with the above vectors and selecting with G418 exactly as described previously (Niv et al., 1999) for the generation of Rat-1 lines expressing GFP-K-Ras(12V). From each line, two independent representative clones were selected for further analysis; in all cases, both yielded similar results. For experiments using transient expression, cells were transfected with Fugene 6 (Roche Chemicals). Mouse monoclonal pan-Ras antibody-3 (anti-Ras) was purchased from Calbiochem, and mouse anti–caveolin-1 (C37120) from Transduction Laboratories. Peroxidase-conjugated goat anti–mouse IgG was purchased from Sigma-Aldrich. The lipid analogue $DiIC_{16}$ was obtained from Molecular Probes and incorporated into the plasma membrane of live cells as described previously (Niv et al., 1999).

RBD assay for Ras-GTP

Rat-1 cells stably expressing GFP-Ras isoforms (4×10^6 cells in a 10-cm dish) were grown for 48 h and lysed in 1 ml lysis buffer as described previously (Niv et al., 1999). Ras-GTP was precipitated from 950 μl lysate by the GST-fused RBD of Raf-1 coupled to glutathione beads as described previously (Herrmann et al., 1995), whereas 30 μl of the lysate was taken for determination of total GFP-Ras. After SDS-PAGE, GFP-Ras (mol wt ~54,000) was identified by Western blotting using anti-Ras (1:2,000) followed by peroxidase goat anti–mouse IgG (1:7,500) and ECL. GFP-Ras-GTP in transiently expressing Cos-7 cells was assayed similarly, except that fewer cells were used; 3.6×10^5 cells in a 6-cm dish were lysed in 0.5 ml lysis buffer, 450 μl of which was taken for Ras-GTP precipitation and 30 μl for determination of the GFP-Ras level.

For EGF-stimulated GTP loading of GFP-Ras isoforms, cells were plated as described above for 24 h, serum-starved for another 24 h, and incubated with EGF (100 ng/ml, 37°C) for specified periods. This was followed by determination of Ras-GTP as described above.

Fluorescence recovery after photobleaching

Lateral diffusion coefficients (*D*) and mobile fractions were measured by FRAP (Axelrod et al., 1976; Koppel et al., 1976) using previously described instrumentation (Henis and Gutman, 1983). The experiments were performed on Rat-1 cells plated on glass coverslips as described previously (Niv et al., 1999). Studies on transiently expressing cells were conducted 18–20 h after transfection. All experiments were conducted at 22°C, in HBSS supplemented with 20 mM Hepes, pH 7.2, and 2% BSA. The monitoring Argon ion laser beam (488 nm, 1 μW) was focused through the microscope (ZEISS) to a Gaussian radius of 0.85 ± 0.02 μm (63× objective) or 1.36 ± 0.04 μm (40× objective). A brief pulse (5 mW, 7–10 ms for the 63× objective, and 10–15 ms for the 40× objective) bleached 50–70% of the fluorescence in the illuminated region. The time course of fluorescence recovery was followed by the attenuated monitoring beam. *D* and the mobile fraction were determined by nonlinear regression analysis, fitting to the lateral diffusion equation of a single species (single *D* value; Petersen et al., 1986).

Cholesterol depletion and sucrose gradients

Rat-1 cells stably expressing various GFP-Ras isoforms were incubated for 16 h with 50 μM compactin and 50 μM mevalonate in DME containing 10% lipoprotein-deficient serum following established procedures (Hua et al., 1996; Lin et al., 1998). For transient expression studies, cells were treated as described above 6 h after transfection. The cells were homogenized and the total membrane fractions were obtained by centrifugation (100,000 *g*, 30 min, 4°C) as described previously (Haklai et al., 1998). The cholesterol content in the membrane fraction was measured by the F-CHOL kit (Boehringer) and showed 32–35% reduction. The above treatment was preferred over treatment with methyl-β-cyclodextrin (Scheiffele et al., 1997) because the latter reduced the lateral diffusion of raft-resident proteins in a manner unrelated to cholesterol depletion. This was evident from the similar effect mediated by α-cyclodextrin, which does not deplete cholesterol (Rodal et al., 1999). For fractionation of raft and nonraft membrane fractions, we used the detergent-free sucrose gradient flotation procedure described earlier (Song et al., 1996). The total membrane pellet (derived from 12×10^6 cells) was resuspended and sonicated in 2 ml of 500 mM sodium carbonate, pH 11, combined with 2 ml of 90% sucrose in pH 6.5 MES-buffered saline (Song et al., 1996). This was overlaid by 4 ml of 35% sucrose in 250 mM sodium carbonate, pH 11, and another 4 ml of 5% sucrose in the same carbonate buffer. After 16-h centrifugation in an SW41 rotor (Beckman Coulter) at 39,000 rpm, ten 1.2-ml fractions were collected from the top of the gradient. Proteins were precipitated with TCA, resuspended in 100 μl of 0.25 M Tris, pH 8.5, and 10 μl was taken for SDS-PAGE followed by Western blotting with anti-Ras followed by ECL, as detailed for the RBD assay. Densitometry was performed with Image Master VDS-CL (Amersham Pharmacia Biotech) using TINA 2.0 software (Ray Test).

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