Membrane Interactions of a Constitutively Active GFP-Ki-Ras 4B and Their Role in Signaling

EVIDENCE FROM LATERAL MOBILITY STUDIES*

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Membrane anchorage of Ras proteins in the inner leaflet of the plasma membrane is an important factor in their signaling and oncogenic potential. Despite these important roles, the precise mode of Ras-membrane interactions is not yet understood. It is especially important to characterize these interactions at the surface of intact cells. To investigate Ras-membrane interactions in live cells, we employed studies on the lateral mobility of a constitutively active Ras isoform to characterize its membrane dynamics, and examined the effects of the Ras-displacing antagonist S\textsuperscript{-trans, trans-farnesylthiosalicylic acid} (FTS) (Haklai, R., Gana-Weisz, M., Elad, G., Paz, A., Marciano, D., Egozi, Y., Ben-Baruch, G., and Kloog, Y. (1998) Biochemistry 37, 1306–1314) on these parameters. A green fluorescent protein (GFP) was fused to the N terminus of constitutively active Ki-Ras 4B(12V) to generate GFP-Ki-Ras(12V). When stably expressed in Rat-1 cells, this protein was preferentially localized to the plasma membrane and displayed transforming activity. The lateral mobility studies demonstrated that GFP-Ki-Ras(12V) undergoes fast lateral diffusion at the plasma membrane, rather than exchange between membrane-bound and unbound states. Treatment of the cells with FTS had a biphasic effect on GFP-Ki-Ras(12V) lateral mobility. At the initial phase, the lateral diffusion rate of GFP-Ki-Ras(12V) was elevated, suggesting that it is released from some constraints on its lateral mobility. This was followed by dislodgment of the protein into the cytoplasm, and a reduction in the diffusion rate of the fraction of GFP-Ki-Ras(12V) that remained associated with the plasma membrane. Control experiments with other S\textsuperscript{-prenyl anologs} showed that these effects are specific for FTS. These results have implications for the interactions of Ki-Ras with specific membrane anchorage domains or sites.

The small G-proteins of the Ras family are essential components of signaling cascades that regulate important cell functions such as growth and differentiation (1–4). Wild-type Ras isoforms alternate between inactive (Ras-GDP) and active (Ras-GTP) states (5, 6). Mutations at positions 12, 13, or 61 result in constitutively active Ras isoforms; these mutants bind GTP, have transforming activity, and contribute to controlled cell growth (7, 8). The function of Ras proteins as signal transduction regulators and their oncogenic potential require association with the inner leaflet of the plasma membrane (2, 3, 9). Membrane anchorage of Ras proteins is promoted by their C-terminal S-farnesylysteine and by either a stretch of lysines (Ki-Ras 4B) or by S-palmitoyl moieties (Ha- and N-Ras, or Ki-Ras 4A) (10–13). The anchoring moieties of Ras proteins also appear to target them to the plasma membrane (2), possibly to specific membrane domains (14, 15).

Although the essential role of membrane tethering in Ras signaling and transforming activity is well established, the precise mode of Ras-membrane interactions is not yet understood. It is not clear whether Ras proteins are stably associated with the plasma membrane or undergo rapid exchange between membrane-bound and unbound states (16), whether they form tight complexes with putative membrane receptors (17), and whether the Ras anchoring moieties (e.g. farnesylysteine) interact randomly with the membrane lipid milieu or associate preferentially with distinct domains or sites. A possible role for specific membrane domains is implied by the evidence that Ha-Ras is enriched in low buoyant density fractions typical of caveolae or analogous glycosphingolipid/cholesterol-enriched domains (14, 15).

We have recently developed compounds resembling the farnesylysteine of Ras proteins (18–20). One of these compounds, S\textsuperscript{-trans, trans-farnesylthiosalicylic acid} (FTS),¹ inhibited the growth of cells transformed by Ha-Ras; the inhibition is not further downstream, since growth cells transformed by constitutively active v-Raf was not affected (18, 19). FTS specifically dislodged farnesylated Ha-Ras from membranes of Rat-1 cells, but not non-farnesylated N-myristoylated Ras or prenylated G\textsuperscript{b\gamma} of heterotrimeric G-proteins (21), suggesting that it disrupts the interactions of Ras with specific anchoring domains. Since FTS appears to affect directly membrane-bound Ras, it can serve as a tool to investigate Ras-membrane interactions, and has a potential therapeutic value.

Early studies on Ras-membrane interactions either employed cell-free systems or involved cell or tissue fixation. Obviously, it is important to characterize these interactions at the surface of intact cells. To investigate Ras-membrane interactions in live cells, we applied in the current work fluorescence

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¹ The abbreviations used are: FTS, S\textsuperscript{-trans, trans-farnesylthiosalicylic acid}; AFC, N-acetyl-S\textsuperscript{-trans,trans-farnesyl-L-cysteine}; BSA, bovine serum albumin; D, lateral diffusion coefficient; DiIC\textsubscript{18}, 1,1\textsuperscript{-dihexadecyl-3,3\textsuperscript{-diacetoxy} cyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FPR, fluorescence photobleaching recovery; GFP, green fluorescent protein; GFP-Ki-Ras(12V), GFP-tagged constitutively active Ki-Ras 4B(12V); GTP, guanosine triphosphate; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; R\textsubscript{pp}, octadecyl rhodamine B chloride; R\textsubscript{pp}, mobile fraction.
photobleaching recovery (FPR) studies on the lateral mobility of green fluorescent protein (GFP)-tagged constitutively active Ki-Ras 4B(12V) (GFP-Ki-Ras(12V)) protein expressed in Rat-1 cells. In combination with experiments on the effects of the Ras antagonist FTS on Ras membrane anchorage, our results indicate that GFP-Ki-Ras(12V) undergoes fast lateral diffusion at the plasma membrane, rather than exchange between membrane-bound and unbound states. FTS was capable of releasing GFP-Ki-Ras(12V) from some constraints on its mobility during the early phase of FTS treatment, prior to the dislodgment of Ras from the plasma membrane. These results have implications for the nature of the interactions of Ki-Ras with specific membrane anchorage domains or sites.

**EXPERIMENTAL PROCEDURES**

**Materials**—N-Acetyl-S-propargylglycine (APG), S-geranylthioalicylic acid (GTA) and FTS and were prepared and purified as detailed elsewhere (18, 22). 1,1′-Dihexadecyl-3,3,3′,3′-tetratetramethyleneocarbocyanine perchlorate (DiI(+) and octadecyl rhodamine B chloride (R18) were obtained from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA), fatty acid-free BSA, Hank’s balanced salt solution (HBSS), cytochalasin D, peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, and protein A-Sepharose (Sigma) were from Sigma. Mouse monoclonal pan-Ras antibody (anti-Ras) was purchased from Calbiochem, and anti-GFP rabbit polyclonal IgG was from CLONTECH.

**Construction of GFP-Ki-Ras(12V) Chimera**—The entire coding region of human Ki-Ras 4B(12V) cDNA cloned in pBluescript II SK the PstI and BamHI sites of the multiple cloning region was a gift from P. Gierschik (University of Ulm, Ulm, Germany). The insertion was performed by the use of polymerase chain reaction to generate flanking sequences on the 5′ (CTGGGAGCAT, containing a PstI site) and on the 3′ (GGATCC, containing a BamHI site) ends of the Ki-Ras(12V) coding region. It was then excised by the same restriction enzymes, and inserted into PstI/BamHI-digested pEGFP-C3 (CLONTECH), resulting in a chimeric construct of enhanced GFP (a red-shifted enhanced GFP variant) fused in frame to the 5′ end of Ki-Ras(12V) coding regions in the final construct were verified by DNA sequencing.

**Generation of Cell Lines and Cell Culture Procedures**—Cells were routinely grown at 37 °C, 5% CO2, and 100% humidity, in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS; Iatron Labs, Tokyo, Japan). DNA was electrotransfected into the cells using 4 μg of DNA per 1 × 107 cells in a 35-mm dish as described previously (21), using 2,500 μg/ml polybrene. After 48 h, the cells were harvested and counted on day 4 of the treatment.

**Detection of Guanine Nucleotide Binding (see below)**, COS-7 cells were transiently transfected by the DEAE-dextran method (23) with 4 μg of the GFP-Ki-Ras(12V) construct per 1 × 106 cells in a 10-cm dish.

**Detection of Guanine Nucleotide Binding to GFP-Ki-Ras(12V)**—We have basically employed a protocol described previously (24, 25). COS-7 cells were transfected as described above. Eighteen hours after transfection, the medium was replaced by serum-free DMEM, and incubation was continued for 18 h. After washing with DMEM devoid of serum and phosphate, the cells were incubated 4 h at 37 °C with 4 μl of the same medium supplemented with 0.5 mM of carrier-free [32P]orthophosphate (Amersham). The cells were washed three times with ice-cold phosphate-buffered saline (PBS), and lysed (15 min, 4 °C) with 0.5 ml of lysis buffer [50 mM Tris-HCl, pH 7.6, 20 mM MgCl2, 150 mM NaCl, 0.5% Nonidet P-40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol]. After removal of insoluble material (14,000 rpm, 10-min spin in Eppendorf centrifuge), free nucleotides were removed by 1.4% charcoal (24). From each sample, volumes containing equal amounts of radioactivity (5 × 107 cpm) were taken for immunoprecipitation, performed after completing the volume to 500 μl of lysis buffer, 1% BSA, 2 μg/ml anti-GFP. Following incubation for 2 h at 4 °C, 40 μl of protein A-Sepharose (120 μg of protein A) were added, and incubated for 1 h at 4 °C. The beads were washed twice in lysis buffer and once in PBS. The pellets were suspended in 20 μl of 20 mM Tris-HCl, pH 7.6, containing 20 mM EDTA, 2% SDS, 0.5 mM GTP, and 0.5 mM GTP. The suspension was then sonicated (45 sec, 5 min) and centrifuged, and samples (11 μl) were spotted onto a polyethyleneimine cellulose TLC plate (Sigma), which was developed in 0.75 mM KH2PO4, pH 3.4. The TLC plates were analyzed by a phosphorimager (Fuji X BAS1000), and the spots corresponding to guanine nucleotides were quantified using TINA 2.0 software by Ray Test (Staubenhardt, Germany).

**Western Immunoblotting**—Western blotting and ECL were performed as described by us previously (21). Rat-1 cells expressing GFP-Ki-Ras(12V) or GFP were plated at a density of 1 × 105/10-cm dish. After FTS treatment as described above, they were homogenized, and the cytosolic (S100) and total membrane (T100) fractions were obtained by centrifugation (100,000 × g, 30 min, 4 °C) (21). Samples of the total homogenate and of the S100 and P100 fractions were calibrated for similar protein content, and subjected to SDS-polyacrylamide (12.5%) gel electrophoresis followed by electrophoretic blotting onto nitrocellulose filters. The filters were blocked and incubated with antibodies as detailed previously (21), using either mouse anti-Ras (1:2000) or rabbit anti-GFP (1:1000) followed by peroxidase-antigoat anti-mouse (1:7500) or anti-rabbit (1:5000) IgG, respectively. Bands were visualized by ECL and quantified by densitometry on a Bioimaging System 2020 (Dynicon, Jerusalem, Israel), using TINA 2.0 software (Ray Test).

**Labeling of Cells with DiIC16 and R18**—The lipophilic indocarbocyanine lipid analog DiIC16, which distributes equally into both the extranul and internal leaflets of the plasma membrane in cells (26), was incorporated into the plasma membrane of Rat-1 cells expressing GFP-Ki-Ras(12V) following the procedure of Edidin and Stroynowski (27) with minor modifications. The cells were plated on glass coverslips placed in 35-mm dishes at 10,000 cells/dish. A stock solution of DiIC16 (100 μg/ml) was prepared in ethanol, and diluted prior to the experiment to 0.5 μg/ml in HBSS containing 20 mM HEPES, pH 7.4 (HBSS/HEPES). The cells were incubated with this labeling solution 10 min at 37 °C. After washing with HBSS/HEPES supplemented with 0.1 μg/ml fatty acid-free BSA to remove free dye, and immersed in HBSS/HEPES containing BSA (not fatty acid-free; HBSS/HEPES/BSA) and 0.1% Me2SO (same conditions as for FTS treatment) for FPR studies. In experiments where the cells were treated with FTS for 30 min or 24 h, they were washed with HBSS/HEPES prior to labeling, and FTS was added back at the end of the labeling procedure, other than that the DiIC16 labeling was identical to that employed for untreated cells.

**Fluorescence Photobleaching Recovery**—Lateral diffusion coefficients (D) and mobile fractions (RF) were measured by FPR (30, 31) using previously described instrumentation (32). Cells were plated on glass coverslips at 10,000 cells/coverslip; they were treated where indicated with FTS or other drugs and/or labeled with DiIC16 as described above. Nonfluorescent 5 μm coverslips were placed over a serological slide, with a depression filled with HBSS/HEPES/BSA and containing 0.1% Me2SO with or without FTS. The monitoring laser beam (Coherent Innova 70 argon ion laser; 488 nm and 1 microwatt for GFP fluorescence or 529.5 nm and 1 microwatt for DiIC16) was focused through the microscope (Zeiss Universal) to a Gaussian radius of 0.61 μm using 100× oil immersion objective. In some cases, the beam size was...
changed to 1.23 ± 0.04 μm by using 40× water immersion objective. The beam radius was determined as described previously (33, 34). The use of a pinhole in the image plane in the photometer head in front of the photomultiplier makes the light collection confocal, enabling the collection of fluorescence from a narrow depth in the focal plane (31), focusing either on the plasma membrane or in the cytoplasm. A brief pulse (5 milliwatts, 30–40 ms for the 100× 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 R were extracted from the fluorescence recovery curve by nonlinear regression analysis (35). Incomplete fluorescence recovery is interpreted to represent fluorescence-labeled molecules immobile on the FPR experimental time scale (D ≤ 5 × 10−12 cm²/s). All the FPR measurements were conducted at 22 °C.

**RESULTS**

**Expression and Transforming Activity of GFP-Ki-Ras(12V)**—In order to investigate the interactions of Ki-Ras with the plasma membrane of intact cells, we have generated a GFP-tagged constitutively active Ki-Ras 4B (GFP-Ki-Ras(12V)) and stably expressed it in Rat-1 cells (see “ Experimental Procedures”). The orientation of the construct was chosen to retain an intact Ki-Ras C-terminal domain, which is required for post-translational modification and membrane anchorage. The GFP-Ki-Ras(12V) was properly synthesized in the cells, as evidenced by Western blotting using either anti-Ras or anti-GFP antibodies (Fig. 1A). Using anti-Ras, both GFP-Ki-Ras(12V) (54 kDa) and endogenous Ras (21 kDa) were detected, and only the latter was observed in homogenates prepared from cells expressing GFP alone. Upon labeling with anti-GFP, only the 54-kDa band was detected in cells expressing GFP-Ki-Ras(12V), and a 34-kDa band was observed in GFP-expressing Rat-1 cells. No labeling was detected in untransfected Rat-1 cells. The apparent molecular weight of GFP-Ki-Ras(12V) fitted that expected for a GFP-Ras fusion protein, and no smaller fragments were detected. To validate the constitutively active nature of GFP-Ki-Ras(12V), we employed a guanine nucleotide binding assay in intact cells. In this assay, we employed anti-GFP to immunoprecipitate GFP-Ki-Ras(12V), in order to ensure that the binding measured is to the fusion protein and not to endogenous Ras. This experiment (Fig. 1B), performed in transiently transfected COS-7 cells, demonstrated preferential binding of GTP over GDP to GFP-Ki-Ras(12V) (80% versus 20%, respectively), as expected for a constitutively active Ras isofrom (7, 8).

The expression of GFP-Ki-Ras(12V) in the Rat-1 clones was further validated by confocal immunofluorescence microscopy. The fluorescent Ras isoform localized preferentially to the plasma membrane, as opposed to GFP expressed in Rat-1 cells, which distributed mainly to the cytoplasm and the nucleus (Fig. 2, A and B; see also Fig. 3). This indicates that the association of GFP-Ki-Ras(12V) with the plasma membrane is mediated by the Ras protein in this construct and not by the GFP. Importantly, GFP-Ki-Ras(12V) is biologically active, as evidenced by its transforming activity. Thus, the GFP-Ki-Ras(12V)-expressing Rat-1 cell lines exhibited anchorage-independent growth in soft agar (Fig. 2, C and D). Furthermore, these cells were able to develop tumors in nude mice at a rate similar to that of cells expressing a constitutively active Ras (5 out of 5 mice in both cases).

**FTS Mediates Dislodgment of GFP-Ki-Ras(12V) to the Cytoplasm and Inhibits Cell Growth**—We have recently demonstrated that FTS, a compound resembling farnesylcysteine, specifically dislodges farnesylated Ha-Ras(12V) from membranes of Rat-1 cells (21). To examine the effect of FTS on GFP-Ki-Ras(12V), Rat-1 cells stably expressing the fusion protein were subjected to FTS treatment and to analysis by confocal fluorescence microscopy. The results of a typical experiment are depicted in Fig. 3. Prior to FTS treatment, GFP-Ki-Ras(12V) was localized mainly at the rim of the cells, exhibiting typical plasma membrane labeling and co-localization with R18, a fluorescent membrane marker (Fig. 3, panels A–C; see also the insets for optical scanning along the z axis). Following FTS treatment (50 μM, 24–48 h), a significant amount of GFP-Ki-Ras(12V) was dislodged from the membrane into the cyto-
plasm; the dislodgment was not complete, leaving some GFP-Ki-Ras(12V) at the plasma membrane (Fig. 3, panels D–F; see insets for z-scan). As expected, R18 remained at the plasma membrane.

The dislodgment of GFP-Ki-Ras(12V) from the membrane was also followed biochemically, quantifying GFP-Ki-Ras(12V) in the membrane pellet and in the cytosolic fractions by immunoblotting with anti-GFP antibodies. Incubation of Rat-1 cells expressing GFP-Ki-Ras(12V) with 50 μM FTS for 24 h mediated a reduction in the relative amount of the protein associated with the membranes and a parallel increase in its cytosolic fraction, although a significant fraction remained in the membrane pellet (Fig. 4). This suggests that GFP-Ki-Ras(12V) is dislodged by FTS from the membrane and accumulates in the cytoplasm, in accord with the confocal microscopy results (Fig. 3). This differs from our former observations on the fate of Ha-Ras(12V), which was degraded after the dislodgment (21), suggesting that the GFP-Ki-Ras(12V) is more stable. Interestingly, incubation with FTS for periods up to 4 h did not reduce the level of GFP-Ki-Ras(12V) associated with the membrane (Fig. 4), suggesting that relatively long incubation times (>4 h) are required to dislodge GFP-Ki-Ras(12V) from the membrane.

We have formerly demonstrated that FTS mediates dislodgment followed by degradation of Ha-Ras(12V) (21) and Ki-Ras

FIG. 2. Fluorescence microscopy and transforming activity of Rat-1 cells expressing GFP-Ki-Ras(12V). Panels A and B depict confocal fluorescent images of Rat-1 cells stably expressing GFP-Ki-Ras(12V) (A) or GFP (B). In A, stronger fluorescence at the rim is observed, typical of plasma membrane labeling, while, in B, cytoplasmic and nuclear labeling are observed. The cells were grown on glass coverslips and fixed with paraformaldehyde as detailed under “Experimental Procedures.” Images were collected on a laser-scanning confocal microscope (Zeiss model LSM 410) fitted with fluorescein filters. Bar, 10 μm. Panels C and D show colony formation of Rat-1 cells stably expressing GFP-Ki-Ras(12V) in soft agar. The cells (in DMEM containing 10% FCS) were mixed with 0.5 ml of 0.33% Noble agar, and poured (10^4 cells/30-mm dish) onto a layer of 1.5 ml of 0.5% Noble agar in the same medium. The upper layer was covered with 0.25 ml of medium, and placed for 11 days in a CO2 incubator. The dishes were photographed using an inverted microscope (Olympus IX70). C and D are fluorescent (using fluorescein filters) and phase contrast images of the same field, respectively. Bar, 50 μm.

FIG. 3. Confocal microscopy demonstrates a shift of GFP-Ki-Ras(12V) from the plasma membrane to the cytoplasm following FTS treatment. Rat-1 cells stably expressing GFP-Ki-Ras(12V) were plated on glass coverslips, treated with 50 μM FTS for 48 h, and then labeled with R18 as described under “Experimental Procedures.” After fixation with paraformaldehyde, dual images (green fluorescence for GFP, red for R18, and yellow where the two dyes coincide) were collected on the LSM 410 confocal microscope fitted with fluorescein and rhodamine filters. The images were exported in TIFF format to Adobe Photoshop and printed. Bar, 10 μm. The insets in each panel depict a z-scan analysis of the same cell (bar, 20 μm). A and D, GFP (green) fluorescence; B and E, R18 (red) fluorescence; C, superposition of the images in panels A and B; F, superposition of the images in panels D and E. A–C, no FTS treatment (48-h incubation in medium with 0.1% Me2SO). D–F, FTS-treated cells.
4B(12V)

4B(12V) in fibroblasts transformed by these constitutively active Ras proteins. The above phenomena have biological consequences, as evidenced by the ability of FTS to inhibit the growth of these cells (18, 19). Fig. 5 demonstrates that FTS concentrations (10–50 μM), similar to those that dislodge GFP-Ki-Ras(12V) from the membrane, inhibit the growth of Rat-1 cells transformed by the constitutively active fusion protein. This inhibition depends on the structure of FTS, specifically on the length of its farnesyl moiety and its rigid backbone (18, 20), as indicated by the failure of GTS (10-carbon farnesyl chain versus 15-carbon in FTS) and AFC (15-carbon farnesyl chain but no rigid backbone) to inhibit the growth of the transformed cells (Fig. 5). Interestingly, the growth inhibitory response of FTS occurs despite the absence of accelerated degradation of GFP-Ki-Ras(12V), which accumulates in the cytoplasm (Figs. 3 and 4). This suggests that dislodgement, rather than accelerated degradation, of GFP-Ki-Ras(12V) from the membrane is responsible for the FTS growth-inhibitory effect.

GFP-Ki-Ras(12V) Diffuses Laterally in the Inner Leaflet of the Plasma Membrane—Ras proteins do not span the membrane, and are anchored to the cytoplasmic face of the plasma membrane via their farnesyl group and additional moieties. Therefore, they can either show continuous association with the inner lipid leaflet or rapid dynamic exchange between membrane-bound and unbound states. To differentiate between these possibilities, we conducted FPR experiments on GFP-Ki-Ras(12V) expressed in Rat-1 cells, employing laser beams of different sizes. In these experiments, fluorescence recovery after photobleaching may occur either as a result of lateral diffusion of GFP-Ki-Ras(12V) in the plasma membrane (in the case of continuous association), or due to exchange between membrane-bound and cytoplasmic GFP-Ki-Ras(12V) (dynamic exchange). It has been shown (34, 36, 37) that these two mechanisms lead to highly different dependence on the laser beam size in the FPR experiment. The characteristic fluorescence recovery time \( \tau \) (the time required to attain half of the recoverable fluorescence intensity in the case of a Gaussian bleach profile; see Ref. 30) reflects different mechanisms in each case. For lateral diffusion (continuous association), \( \tau \) is essentially the characteristic diffusion time \( \tau_D \), and is proportional to the area illuminated by the beam (\( \tau_D = \omega^2/4D \), where \( \omega \) is the Gaussian radius of the laser beam, and \( D \) is the lateral diffusion coefficient). In the case of dynamic exchange between membrane-bound and cytosolic forms, \( \tau \) reflects the chemical relaxation time due to exchange, which is equal on all surface regions regardless of whether they are illuminated by the beam, and therefore does not depend on the beam size (34, 36, 37).

Typical FPR curves obtained with two different laser beam sizes are shown in Fig. 6. The \( \tau \) values clearly depended on the beam size, and the dependence was proportional to the area illuminated by the beam (increasing \( \omega^2 \) by a factor of 4 resulted in a parallel increase of \( \tau \) by a factor of 3.7 ± 0.5; \( n = 30 \) for each beam size). This clearly demonstrates that GFP-Ki-Ras(12V) diffuses laterally in the plasma membrane, and that the contribution of dynamic exchange to the fluorescence recovery is negligible. Thus, GFP-Ki-Ras(12V) is continuously associated with the plasma membrane, and does not undergo appreciable exchange on the time scale of the FPR measurement (up to 15 s).

The lateral diffusion of GFP-Ki-Ras(12V) (Figs. 6 and 7) is characterized by \( D = (1.9 ± 0.14) \times 10^{-9} \) cm\(^2\)/s and a high mobile fraction (\( R_p = 0.94 ± 0.01 \)). It is significantly faster than the lateral mobility of transmembrane proteins or even of glycosylphosphatidylinositol (GPI)-anchored membrane proteins (27, 38–41). This mobility is slightly but significantly slower than that of the lipid probe DiIC\(_{16}\) in the GFP-Ki-Ras(12V)-expressing Rat-1 cells (\( D = (3.3 ± 0.37) \times 10^{-9} \) cm\(^2\)/s, \( R_p = 0.84 ± 0.04, n = 29, 22 \) °C). The lateral diffusion rate of GFP-Ki-Ras(12V) is in the range expected for a lipid-anchored protein; a soluble cytoplasmic protein diffuses at a much faster rate, as we observed for GFP expressed in Rat-1 cells, whose fluorescence recovered on a much faster time scale. The extremely fast recovery (\( \tau \) around 0.015 s) prevented an accurate determination of the diffusion rate of free GFP, but suggests a lower limit for \( D \) (\( D ≥ 4 \times 10^{-7} \) cm\(^2\)/s).

**FTS Induces a Transient Increase in the Lateral Diffusion**

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\(^{2}\) G. Elad, A. Paz, R. Haklai, D. Marciano, and Y. Klog, unpublished observations.
Mobility and Signaling of GFP-Ki-Ras(V12)

Rate of GFP-Ki-Ras(12V)—To examine the effects of FTS on the interactions of GFP-Ki-Ras(12V) with the plasma membrane, we studied the lateral diffusion of GFP-Ki-Ras(12V) in the plasma membrane of Rat-1 cells as a function of the time of incubation with 50 μM FTS (Fig. 6). FTS induced a rapid (within 30 min) and transient (lasting 2–4 h) increase in the lateral diffusion rate (D) of GFP-Ki-Ras(12V) without affecting its high mobile fraction. This increase occurs prior to the dislodgment of GFP-Ki-Ras(12V) to the cytoplasm (see Figs. 4 and 7). At longer incubations (6–48 h), D of GFP-Ki-Ras(12V) was reduced to about 30% below the value measured prior to FTS treatment, with no effect on RF (Fig. 7A). The effects of FTS on the lateral mobility of GFP-Ki-Ras(12V) are not due to changes in the dynamic properties of the bulk membrane lipids, since neither the D nor the RF values of the DiIC16 lipid probe were altered by FTS (Fig. 7B). It should be noted that the 30-min FTS treatment raised the D value of GFP-Ki-Ras(12V) to the same value measured for DiIC16 in accord with the notion that GFP-Ki-Ras(12V) is released from some mobility constraints altered by FTS. However, FTS treatment raised the RF value of GFP-Ki-Ras(12V) to the same value measured for DiIC16, in accord with the notion that the differences between the D values measured on FTS-treated cells as compared with untreated cells (0° incubation time) were significant (p < 0.001 for all incubation times except the 6- and 48-h time points, where p < 0.005 was obtained. B, DiIC16 mobility. The mobile fractions were similar in all cases (RF = 0.84 ± 0.04). The use of Student’s t test indicated that the differences between the D values on FTS-treated as compared with untreated cells were not significant (p > 0.25 in all cases).

Fig. 7. Time-dependent alterations in the lateral mobility of GFP-Ki-Ras(12V) by FTS. The experiments were conducted as described under “Experimental Procedures” and in Fig. 6, using 100X objective. The times indicate incubation periods with 50 μM FTS. All measurements were at 22 °C. Each bar is the mean ± S.E. of 30–70 measurements. A, GFP-Ki-Ras(12V) mobility. The mobile fractions were high throughout and were not affected by the treatment (RF = 0.94 ± 0.01 in all cases). Student’s t test showed that the differences between the D values measured on FTS-treated cells as compared with untreated cells (0° incubation time) were significant (p < 0.001 for all incubation times except the 6- and 48-h time points, where p < 0.005 was obtained. B, DiIC16 mobility. The mobile fractions were similar in all cases (RF = 0.84 ± 0.04). The use of Student’s t test indicated that the differences between the D values on FTS-treated as compared with untreated cells were not significant (p > 0.25 in all cases).

Ras inhibitors (45) and FTS3 are capable of restoring actin stress fibers in Ras-transformed Rat-1 cells. To examine the possible dependence of the FTS-mediated modulation of GFP-Ki-Ras(12V) mobility on actin stress fibers, we employed cytochalasin D. The results depicted in Fig. 8 show that the transient increase in D of GFP-Ki-Ras(12V) after a short incubation with FTS persisted in cytochalasin D-treated cells. Cytochalasin treatment by itself mediated a small but significant reduction in D of GFP-Ki-Ras(12V), most likely due to the morphological changes in the cell membrane and/or the formation of actin aggregates. However, FTS treatment for 30 min increased D of GFP-Ki-Ras(12V) by the same factor as in the absence of cytochalasin (Fig. 8), suggesting that at the early stages the FTS effect is independent of stress fibers. On the other hand, the reduction in the lateral mobility of GFP-Ki-Ras(12V) after prolonged incubation (24 h) with FTS was not detected after cytochalasin D treatment (Fig. 8). Thus, the long-term reduction in GFP-Ki-Ras(12V) mobility by FTS may depend to some extent on cytoskeletal interactions.

Different S-prenyl analogs vary in their ability to inhibit the growth of Ras- or GFP-Ki-Ras(12V)-transformed cells (Refs. 18 and 19; see Fig. 5). It was therefore important to examine whether the effects of S-prenyl analogs on the lateral diffusion of GFP-Ki-Ras(12V) correlate with their ability to inhibit cell growth. Fig. 9 demonstrates that AFC and GTS, which fail to inhibit the growth of Rat-1 cells expressing GFP-Ki-Ras(12V), have no effect on the lateral diffusion of GFP-Ki-Ras(12V) at either short or long incubation periods. This contrasts with the modulation of GFP-Ki-Ras(12V) mobility by FTS, which effectively inhibits cell growth (Figs. 5 and 7).

DISCUSSION

In the current studies, we employed FPR and the Ras-displacing antagonist FTS to characterize directly and quantita-

3 Y. Egozi, M. Gana-Weisz, and Y. Kloog, unpublished results.
Ras(12V) persist after cytochalasin D treatment. The experiments were conducted as described under “Experimental Procedures” and in Fig. 7. Cells were incubated with 50 μM FTS for 30 min or 24 h, and cytochalasin D (cyto; 10 μg/ml) was added 15 min prior to the FPR measurements, which were performed at 22 °C. Each bar is the mean ± S.E. of 30–34 measurements. The mobile fractions were not significantly affected by the FTS treatments, but were slightly reduced by cytochalasin D ($R_m = 0.94 ± 0.01$ and $R_m = 0.90 ± 0.02$ in the absence and presence of cytochalasin D, respectively). For the 30-min FTS treatment, Student’s $t$ test showed that the $D$ values of cells treated with cytochalasin D and FTS were significantly different from those of cells treated with cytochalasin alone ($p < 0.001$). No significant difference ($p > 0.25$) was observed between cytochalasin-treated cells incubated with or without FTS for 24 h.

The effects of FTS on the lateral mobility of GFP-Ki-Ras(12V) persist after cytochalasin D treatment. The experiments were conducted as in Fig. 7. The times indicate the incubation periods with 50 μM AFC or GTS, or with 0.1% Me2SO in the control (−). The $R_m$ values were similar in all cases (0.94 ± 0.01). Each bar is the mean ± S.E. of 50–80 measurements. Student’s $t$ test revealed no significant differences between the $D$ values measured on control versus drug-treated cells ($p > 0.25$ in all cases, except for control versus AFC-treated cells after 30 min, which yielded $p > 0.1$).

The lateral diffusion of GFP-Ki-Ras(12V) is less restricted than that of transmembrane or GPI-anchored proteins (Fig. 6; see also Refs. 27 and 38–41). However, its $D$ value is 1.6-fold lower than that of the lipid probe DiIC16, suggesting that it experiences some mobility restricting interactions. GFP-Ki-Ras(12V) is released from these constraints during the early phase of FTS treatment (within 30 min), as indicated by the elevation of its diffusion rate to that of DiIC16 (Fig. 7B). This effect is clearly due to modulation of GFP-Ki-Ras(12V) lateral diffusion, as demonstrated by the lack of effect on its exchange rate using the FPR beam-size test, and by the lack of FTS effect on the dynamic properties of the bulk membrane lipids (see “Results”). A plausible mechanism that could give rise to the FTS early-phase effect is competition by the farnesyl-like FTS for sites that interact with GFP-Ki-Ras(12V) and retard its lateral mobility. This notion is supported by the lack of effect of structurally different S-prenyl analogs (Fig. 9). These interactions appear to be independent of the actin cytoskeleton, since they persisted in cytochalasin D-treated cells (Fig. 8).

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**Note:** H. Niv and Y. Kloog, unpublished observations.
ized to glycosphingolipid/cholesterol enriched domains exhibit large immobile fractions in FPR studies (27, 41). Together with the finding that GFP-Ki-Ras(12V) displays high mobile fractions in FPR studies (27, 41), it is important for its activity. Furthermore, FTS inhibited the release of GFP-Ki-Ras(12V) from the initial complexes of a labeled membrane component with an immobile entity reduce its apparent diffusion rate (7A). A plausible mechanism is that once a bound labeled molecule remains associated with the immobile component throughout the measurement.

The FTS effect on GFP-Ki-Ras(12V) mobility is biphasic, shifting from an initial elevation in the diffusion rate to a reduction in D (Fig. 7A). A plausible mechanism is that once GFP-Ki-Ras(12V) is released from its original sites of interaction due to FTS competition (resulting in the early-phase elevation in its D value), its association with the membrane is altered, enabling enhanced interactions with novel domains or elements. The long-term FTS treatment does not affect the bulk of the membrane lipids (no effect on DiIC16 mobility; Fig. 7B), however, FTS may induce the formation of new membrane domains or stabilize pre-existing ones that interact with Ras. Interactions with actin stress fibers may also contribute to the reduction in GFP-Ki-Ras(12V) mobility, since cytochalasin D inhibited of cells expressing GFP-Ki-Ras(12V), which accumulated to glycosphingolipid/cholesterol enriched domains exhibit large immobile fractions in FPR studies (27, 41). Together with the finding that GFP-Ki-Ras(12V) displays high mobile fractions in FPR studies (27, 41), it is important for its activity. Furthermore, FTS inhibited the release of GFP-Ki-Ras(12V) from the initial complexes of a labeled membrane component with an immobile entity reduce its apparent diffusion rate (7A). A plausible mechanism is that once a bound labeled molecule remains associated with the immobile component throughout the measurement.

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