The Efficacy of Raf Kinase Recruitment to the GTPase H-ras Depends on H-ras Membrane Conformer-specific Nanoclustering*‡1

Received for publication, November 26, 2013, and in revised form, February 6, 2014. Published, JBC Papers in Press, February 25, 2014. DOI 10.1074/jbc.M113.537001

Camilo Guzmán11,1, Maja Šolman11,1, Alessio Ligabue9, Olga Blažević9, Débora M. Andrade9, Luc Reymond9, Christian Eggeling9,10, and Daniel Abankwa12

From the 4Turku Centre for Biotechnology, Åbo Akademi University, Tykistökatu 68, 20520 Turku, Finland, the 5Department of Nanobiophotonics, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany, the 6Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland, and the 7MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Headley Way, OX3 9DS Oxford, United Kingdom

Background: Ras nanoclusters contain 6–8 Ras proteins on the plasma membrane and serve as indispensable signaling platforms for Ras-MAPK signaling.

Results: Ras membrane conformer mutants impart specific galectin-1-dependent nanoclustering responses.

Conclusion: Mutations in Ras can affect its nanoclustering response and thus allosterically effector recruitment and downstream signaling.

Significance: Disease-associated mutations that perturb Ras membrane conformers may alter signaling through nanoclustering.

Solution structures and biochemical data have provided a wealth of mechanistic insight into Ras GTPases. However, information on how much the membrane organization of these lipid-modified proteins impacts on their signaling is still scarce. Ras proteins are organized into membrane nanoclusters, which are necessary for Ras-MAPK signaling. Using quantitative conventional and super-resolution fluorescence methods, as well as mathematical modeling, we investigated nanoclustering of H-ras helix 4 and hypervariable region mutants that have different bona fide conformations on the membrane. By following the emergence of conformer-specific nanoclusters in the plasma membrane of mammalian cells, we found that conformers impart distinct nanoclustering responses depending on the cytoplasmic levels of the nanocluster scaffold galectin-1. Computational modeling revealed that complexes containing H-ras conformers and galectin-1 affect both the number and lifetime of nanoclusters and thus determine the specific Raf effector recruitment. Our results show that mutations in Ras can affect its nanoclustering response and thus allosterically effector recruitment and downstream signaling. We postulate that cancer- and developmental disease-linked mutations that are associated with the Ras membrane conformation may exhibit so far unrecognized Ras nanoclustering and therefore signaling alterations.

The Ras-MAPK pathway is spatiotemporally regulated on many levels (1, 2). Its central switch Ras toggles between GTP-on- and GDP-off-states, which are characterized by marked conformational differences of the so-called switch I and II regions. A wealth of structural and biochemical data of Ras have been accumulated, which describe the thermodynamics of its interactions and explain Ras activity, however, only of the soluble portion of the protein (3). Thus, the mechanistic basis for the most frequent oncogenic Ras mutations in codons 12, 13, and 61, as well as mutations in its regulators, guanine nucleotide exchange factors, which activate Ras or GTPase-activating proteins, that critically accelerate deactivation were understood (4). However, Ras proteins are dynamically membrane anchored by a farnesyl group in conjunction with palmitoyl groups or a stretch of basic amino acids that are all located at its C terminus (2, 5). Relatively little is known about the Ras reaction system in its native membrane environment. In the plasma membrane, ∼40% of Ras proteins laterally segregate into distinct nanoscopic proteolipid domains, the so-called nanoclusters. Nanoclusters are only 6–20 nm in dimension and contain 6–8 Ras proteins (6–8). Current evidence suggests that nanoclusters are immobile with lifetimes broadly estimated to be as short as microseconds to reach up to 0.5 s (7, 9, 10).

Although nanoclustering is an intrinsic property of membrane-bound Ras polypeptides (9), its stability may be regulated by nanocluster scaffolding proteins such as galectin-1 (Gal-1)3 and -3 (10–13). Structural modeling and experimental data

* This work was supported by an Academy of Finland fellowship grant, the Sigrid Juselius Foundation, the Cancer Society of Finland, and a Marie-Curie Reintegration Grant (to D. A.).
† This article was selected as a Paper of the Week.
‡ This article contains supplemental Tables 1 and 2.
1 Both authors contributed equally to this work.
2 To whom correspondence should be addressed. Tel.: 358-2-333-6969; E-mail: daniel.abankwa@btk.fi.
3 The abbreviations used are: GAL-1, galectin-1; BHK, baby hamster kidney; FCS, fluorescence correlation spectroscopy; FLIM, fluorescence-lifetime imaging microscopy; FRAP, fluorescence recovery after photobleaching; MEF, mouse embryonic-fibroblast; RBD, ras binding domain of c-Raf; STED-FCS, fluorescence correlation spectroscopy enhanced with stimulated emission depletion; mGFP, monomeric green fluorescent protein; mRFP, monomeric red fluorescent protein.
suggest that Gal-1 can accommodate the farnesyl moiety on the C terminus of H-ras, yet it must recognize the G-domain directly or indirectly, as it binds only to active GTP-loaded H-ras (14, 15). The exact structural mechanism, stoichiometry, and complex composition of H-ras- and Gal-1-containing nanoclusters are, however, unknown. Overexpression of Gal-1 enhances the stability of H-ras nanoclusters, which can be followed by an increased immobilization of Ras and downstream MAPK signaling (10, 11, 14), whereas its down-modulation allows by an increased immobilization of Ras and downstream MAPK signaling (10, 11, 14), whereas its down-modulation decreases H-ras nanoclustering (12). Importantly, nanoclustering does not only lend the Ras reaction system new properties, such as signaling robustness and noise resistance, but is required for proper signaling (16).

We previously described a novel mechanism of how Ras operates on the membrane (17–19). Computational simulations of membrane-bound H-ras in combination with Förster/Fluorescence resonance energy transfer (FRET) experiments in intact mammalian cells suggested that H-ras exists in a nucleotide-dependent conformational equilibrium on the membrane that is guided by the novel switch III. Switch III is formed by the β2-β3 loop (also known as interswitch in Rab and Arf proteins (20)) in conjunction with helix α5 and exhibits less overt conformational changes than the classical switch I and II regions (21, 22). When structural simulations were carried out with GTP-bound H-ras, an unexpected conformer with a reoriented G-domain was found, which was stabilized by membrane contacts of Arg-128/Arg-135 on helix α4 (Fig. 1A, right). Conversely, when simulated with GDP-H-ras, a conformer with a more conventional membrane anchorage was observed, which was specifically stabilized by membrane contacts of residues Arg-169/Lys-170 in the hypervariable region (hvr) (Fig. 1A, left). These results suggested a GTP-dependent conformational equilibrium of H-ras on the membrane.

We subsequently showed that the Ras isoform/paralog-specific variations of the helix α4 and the hvr have a systematic impact on effector recruitment (18), suggesting that the conformational equilibrium that is modulated by the properties of helix α4 and the hvr is important for Ras paralog-specific activity. Recent work by others supported G-domain membrane interactions of membrane-bound H-ras (25, 26).

Here, we investigated the mechanism by which the H-ras membrane conformers realize differential effector recruitment. Using in vitro binding of H-ras membrane conformers and the RBD, we confirmed that outside of the membrane, binding is identical. We then showed that differences in the Gal-1-dependent nanoclustering response determine effector recruitment. Using STED-FCS, FRAP, and FLIM-FRET, we followed the emergence, manifestation, and the activity of the mutant-specific H-ras signaling nanocluster. Our results significantly extend the previous model by revealing a complex, allosteric coupling between H-ras membrane conformers, Gal-1, and nanoclustering that dictates effector recruitment and signaling.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Molecular Cloning**—Different expression plasmids for in vitro and cellular experiments were used. For protein expression in Escherichia coli, the pQE-A1 plasmid was generated from pQE-30 Xα (Qiagen, Hilden, Germany) with the following modifications; the sequence coding for the PreScission protease (GE Healthcare) recognition site and a sequence coding for the 12-amino acid A1 tag followed by a BglII restriction site were introduced between the original factor Xa recognition site and the multiple cloning site to allow for in-frame BglII/KpnI cloning of H-ras mutant cDNAs. The PreScission protease recognition site was introduced for an efficient removal of the His tag. The A1 tag (GDSLDMLEWSLM) is an alternative acyl carrier protein tag (New England Biolabs, Ipswich, MA) and is used for selective fluorescent protein labeling in vitro (27). To construct the pQE-A1 plasmid, the sequence of N-terminally HA-tagged H-ras wild type (WT) was amplified from the pmGFP-H-ras(WT) plasmid (17) by two consecutive PCRs. First, the A1 tag (27) and the BglII restriction site were added with a forward primer, 5′–ATCGAGGGAGGCCTCTGGAGTTCTGTTCACAGGTCTCGTGATG- CCTGATATGCTGGAATGGAGCCTGATGGGAGATCTCGA- GCTCAACCACATGTACC-3′ (Sigma), and next the Stul restriction and PreScission protease recognition sites were added using a second forward primer 5′–GGCGATACCCGGAATGTTTCAGGGTCCTGGCGATAG- CCTGATATGCTGGAATG-3′. For both PCRs the same reverse primer, 5′–CATTTTATTTTTCAGGGTTC AGGD-3′, was used. PCR products were purified and subcloned into pCR™ II-Blunt-TOPO (Invitrogen). From there, the fragment was cloned into the pQE-30 Xα vector using Stul and KpnI restriction sites to produce pQE-A1-H-ras (WT). A1-tagged H-ras-R169A/K170A and H-ras-R128A/R135A plasmids were generated from corresponding pmGFP plasmids (17) by cloning via BglII and KpnI restriction sites into the pQE-A1 vector. For the RBD of c-Raf expression plasmid, the open reading frame of the RBD was amplified by PCR with addition of appropriate restriction sites from the pmRFP-RBD plasmid (17), and the insert was cloned in-frame with the A1 tag (27) into the BglII and KpnI sites of the pQE-A1 vector. All final constructs were verified by DNA sequencing (GATC, Cologne, Germany). pPINE2-Gal-1- His prokaryotic expression plasmid for galec- tin-1 was constructed in the protein expression facility of the University of Queensland, Brisbane, Australia, via in vitro ligation (28). Mammalian expression plasmids encoding N-terminally mGFP-tagged H-ras orientation mutants, pmGFP-H-rasG12V-R169A/K170A, pmGFP-H-rasG12V-R128A/R135A, and pmGFP-H-rasG12V, as well as mRFP-tagged RBD of c-Raf (pmRFP-RBD) and galec- tin-1 (pmRFP-Gal-1) that were used for cellular experiments, have been described previously (17, 18). Plasmids used for either knockdown (pcDNA3-asGal-1) or overexpression of untagged galec- tin-1 (pcDNA3-Gal-1) were described elsewhere (14). To generate SNAP-tagged orientation mutants for fluorescence correlation spectroscopy enhanced with stimulated emission depletion (STED-FCS) experiments, H-rasG12V, H-rasG12V-R169A/K170A, and H-rasG12V-R128A/R135A sequence fragments from pmGFP-H-rasG12V, pmGFP-H-rasG12V-R169A/K170A, and pmGFP-H-rasG12V-R128A/R135A were subcloned into the Xhol and PstI restriction sites of the pSNAP vector. The backbone of pSNAP vector is a pEGFP-C1 vector (Clontech) in which enhanced GFP was replaced by the SNAP tag (New England Biolabs, Ipswich, MA).
between Nhel and BsrGI restriction sites, while preserving the original reading frame.

**Protein Expression and Purification**—All A1-tagged proteins used in this study were expressed in the *E. coli* strain M15 (pREP4) (Qiagen, Hilden, Germany). Competent *E. coli* M15 (pREP4) cells were transformed with corresponding plasmids, and cultures were supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/ml) and induced with 100–500 µM isopropyl β-D-thiogalactopyranoside at an A600 of 0.6–0.8. Cells were cultured for 3 h at 37 °C, collected by centrifugation at 6000 × g for 20 min, and resuspended in 20 mM sodium phosphate, 5 mM MgCl₂, 5 mM imidazole, 150 mM NaCl, pH 8.0. The bacteria were disrupted by freezing/thawing three times followed by sonication. Nondisrupted cells and large debris were removed by centrifugation at 49,000 × g for 60 min at 4 °C. The His-tagged proteins were further purified using a HiTrap nickel-nitrilotriacetic acid column (GE Healthcare) and eluted with 150–400 mM imidazole. The Histag was then removed by proteolysis with PreScission protease (GE Healthcare), according to the manufacturer’s instructions (2 units of protease per 100 µg of protein overnight), and the final protein was further separated from uncleaved, His-tagged protein by nickel-nitrilotriacetic acid chromatography. The removal of salt was carried out by dialysis against 25 mM HEPES, 40 mM NaCl, 3 mM MgCl₂, pH 7.2, for 16 h at 4 °C. Nucleotide exchange on the H-ras molecule was performed according to Tucker et al. (29) with minor modifications. In brief, the bound GDP present in purified samples of H-ras could be exchanged against other nucleotides (GTP and mant-GTP/GDP) in the presence of EDTA. The mant-GTPγS-loaded H-ras was obtained by titration of 100 nM mant-GTPγS (or 500 nM mant-GDP) with purified H-ras in the presence of 5 mM EDTA. The reaction was monitored by following the fluorescence anisotropy of the mant-nucleotide. Once the binding was saturated, the titration was stopped by addition of 20 mM MgCl₂. Mant-GTPγS-loaded H-ras was immediately used to study the affinity between the RBD and H-ras mutants in solution. No further purifications were necessary as the nucleotide-free H-ras has a much lower affinity for the RBD (30, 31). The change in anisotropy due to a loss of mant-GTPγS from H-ras was negligible for the duration of a typical experiment (less than 1% in 2 h), as established in control experiments in the absence of the RBD (*k_off ~ 1.5 × 10⁻⁴ s⁻¹; data not shown).

**Fluorescence Anisotropy Measurements**—Fluorescence anisotropy was used to study the affinity between the RBD and H-ras mutants in solution. Typically, 100 nM mant-GTPγS or 500 nM mant-GDP-loaded H-ras were used for each assay. Steady-state anisotropy measurements were performed with a Synergy H1 hybrid fluorescence plate reader (BioTek, Winooski, VT) equipped with a polarization filter cube. Fluorescence measurements were done using a bandpass 340/30-nm excitation filter and by monitoring the emission using a bandpass 485/20-nm filter. Parallel and perpendicular excitations were used, and the emission intensities were acquired sequentially. Fluorescence anisotropies were calculated from the measured fluorescence intensities, according to Equation 1,

\[
\frac{I_{vv} - G(\lambda)I_{hv}}{I_{vv} + 2G(\lambda)I_{hv}} = \frac{I_{vv} - B_{max}}{I_{vv}} = \frac{Y}{2L_t}
\]

where \(r\) is the fluorescence anisotropy; \(I_{vv}\) is the fluorescence emission intensity detected with vertically polarized excitation and horizontally polarized emission; \(I_{hv}\) is the fluorescence emission intensity detected with horizontally polarized excitation and vertically polarized emission, and \(G(\lambda)\) is the correction factor (\(G(\lambda) = 1\) for \(\lambda\) between 300 and 700 nm, BioTek communication) for the Synergy H1 hybrid reader (32). Measurements were conducted at room temperature in buffer containing 25 mM HEPES, pH 7.2, 100 mM NaCl, and 3 mM MgCl₂. Data processing was done using Gen5 software (version 2.01, BioTek), and \(K_d\) values were determined using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA). The global fitting of anisotropy data were done taking the RBD depletion into account as shown in Equation 2,

\[
Y = \frac{B_{max}(1 - \frac{I_{hv}}{I_{vv}})}{2L_t} = \frac{B_{max}(1 - Y)}{2L_t}
\]

where \(Y\) is the change in anisotropy after the addition of the chosen concentration of RBD; \(B_{max}\) is the maximum change in anisotropy due to the specific binding, and \(D\) is calculated as shown in Equation 3,

\[
D = L_t + K_d + X
\]

where \(L_t\) is the concentration of the fluorescently labeled H-ras (100 nM); \(K_d\) is the dissociation constant of the complex (fluorescently labeled H-ras · RBD), and \(X\) is the concentration of RBD (33, 34).

**Cell Culture**—BHK cells and Gal-1 knock-out mouse embryonic fibroblasts (Gal-1⁻/⁻ MEFs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, l-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). They were grown to a confluency of 80% (8 × 10⁶ cells/ml) and subcultured every 2–3 days. Gal-1⁻/⁻ MEFs were a kind gift from the laboratory of Prof. Yoel Kloog (35). For Western blotting analysis and fluorescence recovery after photobleaching (FRAP) experiments, 200,000 cells were seeded in a 6-well plate (Cellstar catalog no. 657160, Greiner Bio-one, Stonehouse, Gloucestershire, UK), and after 24 h, they were transfected using JetPrime transfection reagent (Polyplus Transfection, New York) with the following plasmids: pmGFP-H-rasG12V, pmGFP-H-rasG12V-R169/K170A, or pmGFP-H-rasG12V-R128/135A. Plasmids were transfected alone or together with galectin-1 cDNA containing plasmids pcDNA3-asGal-1 in the case of depletion or with plasmids pMRFP-Gal-1 or pcDNA3-Gal-1 in the case of overexpression. For FLIM experiments, 100,000 cells were seeded on a 6-well plate on glass coverslips, and transfection with corresponding plasmids was done after 24 h using FuGENE 6 transfection reagent (Promega, Fitchburg, WI).
Raf Recruitment Depends on Ras Conformer Nanoclustering

pmGFP-H-rasG12V-R169A/K170A, and pmGFP-H-rasG12V-R128A/R135A alone or with pmRFP-RBD, pcDNA3-Gal-1, or pmRFP-Gal-1 were fixed in 4% paraformaldehyde/PBS for 20 min and washed in PBS, and coverslips with cells were then mounted with Mowiol 4-88 (81381, Sigma) on microscope slides. Samples were excited with sinusoaidally modulated (40 MHz) epi-illumination at 3 watts and 470 nm, using a temperature-stabilized multi-LED system (Lambert Instruments). Cells were imaged with a ×63 NA 1.4 oil objective using an appropriate GFP filter set (excitation, bandpass 470/40; beam splitter, FT 495; emission, bandpass 525/50). The phase and modulation fluorescence lifetimes were determined per pixel from images acquired at 12 phase settings using the manufacturer’s software. Fluorescein at 0.01 mM, pH 9, was used as a lifetime reference standard (lifetime 4.0 ns). The phase lifetime of the donor (mGFP constructs) was determined for regions of interest containing 1–5 cells, coexpressing the indicated constructs. The percentage of the apparent FRET efficiency (E_app) was calculated using the measured lifetimes of each donor-acceptor pair (τD, A) and the average lifetime of the donor only (τD) samples. The formula employed for this was taken from (36) Equation 4,

\[
E_{\text{app}} = \left(1 - \frac{\tau_{\text{D(A)}}}{\tau_{\text{D}}} \right) \cdot 100\%
\]  

Confocal FLIM (TCSPC-FLIM)—Fluorescence lifetime imaging with subcellular resolution was done on a confocal microscope by means of time-correlated single photon counting (TCSPC). FLIM was performed on an inverted confocal laser-scanning microscope (Leica TCS STED, Leica Microsystems GmbH, Wetzlar, Germany) using a ×100 silicone oil immersion objective (NA 1.4). Samples were excited using a tunable Titanium:Sapphire Mai Tai laser (Spectra-Physics, Santa Clara, CA) delivering femtosecond pulses at a rate of 80 MHz with an output power of 1.9 watts for the selected wavelength of 900 nm. This wavelength was found to be optimal for the two-photon excitation of mGFP in mGFP/mRFP FRET pairs. Collection of the fluorescence was done by employing an avalanche photodiode detector and a bandpass filter to select the signal in the range 500–530 nm. Emitted photons from the donor fluorophore were collected and counted using the Time-Harp 200 (PicoQuant, Berlin, Germany) time-correlated single photon counting board. Laser power was adjusted to achieve a photon collection count of 150 photons/s. Images were acquired during 7 min in a 256 × 256 format, and the fluorescence lifetime was calculated for each pixel. Data acquisition and processing was done using the SymPhoTime software (PicoQuant).

STED-FCS Measurements in Cells—A combination of STED and FCS was used to assess the diffusion dynamics of H-ras. The STED-FCS setup used and its calibration procedure include minor changes from the setup and calibration described in detail elsewhere (37). BHK-21 cells were seeded on microscopy grade glass coverslips (diameter 18 mm, number 1 thickness) to a confluency of about 90% and grown at 37 °C in a water-saturated atmosphere of 5% CO2 in air. SNAP-tagged H-rasG12V proteins expressed in those cells were fluorescently labeled with the silicon-containing rhodamine (SiR) dye (38). The SNAP tag is a 20-kDa mutant of the DNA repair protein O6-alkylguanine-DNA alkyltransferase. O6-Alkylguanine-DNA alkyltransferase binds irreversibly and covalently benzylguanine derivatives. In the presence of a benzylguanine derivative that is bound to a synthetic probe, such as the fluorophore SiR, the SNAP-tagged protein becomes labeled with that synthetic probe (39).

During measurements cells were kept at 23 °C in Dulbecco’s modified Eagle’s medium (DMEM) without phenol red and buffered with 10 mM HEPES (HDMEM). We assessed the H-ras dynamics by placing the co-centered excitation and STED beams on random positions in the plasma membrane adherent to the surface and completed all measurements before any significant morphological changes of the cell could occur. Acquisition times were 10 s, which were thus 2 orders of magnitude longer than the typical transient times of H-ras proteins through the confocal observation area. The apparent diffusion coefficients for each H-ras orientation mutant were calculated from at least 7 and up to 30 measurements per given observation diameter. Repeated measurements were performed on the same cell, as well as on different cells. Results are given as the average ± S.E. of measurements acquired under identical treatment conditions.

Western Blot Analysis—Twenty four hours after transfection, cells were harvested using a buffer containing 50 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1 mM bromphenol blue, and 10 mM Tris-HCl, pH 6.8. Proteins from 20 μl of cell extract were first separated using SDS-PAGE (14%) and then electroblotted on a nitrocellulose membrane (GE Healthcare). After blocking with 5% milk powder in TBS + 0.1% Tween 20, membranes were probed with primary antibody for galecin-1 (sc-28248 Santa Cruz Biotechnology, Dallas, TX) diluted 1:3000 and then with secondary peroxidase-conjugated bovine anti-rabbit IgG antibody (sc-2370 Santa Cruz Biotechnology) diluted 1:3000. β-Actin or GAPDH was used for normalization. Membranes were first probed with primary antibody for β-actin (A1978 Sigma) or GAPDH (SAB1405848 Sigma) diluted 1:500,000 and then secondary peroxidase-conjugated bovine anti-mouse IgG antibody (Santa Cruz Biotechnology, sc-2954) diluted 1:3000. Secondary antibodies were detected using enhanced chemiluminescence (ECL Prime Western Blotting Detection Reagent, GE Healthcare). Quantification of the signal intensities was performed using Quantity One software (Bio-Rad). Endogenous levels of Gal-1 in BHK cells were determined from the Western blot standard curve of purified Gal-1 that was expressed from the pOPINE2-Gal-1-His plasmid.

Fluorescence Recovery after Photobleaching—FRAP was used to monitor diffusion properties of H-rasG12V mutants, under three different conditions: endogenous cellular Gal-1, mRFP-Gal-1 overexpression, and Gal-1 depletion (see under “DNA Constructs and Molecular Cloning” for details). Experiments were performed 24 h after transfection. Cells were kept in Ringer’s buffer (10 mM HEPES, 10 mM glucose, 2 mM NaH2PO4·H2O, 0.1 mM MgCl2·6H2O, 2 mM CaCl2, 5 mM KCl, 155 mM NaCl, pH 7.2) at 22 °C. All data were acquired on a Leica TCS SP5 STED microscope (Leica Microsystems GmbH, Wetzlar, Germany) using the FRAP wizard from the manufacturer. Cells were imaged using confocal microscopy (excitation, 488
Raf Recruitment Depends on Ras Conformer Nanoclustering

nm; detection, 497–568 nm) with bidirectional scanning at a 700 Hz frequency, 512 × 512 resolution, and ×15 zoom (pixel size 0.0201 μm). Under these conditions, we obtained a time between frames of 1.514 s. The first five frames were taken with only 10% of the laser intensity (laser nominal power is 65 milliwatts) and were used as a reference for normalization. In the next 20 frames, a square region of interest of 2.5 × 2.5 μm² was bleached using the full laser power to reduce the fluorescence signal in the region to 30–50% of the initial intensity. To monitor the recovery, an additional 75 frames were taken with 10% of the laser intensity. Fluorescence signals were quantified in ImageJ 1.47g (National Institutes of Health, Bethesda, and recovery curves were normalized using the fluorescence intensity obtained in the first five frames, before photobleaching. The apparent characteristic half-time of recovery and the recovery curves were normalized using the fluorescence intensity obtained in the first five frames, before photobleaching. The apparent characteristic half-time of recovery and the immobile fraction were determined using curve fitting analysis in IGOR Pro 6 (WaveMetrics, Tigard, OR) according to Equation 5 by Feder et al. (40).

\[
F(t) = \frac{F_0 + F_\infty \left( \frac{t}{t_{1/2}} \right)}{1 + \left( \frac{t}{t_{1/2}} \right)}
\]

(Eq. 5)

In this equation, \(F(t)\) represents the normalized fluorescence intensity; \(t\) is the time; \(F_\infty\) is the normalized intensity after an infinite time; \(t_{1/2}\) is the half-time of recovery, and \(F_0\) is the fluorescence intensity at \(t = 0\), immediately after bleaching. Immobile fractions \(Q\) were calculated using

\[
Q = \frac{(1 - F_\infty)/(1 - F_0)}{F_0 + F_\infty \left( \frac{t}{t_{1/2}} \right)}
\]

For each sample ~10 experiments were performed.

Statistical Analysis—Statistical differences between the different samples studied by FLIM, FRAP, and fluorescence anisotropy were established using analysis of variance. The analysis of variance test was complemented by Tukey’s honestly significant difference test to establish which pairs of samples were significantly different. Analysis was performed using the software R version 2.15.2 (R Development Core Team, Vienna, Austria).

Simulations of Nanocluster Formation—Simulations of nanocluster formation were performed according to the schematic representation of the different reaction paths presented in Fig. 8, and using the variables and equations introduced in supplemental Tables S1 and S2. In our nanocluster model system, we had 1000 H-ras-GTP (in the following short H) molecules and observed the changes in the number of nanoclusters, depending on the number of Gal-1 molecules (in the following short G) and most importantly depending on the orientation mutant-specific HG complex stability, as defined by its lifetime. The concentration changes of the individual complexes were described using ordinary differential equations, which were solved by numerical simulation using the software R version 2.15.2 (R Development Core Team) and the ordinary differential equation solver package “deSolve” created by Karline Soetaert, Thomas Petzoldt, and R. Woodrow Setzer. Simulations were run until stability of the concentrations of all individual complexes was reached, typically 100 steps of 0.1 s. Each of these concentrations at stability was recorded as the final concentrations for the particular set of initial conditions. The initial concentration (number of molecules) for H-ras was always the same (\(H(t = 0) = 1000\)), whereas different initial concentrations of Gal-1 (\(G(t = 0)\)) were evaluated between 0 and 15,000 with 100-unit increments. Initial concentrations for any other individual complex was always inputted as zero.

To find the specific conditions for the model to reproduce the experimental data, eight fitting parameters were used as follows: dissociation rates \(r_1\) and \(r_2\); association rates \(a_1\) and \(a_2\); collapse rate \(c_1\); and parameters \(Ras_{Gal}, Gal_{Gal}\), and \(Coll_{Gal}\) that describe the changes of those rates as higher \(n\)-mer clusters are reached. Two of these parameters have constraints. One is due to the fact that \(r_1\) and \(a_1\) are linked through the dissociation constant of two Ras proteins \(K_d\), and the other one is due to the fact that \(r_2\) and \(a_2\) are linked through the dissociation constant of the HG complex \(K_{d2}\). This left a total of six unconstrained parameters. Final fitting parameters are presented and described in detail in supplemental Tables S1 and S2.

Fits have been performed first for H-rasG12V using as starting point for the association, dissociation, and collapse rates, the experimentally derived formation time of a nanocluster (<1 s) (41) or the lifetime of nanoclusters (<0.5 s) (7, 41, 42). For \(Ras_{Gal}, Gal_{Gal}\), and \(Coll_{Gal}\), initial values were chosen as 1, meaning identical conditions of binding at all levels of clustering (monomer, dimer, trimer, etc.). For \(Gal_{Gal}\) and \(Coll_{Gal}\) values higher than 1 establish the positive cooperative effect present when more molecules of Gal-1 are involved (Fig. 8, box i). \(Gal_{Gal}\) leads to higher association rates (both for binding of additional Gal-1 or H-ras), and \(Coll_{Gal}\) decreases the collapse rate of higher \(n\)-mer clusters. In the case of \(Ras_{Gal}\), values higher than 1 represent negative cooperativity as \(Ras_{Gal}\) decreases the association rates of H-ras at higher levels of organization in the nanocluster formation process (Fig. 8, box ii). This negative cooperativity is necessary to prevent all H-ras from being bound into nanoclusters.

To obtain the fits for mutants H-rasG12V-R169A/K170A and H-rasG12V-R128A/R135A, parameter results obtained from H-rasG12V were used as starting values. Most importantly, these were adjusted to reflect the experimentally determined relative affinities between H-ras and Gal-1, which increase in the order H-rasG12V-R169A/K170A > H-rasG12V-R128A/R135A > H-rasG12V-R169A/K170A. Although this assumption cannot be verified experimentally, it is in line with a recent coarse grain structural computational simulation of the two H-ras conformers that are represented by our orientation mutants, showing that the two conformers interacted differently in their clusters, which is consistent with the modeled different affinities (43).

An immobile and functional nanocluster is formed, once four H molecules have assembled. This H-ras-GTP 4-mer represents the smallest nanocluster unit size found by both structural and Monte-Carlo computational simulations of Ras polypeptides (44, 45) and agrees with the minimal number of Ras found experimentally in a nanocluster (6). To lower computational efforts, we had to exclude higher cluster oligomers. Any immobile (4-mer) H-ras-GTP nanocluster will recruit RBD molecules. This incorporates evidence from single molecular
Raf Recruitment Depends on Ras Conformer Nanoclustering
data that suggest that the effector Raf is recruited only to immo-
obilized Ras-GTP (7, 41, 42). For this recruitment step, which we
do not explicitly model, we assume that the average time for an
RBD to find an H-ras-GTP in a nanocluster is smaller than the
lifetime of a nanocluster. Thus, the RBD can “sense” the different
lifetimes of nanoclusters. Also, the lifetime of an H-ras-
GTP-RBD complex has to be of similar magnitude as that of
the nanocluster, to prevent that the RBD remains bound to H-ras-
GTP after the nanocluster has collapsed. Both assumptions are
based on published data, such as the relation of the lifetime of a
nanocluster (<0.5 s) (7, 41, 42), compared with the binding
time and the lifetime of the highly dynamic H-ras-GTP/RBD
binding process (binding time 0.007–0.05 s; lifetime 0.1–0.5 s).
These values were calculated from literature values of
$k_{on} \text{(30–35 } \mu \text{M}^{-1} \text{s}^{-1}) \text{ and } k_{off} \text{(2–6 s}^{-1}), \text{ respectively (46–48). In}
the case of the binding time, we are assuming an equilibrium
concentration of the H-ras-GTP-RBD complex in the range
0.5–5 μM.

RESULTS
H-ras Orientation Mutants Interact Identically with the Ras
Binding Domain of the Effector c-Raf in Solution—Our previous
structure of focused computational and cell biological work
established different Raf effector recruitment and MAPK sig-
naling strengths for H-rasG12V mutants that represent differ-
ent conformational states of Ras on the membrane (Fig. 1A) (17,
19). H-rasG12V with mutations R169A/K170A in the hvr
increases, whereas mutations R128A/R135A on helix α4
decreases effector recruitment and MAPK signaling. Because of
their bona fide impact on the conformation or orientation of
H-ras on the membrane, we here refer to these mutations as
“orientation mutations.” H-ras orientation-mutations are dis-
dtant from the effector interaction surface (Fig. 1A); therefore,
the structural basis for their specific Raf effector recruitment
levels and ensuing signaling changes remained unresolved.

To understand how they exhibit their specific biological activity,
we first verified our previous assumption that effector
binding to nonmembrane-bound H-ras is thermodynamically
unaffected by the orientation mutations on helix α4 or the hvr.
We therefore studied the interaction of purified H-ras orientation
mutants with the Ras binding domain of c-Raf (in the following
short: RBD) in solution, using a fluorescence anisotropy binding
assay. Both H-ras orientation mutations showed RBD binding identi-
tical to that of WT H-ras in vitro (Fig. 1B and Table 1).

We next wanted to confirm that this was also true in cells,
by analyzing these interactions using FRET between mGFP-la-
beled H-ras (mGFP-H-rasG12V, donor) and mRFP-labeled
RBD (mRFP-RBD, acceptor). Confocal FLIM-FRET images
showed increased FRET mainly at the cell periphery consistent
with the recruitment of the RBD to plasma membrane-bound
active Ras (Fig. 2A, middle). We then analyzed cells that were
treated with compactin, a HMG-CoA reductase inhibitor that
efficiently blocks prenylation and thus membrane anchorage of
Ras (49, 50). This led to a cytoplasmic redistribution of the
Ras-RBD complex (Fig. 2A, right), which to our surprise was
accompanied by a reduction of FRET throughout the cyto-
plasm. Therefore, compactin treatment allowed us to study the
interaction of the H-ras orientation mutants with the RBD in
solution, while remaining in the intact cellular environment
that contained any putative binding modulators.

We next used this assay to establish quantitative differences
between the orientation mutations, using wide-field FLIM-FRET
measurements. In the absence of compactin, the FRET levels of
the hyperactive H-rasG12V-R169A/K170A and H-rasG12V or
H-rasG12V-R128A/R135A were significantly different, as re-
ported previously (17). Consistent with our in vitro data,
compactin treatment abolished these differences (Fig. 2B), con-
firming that different RBD recruitment arises only if H-ras
orientation mutants are membrane-bound. As already ob-
served in the confocal FLIM data (Fig. 2A, right), the apparent

FIGURE 1. Identical binding of H-ras orientation mutants to the RBD in
solution. A, structures of the c-Raf RBD (green) bound to the computationally
generated models of membrane-bound H-ras (blue) in the GDP (left) and GTP
(right) conformations. These conformers are stabilized by residues Arg-169/
Lys-170 in the hvr and Arg-128/Arg-135 of the helix α4, respectively. The
structure 1GU was overlaid onto the previously described computational
models of membrane-bound H-ras (19). Note that mutations of these resi-
dues modulate effective RBD interaction in cells, but they are distant from the
effector binding site. B, fluorescence anisotropy binding assay of the RBD and
H-ras wild type (residues 2–189) (gray circles), H-rasG12V (red triangles),
or H-ras-G12V/R135A (black squares) loaded with mant-GTPγS. The latter
two orientation mutants, where membrane-contacting residues were
neutralized, represent the two conformers shown (A, right and left, respec-
tively). Binding curves for the mant-GTPγS condition were obtained using
the global fitting function reported under “ Experimental Procedures.” Data for
mant-GDP (data not shown) did not suggest any significant interaction. Error
bars represent the mean ± S.E. (n ≥ 4).

TABLE 1
Dissociation constants of the interaction of H-ras orientation mutants
with the Ras binding domain of C-Raf

| H-ras, WT | H-ras-R169A/K170A | H-ras-R128A/R135A |
|----------|-------------------|-------------------|
| Mant-GTPγS $K_d$ (nM) | 412 ± 71 | 327 ± 72 | 422 ± 76 |

In vitro dissociation constants ($K_d$) of the C-Raf-RBD and mant-GTPγS-bound
H-ras (residues 2–189; 100 nm) with or without orientation mutations determined
from fluorescence anisotropy measurements. $K_d$ values ± S.E. are listed (n ≥ 4). Statistical
analysis did not reveal any significant difference between the three mant-
GTPγS-bound H-ras orientation mutants; see under “ Experimental Procedures” for
details about statistical analysis.
FRET efficiency dropped significantly, while still being high enough to suggest that a complex containing Ras and the RBD was relocalized to the cytoplasm after compactin treatment. In conclusion, outside of the membrane H-ras orientation mutants bind identically to the c-Raf-RBD, consistent with our model in which we propose different conformational states on the membrane that dictate the downstream activity of H-ras by an unknown mechanism.

**STED-FCS Experiments Reveal Specific Gal-1-dependent Transient Nanoclustering of H-ras Orientation Mutants**—We hypothesized that the higher FRET efficiency of membrane-anchored H-ras orientation mutants with the RBD, as compared with the cytoplasmically redistributed complex, was due to an increase in the recruitment efficiency of Ras up-concentrated in the membrane. In the plasma membrane, Ras is organized in nanoclusters (8). We showed previously that the nanocluster scaffolding protein galectin-1 (Gal-1) interacts differently with the H-ras orientation mutants (18). Gal-1 can increase H-rasG12V nanoclustering and effector recruitment (11). This led us to investigate in detail whether orientation mutant-dependent differences in Gal-1 interaction could give rise to specific changes in H-ras nanoclustering and subsequent effector recruitment.

We reasoned that this interaction would lead to specific incorporation probabilities of H-ras into an immobile nanocluster (Fig. 3A). To assess specific membrane diffusion dynamics of different H-ras orientation mutants due to transient nanoclustering, we employed STED-FCS. Conventional FCS can be applied to study membrane dynamics by giving insight into diffusion characteristics of membrane proteins and lipids (51, 52). Nevertheless, being limited by the diffraction of light, this technique cannot resolve dynamics at the nanoscale. By combining it with STED microscopy, a method that breaks the diffraction limit of light in fluorescence microscopy, STED-FCS is able to resolve membrane dynamics with unprecedented resolution (53). In STED-FCS, the diffusion coefficient of a target molecule is measured at different scales. How the apparent diffusion coefficient varies with the area of observation defines the diffusion characteristics of the target molecule. For example, free diffusion is characterized by a constant diffusion coefficient, which is independent of the scale of observation. Transient clustering, however, is characterized by an apparent dif-
Raf Recruitment Depends on Ras Conformer Nanoclustering

With normal/endogenous Gal-1, all of the mutants had decreasing apparent diffusion coefficients ($D$) with decreasing observation spot size (diameter $d$), indicating transient nanoclustering (Fig. 3B). Overexpression of Gal-1 further enhanced the decrease of $D$ with smaller $d$, while decreasing Gal-1 levels render the $D(d)$ dependences for all orientation mutants more consistent with free diffusion, i.e. very low levels of Gal-1 do not affect transient nanoclustering sufficiently; therefore, the diffusion of Ras proteins resembles more free Brownian motion (Fig. 3, B and C).

We quantified the hindered diffusion using the slopes of the $D(d)$ curves in the approximate linear regime with $d$ ranging between 150 and 240 nm (Fig. 3C). Thus, we obtained three different slope profiles for the three different orientation mutants, which act like fingerprints of the three different molecular (conformational) states. H-rasG12V-R169A/K170A showed the highest slope already at normal Gal-1 levels, although this was reached by H-rasG12V only when Gal-1 was overexpressed. However, even in this Gal-1 overexpression condition, H-rasG12V-R128A/R135A did not reach the highest slope. This evolution of increasing Gal-1-dependent hindered diffusion in the order H-rasG12V-R169A/K170A > H-rasG12V > H-rasG12V-R128A/R135A is consistent with the order of membrane-dependent Gal-1 binding of these mutants (18) and their biological activity profiles (17, 19). Therefore, our STED-FCS data suggested different Gal-1-dependent nanoclustering propensities of H-ras orientation mutants.

**H-rasG12V Orientation Mutants Show Specific and Gal-1 Dose-dependent Immobilization Responses in FRAP Experiments**—After we had shown that Gal-1 mutant-specifically affects the emergence of nanoclusters, we next addressed whether this is also true in established nanoclusters (Fig. 5A). Single molecule data showed that active Ras is immobilized in nanoclusters (10, 54, 55), which is why the immobile fraction determined in FRAP experiments corresponds to the nanoclustered fraction of Ras (10). Hence we used a FRAP assay to detect Gal-1-dependent differences in nanoclustering of H-ras orientation mutants (Fig. 5, B and C).

With normal Gal-1 levels, both H-rasG12V and the more active H-rasG12V-R169A/K170A have a high immobile fraction of ~0.5 (Fig. 5C). Conversely, the less active mutant H-rasG12V-R128A/R135A was significantly ($p < 0.1$) less immobile. Interestingly, with high overexpression of Gal-1 (this time ~5-fold over endogenous; Fig. 4, E and G, and Table 2) we could only achieve a maximum of ~60% immobile H-ras, irrespective of which mutant we studied (Fig. 5B). With Gal-1 depletion, the immobile fraction of H-rasG12V and H-rasG12V-R128A/R135A significantly decreased to ~0.26, whereas that of H-rasG12V-R169A/K170A decreased a little further down to ~0.17 (Fig. 5C). For comparison, the immobile fraction of H-rasG12V in Gal-1 knock-out MEFs was ~0.08. These data are in line with our STED-FCS data, which indicated that the nanoclustering behavior of H-rasG12V-R169A/K170A is more sensitive to Gal-1 levels than that of H-rasG12V or H-rasG12V-R128A/R135A. In summary, FRAP data revealed a mutation-specific dependence of the immobilization (nano-
clustering) response of our H-ras mutants on cellular Gal-1 levels.

c-Raf-RBD Recruitment Is Mutant-specifically Modulated by Gal-1 Levels—If changes in nanoclustering underlie the differential recruitment of the effector fragment RBD to H-ras orientation mutants, then Gal-1 levels should also modulate this process (Fig. 6A). We therefore again performed FLIM-FRET experiments in BHK cells and measured FRET of mGFP-tagged H-ras orientation mutants when coexpressed with the mRFP-tagged RBD at increasing Gal-1 levels (Fig. 6B and C).

As compared with the normal Gal-1 condition, ~2-fold overexpression of Gal-1 (Fig. 4, F and G) led to a small but significant increase of FRET for all mutants, indicating increased recruitment of the RBD. Importantly, the relative difference between the orientation mutants remained the same, i.e. the hyperactive mutant H-rasG12V-R169A/K170A showed the highest FRET (Fig. 6C). However, knockdown of Gal-1 drastically decreased the FRET to an extent that there were no more significant differences between the H-ras orientation mutants. Additional treatment with compactin did not further decrease FRET.

Thus, three Gal-1-dependent RBD recruitment responses were observed. Similar to the FRAP and STED-FCS data, we found that H-rasG12V-R169A/K170A displayed the largest change in FRET, when moving from low to normal Gal-1 levels. In addition, H-rasG12V FRET changes were smaller and identical to those of H-rasG12V-R128A/R135A. In conclusion, these FRET data confirmed our hypothesis that Gal-1-dependent differences in nanoclustering direct the H-ras orientation mutant-specific effector recruitment from the cytosol.

Computational Modeling Reveals That RBD Recruitment Depends on the Fraction and the Lifetime of H-ras Nanocluster—It was previously observed that the nanoclustered fraction of K-ras is similar to the fraction of the RBD recruited to Ras on the plasma membrane, consistent with recruitment of the RBD only to nanoclustered Ras (16). However, in addition to the fraction of Ras in nanoclusters, also the stability or lifetime of nanoclusters would affect how many Raf molecules can be recruited. For H-ras it is known that Gal-1 strongly regulates these parameters (10, 11), and it is plausible that they vary depending on the H-ras-GTP-Gal-1 complexation (18).

Because of the complexity of the nanocluster–reaction system with three interaction partners and many unknown molecular mechanistic details, we developed a computational model, which would help to understand the quantitative relationship between the fraction of nanoclustered H-ras mutants (Fig. 5C) and that of the recruited c-Raf-RBD (Fig. 6C). We incorporated current knowledge on Ras nanoclustering in our model system, which we otherwise tried to keep as simple as possible (Fig. 8 and supplemental Tables S1 and S2). In brief, we used a deterministic set of ordinary differential equations and solved them by numerical simulation. In our mathematical nanoclustering model, we assumed that an immobile nanocluster is formed.

### TABLE 2

| H-ras mutation                  | Overexpressed Gal-1 | Ratio of overexpressed Gal-1/endothelial Gal-1 ± S.E. |
|-------------------------------|---------------------|---------------------------------------------------|
| H-rasG12V-R169A/K170A         | mRFP-Gal-1          | 5.03 ± 1.02                                      |
| H-rasG12V                     | mRFP-Gal-1          | 6.36 ± 0.35                                      |
| H-rasG12V-R128A/R135A         | mRFP-Gal-1          | 4.69 ± 0.47                                      |
| Average                       | mRFP-Gal-1          | 5.44 ± 0.45                                      |
| Endogenous Ras                | pcDNA3-Gal-1        | 2.38 ± 0.16                                      |

| Mutants                      | Relative amount of Gal-1 | FRET, when moving from low to normal Gal-1 levels |
|------------------------------|--------------------------|--------------------------------------------------|
| H-rasG12V-R169A/K170A        | 10.0 ± 0.4               |                                                    |
| H-rasG12V-R128A/R135A        | 8.0 ± 0.4                |                                                    |
| H-rasG12V                    | 6.0 ± 0.4                |                                                    |
| H-rasG12V                   mRFP-Gal-1 | 4.0 ± 0.4               |                                                    |
| H-rasG12V-R169A/K170A        mRFP-Gal-1 | 2.0 ± 0.4               |                                                    |
| H-rasG12V-R128A/R135A        mRFP-Gal-1 | 1.0 ± 0.4               |                                                    |

*Average corresponds to the combination of the three mutants coexpressed with mRFP-Gal-1. ANOVA test showed that differences between mutants were statistically not significant (p = 0.63, n = 3).
With our model, we could reproduce several important experimental observations. We could capture the ratio between H-rasG12V and endogenous Gal-1 (1:2.5 experimentally and 1:2.3 by modeling) that was required to realize the same immobile fraction (Fig. 7A). The experimentally observed lower and upper limits in the immobile (nanoclustered) fraction were also found in the simulations. For all mutants, the simulations showed an immobile fraction obeying $0 < \text{immobile fraction} < 0.1$ in the absence of Gal-1. This is supported by our experimental FRAP data in Gal-1 knock-out MEFs, where we found an immobile fraction of $0.08 \pm 0.04$ (Fig. 5C). Also, the observed maximal immobile fraction of $\sim 0.6$ was properly reflected in our model. Most importantly, our model evidenced that small or even no differences in the nanoclustered fraction could lead to large differences in RBD recruitment (Fig. 7, A and B).

This explains our observation of the up- and down-modulated nanoclustering of the orientation mutants as compared with H-rasG12V. This can be seen especially when focusing on results obtained at low Gal-1 concentrations; H-rasG12V-R128A/R135A has low binding rates with Gal-1, as compared with its rate of association with another Ras into a growing nanocluster. This results in preferential incorporation of Gal-1 not into growing nanoclusters ($<0.1$ H-ras-GTP; Fig. 8) but into intermediate, less stable (Gal-1 devoid or poor) four H-ras-GTP-containing nanocluster configurations ($H_4G_n$; $n < 4$; Figs. 8 and 9A). Thus, relatively few fully stabilized and few less stable intermediate nanoclusters are formed by H-rasG12V-R128A/R135A under low Gal-1 conditions (Fig. 9B). Conversely, high Gal-1 binding rates favor binding of Gal-1 to free H-rasG12V-R169A/K170A and allow for the formation of many new nanoclusters (Fig. 9B) that include Gal-1 from early steps (Fig. 8, box ii). This H-ras mutant therefore effectively bypasses intermediate nanocluster configurations and goes straight to fully stabilized ($H_4G_n$) nanoclusters (Fig. 9B). At higher Gal-1 concentrations our simulation was allowed to follow the development of intermediate nanocluster number and stability, both of which decrease due to the formation of more and more fully stabilized nanoclusters (Fig. 9, C and D).

In conclusion, our computational model could capture essential quantitative features of our experimental data. It clarified that Gal-1 induced stabilization of nanoclusters and the H-ras mutant, specifically affect the fraction and lifetime of the various nanocluster configurations. This allows for specific recruitment rates of the RBD, even if the overall nanoclustered fraction is identical.

**DISCUSSION**

We have demonstrated H-ras membrane conformation/orientation-specific nanoclustering responses that allosterically regulate effector recruitment. We here use allosterism following the concept advanced by Kuriyan and Eisenberg (56), who proposed a broader definition that includes any structural and organizational changes that impact on the conformation or active site organization (57). Classical signaling defects of Ras activity can be readily detected by biochemical methods in solution. However, our findings would have gone unnoticed by classical biochemical approaches, due to the membrane-associated re-tuning of the Ras nanocluster system-response that leads to

---

**FIGURE 5.** H-ras orientation mutants display different galectin-1-dependent immobilization responses in FRAP experiments. A, schematic representation of nanocluster formation and effector recruitment. Gray box highlights the events that are studied by FRAP analysis. The fraction of H-ras present in (immobile) nanoclusters is identified by the immobile fraction of the fluorescence recovery. B, FRAP analysis was performed on BHK cells transiently expressing pmGFP-H-rasG12V-R169A/K170A, pmGFP-H-rasG12V, or pmGFP-H-rasG12V-R128A/R135A. For each mutant, measurements with endogenous Gal-1 levels were compared with those with Gal-1 depletion or mRFP-Gal-1 overexpression. An example of fluorescence recovery traces for endogenous Gal-1 levels were compared with those either depleted of Gal-1 or overexpressing mRFP-Gal-1. See under "Experimental Procedures" for details about statistical analysis (**, $p < 0.05$; ***, $p < 0.01$; ****, $p < 0.001$).

---

step-by-step and contains four H-ras-GTP molecules (Fig. 8). The rate of nanocluster formation depends on the number of proteins of the nanocluster scaffold Gal-1 and most importantly on the known H-ras-GTP orientation mutant-Gal-1 complexation tendencies (18). This was basically observed in the STED-FCS experiments (Fig. 3), which showed that Gal-1-dependent incorporation of our H-ras variants into nanocluster follows the order H-rasG12V-R169A/K170A > H-rasG12V $\geq$ H-rasG12V-R128A/R135A. Finally, any immobile nanocluster was assumed to be capable of recruiting the RBD for the duration of its lifetime.
profound and systematic alterations of Ras/MAPK signaling strength (17, 18).

Based on our results, we suggest the following model. Different H-ras orientation mutants that shift the conformational equilibrium of H-ras on the membrane have different abilities to form specific complexes that contain the nanoclustering scaffold protein Gal-1 (18). The exact structural details of this interaction are unknown, but the fact that membrane anchorage is required for a clear discrimination of the mutants is in agreement with different conformers having different complexation abilities. However, we cannot exclude at this point that the mutations on helix α4 or in the hvr directly perturb contacts between the two proteins. Alternatively, any complex that brings H-ras and Gal-1 into close proximity, i.e. within a FRET distance of ~5–7 nm, could be changed. The stability of these complexes determines the incorporation probability of H-ras into nanocluster and the lifetime of nanocluster, as evidenced by the combination of our experimental and simulation data. Longer lived nanoclusters can recruit more effectors to the membrane (Fig. 6), which determines the input into the downstream MAPK signaling cascade. This implies that Ras signaling scales with the number and stability of nanoclusters. Thus, our results significantly extend the previous model (10, 16, 58) by revealing a complex, allosteric coupling between H-ras membrane conformers, Gal-1, and nanoclustering that dictates effector recruitment and signaling. The distinct dependence of H-ras orientation mutant nanoclustering on cellular Gal-1 levels may explain why differences in nanoclustering were not found significant by electron microscopic analysis (17). Only our titration of cellular Gal-1 levels clearly revealed the different nanoclustering response profiles of the H-ras mutants.

It is generally conceivable that the fluorescent protein tags affect the affinity (here assessed by the total FRET) of interac-

FIGURE 6. Effector recruitment to H-ras orientation mutants correlates with Gal-1-dependent nanoclustering. A, schematic representation of nanocluster formation and effector recruitment. Gray box highlights the interaction between H-ras orientation mutants in nanoclusters and the c-Raf Ras binding domain (RBD) that is studied here. B, BHK cells transiently expressing pmGFP-H-rasG12V-R128A/R1365A and the acceptor mRFP-RBD were analyzed. Three different levels of galectin-1 were tested as follows: Gal-1 depletion, with or without 5 μM compactin treatment, normal/endogenous cellular Gal-1 level, and Gal-1 overexpression. Examples of FLIM-FRET images of cells, expressing H-rasG12V-R128A/R1365A under the above-described conditions. Image color look-up table on the right shows fluorescence lifetimes. C, apparent FRET efficiency was calculated from obtained fluorescence lifetimes. Blue vertical band represents the average ± S.D. of the FRET efficiencies of our three mutants with Gal-1 depletion combined with compactin treatment. The cellular total Gal-1 concentration relative to endogenous galectin-1 in control BHK cells ([Gal-1]rel) is displayed to the left of the data. Error bars correspond to the mean ± S.E., and numbers inside the bars correspond to the total number of cells imaged in each case. Three independent biological repeats were analyzed. Statistical differences between treated and untreated cells were tested; see under “Experimental Procedures” for details about statistical analysis (NS, nonsignificant, *, p < 0.05; **, p < 0.01; ***, p < 0.001).
Raf Recruitment Depends on Ras Conformer Nanoclustering

FIGURE 7. Computational modeling explains that the nanoclustered fraction and the nanocluster lifetime dictate specific RBD recruitment levels. We developed a mathematical model of nanocluster formation, which incorporates essential published experimental parameters. Thus, we could show that the RBD recruitment is determined by the nanoclustered fraction in conjunction with the nanocluster lifetime, which both depend on the H-ras orientation mutant-Gal-1 complexation. Our previous results suggest that RBD recruitment (input into the MAPK-pathway) correlates exactly with MAPK signaling output. A, nanoclustered (immobile) fractions of H-rasG12V-R169A/K170A (blue), H-rasG12V (gray), and H-rasG12V-R128A/R135A (green) displayed as a function of the ratio between the Gal-1 concentration and the H-ras concentration. Symbols correspond to the experimental data already presented in Fig. 5, and dotted lines to the simulation results. Error bars on the experimental data correspond to the mean ± S.E. of the nanocluster fraction (vertical) and of the Gal-1 concentration (horizontal). B, RBD recruitment (simulation, left axis) and apparent FRET efficiency (experimental, right axis) are displayed together as a function of the ratio between Gal-1 concentration and H-ras concentration. Symbols correspond to the experimental data already presented in Fig. 6, and dotted lines to the simulation results. Error bars on the experimental data correspond to ± S.E. of the apparent FRET efficiency (vertical) and of the Gal-1 concentration (horizontal).

FIGURE 8. Schematic representation of the different reaction paths that were used to computationally describe nanocluster formation. Starting from single H-ras-GTP and galectin-1 (Gal-1) molecules, we present the different paths studied with our simulation leading to nanoclustering. An immobile and functional nanocluster is assumed to be reached once there are four H-ras-GTP molecules bound together, independent of the number of bound galectin-1 molecules. The presence of galectin-1 on a nanocluster increases its stability. Buildup of nanoclusters takes place in a step-by-step fashion, where single molecules are added one at a time depending on the association and dissociation rates (a and b, respectively) of the respective complexation reaction. Not displayed here are collapse rates (c) for every multimer. Association, dissociation, and collapse rates vary systematically if additional molecules of H-ras-GTP (box i) or galectin-1 (box ii) are present in a given multimer. See supplemental Tables S1 and S2 and under “Experimental Procedures” for details.
can only be explained by the introduced mutations. Moreover, results obtained with the SNAP tag, which is not known to have a dimerization tendency, lead to comparable results as those with the mGFP tags (compare Figs. 3C and 5C). We therefore conclude that the fluorescent protein tags do not affect our conclusions on the H-ras orientation mutant-specific RBD recruitment.

Importantly, our data suggest that Ras mutations can alter signaling not only by classical alterations of its biochemical properties but also by affecting nanoclustering. This has two broad implications for Ras biology. First, we previously showed that the systematic change of the activity of the H-ras orientation mutants is representative of several if not all Ras paralogs (18). Therefore, our current results corroborate the exciting possibility that Ras paralog-specific signaling is critically affected by allosteric processes associated with membrane nanoclustering.

Second, we formerly also showed that orientation mutations on helix α4 and the hvr are linked to mutations in a novel switch III region, suggesting that the switch III is also involved in mediating membrane orientation of H-ras (17, 21, 22). Therefore, based on the results presented here, mutations in this switch III region may lead to differences in Ras nanoclustering. Intriguingly, a number of cancer and RASopathy associated mutations can be found in the β2-β3 loop and on helix α5 of Ras, which constitute the switch III (61).
mutations lead to increased nanoclustering and therefore augmented signaling output of Ras in a tumor growth-promoting fashion.

In summary, our results are significant, as they suggest that the Ras nanoclustering system broadly impacts Ras/MAPK signaling output in the physiological and pathophysiological context. Recent evidence suggests that certain approved drugs can have an impact on Ras nanoclustering (41, 62). Therefore, we propose that targeting Ras nanoclustering to block or modulate aberrant Ras functioning may have exciting therapeutic potential for cancer and other diseases.

Finally, we anticipate that our findings on H-ras will also have relevance for other membrane-anchored signaling proteins. We have provided evidence for nanoclustering of heterotrimeric G proteins, Rho and Rab small GTPases, as well as Src family kinases (50, 63, 64). Therefore, our results may represent another case, where insight into allosteric regulation of Ras by nanoclustering will be paradigmatic for other signaling proteins.

Acknowledgments—We thank Christina Oetken-Lindholm for technical help. We acknowledge Veronika Mueller and Alf-Honigmann (Max Planck Institute, Goettingen, Germany) for help with the STED-FCS measurements; Vladimir Belov and Gyuzel Mitronova (Max Planck Institute, Goettingen, Germany) for synthesis of the fluorescent lipid used for STED-FCS calibration, and Stefan Hell (Max Planck Institute, Goettingen, Germany) for ongoing generous support. We thank Alemayehu Gorfe (University of Texas at Houston) for the images in Fig. 1A.

REFERENCES
1. Mor, A., and Philips, M. R. (2006) Compartmentalized Ras/MAPK signaling. Annu. Rev. Immunol. 24, 771–800
2. Rocks, O., Peyer, A., and Bastiaens, P. I. (2006) Spatio-temporal segregation of Ras signals: One ship, three anchors, many harbors. Curr. Opin. Cell Biol. 18, 351–357
3. Vetter, I. R., and Wittinghofer, A. (2001) The guanine nucleotide-binding switch in three dimensions. Science 294, 1299–1304
4. Downward, J. (2003) Targeting RAS signalling pathways in cancer therapy. Nat. Rev. Cancer 3, 11–22
5. Ahearn, I. M., Haigis, K., Bar-Sagi, D., and Philips, M. R. (2012) Regulating the regulator: Post-translational modification of RAS. Nat. Rev. Mol. Cell Biol. 13, 39–51
6. Plowman, S. J., Muncke, C., Parton, R. G., and Hancock, J. F. (2005) H-ras, K-ras, and inner plasma membrane raft proteins operate in nanoclusters with differential dependence on the actin cytoskeleton. Proc. Natl. Acad. Sci. U.S.A. 102, 15500–15505
7. Hancock, J. F., and Parton, R. G. (2005) Ras plasma membrane signalling platforms. Biochem. J. 389, 1–11
8. Abankwa, D., Gorfe, A. A., and Hancock, J. F. (2007) Ras nanoclusters: Molecular structure and assembly. Semin. Cell Dev. Biol. 18, 599–607
9. Janosi, L., Li, Z., Hancock, J. F., and Gorfe, A. A. (2012) Organization, dynamics, and segregation of Ras nanoclusters in membrane domains. Proc. Natl. Acad. Sci. U.S.A. 109, 8097–8102
10. Rotblat, B., Belanis, L., Liang, H., Haklai, R., Elad-Zafia, G., Hancock, J. F., Kloog, Y., and Plowman, S. J. (2010) H-Ras nanocluster stability regulates the magnitude of MAPK signal output. PLoS ONE 5, e11991
11. Belanis, L., Plowman, S. J., Rotblat, B., Hancock, J. F., and Kloog, Y. (2008) Galectin-1 is a novel structural component and a major regulator of H-Ras nanoclusters. Mol. Biol. Cell 19, 1404–1414
12. Prior, I. A., Muncke, C., Parton, R. G., and Hancock, J. F. (2003) Direct visualization of Ras proteins in spatially distinct cell surface microdomains. J. Cell Biol. 160, 165–170
13. Shalom-Feuerstein, R., Plowman, S. J., Rotblat, B., Ariotti, N., Tian, T., Hancock, J. F., and Kloog, Y. (2008) K-ras nanoclustering is subverted by overexpression of the scaffold protein galectin-3. Cancer Res. 68, 6608–6616
14. Paz, A., Haklai, R., Elad-Sfadia, G., Ballan, E., and Kloog, Y. (2001) Galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation. Oncogene 20, 7486–7493
15. Rotblat, B., Niv, H., André, S., Kaltnner, H., Gabisius, H., and Kloog, Y. (2004) Galectin-1 (L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectively inhibits Ras-GTP. Cancer Res. 64, 3112–3118
16. Tian, T., Harding, A., Inder, K., Plowman, S., Parton, R. G., and Hancock, J. F. (2007) Plasma membrane nanoswitches generate high-fidelity Ras signal transduction. Nat. Cell Biol. 9, 905–914
17. Abankwa, D., Hanzal-Bayer, M., Ariotti, N., Plowman, S. J., Gorfe, A. A., Parton, R. G., McCammon, J. A., and Hancock, J. F. (2008) A novel switch region regulates H-ras membrane orientation and signal output. EMBO J. 27, 727–735
18. Abankwa, D., Gorfe, A. A., Inder, K., and Hancock, J. F. (2010) Ras membrane orientation and nanodomain localization generate isoform diversity. Proc. Natl. Acad. Sci. U.S.A. 107, 1130–1135
19. Gorfe, A. A., Hanzal-Bayer, M., Abankwa, D., Hancock, J. F., and McCammon, J. A. (2007) Structure and dynamics of the full-length lipid-modified H-Ras protein in a 1,2-dimyristoylgllycerol-3-phosphocholine bilayer. J. Med. Chem. 50, 674–684
20. Amor, J. C., Horton, J. R., Zhu, X., Wang, Y., Sullards, C., Ringe, D., Cheng, X., and Kahn, R. A. (2001) Structures of yeast ARF2 and ARL1: distinct roles for the N terminus in the structure and function of ARF family GTPases. J. Biol. Chem. 276, 42477–42484
21. Grant, B. J., McCammon, J. A., and Gorfe, A. A. (2010) Conformational selection in G-proteins: Lessons from Ras and Rho. Biophys. J. 99, L87–189
22. Grant, B. J., Gorfe, A. A., and McCammon, J. A. (2009) Ras conformational switching: Simulating nucleotide-dependent conformational transitions with accelerated molecular dynamics. PLoS Comput. Biol. 5, e1000325
23. Güldenhaupt, J., Rudack, T., Bacher, P., Mann, D., Triola, G., Waldmann, H., Köttig, C., and Gerwert, K. (2012) N-Ras forms dimers at POPC membranes. Biophys. J. 103, 1585–1593
24. Mazhab-Jafari, M. T., Marshall, C. B., Stathopulos, P. B., Kobashigawa, Y., Stambolic, V., Kay, L. E., Inagaki, F., and Ikura, M. (2013) Membrane-dependent modulation of the mTORC1 activator Rheb: NMR observations of a GTPase tethered to a lipid-bilayer nanodisc. J. Am. Chem. Soc. 135, 3367–3370
25. Kapoor, S., Triola, G., Vetter, I. R., Erkamp, M., Waldmann, H., and Winter, R. (2012) Revealing conformational substrates of lipidated N-Ras protein by pressure modulation. Proc. Natl. Acad. Sci. U.S.A. 109, 460–465
26. Kapoor, S., Weise, K., Erkamp, M., Triola, G., Waldmann, H., and Winter, R. (2012) The role of G-domain orientation and nucleotide state on the Ras isoform-specific membrane interaction. Eur. Biophys. J. 41, 801–813
27. Zhou, Z., Cironi, P., Lin, A. J., Xu, Y., Hrvatin, S., Golan, D. E., Silver, P. A., Walsh, C. T., and Yin, J. (2007) Genetically encoded short peptide tags for orthogonal protein labeling by Sfp and AcpS phosphopantetheinyl transferases. ACS Chem. Biol. 2, 337–346
28. Berrow, N. S., Alderton, D., Sainsbury, S., Nettleship, J., Assenberg, R., Rahman, N., Stuart, D. I., and Owens, R. J. (2007) A versatile ligation-independent cloning method suitable for high-throughput expression screening applications. Nucleic Acids Res. 35, e45
29. Tucker, J., Szakiel, G., Feuerstein, J., John, J., Goody, R. S., and Wittinghofer, A. (1986) Expression of p21 proteins in Escherichia coli and stereochemistry of the nucleotide-binding site. EMBO J. 5, 1351–1358
30. Taylor, S. J., and Shalloway, D. (1996) Cell cycle-dependent activation of Ras. Curr. Biol. 6, 1621–1627
31. Wong, K. A., Russo, A., Wang, X., Chen, Y.-J., Lavie, A., and O’Bryan, J. P. (2012) A new dimension to Ras function: a novel role for nucleotide-free Ras in class II phosphatidylinositol 3-kinase β (PI3KCIβ) regulation. PLoS ONE 7, e45360
32. Lakowicz, J. R. (2006) Principles of Fluorescence Spectroscopy, 3rd Ed, pp. 353–366, Springer, New York
Raf Recruitment Depends on Ras Conformer Nanoclustering

33. Zhang, X.-D., Dou, S.-X., Xie, P., Hu, J.-S., Wang, P.-Y., and Xi, X. G. (2006) Escherichia coli RecQ is a rapid, efficient, and monomeric helicase. J. Biol. Chem. 281, 12655–12663
34. LiCata, V. J., and WOWor, A. I. (2008) Applications of fluorescence anisotropy to the study of protein-DNA interactions. Methods Cell Biol. 84, 243–262
35. Levy, R., Biran, A., Poirier, F., Raz, A., and Kloor, Y. (2011) Galectin-3 mediates cross-talk between K-Ras and Let-7c tumor suppressor microRNA. PLoS ONE 6, e27490
36. Majoul, I., Jia, Y., and Duden, R. (2006) Handbook of Biological Confocal Microscopy, pp. 788–808, Springer, New York
37. Mueller, V., Ringemann, C., Honigmann, A., Schwarzmann, G., Medda, R., Leutenegger, M., Polyakova, S., Belov, V. N., Hell, S. W., and Eggeling, C. (2011) STED microscopy reveals molecular details of cholesterol- and cytoskeleton-modulated lipid interactions in living cells. Biophys. J. 101, 1651–1660
38. Lukinavicius, G., Umezawa, K., Olivier, N., Honigmann, A., Yang, G., Plass, T., Mueller, V., Reymond, L., Correa, I. R., Luo, Z.-G., Schultz, C., Lemke, E. A., Heppenstall, P., Eggeling, C., Manley, S., and Johnsson, K. (2013) A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. Nature Chemistry 5, 132–139
39. Kepper, A., Pick, H., Arrivoli, C., Vogel, H., and Johnsson, K. (2004) Labeling of fusion proteins with synthetic fluorophores in live cells. Proc. Natl. Acad. Sci. U.S.A. 101, 9955–9959
40. Feder, T. J., Brust-Mascher, I., Slattery, J. P., Baird, B., and Webb, W. W. (1996) Constrained diffusion or immobile fraction on cell surfaces: a new interpretation. Biophys. J. 70, 2767–2773
41. Cho, K.-J., Kasai, R. S., Park, J.-H., Chigurupati, S., Heidorn, S. J., van der Sloot, A. M., Garcia-Olivas, R., Mallabiabarrena, A., Sanjuan, X., Zimmermann, T., and Serrano, L. (2013) Real-time single-molecule co-immunoprecipitation analyses reveal cancer-specific Ras signalling dynamics. Nat. Commun. 4, 1505
42. Hibino, K., Watanabe, T. M., Kozuka, J., Iwane, A. H., Okada, T., Kataoka, T., Yanagida, T., and Sako, Y. (2003) Single- and multiple-molecule dynamics of the signaling from H-Ras to cRaf-1 visualized on the plasma membrane of live cells. Chem. Phys. 281, 775–793
43. Li, H., and Gorfe, A. A. (2013) Aggregation of lipid-anchored full-length H-Ras in lipid bilayers: simulations with the MARTINI force field. PLoS ONE 8, e71018
44. Gurry, T., Kuharamanooglu, O., and Endres, R. G. (2009) Biophysical mechanism for ras-nanocluster formation and signaling in plasma membrane. PLoS ONE 4, e6148
45. Li, Z., Janosi, L., and Gorfe, A. A. (2012) Formation and domain partitioning of H-Ras peptide nanoclusters: effects of peptide concentration and lipid composition. J. Am. Chem. Soc. 134, 17278–17285
46. Spoermer, M., Herrmann, C., Vetter, I. R., Kalbitzer, H. R., and Wittinghofer, A. (2001) Dynamic properties of the Ras switch I region and its importance for binding to effectors. Proc. Natl. Acad. Sci. U.S.A. 98, 4944–4949
47. Kiel, C., Aydin, D., and Serrano, I. (2008) Association rate constants of ras-effector interactions are evolutionarily conserved. PLoS Comput. Biol. 4, e1000245
48. Lee, H.-W., Kyung, T., Yoo, J., Kim, T., Chung, C., Ryu, J. Y., Lee, H., Park, K., Lee, S., Jones, W. D., Lim, D.-S., Hyeon, C., Heo, W. D., and Yoon, T.-Y. (2013) Raf inhibitors target ras spatiotemporal dynamics. Proc. Natl. Acad. Sci. U.S.A. 110, 1799–1809
49. Nussinov, R. (2013) The spatial structure of cell signaling systems. Phys. Biol. 10, 045004
50. Tian, T., Plowman, S. J., Parton, R. G., Kloor, Y., and Hancock, J. F. (2010) Mathematical modeling of K-Ras nanocluster formation on the plasma membrane. Biophys. J. 99, 534–543
51. Prior, I. A., Lewis, P. D., and Mattos, C. (2012) A near-infrared fluorophore for live-cell super-resolution microscopy. Methods Enzymol. 519, 277–302
52. Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S., Belov, V. N., Hein, B., von Middendorff, C., Schiète, A., and Heil, S. W. (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. Nature 457, 1159–1162
53. Murakoshi, H., Iino, R., Kobayashi, T., Fujiwara, T., Ohshima, C., Yoshimura, A., and Kusumi, A. (2004) Single-molecule imaging analysis of Ras activation in living cells. Proc. Natl. Acad. Sci. U.S.A. 101, 7317–7322
54. Lommerse, P. H., Snaar-Jagalska, B. E., Spalink, H. P., and Schmidt, T. (2005) Single-molecule diffusion measurements of H-Ras at the plasma membrane of live cells reveal microdomain localization upon activation. J. Cell Sci. 118, 1799–1809
55. Kuriyan, J., and Eisenberg, D. (2007) The origin of protein interactions and allostery in colocalization. Nature 450, 983–990
56. Grunberg, R., Burnier, J. V., Ferrar, T., Beltran-Sastre, V., Stricher, F., van der Sloot, A. M., Garcia-Olivas, R., Mallabiabarrena, A., Sanjuan, X., Zimmermann, T., and Serrano, L. (2013) Engineering of weak helper interactions for high-efficiency FRET probes. Nat. Methods 10, 1021–1027
57. Prior, I. A., Lewis, P. D., and Mattos, C. (2012) A comprehensive survey of Ras mutations in cancer. Cancer Res. 72, 2457–2467
58. Zhou, Y., Cho, K.-J., Plowman, S. I., and Hancock, J. F. (2012) Nonsteroidal anti-inflammatory drugs alter the spatiotemporal organization of Ras proteins on the plasma membrane. J. Biol. Chem. 287, 16586–16595
59. Abankwa, D., and Vogel, H. (2007) A FRET map of membrane anchors suggests distinct microdomains of heterotrimeric G proteins. J. Cell Sci. 120, 2953–2962
60. Najumudeen, A. K., Köhne, M., Solman, M., Alexandrov, K., and Abankwa, D. (2013) Cellular FRET-biosensors to detect membrane targeting inhibitors of N-myristoylated proteins. PLoS ONE 8, e66425