N-Ras Forms Dimers at POPC Membranes

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ABSTRACT Ras is a central regulator of cellular signaling pathways. It is mutated in 20–30% of human tumors. To perform its function, Ras has to be bound to a membrane by a posttranslationally attached lipid anchor. Surprisingly, we identified here dimerization of membrane-anchored Ras by combining attenuated total reflectance Fourier transform infrared spectroscopy, biomolecular simulations, and Förster resonance energy transfer experiments. By analyzing x-ray structural models and molecular-dynamics simulations, we propose a dimerization interface between α-helices 4 and 5 and the loop between β2 and β3. This seems to explain why the residues D47, E49, R135, R161, and R164 of this interface are influencing Ras signaling in cellular physiological experiments, although they are not positioned in the catalytic site. Dimerization could catalyze nanoclustering, which is well accepted for membrane-bound Ras. The interface could provide a new target for a seemingly novel type of small molecule interfering with signal transduction in oncogenic Ras mutants.

INTRODUCTION

More than 150 different small GTPases regulate a great variety of cellular processes (1). They all share a common G domain that usually exists in a signaling (GTP-bound) state or an inactive (GDP-bound) state. Small GTPases are switched on by exchanging GDP for GTP, a process catalyzed by guanine nucleotide exchange factors, and are switched off by GTP hydrolysis, which is catalyzed by GTPase-activating proteins. The molecular reaction mechanism of small GTPases, especially Ras, has been analyzed in detail using a large variety of experimental techniques such as x-ray structure analysis (reviewed recently in Wittinghofer and Vetter (2)), NMR (3), Fourier transform infrared (FTIR) spectroscopy (reviewed recently in Kötting et al. (4)), and other biophysical techniques (5,6). To complement these studies, the molecular reaction mechanism has also been theoretically studied using biomolecular simulations (7–9). These provide significant insight into the switch mechanism and its catalysis by effector proteins.

However, for GTPases to perform their functional roles within the living cell, membrane targeting plays an additional and important role. Most small GTPases are membrane-anchored. In the case of Ras, the various attached lipid anchors at the C-terminus of different isoforms (H-, N-, and K-Ras) lead to specific compartmentalization of the molecule, and the dynamics of this compartmentalization is influenced by enzymes that reverse the anchorage by cleaving certain lipid moieties (10). The availability of lipated Ras proteins (11–13) has triggered many studies of Ras-membrane interactions. It has been shown that the structure of the G domain and its molecular switch mechanism is conserved even when Ras is membrane-bound (4,14). N-Ras containing one farnesyl and one palmitoyl group is shown to cluster at the boundary of lipid rafts (15), and pressure-driven conformational substrate selection was found to be influenced by the membrane (16).

Additionally, molecular-mechanics (MM) simulations of an N-Ras peptide (17), H-Ras (18), and K-Ras (19) were performed. The H-Ras and K-Ras simulations show possible orientation changes of the G domain between the GDP- and GTP-bound states, which might be relevant to the activation of signaling pathways. This movement results in different membrane interactions of the amino acids R128, R135, R169, and K170 in H-Ras. These residues are therefore believed to be functionally important for the activation mechanism. In vivo experiments confirm that mutations at these residues affect signal transduction (19,20).

In this study, we have extended our attenuated total reflectance Fourier transform infrared (ATR-FTIR) experiments on membrane-anchored Ras. We analyzed the orientation of a lipated Ras molecule bound to a single membrane bilayer using polarized light with a novel experimental setup (4). This setup allows simultaneous Förster resonance energy transfer (FRET) measurements in addition to the infrared measurements. Furthermore, FRET provides spatial resolution because it enables distance measurements between neighboring Ras proteins. The study is complemented by the analyses of the orientation of membrane-anchored Ras during MM simulations. All three applied techniques are in full agreement and indicate that Ras is
bound to the lipid bilayer as a dimer or oligomer, in an orientation perpendicular to the membrane, in contrast to an earlier proposal (18). Mutations in the amino acids D47, E49, R135, R161, and R164 that affect signaling seem to influence Ras dimerization, but not the interaction of Ras with the membrane. This is an alternative explanation for previous in vivo experimental observations (19,20). Interestingly, membrane-driven dimerization of Ras was proposed more than a decade ago (21). Dimerization could catalyze nanoclustering, which is well accepted for membrane-bound Ras. These nanoclusters enable strong signal bursts that lead to high-fidelity signal transmission (22).

MATERIALS AND METHODS

Materials

We purchased 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; Lipoid, Ludwigshafen, Germany). Lipid solutions were prepared to a concentration of 32 mM in chloroform. Lipid vesicle solutions were prepared in buffer (10 mM Tris/HCl, pH 7.4, 5 mM MgCl2). A 9.5 g/L protein stock solution was used for the injection of N-Ras protein into the binding buffer solution (20 mM Tris/HCl, pH 7.4, 5 mM MgCl2, 1 mM DTT, 0.1 mM GDP) that bathed the adsorbed POPC model membrane. The nucleotide exchange was performed in buffer E (50 mM 2-(O-morpholinoo)ethanesulfonic acid, 100 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine, pH 6.5). The semisynthetic N-Ras protein used in these experiments was prepared as described elsewhere (11,13,14,23).

Preparation of ATR crystals

Before each measurement, both sides of the germanium internal reflection element (IRE) were polished for ~10 min by machine (Logitech CP50; Logitech, Old Kilpatrick, Scotland) with a diamond-polishing suspension (0.1 μm, Logitech) on a hard cloth (Microtex 500 HC-W; Logitech) that rotated at 40 rpm. After rinsing the IRE with Milli-Q water (Millipore, Billerica, MA), the hydrophilic character of the IRE surfaces was attained by dipping the IRE for 10 min into a concentrated sulfuric acid solution, followed by rinsing, and then drying under a nitrogen gas flow. To further enhance the hydrophilic character of the IRE and to remove small traces of organic compounds, we finally treated the IRE for 15 min with an air plasma (Harrick Scientific, Pleasantville, NY).

Bilayer preparation and protein immobilization

The solid-supported-lipid bilayer was prepared by spreading vesicles onto hydrophilic IRE surfaces. After solid-supported-lipid-bilayer preparation and the subsequent washing steps, the lipid-anchored N-Ras was added to the circulating flow-through system. This resulted in a slow immobilization of the protein. Both procedures have been described in detail previously (4,14).

ATR measurements and spectra analyses

ATR-FTIR measurements were performed with a Vertex 80V-spectrometer (Bruker Optik, Ettlingen, Germany) at 293 K, with a spectral resolution of 2 cm⁻¹ and a scanner velocity of 80 kHz; scans were performed in the double-sided forward-backward mode. The resulting interferograms were apodized with the Blackman-Harris three-term function and with a zero filling factor of 4. The IRE was a 52 mm × 20 mm × 2 mm trapezoidal germanium ATR plate with an aperture angle of 45°. Only one side of the IRE was used, which resulted in 13 active reflections.

Nucleotide exchange

Before the nucleotide exchange, the binding buffer in the cuvette was replaced by the exchange buffer E, we took the following steps:

Step 1. The system was washed with buffer E supplemented with 5 mM MgCl₂ and 0.1 mM GDP. After 10 min, the entire binding buffer in the cuvette was replaced, and a reference spectrum was recorded.

Step 2. The surface was incubated with buffer E supplemented with 1 mM EDTA + 0.1 mM of the desired nucleotides (Mnt-GDP, TNP-GDP, or GDP) for 30 min. This lowered the nucleotide affinity of Ras, because Mg²⁺ was chelated by EDTA (25). Due to the excess of available nucleotides, the former bound nucleotides are exchanged for the desired nucleotides.

Step 3. The cuvette was flushed with buffer E supplemented with 5 mM MgCl₂ and the used nucleotides for 6 min. This ensured strong binding of the nucleotides.

Step 4. The buffer was exchanged with the same buffer used for the reference spectra in Step 1, and time-correlated single-photon counting (TCSPC) measurements were performed in parallel to the ATR-FTIR measurements.

MM simulations

MM simulations were performed with GROMACS 4.0.7 (26,27) and the optimized-potentials-for-liquid-simulations all-atom force field (28). Quantum mechanics (QM) simulations were performed with Gaussian03 (29), and QM/MM simulations with the GROMACS/Gaussian interface (30) and the normal QM/MM scheme (31). The simulation system consisted of a full-length N-Ras protein attached to a POPC membrane in a triclinic box filled with TIP4P water and physiological sodium chloride concentration.

Further details about the theoretical methods can be found in the Supporting Material.

TCSPC measurements and analysis

Lifetime measurements were carried out by means of TCSPC. Data acquisition was established with a setup from PicoQuant (Berlin, Germany) consisting of a pulsed laser-diode, emitting at 375 nm (LDH-P-C-470B; pulse-width at full width at half-height <70 ps) driven by a PDL 800-D laser driver, a photomultiplier detector module (PMA 182-N-M) equipped with a filter-holder, and a Pico Harp 300 Photon-Counting System. To avoid pile-up effects, the excitation rate (10 kHz) and the applied laser intensity were coordinated such that the detected photon count rate did not exceed 1% of the excitation rate during the measurements. Photon events were stored in histogram bins of 32-ps width and the integration time for each histogram was 10 s. A fiber-bundle (Reflection Probe FCR-7-UV-400-2 Meter; Avantes, Apeldoorn, Netherlands) exhibiting 6±1 fibers, two FC/APC-terminations, and one probe-end was used. The sample was excited via the single-fiber-channel; photon detection was utilized by the six-fiber channel. The probe-end was fed through the side-plate of the spectrometer and fixed tightly above the sample surface, facing it at ~45°. Thereby, sample excitement and fluorescence detection could be achieved simultaneously.

To avoid internal back-reflection artifacts, all three endings of the fiber-bundle were ground by 8°. A proper bandpass-filter (ET450/50m; AHP Analysetechnik, Tübingen, Germany) was used to ensure the detection of the donor fluorescence without disturbance from acceptor fluorescence, scattered light, or autofluorescence.
Because the quantum-yield of TNP is approximately three orders-of-magnitude lower than that of Mant, the FRET efficiency was obtained solely by detecting the lifetime of donor fluorescence in both the presence \((\tau_{DA})\) and absence \((\tau_{D})\) of the acceptor without any observations of the fluorescence lifetime of the acceptor.

Fluorescence measurements were performed for three different nucleotide-bound states of Ras that were produced by nucleotide exchange, described above. A reference-state \(G\) (GDP), a donor-state \(GxM\) (Mant-GDP and GDP), and a FRET-state \(GxT\) (Mant-GDP and TNP-GDP) were measured in buffer E supplemented with 5 mM MgCl\(_2\). Histograms (10 s integration time, 20 min recording time) of the respective sample states (reference, donor, and FRET) were averaged, yielding a single histogram for each state. Measurements were performed twice in sequence, resulting in the following experiment chronology:

\[
G – MxG – MxT – G – MxG – MxT – G.
\]

In this way, two consecutive MxG and MxT measurements were preceded and followed by two G reference measurements. The G steps were linearly interpolated to approximate reference histograms at the time when MxG and MxT were measured.

Reference histograms were subtracted from the histograms of MxG and MxT to compensate for autofluorescence and scatter-light. Data were analyzed using FluoFit Pro software (PicoQuant). Reference-corrected histograms were iteratively convoluted, using an instrument response function (IRF), and fitted biexponentially using Eq. 1,

\[
I(t) = \int_{-\infty}^{t} IRF(t') \sum_{i=1}^{2} A_i e^{-t'/\tau_i} dt',
\]

where \(t\) is the time [ns], \(A_i\) is the amplitude of the respective exponential term, and \(\tau_i\) is the corresponding lifetime.

The IRF was obtained by TCSPC by illuminating a diluted Ludox solution (Ludox PX 30 Kieselsol; Deffner & Johann, Röthlein, Germany) at 10 MHz in an absolutely dark environment. IRF measurements were performed with similar laser driver settings and the same fiber as used in the remaining FRET experiments presented here, but without filters.

For all states, biexponential fitting yielded good agreement with the data. The two obtained lifetimes of ~3 ns and ~8 ns were averaged using amplitude weighting as determined by Eq. 2:

\[
\tau_{aw} = \frac{A_1 \cdot \tau_1 + A_2 \cdot \tau_2}{A_1 + A_2}.
\]

The FRET efficiency, \(E\), was calculated using the amplitude-weighted lifetimes with (state MxT, \(\tau_{MxT}\)) and without (state MxG, \(\tau_{MxG}\)) the acceptor, according to Eq. 3:

\[
E = 1 - \frac{\tau_{DA}}{\tau_D} = \frac{R_0^6}{R_0^6 + r^6} \Rightarrow r = \left(\frac{R_0^6 (1 - E)}{E}\right)^{\frac{1}{6}}.
\]

Measurements were performed twice in sequence and averaged. The differences of the efficiencies in between single measurements are <1.5%.

**Determination of the Förster radius \(R_0\)**

The radius is given as

\[
R_0 = 0.211 \cdot (\kappa^2 n^{-4} Q_0 J)^{\frac{1}{6}}.
\]

Here, \(\kappa^2\) is the orientation factor of the transition dipoles, \(Q_0\) is the quantum yield of donor fluorescence in the absence of FRET, \(J\) is the integral spectral overlap of the normalized donor emission and the molar acceptor absorption, and \(n\) is the refractive index of the medium between donor and acceptor, which was set to 1.4.

\(Q_0\) and \(J\) were derived experimentally whereas \(\kappa^2\) was obtained by MD simulations. Details are given in the Supporting Material.

**RESULTS AND DISCUSSION**

**Dichroitic-ATR FTIR experiments on lipidated Ras**

Dichroitic ATR-FTIR spectroscopy is an excellent technique to determine the orientation of lipid-anchored Ras. A single POPC bilayer is immobilized at a germanium surface and subsequently N-Ras is bound via a semisynthetic anchor (4,14) (see Fig. S1 A in the Supporting Material). The monolayer of membrane-bound Ras proteins is stable for several days. To determine the orientation of lipid-bound Ras, we used polarized infrared light. The amide I mode of an \(\alpha\)-helix (C=O) is almost parallel to the helix, whereas the amide II mode (NH) is almost perpendicular to the helix (32). Therefore, if an \(\alpha\)-helix is oriented perpendicular to the membrane, parallel-polarized light interacts more strongly with the amide I mode than vertical polarized light, resulting in a positive dichroic difference \(D^*\) (see Fig. S1 B in the Supporting Material). Just the opposite effect is expected for the amide II mode, because it is almost perpendicular to the amide I mode (32–34). The resulting dichroic difference spectrum \(D^*\) for amide I and amide II has a characteristic pattern that clearly indicates the orientation of the \(\alpha\)-helix.

Polarized absorption spectra and the dichroic difference spectrum \(D^*\) for the amide I and amide II bands of N-Ras were recorded (Fig. 1). The amide I band at 1661 cm\(^{-1}\), typical for an \(\alpha\)-helix, is positive, and the amide II at 1551 cm\(^{-1}\), typical for \(\beta\)-sheet, is negative. The dichroic difference spectrum \(D^*\) for amide I and amide II has a characteristic pattern that clearly indicates the orientation of the \(\alpha\)-helix.

**FIGURE 1** Binding of lipidated Ras to a solid supported POPC bilayer. Calculating the dichroitic difference spectra \(D^*\) allows the determination of the orientation of the absorbing groups, e.g., \(\alpha\)-helices. The calculation of \(D^*\) of Ras shows a positive band in the amide I and a negative band in the amide II regions; these band positions are indicative of \(\alpha\)-helices. Therefore, the average of all Ras \(\alpha\)-helices is oriented more perpendicular to the membrane.
1545 cm\(^{-1}\), also typical for an \(\alpha\)-helix (32), is negative. This pattern clearly indicates that the majority of the helical content of Ras is oriented perpendicular to the membrane.

Seventy-percent of the helical content of Ras is found in helices 3, 4, and 5, all of which have the same orientation. To find an overall measure for the average orientation of Ras relative to the membrane, we defined the angle \(\alpha\) (Fig. 2). It is the angle between the membrane normal and a vector that represents the mean orientation of all helices. The latter is obtained by the simple addition of the vectors for each helix. The length of each vector corresponds to the length of the helix, while the direction is obtained by a regression line through the backbone atoms along the helix. A perpendicular orientation with a small angle \(\alpha\) (Fig. 2 A (I)) represents our experimental result. Unfortunately, an orientation with a large angle \(\alpha\) (Fig. 2 A (II)) as proposed by Gorfe et al. (17) does not agree with our experimental results.

**Molecular-mechanics simulations of Ras monomers**

The proposal of a parallel-oriented Ras results from MM simulations of H-Ras (18) and K-Ras (19) bound to a DMPC membrane with a maximum simulation time of 40 ns. Here, we extended the approach of Gorfe et al. (18) and Abankwa et al. (19) and performed several longer (200 ns) MM simulations of the full-length N-Ras bound to a POPC membrane which matches the conditions of our experimental setup. We analyze the angle \(\alpha\) of our simulations, which is revealing the orientation of the protein regarding to the membrane. This orientation is the only available observable, which can be compared directly to experimental results. To create independent simulations runs, we started our simulations with three different orientations (\(\alpha = 12^\circ, 36^\circ, \text{or} 82^\circ\)) of the N-Ras G domain including the structures I and II (Fig. 2 A). In the start structures, there are no contacts between the G domain and the membrane. We deleted one lipid from the membrane. Close to this missing membrane lipid, we positioned the free ends of the side chains of the two lipid moieties of the protein anchor. The last carbon atom of each lipid protein anchor is placed directly above the plane through the phosphatidycholines of the membrane. Thus, the penetration depth of the anchor side chains is zero. This is necessary because it is impossible to achieve a spontaneous peptide insertion within the accessible timescales (i.e., those available as of this writing) of MM simulations (17). After 50 ns, the protein is stably attached to the membrane (see Fig. S2 in the Supporting Material). This attachment process is artificial but essential to prevent any predetermined attachment to the membrane.

The orientation of Ras monomers during the simulation can be followed by the angle \(\alpha\) (Fig. 2 A). Independent of differences in the starting structure, the orientation becomes stable after ~50 ns and results in all cases in a G domain orientation of ~80°, thus parallel to the membrane. The N-Ras monomer shows an orientation parallel to the POPC membrane, in agreement with the earlier simulations of K-Ras and H-Ras attached to a DMPC membrane by Gorfe et al. (18) and Abankwa et al. (19). This is still in sharp contrast to the experimental value of \((23 \pm 2)^\circ\) from the

**FIGURE 2 Orientation of N-Ras-GDP at a POPC membrane. The angle \(\alpha\) between the \(\alpha\)-helix vector (red arrow) and the normal of the membrane (black arrow) defines the orientation. The \(\alpha\)-helix vector is the vector sum of the regression line through the backbone atoms of each helix of the protein. According to our MM simulations, a Ras monomer (A) always orients with a large \(\alpha\) as shown in orientation (I). Only a Ras dimer (B) is stable with a small angle \(\alpha\), which is in accordance with our dichroic FTIR measurements.**
Molecular-mechanics simulations of Ras dimers

An evaluation of all published x-ray structural models of Ras showed that Ras forms dimers in 50 out of the 71 structures. Even among crystals with four different space groups, the same Ras dimer is formed (see Fig. S3 in the Supporting Material). Can Ras dimerization resolve the contradiction?

To elucidate the influence of dimerization on the orientation of membrane-bound Ras, MM simulations with Ras dimers based on the crystal structure PDB:1QRA were performed (Fig. 2 B). We set up the simulation system for the Ras dimer by following the same protocol as for the Ras monomer. But, due to dimerization, there are four lipid moieties that have to be positioned close to the membrane. Therefore, the range of possible starting structures without direct G domain membrane contact is smaller for the dimer than for the more flexible monomer. The simulations were started with \( \alpha \)-values of 3°, 5°, and 17° and run for 200 ns each. After 50 ns, a stable attachment (see Fig. S2 in the Supporting Material) and orientation at the membrane were obtained. The last 150 ns of the simulation trajectories were used to analyze the dimer structure. Independent of the start orientation, the Ras dimer has an averaged angle \( \alpha \) of 21°, in full agreement with the experimentally observed value of (23 ± 2)° meaning a perpendicular orientation (structure III in Fig. 2). Dimerization resolves the contradiction; now our MM simulations and dichroitic IR measurements are in full agreement.

Dimerization measured by FRET experiments

To provide additional independent experimental evidence for dimerization, FRET experiments were performed. In FRET experiments, the distance between two neighboring Ras proteins is determined, by measuring the energy transfer between two fluorescence labels attached to individual Ras monomers (Fig. 3). In these experiments, we used the same setup for membrane-anchored Ras as was previously used for dichroitic ATR-FTIR measurements. For FRET analysis, the fluorescence lifetimes were measured by the mentioned time-correlated single-photon counting setup. The fluorescence lifetimes of Ras-Mant-GDP in the presence (\( \tau_{DA} \)) and absence (\( \tau_D \)) of the acceptor (Ras-TNP-GDP) were determined. From these values, the FRET efficiency \( E \) was determined according to

\[
E = 1 - \frac{\tau_{DA}}{\tau_D}
\]  

(5)

The obtained histograms (Fig. 4) clearly show that the lifetime is reduced in the presence of the acceptor. For a quantification of the FRET effect, the histograms were analyzed as described above (Eqs. 1 and 2). The average lifetime decreased in the presence of an acceptor from 5.5 ns to 4.9 ns. This corresponds to a measured FRET efficiency of ~11%. Assuming a dimer, the actual FRET effect is even larger than that measured. Due to the statistical distribution of the nucleotides, only a certain fraction of the Ras dimers carry both a Mant- and a TNP-GDP (Fig. 3), whereas others carry two Mant or two TNP nucleotides. Therefore, the measured FRET efficiency is reduced by
a factor of 2.57. Taking this into account, the FRET efficiency in the actual Mant-TNP-dimers is calculated to be 28% (see the Supporting Material for a detailed analysis). The observed FRET effect can only be explained by a neighboring Ras in a dimer or oligomer, thereby confirming our experimental and theoretical observation of perpendicularly oriented Ras dimers.

In contrast to membrane-bound lipidated Ras, Ras without lipid anchor in solution (10 μM) exhibits a FRET efficiency of only 3.5%. Therefore, dimerization in solution can be excluded under physiological concentrations. The results in solution point to a low-affinity complex. Dimerization clearly depends on the high local concentrations created due to membrane binding. To exclude surface concentration effects under our experimental conditions, dichroitic binding kinetics (see Fig. S4 in the Supporting Material) were measured. They showed that D* is independent of surface loading, and therefore the perpendicular orientation is not a nonspecific effect of molecular crowding. Additionally, in the FRET measurements, we used only 8% surface loading to avoid FRET due to crowding. However, even a random distribution of this surface concentration may induce a FRET signal. A theoretical FRET efficiency analysis according to Wolber and Hudson (35) for a random distribution at the experimental surface concentration of Ras (see the Supporting Material) results in a 6% FRET efficiency. This is much smaller than the observed efficiency of 11%, confirming that the observed FRET effect is caused by Ras dimerization and not by randomly concentrated Ras on the ATR surface.

The Ras-Ras distance

From the Förster efficiency, we can calculate the FRET distance \( r_{\text{exp}} \) according to Eq. 6,

\[
E = 1 - \frac{\tau_D}{\tau_A} = \frac{1}{1 + \left( \frac{r_{\text{exp}}}{R_0} \right)^6},
\]

where \( R_0 \) is the Förster radius and \( r_{\text{exp}} \) is the distance between donor and acceptor (Fig. 3). \( R_0 \) is calculated by applying Eq. 4.

Here, \( \kappa^2 \) is determined by the overlap of the fluorescence labels and depends on their dynamics (36). Often a value of 2/3, which is the value for isotropic distribution, is assumed for \( \kappa^2 \). Under the experimental conditions, the dynamics of the labels might be restricted at the surface. Therefore, we performed MM simulations using the experimental conditions and arrived at a lower \( \kappa^2 \) of 0.41. The corresponding standard deviation of 0.31 indicates orientational flexibility, but the mean value is slightly smaller than the commonly used value of 2/3, which shows that a small part of the configuration space is not populated. Further details on the calculation of \( \kappa^2 \) and \( R_0 \), including the quantum yield \( Q_0 \) and the overlap integral \( J \), are explained in detail in the Supporting Material. The Förster radius is calculated to be 40 Å. For the measured FRET efficiency of 28%, this translates to a FRET distance of 46 ± 3 Å. The MM simulation of the proposed dimer results in an \( r_{\text{calc}} \) of 46 ± 3 Å (average of three dimer simulations). This result confirms the presence of lipid-anchored Ras dimers and agrees nicely with the structural dimer model (Fig. 3), as observed in most x-ray structures.

The Ras dimer interaction surface

The last 150 ns of the simulation trajectories (Fig. 2 B) were used to analyze the dimer structure. The interaction surface of the dimer is mainly composed of α-helices 4 and 5 and the loop between β2 and β3. A scheme of stable amino-acid interactions of the equilibrated dimer at the membrane was acquired (Fig. 5). The salt bridge between Arg161 and Asp154 remains stable during our simulations. Some additional interactions that are not observed in the x-ray structural models are formed (Fig. 3), namely interactions of Lys135 with Glu49 and the π-π interaction between Glu49 and His131. The interface containing both salt bridges is stabilized by an intramolecular connection between β2, β3 loop and α-Helix 5 through the salt bridge of Asp47 with

![FIGURE 5 Ras dimerization interface. Stable amino-acid interactions include the salt bridges (magenta circles) between Arg and Asp and between Lys and Glu. Arg further participates via π-π interactions with His and Met and Gly by van der Waals interactions (cyan squares). Intramolecular interactions stabilizing the network are found between Glu and Arg (light blue and light green circles). Amino acids that show impact in cell experiments (red).](image_url)
Arg$^{164}$ and Arg$^{161}$ of the same Ras protein. The dimer is further stabilized by van der Waals interaction between Met$^{168}$ and Gly$^{138}$. The surface representation of the interface is given (see Fig. S10 in the Supporting Material). While His$^{131}$ is found only in N-Ras, all the other residues are not isoform-specific. Thus, a similar interaction surface should exist in H-Ras and K-Ras. Mutations in a neighboring residue, Asn$^{153}$, are related to the Noonan (K-Ras) and Costello (H-Ras) syndromes (37). In summary, the residues in helices a, α5 and the loop between β2 and β3 form a stable interaction network, which results in a stable interface. Mutations of the crucial amino acids should destroy the dimer and lead to a parallel Ras orientation.

Impact of dimer mutants on the situation in the cell

In principle, the experimental result shows a dimer, but oligomerization of Ras cannot be excluded. However, the crystal contacts and the results of the MM simulations clearly favor a dimer. Although the observation of Ras dimers in highly concentrated x-ray crystals does not automatically imply that dimer formation occurs in vivo, it has to be taken into account that the local protein concentration at the membrane in vivo may be comparable to crystallization conditions. Considering the height of Ras (~5 nm height) as the third dimension and thereby approximating the accessible diffusion volume of membrane-bound Ras, the local concentration of Ras is roughly 1–5 mM within nanoclusters and 10–50 μM in average for the whole plasma membrane in vivo (38). These values are in the same region as the protein concentration of 0.7 mM used for crystallization of Ras (39). Interestingly, residues that are known to have an impact on Ras signaling are located in the proposed dimer interface. These residues, located in α-helices 4 and 5 and in the loop between β2 and β3, have been proposed to interact with the lipid membrane by acting in a nucleotide-dependent membrane interaction as the so-called switch III (20). However, in light of our findings we now put forth an alternative explanation. The residues at positions 135 and 161 are crucial for dimerization due to electrostatic interactions (Fig. 5). Therefore, mutations at these positions to alanine result in decreased signaling in cell-based assays (20).

Dimer effects on interacting signaling proteins

The dimer might influence the activation state in several ways. One possibility is that Ras dimers facilitate the binding of other proteins, such as guanine nucleotide exchange factor. Ras activation by Son of Sevenless (SOS), which possesses two binding sites for Ras, one catalytic and one allosteric binding site, could be enforced. To explore whether the dimer influences effector proteins, molecular modeling of the Ras dimer in complex with the Ras-binding domain of Raf-1, the GTPase-activating protein Neurofibromin 1 (NF1(1–333)), and the catalytic domain of SOS (SOScat) was performed. In all cases, interaction with at least one Ras is possible.

Furthermore, it is known that nucleotide loading and the type of lipid mixture can influence the partition and orientation of Ras at the membrane. Here, we have used Ras-GDP and a POPC membrane. It is obvious that the experiments have to be expanded to mimic in vivo conditions for GDP and GTP. Most interesting is the question of whether the orientation is affected by GTP/GDP exchange as proposed. Interestingly, Inouye et al. (21) have found evidence for Ras dimerization upon lipid interaction using a bifunctional amine-reactive cross-linker. Using lipid-modified H-Ras purified from SF9 cells in a Raf-1 activation assay, they found that Ras forms dimers in liposomes and also in intact cells, which is essential for Raf-1 activation.

Potential in vivo role of the Ras dimer

Ras dimerization could contribute to nanoclustering. Small clusters of ~6 Ras proteins have been found (40). Their size and occurrence vary depending on the isoform and activation state. Whereas several scaffolding proteins are known to influence these clusters (41), the driving forces of such clustering are, to our understanding, still not known. Transient dimerization of Ras could also drive such a clustering. It is known that reversible dimerization alone can cause membrane proteins to cluster into oligomer-like structures (42). This is caused by diffusion-limited partner switching of the dimerizing molecules, and the cluster size depends on the lateral diffusion rate and the stability of the dimers.

CONCLUSION

Lipidated Ras-GDP bound to a POPC bilayer shows a perpendicular orientation relative to the membrane as revealed by dichroic ATR-FTIR experiments. This perpendicular orientation is only stable when Ras dimerizes, as revealed by MM simulations. The presence of Ras dimers, as observed in numerous x-ray crystals, was confirmed by FRET experiments. The FRET results are in agreement with the MM simulations of these Ras dimers, yielding an intermolecular distance of 46 Å. The residues identified as crucial for dimer formation are also crucial in Ras signaling in cellular physiological experiments, as indicated by their respective mutations to alanine (20). The dimerization should be confirmed in future experiments at a physiological membrane. The proposed dimer interface might open an avenue for influencing signal transduction by a seemingly novel type of small molecule that inhibits dimer formation.

SUPPORTING MATERIAL

Parameters, equations, 10 figures, and references (43–56) are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)00970-8.
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N-Ras forms dimers at POPC membranes

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Supporting Material

Simulation parameters

MM simulations were performed with GROMACS 4.0.7 (1, 2) and the optimized potentials for liquid simulations all atom (OPLS/AA) force field (3), with guanosine-5’-triphosphate (GTP) and guanosine-5’-diphosphate (GDP) parameters, which have been improved regarding the reproduction of the dihedral energy surface. The TIP4P water model (4) and a POPC bilayer with the parameters of Ulmschneider et al. (5) were used. The farnesyl and palmitoyl anchors were parameterized by adopting parameters from comparable groups of the POPC membrane. During MM simulations, the electronic interactions of the system were evaluated using the Fast Particle-Mesh Ewald method (6) with a grid spacing of 0.12 nm and fourth-order spline interpolation. The van der Waals interactions between atom pairs were truncated within a cut-off value of 1.4 nm. A Berendsen thermostat with a coupling constant of 0.1 ps and a Berendsen barostat with a coupling constant of 1.0 ps were used to keep the system at a constant temperature and pressure of 310 K and 1 standard atmosphere, respectively (7). For the simulation system, a triclinic box was used with periodic boundary conditions. The simulations were run with an integration step of 2 fs and therefore, all bonds were constrained to their equilibrium distance by the LINCS algorithm (8). Quantum mechanics (QM) simulations were performed with Gaussian03 (9), and QM/MM simulations with the GROMACS/Gaussian interface (10) and the normal QM/MM scheme (11). All QM simulations were carried out with the B3LYP functional (12, 13) combined with the 6-31G* basis set.
**Simulation systems**

A homology model for N-Ras with the template X-ray structure of H-Ras·GDP (4Q21 AA 1-168 resolved was built using the SCRWL algorithm (14). N-Ras and H-Ras are 93% homologous with 86% identity. The main differences are in the hypervariable region (HVR) and the anchor region, neither of which have been resolved by X-ray or NMR analysis. The homology for the G domain alone is even higher with 97% identity. Therefore, the homology model for the G domain is very reliable. There is one X-Ray structure of the N-Ras G-domain (3CON) (15) available, but in this structure α-helix 2 is not resolved. The resolved parts of the X-ray structure are very similar compared to our homology model. The remaining regions of full-length N-Ras were modeled based on other experimental hints, as follows.

Comparison of the secondary structure analysis of the alpha-helical region of simulated full length N-Ras and H-Ras with the results from FTIR spectroscopic measurements (16) have shown that the helix 5 has to be extended from amino acid 168 to 172. Now, α-helix 5 ends at Ser173, which is followed by another serine. This is a typical motive for a helix ending. The dihedrals of the anchor region 180–186 are known from NMR studies (17), and indicate a horseshoe conformation for this region. The structure of the HVR, which has not yet been experimentally solved, was modelled using an all-trans conformation of the amino acid backbone.

The full-length N-Ras·GTP and N-Ras·GDP structures were then protonated, checked, and amended using the MAXIMOBY algorithm (18). These prepared structures were placed into a triclinic simulation box, filled with TIP4P water, and a POPC bilayer. To mimic the physiological salt concentration of 154 mmol/l, sodium cations and chlorine anions were added.

**The quantum yield Q₀**

Hiratsuka (1983) determined the quantum yield of Mant-GTP in water to be 0.24 (19). We expected the same quantum yield for Mant-GDP in water. To investigate the influence of binding of Mant-GDP to Ras on the quantum yield, we compared the static fluorescence emission spectra of Ras-bound and free Mant-GDP.

For this, Mant-GDP was bound to Ras via the EDTA method (20), followed by a 1-min gel filtration step (Zeba Spin Desalting Columns, Pierce Biotechnology, Rockford, IL, USA) in
which the buffer was exchanged to buffer E supplemented with 5 mM MgCl$_2$. Static fluorescence measurements were performed using a Jasco Spectrofluorometer FP 6500 (Gross-Umstadt, Germany). Ras-Mant-GDP (1 µM) in buffer E supplemented with 5 mM MgCl$_2$ was excited at 355 nm, and emission was detected from 390 to 600 nm. Mant-GDP was released from Ras by the addition of 10 mM EDTA and 100 µM non-fluorescent GDP. Emission spectra were successively recorded, reference-corrected, and integrated.

To compensate for bleaching, the solution was constantly stirred in a quartz cuvette. The overall emission (F) before the release of Mant-GDP from Ras (first spectrum) was compared to that after almost complete exchange (last spectrum) (Fig. S5), yielding Eq. S1

$$\Delta F = \frac{F_{\text{bound}}}{F_{\text{unbound}}} \approx \frac{29320}{19387} = 2.19.$$  \hspace{1cm} (S1)

This is why the assumed quantum yield of Mant-GDP (0.24 in solution, (19)) was adapted to be $0.24 \cdot 2.19 = 0.53$ when bound to Ras; this is established for the experiments presented here. Similar changes of $Q_0$ have been observed for Mant-labeled guanosine nucleotides due to binding to Ras (21).

**Determination of the overlap integral J between Mant and TNP**

The overlap integral, which represents the spectral overlap between the donor fluorescence and the acceptor absorbance in units [nm$^4$ M$^{-1}$ cm$^{-1}$], is another important parameter for the calculation of $R_0$ and is derived from Eq. S2:

$$J(\lambda) = \frac{\int_{-\infty}^{\infty} F_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 d\lambda}{\int_{-\infty}^{\infty} F_D(\lambda) d\lambda}.$$  \hspace{1cm} (S2)

Here, $F_D(\lambda)$ and $\varepsilon_A(\lambda)$ are the donor emission and the extinction coefficient of the acceptor at a given wavelength $\lambda$, respectively. Absorbance spectra of 10 µM TNP-GDP in buffer E supplemented with 5 mM MgCl$_2$ were recorded between 370 and 650 nm in steps of 2 nm using an Uvikon 810-Spectrophotometer (Bio-Tek Kontron Instruments, Neufahrn, Germany). The solution was kept in a quartz cuvette with a path length, d, of 1 cm. Extinction coefficients, $\varepsilon(\lambda)$, were calculated using Lambert-Beer’s Law (S5). Emission spectra of 1 µM Mant-GDP in buffer E supplemented with 5 mM MgCl$_2$ were recorded between 370 and 650 nm in steps of 2 nm (excitation around 355 nm) using a Spectrofluorometer FP 6500 (Jasco,
Grosse-Umstadt, Germany). The solution was kept in a quartz cuvette and stirred constantly to compensate for photo-bleaching. Spectra were reference-corrected (Fig. S6).

The denominator of Eq. S2 is used for normalization. The numerator was calculated by stepwise (2-nm) multiplication of the respective parameters and subsequent integration between 370 and 650 nm. Values exceeding the boundaries of the integration were negligible.

Finally, \( J \) was determined to be:

\[
J(\lambda) = \frac{\int_{370}^{650} F_D(\lambda) \cdot \varepsilon_\lambda(\lambda) \cdot \lambda^4 d\lambda}{\int_{370}^{650} F_D(\lambda) d\lambda} = \frac{1.9702 \cdot 10^{19}}{2.4645 \cdot 10^{14}} \approx 7.9945 \cdot 10^{14} \text{ } \left[ M^{-1} \text{ cm}^{-1} \text{ nm}^4 \right]
\]

**Determination of \( \kappa^2 \) and \( r_{calc} \) from MM and QM simulations**

For the calculation of \( \kappa^2 \), it is important to consider that both fluorophores are attached to the nucleotide by very short linkers, and in case of the TNP moiety, the linker is even not freely rotatable. This makes it crucial to calculate the value of \( \kappa^2 \) directly from the structure of the simulated dimer and precludes using the value of 2/3 commonly used for a random distribution. In order to calculate \( \kappa^2 \), the transition dipole vector needs to be known. For this, we need to find the possible minimum structures of Mant-GDP and TNP-GDP. Therefore, the structures of Mant-ribose and TNP-ribose were quantum mechanically optimized in vacuum. First, quantum mechanical optimization runs were performed with random starting structures, which are gained by the shaking algorithm of Maximoby (18). Second, all rotatable groups have been scanned with a step size of 5° and all possible minima combinations have been used as starting structures for optimization. TNP-ribose results in only one structure with a reasonable energy whereas the Mant-ribose is more flexible, resulting in 10 possible minimum structures (Fig. S8). In addition, the Mant group can flip between the 2' and the 3' hydroxyl group of the ribose. Therefore, there are 20 possible structures for Mant-GDP and only one fixed structure for TNP-GDP (Fig. S9). In order to get a first impression if these vacuum structures would fit into the protein environment without steric clashes, the TNP-GDP was included on one site of the monomer and the different Mant-GDP structures (Fig. S8) on the other side of the dimer. The snapshot after 100 ns of the dimer simulation run was used as starting structure. During MM equilibration runs with fixed Mant and TNP we equilibrated the protein environment with the labeled substrate. Because all analyzed Mant and the TNP conformations point out of the binding niche they do not have clashes with the protein or are significantly influenced by the protein environment during our simulations. Thus, we assume that all Mant-GDP conformations obtained in vacuum are also possible in
the protein environment. This result is confirmed by the X-Ray structure of H-Ras with bound Mant-GPPNHP (1GNP) (22). In this structure there is no contact between the Mant-group and the Ras protein. In order to get an impression of the flexibility of the labeled structures we equilibrate the Mant-GDP and TNP-GDP structures in the protein environment. All 20 structures are stable during 5 ps QM/MM simulations. The Mant, the TNP and the ribose were treated quantum mechanically and the rest of the protein by molecular mechanics. All these tests are arguments for the reasonability of the vacuum structures. Only longer MM simulations including Mant and TNP would bring a clear proof of the flexibility. Therefore, validated MM parameters for Mant and TNP are needed, which is still a big challenge in computer science. Because there are no force-field parameters of Mant and TNP available, and we merely want to know the range of possible distances, we aligned the 20 structures of Mant-GDP and TNP-GDP combinations with the GDPs of the MM trajectories of the dimer runs. After an equilibration time of 50 ns, the distance between the middle of the aromatic ring of the Mant- and the TNP group was calculated for each 1-ns interval of the last 150-ns trajectories of the three independent runs. From this calculation, an overall averaged distance of \( r_{\text{calc}} = 46.1 \pm 3.0 \text{ Å} \) (\( r_{\text{calc,1}} = 46.4 \pm 3.0 \text{ Å} \); \( r_{\text{calc,2}} = 45.8 \pm 3.0 \text{ Å} \); \( r_{\text{calc,3}} = 46.0 \pm 2.9 \text{ Å} \)) was predicted for the dimer (Fig. 4). The value \( \kappa^2 \) is required for the experimental estimation of the distance, \( r_{\text{exp}} \), based on the FRET efficiency. By QM calculations of Mant and TNP in vacuum we obtain the transition dipole moment vector of each structure. With this we are able to calculate \( \kappa^2 \) by

\[
\kappa^2 = \left(\cos \theta_T - 3 \cos \theta_B \cos \theta_A \right)^2.
\]

In which \( \theta_A \) is the angle between the vector \( \vec{r}_{\text{calc}} \) and the transition dipole moment of TNP, \( \theta_B \) is the angle between \( \vec{r}_{\text{calc}} \) and the transition dipole moment of Mant and \( \theta_T \) is the angle between the transition dipole moment of Mant and TNP given by

\[
\cos \theta_T = \sin \theta_B \sin \theta_A \cos \phi + \cos \theta_B \cos \theta_A.
\]

Here \( \phi \) is the azimuth between the planes given by the transition dipole moment of Mant and \( \vec{r}_{\text{calc}} \) and by the transition dipole moment of TNP and \( \vec{r}_{\text{calc}} \). A detailed Figure can be found in Höfling et al. (23) (Fig. 3). Following the same scheme by which the distance \( r_{\text{calc}} \) was determined, an overall averaged \( \kappa^2 \) of 0.41 ± 0.31 (\( \kappa_1^2 = 0.42 \pm 0.33 \); \( \kappa_2^2 = 0.39 \pm 0.30 \); \( \kappa_3^2 = 0.42 \pm 0.33 \)) was obtained from the simulations; this is slightly smaller than the commonly used value of 2/3 when an isotropic orientation is assumed. The very high value for the standard deviation of \( \kappa^2 \) reveals that we have almost an isotropic orientation. Due to sterical
restriction a small part of the configuration space is not populated and therefore the $\kappa^2$ is slightly smaller than $2/3$.

Statistics in nucleotide exchange

For measuring $\tau_{DA}$, Mant-GDP and TNP-GDP were added to the exchange buffer in equal concentrations (50 $\mu$M). In this case, the two different nucleotides are expected to bind Ras statistically, meaning that in case of a dimer-like formation of Ras, both heterogeneous pairs of nucleotides (MT) and homogenous pairs (MM and TT) will be bound within one dimer. Consequently, the labeling of Ras dimers, assuming exactly equal affinities for GDP, Mant-GDP, and TNP-GDP, would result in four different dimers:

| No FRET | FRET | FRET | No FRET |
|---------|------|------|---------|
| MM      | MT   | TM   | TT      |

For the first dimer (MM), no FRET is possible and the fluorescence lifetime of Mant-GDP is $\tau_u$ (Fig. S6, right). Two of the shown dimers (MT, TM) will exhibit FRET and a lifetime $\tau_q$. The last dimer (TT) carries two TNP-fluorophores which will not contribute to the detected signal since suitable band-pass-filters were used.

To extract $\tau_{DA}$ from the composed, measured signal, one has to investigate the distribution of the differently labeled GDPs on the Ras-molecules/dimers. If

\[ X_1 \rightarrow \text{number of unquenched Mant-GDP's, revealing } \tau_u \]
\[ X_2 \rightarrow \text{number of quenched Mant-GDP's, revealing } \tau_q \]

Using Eq. S3

\[ E = 1 - \frac{\tau_q}{\tau_u} \implies -\tau_q = (E - 1) \cdot \tau_u \implies \tau_q = (1 - E) \cdot \tau_u \]  \hspace{1cm} (S3)
a correlation between the measured and the theoretical Förster efficiency can be deduced as expressed by Eq. S4:

$$E_{\text{meas}} = 1 - \frac{\tau_{D4}}{\tau_D} = 1 - \frac{x_1 \cdot \tau_u + x_2 \cdot \tau_u}{(x_1 + x_2) \cdot \tau_u} = 1 - \frac{x_1 \cdot \tau_u + x_2 \cdot (1 - E_{\text{theo}}) \cdot \tau_u}{(x_1 + x_2) \cdot \tau_u} \quad (S4)$$

let be $$x_1 + x_2 = x_3$$,

$$\Rightarrow 1 - \frac{x_3 \cdot \tau_u - x_2 \cdot E_{\text{theo}} \cdot \tau_u}{x_3 \cdot \tau_u} = 1 - \frac{x_2 \cdot (x_3 - x_2 \cdot E_{\text{theo}})}{x_3} = 1 - \frac{x_3 - x_2 \cdot E_{\text{theo}}}{x_3} = \frac{x_2}{x_1 + x_2} \cdot E_{\text{theo}}$$

Since Ras binds Mant-GDP and TNP-GDP with different affinities (see below), $$x_1$$ and $$x_2$$ deviate from 2. By use of the determined probabilities (see below), the average amounts of homo- and hetero-dimers can be calculated as follows.

$$W(MM) = 0.61 \cdot 0.61 = 0.37 \quad \Rightarrow \quad 0.37 \cdot 4 = 1.48 \cdot MM \quad x_1 = 2.96$$
$$W(MT) = 0.61 \cdot 0.39 = 0.24 \quad \Rightarrow \quad 0.24 \cdot 4 = 0.96 \cdot MT$$
$$W(TM) = 0.39 \cdot 0.61 = 0.24 \quad \Rightarrow \quad 0.24 \cdot 4 = 0.96 \cdot TM$$
$$W(TT) = 0.39 \cdot 0.39 = 0.15 \quad \Rightarrow \quad 0.15 \cdot 4 = 0.60 \cdot TT$$

This leads to a correction-factor of around 2.57. Thus, the measured FRET efficiency $$E_{\text{meas}}$$ was multiplied by the correction-factor 2.57 to compensate for the statistics due to the flow-through-system.

**Homo-FRET**

When measuring $$\tau_D$$, the fluorophore emission could possibly be decreased by homo-FRET. Experiments were performed to check for this, whereby either half or all of the GDP was exchanged for Mant-GDP. In the latter case, only homo-dimers are present. Corresponding lifetimes were compared to $$\tau_D$$, but no decrease due to homo-FRET was observed. Additionally, there is no significant spectral overlap relevant to Mant-GDP (Fig. S6), indicating the absence of homo-FRET.
**Determination of the nucleotide affinities to Ras**

To check for differences in the nucleotide-affinities, we performed HPLC measurements of Ras that was previously exchanged with a mixture of Mant-GDP and TNP-GDP, as well as of the nucleotide mixture alone without protein. The nucleotide exchange was done as described above (see the section on quantum yield $Q_0$). HPLC measurements were performed using a reverse-phase column (ODS Hypersob 5 µm, 250 mm, 4.6 mm) on a Beckman System Gold Modules 125, 166 (Beckman Coulter, Krefeld, Germany) instrument with 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$, 5 mM tetrabutyl ammonium bromide, 25% acetonitrile at pH 6.5. The areas under the related peaks (Mant-GDP and TNP-GDP) were integrated to obtain the overall absorbance (extinction) of the labeled nucleotides at 254 nm (Fig. S7). Based on the respective extinction-coefficients, the concentration of the treated nucleotides can be calculated by rearranging Lambert-Beer’s Law, Eq. S5:

$$E = \varepsilon \cdot c \cdot d \Rightarrow c = \frac{E}{\varepsilon \cdot d}$$  \hspace{1cm} (S5)

Here, $c$ is the concentration of the nucleotide, $E$ is the measured extinction at a given wavelength $\lambda$ (here 254 nm), $d$ is the path length of the transmitting light, and $\varepsilon$ is the extinction coefficient of the nucleotide at $\lambda$.

The labeled nucleotides were received from Jena BioScience (Jena, Germany) and were delivered at pH 7.5. Exact concentrations of the delivered material were calculated using Eq. S5. For this, the respective extinctions were measured at 252 nm with a V-650 Spectrophotometer (Jasco, Grosse-Umstadt, Germany). For $\varepsilon$, the literature values (252 nm, pH 7.5) of Mant-GDP (22600 M$^{-1}$cm$^{-1}$) and TNP-GDP (24100 M$^{-1}$cm$^{-1}$) were used.

Subsequently, for a defined concentration of the labeled nucleotides (in buffer E, supplemented with 5 mM MgCl$_2$), the absorbance at 254 nm was measured with a V-650 (Jasco). The respective extinction coefficients (Mant-GDP and TNP-GDP at 254 nm, pH 6.5) were obtained by applying Eq. S5. These values of $\varepsilon_{\text{Mant-GDP, pH6.5}}$ (25400 M$^{-1}$cm$^{-1}$) and $\varepsilon_{\text{TNP-GDP, pH6.5}}$ (26350 M$^{-1}$cm$^{-1}$) were used to determine the concentrations of Mant-GDP and TNP-GDP corresponding to the integrated areas (absorbance) obtained from HPLC measurements (Fig. S7). Comparing the relationship between these concentrations (Mant-GDP to TNP-GDP) in the solved and the Ras-bound cases delivers the ratio of the affinities of Ras for Mant-GDP and TNP-GDP. For the sake of simplicity, the following values were rounded.

The calculated ratio of 1.57 Mant-GDP : 1 TNP-GDP leads to different probabilities, W, for Mant-GDP and TNP-GDP to be bound by Ras, as follows.
\[ W(\text{Mant}) = 1.57 : 2.57 = 0.61 \quad \quad \quad W(\text{TNP}) = 1 : 2.57 = 0.39 \]

**Error Propagation for Calculation of Förster Radius \( R_0 \)**

\( R_0 \) depends on a variety of parameters. In general, in a function of variables \( y = y(x_1, x_2, \ldots, x_n) \) with inherent uncertainties \( \Delta x_i \), the overall uncertainty is given by Eq. S6:

\[
\Delta y = \left\{ \sum_{i=1}^{n} \left( \frac{\partial y}{\partial x_i} \cdot \Delta x_i \right)^2 \right\}^{1/2}.
\]

(S6)

In the present situation, the function is the distance between the donor and acceptor \( r = r(R_0, E) \), which was calculated by Eq. S7:

\[
r = \left( \frac{R_0^6 \cdot (1 - E)}{E} \right)^{1/6}
\]

(S7)

Including the Förster Radius \( R_0 = 0.211 \cdot (\kappa^2 \cdot n^4 \cdot Q_0 \cdot J)^{1/6} \) and the FRET-Efficiency \( E = (E_1 + E_2)/2 \) with \( E_i = 1 - \tau_{DA(i)}/\tau_{D(i)} \), using values of \( \kappa^2 = 0.41 \pm 0.31 \), \( n = 1.4 \pm 0.05 \), \( Q_0 = 0.53 \pm 0.05 \), \( J = 7.99 \cdot 10^{14} \pm 8 \cdot 10^{13} \), \( \tau_{DA(1)} = 4.83 \pm 0.033 \), \( \tau_{D(1)} = 5.48 \pm 0.039 \), \( \tau_{DA(2)} = 4.57 \pm 0.031 \), \( \tau_{D(2)} = 5.1 \pm 0.043 \), this yields a distance of \( 46.4 \pm 6.1 \) Å between donor and acceptor. The high overall uncertainty is due to the moderate accuracy of \( \kappa^2 \). Note that the use of \( \kappa^2 = 2/3 \) would lead to a distance of \( 50.3 \) Å which is within the error margin.

**Expected FRET efficiency for randomly distributed Ras**

Wolber and Hudson deduced an equation to calculate concentration-dependent FRET efficiencies for randomly distributed donors and acceptors on a two-dimensional surface (24). This takes into account that the fluorophores are attached to proteins with finite dimensions, and is given by Eq. S8:
$$E_{\text{stat}} = 1 - \left( A_1 \cdot e^{-k_1 \cdot r} + A_2 \cdot e^{-k_2 \cdot r} \right)$$  \hspace{1cm} (S8)

Here, $A_i$ and $k_i$ are fitting parameters depending on the system and $c_A = (R_0)^2 \cdot C$ is the reduced acceptor surface density with $C$ being the acceptor surface density. The parameters depend on the distance of the closest potential approach ($R_c$) of the donors and acceptors. This takes into account the fact that donors and acceptors are attached to proteins of finite size. A typical value of $R_c$ for energy transfer in the case of protein-protein interactions can be approximated by the protein diameter. Similar approaches have been used regularly in literature (25).

According to Wolber and Hudson (24), the following parameters are best suited to approximate $E_{\text{stat}}$ properly.

$$A_1 = 0.6414, k_1 = 1.7400, A_2 = 0.3586, k_2 = 0.1285$$

These parameters arise from numerical fits performed by them and are valid only if $R_c/R_0$ is around 1.0 and the concentration of fluorophores per Förster radius is within 0 and 0.6.

Since $R_c$ was set to 4 nm, which is the diameter of Ras derived from the X-ray structure 1QRA, and $R_0$ was calculated to be 3.98 nm, $R_c/R_0$ indeed is almost exactly 1.0. For FRET measurements the surface concentration of Ras was adjusted to be in the range of 1.5 pmol per cm$^2$. The surface concentration of Ras was calculated using the area of the amide II band together with the absorption coefficient described in (26). A short calculation yields:

$$c_A = R_0^2 \cdot C = R_0^2 \cdot 1.5 \cdot 10^{-12} \text{ mol/cm}^2 \text{ Ras} \approx 14.31 \cdot 10^{-2} \text{ Ras per } R_0^2$$

when applying $R_0 = 39.8\text{Å}$. Because of different affinities of Ras for Mant-GDP and TNP-GDP (1.57 : 1) \,$\rightarrow$\, $c_A \approx 0.056$.

Therefore, Eq. S8 can be used here. Applying the appropriate parameters results in a FRET efficiency $E_{\text{stat}}$ of 6.2%.

**No Ras dimer in solution**

The fluorescent nucleotides Mant-GDP and TNP-GDP were bound to Ras via the EDTA method (20), followed by a 1-min gel filtration step (Zeba Spin Desalting Columns, Pierce Biotechnology), in which the buffer was exchanged to buffer E supplemented with 5 mM MgCl$_2$. Three different TCSPC experiments were performed using the same devices and software as described above. In all three measurements, labeled Ras (solved in Buffer E + MgCl$_2$) was stirred within a quartz cuvette. Histograms were accumulated for the cases of (1.)
only Mant-GDP, (2.) Mant-GDP and GDP, or (3.) Mant-GDP and TNP-GDP bound to Ras. Lifetimes were calculated as described above and a FRET-efficiency of 3–4% was found.

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Supporting Figures

**Fig. S1:** (A) Binding of lipidated Ras to a solid supported POPC bilayer. (B) Calculating the dichroitic difference spectra $D^*$ allows for the determination of the orientation of the absorbing groups e.g. $\alpha$-helices. The scaling factor $R_{iso}$ was calculated using wavenumber-dependent refractive indices of germanium and $H_2O$. $n(Ras)$ was taken as 1.45.

**Fig. S2:** The attachment of the N-Ras-GDP G-Domain at the membrane during three independent 200-ns MD simulations of a Ras monomer (A) and a Ras dimer (B) are depicted. The graphs show the distance $d$ between the center of mass of the protein and the membrane (see Fig. 2) during the simulation time. After 50 ns the protein is stably attached at the membrane.
Fig. S3: Analysis of the available X-ray structures of Ras. Fifty of the 71 structures show the dimer.

Fig. S4: Normalized kinetics of the amide II band area and the area of D* in the amide I region of Ras during immobilization to the membrane evolve with similar time constants. This shows that the D* signal is independent of surface coverage, eliminating crowing as a driving force.
**Fig. S5:** Time-course of overall Mant-GDP-emission (left), Measurement series of Mant-GDP-emission-spectra (right).

**Fig. S6:** Relationship between the wavelength-dependent molar extinction coefficient \( \varepsilon(\lambda) \) of a FRET-acceptor and the emission-spectrum of a corresponding FRET-donor. TNP-GDP is the acceptor and Mant-GDP donor (left); Mant-GDP is both the acceptor and the donor (right).

**Fig. S7:** Absorbance spectra recorded at 254 nm via HPLC; labeled GDP (50 mM MES, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM TCEP, pH 6.5) in solution (left) and bound to Ras after nucleotide exchange (right).
Fig. S8: Minimum conformations of Mant ribose in vacuum with their transition dipole moments. Shown are the 10 structures with the lowest conformational energies reached by quantum mechanical optimization with the B3LYP/6-31G* level of theory. The given conformational energy value in each case is the energy difference relative to the lowest observed conformational energy.
**Fig S9:** Minimum conformation of TNP ribose in vacuum with its transition dipole moment. Shown is the structure with the lowest conformational energy reached by quantum mechanical optimization with the B3LYP/6-31G* level of theory.

**Fig S10:** Surface representation of the dimer interface. From our MM simulations, we identified the highlighted amino acids participating in the intermolecular stabilization of the dimer. Magenta means interaction by hydrogen bonds, cyan by van-der-Waals interactions, and blue by both.