Ras membrane orientation and nanodomain localization generate isoform diversity

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The structural elements encoding functional diversity among Ras GTPases are poorly defined. The orientation of the G domain of H-ras with respect to the plane of the plasma membrane is recognized by the Ras binding domain of C-Raf, coupling orientation to MAPK activation. We now show that two other proteins, phosphoinositide-3-kinase- α and the structurally unrelated galectin-1, also recognize G-domain orientation. These results rationalize the role of galectin-1 in generating active GTP-H-ras signaling nanoclusters. However, molecular dynamics simulations of K-ras membrane insertion and fluorescence lifetime imaging microscopy (FLIM)-Förster resonance energy transfer (FRET) imaging of the effector interactions of N-Ras, K-Ras, and M-Ras suggest that there are two hyperactive, signaling-competent orientations of the Ras G domain. Mutational and functional analyses establish a clear relationship between effector binding and the amphipathicities of helix $\alpha 4$ and the C-terminal hypervariable region, thus confirming that these structural elements critically tune the orientation of the Ras G domain. Finally, we show that G-domain orientation and nanoclustering synergize to generate Ras isoform specificity with respect to effector interactions.

FRET | nanocluster | signal transduction | small G protein

Few of the 39 Ras family GTPases that operate as membrane-anchored molecular switches have been characterized in structural and functional detail (1). It is clear, however, that Ras proteins share overlapping sets of effectors and activators (2–5) as well as have specific biological functions (6–9). A fundamental question is how these different biological functions are realized. Some differences arise from differential expression during ontogenesis and within tissues (10); however, variations in the protein sequences of Ras isoforms suggest structural and therefore, molecular mechanistic differences. This hypothesis is supported by studies showing that different Ras isoforms activate common signaling pathways to various extents (11–13). Signal output is a complex spatiotemporal integration of the activities of many components, such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and effectors (14). Despite this complexity, it is probable that there is a Ras structural component that contributes to distinct preferences for GEFs, GAPs, and effectors (4, 5).

The C-terminal hypervariable region (HVR), which is modified by lipids and anchors the G domain to the membrane, is one structural component of Ras that contributes to biological differences among isoforms. This region is associated with differences in trafficking, subcellular localization, and lateral segregation to specific nanoscale compartments, or nanoclusters, on the plasma membrane (14, 15). Lateral segregation of H- and K-ras4B (K-ras) into distinct nanoclusters of the plasma membrane may in part underlie the observed isoform specific differences in MAPK signaling (11), because C-Raf is selectively retained in K-ras but not in H-ras nanoclusters (16–18).

Using molecular dynamics simulations in conjunction with a lateral segregation FRET analysis, we recently showed that membrane-bound H-ras exists in a guanine-nucleotide-dependent conformational equilibrium (19, 20). In the GTP-bound state, helix

$\alpha 4$ contacts the membrane, stabilizing H-ras in an orientation that facilitates Raf binding and MAPK activation. In an alternative orientation, GDP-Ras is stabilized by membrane contacts of the HVR. Switch III, comprising the $\beta 2$ - $\beta 3$ -loop and helix $\alpha 5$, controls switching between the two states. A simple balance model can readily explain these observations; the HVR and helix $\alpha 4$, which we have defined as the switched elements, operate H-ras like a balance (Fig. 1) (20, 21). An alignment of 13 Ras isoforms shows that sequences for both helix $\alpha 4$ and the HVR are highly divergent (21). These two structural elements could, therefore, contribute to Ras isoform diversity through a similar balance mechanism (20).

Using fluorescence lifetime imaging of intact cells, molecular dynamics simulations, and biochemistry, we provide evidence that G-domain orientation is a key codec for Ras isoform diversity. We show that interactions of H-ras with phosphoinositide-3-kinase α (PI3K α), another major effector, and the scaffolding protein galectin-1 (22, 23) are orientation sensitive. Conversely, N-ras, K-ras, and M-ras interactions with the C-Raf-binding domain (RBD) are differentially affected by mutations in their switched elements, suggesting different, isoform-specific orientations. These data are supported by molecular dynamics (MD) simulations, which reveal that the active G-domain orientation of K-ras is inverted with respect to that of H-ras. Finally, we show that nanodomain localization and membrane orientation combine to define selective access of different Ras isoforms to C-Raf.

Results and Discussion

Membrane Orientation of H-ras Is Recognized by PI3K α and Galectin-1

To test for a general relevance of the balance model for Ras interactions, we explored whether or not interaction partners other than C-Raf recognize H-ras membrane orientation. Recently, we showed that the soluble RBD of C-Raf can be used to probe the membrane orientation of the H-ras G domain (21). Therefore, we coexpressed a set of previously characterized mGFP-H-rasG12V switched element mutants with the monomeric red fluorescent protein (mRFP)-labeled soluble RBD of the effector PI3K α (Fig. S34) or the scaffolding protein galectin-1 (22, 23). To quantify the extent of binding between mGFP-Ras and mRFP-PI3K α -RBD or mRFP-galectin-1, we measured the decrease in the fluorescence lifetime of the donor fluorophore (mGFP) caused by FRET in a large number of intact cells (21). Because the mRFP FRET-acceptor constructs were always present in comparable excess for all FRET pairs (Figs. S1 and S2), the decrease in mGFP fluorescence lifetime correlated closely with the extent of complex

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Fig. 1. The balance model explains G-domain orientation-activity relationships. (A) The balance model shows how orientation of the Ras G domain with respect to the plasma membrane is controlled by the switched elements, helix $\alpha 4$ and the HVR, and how orientation correlates with activity. A conformational equilibrium between the two orientations is assumed based on computational simulations (19). The steepness of the balance signifies the probability of finding Ras in that orientation. The equilibrium is regulated by the activation state and mediated by the specific residues of the switched elements. Mutation of the switched elements shifts the probability of the G domain assuming a given orientation. In H-ras, for example, (B) the mutation R128A/R135A in helix $\alpha 4$ shifts the balance toward stabilization by the HVR, whereas (C) mutation R169A/K170A in the HVR shifts the balance toward stabilization by helix $\alpha 4$.

formation between mGFP-Ras and the cognate mRFP-interaction partner. In this assay, the primary variable determining the Ras interaction-partner association constant is G-domain orientation. This is because cocrystal structures show a circumscribed interaction surface between the effector loop and the minimal RBD (24, 25) that does not involve helix- $\alpha 4$ or the proximal HVR. In addition, the minimal RBDs and galectin-1 lack any intrinsic membrane-interaction motifs, and so, Ras binding will be insensitive to the nanoscale lipid environment of different types of Ras nanocluster (*SI Text*) (17, 18, 21).

For all mGFP-H-rasG12V mutants, we observed a significant reduction in the fluorescence lifetime of mGFP when mRFP-PI3K α -RBD or mRFP-galectin-1 were coexpressed, indicating a molecular interaction (Fig. 2 *A* and *B*). For both mRFP-constructs, these interactions were increased by the mutations R169A/K170A, which relieve contacts of the HVR with the plasma membrane and shift the G-domain equilibrium toward the conformation that is stabilized by helix $\alpha 4$ (Fig. 1C). This increased binding was almost completely abrogated by additional mutations R128A/R135A in helix $\alpha 4$ (Fig. 2 *A* and *B*). The R128A/R135A mutations in isolation, which relieve contacts of helix $\alpha 4$ (Fig. 1B), had no effect on PI3K α -RBD or galectin-1 interactions relative to the H-rasG12V controls (Fig. 2 *A* and *B*). The strongest lifetime reductions were seen with mGFP-H-rasG12V- $\alpha 4$ MARCKS (Fig. 2 *A* and *B*); in this construct, a membrane-anchoring sequence from the myristoylated alanine-rich C-kinase substrate (MARCKS) protein replaces helix $\alpha 4$ and strongly reorients the G domain (Fig. 1C) (21).

Given that the PI3K α -RBD and galectin-1 interaction profiles of the H-rasG12V switched element mutants (e.g., R169A/K170A and R128A/R135A) in intact cells are qualitatively identical to that of the C-Raf-RBD (Fig. 24, red overlay) (21), we conclude that the C-Raf-RBD, the PI3K α -RBD, and galectin-1 all sense orientation of the H-ras mutants in a very similar manner. The absolute quantitative differences in fluorescence lifetime imaging microscopy (FLIM) measurements may reflect known structural differences between the two Ras-RBD complexes and the different regions of Ras that interact with galectin-1 (*SI Text*) (4, 5, 23–26). The recognition of the same G-domain orientation by both galectin-1 and Raf is, therefore, consistent with the proposed

Fig. 2. Recognition of H-ras membrane orientation by PI3K α -RBD and galectin-1. (A) BHK cells transiently coexpressing the indicated mGFP-H-rasG12V mutants and an excess of mRFP-PI3K α -RBD (PI3K α -RBD) or (B) mRFP-galectin-1 (galectin-1) at comparable levels were analyzed using FLIM-FRET. For comparison, the profile of the interaction with mRFP-C-Raf-RBD from our previous publication (21) is shown as a red overlay in A. Mean mGFP fluorescence lifetimes ($\tau_{\phi} \pm \text{SEM}$) were determined from four independent experiments. The number of cells analyzed is given in brackets. On coexpression of mRFP-PI3K α -RBD or mRFP-galectin-1, all mGFP fluorescence lifetimes were significantly reduced, consistent with binding of PI3K α -RBD or galectin-1 to Ras. Statistically significant changes in the fluorescence lifetimes of mGFP-H-rasG12V mutants compared with mGFP-H-rasG12V are shown (*** $P < 0.001$; ns, not significant; two-tailed Student's t test).

role for galectin-1 in stabilizing the active G-domain orientation. This stabilization allows assembly of H-ras-GTP monomers with this orientation into nanoclusters that then serve as the sole sites of Raf recruitment to the plasma membrane (17, 26). More broadly, the coevolution of the recognition of Ras membrane orientation by the RBDs and galectin-1 is intriguing, because it supports a general relevance of G-domain orientation for regulating Ras interactions on the plasma membrane.

C-Raf-RBD Interaction Profiles of K-ras, N-ras, and M-ras Chimera Suggest Isoform-Specific G-Domain Orientations. We next tested whether or not G-domain orientation is relevant for isoforms other than H-ras. We first used FLIM-FRET to determine C-Raf-RBD interaction profiles of a set of N-ras (H-N) and K-ras4B-like (H-K) H-rasG12V chimeras (Fig. 3A) to characterize their membrane orientations. We used chimeras to exactly preserve the RBD-Ras interaction surface and associated structural and thermodynamic properties of the G-domain and to allow ready comparison with our previous H-rasG12V data (21) (Fig. 3). Both, the H-N and H-K chimeras had the same subcellular distributions and similar functional properties as their wild type counterparts, N- and K-rasG12V (Fig. S3C).

Interestingly, the K-ras chimera showed an inverted interaction profile compared with H-ras. The H-K- $\alpha 4$ MARCKS chimera interacted significantly less with the C-Raf-RBD than did H-K (Fig. 4A). However, alanine mutation of the basic residues R128 and R135 on helix $\alpha 4$ significantly enhanced the interaction. The implied dominance of the HVR membrane anchorage of K-ras suggested by these data was supported by MD simulations of the full-length K-ras in complex with a 1,2-dimyristoylglycerol-3-phosphocholine (DMPC) lipid bilayer. We found that whereas K-ras was stably anchored to the bilayer through its farnesylated and positively charged C terminus, the rest of the protein

Fig. 3. Properties of K-ras, M-ras, and N-ras chimeras. (A) The G domains of H-ras, N-ras, and K-ras are 90–100% conserved, and we, therefore, facilitated construction of switched element mutants in N-ras and K-ras by generating chimeras with existing H-ras mutants (21). This also ensured that the interaction surface between Ras and the RBD and the whole G domain were identical, allowing ready comparison among different datasets (see Fig. 5). To this end, we replaced the HVR of various H-ras mutants with the HVR of N-ras or K-ras4B to generate H-N and H-K chimeras, respectively. In the M-K chimera, the HVR of M-ras was replaced with that of K-ras4B. (B) The upper figure shows a ClustalW sequence alignment of residues on helix α4 of the Ras isoforms and the polybasic membrane anchor of the MARCKS protein. Sequences from H-ras, K-ras, and N-ras are nearly identical but diverge from M-ras and the MARCKS sequence. These relationships also translate into different amphiplicity scores, which are calculated using the Bio3D package developed by Grant et al. (41) (<http://mccammon.ucsd.edu/~bgrant/bio3d/>). The lower figure shows the similarity of the HVRs of K-ras and M-ras in terms of amphiplicity scores and numbers of basic residues.

exhibited extensive fluctuations. As shown in Fig. 4B, the orientation of the catalytic domain with respect to the membrane plane was similar in the GDP and GTP states; neither led to a reorientation of helix α4 toward the membrane within the timeframe of the simulation. Thus, whereas the most active orientation of H-ras was stabilized by helix α4 (Fig. 1C), K-ras was stabilized by the HVR (Fig. 1B).

To validate the inverse hyperactive orientation of K-ras, we made use of another Ras isoform, M-ras, that has a similar C-terminal polybasic domain to K-ras; however, it has a helix α4 that is more similar to the polybasic sequence in the H-K-α4MARCKS chimera (Fig. 3A and B) than it is to helix α4 in K-ras. We, therefore, expected that an M-K chimera would have a stronger interaction with the C-Raf-RBD than H-K-α4MARCKS and a weaker interaction than that of H-K-R123A/R135A. Fig. 4A and Fig. S4 show that these predictions hold. Similar results were obtained with full-length M-ras, which was expected given the similarity of the M-ras and K-ras4B HVR (Figs. 3B and 4A).

We next analyzed the orientation dependent interaction of N-ras with the C-Raf-RBD. Mutations of the conserved residues R169A/K170A in the HVR of the H-N chimera increased the interaction with the C-Raf-RBD (Fig. 4A), as observed previously for H-rasG12V (21). However, the mutations R128A/R135A, on the

Fig. 4. Different switched elements in K-ras, N-ras, and M-ras affect the interaction probability with C-Raf-RBD. (A) Interaction profiles of mGFP-Ras and mRFP-C-Raf-RBD assayed using FLIM-FRET in BHK cells. Average mGFP fluorescence lifetimes ($\tau_{\phi} \pm \text{SEM}$) were determined from four independent experiments. The number of cells analyzed is given in brackets. The donor-only lifetimes of H-N and H-K shown are averages of donor-only lifetimes of all cognate mutants. Donor lifetimes of individual constructs are given in Fig. S3B. Average fluorescence lifetimes of donor-only samples of M-K ($\tau_{\phi} = 2.083 \pm 0.008$) and M-rasG22V ($\tau_{\phi} = 2.033 \pm 0.005$) were comparable. For comparison with our previous data (21), corresponding lifetimes of H-rasG12V (black dashed) and the hyperactive mutant H-rasG12V-R169A/K170A (light gray dashed) are shown. Qualitatively identical results were obtained with corresponding mutants of full-length N-ras and K-ras4B (Fig. S3D). On coexpression of mRFP-C-Raf-RBD, all mGFP fluorescence lifetimes were significantly reduced compared with the donor-only samples, which is consistent with RBD binding to Ras. Statistically significant changes of the mGFP lifetimes of each mutant from the nonmutated H-N/ or H-K/ RBD FRET pairs are indicated (*** $P < 0.001$; 2-tailed Student's t test). (B) Snapshots of GDP- (Left) or GTP-loaded (Right) K-ras were inserted into a DMPC bilayer and were obtained by molecular-dynamics simulations (30 ns and 31.5 ns, respectively). Note that in K-ras, helix α4 (arrow) does not stably contact the membrane in contrast with GTP-H-ras (19).

other switched element, helix α4, also increased C-Raf-RBD binding. Interestingly, whereas H-N did not interact as well with the C-Raf-RBD as H-rasG12V, the H-N switched element mutants interacted more strongly than the hyperactive H-rasG12V-R169A/K170A (Fig. 4A). This suggests some bistability in the membrane interactions of N-ras, which allows either of the opposite hyperactive orientations of H- and K-ras4B to be assumed.

The easier release of the HVR side of the balance by the R169A/K170A mutation in the N-ras like chimera, which then results in a stronger reorientation than in H-ras, is supported by structural simulations. Computer simulations of the minimal membrane anchors of N-ras and H-ras have shown that the insertion depth into a zwitterionic model membrane of the double lipid-modified N-ras is less than that of the triple lipidated H-ras (19, 27). Furthermore, a mutant H-ras anchor in which the palmitoylated residue at position 184 has been mutated to emulate monopalmitoylated N-ras was found to be more interfacial than the wild-type H-ras anchor (19). Unfortunately, variably low-expression levels precluded a detailed analysis of the effect of mutational reorientation on MAPK signaling for N- and K-ras (Fig. S3E), although we have shown previously for a set of

resulting in better retention of the RBD-CRD and Raf in K-ras nanoclusters (18, 34, 35). Thus, both the increased selectivity of the RBD-CRD and Raf interaction and the overall reduced FRET are a consequence of this preferential retention in K-ras nanoclusters (17, 18) after efficient recruitment by the Ras G-domain. FRET is higher for the RBD, because the only available membrane-interaction mechanism is by direct Ras binding, resulting in high fluorophore proximity; however, proximity in a nanocluster, not mediated by direct protein–protein interaction, will result in lower FRET, because interfluorophore distances are greater. Taken together, these results show that G-domain orientation and the lateral segregation of different Ras isoforms into distinct types of nanocluster combine to generate isoform diversity. Integration between orientation and nanoscale environment is likely achieved at the level of the full-length effector, where the effector possesses membrane-interaction motifs.

In conclusion, we propose that the switched elements, helix $\alpha 4$ and the HVR, tune the membrane orientation of the Ras G domain. Membrane orientation of the G domain constitutes a first-level selectivity filter, which determines the recruitment rate of effectors from the cytosol or possibly also from within the membrane. On the membrane, intrinsic membrane-binding domains of the effector, such as the CRD, come into play as a second-level selectivity filter, by modulating orientation-sensitive interactions through differential retention in and partitioning to the cognate Ras nanodomain or nanocluster (16, 20, 36). Such selective association with certain Ras isoforms in specific nanodomains is likely to be mediated by polybasic HVRs and the lipid anchors of Ras proteins (34, 35). More generally, our data highlight the importance of the nanoscale lipid environment of signaling proteins that can be recognized and remodeled by basic and amphipathic sequence stretches (37).

In the future, it will be interesting to determine which Ras isoform-associated nanodomains are targeted by the membrane-anchoring domains of different Ras effectors and other Ras regulators. Although here we have focused on the interactions of GTP-Ras, orientation sensitivity may also apply to GDP-Ras. For example, in the complex of Ras and the SOS-Cdc25 domain, the GEF domain has a conspicuous curved shape that may be adapted to sense orientation (38). It will be interesting to determine whether or not the GEF/GDP-Ras interactions are also orientation sensitive. On a similar note, the relatedness of G domains in the small G-protein superfamily (1) suggests that other G proteins, such as Rho and Rab, have similar additional structural switches that operate in the context of their native membrane environment and impact on isoform-specific behavior.

Methods

Molecular Cloning. Construction of switched element H-ras mutants was described previously (21). N-ras- and K-ras-like chimera and mutants were constructed using corresponding H-ras mutants as templates, and the sequences encoding for the isoform-specific HVRs using PCR were added. The M-K chimera was constructed by the same method. The Ras binding domain (residues 173–292) of the catalytic subunit of bovine PI3K α or the RBD together with the cysteine-rich region (residues 51–200) of human C-Raf were PCR amplified and cloned in frame with the mRFP cDNA.

Cell Culture. BHK cells were cultured as described (39) and transfected using Lipofectamine (Invitrogen) or Fugene5 (Roche) with donor and acceptor constructs 48 h before analysis. For experiments with wild-type H-ras proteins, cells were starved of serum overnight.

FLIM and Confocal Imaging. FLIM-FRET experiments were carried out using a lifetime fluorescence imaging attachment (Lambert Instruments) on an inverted microscope (Olympus IX71). BHK cells expressing mGFP-H-ras constructs for 2 days, alone or with mRFP-tagged galectin-1 or RBD constructs (acceptors), were excited using a sinusoidally modulated 3-W 470 nm LED (light-emitting diode) at 40 MHz under epi-illumination. Cells were imaged with a 60 $\times$ NA 1.45 oil objective using an appropriate GFP filter-set. The phase and modulation were determined from a set of 12 phase settings using the manufacturer's software. Fluorescein at 0.1 mM was used as a lifetime reference standard and to fix an expression window for the donor. The phase lifetime of the donor (mGFP constructs) was determined for multiple cells across three independent experiments. Confocal imaging was carried out as described previously (40).

MD Simulations. Multiple simulations were performed on the GDP- and GTP-bound full-length K-rasG12V in a DMPC bilayer. The structure of K-rasG12V catalytic domain was modeled based on the corresponding structure of H-rasG12V as described before (28). The hypervariable region was modeled as an extended chain and then ligated onto the catalytic domain structure (19). The simulation protocols followed those previously reported simulations for H-rasG12V (19).

Statistical Analysis. Student's *t* tests (two-tailed) were used to assess significant differences between mean values.

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