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Study of Förster Resonance Energy Transfer to Lipid Domain Markers Ascertains Partitioning of Semisynthetic Lipidated N-Ras in Lipid Raft Nanodomains

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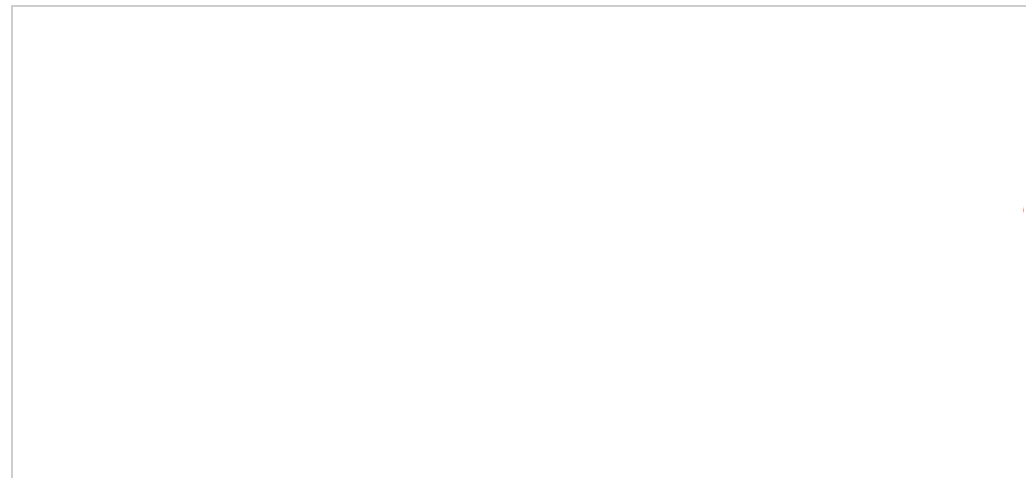
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Abstract



Cellular membranes are heterogeneous planar lipid bilayers displaying lateral phase separation with the nanometer-scale liquid-ordered phase (also known as “lipid rafts”) surrounded by the liquid-disordered phase. Many membrane-associated proteins were found to permanently integrate into the lipid rafts, which is critical for their biological function. Isoforms H and N of Ras GTPase possess a unique ability to switch their lipid domain preference depending on the type of bound guanine nucleotide (GDP or GTP).

This behavior, however, has never been demonstrated *in vitro* in model bilayers with recombinant proteins and therefore has been attributed to the action of binding of Ras to other proteins at the membrane surface. In this paper, we report the observation of the nucleotide-dependent switch of lipid domain preferences of the semisynthetic lipidated N-Ras in lipid raft vesicles in the absence of additional proteins. To detect segregation of Ras molecules in raft and disordered lipid domains, we measured Förster resonance energy transfer between the donor fluorophore, mant, attached to the protein-bound guanine nucleotides, and the acceptor, rhodamine-conjugated lipid, localized into the liquid-disordered domains. Herein, we established that N-Ras preferentially populated raft domains when bound to mant-GDP, while losing its preference for rafts when it was associated with a GTP mimic, mant-GppNHp. At the same time, the isolated lipidated C-terminal peptide of N-Ras was found to be localized outside of the liquid-ordered rafts, most likely in the bulk-disordered lipid. Substitution of the N-terminal G domain of N-Ras with a homologous G domain of H-Ras disrupted the nucleotide-dependent lipid domain switch.

Lipid rafts, the nanoscale lipid domains, in a plasma membrane of living cells play a crucial role in organizing cellular signaling and regulatory cascades.¹⁻⁶ Micrometer-sized lipid domains with a liquid crystal-like order may be easily observed by optical fluorescence microscopy in model membranes constituted from heterogeneous lipid mixtures.^{4,7-10} However, their cellular counterparts are expected to be much smaller, nanometer-sized, meaning they can be resolved only by electron and atomic force microscopy techniques.¹¹⁻¹⁴ In a cell, many membrane proteins permanently reside in raft membrane domains, which is essential for their function.^{5,15-19} Ras, a small monomeric GTPase, provides an intriguing example of a membrane protein that dynamically switches its nanodomain affinity upon the transition between its active and inactive functional states (bound to GTP and GDP, respectively).²⁰⁻²³

Ras is a small monomeric GTPase involved in the regulation of cell growth, proliferation, and differentiation.²⁴ Mutations in the Ras genes are observed in $\leq 25\%$ of all human cancers, which makes Ras one of the major targets for cancer therapy.²⁵⁻²⁸ Ras consists of a GTPase catalytic domain (G domain) binding guanine nucleotides and the C-terminal peptide anchored to the inner leaflet of the plasma membrane through a posttranslational lipidation motif.²⁹⁻³¹ Membrane attachment is crucial to Ras function; i.e., most effector proteins can be activated by Ras-GTP only when it is associated with the membrane surface.^{25,32}

Ras proteins are represented by three Ras isoforms with a high degree of homology and nearly 90% sequence identity in the N-terminal GTPase domain.³³ The 22 or 23 remaining C-terminal amino acids, known as the hypervariable region, have no sequence similarity except for the conserved CAAX motif necessary for membrane targeting.³⁴ The variability of the C-terminal sequences of the Ras isoforms leads to different processing patterns in the cell. All Ras isoforms are modified by attachment of a prenyl (farnesyl) chain at the extreme C-terminal cysteine. H-Ras and N-Ras additionally get two palmitoyl chains and one palmitoyl chain, respectively, while K-Ras4B features a polybasic domain as an alternative membrane-anchoring mechanism.^{29,35} It was demonstrated that the membrane-targeting region is responsible for partitioning of proteins between membrane domains.³⁶

A dynamic change in H-Ras localization from cholesterol-rich rafts to the disordered lipid domains upon activation (GTP binding) was first observed using density gradients and immuno-gold electron microscopy in native cellular membranes.^{22,37-40} An explanation of this behavior of H-Ras was proposed when scaffolding protein galectin-1 was found to associate with activated H-Ras nanoclusters in

disordered lipid domains.⁴¹ The K-Ras isoform was found to reside in the disordered phase irrespective of its activation status (bound to GDP or GTP).^{22,38} The lipid domain preferences of N-Ras remain controversial as it was observed in a raft phase of COS-7 cell membranes when in the GDP form,⁴² while Roy reported that N-Ras-GDP was localized in the disordered lipid phase of BHK cells and moved to raft domains upon GTP binding.⁴³ Experiments in model membranes recapitulated none of these findings; i.e., N-Ras was found concentrated at the raft–disordered domain boundary in model lipid bilayers irrespective of the bound nucleotide.⁴⁴⁻⁴⁷ The behavior of N-Ras has been studied in these reports in very different systems ranging from natural plasma membranes of BHK and COS-7 cells to synthetic lipid mixtures, which might be one of the causes of observed differences. The dynamic shift from one phase to another upon activation of N-Ras observed by Roy et al.⁴³ could be due to binding to yet unidentified protein scaffolds (by analogy with H-Ras).

In this report, we make use of a full-length semisynthetic lipidated N-Ras to establish for the first time that it is capable of changing its nanodomain localization in model lipid membranes in a nucleotide-dependent manner in the absence of any other proteins.

Materials and Methods

The Materials and Methods section in its entirety is included in the [Supporting Information](#).

Results

Our goal was to assess relative affinity of N-Ras lipoprotein for raft and disordered lipid domains in a model lipid system and determine whether the raft affinity of N-Ras is dependent on the nature of a bound nucleotide (hence, the biologically active/inactive protein conformation) in the absence of “helper” proteins. Because of the nanoscale dimensions of rafts, we relied on measurements of FRET between the Ras-attached fluorophore and fluorescent lipid domain markers.⁴⁸⁻⁵⁰ H-Ras localization was previously probed by FRET to lipid domain markers, but those reports did not include N-Ras.^{51,52}

In the following subsections, we (1) evaluated the model lipid bilayers to confirm that they form nanometer ordered domains mimicking the size of cellular rafts, (2) detected nonraft localization of the C-terminal lipidated peptide of N-Ras, (3) evaluated a hypothesis that the C-terminal peptide may be attracted to the raft boundary, (4) established lifetime-based detection of nanodomain localization, and (5) determined nanodomain preferences of N-Ras and the H/N-Ras chimera in the active and inactive states (bound to GTP mimic or GDP).

Lipid Membrane Mimic with Nanoscale Lipid Domains

To create lipid bilayers that spontaneously form nanometer-sized raft domains (ranging from approximately 4 to 15 nm), we followed the method of Pathak and London⁵⁰ and utilized a lipid mixture of sphingomyelin (SM), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and cholesterol in an equimolar ratio (known hereafter as the raft lipid mixture). A pure POPC lipid was used to make homogeneous (nonraft) control bilayers. Confocal fluorescence microscopy of a supported lipid bilayer made of the raft lipid mixture confirmed that these bilayers do not form nonphysiological micrometer-sized rafts ([Figure 1](#)). We used NBD-DPPE (green fluorescence) as a lipid raft marker and Rhod-DOPE (red fluorescence) as a disordered domain marker to detect micrometer-sized domains. [Figure 1](#) reveals the absence of significant areas of green and red color, confirming that the size of rafts and disordered

(~30%) both the catalytic rate and nucleotide exchange kinetics in H-Ras.⁷² By comparison, another relatively small fluorophore, BODIPY, was observed to significantly accelerate spontaneous dissociation of the nucleotides,⁷³ which would lead to a loss of the label during sample preparation. In contrast, a slight inhibition of spontaneous nucleotide dissociation and hydrolysis by the presence of mant ensured the relative stability of Ras–mant-nucleotide complexes in our experiments.

To prepare protein/LUV samples, Ras lipoproteins were loaded with mant-nucleotides and associated with LUVs via overnight incubation. However, lipoproteins, such as lipidated full-length Ras, tend to aggregate in aqueous buffers because of their hydrophobic lipid modifications. Therefore, it was essential to ensure that any aggregated N-Ras that was not incorporated into LUVs was removed before fluorescence measurements. Using size-exclusion chromatography, we achieved complete separation of N-Ras associated with LUVs (eluted near the exclusion limit of the column, >800 kDa) from the N-Ras aggregates (~500 kDa) as well as from any unbound mant-nucleotides (Figure S1 and Table S2).

To establish the predominant lipid domain localization of the N-Ras bound to mant-nucleotides, we determined the efficiency of FRET between the mant group and Rhod-DOPE in homogeneous and raft LUVs. Because the thermal stability of Ras is relatively limited, we performed all experiments at low temperatures. Figure 7A shows FRET efficiencies observed for mant in N-Ras-mGDP and N-Ras-mGppNp in homogeneous and raft LUVs (for a complete summary of the lifetime measurements, see Table S1). N-Ras-mGDP and N-Ras-mGppNHp associated with homogeneous lipid bilayers demonstrated relatively high FRET efficiencies, indicating significant energy transfer from the mant fluorophore to the rhodamine of Rhod-DOPE. This is expected on the basis of the fact that the donors are readily accessible to acceptors in the homogeneous bilayer (no domains). In raft LUV samples, N-Ras-mGDP exhibited very low FRET values, indicating effective segregation of mant-labeled Ras-GDP from Rhod-DOPE at 5 °C. These samples also exhibited relatively shorter lifetimes, which might be explained by homotransfer^{74,75} between mant groups due to protein molecules crowding and/or clustering in the rafts.^{22,76,77} In contrast, Ras-mGppNHp exhibited relatively high FRET values that reported on the easy accessibility of mant to Rhod-DOPE.

in vivo observations of Matallanas et al., though the lipid domain preferences of N-Ras-GTP were not explored in this study.⁴² In contrast, Roy et al. observed the G12V N-Ras mutant clustered in cholesterol-dependent rafts (the G12V mutation ensures that the proteins were predominantly bound to GTP) while the wild type (GDP-bound) was not found to be clustered (remained outside of lipid rafts).⁴³ It should be noted that these research groups performed experiments on different types of cells: MDCK and COS-7⁴², and BHK.⁴³ Variability in the lipid membrane composition and the presence of cell-type specific membrane proteins may be, in part, responsible for the observed opposite localization patterns (the cell-type dependence of localization was recently reported for H-Ras⁷⁸).

In our study, we made it our goal to separate interaction of N-Ras with lipids from possible interactions with other cellular membrane proteins. Choosing an adequate lipid raft model for an *in vitro* study is difficult because the cellular bilayers are asymmetric with their inner leaflet (where Ras proteins are) enriched with negatively charged phosphatidylserine and completely lacking SM.^{79,80} The inner leaflet mixture, however, cannot spontaneously form rafts; instead, raft formation must be triggered by the cross-leaflet interdigitation with the lipids of the outer side of the plasma membrane (rich in SM).^{81,82} Preparation of such asymmetric LUVs was recently reported.^{82,83} However, performing experiments with asymmetric bilayers to study protein–nanodomain interactions is not straightforward as the lipid asymmetry is relatively short-lived; cells maintain it by a continuous action of lipid transporters, and the loss of lipid asymmetry is a signature of cell death.⁸⁴ LUVs in our work and all other Ras reports were inherently symmetric, which makes it difficult to evaluate which study made use of a more relevant lipid bilayer. Nicolini et al. used DMPC/DSPC/cholesterol LUVs and did not recapitulate any of the *in vivo* observations.⁴⁴ Larsen et al. analyzed the distribution of the N-Ras C-terminal lipopeptide in DOPS/PSM/cholesterol bilayers and found it to be populating raft domains.⁸⁵ This is contrary to the observation of Nicolini and others,⁴⁴ as well as our own data reported in this paper, suggesting that the choice of the lipid system is crucial and far from settled.

In our study, the lipid mixture with an equimolar ratio of SM, POPC, and cholesterol was used to create raft nanodomains most closely mimicking the size of cellular raft domains⁵⁰ and helped reveal the “raft affinity switch” in the N-Ras macromolecule. Obtained data allow us to conclude that while interactions with the cellular protein binding partners might be important for regulation of Ras domain preferences, the G domain itself controls the interaction with the raft phase, while the C-terminal lipopeptide “pulls” the protein outside of the raft. We do not consider our evidence of nonboundary localization of the C-terminal lipopeptide particularly strong due to the lack of a readily available positive control, such as a well-characterized lineactant that would serve as a calibration for the N-Ras peptide action in [Figure 5](#). Therefore, our most accurate finding concerning the N-Ras lipid domain preferences would be that G domain in the GDP-bound state is strongly attracted to the lipid rafts, overcoming opposite preferences of the C-terminal lipopeptide; the Ras–raft interaction is weakened or absent in the GTP-bound form, allowing the C-terminal lipopeptide to draw N-Ras outside of the raft (to the boundary or into the disordered membrane). Molecular dynamics simulation by the Gorfe group determined that H-Ras C-terminal lipids favored localization at the raft boundary (due to palmitoyl chains favoring the ordered raft phase with the farnesyl lipid being excluded from it).⁸⁶ Because the N-Ras lipoprotein also has farnesyl and palmitoyl lipids, one might expect similar boundary localization for both the truncated C-terminal N-Ras lipopeptide and the full-length N-Ras when it is bound to GTP.

Gorfe et al. identified a set of basic residues in the H-Ras sequence that made specific contacts with the lipid bilayer in molecular dynamics simulations: R128, R135, R169, and K170.⁸⁷ Abankwa and co-workers

found these sites modulating Ras signaling function and proposed that conformations of these residues and the overall orientation of the G domain must be affected by the nucleotide binding site via an allosteric coupling mechanism.^{51,52,88} These positively charged sites are presented in N-Ras by conservative substitutions (K128, K135, and K169), while K170 is identical. At approximately the same time, using spin relaxation nuclear magnetic resonance measurements, we reported that G domains of H-Ras and K-Ras possessed global conformational exchange dynamics connecting the effector interface of Ras with the rest of the molecule.^{89,90} We demonstrated that the novel ion binding pocket on the membrane-facing side of the G domain in H-Ras described by Buhrman and co-workers⁹¹ was thermodynamically coupled to the nucleotide binding site while being nearly 20 Å away: its affinity for a divalent ion changes by a factor of 5 upon replacement of GDP with the GTP mimic.⁹² Because of the high level of sequence identity of the G domains, we reasonably expect N-Ras to possess the same allosteric coupling mechanism. However, our observation that the chimeric H/N-Ras construct does not discriminate the lipid nanodomains indicated the conserved features described above are not sufficient; the interactions between the N-terminal G domain and the C-terminal lipopeptide must also involve isoform-specific contacts.

The plausible molecular mechanism of the lipid domain recognition by Ras isoforms has not been resolved experimentally. Instead, Werkmuller et al. provided evidence that G domains of N and K-Ras are experiencing similar rotational freedom next to the membrane regardless of the bound nucleotide or the type of lipid bilayer (raft or homogeneous).⁹³ The similarity of rotational diffusion under all conditions implied that their G domains do not interact with the lipid other than through a C-terminally lipidated peptide acting as a tether (however, it was not clear if the Ras/LUV samples were separated from aggregated lipidated Ras or the authors simply assumed 100% binding and a negligible contribution from lipoprotein aggregates). This is why the view that other cellular proteins like galectins interact with the GTP-bound Ras and cause redistribution between lipid domains remains an attractive alternative.³⁸ However, N-Ras was not found to bind galectins, and most importantly, galectin 1 (interacting with H-Ras) is a cytosolic protein⁽⁹⁴⁾ recruited by H-Ras to the plasma membrane,⁴¹ making it less likely to be the domain recognition driver. In our experiments, the N-Ras-mGppNHp complex demonstrated association with rhodamine-labeled disordered domains while the N-Ras-mGDP complex was concentrated in rafts, all in the absence of other cellular components, which forces us to reconsider the role of a G domain in lipid raft recognition by Ras proteins.

Finally, we note an important caveat in the differences that may be reported in studies using bulk fluorescence measurements of LUVs versus confocal microscopy experiments (including single-molecule tracking). Confocal microscopy, by design, involves focusing of a very intense laser light into a very small area to ensure effective excitation. This mode of observation was demonstrated to create artifacts due to overly intense illumination, particularly when observing Ras, which is a tyrosine-rich molecule prone to irreversible photoactivated cross-linking.⁹⁵ Previously, we established that the light intensity used in a solution fluorescence measurement in a conventional spectrofluorometer did not lead to cross-linking artifacts in a highly homologous Ras construct;⁹⁶ therefore, oxidative cross-linking of N-Ras was not expected to negatively affect the results of this report.

Conclusions

In this study, we demonstrated that the N-Ras lipoprotein changes its lipid nanodomain preferences in a nucleotide-dependent manner in the absence of other membrane proteins (in a model lipid membrane).

The signaling-inactive, GDP-bound N-Ras was found to have preferential affinity for lipid rafts. N-Ras in its activated conformation (bound to the GTP mimic) was localized at the raft boundary or in a disordered lipid phase. The molecular mechanism must rely on specific interactions between the G domain and the C-terminal lipopeptide as the chimeric construct, including the G domain from H-Ras, did not reveal the nucleotide-dependent change in localization. Thus, we established that the specific nanodomain preference is an intrinsic property of the full-length N-Ras lipoprotein, which may further be modulated by specific protein–protein interactions in the cell.

Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://pubs.acs.org/doi/10.1021/acs.biochem.7b01181) at DOI: [10.1021/acs.biochem.7b01181](https://pubs.acs.org/doi/10.1021/acs.biochem.7b01181).

- Complete Materials and Methods section, Tables S1 and S2 with lifetime and size-exclusion data, supporting figures, and estimates of protein surface densities

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