Membrane Anchoring

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The Lipid Modifications of Ras that Sense Membrane Environments and Induce Local Enrichment**

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The transduction of an external stimulus from the outside of a cell into its nucleus is one of the most important mechanisms for the regulation of numerous biological processes. External signals activate receptors that transmit the information across the membrane, where it is transducted by a set of proteins that activate ion channels, phosphokinases, or other downstream effectors. GTP binding proteins that pick up the signal at the receptor, such as heterotrimeric G proteins or Ras, are membrane-associated by post-translationally acquired lipid modifications.[1] These lipid chains provide the hydrophobic free energy for membrane association and their lack releases the proteins to the cytosol, rendering them inactive. Thus, through membrane binding, Ras increases its effective concentration to optimize the interaction both with the receptor and downstream effectors.[2] Ras is an important molecular switch that regulates cell proliferation, differentiation, and growth.[3]

The highly specific membrane binding of Ras can be appreciated by comparing the members of the Ras family: Two lipid modifications are required for N-Ras and K-Ras4A, whereas H-Ras carries three lipid chains.[4] In contrast, K-Ras4B requires the concerted action of one lipid chain and favorable electrostatics for membrane binding.[4] Although inserted into the membrane, the lipid modifications experience a high degree of motional freedom that is also transmitted to the adjacent polypeptide chain.[5,6]

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Although the highly homologous Ras proteins interact with the same effectors in vitro, they produce distinctly different output signals in vivo, which suggests that these differences are imparted by the lipid-modified C termini of the proteins, where the homology is very low.[7] Moreover, depending on the nucleotide binding state, the localization of Ras in liquid-crystalline or raft domains[8] of the membrane appears to be regulated. Only active H-Ras*GTP interacts with the respective set of effectors; the non-activated form, H-Ras*GDP, is constrained to rafts, where the signal is not further transmitted. An alternative model suggests that the difference in signaling of the Ras isoforms is imparted from the altered access and residence time in a specific compartment.[9,10] This model suggests that interactions of Ras and its lipid modifications with rafts or fluid membrane domains determines the membrane localization and the biological function of the molecule, which is investigated herein.

^{2}H NMR is a useful tool for the investigation of lipid rafts. It is applicable to each component of a lipid mixture, and only requires the synthesis of the relevant molecule with a deuterated chain. First, we investigated the adaptation of the lipid modifications of a N-Ras heptapeptide, which was hexadecylated at Cys 181 and Cys 186, to the membrane thickness. Four different membranes composed of lipids with varying hydrocarbon chains were chosen to constitute the host membrane. Membrane thicknesses studied by ^{2}H NMR varied from 21.0 Å (DLPC) to 38.8 Å (DPPC/cholesterol 10:6, Table 1). The high cholesterol content leads to condensation of the lipids, which increases their length[11] and abolishes the phase transitions of DPPC such that all lipid mixtures could be studied at 30 °C.

Table 1: Structural parameters for the lipid membranes and the Ras peptide at 30 °C with a 10:1 lipid/peptide molar ratio.

Sample	A [Å^2][a]	D_C [Å][b]	L_C [Å][c]
[D_{62}]DPPC/Chol	22.7	38.8	16.2
[D_{62}]DPPC/Chol/Ras	22.8	38.6	16.1
DPPC/Chol/[D_{66}]Ras	23.6	37.2	15.5
[D_{31}]POPC	30.8	28.6	11.6
[D_{31}]POPC/Ras	30.6	28.8	11.6
POPC/[D_{66}]Ras	33.3	26.4	10.0
[D_{54}]DMPC	29.9	25.8	10.5
[D_{54}]DMPC/Ras	29.2	26.4	10.7
DMPC/[D_{66}]Ras	33.6	26.2	10.0
[D_{46}]DLPC	31.5	21.0	8.2
[D_{46}]DLPC/Ras	30.9	21.4	8.4
DLPC/[D_{66}]Ras	35.4	24.8	8.7

[a] Cross-sectional area of one hydrocarbon chain. [b] Hydrocarbon thickness of the membrane. [c] Chain extent of a single chain.

^{2}H NMR spectra were recorded for deuterated membranes in the absence and presence of Ras and also for a protonated membrane in the presence of deuterated [D_{66}]Ras. Representative ^{2}H NMR spectra for Ras in DLPC are shown in Figure 1 a–c. Insertion of Ras did not alter the order parameters of the membrane phospholipids significantly; however, the NMR spectrum of the Ras lipid chains is drastically narrower, which indicates lower chain order parameters.

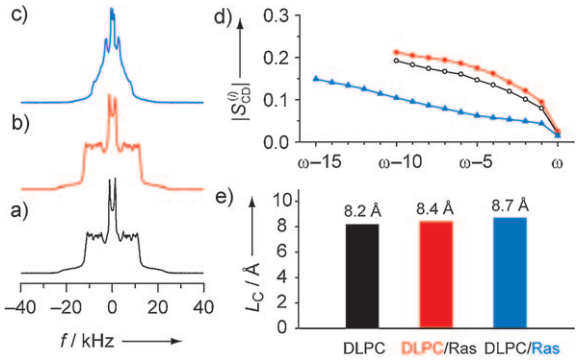

Figure 1. ^{2}H NMR spectra of deuterated DLPC (a), deuterated DLPC/Ras 10:1 (b), and deuterated [D_{66}]Ras in DLPC (c). d) Order parameters of DLPC in the absence (black) and presence (red) of Ras and of the Ras lipid chains (blue). e) Plot of the chain lengths (L_C) of the DLPC and Ras chains.

From the chain order profiles (Figure 1 d), the geometric parameters for the lipid chains of the host membrane and of the lipid modification of Ras were calculated[12] (Table 1). In DLPC, a small increase in the chain lengths upon addition of Ras was detected (Figure 1 e). Surprisingly, the Ras chain exhibits an almost identical length although it contains four additional methylene groups. Thus, almost perfect chain-length matching is observed for Ras and DLPC. The 16:0 Ras chain is accommodated in the 12:0 DLPC bilayer by increasing its cross-sectional area to 35.4 Å^2, whilst the lauroyl chains of the host membrane occupy only 30.9 Å^2 each.

We then investigated whether this chain-length adaptation was also encountered in membranes of larger hydrophobic thickness. ^{2}H NMR spectra and order parameters for DMPC, POPC, and DPPC/cholesterol are given in the Supporting Information. The chain lengths of the membranes and the Ras chains are shown in Figure 2. In all cases, an almost perfect chain-length adaptation between Ras and its host membrane was found. Considering all the ^{2}H NMR data, we suggest that the length of the Ras lipid chain adapts to that of the host membrane. Depending on the hydrophobic thickness of the lipid membrane, Ras lipid chain lengths between 8.7 Å and 15.5 Å were observed; in other words, the length of the lipid modification of Ras can almost double to match the thickness of the host membrane. Concomitantly, the chain length adaptation of Ras is accompanied by a variation of its cross-sectional area between 35.4 Å^2 and 23.6 Å^2. In contrast, the lipid membrane alters its thickness insignificantly upon Ras insertion. Therefore, instead of the membrane adapting to Ras, the Ras chains adapt to the

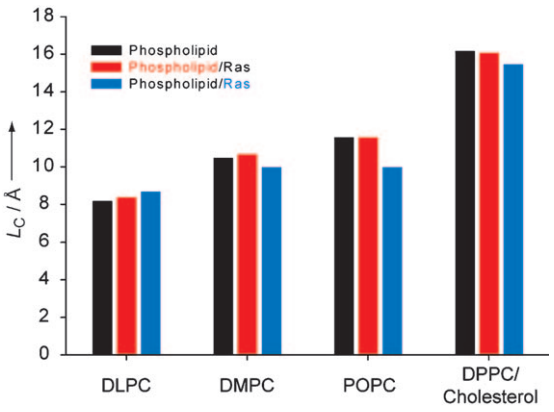

Figure 2. Chain lengths of the phospholipids (black and red) and Ras peptides (blue) in host membranes of varying composition (30 °C).

membrane, which appears to be the energetically more favorable process. This situation is opposite to the insertion of stiff transmembrane α helices, where the membrane adapts to reduce the hydrophobic mismatch.[13] Such a process involves many lipid molecules, which likely makes it energetically more costly.

The adaptation of the Ras chains to the length of the host membrane requires either their compression or dilation. A compressed lipid chain increases its number of *gauche* defects, thereby varying the chain enthalpy and entropy.[14] A single *gauche* defect decreases the chain length by 1.1 Å,[15] which allows an estimation of the number of *gauche* defects associated with the Ras chain adaptation in different host membranes. An *all-trans* 16:0 hydrocarbon chain has a length of 17.8 Å (14 C–C bonds between protonated carbon atoms). The most extended Ras chain was observed in the DPPC cholesterol (15.5 Å), for which we calculate two *gauche* defects on average in the Ras chain. In contrast, in the DLPC membrane, the chain extends to only 8.7 Å, which requires the presence of about eight *gauche* defects.

What appears to be an interesting physicochemical chain-matching phenomenon should also be of biological importance. In the cell, Ras travels between the plasma and Golgi membranes and within different compartments of the plasma membrane. Cellular studies have shown that the localization of Ras proteins is in part regulated by the different post-translational lipid modifications.[16,17] The lipid anchor on the N-Ras protein controls the fast and reversible distribution of the molecule over the various membranes.[18]

Protein palmitoylation is considered to be a raft targeting signal, and Ras may be targeted to rafts as well.[19] The raft (liquid-ordered, l_o) and liquid-disordered (l_d) domains of the membrane are characterized by different thicknesses.[20–22] Mostly long-chain saturated sphingomyelin (SM) and cholesterol are segregated into l_o domains, whereas l_d domains contain mostly unsaturated lipids, which are more disordered and therefore shorter. Recently, it was shown that the N-Ras proteins exhibited diffusion and subsequent clustering in the l_o/l_d phase boundaries.[10] Computer simulations have suggested interactions of the G-domain of H-Ras with the membrane.[23] Therefore, instead of using the N-Ras hepta-

peptide, the experiments in raft-forming membranes were carried out using full-length lipidated N-Ras.[24] This fully functional construct contained a hexadecyl chain at Cys 181 and a farnesyl chain at Cys 186.

We studied the lateral distribution of Ras in giant plasma-membrane vesicles (GPMV) of HeLa cells.[25] The l_d phase was labeled with R18 (red fluorescence). Bodipy-labeled N-Ras (green fluorescence) is only localized in this domain (Figure 3a).

As GPMVs cannot be prepared in sufficient quantities for NMR studies, we used bilayers made from the envelope membrane of influenza viruses that mimic the lipid composition of biological membranes. Small amounts of synthetic phospholipids were added to provide ^{2}H NMR sensitive probes. Confocal fluorescence microscopy of giant unilamellar vesicles (GUV) made of this biological mixture and AFM on corresponding supported bilayers indicated the coexistence of l_o and l_d domains over a broad temperature range (Figure 3b–d and Supporting Information). As observed in the GPMVs, N-Ras is also localized in the l_d domain of the virus membranes. Furthermore, both fluorescence microscopy and AFM showed that a significant portion of N-Ras is localized in the l_o/l_d phase boundary of this membrane (Figure 3b–d). However, the marker for the l_d phase is not found in the phase boundary.

For ^{2}H NMR spectroscopy, we added 10 mol% of deuterated POPC and palmitoyl sphingomyelin (PSM) as probes for the l_d and l_o phases, respectively. As either POPC, PSM, or N-Ras was deuterated, characteristic ^{2}H NMR spectra and order parameters could be measured (Figure 4a–c). The ^{2}H NMR spectra of the lipid components vary considerably

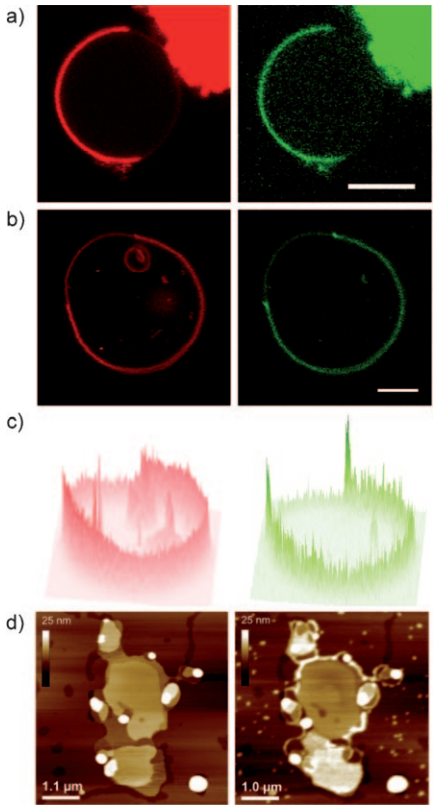

Figure 3. N-Ras localization in l_d domains. a) Confocal laser scanning microscopy images of GPMVs prepared from HeLa cells that exhibit lateral lipid domains at 4°C. Bodipy-labeled N-Ras protein (green fluorescence) is co-localized with R18 (red fluorescence), which is enriched in the l_d phase (scale bar 5 μm). The fluorescence in the upper right corner is due to cellular residues. b) GUVs (prepared from lipid extracts of influenza virus containing 10 mol% PSM, 10 mol% POPC, and 1 mol% N-Rh-DOPE) incubated with Bodipy-*N*-Ras protein for 24 h at 20°C exhibit localization of Ras in the l_d domain and particularly at the l_o/l_d phase boundary at 4°C. c) Fluorescence intensity profiles of the images in b) emphasizing the preferred localization of Bodipy-*N*-Ras at the l_o/l_d phase boundary (green fluorescence). d) AFM images of lipid bilayers consisting of the viral membranes, before ($t = 0$ h, left) and after ($t = 3.5$ h, right) addition of N-Ras. A pronounced incorporation of N-Ras in the domain boundary is detected.

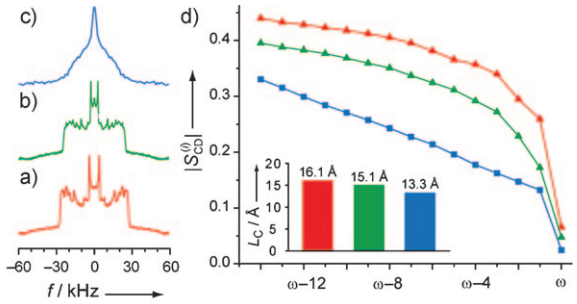

Figure 4. ^{2}H NMR spectra of PSM (a), POPC (b), and the full-length Ras protein (c) in membranes formed from influenza virus lipids at 30°C. The virus membranes contained 10 mol% POPC and PSM and 7 mol% Ras protein. d) Order parameter plot and the full-length Ras protein of the respective lipids in virus membranes. The inset shows the chain lengths of the PSM (red), POPC (green), and Ras (blue).

according to the different environments, and the ^{2}H NMR spectrum of the N-Ras hexadecyl chain in the mixture is narrower, shows poor spectral resolution, and does not resemble that of either lipid component. Such ^{2}H NMR spectra, which are indicative of slower motions with microsecond correlation times, are similar to a critical behavior with significant fluctuations at the critical point of ternary lipid mixtures.[26] Furthermore, the Pake spectrum is superimposed with an isotropic signal that accounts for about 8.5% of the intensity. Such phenomena are often encountered in biological membranes and can be explained by highly mobile lipid chains.[27] This result would suggest that about 8.5% of the Ras chains are isotropically mobile and are most likely not inserted into the membrane.

Figure 4d shows a plot of the order parameters of the phospholipids in the mixture and of N-Ras. Consistent with the differences of l_o and l_d domains,[20–22] the order parameters of PSM are higher than those of POPC. Nevertheless, the lengths of the chains are relatively similar (15.1 Å for POPC and 16.1 Å for PSM). The N-Ras chains show somewhat lower order, which corresponds to a shorter chain length (13.3 Å), indicating that N-Ras is essentially surrounded by lipid molecules in the liquid-crystalline state.

Raft and l_d domains of the plasma membrane vary not only in their lipid distribution but also in their physical properties. This variation has a profound impact on the hydrophobic thickness of the membrane compartments, which could be sensed by the lipid modifications of Ras. Considering the Ras chain adaptation to the thickness of the host membrane, the lateral diffusion of Ras into or out of a raft would be related to quite significant structural and dynamical alterations. In the membranes prepared from lipid extracts of influenza virus membranes, the marker lipids (POPC for the l_d and PSM for the l_o phase) clearly showed the different hydrophobic thicknesses of these domains. More importantly, the lipid modification of N-Ras is clearly disordered, showing a chain extent of 13.3 Å, which corresponds to approximately four *gauche* defects. This chain length is larger than in DLPC, DMPC, or POPC membranes, but shorter than the POPC in the viral lipid bilayers, which is the probe for the l_d phase. This result is a strong indication for a residence of the N-Ras protein in the l_d domain. Furthermore, the ^{2}H NMR spectra indicate the characteristics of intermediate timescale motions that might be due to long-lived fluctuations of correlated N-Ras molecules on the submicrometer length scale. Such features are in agreement with the sequestration of N-Ras in the l_o/l_d phase boundary, where it experiences a favorable decrease in line tension associated with the rim of the demixed phase, as verified in the viral membrane system using AFM and fluorescence microscopy. This result suggests that localization of Ras in the phase boundary may allow a faster and more flexible redistribution of the molecules between the compartments of the plasma membrane.

In summary, the lipid chain modifications of membrane-associated N-Ras undergo a remarkable adaptation to the hydrophobic thickness of the host membrane. A saturated 16:0 chain of N-Ras can easily halve its length by introducing up to six additional *gauche* defects. Depending on the host membrane, the N-Ras lipid anchors undergo large amplitude motions and are highly flexible. We may assume that the flexibility in the adaptation to the properties of the host membrane compartment are a prerequisite for the sorting and trafficking of the molecule in the plasma membrane and other cellular membranes.

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