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Review

Membrane binding of lipidated Ras peptides and proteins — The structural point of view

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ABSTRACT

Biological membranes are interesting interfaces, at which important biological processes occur. In addition to integral membrane proteins, a number of proteins bind to the membrane surface and associate with it. Posttranslational lipid modification is one important mechanism, by which soluble molecules develop a propensity towards the membrane and reversibly bind to it. Membrane binding by insertion of hydrophobic lipid moieties is relevant for up to 10% of all cellular proteins. A particular interesting lipid-modified protein is the small GTPase Ras, which plays a key role in cellular signal transduction. Until recently, the structural basis for membrane binding of Ras was not well-defined. However, with the advent of new synthesis techniques and the advancement of several biophysical methods, a number of structural and dynamical features about membrane binding of Ras proteins have been revealed. This review will summarize the chemical biology of Ras and discuss in more detail the biophysical and structural features of the membrane bound C-terminus of the protein.

Contents

1. Introduction

The continued existence of cells crucially depends on their ability to receive signals from the environment and to respond to them in a suitable way. This process of signal transduction, i.e., the forwarding of signals from the extracellular matrix via the cytoplasm into the nucleus, involves, among others, proteins that are modified with hydrophobic structural features attached to the peptide backbone. Posttranslational lipidation by acylation with fatty acids, alkylation with prenyl moieties, esterification with cholesterol, and conjugation of the more complex lipid glycosylphosphatidylinositol (GPI) (Fig. 1), occurs on a wide variety of intracellular and extracellular signaling proteins [1]. These hydrophobic modifications typically result in membrane binding affinity of the lipidated proteins, thereby affecting protein localization and functioning. Whereas classical transmembrane proteins span the membrane and are occasionally lipidated, the majority of lipidated proteins typically reside on only one side of the membrane. After trafficking along intracellular sorting pathways, the

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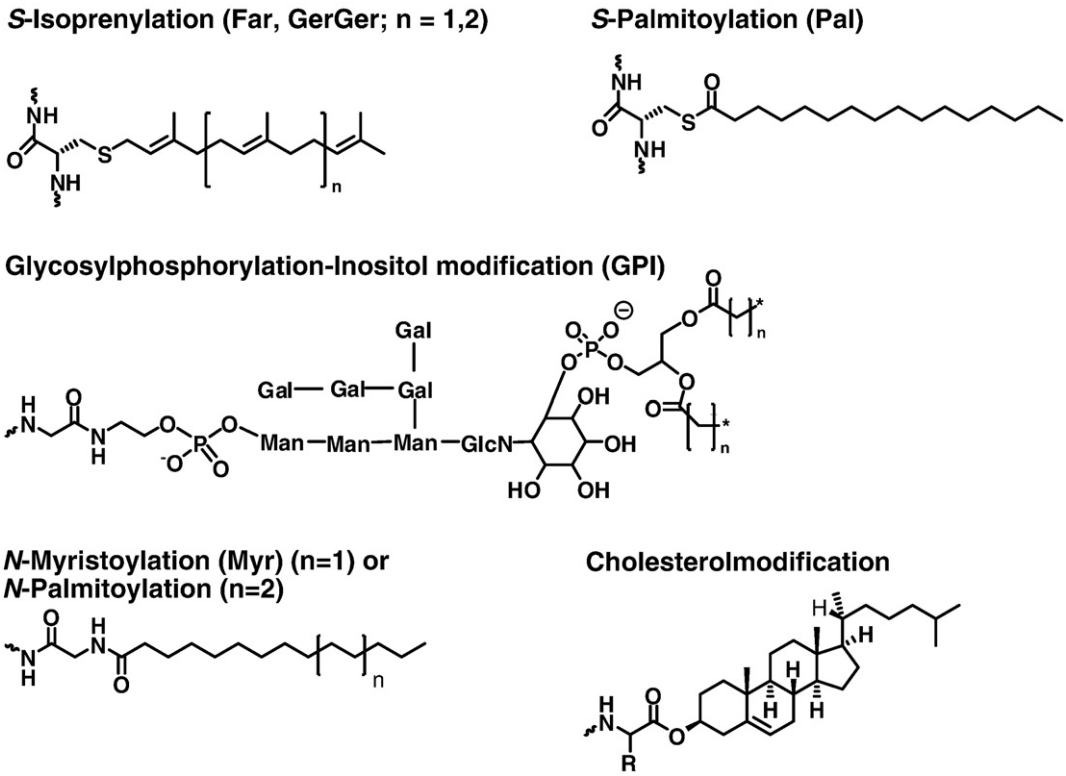

Fig. 1. Structural overview of different lipidation motifs found on membrane-binding proteins.

final destiny of GPI-anchored proteins is the extracellular leaflet of the plasma membrane [2], prenylated and/or acylated proteins typically reside at the inner leaflet of the plasma membrane or at the surface of endomembranes [1]. The posttranslational lipidation of proteins is of special physiological importance as it does not only control the function of these proteins in signal transduction, but for example also in vesicular transport [3].

The modification of soluble proteins with lipids facilitates the membrane binding of these proteins, thereby restricting the diffusional mobility of the proteins from three to two dimensions, with concomitant significant effects on the biomolecular interactions of these molecules with both lipids and effector proteins. Typically, a single lipid modification, for example farnesylation or myristoylation, confers only limited membrane affinity to the proteins, resulting in rapid dissociation of the molecule from membrane. Farnesylated or myristoylated proteins therefore mostly feature more than one membrane targeting element [4,5]. These can be for example one or more lipids such as an additional one or two palmitoyl cysteine thioester functionalities as present in K-Ras4A, N-Ras or H-Ras, or a polybasic amino acid stretch, as for example operative in K-Ras4B, MARCKS or Src [4,6–9]. Interestingly, this second membrane targeting element alongside the prenyl functionality is often of a reversible nature; a cysteine thioester can be hydrolyzed and a polybasic amino acid stretch can be partly counterbalanced via phosphorylation of neighboring serines [1,6,10]. As such lipidation of proteins also provides a way to reversibly influence protein localization and protein interactions. Protein lipidation is thus a diverse control mechanism for the biological function of these proteins.

The lipidation of proteins targets them to membranes, including the plasma membrane. At the plasma membrane, but not solely there, these lipidated proteins often function as signal-transducing proteins, relaying extracellular signals that stimulate transmembrane cell surface receptors into non-membrane bound intracellular effectors [11]. The localization of these proteins at the membrane surface allows an efficient complex formation due to the increased effective concentration of the proteins and the aforementioned two-dimensional nature of the membrane. Examples of classes of proteins in which the signal transduction is regulated via their membrane bound state are the (trimeric) G-proteins, the Src family tyrosine kinases, and small monomeric G-proteins.

An example of an important small subfamily of the monomeric G-proteins, with a major role in cellular signaling, are the Ras proteins [12,13]. The signal transduction process, in which Ras is involved, occurs on membranes [3]. At the plasma membrane, this includes for instance the binding of a signaling hormone to a transmembrane receptor tyrosine kinase (RTK) and subsequent receptor dimerization, autophosphorylation, and recruitment of exchange factors (e.g. Sos that is recruited via the adaptor protein Grb2, growth-factor-receptor-binding protein 2), which then stimulate the nucleotide exchange of Ras and as such 'turns on' Ras. This is then followed by the interaction at the membrane of Ras with effectors such as Raf kinase or phosphatidylinositol 3-kinase and subsequent downstream signaling (Fig. 2).

The introduction above illustrates that membrane localization of lipidated proteins is a highly important feature. Apart from regulating signal transduction, it also plays a major role in vesicular transport, protein–protein interactions, and membrane composition. In spite of the tremendous biological significance of membrane binding of lipidated proteins and Ras in particular, precise information about details determining this process is very limited. Several studies have addressed for example the dynamics of the soluble N-terminus of Ras such as X-ray crystallographic studies [14], but molecular information on the lipidated C-terminus has been lagging behind [15]. In recent years however, significant progress has been obtained concerning the structural insights in the membrane binding of lipidated Ras peptides and proteins. This has in part been due to new chemical biology approaches combining the generation of the lipidated Ras model peptides and the completely processed Ras

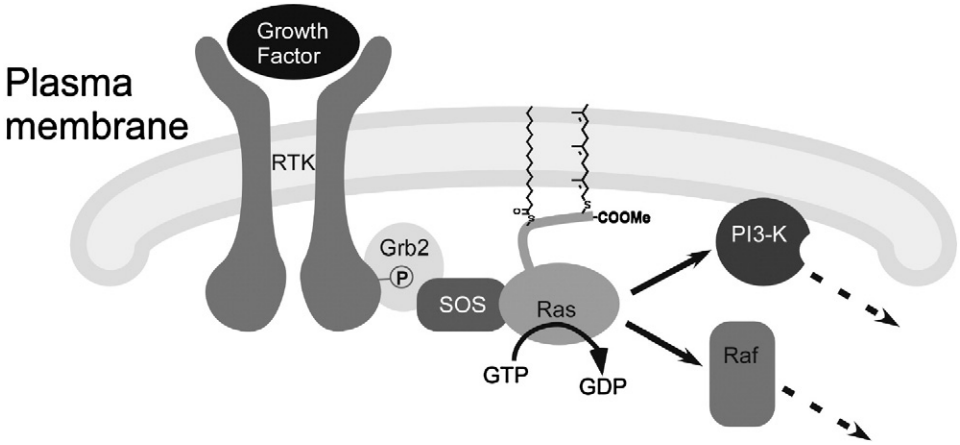

Fig. 2. Schematic overview of signal transduction at the plasma membrane by Ras. RTK: receptor tyrosine kinase, Grb2: growth-factor-receptor-bound protein 2, SOS: son of sevenless, Raf: Raf kinase, PI3-K: phosphoinositide 3-kinase.

proteins with advanced biophysical characterization techniques [15,16].

2. Chemical biology of Ras

Ras GTPases belong to the class of monomeric G-proteins, involved in cellular processes such as vesicle transport, the cell cycle, nuclear import and signal transduction [13]. These G-proteins adopt their so-called active state by binding guanosine-5′-triphosphate (GTP), while binding of guanosine-5′-diphosphate (GDP) stabilizes their inactive state (GTP-GDP switch). The active form acts by stimulating or inhibiting cellular processes via interactions with effectors. Hydrolysis of the GTP to GDP results in the deactivation of the proteins. Due to the importance of these molecular switches, their misregulation often has deleterious consequences. Prominent examples can be found in many cancers, where mutated Ras proteins "arrested in the GTP-bound state" are often encountered [17]. These Ras proteins are thus continuously in their active form, which results–together with additional disturbances in signal transduction processes–in uncontrolled cell growth.

Members of the Ras superfamily are posttranslationally modified via prenylation [18] and in some cases acylation reactions [19]. The two most common types of posttranslational lipid modifications encountered in the Ras superfamily are *S*-palmitoylation and *S*-isoprenylation. Isoprenylation is a stable (non-reversible) modification, whereas palmitoylation is a reversible process [20,21]. Additionally, some of the proteins belonging to the Ras superfamily are posttranslationally converted into C-terminal methyl esters.

The Ras proteins are a subgroup of the Ras superfamily and the three isoforms H-, N- and K-Ras are the most prominent examples of this subgroup (Fig. 3) [12]. The differences in amino acid sequence between these Ras proteins are mainly in their C-terminal hypervariable region. This apparently random-coiled terminal part contains the signal sequence for the posttranslational modifications and regulates the membrane binding of the proteins. As for other lipidated proteins, until recently, most experiments with these molecules focusing on the molecular/structural understanding of the Ras proteins and their interactions were performed on the soluble part of the molecules, i.e., the protein without the C-terminus, or with low resolution methods. The results obtained via these approaches can, however, at best only

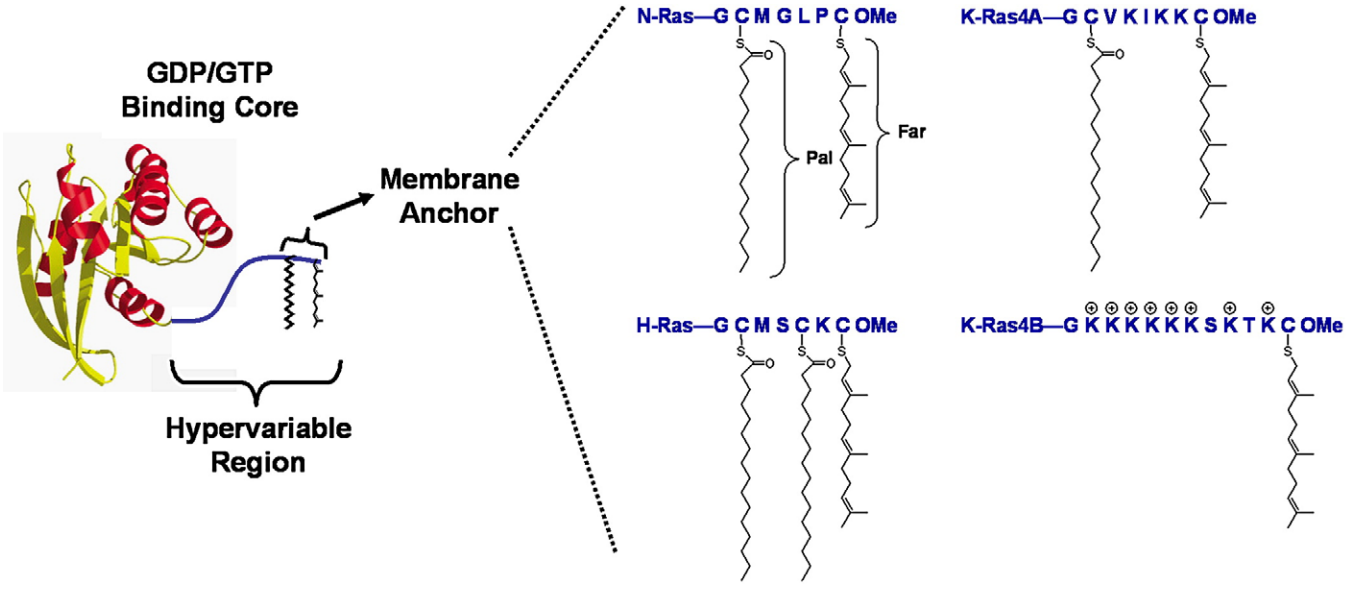

Fig. 3. Ras core structure and C-terminal lipidation motifs of posttranslational completely processed N-Ras, H-Ras, K-Ras4A, and K-Ras4B. For N-Ras, H-Ras, and K-Ras4A the cysteine palmitoyl thioesters are reversible posttranslational modifications, regulating membrane affinity and localization in cooperation with the farnesyl thioether. For K-Ras4B the polybasic lysine stretch is the second membrane targeting sequence, additional to the farnesyl thioether. HVR: hypervariable region.

give an approximation of the real situation. Especially the important membrane-binding mechanism could not be addressed by these studies. This is even more required, as the main molecular diversity between the individual Ras members is encountered in the hypervariable region (HVR) including the lipidated part of the protein at the C-terminus.

The biochemical generation of fully functional and modified lipidated proteins is, however, difficult and time-consuming. In the case of *S*-palmitoylation, it leads to heterogeneous mixtures and is, therefore, in most cases, not practical or applicable. Therefore, in recent years, chemical biological approaches have been developed that give access to fully functional lipidated Ras peptides and proteins, together with additional non-natural modifications. This was achieved via two techniques that were developed more or less in parallel: (*i*) progress in the field of protein ligation and chemical synthesis of proteins [22,23] and (*ii*) progress in the field of lipidated peptide synthesis [15,16,24–27]. These developments and their application in chemical biology have been used to investigate and explain unsolved questions regarding the membrane binding of Ras proteins. An example how such a chemical biology approach was also used to elucidate aspects of the reversible palmitoylation of Ras is given below.

S-palmitoylation of proteins entails connection of a (C16) fatty-acid chain to the thiol functionality of a cysteine via a thioester bond [28]. Although *S*-acylation also occurs by other types of fatty acids, palmitoylation is by far the more dominant. In contrast to the other protein lipidation motifs, *S*-palmitoylation is a reversible posttranslational modification [20,29]. The palmitoylation of proteins does not seem to be confined to specific sequence motifs. Even though some recurring elements can be found in the proteins that are being palmitoylated [11], the major requirement seems to be the presence of a cysteine, located close to the membrane. Typically, palmitoylated proteins feature more than one lipid modification. It is assumed that the other lipid modification provides the affinity to the membrane that enables palmitoylation of the respective cysteine(s).

The reversible palmitoylation of proteins has regulatory functions [30]. These regulatory mechanisms and *S*-palmitoylation enzymology [28] are on the forefront of the scientific interest in functioning and regulation of membrane bound proteins [1,31]. The importance of Ras palmitoylation has been recently highlighted in studies on the regulation of its localization and activity [3,8,32–35].

In the Ras superfamily, H-Ras and N-Ras are examples of proteins that are both prenylated and palmitoylated, with N-Ras featuring one and H-Ras two possible palmitoyl functionalities (Fig. 3). It has been shown for example that the two individual palmitoyl residues of H-Ras have a distinct role in protein trafficking, localization and signaling [36].

Cellular studies have shown that the localization of Ras proteins to different subcellular membrane compartments is in part regulated by the different posttranslational lipid modifications of the different isoforms [35,37–39]. Using a chemical biology approach with semi-synthetic proteins it could be shown that the lipid anchors on the N-Ras protein control the fast and reversible distribution over the different membranes [10,33]. The use of semi-synthetic Ras proteins showed that Ras rapidly cycles between the plasma membrane (PM) and the Golgi and that this cycling of Ras regulates the spatiotemporal distribution and activity of H- and N-Ras. These experiments furthermore showed that retrograde PM-Golgi trafficking of N-Ras is mediated by depalmitoylation/repalmitoylation activities that act on Ras in different subcellular localizations. Together with the anterograde transport a cycle is generated that determines the specific PM and Golgi localization of Ras (Fig. 4). The palmitoylation status drives rapid exchange of Ras between the plasma membrane and the Golgi apparatus; depalmitoylated Ras protein exchanges rapidly between the membranes, and repalmitoylation occurs at the Golgi, where Ras signals and is redirected to the plasma membrane. Without the de/reacylation cycle, palmitoylated Ras would incorrectly localize to any membrane compartment and lose its specific activity.

Apart from the distribution over different subcellular compartments, Ras additionally compartementalizes within specific membranes, such as the plasma membrane. Here, the localization within raft and non-raft domains is of interest. For instance, protein palmitoylation is a well-known raft targeting signal [40,41]. Although the branched prenyl groups may not fit well into the highly ordered raft domains, it has been shown that H-Ras is also targeted to raft domains [42]. Interestingly, the partitioning of Ras into raft and non-raft membrane structures depends on GTP-GDP loading [38]. The isoform-specific nano-clustering [39] relies on a complex interplay of the different types of lipid anchors, the hypervariable region, scaffolding proteins, the membrane, and possibly membrane receptors. H-Ras and K-Ras for example feature slower diffusion upon activation, as illustrated by a different behavior of H-Ras vs. N-Ras upon activation by GTP [43]. H-Ras has been most intensively studied and shown to have a high affinity for raft domains in the GDP-loaded state [35]. K-Ras is thought to have little affinity to cholesterol-rich regions in the membrane, whereas N-Ras seems to increase its affinity to rafts upon GTP loading [36]. The different affinities for raft domains of the GDP and GTP-bound Ras proteins would drive the different processes Ras is involved in at the plasma membrane (activation with GTP and downstream signaling) to specific sub-compartments on the plasma membrane [35]. The clustering of the different Ras proteins in different membrane micro-environments as such may be an explanation for the significantly different biological response of the different Ras isoforms [44]. Depending on the sub-compartment of the plasma membrane, where the Ras isoform of interest localizes and clusters, different interactions with other membrane-associated proteins, effectors and scaffolding proteins are made possible and may so explain the different biological effects of the three Ras isoforms.

3. Physical and structural biology of Ras

3.1. Biophysical studies of membrane binding of lipidated peptides

Before the discussion of the particular structural and dynamical features of Ras membrane binding the physical principles of membrane binding of lipidated proteins are briefly reviewed. For a more extensive discussion, the reader is referred to an excellent book chapter on this topic [45].

The thermodynamics of membrane binding of lipidated proteins is well-understood. The starting point for the discussion is the studies by Tanford on the hydrophobic effect [46]. The free energy of transfer of fatty acids comprising 8 to 22 carbons from aqueous solution into *n*-heptane provided a linear relationship of the unitary Gibbs free energy of transfer with the number of carbons of the chain that could be described by the equation $\Delta G^0 = (17.81 - 3.45 \times n_C)$ kJ/mol. This indicates that for each carbon that partitions into the hydrophobic phase, a constant decrease in the free energy by −3.45 kJ/mol is observed, which makes the partitioning an energetically favorable process. This favorable Gibbs free energy represents the basis for the membrane affinity of lipidated proteins. The 17.81 kJ/mol term reflects the resistance of the COOH group to partition into the *n*-heptane phase of low dielectric constant. If we consider an identical expression for the Gibbs free energy of transfer of a lipidated protein from the aqueous phase into the membrane, a significant binding energy arises, which binds a large portion of the lipid-modified protein to the membrane as shown in Fig. 5. For lipid modifications with 12, 14, and 16 carbons, approximately 5%, 44%, and 93% of the protein would be bound to the cellular membrane under these conditions.

A number of experimental studies on small lipidated peptides and proteins have investigated the thermodynamics of their membrane binding [47–52]. Most interestingly, these studies could confirm that partitioning of each methylene group into the lipid membrane

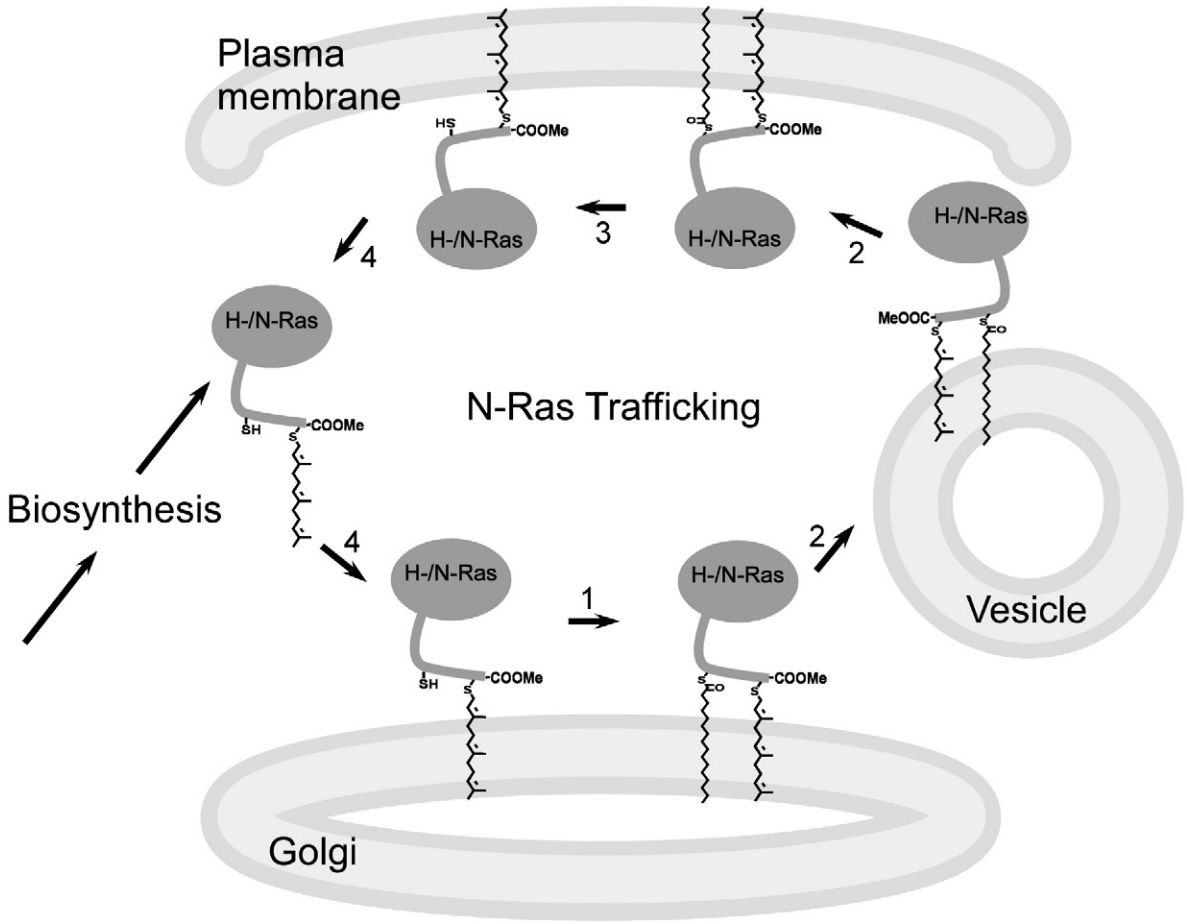

Fig. 4. N-Ras trafficking cycle from the Golgi complex to the PM and back. *S*-Palmitoylation of the N-Ras occurs at the Golgi (1), followed by directed vesicular transport to the PM (2) where the protein is released after hydrolysis of the thioester (3) and transported back via a non-vesicular pathway to the Golgi (4).

decreases the Gibbs free energy of transfer by approximately −3.45 kJ/mol as observed by Tanford resulting in a linear dependence of the free energy on the number of carbons in the peptide lipid chain [47,48,50]. However, the experimentally found absolute values for the binding energy were significantly smaller than for partitioning of free fatty acids into *n*-heptane (see Fig. 5). This can be understood from the fact that a *n*-heptane/water interface mimics the complicated nature of the lipid membrane and its well-tuned lipid water interface [53,54] only insufficiently. Further, the simple Tanford formula ignores the mass-dependent entropy price that must be paid when a protein binds to the membrane [50]. As the membrane-associated protein significantly looses degrees of freedom resulting in less favorable entropy the binding energy can actually be much lower. This physically very simple point has a number of significant biological consequences for the membrane binding of lipid-modified proteins.

First, a single unbranched lipid modification is not sufficient to anchor a lipidated protein in the membrane. The binding energy arising for a protein with a single lipid modification only provides binding for about 2% of a myristoylated and 23% of a palmitoylated protein. The situation is a bit more favorable for farnesylated or geranylgeranylated proteins, where a fraction of 58% or 98% is bound, respectively (see Fig. 5). As a consequence of the single lipid modification, these proteins dissociate from and associate with the membrane with rapid half-times (typically seconds or less) [55,56].

Second, for stable membrane association, an additional membrane-binding mechanism is required that further decreases the total free energy of transfer from water to the membrane surface. Several such mechanisms have evolved. (*i*) A simple mechanism that actually increases binding of lipidated peptides is the *O*-methylation of the carboxyl terminus of the terminal cysteine that occurs additionally to the *S*-isoprenylation of the CAAX motif at the C-terminus of the protein. On average, *O*-methylation provides a decrease in the free energy by ~6 kJ/mol, which increases the fraction of membrane bound

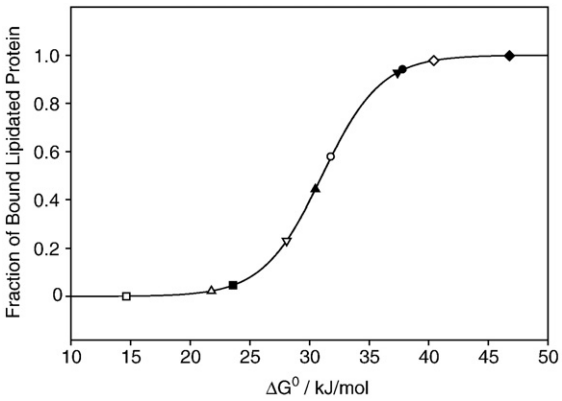

Fig. 5. Fraction of bound lipidated protein as a function of the unitary Gibbs free energy of transfer from the aqueous phase into a membrane at 25 °C. The solid line represents $\Delta G^0 = RT \ln K_p$, where R is the gas constant, T the absolute temperature, and K_p the partition coefficient. The filled square, triangle, and inverted triangle are calculated for free fatty acid partitioning of C12, C14, and C16 fatty acids, respectively [46]. The open square, triangle, and inverted triangle show experimental membrane-binding data of model acylated proteins modified with a C12, C14, or C16 lipid chain [47]. Circles and diamonds show membrane binding of farnesylated and geranylgeranylated proteins, respectively, where open symbols indicate just lipid modification and filled symbols show lipid modification plus *O*-methylation [48].

farnesylated protein by about 60% (see Fig. 5) [48]. (*ii*) A second or even third lipid modification provides sufficient binding energy for permanent membrane association of a lipidated protein. For instance, the N- and H-Ras proteins receive one or two additional palmitoyl groups at further cysteine residues (see Fig. 3). Other GTP binding proteins obtain additional geranylgeranyl or farnesyl lipid modifications [3]. An additional lipid modification also prevents the spontaneous diffusion between different membranes and may also increase the probability of a lipid-modified protein to partition into lipid rafts [45]. Experimentally, the average half-life of doubly lipid-modified proteins is on the order of 100 h [49]. (*iii*) In addition to lipid chain insertion, the binding energy of lipidated proteins can be increased by hydrophobic [57] or basic residues in the sequence [58]. In particular electrostatic interactions between the negatively charged inner leaflet of the plasma membrane and clusters of basic amino acids provide an additional binding motif for permanent membrane binding [58]. Several singly lipid-modified proteins feature such basic clusters either in the direct vicinity of the lipid modification (K-Ras4B [59] or pp60^{src} [7]) or further away as in the case of MARCKS [6]. The electrostatic contribution to the binding energy is normally much smaller than what is gained by the insertion of the lipid chains. Depending on the fraction of acidic lipids in the membrane and the number and distribution of charged amino acids on the protein [51], typically just 1–5 kJ/mol binding energy arise from electrostatics [60]. However, as seen from Fig. 5, even small contributions to the binding energy can shift the fraction of bound lipidated protein significantly to higher values.

Third, due to the polar nature of the polypeptide backbone and in particular the charged amino acid side chains lipidated peptides may not approach the membrane surface completely or insert into the lipid water interface of the bilayer. This phenomenon called Born effect is well-known and arises from the image charge that repels the peptide from the low dielectric membrane surface [60]. This may actually mean that a lipid modification of a protein is not completely inserted into the membrane. Indeed, such a behavior has been suggested for pp60^{src} [7,51]. From binding and surface potential measurements, McLaughlin et al. concluded that only 10 methylene groups of myristoylated pp60^{src} penetrate into the hydrocarbon interior of the membrane and the polar N-terminal glycine of the peptide remains in the aqueous phase [51]. In addition to the polar peptide backbone, pp60^{src} contains three cationic and one anionic residues in the first 10 *N*-terminal amino acids, which experience a strong Born repulsion when approaching the lipid membrane surface.

The fourth and physiologically most relevant is that the diffusion of a membrane-associated lipidated protein is restricted to two dimensions as opposed to the three-dimensional diffusion the molecule undergoes in the cytosol. This effectively increases the concentration of the protein at the membrane by a factor of ~1000 [58]. Thus, the likelihood to interact with downstream effectors and other physiologically important modifications increases providing the physical basis for biological signal transduction events [3,6,15].

All these interesting findings and models about membrane binding of lipidated proteins and peptides so far were based on binding studies, low resolution methods (CD, fluorescence spectroscopy), and electrostatic calculations. However, within the last decade, a number of groups applied solid-state NMR techniques to study the structure and dynamics of lipid-modified membrane-associated peptides and proteins to provide a first structural basis [61–63]. Solid-state NMR is a particularly well-suited method to study structure and dynamics of membrane-associated peptides in the liquid-crystalline phase state [64–66]. In addition, explicit all-atom molecular dynamics simulations have caught up with experimental techniques providing insight into specific aspects of the interaction of proteins with membranes, which complements the results from the experimental ensemble techniques [67–69]. In the following sections, we will discuss structure and dynamics of membrane-associated lipidated proteins and peptides. We will restrict the discussion to data for Ras proteins that were primarily determined using solid-state NMR spectroscopy and other biophysical techniques as well as molecular dynamics simulations.

3.2. Structural and dynamical studies of the Ras lipid chains

The first obvious point of interest in a structural investigation of a lipid-modified protein is the lipid modification itself. However, the experimental study of the lipid chain of a lipidated protein is complicated since the surrounding phospholipid membrane consists of exactly the same or very similar hydrocarbon chains, which makes them indistinguishable for experimental techniques. To circumvent this problem, either fluorescence or EPR probes have to be applied, which may provide the problem of influencing the properties of the molecule under study [70–73]. Alternatively, isotopic labeling has to be exploited, which should be non-invasive by nature. A relatively simple ^{2}H-labelling of the lipid chains of a protein makes them amenable to techniques that can distinguish between protons and deuterium, i.e. between the lipid chains of the protein or those of the surrounding membrane. Such labeling can be carried out chemically [49,50,63,74,75] or enzymatically using an appropriate expression system [52,76]. By switching the ^{2}H label between the lipidated protein and the phospholipids, the properties of both molecules can be studied separately and compared to yield biophysical information.

Three biophysical methods are sensitive to the ^{1}H/^{2}H contrast: neutron diffraction, FTIR spectroscopy, and ^{2}H (solid-state) NMR spectroscopy. All these methods have been applied to membrane-associated Ras peptides and proteins.

In the first set of experiments, a heptapeptide (see Fig. 6A) with the sequence of the C-terminal amino acids of N-Ras with two hexadecyl lipid modifications was used [77]. The neutron scattering length density profiles of chain deuterated DMPC-d_{54} in the presence and in the absence of a protonated doubly lipid-modified Ras heptapeptide showed contrast differences only in the hydrocarbon region suggesting that the Ras lipid modifications are well inserted into the hydrophobic core of the membrane [77].

Attenuated total reflection FTIR spectra of the protonated lipidated Ras heptapeptide in perdeuterated DMPC-d_{67} showed well-resolved bands for the symmetric and antisymmetric methylene stretching modes of the peptide and the lipid chains providing individual information on each component [77]. Temperature scans on these spectra revealed a significant shift to larger wavenumbers at the phase transition of DMPC. As typical for collective phase transition phenomena, the order-disorder melting transition of the Ras peptide occurs exactly parallel to that of the DMPC membrane, which indicates that the peptide was well incorporated into the membrane. From the FTIR spectra, order parameters of the lipid and peptide chains could be calculated, respectively. The most interesting result of this calculation was the fact that the order parameter of the Ras 16:0 chain was much smaller than that of the surrounding DMPC membrane featuring 14:0 chains [77]. This is remarkable since the FTIR spectra did not provide an indication that Ras peptides were clustered or segregated into a separate domain.

To gain more insight into this interesting behavior, intensive ^{2}H solid-state NMR experiments were carried out. ^{2}H NMR of perdeuterated phospholipid molecules is an extremely precise and versatile method to study membrane structure and dynamics [78–81]. In particular, ^{2}H NMR provides information on the order parameter of each resolved segment of an acyl chain and in combination with relaxation measurements also insights into the correlation times of motion.

First ^{2}H NMR studies on the Ras heptapeptide in DMPC could confirm the order parameter differences between the Ras and the DMPC chains, which were initially observed by FTIR [77]. Fig. 7 shows the ^{2}H NMR spectra of DMPC-d_{54} in the absence (A) and in the

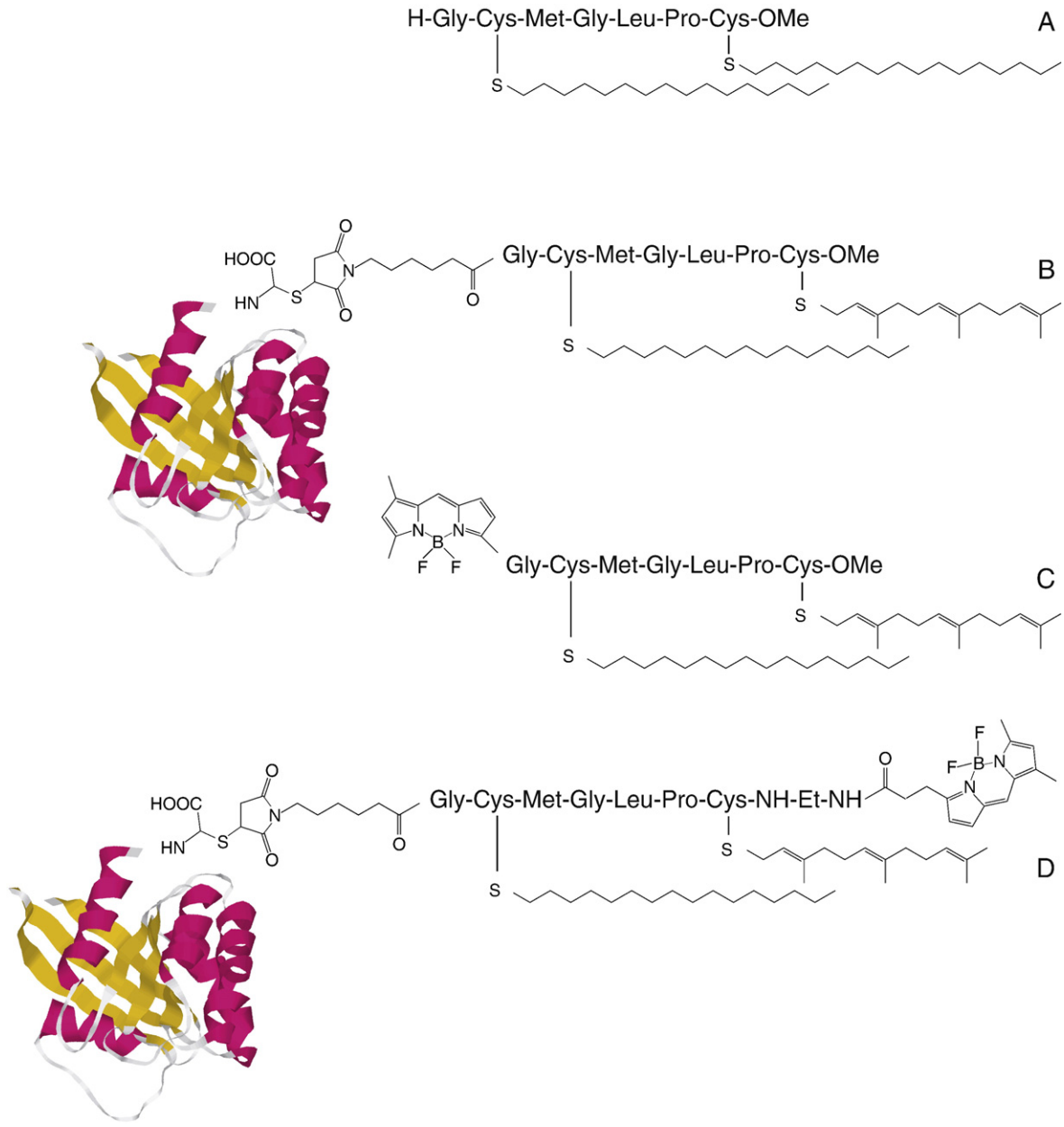

Fig. 6. Structure of the N-Ras proteins and peptides for which structural studies have been conducted. (A) Lipidated C-terminal N-Ras heptapeptide vor NMR studies, (B) lipidated full-length N-Ras protein with a malemidocaproyl (MIC) linker between the N-terminal domain of Ras and the C-terminal heptapeptide, (C) BODIPY labeled lipidated C-terminal N-Ras heptapeptide for fluorescence studies, (D) BODIPY labeled lipidated full-length N-Ras protein with the MIC linker for fluorescence studies.

presence of Ras (B). While almost identical NMR spectra were measured for the phospholipids in the absence an in the presence of Ras, the ^{2}H NMR spectrum of chain deuterated Ras-d_{66} in DMPC varies considerably (Fig. 7C). From the ^{2}H NMR order parameters (Fig. 7D), geometrical parameters such as the length and the area per molecule of the acyl chains can be calculated using the mean torque model [82]. Applying such sophisticated models, the apparent disagreement of the order parameters between the Ras and the phospholipid chains could be understood. In fact, the quantitative analysis of the ^{2}H NMR spectra indicated that the Ras lipid modifications and the acyl chains of the surrounding DMPC matrix had almost exactly the same length (10.1 Å vs. 10.3 Å) for a perfect fit of the Ras chains into the surrounding lipid matrix [83]. In other words, for the alignment of the lengths of the Ras 16:0 chains with the DMPC 14:0 chains, a drastic decrease of the order parameter of the Ras chains had to occur in order to match the length of the surrounding lipid matrix. In contrast, the area per chain varies drastically. As a consequence of the shorter lengths, the Ras chains occupy a much larger cross-sectional area (33.5 Å^2) than the DMPC chains (29.2 Å^2), which appear to remain close to their equilibrium value for an undisturbed bilayer.

The adaptation of the Ras lipid chain lengths to the surrounding phospholipid matrix appears to be a general feature of Ras. We are currently analyzing a series of ^{2}H NMR experiments of Ras in phospholipid membranes of varying hydrophobic thickness (Vogel and Huster, unpublished results). From the preliminary results, we can conclude that Ras adopts the lengths of its lipid modifications to that of the surrounding membrane.

In addition to the structure and the packing properties of the lipid modifications of membrane-associated Ras peptides, ^{2}H NMR has revealed insights into their molecular dynamics. In NMR spectroscopy, dynamics information is available from relaxation measurements. For lipid bilayers, plots of the ^{2}H Zeeman order spin-lattice relaxation rate

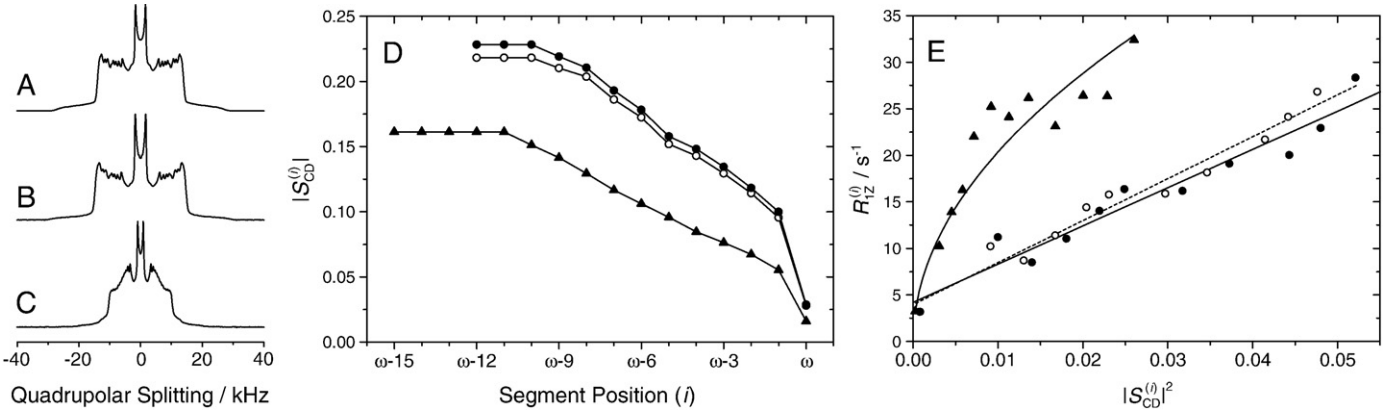

Fig. 7. Summary of ^{2}H NMR data on lipid-modified N-Ras peptides. ^{2}H NMR spectra of DMPC-d_{54} (A), DMPC-d_{54} in the presence of the Ras peptide (B), and DMPC in the presence of the deuterated Ras-d_{66} peptide (C). (D) Order parameter profile calculated from the ^{2}H NMR spectra shown in (A–C). Open circles show the order parameters of DMPC-d_{54} in the absence of Ras and filled circles represent the order parameters of DMPC-d_{54} in the presence of Ras at a molar ratio of 1:10 (Ras:DMPC). The filled triangles illustrate the order parameters of the deuterated Ras-d_{66} peptide in DMPC. (E) Square law plots of the phospholipid chains in the absence and in the presence of Ras peptides as well as for the lipid modifications of Ras. Symbols as in (D). Reproduced from Ref. [83] with permission.

(R_{1Z}) vs. the square of the order parameter ("square law plots") have proved useful as they report the elastic properties of the respective membranes [84,85]. Saturated membranes that are characterized by a certain amount of conformational flexibility usually produce linear square law plots with a characteristic slope. In the presence of cholesterol, which makes the membrane stiffer, saturated phospholipids still produce linear square law plots but the slope is shallower. In contrast, saturated phospholipids in the presence of detergents or unsaturated phospholipids produce square law plots that depart from linearity. These plots show some curvature and feature a steeper slope [83].

For the DMPC/Ras preparations the DMPC molecules show linear square law plots and only marginal alterations in the presence and in the absence of Ras are detected (Fig. 7E). However, the square law plots of the lipid modifications of membrane-associated Ras provide the features of a lipid membrane in the presence of a detergent, which are characterized by large molecular dynamics. This suggests that the lipid modifications of Ras are highly dynamic and flexible while inserted into the lipid membrane.

With the limited amount of data it is not yet not possible to assess if the particularly highly dynamic character of the protein-attached lipid chains is specific for Ras. We have studied the dynamics of the myristoyl moiety of GCAP-2 [52] and Src (Scheidt and Huster, unpublished results) and found both chains to show a similar mobility as the surrounding host matrix. In a very recent paper the palmitoyl chain of the N-terminal segment of pulmonary surfactant protein SP-C was studied by ^{2}H NMR [86]. Similarly to Ras, the authors found much narrower quadrupolar splittings for the protein lipid chain compared to the DPPC host matrix.

The interesting molecular dynamics of Ras was further investigated using ^{2}H NMR spectroscopy of oriented membrane layers with incorporated Ras peptide [87]. In particular the angular dependence of the ^{2}H relaxation rate contains a wealth of information with regard to the dynamics of deuterated molecules [88–91]. Depending on the correlation time and amplitude of molecular motions, a characteristic dependence of the relaxation rate from the macroscopic orientation of the sample is measured. In powder samples (i.e. multilamellar vesicles) this information is averaged out. Qualitatively, a very pronounced angular dependence of the relaxation rate is characteristic for rigidity or only small amplitude motions, while little angular dependence is indicative of motions with large amplitudes. In the limiting case of isotropic tumbling of a molecule, no angular dependence of the relaxation rate is measured as typical for molecules in solution.

For oriented lipid layers with the Ras heptapeptide incorporated, a rather flat angular dependence of the R_{1Z} relaxation rate on the orientation of the membrane was measured (Fig. 8). A model that considered order fluctuations as well as collective motions of the membrane [89,92] was able to describe the experimental data very well using an anisotropic axially symmetric diffusion tensor with the principle values of $D_{||}=2.1\times10^9$ s^{-1} and $D_{\perp}=4.5\times10^5$ s^{-1} [87]. A viscoelastic fitting parameter that describes the membrane elasticity, viscosity, and temperature was found very similar to that of the surrounding DMPC host matrix.

All structural information on the conformation, packing, and dynamics of the Ras acyl chains described above was derived from a doubly hexadecylated heptapeptide. Although there are some good arguments in favor of the suggestion that the structure and dynamics of the Ras lipid modifications should not be influenced by the N-terminal domain of Ras, an experimental study is always preferred. Further, the issue whether or not the farnesyl lipid modification of Cys 186 would influence the palmitoylation/hexadecylation of Cys 181 should be studied experimentally. To this end, a full-length Ras protein comprising a deuterated hexadecyl thioether in position 181 and the farnesylated Cys 186 was prepared by hybrid synthesis (see Fig. 6B) [74] and studied by ^{2}H NMR [93]. As expected, basically all experimental results obtained for the membrane bound lipidated heptapeptide could be confirmed: (*i*) the Ras chain order parameters are significantly lower than those of the host membrane, (*ii*) the length of the Ras chains is equivalent to that of the DMPC host membrane in spite of the two carbon difference in length, while the cross-sectional area of a DMPC chain is much lower (29.2 Å^2) than that of the Ras chain (33.8 Å^2), and (*iii*) square law plots of the Ras chains show a bent shape with a steep slope indicative of large amplitude motions while the square law plots of the host membrane are linear featuring a shallower slope.

It should be emphasized that the higher mobility of the Ras lipid chains is not just the result of the length mismatch with the surrounding DMPC matrix. We have also studied mixtures of DMPC and DPPC at a 150:1 mixing ratio (identical to that of the Ras protein in DMPC). In this preparation, the chain length mismatch is identical to that of Ras in DMPC. However, no chain length matching was observed, DMPC has a chain length of 10.6 Å and DPPC a chain length of 12.4 Å. However, the area per chain was very close for both lipids, 29.7 Å^2 for DMPC and 29.0 Å^2 for DPPC (Reuther and Huster, unpublished results).

So experimentally, the hexadecyl lipid modifications of the Ras protein are highly dynamic and flexible undergoing large amplitude

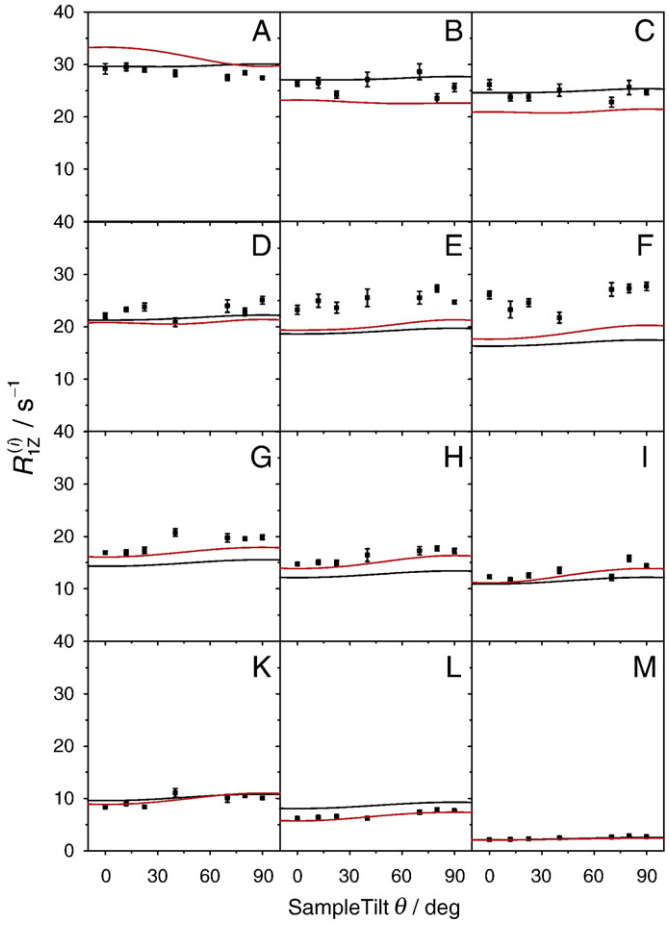

Fig. 8. Simultaneous fit of analytical models for experimental ^{2}H NMR spin-lattice relaxation rates $R_{1Z}^{(i)}$ determined for the Ras lipid chains in oriented DMPC bilayers as a function of angle between the lamellar normal and external magnetic field. Experimental relaxation rates are given as data points, and best fits to a composite membrane deformation models correspond the black lines. Panels refer to the residual quadrupolar couplings, i.e. representing different carbon positions. Panel (A) refers to the largest quadrupolar coupling followed by the next largest (B) and so on. Panel (M) illustrates the relaxation rate for the terminal methyl groups of the Ras chains. The angular dependence of the relaxation rates obtained from molecular dynamics simulations are given in red lines. Reproduced from Ref. [87] with permission.

motions in a fast correlation time window. Unfortunately, no direct experimental information on the farnesyl chains of Ras could be obtained because deuterated farnesyl chains are not available. Even in the computational models and molecular dynamics simulations on Ras peptides and proteins the farnesyl chain has not been explicitly treated. Nevertheless, a number of very interesting results were obtained in computational work that nicely complemented the experiments.

The free energy of transfer of the H-Ras membrane anchor (with three hexadecyl chains) from water into a DMPC membrane was calculated using the adaptive-biasing-force method [94]. The calculation showed that the insertion of H-Ras into the membrane is spontaneous and nonspecific. It produces a gain in free energy of ~120 kJ/mol. This is in good agreement with the prediction from the Tanford formula discussed above. From this and other molecular dynamics simulations (20 to 40 ns in length), further insights into the structural and dynamical properties of the H-Ras and N-Ras lipid chains are available [95–97].

In agreement with the experimental work on N-Ras, the order parameters of the lipid matrix are only marginally influenced by insertion of H-Ras [96]. The order parameters for the lipid modification on Cys 181 (palmitoyl), Cys 184 (palmitoyl), and Cys 186 (hexadecyl to mimic farnesyl) show dramatic differences in mobility with the palmitoyl chain on Cys 181 being the most flexible [95]. Further, the structure of DMPC in the vicinity of the longer Ras lipid chains is disturbed; also a feature that cannot be observed by bulk methods such as NMR or FTIR spectroscopy. Distinct alterations of the order parameter of the interaction lipids were observed for H-Ras*GTP (higher order) and H-Ras*GDP (lower order) [95]. Finally, binding of H-Ras to the inner monolayer of the plasma membrane also affects the outer leaflet of the bilayer.

In additional simulations on the doubly hexadecylated N-Ras heptapeptide in DMPC (total simulation time of 150 ns) [97] a number of simulation results could directly be compared to the experimental work on the same system. In agreement with experiment, the peptide–lipid chains show drastically lower order parameters. Quantitatively, the simulation nicely reproduced the experimentally found difference of the average order parameter (0.060 vs. 0.057). Accordingly, the experimental chain length difference was also reproduced in the simulation. Further, the Ras lipid chains were found to be inserted in the hydrophobic core of the membrane in agreement with the experimental neutron scattering length profiles.

Finally, a >100 ns simulation of the N-Ras heptapeptide in DMPC could very well reproduce the angular dependence of the R_{1Z} relaxation rates that were experimentally determined (see Fig. 8) [87]. This is particularly noteworthy since NMR relaxation is sensitive to various motional processes that occur on different timescales. Naively, this result may suggest that the molecular motions that drive the ^{2}H relaxation are sufficiently fast to be contained in a >100 ns simulation.

However, there is also some disagreement between experimental data and the simulations. First, the experimental and simulated order parameter plots differ from one another although the average order parameters are rather comparable. The trivial reason could be that the assignment of experimental order parameters rests on the assumption that order gradually decreases from the upper chain to the end of the chain, which is not correct due to geometric constraints [98]. More seriously, the still relatively short simulations and the limited number of peptides in a simulation box may mean that the sampling of the trajectories is not completed within the simulation time. Further, the amplitudes of the methylene motions may also be influenced by collective motions of the entire bilayer that are not contained in the simulation. In fact, for the analytical description of the relaxation date of the Ras chains, such collective motions were required to yield a reasonable fit [87]. Slow motions can produce amplitudes of up to 30°, which would significantly lower the order parameters of the peptide chains. These motions may not play a big role for the relaxation times, which agree well between simulation and experiment, but could be of great importance for the amplitudes of motion, i.e. the order parameters.

In spite of these problems that may very well disappear as longer trajectories and larger systems will become available, computational results represent a great complementary method to experimental studies on membrane binding of lipidated proteins. In particular, the site resolution that is not available from the bulk experimental techniques helps understanding the complicated structure and dynamics of the lipid chains of lipid-modified proteins to provide very descriptive snapshots of the membrane bound molecules (Fig. 9). As will be discussed in the next paragraph, also valuable information on the structure and dynamics of the polypeptide backbone and sidechains as well as membrane–protein interaction is available from simulation work.

3.3. Structural studies of the membrane binding of Ras

Until recently, no structural models were available for the membrane anchor of lipidated membrane-associated proteins. Both the complicated biosynthesis of lipid-modified proteins that typically requires eukaryotic cells for expression, which normally correlates

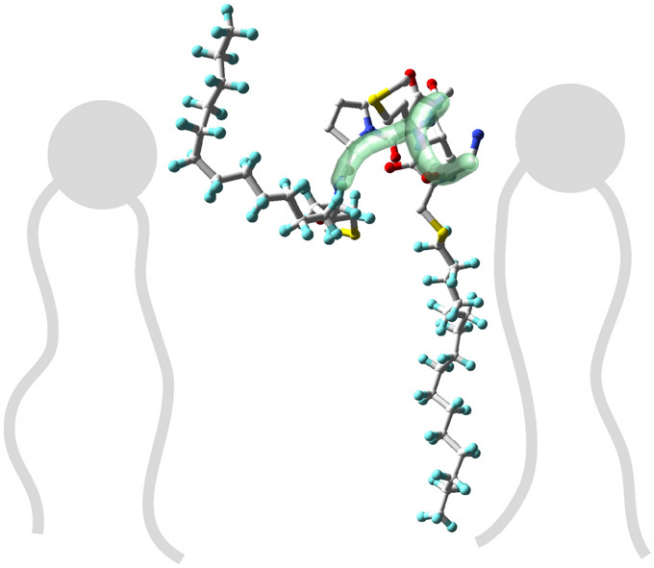

Fig. 9. Cartoon snapshot derived from molecular dynamics simulations of Ras heptapeptide in a DMPC bilayer matrix. Two lipid molecules are schematically drawn. Reproduced from Ref. [87] with permission.

with low yields and the failure of standard methods to routinely carry out structure determinations of membrane proteins have hampered structural studies on these molecules [99]. However, recent progress in the synthesis of lipid-modified proteins using peptide ligation either with artificial linkers [74] or intein methods [3] allows production of sizable amounts of lipidated proteins. Further, solid-state NMR, though not yet a routine technique for structure determination, has significantly advanced to provide three-dimensional structures of crystalline [100], fibrillar [101,102], and membrane proteins [103]. Also, the method has unique potential to characterize the molecular dynamics of membrane proteins in a very broad correlation time window.

We have used ^{13}C magic-angle spinning (MAS) solid-state NMR techniques to determine a structural model of the membrane anchor of full-length N-Ras protein bound to DMPC membranes [104]. To this end, two Ras constructs with alternating labeling scheme of the terminal seven amino acids were prepared and reconstituted into DMPC membranes. The ^{13}C MAS NMR spectra showed a good resolution and the full assignment of all seven amino acids could be carried out using ^{1}H–^{13}C and ^{13}C–^{13}C correlation experiments. In NMR spectroscopy, protein structure information is available from the isotropic chemical shifts of the backbone and sidechain atoms [105,106]. Characteristic chemical shift changes are indicative of secondary structure motifs and can yield full structural models. To date, database approaches are available that convert chemical shift information into backbone torsion angle information that provide the basis for structure calculations. We used the TALOS database program that contains chemical shift information from 186 proteins of known X-ray structure [107]. On the basis of the isotropic chemical shifts, TALOS provides the backbone torsion angles from structure and sequence similarities. On the basis of the TALOS torsion angles, a first structural model of the backbone of membrane anchor of full-length lipidated Ras protein in DMPC membranes was calculated (Fig. 10). The backbone structure of N-Ras resembles a horseshoe. There are no intramolecular hydrogen bonds to stabilize that structure, which agrees with previous FTIR data [77].

While TALOS provides reasonable models for membrane bound proteins [108], fibrillar proteins [102], or receptor ligands [109,110], a number of issues cannot be answered from secondary chemical shift data only. In particular, no information about the sidechain conformation and the topology of the protein with regard to the membrane can be given. This information can be derived from additional solid-state NMR experiments. In case of Ras, where only a small fraction of the

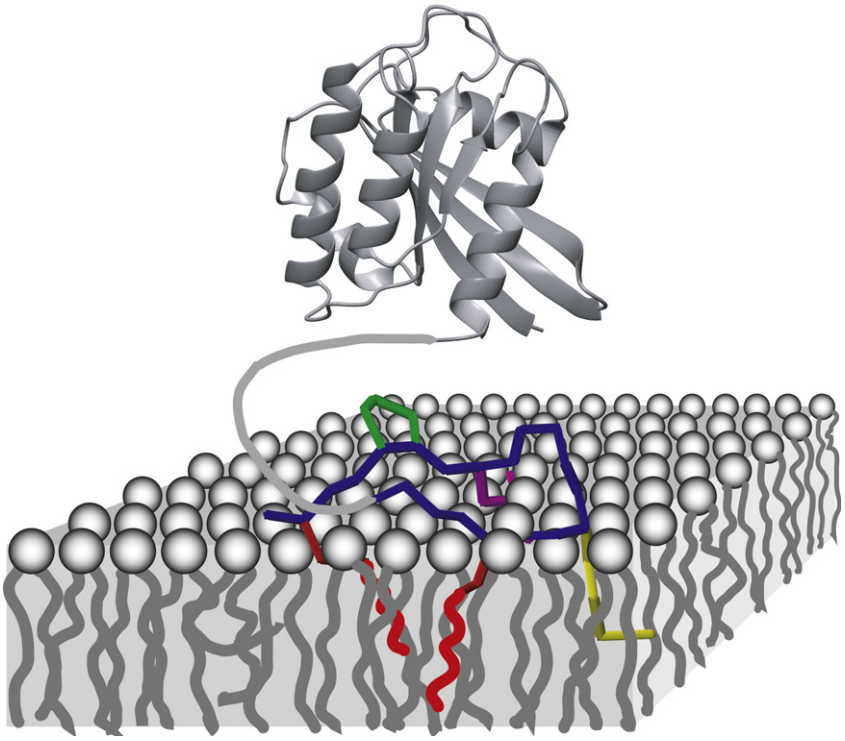

Fig. 10. Model of the membrane structure and topology of lipid-modified N-Ras protein (not to scale). The soluble N-terminus is connected to the membrane anchor via a flexible linker domain. The structure of the N-terminus (residues 1–166) was adapted from the crystal structure [119]. The protein backbone of the C-terminal segment is shown in blue, the hexadecylated cysteine sidechains are in red, proline is in green, leucine in magenta, and methionine in yellow. Reproduced from Ref. [104] with permission.

protein was isotopically labeled and the sample also contained large amounts of lipid molecules and water, the sensitivity was not sufficient to carry out such extensive NMR studies. Instead, additional NMR experiments were run on the doubly lipid-modified heptapeptide from the C-terminus of the protein. Under these circumstances, sufficient sensitivity for a number of complicated NMR experiments could be gathered to refine the previous structural model. Further, small membrane-associated peptides provide high mobility in the membrane, which allows to acquire ^{1}H MAS NMR spectra and to use the nuclear Overhauser effect (NOE) for additional structural constraints.

^{1}H MAS NMR spectra of the N-Ras heptapeptide in DMPC membranes provided well-resolved NMR spectra, in which side chain signals could be resolved and assigned [111]. The Hα signals were superimposed with the glycerol lipid signals that are present even in the most deuterated DMPC-d_{67} that is commercially available. Two-dimensional NOESY spectra showed a number of well-resolved intramolecular crosspeaks as well as intermolecular peptide–lipid correlations indicative of molecular contacts over distances of less than 5 Å. Further, strong water exchange of the backbone amides was detected suggesting that Ras is localized on the membrane surface or in the membrane interface to have access to the solvent.

Intermolecular peptide–lipid crosspeaks were analyzed quantitatively suggesting that the hydrophobic sidechains of leucine and methionine as well as the lipidated cysteines are inserted into the hydrophobic core of the membrane. In contrast, the more polar proline and the backbone amides were localized in the lipid water interface of the membrane [111]. This information helped to construct a structural model of the membrane bound Ras protein as shown in Fig. 10, where the lipid chains and the hydrophobic side chains are inserted into the membrane. It should be emphasized that the NOESY data of membrane-associated Ras peptide reveal a broad distribution of the molecule in the lipid water interface of the membrane. This would agree with the high dynamics and flexibility of the Ras lipid modifications as well as with the dynamic nature of the lipid water interface of the membrane. This highly specific interface region provides various modes of interactions for the Ras membrane anchor to bind including the hydrophobic effect, hydrogen bonds, dipolar and van der Waals interactions. In addition, a rather flexible structural arrangement would provide favorable configurational entropy that also contributes to the minimum in free energy.

For additional information on the side chains conformation of Ras, ^{13}C detected ^{1}H–^{1}H-NOE measurements were carried out (Reuther and Huster, unpublished results). Overall, 17 unique intramolecular NOEs were detected that further constrained the membrane structure of membrane bound Ras and provided explicit information about its sidechain conformation. A preliminary model of the membrane anchor of N-Ras is given in Fig. 11. This model now contains explicit

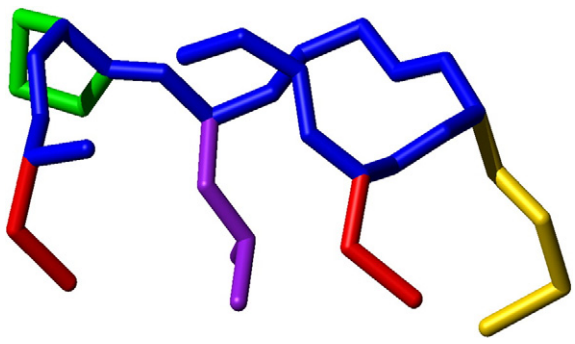

Fig. 11. Structural model of the membrane anchor of the terminal seven amino acids of N-Ras determined from solid-state NMR results on the Ras heptapeptide on the basis of isotropic chemical shifts and NOEs. The colour code is identical to Fig. 10. The lipid chain modifications of the cysteines are omitted for clarity.

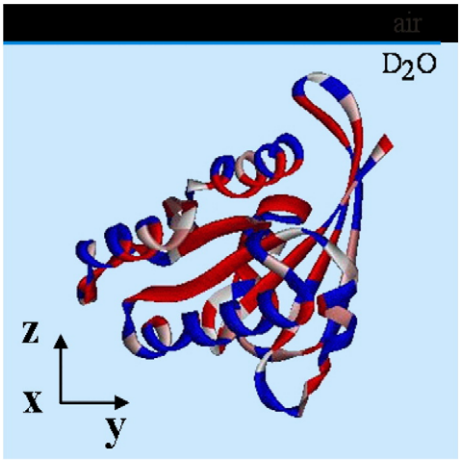

Fig. 12. Orientation of the N-terminal domain of Ras at the membrane surface relative to the normal of the air water interface. The model was determined from IRRAS results on Ras adsorption on phospholipid monolayers [112]. The protein structure was drawn from the crystal structure [120] and is lacking the lipidated C-terminus. The hydrophilic residues are shown in blue, hydrophobic residues in red. The air water interface is symbolized as a blue line.

sidechain information and the membrane topology, which are both required for the design of drugs targeting the membrane anchor of Ras.

More structural information on Ras bound to lipid surfaces is available from monolayer studies using infrared reflection absorption spectroscopy (IRRAS) [112] as well as X-ray diffraction and specular reflectivity [113]. Full-length lipid-modified N-Ras protein was added to a POPC/sphingomyelin/cholesterol monolayer from the subphase [112]. Sufficient insertion of farnesylated/hexadecylated Ras could only be shown at low lateral pressure of 10 mN/m. Doubly hexadecylated N-Ras inserted at 30 mN/m and from the amide bands of the IRRA spectra the orientation of the secondary structure elements of the soluble domain with respect to the monolyer surface could be determined (Fig. 12).

An X-ray monolayer study revealed that N-Ras insertion does not influence the lipid tilt angles and the structure of the lipid molecules in the DPPC monolayer but affects its electron density [113]. In particular, the headgroup/glycerol/upper chain region of the monolayer showed contrast variations indicative of Ras binding.

A number of molecular dynamics simulations have studied Ras peptides and proteins in DMPC membranes. For the N-Ras heptapeptide, no regular hydrogen bond stabilized secondary structure was found in agreement with experiment [97]. Some snapshots of the backbone structure of the Ras peptide from the simulation are shown in Fig. 13, some of those agree well with the experimentally found horseshoe model. The structure was pronounced 'extended'. Backbone conformations differing by up to 4 Å were observed in different trajectories. Further, the simulation could reproduce the deep insertion of the peptide into the lipid water interface of the membrane, which is possible by hydrogen-bonding between the backbone amides and the phosphate oxygen. Further, the insertion of methionine and leucine sidechains into the bilayer was seen as well as the orientation of the proline sidechain towards the aqueous phase.

Two reports on molecular dynamics simulations on the membrane binding on H-Ras provided more insight into the protein-membrane interaction of Ras [95,96]. In particular, very interesting differences between H-Ras*GTP und H-Ras*GDP were found. The insertion depth and the backbone localization of the membrane anchor of H-Ras are modulated by the catalytic domain and the hypervariable linker region. The authors analyzed the number of Ras-DMPC contacts that occurred within 4 Å (similar to the intermolecular NOE measured by ^{1}H MAS NOESY experiments). The backbone of the membrane anchor

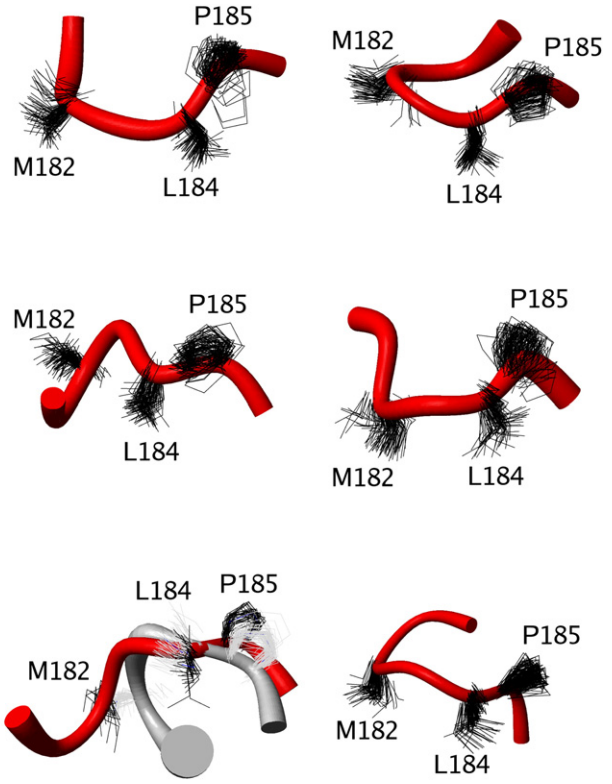

Fig. 13. Average backbone structures of the N-Ras peptides from a molecular dynamics simulation of the peptide in DMPC. The thickness of the tube represents the root mean square fluctuations in the backbone during the simulation. For details see [97]. Reproduced from Ref. [97] with permission.

of H-Ras*GTP is localized approximately 16 Å from the bilayer center. In contrast, H-Ras*GDP inserts deeper and populates a region in the lipid water interface at around 12 Å from the bilayer center. Also in agreement with the NOESY results, the simulation shows a broad distribution of the membrane inserted protein segments parallel to the membrane normal.

In addition to the lipid anchor, direct interactions of residues in the G-domain and the linker with phosphate headgroups of the DMPC membrane have been observed by molecular dynamics simulation [96]. It was found that two possible modes of membrane binding are found for H-Ras and these could be modulated by GDP/GTP exchange. In model I (dominant conformation of the GDP-bound conformation), the protein interacts with the membrane surface through several residues of the HVR and the N-terminus and in particular residues at the β2–β3 loop of the G-domain. In model II (dominant conformation of the GTP-bound conformation), the protein makes a larger interaction surface involving parts of α4 and α5 (Fig. 14).

3.4. Interaction of Ras with cholesterol and localization in rafts and liquid-disordered domains

The interesting differences in the membrane interaction of H-Ras*GDP and H-Ras*GTP were already mentioned in the previous chapters of this article. The search for such differences is motivated by cell biology experiments on the cellular localization of Ras [44,59,114]. The work from the Hancock group has shown that highly homologous Ras isoforms interact *in vitro* with the same set of effectors but generate very distinct signaling outputs *in vivo* [114]. So it was concluded that the biological differences of Ras signaling are imparted by the C-termini of the proteins. From these studies, it was suggested that H-Ras exists in a dynamic equilibrium between lipid raft and the disordered plasma domain. Activation of H-Ras on GTP loading shifts the equilibrium in favor of residence in the disordered plasma membrane [59].

More recently, a modified model was proposed for Ras isoform-specific signaling [10]. According to this model, all Ras isoforms have similar preferences for their effector proteins and regulators. In any given compartment, all Ras isoforms are subject to the same regulation and encounter identical effectors [115]. The difference in Ras signaling of the individual isoforms is imparted from the altered access and residence time in a specific compartment. For N-Ras and H-Ras it is the stability of the palmitoylation that allows fine tuning of the onset, the duration, and the amplitude of signaling.

Although biophysical model systems do by far not consider the complex biology of Ras compartmentalization in the cell, such studies can help understanding specific aspects of the complicated dynamical

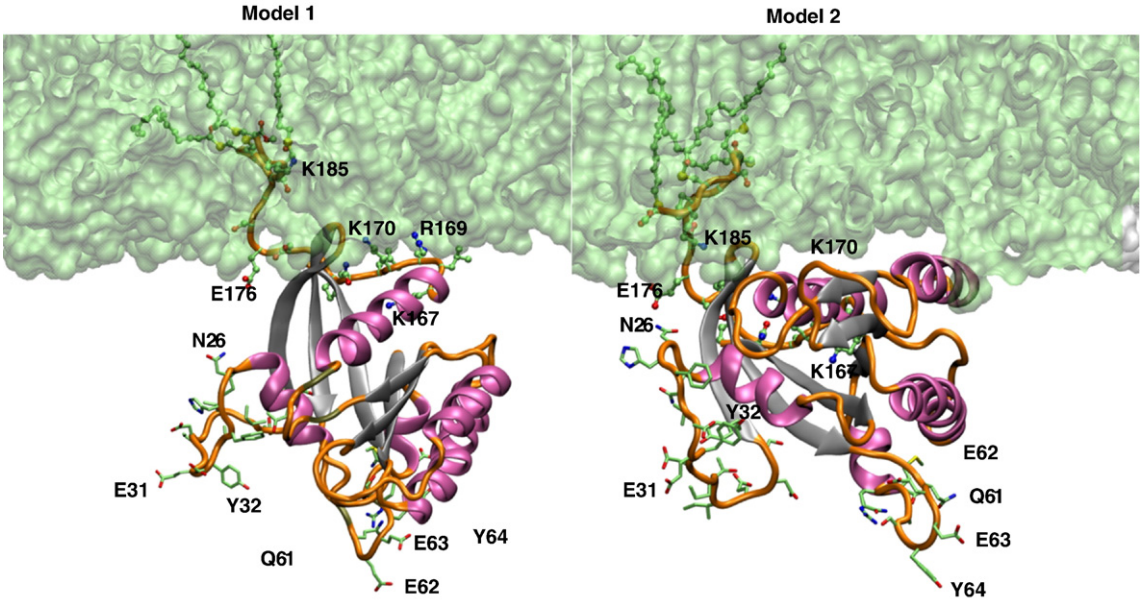

Fig. 14. Molecular representation of the two different modes of H-Ras binding to DMPC membranes determined from molecular dynamics simulations. The HVR residues are shown as ball-and-stick models. Reproduced from Ref. [96] with permission.

shuttling of Ras. For instance, the issue whether or not Ras preferentially interacts with cholesterol or partitions into liquid-disordered or raft domains has been addressed in a number of experimental and computational studies.

In one publication, the partitioning of the N-Ras heptapeptide was studied in DMPC/DSPC/cholesterol mixtures using fluorescence microscopy [116]. Addition of the fluorescently labeled Ras peptide (see Fig. 6C) to the giant unilamellar vesicles (GUV) led to a phase separation, which was explained by the high affinity of the lipidated peptide to a fluid environment. The peptide-rich domains decreased their size with decreasing temperature and peptide aggregation occurred at low temperature. The association of the palmitoylated/farnesylated heptapeptide with liquid-ordered or gel-like domains was not observed.

Further, the association of full-length N-Ras protein in lipid microdomains was studied using the maleimidocaproyl (MIC) Ras construct (see Fig. 6D) in a canonical raft mixture of POPC/sphingomyelin/cholesterol [117]. Fluorescence microscopy and tapping mode atomic force microscopy indicated that N-Ras*GDP proteins partition preferentially into liquid-disordered microdomains in contrast to the predictions of the Hancock model. The uptake of Ras to these liquid-disordered domains occurs on the order of minutes. The better spatial resolution of atomic force microscopy also allowed detection of a large portion of the protein located at the liquid-ordered/liquid-disordered phase boundary, where it experiences a favorable decrease in line tension associated with the rim phase of the demixed phase.

Although the molecular dynamics simulations did not contain explicit cholesterol, the observed differences in membrane binding of H-Ras*GDP and H-Ras*GTP suggested three mechanisms, by which a preferential interaction between H-Ras and cholesterol-rich and cholesterol-poor domains could come about [95]. First, membrane thinning by H-Ras*GDP may force the protein to segregate into cholesterol-rich domains. Second, the backbone of H-Ras*GDP and the hydroxyl groups of cholesterol may interact with the carbonyls of the membrane, which may mean that cholesterol and H-Ras*GDP may have to cluster together. Third, both cholesterol and H-Ras*GDP induce negative curvature which may explain their mutual preference for such regions. However, up to now, no simulations of Ras in membranes that contained cholesterol are available in the literature.

3.5. The molecular dynamics of the Ras membrane anchor

As already discussed in the previous chapters, the membrane anchor of Ras represents a rather dynamical part of the protein. So far, this has been learned from the ^{2}H NMR data on Ras proteins and peptides comprising selectively deuterated lipid modifications, the ^{1}H MAS NOESY results, and the molecular dynamics simulations. In particular the relaxation rates of oriented Ras containing samples revealed large order fluctuations of the membrane anchor of Ras (Fig. 8) [87]. Theses results suggest that not only the Ras lipid modifications but also the entire polypeptide chain of the Ras membrane anchor must be very dynamic. In order to quantitatively study the molecular motions of membrane bound Ras proteins, the amplitude and correlation times of motions were determined for the terminal seven amino acids in N-Ras proteins [93].

In NMR spectroscopy, motional amplitude information can be easily derived from motionally averaged anisotropic interactions (for instance from the quadrupolar interaction in the case of ^{2}H). The ratio of the motional averaged anisotropic interaction and the full interaction strength for the rigid limit defines an order parameter that provides the amplitude of motion. In addition, ^{1}H–^{13}C dipolar couplings can be measured with good precision providing order parameters for all protonated carbons in each amino acids. Most efficiently, such parameters are measured in a separated local field experiment under MAS [118]. Using the two MIC Ras constructs (Fig. 7B), for which the membrane bound structure was determined previously, we could map the motional amplitudes of the backbone and sidechains of the terminal seven amino acids of N-Ras. In general, the order parameters of the backbone of the Ras membrane anchor are relatively low suggesting large amplitude motions. Order parameters decrease further into the sidechains except for proline 185, where the five membered ring folds back onto the main chain. It is remarkable that the lipid-modified Cys 181 and 186 show rather different order parameters, the hexadecylated Cys 181 is much more flexible than the farnesylated Cys 186 (Fig. 15). To corroborate this intriguing difference, the ^{1}H–^{13}C dipolar coupling measurement was repeated for doubly hexadecylated Ras heptapeptide (Reuther and Huster, unpublished results). In general, order parameters are somewhat smaller for the peptide, indicative of even more molecular mobility of the smaller heptapeptide. Most interesting is the tremendous difference in the order parameter of Cys 186, which is 0.44 for the farnesylated Ras proteins and only 0.10 for the hexadecylated Ras peptide. Unfortunately, there is no ^{2}H NMR data available for the farnesyl chain and even the molecular dynamics simulations used hexadecyl chains instead of farnesyl [95]. This first experimental result would suggest that the farnesylated Cys provides the stronger membrane anchor than the palmitoylated Cys, which would also be in agreement with physicochemical studies of peptide partitioning into membranes [49]. It is reasonable to assume that the branched farnesyl chain can anchor more stably in the liquid-crystalline membrane providing “hooks and eyes”. This could mean that the farnesyl chain provides the majority of the binding free energy while the palmitoyl moiety contributes only some binding energy but provides a more significant entropic contribution, which also results in a very favorable free energy [93].

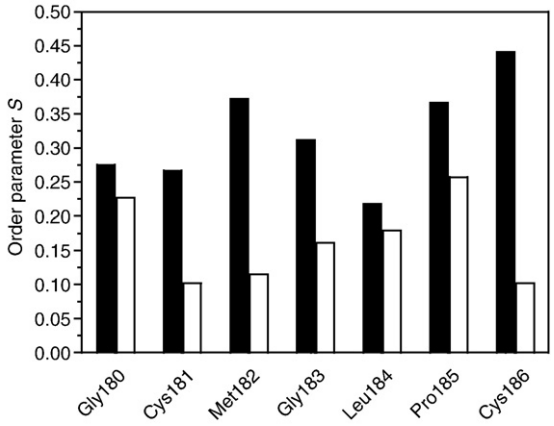

Fig. 15. ^{1}H–^{13}C order parameters of the C-terminal amino acids of the lipidated human N-Ras protein associated with DMPC membranes. Black bars represent the order parameters of the full-length Ras protein while white bars show the order parameters of the Ras heptapeptide.

Finally, the correlation time of motion was determined from ^{13}C relaxation time measurements [93]. For the interpretation of the relaxation data, a superposition of two anisotropic molecular motions, each described by an order parameter and a correlation time was used. At 37 °C, a slow correlation time of 143.9 ns and a fast correlation time of 1.9 ns were found. The order parameters were 0.445 and 0.70 for the slow and the fast process, respectively. These results were interpreted as a superposition of a slow axially symmetric rotation of Ras on the membrane surface and fast fluctuations of the C–H bond vectors, respectively.

4. Conclusions

In the field of Ras GTPases, chemical biology has significantly contributed to the elucidation of the structural evaluation of membrane binding as illustrated in this review. The combination of

protein semi-synthesis and biological and biophysical evaluation allows the study of the phenomena previously not possible via chemical or biological approaches alone. This particularly applies to the structural and dynamic features of the membrane anchor of Ras as well as the localization of the molecule in the membrane and its compartments. Together with the wealth of structural information that is available on the soluble N-terminus of Ras the knowledge about the structural biology of the molecule has significantly advanced over the last decade. In addition to structural features, molecular dynamics is a prerequisite to understand protein function. Both experimental and *in silico* methods have revealed interesting insights into the molecular motions of the membrane-associated protein. The conformational flexibility that is a decisive feature of fluid lipid membranes is also encountered in the membrane anchor of the Ras molecule.

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