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Review

Interactions of Ras proteins with the plasma membrane and their roles in signaling

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Abstract

The complex dynamic structure of the plasma membrane plays critical roles in cellular signaling; interactions with the membrane lipid milieu, spatial segregation within and between cellular membranes and/or targeting to specific membrane-associated scaffolds are intimately involved in many signal transduction pathways. In this review, we focus on the membrane interactions of Ras proteins. These small GTPases play central roles in the regulation of cell growth and proliferation, and their excessive activation is commonly encountered in human tumors. Ras proteins associate with the membrane continuously via C-terminal lipidation and additional interactions in both their inactive and active forms; this association, as well as the targeting of specific Ras isoforms to plasma membrane microdomains and to intracellular organelles, have recently been implicated in Ras signaling and oncogenic potential. We discuss biochemical and biophysical evidence for the roles of specific domains of Ras proteins in mediating their association with the plasma membrane, and consider the potential effects of lateral segregation and interactions with membrane-associated protein assemblies on the signaling outcomes.
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Keywords: Ras; Membrane association; FRAP; Lateral domains; Membrane-cytoplasm exchange; Transbilayer signaling

Contents

1. Introduction

Many cellular responses require the tethering of signaling proteins to the membrane. The biophysical nature of this association, which depends on interactions between several cellular constituents including lipids, membrane proteins, protein–lipid assemblies/scaffolds and cytoskeletal elements [1–4], is inseparable from the biological regulation of their

Abbreviations: D, lateral diffusion coefficient; DMPC, 1,2-dimyristoylglycero-3-phosphocholine; EM, electron microscopy; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; HA, influenza hemagglutinin; HVR, hypervariable region; PLCγ, phospholipase Cγ; wt, wild-type.

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responses. In the absence of a higher level of organization and compartmentalization endowed by the plasma membrane, signaling specificity and fidelity would be largely hampered. Signaling proteins interacting with the internal face of the plasma membrane can be divided into several classes. One class, which includes many of the small GTPases, exhibits continuous dynamic interactions with the internal leaflet of the plasma membrane by specific lipid modifications and/or polybasic clusters. We focus here on the small GTPases H-, K- and N-Ras, which are essential components of signaling cascades that regulate cell growth, differentiation and apoptosis [5–8].

Ras GTPases constitute molecular switches anchored in the internal leaflet of the plasma membrane, regulating multiple signaling pathways; the major Ras-activated effectors in the different pathways are Raf kinases (the Mek/Erk pathway), phosphatidylinositol 3-kinases (PI3K) and Ral guanine nucleotide exchange factors (Ral-GEF) [5–10]. Ras proteins alternate between a GDP-bound, inactive form and a GTP-loaded, activated conformation [11], and control cell growth, proliferation, apoptosis and differentiation [6–8,12–16]. Oncogenic Ras can transform cells both in vitro and in vivo [15], and was shown to contribute to neoplastic processes by overactivation of several pathways [6,9,10,17]; the importance of specific Ras-activated pathways to transformation appears to depend on the species [18,19].

There are four mammalian Ras proteins, encoded by three *ras* genes: H-Ras, N-Ras, K-Ras4A and K-Ras4B. The latter of the last two is the more abundant in mammalian cells, and will be referred to hereafter as K-Ras, The Ras isoforms are highly homologous; their G-domain (residues 1–165; Fig. 1), which binds guanine nucleotides and is required for the switch function and for effector binding, is nearly identical. On the other hand, their C-termini (last 24–25 amino acids), termed "the hypervariable region" (HVR), are highly varied between

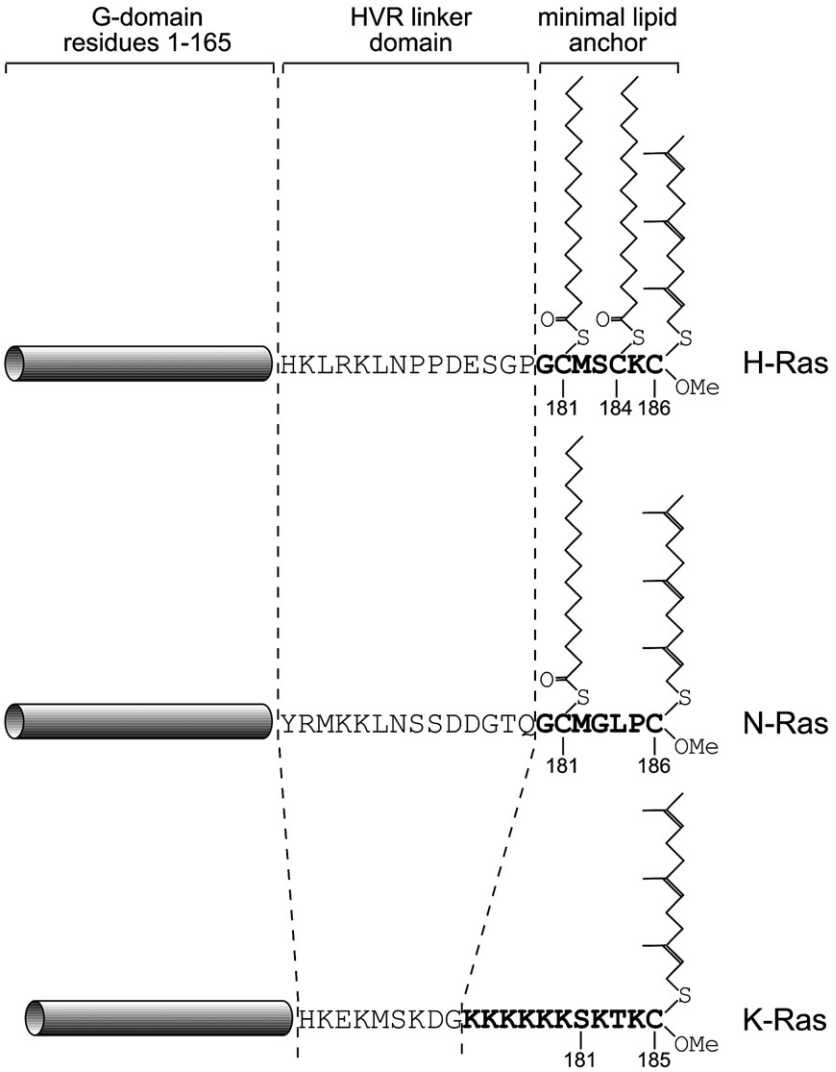

Fig. 1. Domain structure of Ras isoforms. The fully processed forms of the proteins are shown. The minimal lipid anchor (bold) is at the C-termini, containing a farnesyl moiety (all Ras isoforms), one (N-Ras) or two (H-Ras) palmitoyl moieties, or a six-lysine polybasic cluster (K-Ras). The numbers of amino acid residues that undergo specific modifications are depicted below the relevant residues. The lipid anchor is connected to the HVR linker domain, and together they comprise the complete HVR domain. The HVR linker is preceded by the G-domain (residues 1–165), which is highly homologous in all Ras isoforms.

the different Ras isoforms [20]. Importantly, the HVR region, which can be divided into the lipid anchor (the most C-terminal region) and the preceding linker domain (Fig. 1), contains the sequences responsible for the membrane anchoring of the various Ras isoforms, as well as for Ras intracellular trafficking [7,8,21–23].

In this review, we discuss the importance of the membrane interactions of Ras proteins for their activity, the domains and factors that mediate and regulate their membrane association, and the potential importance of recruitment to membrane subdomains for signal regulation. Notably, a significant body of recent data suggests that although Ras activation is usually initiated at the plasma membrane, Ras proteins can also signal to different extents from the membranes of intracellular organelles (the Golgi, endosomes, mitochondria, ER). Ras signaling from endomembranes has been covered by several recent reviews [8,24–27], and will therefore be referred to only marginally in the current review, which focuses on the plasma membrane.

2. Membrane targeting signals in Ras isoforms

The membrane interactions of Ras proteins with the cytosolic membrane leaflet are essential for their normal signaling and their transforming activities [28–31]. Yet, despite the similarity between the different Ras isoforms in their response to hormonal stimuli and signaling, there are distinct differences between the isoforms in the extent to which they activate specific downstream signaling pathways [32–34], and there is evidence that the isoforms are not functionally redundant [35,36]. This view is supported by the fact that different *ras* genes are mutated or overactivated in different human tumors [14,37]. A potential mechanism that could give rise to some of these differences is distinct membrane anchorage of each Ras isoform, resulting in dissimilar spatial organization in the plasma membrane and/or different subcellular localization [7,8].

Several regions of Ras proteins have distinct functions in their interactions with the membrane; some are common to all Ras proteins, but some are distinct to specific isoforms. All Ras proteins share a conserved C-terminal CAAX motif, which undergoes post-translational prenylation by cytosolic farnesyl transferase to generate *S*-farnesyl cysteine thioester, followed by proteolytic cleavage of the AAX sequence and methyl esterification of the resulting C-terminal isoprenylated cysteine in the ER [38–41]. The resulting *S*-farnesyl-carboxymethyl ester (Fig. 1) is not sufficient for effective membrane association, and a second signal is required. The second signals are located in the HVR region, and differ between Ras isoforms. Thus, the HVR regions of H-Ras, N-Ras and K-Ras4A contain one or two cysteines that undergo palmitoylation (C^{181} and C^{184} in H-Ras, C^{181} in N-Ras, and C^{180} in K-Ras4A), while in K-Ras4B (K-Ras) the second signal is provided by a six-lysine polybasic motif (K^{175}–K^{180}) that interacts electrostatically with negatively-charged phospholipids in the internal membrane leaflet [30,31,42–46] (Fig. 1). The C-terminal domains up to the *S*-palmitoyl residue (C^{181} or C^{180}), or up to K^{175} in K-Ras, are termed minimal lipid (or membrane) anchors, and their grafting onto foreign proteins is sufficient to promote traffic and anchorage to the plasma membrane [29,47,48]. The remainder of the HVR region upstream of the anchor (19–20 residues) is termed the HVR linker region [47]; however, it is not merely a linker, and at least in H-Ras it modulates membrane association and targeting (see below).

The predominantly membranal localization of Ras proteins in cells [7,8,24–26] suggests that their steady-state distribution favors the membrane-associated fraction. However, this does not provide information on the dynamics of their membrane interactions, which may be stable or transient. To study this issue, we developed a variation of the fluorescence recovery after photobleaching (FRAP) method, termed FRAP beam-size analysis [49,50]. This approach is based on the realization that for proteins interacting with the cytoplasmic membrane leaflet, FRAP can occur not only by lateral diffusion but also by exchange between membrane and cytoplasmic pools. FRAP beam-size analysis characterizes the membrane interaction dynamics of these proteins based on their lateral diffusion rates (lipid-like, or slower protein-like diffusion due to association with membrane protein targets) and on the relative contribution of membrane-cytoplasmic exchange to their FRAP recovery kinetics. The FRAP experiments are performed twice, using two different Gaussian laser beam sizes. In the case of membrane association which is stable on the time scale of the FRAP experiment, only the lateral diffusion contributes significantly to the FRAP. Therefore, the characteristic fluorescence recovery time τ ($t_{1/2}$ for fluorescence recovery) is the characteristic diffusion time τ_D, proportional to the area illuminated by the laser beam ($\tau = \tau_D = \omega^2 / 4D$, where ω is the Gaussian radius of the beam and D is the lateral diffusion coefficient [51,52]). On the other hand, if the exchange kinetics are much faster than the lateral diffusion rate, FRAP will occur by exchange; under such conditions, τ is the chemical relaxation time, which is independent of the laser beam size [50]. Thus, the $\tau(40\times)/\tau(63\times)$ ratio expected for the two beam sizes generated using 40× and 63× objectives in our FRAP setup is 2.56 (the measured ratio between the areas illuminated by the two beams) for recovery by pure lateral diffusion, versus 1 for exchange; intermediate values suggest a mixed recovery mode, where the faster process has a higher contribution [50]. Using this method to characterize the membrane interactions of biologically-active green fluorescent protein (GFP)-tagged Ras isoforms in live cells, we have demonstrated that both H-Ras and K-Ras, whether GDP-loaded (unactivated wild-type Ras) or GTP-loaded (constitutively active G12V mutants), display FRAP by nearly pure lateral diffusion [53,54]. The negligible contribution of exchange on the time scale of the lipid-like lateral diffusion of these Ras proteins (7–15s for complete FRAP curves) demonstrates that their association with the plasma membrane is relatively stable (no significant exchange on a time scale of seconds). However, while this indicates that the exchange kinetics is slow relative to their lateral diffusion, it does not exclude the existence of slower exchange. Indeed, several studies on K-Ras indicated that, on a scale of minutes, it can translocate from the plasma membrane to internal organelles. Thus, in hippocampal neurons, reversible translocation of K-Ras to the Golgi and to endosomal membranes was induced by Ca^{2+}/calmodulin [55]. Moreover, using rapamycin-regulated protein dimerization to trap K-Ras derivatives by

binding to a mitochondrial outer-membrane protein incorporating a complementary heterodimerization module, K-Ras was shown to undergo rapamycin-induced transfer to the mitochondrial trap within several minutes [56]. Interestingly, PKC-dependent phosphorylation of S^{181} in K-Ras was also reported to enable its translocation to mitochondria, ER and Golgi, probably by the weakening of its charge-based interactions with plasma membrane lipids due to partial neutralization of the positively-charged polybasic cluster [57]. There are also indications that H-Ras and N-Ras may undergo exchange at a rate slower than their lateral diffusion. Thus, mutations in the HVR linker region of H-Ras, in which the intact lipid anchor was retained, resulted in a shift to exchange in FRAP beam-size analysis and reduced the membrane-associated fraction [23]. Moreover, FRAP studies demonstrated that mutationally depalmitoylated H- and N-Ras translocate from the plasma membrane to the Golgi via a non-vesicular pathway [58–60]. Finally, exchange of H- and N-Ras from plasma membrane sites may be mediated in part by association of a small fraction of the membrane-resident Ras proteins with "rasosomes", proteinacious nanoparticles that diffuse randomly in the cytoplasm and attach/detach to and from the plasma membrane [61].

3. Association of Ras proteins with lateral domains/clusters in the plasma membrane

The plasma membrane is not homogeneous laterally, and contains many sub-micron domains, protein clusters and membrane-associated scaffolds [2,3,62,63]. In the following sections (Sections 3–5), we will discuss the interactions of Ras isoforms with laterally segregated clusters or domains, and their potential effects on membrane association and signaling.

Membrane domains enriched in cholesterol and sphingolipids, termed "lipid rafts", were demonstrated in artificial lipid bilayers and proposed to exist also in cell membranes [7,64–67]. However, their properties and even their very existence in cells are still under debate [2,4,64,68–70]. The recent view of rafts is as small transient cholesterol-dependent assemblies complexed with proteins; the lipid–protein interactions in these assemblies are mutual, and determine their formation, size and stability [2,64,71,72]. The first tentative evidence that H- and K-Ras may differ in their association with raft domains came from biochemical fractionation experiments [73,74]. These were followed by studies based on electron microscopy (EM) and biophysical methods that measured the clustering, diffusion and interactions of Ras proteins in cell membranes. Taken together, these studies provided compelling evidence that the affinity of H- and K-Ras isoforms to cholesterol-sensitive assemblies (rafts) is highest for GDP-loaded wild-type (wt) H-Ras, decreases (but still exists) for constitutively active (GTP-loaded) H-Ras(G12V), and is essentially negligible for either K-Ras(wt) or K-Ras(G12V) [23,47,54,74,75]. These conclusions are in agreement with the demonstration that prenylation alone excludes proteins from raft-like domains, while dual acylation (*S*-palmitoyl and/or *N*-myristoyl residues) leads to association with raft domains [76,77]. Such partitioning may also affect the susceptibility of Ras isoforms to specific modifications. For example, in spite of their highly similar G-domains, the G-domain of H-Ras (but not K-Ras) undergoes ubiquitination that targets it to endosomes, and this selectivity is conferred by the H-Ras membrane anchor [78]. It should be noted that although some studies did not detect effects of cholesterol depletion on the lateral diffusion of H-Ras [79,80], this discrepancy appears to arise due to cholesterol-independent effects of the methyl-β-cyclodextrin treatment employed to deplete cholesterol from the plasma membrane; an alternative cholesterol depletion method (metabolic inhibition of cholesterol synthesis by compactin) did not produce such artifacts and increased selectively (nearly 2-fold) the lateral diffusion rate of H-Ras but not K-Ras [54,81,82]. The association of N-Ras with cholesterol-sensitive domains was not investigated as extensively; however, there are indications that unlike H-Ras, it is the activated (GTP-loaded) form of N-Ras that has a higher affinity for raft-like domains [60].

While GTP-loaded activated H-Ras exhibits reduced affinity to raft domains, both constitutively active H- and K-Ras associate with distinct clusters/assemblies in the cell membrane that are insensitive to cholesterol depletion. Independent studies based on FRAP of GFP-tagged H- and K-Ras isoforms in live cells [54] and on EM cluster analysis of these proteins in intact plasma membrane sheets [47] demonstrated that H-Ras(G12V) and K-Ras(G12V) largely associate with spatially distinct, cholesterol-insensitive (nonraft) clusters. The FRAP live-cell studies further demonstrated that these interactions are transient [54]. In line with these findings, single-molecule diffusion measurements of either GFP-H-Ras(G12V) or of YFP-tagged H- and K-Ras visualized by FRET with a fluorescent GTP analogue indicated that the activated (GTP-loaded) H- and K-Ras interact transiently with cholesterol-independent clusters or microdomains [80,83].

Notably, the clusters/assemblies with which activated H-Ras and K-Ras interact appear to be dissimilar [47,54]. Interactions with distinct assemblies containing nonidentical sets of signaling molecules may serve as a mechanism to induce selective activation of certain signaling pathways by one Ras isoform but not by the other [32,34,84,85]. The identity of Ras-interacting proteins that direct activated Ras proteins to different isoform-specific clusters is still unclear, but proteins such as galectin-1 and galectin-3 are likely candidates. These proteins, which possess a prenyl-binding pocket [86], interact with H- and K-Ras [87–89]. Both galectins show preferential binding to the activated, GTP-loaded Ras proteins. Moreover, they exhibit selectivity for specific Ras isoforms; galectin-1 binds both H- and K-Ras [87,88], while galectin-3 interacts only with K-Ras [89]. These interactions were reported to prolong Ras signaling and to modulate the balance between the relative levels of activation of different Ras effector pathways [88,89].

4. Distinct roles of specific domains in lateral partitioning of Ras isoforms

The efficacy of the association of Ras proteins with the membrane, their targeting to specific lateral domains/clusters and their signaling outcome are intertwined. The relations

between these parameters were thoroughly investigated in H-Ras. A striking example for the dual role of the HVR linker segment (Fig. 1) in H-Ras membrane association and lateral segregation was supplied in a study that combined EM spatial mapping with FRAP beam-size analysis [23]. The FRAP studies demonstrated that deletion of the HVR linker (14 amino acids; Fig. 1) to generate GFP-H-Ras(G12V)-Δhvr [23], whose lipid anchor (including the farnesyl and two palmitoylation sites) is intact, reverted the FRAP mechanism from lateral diffusion to exchange. Together with the fast FRAP kinetics of the Δhvr mutant, this indicates a fast rate of dissociation from the plasma membrane. The reduced membrane association was also evident in biochemical fractionation studies [23], and demonstrates that the HVR linker has a significant contribution to the membrane association of H-Ras. In line with these findings, a recent simulation-based study suggested a role for the HVR linker in H-Ras binding to 1,2-dimyristoylglycero-3-phosphocholine (DMPC) bilayer [90]. In parallel, the EM data [23] demonstrated that while GFP-H-Ras (G12V) resided mainly in cholesterol-insensitive clusters, the clustering of its Δhvr mutant in the plasma membrane was abolished by cholesterol depletion. These findings indicate that the membrane interactions contributed by the HVR linker domain are largely dependent on cholesterol; indeed, cholesterol depletion resulted in the loss of the membrane association of the Δhvr mutant, but not of H-Ras(G12V), in live cells [23]. Taken together, these studies suggest that the interactions of the HVR linker domain with the plasma membrane serve a dual role: they are essential for stable membrane binding of H-Ras, but at the same time their preference for nonraft domains (evidenced by the loss of nonraft binding in the Δhvr mutant) regulates the dynamic distribution of H-Ras between raft and nonraft sites (Fig. 2). The transient interactions with raft and nonraft sites may be relevant for the regulation of H-Ras signaling [47,54,74,75], as discussed below.

The reduction in the membrane association of H-Ras(G12V) upon deletion of the HVR linker domain contrasts with the ability of the minimal lipid anchors of Ras proteins to confer relatively stable membrane association [29,47,48]. Indeed, the H-Ras minimal lipid anchor (including the CAAX motif and two palmitoylation sites, termed tH) fused directly to GFP

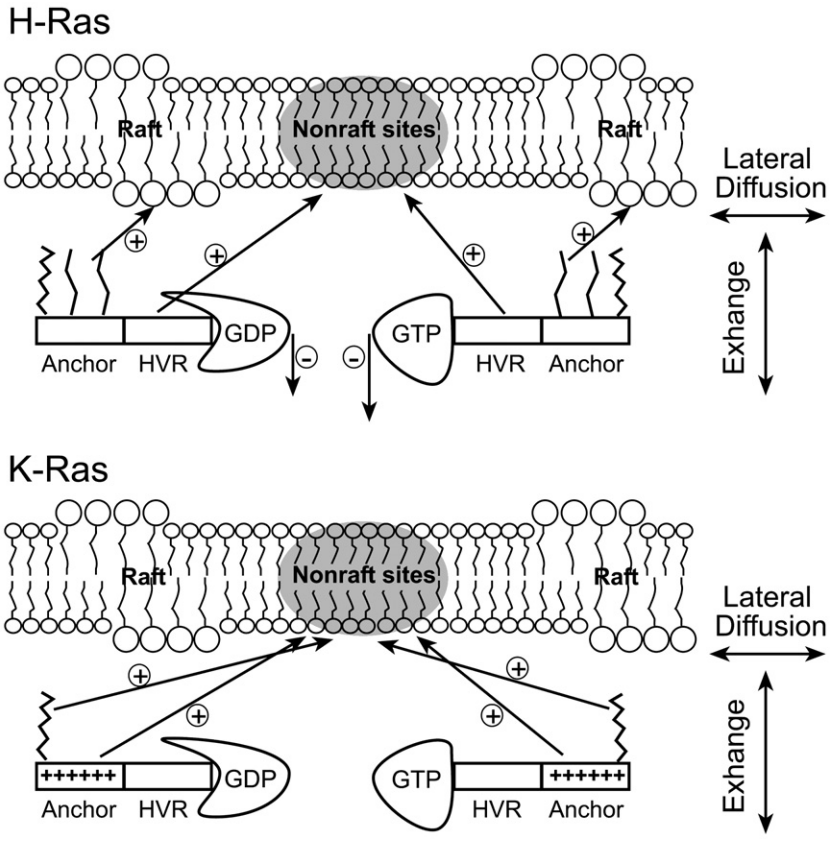

Fig. 2. Schematic representation of the distinct contribution of specific domains in Ras proteins to their interactions with the plasma membrane. In the case of H-Ras, the lipid anchor contributes positive interactions (⊕) favoring association with raft domains; the preference to cholesterol-sensitive (raft-like) assemblies is mainly due to the palmitoyl residues, with a stronger contribution by the palmitoylation at C^{181}. The HVR linker region is also important for stabilizing the membrane association of H-Ras, but is targeted preferentially to nonraft domains and assemblies. On the other hand, the G-domain has a negative (−) contribution (weakening of the membrane association), whose extent depends on the activation state of H-Ras; this repulsion, indicated by downward arrows, is stronger (longer arrow) at the GTP-loaded conformation [23]. N-Ras (not shown in the scheme) resembles H-Ras monomyristoylated at C^{181}, and is targeted to raft domains mainly while at the GTP-loaded conformation [60]. K-Ras lacks palmitoyl residues, and its C-terminal farnesyl moiety as well as the six-lysine polybasic stretch (++++++) interact mainly with nonraft membrane regions and assemblies [47,54].

(GFP-tH) exhibited stable membrane association (FRAP by lateral diffusion) [23]. Thus, the contribution of the HVR linker domain is essential for stable membrane association in the context of the full-length GFP-H-Ras(G12V), but not in the context of direct linkage to GFP, suggesting that the catalytic N-terminal G-domain of H-Ras has a negative contribution to its membrane association. Interestingly, analogous studies on GDP-bound H-Ras(wt) and its Δhvr mutant [23] indicated that the negative contribution of the G-domain is weaker in the inactive, GDP-loaded conformation. Indeed, simulation studies on H-Ras binding to DMPC bilayers suggested a dependence of the interactions on the GDP/GTP-loading state of H-Ras, with the GDP-loaded conformation showing a deeper insertion into the bilayer [90]. Thus, the affinity of H-Ras to the membrane, its activation state (GDP- versus GTP-loaded) and its spatial (lateral) localization in the plasma membrane are correlated and provide intricate means to regulate its activity. These findings are summarized in the model depicted in Fig. 2.

The palmitoylation sites in the C-terminal lipid anchor of H-Ras, C^{181} and C^{184}, also have distinctive effects on its lateral segregation in the plasma membrane, as well as on its transport to membranes of intracellular organelles [8,29,58–60]. The roles of the palmitoyl residues in anchoring H-Ras to the plasma membrane were studied by FRAP beam-size analysis of GFP-H-Ras mutants lacking either palmitoylation site (C181S or C184S mutations). These studies demonstrated that palmitoylation at C^{181}, but not at C^{184}, is sufficient to stabilize the membrane interactions of H-Ras(wt) or H-Ras(G12V) [60]. Importantly, C^{181} is also the position of the palmitoylated cysteine in N-Ras. The H-Ras palmitoylation-site mutants differed also in the extent to which they were affected by cholesterol depletion; although elimination of either site (C181S or C184S mutations) resulted in a significant weakening of the mutant's membrane association following cholesterol depletion (elevated exchange rates), the effect was markedly higher on the C184S mutants (H-Ras(wt) and H-Ras(G12V); these mutants retain a single palmitoylation site at C^{181}) [60]. Concomitantly, EM cluster analysis revealed that the C184S mutation (monopalmitoylation at C^{181}) drives GTP-loaded H-Ras(G12V), which normally has a weaker affinity to raft domains than unactivated H-Ras, into cholesterol-sensitive sites [60]. Thus, palmitoylation of C^{181} is not only important for anchoring H-Ras in the bulk plasma membrane, but also preferentially targets H-Ras to cholesterol-sensitive domains/clusters (Fig. 2). In line with the role of the palmitoyl residues of H-Ras in conferring its association with cholesterol-sensitive assemblies, K-Ras, which lacks palmitoylation sites, is not targeted to raft-like domains [47,54,74,75,81]. Rather, its six-lysine polybasic cluster in the HVR region interacts with negatively-charged lipids in the internal membrane leaflet [91–93], apparently at nonraft regions (Fig. 2). Recently, using a rapamycin heterodimerization-dependent method to rapidly recruit to the plasma membrane specific phosphatases that deplete phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, it was demonstrated that these two negatively-charged inositol phospholipids, which are less abundant and serve as second messengers, target K-Ras to the plasma membrane [46].

5. Targeting of Ras proteins to distinct assemblies and modulation of Ras signaling

The different targeting of Ras isoforms to cholesterol-dependent assemblies may affect their responsiveness to extracellular signals. Such differential effects selective for specific Ras isoforms were recently exemplified by the effects of external clustering of raft-associated influenza hemagglutinin (HA) variants on the lateral diffusion and signaling of H-Ras, but not K-Ras [75]. The studies compared systematically the transbilayer effects of clustering outer-leaflet HA proteins that are either raft-resident, such as glycosylphosphatidylinositol (GPI)-anchored HA embedded only in the external leaflet and HA(wt), and the nonraft mutant HA(2A520) [94,95] on the properties of raft or nonraft inner-leaflet Ras proteins (H-Ras and K-Ras). The effects of the clustering on the lateral diffusion of the Ras proteins and on their signaling were measured. The clustering of raft-associated (but not nonraft) HA proteins retarded the lateral diffusion of H-Ras, with a stronger effect on GDP-loaded H-Ras(wt) than on GTP-loaded H-Ras(G12V); nonraft K-Ras proteins were not affected [75]. In parallel experiments on EGF-stimulated Ras signaling, clustering raft-associated but not nonraft HA proteins facilitated the initial activation step (GDP-to-GTP exchange) of H-Ras without affecting K-Ras. In spite of this enhancement, the ensuing downstream signaling by H-Ras (Erk phosphorylation) was inhibited. These findings led to a model (Fig. 3) where cholesterol-sensitive assemblies serve as "catalytic centers" with which H-Ras interacts dynamically. The entry of inactive H-Ras into these assemblies facilitates its activation (shift to the GTP-loaded state); however, once activated, H-Ras has to exit these centers and associate with nonraft assemblies for efficient downstream signaling, making its dynamic entry/exit into and out of the "catalytic centers" an essential feature of the signal propagation. Clustering of external raft-resident proteins stabilizes the cholesterol-sensitive assemblies also in the internal leaflet, resulting in a transbilayer modulation that "traps" H-Ras in the raft clusters. This, in turn, enhances the step of H-Ras activation (GTP loading); however, since the departure of activated Ras from the clusters is retarded, downstream signaling is inhibited. This model is supported by biochemical and EM studies, which suggested that activated H-Ras has to exit raft-like domains in order to induce effective activation of Raf [47,74]. It should be noted that a study based on chimeric constructs artificially tethered to raft or to nonraft domains failed to detect a requirement for dissociation of activated H-Ras from rafts to induce signaling [22]; however, alteration of raft association kinetics in the artificial constructs could have critical effects on their signaling, especially since it is the dynamic aspect of the lateral segregation which appears to be a key feature of Ras activation and downstream signaling cascade. Furthermore, the aspect of raft association in this study, as measured by the inhibitory effects of dominant negative H-Ras chimeras, was ambiguous due to cross-reactivity between the dominant negative constructs.

The relevance of the model depicted in Fig. 3 is not limited to modulation of Ras signaling. It presents a simple mechanism

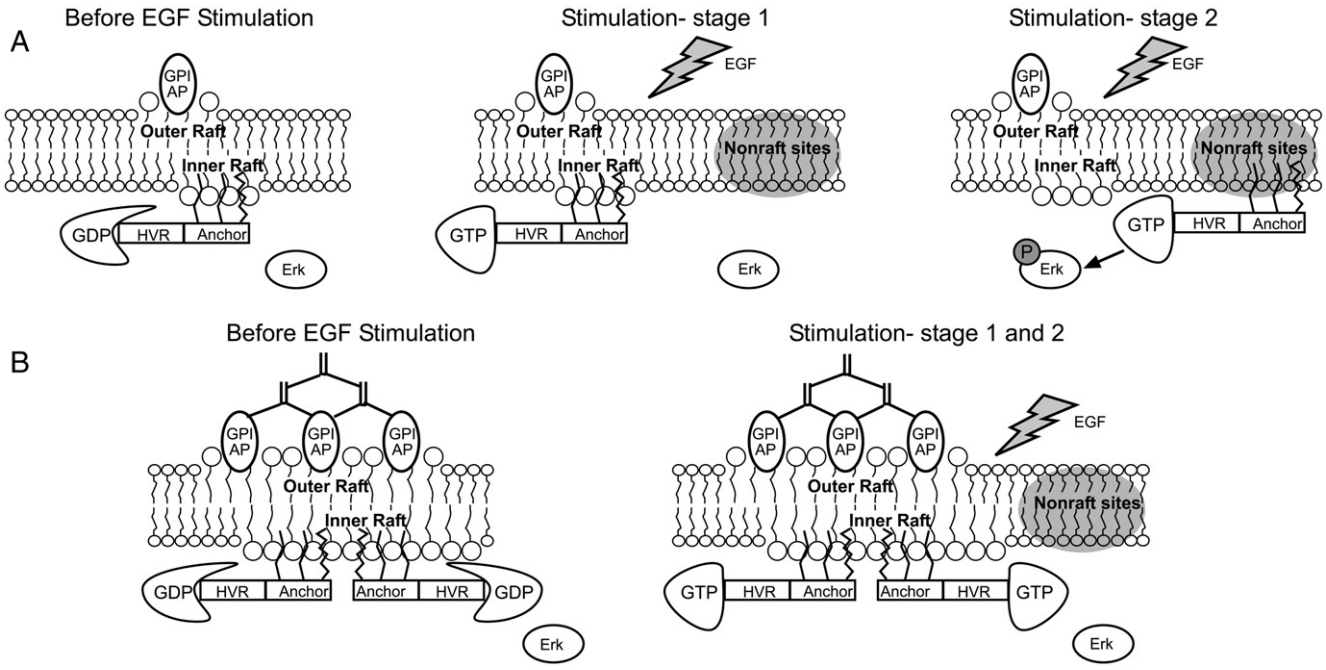

Fig. 3. Transbilayer modulation of H-Ras intracellular signaling following the extracellular clustering of unrelated GPI-anchored proteins. The various domains of H-Ras are defined as in Figs. 1 and 2. (A) No clustering. Before activation, GDP-loaded H-Ras(wt) interacts dynamically with cholesterol-dependent domains (rafts) in the inner plasma membrane monolayer. Upon stimulation by EGF (stage 1), it is activated and exchanges GDP for GTP; the GTP-loading step is proposed to be facilitated by association with inner-leaflet raft domains, which serve as "catalytic centers" that catalyze H-Ras activation. However, for downstream signaling, the interaction with the catalytic centers has to be dynamic, as the GTP-loaded H-Ras has to exit them and journey to other sites or assemblies designated here as "nonraft sites". At these sites, specific signaling cascades (here, Erk phosphorylation in the MAPK pathway) are facilitated. (B) Effects of extracellular clustering of raft-resident proteins. In the example studied, GPI-anchored proteins (GPI-AP) that associate with raft-like domains and are embedded only in the external membrane leaflet are subjected to clustering (by IgG crosslinking, or by natural ligands) prior to stimulation. The clustering stabilizes outer-leaflet raft domains, and enhances their loose interactions with inner-leaflet rafts; the bridging between the raft-like domains in the two leaflets may involve both lipid interdigitation and mutual affinity to raft-interacting cellular transmembrane proteins. Following the clustering-mediated stabilization, H-Ras association with raft-like domains is stabilized, and the kinetics of its dynamic association–dissociation kinetics with these domains is slowed down. The longer residence times in the stabilized raft clusters enhance the first stage (GTP loading), but inhibit the departure of the activated H-Ras from these clusters and its journey to the nonraft sites where Erk phosphorylation is facilitated. Reproduced from [75] with permission from the American Society for Microbiology.

that may act in many other cases to achieve transbilayer modulation between proteins associated with cholesterol-enriched assemblies in the external leaflet, and signaling proteins in the internal membrane monolayer. This notion is supported by recent studies [96,97] where limited clustering of GPI-anchored receptors by antibodies conjugated to colloidal gold nanoparticles was shown to transiently recruit Lyn, phospholipase Cγ (PLCγ) and Gαi2 to the clusters, activating Lyn and PLCγ. Importantly, the clustering-mediated facilitation of Lyn and PLCγ signaling depended on the association of the crosslinked GPI-anchored proteins with raft domains [96,97]. These studies proposed an extension of the model to explain also how the clustering may lead to stimulation-induced transient immobilization (stall) of the clusters with actin filaments via putative linker proteins, a process that may be linked to the activation of some intracellular signaling events [96–98].

6. Concluding remarks

The interactions between signaling proteins and cellular membranes are emerging as important modulators of cellular signaling. The spatio-temporal organization in cells is largely dependent on both the nature and the dynamics of the association of proteins with specific membranes and on their targeting to defined domains or regions within these membranes. The dynamic association–dissociation of signaling proteins to and from specific membranes, as well as their ability to move within the membrane (lateral diffusion and segregation in specific domains/clusters), enables intricate control and fine-tuning of biological processes. A multitude of protein domains and post-translational modifications contribute to the membrane interactions of signaling proteins, and these moieties are in turn subject to regulation by other cellular enzymes, which in many cases can be activated by extracellular signals. A typical example is provided by the Ras proteins, whose membrane association and targeting depend on several moieties subject to numerous enzymatic modifications that differ in their cellular dynamics (e.g., stable thioester linkage of the farnesyl group versus the much faster hydrolysis of the palmitoyl thioester bonds in H- and N-Ras by thioesterases). This situation gave rise to the notion that compartmentalization, both between and within specific membranes, can have a strong impact on central signaling pathways. Importantly, modulations in the dynamics of the interactions between signaling proteins and cholesterol-sensitive assemblies in the internal leaflet have the potential to be either stimulatory or inhibitory, depending on whether the

stabilization of the specific signaling protein in these assemblies facilitates or inhibits its activity. Potential association with linker proteins bridging these assemblies with e.g. cytoskeletal components may yet add another level of regulation to cellular signaling events. The merging of biochemical approaches with biophysical methods that enable studies on the dynamics of the membrane interactions of signaling proteins in live cells has the potential to yield the quantitative information necessary to gain new insights into the role of membrane interaction dynamics in signal transduction.

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