Plasma membrane regulates Ras signaling networks

Tanmay Sanjeev Chavan¹, Serena Muratcioglu², Richard Marszalek³, Hyunbum Jang⁴, Ozlem Keskin², Attila Gursay², Ruth Nussinov^{4,5}, and Vadim Gaponenko^{3,*}

¹Department of Medicinal Chemistry; University of Illinois at Chicago; Chicago, IL USA; ²Center for Computational Biology and Bioinformatics; Koc University; Istanbul, Turkey; ³Department of Biochemistry and Molecular Genetics; University of Illinois at Chicago; Chicago, IL USA; ⁴Cancer and Inflammation Program; Basic Science Program; Leidos Biomedical Research, Inc.; Frederick National Laboratory for Cancer Research; National Cancer Institute at Frederick; Frederick, MD USA; ⁵Sackler Institute of Molecular Medicine; Department of Human Genetics and Molecular Medicine; Sackler School of Medicine; Tel Aviv University; Tel Aviv, Israel

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Abbreviations: EGF, extracellular growth factor; EM, electron microscopy; FRET, forster resonance energy transfer; GAPs, GTPase activating proteins; GDP, guanosine diphosphate; GEFs, guanine nucleotide exchange factors; GTP, guanosine triphosphate; PI3-K, phosphoinositide 3-kinase; SOS, son of sevenless

Ras GTPases activate more than 20 signaling pathways, regulating such essential cellular functions as proliferation, survival, and migration. How Ras proteins control their signaling diversity is still a mystery. Several pieces of evidence suggest that the plasma membrane plays a critical role. Among these are: (1) selective recruitment of Ras and its effectors to particular localities allowing access to Ras regulators and effectors; (2) specific membrane-induced conformational changes promoting Ras functional diversity; and (3) oligomerization of membrane-anchored Ras to recruit and activate Raf. Taken together, the membrane does not only attract and retain Ras but also is a key regulator of Ras signaling. This can already be gleaned from the large variability in the sequences of Ras membrane targeting domains, suggesting that localization, environment and orientation are important factors in optimizing the function of Ras isoforms.

Introduction

An intriguing phenomenon in biology is the astonishing number of functions some proteins, such as Ras GTPases, tend to perform. Ras activates more than 20 effectors, initiating complex cellular responses to diverse environmental stimuli. Ras signaling is initiated by growth factor, antigen, chemokine and cytokine ligands. The current paradigm states that the receptor-ligand interaction activates Ras at the plasma membrane through Guanine exchange factors (GEFs), which promote the displacement of GDP for GTP.¹ In its active state, Ras interacts with effector proteins to regulate cell proliferation, survival, migration, invasion, and apoptosis.²⁻⁴ Of all Ras effectors, Raf and phosphoinositide-3 kinases (PI3-Ks) are the best studied. Signaling through

Ras ceases when its GTPase domain hydrolyses GTP to GDP (Fig. 1).

GTP hydrolysis is a slow process that needs to be catalyzed by GTPase activating proteins (GAPs).⁵ Ras GTPases are small 21 kDa proteins and many of Ras binding partners seem to share the same binding site. This site is a short extension of the Switch I region in the Ras GTPase domain, named the effector binding region⁶⁻⁸ (Fig. 2). Binding of multiple effectors to a short stretch of amino acids in a small protein at the same time seems inconceivable. How Ras achieves signaling diversity is a conundrum waiting to be fully resolved.

Accumulating evidence suggests that Ras association with the plasma membrane plays a significant role in deciding the number and the type of signaling outputs. Like a circuit board, the plasma membrane organizes different types of circuits or platforms, through which Ras switches on an intricate network of signals. The principles for the circuit formation are becoming clearer as we discover the complex organization of lipids within the plasma membrane. Lateral segregation of lipids into distinct microdomains within the membrane has led to the hypothesis that these microdomains serve as signaling platforms.⁹⁻¹¹ The interaction of Ras with different membrane microdomains can affect its access to GEFs and effectors.¹² The membrane microdomains can promote distinct changes in Ras structure and orientation, which influence the preferred oligomerization states of Ras isoforms. Understanding the mechanisms of regulation of Ras signaling is the focus of research in many labs. Regulation of Ras function by post-translational modifications has been analyzed in an excellent review by Ahearn et al.¹² Many of these post-translational modifications affect Ras interactions with the plasma membrane. Here, we provide an overview of recent advances in Ras biology underscoring the role of the plasma membrane in Ras signaling.

Unique microdomains are present in the plasma membrane

The composition and lateral segregation of lipids, the shifts in protein localizations, and the coupled endocytic or exocytic processes result in a highly heterogeneous and dynamic plasma membrane. This heterogeneity leads to the formation of several

*Correspondence to: Vadim Gaponenko; Email: vadimg@uic.edu
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Figure 1. Growth factor (GF, purple boxes) binding to its receptor (RTK dimer, long purple rectangles) initiates recruitment of GRB2 (a dark blue circle) and SOS (light blue triangles) to the membrane. SOS is a guanine exchange factor (GEF), which binds Ras-GDP and converts it to active Ras-GTP. GTPase activating proteins (GAPs) catalyze conversion of Ras-GTP to its GDP loaded form. The balance between GEFs and GAPs determines the amount of active Ras in cells. Active Ras proteins bind more than 20 known effectors (arrows followed by circles indicate the effectors, e.g. interaction with TIAM1 leads to cell migration, and that with RIN leads to endocytosis, etc.), which produce diverse cellular effects.

types of microdomains functioning as signaling platforms for membrane proteins.⁹⁻¹¹ These microdomains can be either transient or long-lasting relatively stable assemblies.¹³ A lipid raft is

Figure 2. Active Ras GTPases interact with and switch on other proteins in the signal transduction chain. Most binding partners of Ras interact with the effector region or switch I region (dashed circle) on the protein through their Ras binding domains (RBDs). Thus, binding is highly competitive for these proteins. The ones with higher binding affinities can compete out the others and transduce the signal through the downstream pathways. Raf-1 (red, PDB ID: 4G0N), PLC ϵ (orange, PDB ID: 2C5L), PI3K (magenta, PDB ID: 1e8y), and RASSF1 (green, Homology model), are major Ras partners that bind to the effector region on H-Ras (cyan, PDB ID: 1QRA). Each association activates a specific pathway/pathways and induces distinct biological responses including cell cycle progression, Ca²⁺ signaling, cell migration, and apoptosis/cell cycle arrest, respectively.

one such microdomain, which is rich in cholesterol and saturated lipids, like sphingolipids. These components are packed tightly together, forming a highly ordered, high density structure that is distinct from the surrounding low density lipids. One example of a high density microdomain is caveolae. Caveolae are invaginations present in cells consisting of components similar to those of lipid rafts.¹⁴ Several unclassified lipid microdomains have been found in different studies. Cholesterol-independent non-raft microdomains have been observed using electron microscopy.¹⁵ Another such non-raft microdomain contains primarily acidic head-groups present on phosphatidylserine and phosphatidic acid. Positively charged proteins bind to such microdomains through electrostatic interactions.¹⁶ Thus, it is becoming evident that the organization of the plasma

membrane is complex, consisting of a number of entities, which differ in lipid composition and consequently in physical properties. Membrane associating proteins like Ras exploit this heterogeneity of the membrane to diversify and optimize their functions. An example of this is the enhanced K-Ras clustering and MAPK activation present in caveolin deficient cells.¹⁷ Another example suggests that membrane fluidity, dependent on the relative populations of disordered and rigid microdomains, dictates the insertion of farnesyl group of K-Ras into the membrane phospholipids.¹⁸ Disordered lipid microdomains of the membrane allow for the insertion of farnesyl group whereas the rigid microdomains restrict its insertion into the membrane phospholipids.

Protein-membrane interactions can no longer be viewed as binary switches turning on and off protein activity. The emerging concept is that the membrane uses multiple ways for fine tuning of protein function. Understanding of the regulatory mechanisms employed by the plasma membrane is important for the future design of therapeutic approaches modulating the activity of membrane proteins in disease.

Interaction of Ras GTPases with different membrane microdomains

Ras isoforms, H-Ras, N-Ras, K-Ras4A and K-Ras4B, contain a highly conserved N-terminal catalytic domain. The C-terminal 22–25 amino acid-long portion of Ras is named the hypervariable region (HVR), since it differs significantly across isoforms in its amino acid sequence.¹⁹ Although the N-terminal domains contribute to membrane binding,²⁰ Ras proteins primarily interact with the membrane through their HVRs.²¹ The differences in amino acid sequence of Ras HVRs are augmented by different post-translational modifications. For instance, H-Ras, N-Ras and K-Ras4A are modified with farnesyl and palmitoyl lipid groups

on their HVRs, which anchor the proteins to the plasma membrane. The palmitoylation-depalmitoylation cycle allows them to shuttle between the plasma membrane and the endomembranes of the Golgi apparatus and Endoplasmic Reticulum.²² K-Ras4B, on the other hand, lacks palmitoyl groups in its HVR. Instead, a polylysine patch in the K-Ras4B HVR, in addition to the farnesyl group, assists in binding the membrane. In addition, K-Ras4B has an alternative cycling mechanism. K-Ras4B undergoes protein kinase C (PKC)-mediated phosphorylation at a serine residue (S181) on its HVR. This phosphorylation event dissociates K-Ras4B from the plasma membrane through electrostatic repulsion with the negatively charged phospholipid head-groups.²³⁻²⁵ However, a recent study suggests that a significant fraction of phosphomimetic oncogenic K-Ras4B stays associated with the membrane.²⁶ While phosphorylation of S181 causes a shift in membrane localization of K-Ras4B, it is unclear whether the shift is either between the plasma membrane and endomembranes or within the plasma membrane itself. K-Ras4B interaction with endosomal membranes is dynamic, with rapid exchange between the cytosolic and membrane associated pools. The rate of this exchange is modulated by the activation state of the protein, with the inactive state being less mobile than the active form.²⁷ An additional mechanism that allows K-Ras4B to traffic from the membrane to the cytosol is calmodulin binding. Of all Ras isoforms, calmodulin exclusively binds K-Ras4B.²⁸ Upon binding calmodulin, K-Ras4B dissociates from the membrane to the cytosol in a calcium dependent manner.²⁹

Phosphodiesterase δ is also involved in shuttling of prenyl-modified proteins like Ras, Rheb, Rap, and Rho from the membrane to the intracellular compartments by binding to their farnesyl groups.³⁰⁻³² Additionally, phosphodiesterase δ continuously sequesters mislocalized K-Ras from endomembranes and unloads it to perinuclear membranes. Subsequently, electrostatic interactions allow K-Ras enrichment in recycling endosomes, which transport K-Ras to the plasma membrane.³³ The phosphodiesterase δ dependent shuttling mechanism allows for signal propagation and makes phosphodiesterase δ an attractive therapeutic target.^{34,35} An inhibitor of phosphodiesterase δ , called deltarasin, can stop K-Ras signaling by relocating it to endomembranes. However, the lack of specificity toward Ras and the absence of nucleotide dependence in phosphodiesterase δ -Ras binding suggest possible side-effects.

So far, evidence for the localization patterns of Ras proteins on lipid rafts has been contradictory. H-Ras localizes to lipid rafts in a nucleotide dependent manner.³⁶⁻³⁸ Inactive GDP-bound H-Ras is found in lipid rafts,^{39,40} while H-Ras-GTP occupies the disordered membrane regions where it interacts with galectin-1.^{41,42} N-Ras also partitions into lipid rafts, and membrane curvature plays an important role in N-Ras enrichment in the raft-like liquid ordered phases.^{43,44} Moreover, it is possible to alter spatiotemporal organization of H-Ras and N-Ras through liquid-ordered domain stabilization by a nonsteroidal anti-inflammatory drug indomethacin.⁴⁵ K-Ras localization on lipid rafts is a contentious issue. Some groups have observed K-Ras localization on rafts^{39,46}, while others have not.⁴⁰ This inconsistency may be ascribed to differences in the methods of preparation of

lipid rafts or the techniques for protein detection in the membrane microdomains. K-Ras was found in non-raft regions using confocal microscopy,³⁰ immunogold labeling³⁹ and electron microscopy.⁴⁰ However, reports from several groups that use different membrane preparation and detection methods to show K-Ras in lipid rafts, lends credibility to the idea that K-Ras is distributed between raft and non-raft membrane microdomains.^{47,48} K-Ras was co-purified with detergent resistant lipid rafts at physiological pH.³³ Fluorescence microscopic imaging with EGFP and antibody probes was used to discover K-Ras colocalization with a lipid raft marker GM1.³² While purification of lipid rafts from the plasma membrane may significantly affect their composition, most imaging techniques, including confocal microscopy and electron microscopy, are limited by their resolution. If lipid rafts are very small structures within the membrane their detection may be challenging. An example of this comes from the comparison of Lck, which localizes to lipid rafts, and K-Ras using high accuracy position and temporal resolution single-molecule microscopy.⁴⁹ This study revealed only minor differences in the diffusion behavior, suggesting that lipid rafts must be very small (<137 nm in diameter) and do not affect the mobility of Lck and K-Ras. Modern super high resolution imaging techniques, such as photo-activated localization microscopy or stochastic optical reconstruction microscopy that can resolve structures as small as 10 nm in diameter,⁵⁰ will be very useful in addressing the questions in regards to Ras membrane localization. If K-Ras localization and function in the plasma membrane is nucleotide-dependent, like it is for H-Ras, then specific regulation of K-Ras-GTP by drugs altering the membrane composition may provide a possible anti-cancer therapeutic approach.⁵¹⁻⁵³

Membrane microdomains change the access of Ras to its binding partners

Ras proteins have different degrees of access to GEFs, GAPs, and effectors in various microdomains.⁵⁴ This differential access idea (graphically shown in Fig. 3) can explain isoform-specific signaling of Ras. For example, H-Ras activates PI3-K to a greater extent than K-Ras4B whereas K-Ras4B is responsible for activation of Raf-1.⁵⁵ Although the membrane localization pattern of PI3-K has not been studied, a substrate of PI3-K, Phosphatidylinositol 4, 5-bisphosphate (PIP2), is found in the inner leaflet of membrane microdomains.⁵⁶ These PIP2 microdomains can be cholesterol independent and distinct from rafts. It is conceivable that PI3-K may co-localize with its substrate PIP2 in these microdomains. Incidentally, H-Ras nanoclusters are enriched in phosphatidylinositols.⁵⁷ K-Ras-GTP is similar to H-Ras-GTP in its ability to form nanoclusters on PIP3-rich intact plasma membrane sheets.⁵⁷ However, K-Ras-GTP nanoclusters are enriched in phosphatidic acid to a greater extent than either H-Ras-GTP or H-Ras-GDP nanoclusters.⁵⁸ Recently, using a phospholipid array binding assay, we found that while GDP-bound full-length wild type K-Ras4B non-specifically interacted with most anionic phospholipids, the GDP-bound oncogenic G12D and G12V mutants preferentially associated with phosphatidylinositol monophosphates and with phosphatidic acid (Banerjee et al., in press). Preferential binding of oncogenic K-Ras4B-GDP to

Figure 3. Binding of Ras proteins to different membrane microdomains can allow access to specific effectors. This causes differential signaling of Ras proteins from distinct microdomains in the plasma membrane. Examples of membrane microdomains (**A and B**) are indicated in orange and green colors, respectively. Binding of growth factors (purple boxes) to their receptors (dimer shown as long purple rectangles) initiates recruitment of GRB2 (dark blue circle) and GEFs (red diamond in microdomain A and light blue triangle in microdomain B indicate different kinds of GEFs recruited by specific microdomains). This leads to activation of Ras proteins that elicit different downstream signals.

phosphatidic acid-rich microdomains may allow its GTP loading by SOS, which is allosterically activated by phosphatidic acid.^{59,60} Co-localization of K-Ras with phosphatidic acid is consistent with K-Ras's preferential activation of Raf, which has a phosphatidic acid binding domain.⁶¹

Electrostatic interactions between the HVR of K-Ras4B and the plasma membrane lead to higher affinity binding of K-RasG12V to the membrane as compared to H-RasG12V in baby hamster kidney (BHK) cells.²⁵ The higher affinity may stimulate efficient recruitment and activation of Raf-1 molecules only on membrane-anchored K-Ras clusters.⁶² Significantly, when Raf-1 is directed to H-Ras occupied lipid rafts, the activation is minimal.⁶³ This can be attributed to the exposure of Raf-1 to a distinct group of proteins and lipids present in the plasma membrane. Temporal regulation of Raf activation by Ras is an understudied topic. The initial interaction of Raf with Ras is followed by binding of kinases that phosphorylate Raf to achieve its full activation. The plasma membrane is likely to play an important role in this process. Kinetics studies of Raf activation by Ras on the plasma membrane of living cells suggest that Raf fixation to Ras is bimodal, including contributions from the Ras binding domain and the cysteine rich domain.⁶⁴ This allows for Raf to position itself in a conformation suitable for interaction with downstream kinases and also allows extension of the on-times of Raf to interact with the kinases.

Two crucial factors determine the differences in activation by Ras isoforms. The first is the extent of co-localization of Ras isoforms with their corresponding receptor complexes on particular membrane microdomains.⁶⁵ An illustration of this is the fact that K-Ras on disordered microdomains becomes efficiently activated in growth factor dependent cell lines. Conversely, H-Ras or N-Ras bound to lipid rafts are less active in these cells. K-Ras is preferentially activated because epidermal growth factor receptors and components of the activated receptor complex, including GEFs like SOS, also partition to the disordered microdomains.⁶⁵

The second important factor for isoform-specific Ras signaling is the sensitivity of Ras isoforms to different GEFs recruited by activated receptors.⁶⁵ For example, activation of Ras proteins by B-cell receptor (BCR) is independent of the localization patterns of the isoforms. RasGRP, a GEF protein, is responsible for activation of H-, N- and K-Ras proteins through BCR. Conversely, M-Ras, another Ras superfamily protein, is not activated through BCR. This is because M-Ras is sensitive to a different GEF, RasGRF.⁶⁵ Thus, the interplay between different receptors and protein complexes that interact with Ras together modify the protein's exact response. The isoform related differences in access to activators and effects can be exploited for design of specific inhibitors aberrant activation of

Ras in cancer.

Membrane induces conformational and functional alterations in Ras

Differences in orientation of Ras proteins on the plasma membrane can also lead to unique signaling mechanisms. This is predicted to occur through the exposure of different effector-interacting surfaces of membrane-bound Ras toward the cytoplasm (Fig. 4).⁶⁶

Molecular dynamics simulations of Ras proteins, corroborated by fluorescence studies and cell assays, point toward a novel

Figure 4. Different Ras isoforms can adopt distinct conformations on the membrane to diversify effector binding for fine-tuning downstream signals. Binding of growth factors (purple boxes) to their receptors (dimer shown as long purple rectangles) initiates recruitment of GRB2 (dark blue circle) and GEFs (light blue triangles). The activation of Ras proteins also activates their downstream effectors. However, different isoforms of Ras (denoted in orange and light green colors) may adopt unique conformations (shown by distinct indentation in the orange and light green circles, respectively). Different downstream effectors can bind to different conformers of Ras, thus giving rise to distinct signals.

Switch III region involved in reorienting the H-Ras catalytic domain on membrane lipids. The Switch III region is believed to comprise the loop between β -strands 2 and 3, and the α -helix 5 of the catalytic domain. It reorients α -helix 4 of H-Ras upon nucleotide exchange, such that the G-domain in active H-Ras participates in membrane binding. In H-Ras-GDP, however, the HVR plays a more significant role in anchoring the protein in the membrane. This mechanism of orientation-dependent function is called the “balance model”.^{20,67} Interaction of H-Ras with effectors, PI3K and Ras scaffolding protein galectin are sensitive to H-Ras reorientation at the membrane. For example, while PI3-K and galectin interact better with the R169A/K170A mutant of H-RasG12V, their interaction is impaired with the R128A/R135A mutant of H-RasG12V.^{20,67} This is because the former mutant stabilizes and a more active orientation of Ras on the membrane for effector access. Experiments with model membranes further substantiate the role of the plasma membrane in constraining the conformations of the catalytic domains of N-Ras and K-Ras.^{68,69} Interestingly, in H-Ras the differences in the orientation of the catalytic domain on the membrane result in changes in the flexibility of its palmitate groups, which dictate the affinity of H-Ras for lipid rafts.⁷⁰

K-Ras orientation on the plasma membrane, on the other hand, is regulated in a different manner from H-Ras. In K-Ras, the active GTP-bound conformation is stabilized by HVR binding to the membrane. Although the role of a similar to H-Ras Switch III region has not yet been investigated in Ras isoforms, including K-Ras, its existence cannot be overlooked.⁶⁶ An alternative idea is that, in K-Ras, there are only slight differences in orientations of the GTP- and GDP-bound states on the membrane.^{20,67} This is due to the fact that the catalytic domain of K-Ras-GTP is much more flexible than that of H-Ras-GTP. This flexibility causes an almost random orientation of K-Ras-GTP on the membrane.⁶⁸ The structural differences in Ras imposed by the membrane environment can lead to alterations in the Ras oligomerization status, binding of GEFs, GAPs and effectors, ultimately resulting in different signaling outputs.

Ras dimerization, oligomerization and clustering

The possibility of Ras existing in different oligomerization states at the plasma membrane has recently emerged as a new way of regulating Ras function. Ras dimers are observed by X-Ray crystallography.⁷¹ Inouye et al. used a protein-fragmentation complementation assay to demonstrate that Ras-GTP dimers in HEK293 cells mediate Raf-1 activation⁷² (Fig. 5). H-Ras dimerization in lipid bilayers has been demonstrated by time-resolved fluorescence spectroscopy and microscopy, however no higher order oligomers of H-Ras were observed.⁷³ Variations in membrane composition had no effect on H-Ras dimerization. N-Ras also dimerizes in the GDP-bound inactive state in POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) bilayers.⁷¹ Dimerization of K-Ras4B has been studied by NMR⁷⁴ and by photoactivated localization microscopy.⁷⁵ These studies identified contributions from the catalytic and HVR domains in dimer formation. The contribution of HVR to K-Ras4B dimerization is consistent with the involvement of the farnesyl group of HVR

peptides in cooperative dimerization and binding to phospholipid bilayers.¹⁸ Ras proteins bind the POPC bilayers as dimers or oligomers, in an orientation perpendicular to the membrane surface. Although simulations point to a dimer, it has been proposed that oligomerization due to diffusion limited partner switching of dimers cannot be excluded.⁷⁶ K-Ras4B forms nanoclusters that are membrane-anchored through a farnesylated HVR. Anchoring stabilizes the Ras dimers, increasing the Ras effective local concentration and favorably orienting the catalytic domain.⁷⁷ The formation of large, activated Ras signaling complexes has also been observed by Murakoshi H et al. using single molecule FRET in KB cells.⁷⁸ These large, activated signaling complexes recruit GAP molecules to the membrane, which then catalyze GTP hydrolysis and cause dissociation of the complexes. These results suggest that GDP-bound Ras is less prone to oligomerization.⁷⁸ However, several publications propose the formation of Ras-GTP as well as Ras-GDP nanoclusters, which are spatially distinct for each isoform.⁵⁸ Methodological differences used by different labs may have led to alternate conclusions regarding the existence of Ras-GDP oligomers. Murakoshi et al. use FRET to study the formation of complexes whereas the Hancock group employs EM on immunogold labeled plasma membrane sheets and mathematical models toward the same aim. Electron microscopy and single fluorophore tracking indicate that Ras proteins are arrayed in nanoclusters on the inner plasma membrane.^{39,78-80} These nanoclusters are formed through the assembly of the lipid-anchored Ras proteins into transient dynamic structures. They contain 6–8 Ras proteins with a diameter of 12–22 nm, and are highly dynamic structures since each cluster turns over on average every 0.4 s. Each Ras isoform occupies spatially distinct nanoclusters and about 40% of any Ras isoform is arrayed in these nanoclusters.⁷⁹

Recent dramatic advances in single molecule imaging techniques are giving us a deeper insight into interactions of Raf with Ras clusters.^{81,82} Photoactivated localization microscopy with bimolecular fluorescence complementation (BiFC-PALM) allowing detection of protein-protein interactions with 18 nm precision was used to identify K-Ras/Raf clusters of 30 nm in diameter and to observe the nanoscale clustering and diffusion of these complexes from the cell membrane.⁸¹ Similarly, quantitative PALM imaging has allowed visualization of Raf multimers.⁸² This work suggests that the CAAX motif of Ras drives Raf molecules in clusters. These Raf clusters are similar in size to Ras clusters.

Despite these advances, the exact mechanism of Ras dimerization and clustering is not fully understood. Computer simulations suggest that lipid anchors in the HVR of H-Ras and N-Ras are capable of forming dimers and nanoclusters.^{71,73,83} In-cell studies of the K-Ras lipid anchor using Homo-FRET and electron microscopy also demonstrate that the lipid anchor is sufficient for dimerization and clustering on the membrane.⁸⁴ How can the HVR, - that is not involved in nucleotide binding or hydrolysis - affect dimer and nanocluster formation in a nucleotide dependent manner? It is possible that the catalytic domain of Ras has a way to communicate with the HVR. Intriguingly, a conserved water-mediated hydrogen-bonding network linking

Figure 5. Raf activation by Ras. Raf consists of a catalytic domain which has 2 lobes (C- and N-lobes), a cysteine-rich domain (CRD), and Ras-binding domain (RBD). The CRD connects to the catalytic domain via a flexible hinge region. In resting cells (bottom of figure), Raf monomers are autoinhibited by their CRD domain which interacts with the catalytic domain. Interaction with membrane-anchored GTP-bound Ras relieves the autoinhibition. RBD at the N-terminal of Raf interacts with Ras, swinging the CRD away, and exposing the Raf dimerization surface. Raf dimerizes by a side-to-side interaction, creating an asymmetric active dimer. Raf's CRD interacts with the membrane, stabilizing the complex. This architecture effectively creates a Raf-linked Ras dimer. However, in addition, it is currently believed that Ras also forms direct dimers. While dimerization and clustering provide co-localization and leads to cooperativity, here our major tenet is that they drive specificity and that this is the major reason that they evolved. Clustering can help thwart pathway redundancy of Ras isoforms. The protein databank (PDB) codes for the structures shown here are: 1UWH (Raf dimer), 2L05 (RBD), and 3GFT (Ras).

the nucleotide sensor residues R161 and R164 on helix 5 to the active site was discovered in Ras-GTP but not in Ras-GDP.⁸⁵ This hydrogen-bonding network might relay the conformational changes induced by nucleotide binding to helix 5 and subsequently to the directly linked HVR. In this way, the nucleotide-induced conformational changes can affect membrane binding, dimerization and clustering of Ras. Alternatively, nanoclustering of Ras can be mediated by nucleotide dependent interactions

with scaffold proteins, such as galectin.⁸⁶ Finally, it is also possible that the catalytic domain of Ras is directly involved in nucleotide-dependent dimer formation and clustering.

Ras nanoclusters function to efficiently recruit effectors for signaling pathways. The propensity to form nanoclusters is a direct function of the concentration of EGF upon stimulation. As confirmed by single particle tracking, Raf is recruited from the cytosol to Ras clusters.^{78,80} The association of MEK with

both Raf and ERK ensures the recruitment of the entire MAP kinase cascade to Ras nanoclusters.⁸⁷⁻⁸⁹ Studies confirm the significance of Ras nanoclusters in MAPK activation; blocking nanocluster formation disrupts Ras-dependent MAPK signaling.^{45,90-92} In line with this, *in silico* abrogation of Ras nanoclusters leads to failure of Raf recruitment.⁹³ Importantly, the signal output decreases to 3% of its maximal value when Ras proteins function as individual molecules rather than as nanoclusters.^{39,93} A decrease in Ras clustering diminishes the target area of the plasma membrane available for Raf-MEK-ERK recruitment, which in turn, decreases the probability of successful recruitment and activation. Conversely, K-Ras nanoclustering can be increased by membrane depolarization that causes reorganization of phosphatidylserine and PIP2 and amplifies MAPK signaling.⁹⁴ Collectively, these data confirm that cell signaling relies on the formation of functional Ras nanoclusters at the plasma membrane, similar to other membrane-anchored signaling platforms, and suggest that cells can adjust their response to the environmental stimuli by regulating the extent of Ras clustering.⁹⁵ Interestingly, Raf dimerization enhanced by Raf inhibitors increases Ras clustering, suggesting a possible role of effectors in stabilizing Ras oligomers on the plasma membrane.⁹⁶ The emerging model of the role of Ras clustering in activation of Raf is shown in **Figure 6**. Raf is classically activated by the autoinhibition-release mechanism when binding of Ras-GTP to the Ras binding domain (RBD) of Raf allows the cysteine rich domain (CRD) of Raf to move away from the catalytic domain. This exposes the Raf dimerization surface and leads to formation of Raf dimers by a side-to-side interaction. Raf's CRD interacts with the membrane and stabilizes the complex. This creates a Raf-linked Ras dimer.

Thus, the initial Ras dimers and nanoclusters provide co-localization and cooperativity for interactions with effectors. The isoform specific HVRs may participate in Ras clustering and interactions with effectors,⁹⁷ ultimately reducing redundancy in Ras signaling.

The Ras nanocluster system has an intrinsic capability to maintain its downstream functions despite internal and external perturbations. Activation of the MAP kinase module exclusively at the plasma membrane provides resistance against pharmacological inhibitors.⁹⁸ There are 2 possible explanations for this.⁹⁵ The existing nanocluster may act as a digital switch that is completely activated in response to a low Raf input or it could be a high gain amplifier of Raf activation. Experimental studies and molecular simulations suggest that Ras nanoclusters function as high-gain amplifiers for MEK activity.⁹⁵ However, the EGF input is capable of providing a graded ERK output based on the number of activated nanoswitches. A graphical representation of signaling through Ras oligomers is shown in **Figure 6**.

Figure 6. Ras proteins exist in different oligomeric states on certain membrane microdomains. These states function as signaling platforms that activate distinct effectors to produce different signaling outputs. Examples of membrane microdomains (A and B) are indicated in orange and green colored lipids respectively. Binding of growth factors (purple boxes) to their receptors (dimer shown as long purple rectangles) initiates recruitment of GRB2 (dark blue circle) and GEFs (light blue triangle). Different microdomains can allow different oligomeric states of Ras (in the figure microdomain B allows clustering of Ras whereas microdomain A does not). These oligomerization states can lead to different downstream signals.

One proposed function of Ras oligomers is to facilitate homo- and heterodimerization of Raf kinases, which are required for Raf activation.⁹⁹ Thus, Ras oligomers can be expected to increase Raf activation. Potential modulation of SOS activity by Ras dimers can also happen, depending on the 2-dimensional dimerization affinity on membrane surfaces. This effect could contribute to the recently observed mild Ras density-dependence of SOS activity.¹⁰⁰ Thus, interactions of Ras GEFs, GAPs, and other effectors with Ras oligomers are possible but have not yet been well investigated.

Summary

In conclusion, the diverse plasma membrane microdomains appear to play a decisive role in regulating Ras signaling. Membrane association limits the accessibility of Ras to effectors and allosterically alters the conformation of Ras and its oligomerization state. In this way, diversity of Ras signaling responses can be achieved and optimized across Ras isoforms. It is quite possible that the number and distribution of membrane microdomains are affected through cellular mechanisms, which include endosomal trafficking and lipid sorting. Importantly, membrane organization can be controlled extracellularly, for example, by growth factor binding to its receptor or by pharmacological agents. Understanding how regulation of Ras signaling happens at the membrane will offer new opportunities for anti-cancer drug design.

Open Questions

Although it is becoming clear that the plasma membrane regulates Ras signaling in several ways, understanding of the

structural mechanisms underlying this regulation is currently lacking. The structure and oligomerization state of membrane-bound Ras are expected to differ significantly from those without the membrane. New data suggests that the C-terminal HVR can influence the catalytic domain. This suggests that post-translational modifications within HVR are likely to play a role in modulating the structure and dynamics of the catalytic domain. Which regions of Ras are affected by C-terminal carboxymethylation, the farnesyl and palmitoyl groups and how these regions participate in binding the membrane, conformational rearrangements, oligomerization, and interaction with Ras regulators and effectors are the questions that remain to be answered. Solving the structure of Ras bearing relevant post-translational modifications in its membrane-bound state will be a significant step forward in elucidation of the membrane binding mechanism. This structure will allow further research into the mechanisms of Ras oligomerization and regulator and effector access. Finally, the detailed mechanistic insight into the regulation of Ras function by the plasma membrane will lead to development of effective drugs to abate deleterious signaling of Ras in cancer.

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