Compartmentalization of Ras proteins

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Summary

The Ras GTPases operate as molecular switches that link extracellular stimuli with a diverse range of biological outcomes. Although many studies have concentrated on the protein-protein interactions within the complex signaling cascades regulated by Ras, it is becoming clear that the spatial orientation of different Ras isoforms within the plasma membrane is also critical for their function. H-Ras, N-Ras and K-Ras use different membrane anchors to attach to the plasma membrane. Recently it has been shown that these anchors also act as trafficking signals that direct palmitoylated H-Ras and N-Ras through the exocytic pathway to the cell surface but divert polybasic K-Ras around the Golgi to the plasma membrane via an as yet-unidentified-route. Once at the plasma membrane, H-Ras and K-Ras operate in different microdomains. K-Ras is localized predominantly to the disordered plasma membrane, whereas H-Ras exists in a GTP-regulated equilibrium between disordered plasma membrane and cholesterol-rich lipid rafts. These observations provide a likely explanation for the increasing number of biological differences being identified between the otherwise highly homologous Ras isoforms and raise interesting questions about the role membrane microlocalization plays in determining the interactions of Ras with its effectors and exchange factors.

Key words: Ras, Lipid rafts, Caveolae, Trafficking, Plasma membrane, Localization

Introduction

An important question for the Ras biologist is whether the ubiquitously expressed, almost identical Ras isoforms have distinct functions. Because of the high degree of sequence homology (>90%) shared by the three main expressed isoforms (H-Ras, N-Ras and K-Ras4B)*, it has long been assumed that they are functionally redundant. This view is reinforced by observations that all Ras isoforms share common sets of downstream effectors and upstream guanine-nucleotide-exchange factors (GEFs). We have only recently begun to appreciate that differences in the cellular requirements for these Ras isoforms do exist and that signaling through each Ras isoform can result in distinct biochemical outcomes.

Early studies of the frequency of Ras mutation in human cancer revealed that specific Ras isoforms are often preferentially activated in particular malignancies: for example, K-Ras in pancreatic and colonic carcinomas and N-Ras in acute leukaemia (Bos, 1989). More compelling observations came from transgenic experiments showing that only K-Ras is essential for normal mouse development. H-Ras or N-Ras knockouts have no observable side effects (Umanoff et al., 1995; Koera et al., 1997), whereas a K-Ras knockout is embryonic lethal at 12-14 days of gestation: embryos show late-onset growth and hemopoietic defects (Johnson et al., 1997; Koera et al., 1997). Several recent studies have also identified distinct signal outputs from different Ras isoforms. For example, in BHK and COS cells, H-Ras and K-Ras display distinct effector activation profiles: K-Ras is a more potent activator of Raf-1 than is H-Ras, and the converse is true for activation of phosphoinositide 3-kinase (PI3K; Yan et al., 1998). In addition, activated alleles of *H-Ras*, *N-Ras* and *K-Ras* transform NIH3T3 and Rat1 cells with varying potencies (Voice et al., 1999), and N-Ras appears to have a unique role in suppressing apoptosis in cultured primary fibroblasts (Wolfman and Wolfman, 2000).

How can these biological differences be explained? H-Ras, N-Ras and K-Ras have identical sequences covering the effector, exchange factor and guanine-nucleotide-binding domains. The only region of the Ras isoforms that exhibits significant sequence divergence is the final 24 residues of the protein, the hypervariable region (HVR), which exhibits approximately 10-15% conservation compared with >90% identity over the N-terminal 165 residues (see Fig. 1). Ten years ago, the HVR was shown to contain two signal sequences that cooperate in targeting Ras to the plasma membrane. The first signal sequence is the CAAX box (C=cysteine, A=aliphatic amino acid, X=serine or methionine) present at the extreme C-terminus (Willumsen et al., 1984; Hancock et al., 1989). The CAAX box is sequentially post-translationally modified to render it more hydrophobic: the cysteine is farnesylated, the AAX sequence is removed by proteolysis, and then the now-C-terminal cysteine is carboxylmethylated. The second signal sequence consists of a polybasic stretch of six lysine residues (175-180) in K-Ras, or palmitoylation of cysteine 181 in N-Ras, or of cysteines 181 and 184 in H-Ras (Hancock et al., 1990; Hancock et al., 1991). We discuss the role of the remainder of the HVR, the so-called linker domain (see Fig. 1), below. Correct subcellular localization is critical for Ras function, since effectors such as Raf, RalGDS and PI3K must be recruited specifically to the plasma membrane for activation (for reviews, see Morrison and Cutler, 1997; Campbell et al., 1998; Vojtek and Der, 1998). Thus, interfering with Ras plasma membrane localization, by mutating the

*There are two alternatively spliced forms of K-Ras: K-Ras4A and K-Ras4B. It is the 4B isoform that is ubiquitously expressed and for the purpose of clarity we refer to K-Ras4B as K-Ras for the rest of this commentary.

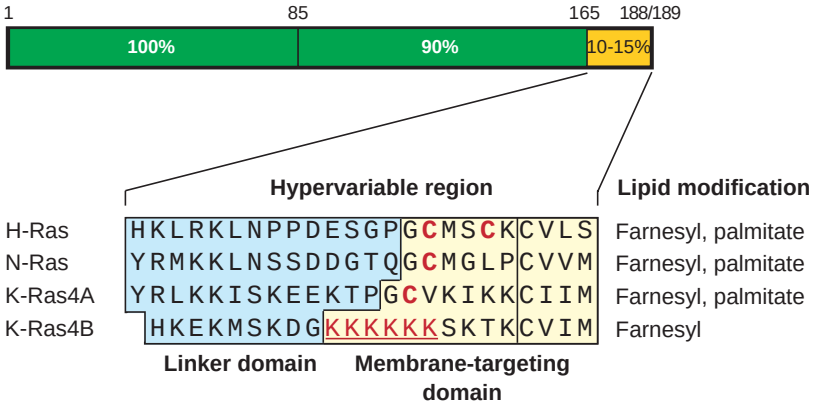

Fig. 1. Ras hypervariable regions. The diagram shows the degree of sequence conservation between isoforms along the Ras protein; all of the effector, exchange factor and nucleotide-binding sites are found in the N-terminal conserved domains. The hypervariable region (HVR) is the only region of significant divergence between the Ras proteins and exhibits <10-15% sequence identity between any two isoforms. The HVR can be divided into two domains: the membrane-targeting domain and the linker domain. The membrane-targeting domain comprises the C-terminal CAAX motif, common to all Ras proteins, plus second signal sequences: cysteine palmitoylation sites (shown in red) in H-Ras, N-Ras and K-Ras4A or a polybasic domain in K-Ras4B (shown in red and underlined). Note that in the text K-Ras refers to K-Ras4B.

CAAX box, or the second signal, or through the use of farnesyltransferase inhibitors, abrogates Ras signaling (Willumsen et al., 1984; Gutierrez et al., 1989; Hancock et al., 1989; Cadwallader et al., 1994; Jackson et al., 1994; Gibbs et al., 1997).

An important consequence of HVR divergence is that the plasma membrane anchors of H-Ras, N-Ras and K-Ras are all different, albeit only in palmitoylation stoichiometry in the case of N-Ras and H-Ras. Biological differences between the Ras isoforms could therefore be due in part to differences in plasma membrane microlocalization. Here, we discuss recent work that has provided substantial support for this hypothesis. In particular, we focus on how Ras proteins are trafficked to the plasma membrane via different routes and how differential localization to distinct plasma membrane microdomains influences Ras function.

The role of the endoplasmic reticulum in Ras trafficking

The CAAX motif of newly synthesized Ras is farnesylated in the cytosol by farnesyl protein transferase, an enzyme cloned and characterized a decade ago. More recently the other mammalian CAAX-processing enzymes have been cloned on the basis of sequence homology shared with their previously identified yeast homologs. AAX proteolysis of farnesylated CAAX peptides is carried out by Rce1 (homologous to Afc1p/Ste24p and Rce1p in yeast; Boyartchuk et al., 1997; Schmidt et al., 1998; Tam et al., 1998; Otto et al., 1999). α-Carboxylmethylation of farnesylated cysteine residues is carried out by Icmt (homologous to Ste14p in yeast; Marr et al., 1990; Hrycyna et al., 1991; Dai et al., 1998). Interestingly, both Rce1 and Icmt are ER-resident proteins; thus the two final steps of post-translational processing of the farnesylated CAAX motif must occur on the cytosolic surface of ER membranes. Rce1 knockouts and Icmt knockouts are lethal late in mouse embryonic development (at 15.5 days or 10.5-12.5 days respectively; Kim et al., 1999; Bergo et al., 2001). No obvious cause of death was observed in Rce1-null embryos, but Icmt-null embryos are much smaller than wild type. The more severe phenotype might be due to the additional role of Icmt in the post-translational processing of Rab proteins. Ras proteins in Rce1-null fibroblasts are partially mislocalized to the cytosol, which is consistent with a role for Rce1 in Ras trafficking.

Protein palmitoyltransferase activity has been identified in multiple cellular locations, depending on the protein substrate used in the assay (Gutierrez and Magee, 1991; Berthiaume and Resh, 1995; Dunphy et al., 1996; Das et al., 1997). Recent data, however, suggest that the enzyme that palmitoylates newly synthesized, CAAX-processed H-Ras may be localized to the ER (Apolloni et al., 2000), although biochemical purification, and hence characterization, of this enzyme is proving extremely difficult (Liu et al., 1996). Indeed, under certain conditions in vitro, non-enzymatic palmitoylation can occur, prompting debate as to whether there is actually a requirement for enzymatic catalysis (Duncan and Gilman, 1996; Bano et al, 1998). Similarly, a Ras palmitoyltransferase has not yet been isolated from yeast, although genetic screens have identified two proteins, Erf2p and Shr5p, that are involved in the palmitoylation process (Jung et al., 1995; Bartels et al., 1999). Deletion of Erf2p or Shr5p impairs Ras palmitoylation and causes partial mislocalization of Ras to the vacuole. Erf2p, which has numerous uncharacterized mammalian homologs, is also localized to the ER (Bartels et al., 1999). Thus although total cellular palmitoyltransferase activity is unaffected in Erf2p- or Shr5p-null cells (Jung et al., 1995; Bartels et al., 1999), a smaller Ras-specific ER pool of palmitoyltransferase might nevertheless be compromised.

A final point about Ras palmitoylation is that it is reversible – palmitate on N-Ras and H-Ras turns over with a half-life of 20 min and 60 min, respectively (Magee et al., 1987 and Hancock, unpublished data) – in contrast to farnesylation, which is irreversible. Experiments with N-Ras have shown that, in the absence of secretory transport, plasma membrane localization is maintained, which suggests that there may be plasma membrane palmitoyltransferase activity that can efficiently re-palmitoylate de-acylated N-Ras (Schroeder et al., 1997).

Trafficking from the ER to the plasma membrane

An important question raised by the localization of CAAX-processing enzymes to the ER is how Ras proteins access the plasma membrane from the ER after post-translational modification. This question has now been solved for H-Ras and N-Ras. Two recent papers showed that N-Ras and H-Ras are distributed throughout the classical secretory pathway (Choy et al., 1999; Apolloni et al., 2000). Brefeldin A (BFA) causes ER accumulation of these proteins, and a 15°C temperature

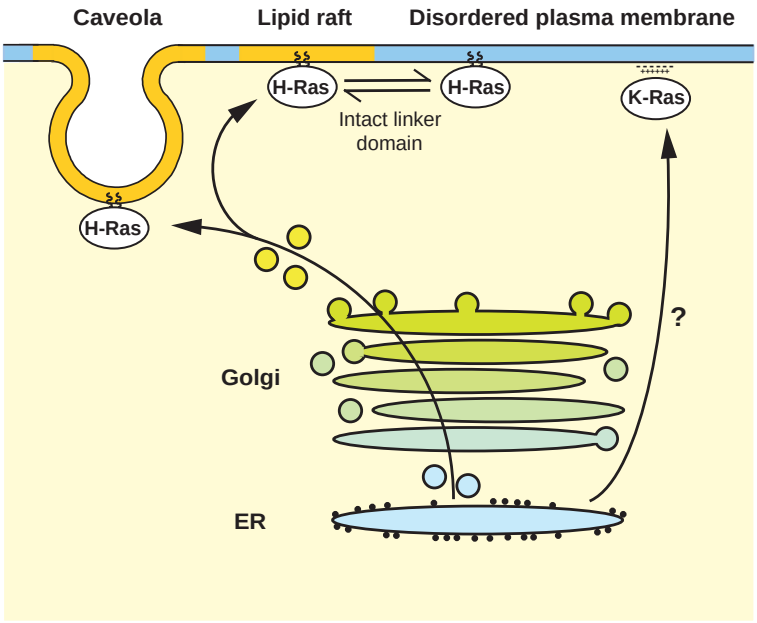

Fig. 2. Trafficking and plasma membrane localization of H-Ras and K-Ras. Following CAAX modification of both isoforms in the ER K-Ras takes an uncharacterized Golgi-independent route to the disordered plasma membrane. By contrast, H-Ras traffics via the classical secretory pathway through the Golgi to caveolae and lipid rafts. H-Ras then exists in a dynamic equilibrium between lipid rafts and the disordered plasma membrane. The HVR linker domain has a critical role in this equilibrium. Activation of H-Ras on GTP-loading shifts the equilibrium in favor of residence in the disordered plasma membrane. N-Ras (not shown) traffics to the plasma membrane, in common with H-Ras, but its plasma membrane microlocalization has not yet been established.

block causes H-Ras to accumulate in the intermediate compartment between the ER and Golgi. These data convincingly demonstrate that palmitoylated Ras proteins access the plasma membrane by vesicular trafficking through the classical exocytic pathway. In contrast to conventional cargo carried in vesicle lumens, however, H-Ras and N-Ras must be transported on the cytoplasmic surface of vesicles.

In contrast to H-Ras and N-Ras, polybasic K-Ras is largely excluded from the Golgi and is transported to the plasma membrane via a route that is insensitive to temperature blocks or BFA. Palmitoylation or a polybasic domain is essential for efficient transport, because non-palmitoylated H-Ras and N-Ras mutants, and K-Ras mutants that lack the polybasic sequence, accumulate in the ER. These and other data strongly suggest that the second targeting signal in the HVR determines the route taken by the different Ras proteins out of the ER to the plasma membrane. The polybasic domain might therefore function as a novel ER-exit signal to divert the K-Ras through an uncharacterized, Golgi-independent pathway to the plasma membrane (see Fig. 2; Choy et al., 1999; Apolloni et al., 2000). Importantly, these studies also reveal the minimal nature of the trafficking signals: chimeras containing the H-Ras, N-Ras and K-Ras plasma membrane targeting sequences fused to green fluorescent protein (GFP) traffic exactly like the cognate full-length Ras proteins.

The nature of the K-Ras trafficking pathway remains elusive and is under intense study. Some intriguing observations have been made, although their significance remains to be established. Treatment of NIH-3T3 cells with the microtubule stabilizer paclitaxel results in accumulation of K-Ras but not H-Ras in an uncharacterized but apparently vesicular compartment (Thissen et al., 1997). In addition K-Ras, but no other Ras or Ras-related protein, binds to taxol-stabilized microtubules in vitro. The polybasic domain of K-Ras mediates this microtubule binding (Chen et al., 2000), but it is currently unclear how microtubules are involved in intracellular trafficking of K-Ras, especially given that electron microscopic and immunofluorescent analysis of taxol-treated cells found no close association of K-Ras with microtubules, but instead showed accumulation of K-Ras in endosomes (Apolloni et al., 2000). The high-affinity electrostatic interactions between K-Ras and the plasma membrane (see below) and the close proximity of the ER to the plasma membrane in most cells have led other investigators to propose that K-Ras reaches the plasma membrane through simple passive diffusion from the ER rather than any specific protein-based transport mechanism (Roy et al., 2000). In this scenario, the polybasic domain does not operate as a formal ER exit signal but merely provides the necessary charge to drive K-ras diffusion to the negatively charged plasma membrane.

Here, we have focused predominantly on Ras processing and trafficking, but note that essentially the same C-terminal signal sequences are present in Rho family small GTPases. All Rho proteins have a C-terminal CAAX motif that is geranylgeranylated*, and then AAX proteolysed and methylesterified by Rce1 and Icmt. RhoB and TC10 are also palmitoylated, Rac1 and RhoA contain strongly polybasic sequences, and Cdc42 and Rac2 are weakly polybasic. Although these proteins contain targeting motifs similar to those of H-Ras, N-Ras or K-Ras, they nevertheless localize to a diverse range of subcellular locations – for example, RhoB localizes to the plasma membrane and Golgi, RhoA localizes to the cytosol, and Cdc42 localizes to the ER and Golgi (Michaelson et al., 2001). Thus, the trafficking routes to the plasma membrane followed by palmitoylated or polybasic Ras proteins represent default pathways dictated by the minimal C-terminal sequences of Ras-related CAAX proteins, but protein-protein interactions occurring elsewhere in the protein must be able to modulate final intracellular localization. For example, the localization of the non-palmitoylated Rho family members, particularly RhoA, is influenced by the cytosolic chaperone RhoGDI (Michaelson et al., 2001). Additional support for this hypothesis comes from observations that Rap1a localizes to the Golgi (Beranger et al., 1991), whereas the C-terminal 16 residues of Rap1a target GFP

*The CAAX motif of RhoB can be farnesylated or geranylgeranylated.

efficiently to the plasma membrane (Roy et al., 2000). In addition, Cdc42 is now known to bind to the γ-subunit of coatomer on COP1-coated vesicles, which could stabilize its interaction with the ER and Golgi compartments rather than the plasma membrane (Wu et al., 2000).

Plasma membrane microdomains and Ras function

One possible consequence of the alternative trafficking pathways used by Ras proteins is that their ultimate microlocalizations within the plasma membrane might also differ. This hypothesis is conceptually more straightforward now that the simple fluid mosaic model of the plasma membrane has been superceded by a more complex structure that includes microdomains that have distinct protein and lipid compositions. The best-studied microdomain is the lipid raft, which comprises sphingolipids and glycosphingolipids packed together with cholesterol (for reviews, see Simons and Ikonen, 1997; Hooper, 1999; Kurzchalia and Parton, 1999). A key biophysical property of lipid rafts is their liquid-ordered structure, which promotes phase separation from the more loosely packed, disordered glycerophospholipids of the bulk plasma membrane. Peripheral membrane proteins anchored by long, saturated acyl chains that readily pack into the liquid domain structure are able to associate with lipid rafts: for example, glycophosphatidylinositol (GPI)-anchored proteins and certain Src family kinases that reside in the outer and inner leaflets of the plasma membrane, respectively (for reviews, see Simons and Ikonen, 1997; Hooper, 1999; Kurzchalia and Parton, 1999; Simons and Toomre, 2000). By contrast, proteins anchored solely by farnesyl or geranylgeranyl groups are excluded from lipid rafts, because the branched, unsaturated prenoid chain cannot incorporate into a liquid-ordered structure (Melkonian et al., 1999; Moffett et al., 2000). This division of the plasma membrane into raft and non-raft compartments clearly presents a framework for differential lateral segregation of the Ras isoforms. However, whereas K-Ras, anchored by a combination of prenylation and electrostatic interaction with the plasma membrane, would not be expected to have any affinity for raft domains, the microdomain localizations of H-Ras and N-Ras, which are anchored by a combination of palmitoylation and prenylation, are much harder to predict.

The first insights into Ras microlocalization really came with observations that disrupting lipid raft structure, by chemically depleting plasma membrane cholesterol, selectively abrogates H-Ras- but not K-Ras-mediated activation of Raf-1 (Roy et al., 1999). Expression of a dominant-negative caveolin mutant, caveolin-DGV, which reduces cell surface cholesterol by interfering with intracellular cholesterol transport (Pol et al., 2001), also blocks H-Ras but not K-Ras signaling pathways (Roy et al., 1999). The simplest interpretation of these results is that H-Ras and K-Ras occupy distinct microdomains of the plasma membrane and that the functional integrity of the H-Ras domain depends on cholesterol.

Another study has now examined the plasma membrane distributions of H-Ras and K-Ras by using a combination of biochemistry and electron and light microscopy (Prior et al., 2001). This showed that GFP targeted to the plasma membrane by the polybasic domain and CAAX motif of K-Ras associates neither with caveolae, a distinct, morphologically identifiable subtype of lipid raft, nor with non-caveolar lipid rafts*. Similarly, full-length K-Ras in the GDP-bound ground state, or activated GTP-bound state, has an identical distribution to that of GFP-targeted by the K-Ras motifs: that is, it resides in the disordered, non-raft plasma membrane (Prior et al., 2001). By contrast, the palmitoylation sequence and CAAX motif of H-Ras targets GFP very efficiently to caveloae and non-caveolar lipid rafts. Intriguingly, although the minimal targeting motif of H-Ras sequesters all of the GFP to lipid rafts, GDP-bound full-length H-Ras is distributed approximately equally between lipid rafts and bulk plasma membrane, and constitutively activated GTP-bound H-Ras is localized predominantly to non-raft bulk plasma membrane. Mutational analysis of H-Ras reveals that the HVR linker domain has a critical role in liberating H-Ras from lipid rafts, since deletion of these sequences completely confines activated H-Ras to lipid rafts (Prior et al., 2001).

The model that best fits the data is shown in Fig. 2. H-Ras is initially trafficked to caveolae and lipid rafts, but then exists in a dynamic equilibrium between lipid rafts and the disordered plasma membrane. GTP loading shifts this equilibrium in favor of residence in the bulk plasma membrane. The precise mechanism regulating H-Ras lateral segregation is unknown, but it does not require interactions with known Ras effectors, and therefore a conformational change in H-Ras, induced by GTP loading, is probably transmitted through to the membrane anchor to reduce raft affinity. Such a mechanism would explain the essential role of the HVR linker domain in the process and in a sense is consistent with recent work showing that prenylation influences the structure of the N-terminal GTPase domains of H-Ras (Williams et al., 2000); thus a ‘reverse’ influence of GTP-induced conformational changes on the C-terminus seems quite plausible. Reversible raft association is not without precedent: the EGF receptor migrates from lipid rafts to clathrin-coated pits upon activation (Mineo et al., 1999), and fluorescent studies of raft dynamics have shown that lipid probes continually exchange between liquid-ordered and disordered membrane domains (Schutz et al., 2000).

The differential localization of H-Ras and K-Ras clearly has important potential consequences for effector interactions and activation of downstream pathways. This is well illustrated by some preliminary data comparing Raf-1 activation by activated H-Ras with that by an H-Ras HVR mutant confined to lipid rafts. Raf-1 can be recruited equally well to disordered plasma membrane and lipid rafts, but Raf activation is much less efficient in raft domains than in bulk plasma membrane (Prior et al., 2001). In addition, Booden et al. have recently shown that mutations in the H-Ras HVR can alter the efficiency with which H-Ras activates its various effectors (Booden et al., 2000). Although this latter study did not evaluate whether these mutations change the plasma membrane microlocalization of H-Ras, it is tempting to speculate that this is the underlying mechanism.

A large body of evidence suggests that the polybasic domain of K-Ras promotes plasma membrane binding by an electrostatic interaction with acidic phospholipids. For example, mutational analyses of the polybasic domain have shown that if the overall net charge and amphiphilic character

*Lipid rafts can exist in the plane of the plasma membrane (as non-caveolar lipid rafts) or be aggregated by the structural protein caveolin into invaginations of the plasma membrane called caveolae (for a detailed review, see Simons and Toomre, 2000).

is retained, the protein will associate with the plasma membrane (Hancock et al., 1990; Hancock et al., 1991; Jackson et al., 1994; Roy et al., 2000). This electrostatic mechanism is supported by biophysical measurements of the interactions between charged prenylated peptides and artificial membranes (Murray et al., 1999; Arbuzova et al., 2000; Roy et al., 2000). Furthermore, measurements of K-Ras lateral mobility in the plasma membrane show that K-Ras movement is relatively unrestricted (Niv et al., 1999). The plasma membrane consists of 20-30 mol% acidic lipids. A recent analysis of protein-lipid electrostatic interactions suggested that polybasic domains could attract acidic lipids and concentrate them in localized areas, which could in turn facilitate the recruitment or activation of other proteins (Murray et al., 1999). Does the presence of K-Ras in the plasma membrane therefore actually create an acidic microdomain in which it then operates? Since acidic phospholipids include phosphatidylserine, which is required for Raf activation, and phosphatidylinositols, the substrates for another Ras effector, PI3K, such K-Ras-defined microdomains within the bulk plasma membrane could turn out to be important 'mini-signaling centers'.

Conclusions and perspectives

Recent insights into the mechanisms of Ras trafficking and plasma membrane localization have answered some important questions but inevitably posed new ones. We now understand how H-Ras and N-Ras traffic to the plasma membrane, but the discovery of the novel Golgi-independent route taken by K-Ras presents a new challenge. K-Ras is the most frequently mutated Ras isoform in human cancers; it is also less susceptible than other Ras isoforms to drugs that target farnesyltransferase. Understanding the mechanisms of K-Ras trafficking could identify new targets for anticancer therapies.

The dynamic association of H-Ras with caveolae and lipid rafts is particularly intriguing. In the light of these discoveries, it will be interesting to investigate whether protein sequences adjacent to lipid anchors and/or protein conformation regulate the association of other acylated proteins with lipid rafts. Important questions regarding the mode of association of H-Ras with lipid rafts and caveolae also still remain. Precursors to these domains are known to form within the TGN, and it will be interesting to discover whether H-Ras associates with lipid rafts at this point.

K-Ras is targeted to non-raft areas of the plasma membrane, but speculation that the polybasic domain can recruit acidic phospholipids and generate de novo signaling centers, if validated, could introduce a new level of complexity to the mechanisms of K-Ras signaling. We must also examine how the distinct microlocalizations of H-Ras and K-Ras and N-Ras (once established) influence their access to exchange factors and their ability to interact with defined effector proteins. The recent studies outlined above represent the starting point for such work. Finally, although there still many unanswered questions, it is quite clear that differential lateral segregation of the Ras isoforms in the plasma membrane underlies the biological differences between these proteins. This highlights the importance in future of considering the cell biology of H-Ras, N-Ras and K-Ras individually rather than collectively.

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