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Topical Review

Mechanisms of Ras Protein Targeting in Mammalian Cells

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Abstract. Many physiological and oncogenic activities of the 'classical' Ras proteins (H-Ras, N-Ras and K-Ras4A and -4B) require their correct localization to the plasma membrane. Nascent Ras proteins, however, initially associate with endomembranes (the ER and in some cases the Golgi) to complete the processing of their farnesylated carboxyl-termini before they are delivered to the plasma membrane. Recent work has revealed the outlines of the intracellular pathways by which Ras proteins reach their ultimate plasma membrane destination and has indicated that these pathways differ for different Ras species. Other studies have demonstrated that mature Ras proteins can transfer between the plasma membrane and intracellular membranes, and that Ras proteins may in some cases signal from intracellular compartments. This review will describe recent progress and still-unresolved questions in these areas.

Key words: Protein isoprenylation — Protein palmitoylation — Protein targeting — Signal transduction — Oncogenes — Membrane traffic

Introduction

The 'classical' Ras proteins (H-Ras, N-Ras and K-Ras4A and -4B) are monomeric guanine nucleotide-binding proteins that play key roles in cellular regulation and that are frequently mutated in human cancers [9]. Early studies of the function and localization of these proteins demonstrated that they all carry carboxy-terminal lipid modifications, through which they associate with cellular membranes and which are essential for their transforming activity [16, 35, 38, 44, 111]. Other studies revealed that the Ras proteins are predominantly associated with the plasma membrane and suggested that this localization is important for the transforming activity of oncogenically mutated Ras [33, 39, 45, 110]. While recent work has suggested that Ras proteins may also localize to and even signal from other cellular compartments, it is clear that plasma membrane targeting of these proteins is a key element in both their physiological and their oncogenic functions [15, 22, 24, 54, 66].

The observation that oncogenic mutants of Ras are transforming only when they are correctly targeted to the plasma membrane has led many researchers to explore the potential therapeutic utility of inhibiting this targeting process. To date most success in this regard has been achieved using farnesyltransferase inhibitors, which block the initial lipid modification that is critical for membrane binding. However, these inhibitors do not entirely block the prenylation of either N- or K-Ras [46, 108], and their biological activities appear to be due at least in part to their actions on other farnesylated proteins. To date, these agents have shown modest efficacy in treatment of human cancers, although they may show greater potential for use in combination therapies [27, 53, 79, 94]. Better understanding of the mechanisms of Ras protein targeting to the plasma membrane may lead to more specific therapies to block the normal subcellular targeting, and hence the transforming activity, of oncogenically mutated forms of these proteins.

This review will discuss recent progress in elucidating the mechanisms by which the different Ras proteins are localized, predominantly but not exclusively to the plasma membrane, in mammalian cells. Beyond advancing our understanding of the mechanisms of plasma membrane delivery of Ras proteins, recent research has shown that these proteins can transfer to and may even signal from other cellular compartments, and that they can associate selectively with particular microdomains within the plasma membrane itself. Understanding the functions of Ras

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Fig. 1. Carboxy-terminal sequences of the 'classical' Ras proteins and selected singly-prenylated members of the Ras protein superfamily. Sequences shown (truncated for R-Ras3) indicate the carboxy-terminal regions that diverge significantly among related members of the same family. Basic residues are indicated in boldface, prenylated cysteine residues are indicated in outline and known or potentially S-acylated cysteine residues (the latter found in positions similar to the palmitoylated cysteines of H- and N-Ras) are underlined.

proteins within the cell will require a sophisticated understanding of these properties, which are emerging as generally important determinants of the function of signal-transducing molecules at the plasma and other membranes.

Basic Properties of the 'Classical' Ras Proteins

The classical Ras proteins are encoded by three genes, H-, N- and K-Ras; the latter species is expressed in two isoforms, designated K-Ras4A and -4B. K-Ras is frequently mutated in colon, lung and pancreatic tumors, and N-Ras in hematologic cancers. Oncogenic mutations of these proteins impair their GTP-hydrolyzing activities, increasing the steady-state proportion of their activated, GTP-bound forms. The Ras proteins play central roles in transducing signals from various plasma membrane receptors, including transmembrane tyrosine kinase-linked receptors and integrins, to diverse downstream effectors [10, 14, 51, 56, 105].

The Ras proteins are highly homologous over roughly the first 85% of their length, including their guanine nucleotide- and effector-binding core. However, the carboxy-terminal sequences of the different Ras proteins, comprising 23–24 amino acids and known as the hypervariable domains, differ greatly (Fig. 1). The carboxy-terminal 10–14 amino acids of each hypervariable sequence, including the terminal -CAAX farnesylation motif, constitute an autonomous targeting signal that can direct heterologous proteins to the plasma membrane in a manner closely resembling that observed for the Ras proteins themselves [19, 39, 40, 97]. Near the terminal farnesylated cysteine residue is found either one or more palmitoylation sites (in H-Ras, N-Ras or K-Ras4A), or a cluster of basic amino-acid residues (in K-Ras4B), which, as described later, are essential for correct plasma membrane targeting.

The amino-terminal half of each Ras hypervariable domain, comprising the 'linker' region (Fig. 1), does not influence the plasma membrane-localizing function of the targeting sequence. However, in the case of H-Ras it has been shown that the linker region can modulate a second targeting function of the carboxyl-terminal sequence, namely the ability of the latter to direct the protein to caveolae and noncaveolar lipid rafts within the plasma membrane [47, 81, 87]. The core, linker and targeting domains of full-length H-Ras interact in such a manner that H-Ras(GDP), but not H-Ras(GTP), associates with

these plasma membrane microdomains. It will be of great interest to determine whether elements within the hypervariable domain interact in a similar manner to regulate the microcompartmentation of other Ras proteins within the plasma membrane.

A final function of the hypervariable domains of Ras proteins is to contribute directly to interactions with effector and regulatory proteins. This property is perhaps most clearly illustrated by reports that the carboxy-terminal farnesyl residue directly influences the interactions of Ras proteins with various effector and modulator proteins in solution [32, 67, 73, 78, 89, 109].

The marked differences in the hypervariable regions of the Ras proteins can be correlated with differences in their function in intact cells and organisms. K-Ras has been shown to be essential for embryonic development in mice [49, 59], while H-Ras/N-Ras double-knockout mice are fertile and show no obvious defects in development [28, 102]. H-ras, N-ras and K-Ras4B likewise differ markedly in their abilities to activate downstream effectors such as Raf kinase, phosphatidylinositol-3-kinase and the rac pathway in intact cells [37, 89, 104, 106, 113] and in their modulation by regulatory molecules such as Ras-GRF/Cdc25Min [50]. It remains uncertain to what degree these differences reflect intrinsic differences in the abilities of the Ras proteins to interact with various effector and regulatory proteins, as opposed to differences in the microcompartmentation of different Ras species within the cell.

Ras Proteins Are Farnesylated, Proteolyzed and Methylated by a Common Pathway

The precursor forms of the Ras proteins, like those of most other singly prenylated proteins, are synthesized on soluble ribosomes with a carboxy-terminal -CAAX motif, where the terminal residue X largely determines the specificity for farnesylation *vs.* geranylgeranylation, and the 'A' residues are typically aliphatic. The Ras precursor proteins are first isoprenylated in the cytoplasm by protein farnesyltransferase (Fig. 2). This enzyme releases the farnesylated protein product only when it binds a new molecule of its substrate farnesyl pyrophosphate [101]. Since the latter may be substantially membrane-bound under intracellular conditions, release of the prenylated precursor protein from farnesyltransferase may occur at membrane surfaces rather than in the cytoplasm.

Following initial farnesylation, the precursors of all Ras proteins are successively processed by two additional reactions at their carboxyl-termini. The terminal -AAX residues are first removed by an endoprotease that has been identified in mammals and in yeast as Rce1p, a multispanning integral membrane protein localized to the endoplasmic reticulum [11, 76, 91]. Disruption of the Rce1p gene in mammalian cells leads to loss of Ras-processing activity in cell extracts and mislocalizes a green fluorescent protein (GFP)/K-Ras4B fusion protein, though not a GFP/H-Ras fusion protein, to cytoplasmic structures [58]. Following removal of their terminal -AAX sequence, ras proteins are carboxyl-methylated on their farnesylated cysteine residue by an enzyme designated Icmt in humans and Ste14p in yeast [21]. This enzyme is also an integral membrane protein of the endoplasmic reticulum. Knockout of Icmt expression is embryonic-lethal in mice [6] and leads to partial mislocalization of K-Ras4B (but not of H-Ras) in isolated fibroblasts [17].

The findings discussed above immediately raise two questions: First, following carboxy-terminal processing (prenylation/-AAX proteolysis/methylation), how do Ras proteins become relocalized from the endoplasmic reticulum to the plasma membrane? Second, can Ras proteins, in either fully or incompletely processed forms, contribute to cellular signaling from compartments other than the plasma membrane? Our current understanding of these issues is discussed in the following sections.

H- and N-Ras Travel to the Plasma Membrane by a Vesicular Mechanism

Early studies of the sequence requirements for plasma membrane targeting of H- and N-Ras revealed that the targeting sequences of these proteins were modified not only by carboxy-terminal farnesylation but also by S-acylation ('palmitoylation') on nearby cysteine residues that are essential for localization to the plasma membrane, though not for membrane binding per se [19, 39, 40, 93, 112]. In more recent studies, nascent H- and N-Ras have been shown to appear initially in association with the ER and with the Golgi, then to reach the plasma membrane from the latter compartment by vesicular transport [2, 19]. Plasma membrane delivery of these species is blocked both by low temperature (15°C) and by brefeldin, classical inhibitors of the constitutive secretory pathway. Association of these proteins (or of chimeric proteins linking GFP to the H- or N-Ras targeting sequences) with transport vesicles requires their S-acylation site(s) as well as the farnesylated cysteine residue.

The mechanism by which H- and N-Ras become stably associated with secretory vesicles for export to the plasma membrane has not yet been clarified at a molecular level. An attractive possibility consistent with present evidence is that these proteins become palmitoylated before packaging into secretory vesicles, and that the combination of palmitoylation and farnesylation provides the 'signal' for stable associa-

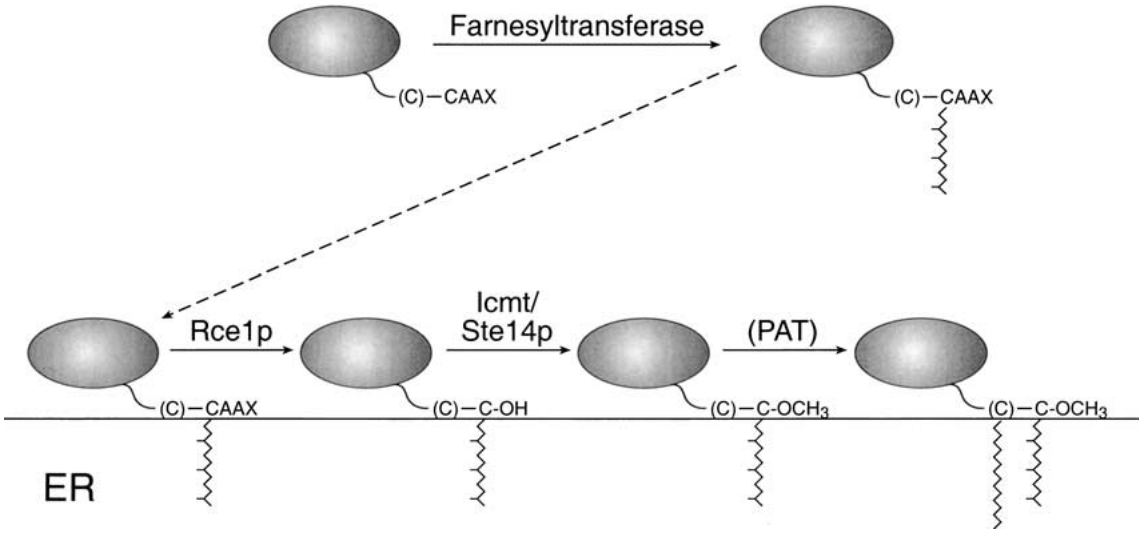

Fig. 2. Processing of the carboxy-terminal sequences of the Ras proteins. After initial farnesylation in the cytosol, all nascent Ras proteins translocate to the endoplasmic reticulum (possibly still bound to farnesyltransferase), where they undergo successive removal of the terminal –AAX residues and methylation of the farnesylated cysteine residue. Nascent H- and N-Ras and K-Ras4A undergo further modification by S-acylation (palmitoylation) on one or more cysteine residues, which, based on recent findings on yeast, may also occur in the endoplasmic reticulum.

tion with transport vesicles [19, 83]. This proposed mechanism of course predicts that the endoplasmic reticulum and/or Golgi compartments of mammalian cells incorporate a protein palmitoyltransferase (PAT, more properly termed a protein S-acyltransferase) that acts upon H- and N-Ras. While findings consistent with this possibility have been reported previously [34, 112], recent studies in yeast have provided the first direct identification of an endomembrane-associated S-acyltransferase that may recognize the Ras proteins as physiological substrates.

S. cerevisiae expresses two Ras homologues, Ras1p and Ras2p, which, like mammalian H- and N-Ras, undergo carboxy-terminal processing on the endoplasmic reticulum before they are transported to the plasma membrane [11, 85, 91]. Screens designed to detect proteins important for Ras palmitoylation identified two candidate genes, SHR5/ERF4 and ERF2, deletion of either of which reduced sharply (but did not wholly abolish) the S-acylation of a modified form of Ras2p [4, 52]. Erf2p exhibits palmitoyltransferase activity toward Ras2p in vitro (63) and is localized to the endoplasmic reticulum [4], consistent with its proposed role in palmitoylating nascent Ras2p. Erf2p exhibits a number of homologues in yeast and in higher organisms; the human homologue Erf2p is localized to the endoplasmic reticulum and Golgi (R. Dechenes and M. Philips, unpublished results). Experiments using peptides representing the farnesylated carboxy-terminus of N-Ras [93] suggest that an S-acyltransferase active upon Ras proteins is associated with the plasma membrane as well. An activity mediating S-acylation of the heterotrimeric G-protein subunit $G_{\alpha s}$ has in fact been found associated specifically with an isolated plasma membrane fraction [25, 26].

The model described above for H- and N-Ras trafficking to the plasma membrane leaves some fundamental mechanistic questions unanswered. It is not yet clear, for example, whether unpalmitoylated H- and N-Ras are retained on endomembranes by binding to a putative protein partner that prevents their export to the plasma membrane. Likewise, it is not known whether palmitoylated H- and N-Ras are escorted by other proteins from the Golgi to the plasma membrane or instead simply follow the bulk flow of membrane material along the constitutive secretory pathway. The latter alternative is plausible in principle, since in their palmitoylated forms the H- and N-Ras targeting sequences are in essence irreversibly anchored to the membrane lipid bilayer [93, 95] and hence could be stably bound to secretory vesicles via their lipidic residues alone. In general, it remains an open question to what extent 'escort' proteins may be required to direct Ras proteins through their intracellular processing and subsequent transit to the plasma membrane. Various identified Ras-binding proteins have been suggested to contribute to Ras protein trafficking [29, 70, 103], although to date none of these has been shown to play a direct or essential role in this process. While such proposals are entirely plausible, it is important to note that there appear to be no inherent *mechanistic* reasons why nascent Ras proteins would need to be more than transiently associated (e.g., as enzyme substrates) with any specific protein partners in the endomembrane system (or the cytoplasm) in order to reach the plasma membrane.

The above discussion has largely ignored the mechanism of plasma membrane transport of the third palmitoylated Ras protein, K-Ras4A. While it is logical to suggest that this Ras species may reach the plasma membrane in a manner similar to H- and N-Ras, this question remains to be addressed experimentally.

K-Ras4B Is Targeted to the Plasma Membrane in a Manner Distinct from H- or N-Ras

The targeting sequence of K-Ras4B lacks palmitoylation sites but features instead a strongly polybasic sequence (–KKKKKKSKTK-) immediately adjoining the farnesylated terminal cysteine. Accordingly, the mechanism of plasma membrane targeting of this Ras protein appears to differ substantially from that of H- and N-Ras. Processing of the K-Ras4B carboxy-terminus, including –CAAX group proteolysis and methylation of the prenylated cysteine at the endoplasmic reticulum, appears to proceed in the same manner as for H- and N-Ras. However, fluorescence and electron microscopy provide no evidence for significant subsequent association of K-Ras4B with either the Golgi compartment or the secretory vesicles that transport H- and N-Ras to the plasma membrane [2, 19]. Consistent with this finding, low temperature- (15°C) or Brefeldin-mediated inhibition of the secretory pathway fails to inhibit plasma delivery of K-Ras4B, in contrast to that of palmitoylated forms of Ras.

Further evidence for a distinct mechanism of plasma membrane targeting of K-Ras4B is provided by the observation that inhibitors of microtubule dynamics impair plasma membrane localization of this Ras species (or of chimeric proteins bearing the K-Ras4B targeting sequence) but not of H- and N-Ras [2, 100]. K-Ras4B also uniquely binds to microtubules in vitro (and can be cross-linked to tubulin) in its –C(farnesyl)AAX and mature (–C(farnesyl)-OCH_3) forms, though interestingly not in its intermediate –C(farnesyl)-OH form [17, 100]. These findings could suggest that K-Ras4B reaches the plasma membrane in a microtubule-directed manner. Welman et al. [107], noting that interaction between the polybasic K-loop of kinesin and the highly anionic carboxy-terminus of tubulin allows rapid one-dimensional diffusion of kinesin along microtubules [74], have in fact suggested that K-Ras4B might similarly move along microtubules to the plasma membrane. However, some experimental observations complicate this appealing model of the role of microtubules in K-Ras4B targeting. First, in Icmt-deficient fibroblasts, a GFP/K-Ras4B fusion protein is still partially targeted to the plasma membrane even though in these cells the end product of K-Ras4B processing is the –C(farnesyl)-OH form, which does not interact with microtubules in vitro [17]. A more serious challenge to the model just noted is the observation of Apolloni et al. [2] that in the presence of paclitaxel a GFP/K-Ras4B chimera is associated with cytoplasmic multilamellar vesicles and tubulovesicular structures but not directly with microtubules themselves. This finding may imply that microtubule-stabilizing agents affect K-Ras4B localization by interfering with mechanisms that normally minimize endocytic uptake of the protein, or that efficiently return it from the endosomal compartment to the plasma membrane.

As in the cases of H- and N-Ras, a variety of proteins has been suggested as potential 'escorts' for nascent K-Ras4B during its processing and ultimate transit to the plasma membrane [29, 41, 55, 70, 103]. To date, however, direct evidence is lacking to assess whether any of these proteins serves an essential role in trafficking K-Ras4B between different membrane compartments. Biochemical and biophysical experiments have revealed that the K-Ras4B targeting sequence can transfer spontaneously between different artificial and natural membranes on a time scale of minutes [30, 61, 86], suggesting that transfer of K-Ras4B from the ER to the plasma membrane could in fact proceed by simple diffusion through the cytoplasm.

Are there Plasma Membrane 'Targeting Receptors' for Ras Proteins?

Several studies have described plasma membrane binding sites for Ras proteins, raising questions whether such sites might play an essential role in targeting Ras to the plasma membrane. Siddiqui et al. [96] showed that the plasma membrane incorporates a protease-sensitive binding activity that binds prenylated H-Ras, N-Ras and K-Ras4B with substantial affinity. However, while these sites show appreciable specificity for a prenylated carboxy-terminal motif, they show relatively modest selectivity for Ras proteins over farnesylated lamin B, which is not plasma membrane-localized. As well, to date it has not been determined whether the isolated carboxy-terminal sequences of the Ras proteins also bind with high affinity to these binding sites, as would be expected if the latter function as Ras targeting receptors. It thus remains to be clarified whether these binding sites play a role in plasma membrane targeting of Ras proteins.

Prior et al. [81] showed, using electron microscopy, that a chimeric protein linking GFP to the carboxy-terminal 10 amino acids of H-Ras (GFP-tH) is concentrated in caveolae, while an analogous construct fusing GFP to the K-Ras4B targeting sequence is not. Interestingly, the density of the GFP-tH chimera found in caveolae was very similar at high

and low expression levels. These findings suggest that caveolae may contain saturable binding sites that specifically recognize the carboxy-terminal targeting sequence of H-Ras. Niv et al. [72] have recently reported evidence from lateral-diffusion measurements that activated H-Ras and K-Ras4B associate with saturable populations of non-raft-localized binding sites in the plasma membrane. In both of these latter studies, however, at high expression levels a large proportion of the Ras proteins (or GFP/Ras chimeras) was not associated with the postulated intramembrane binding sites yet was still correctly targeted to the plasma membrane, suggesting that association with these binding sites may not be essential for plasma membrane targeting.

A different set of observations that could suggest the existence of a Ras 'targeting receptor' at the plasma membrane was provided by reports by Kloog and colleagues that farnesylthiosalicylic acid (FTS) and certain closely related analogues accelerate the degradation of activated H-Ras and K-Ras4B in Rat1 cells and partially shift the distribution of these proteins from the plasma membrane to other compartments [1, 36, 71]. These findings suggest that FTS may disrupt the association of activated K-Ras4B and H-Ras with a protein that normally stabilizes their plasma membrane association. A recent study [77] in fact showed that the soluble protein galectin-1 can be cross-linked in an FTS-sensitive manner to, and co-immunoprecipated with, mutationally activated H-Ras, though not with wild-type H-Ras. From these and other findings it was suggested that galectin-1 acts to stabilize the plasma membrane association of activated H-Ras. It remains to be clarified how galectin-1 fulfills this function. It would be of interest to investigate the relationship between galectin-1 association and palmitoylation of H-Ras, another factor that could stabilize the association of the latter protein with the plasma membrane.

While there is thus intriguing evidence for proteins that bind Ras at the plasma membrane, other experimental results raise questions whether a 'classical' specific protein-protein interaction is required for proper plasma membrane targeting of Ras. First, a surprisingly wide variety of modifications can be introduced into the targeting sequences of the Ras proteins without compromising their plasma membrane-localizing function, so long as certain basic physical properties are preserved. The carboxy-terminal farnesyl group of H-Ras, for example, can be replaced by either an amino-terminal myristoyl residue or a carboxy-terminal polybasic sequence (which allows palmitoylation of nearby cysteine residues), or even with a simple –GCGC(farnesyl)-OCH_3 sequence in the context of a synthetic peptide, without compromising targeting to the plasma membrane [7, 8, 13, 20, 92]. The plasma membrane-targeting motif of K-Ras4B can likewise be replaced by a wide range of variant sequences without loss of function so long as the polybasic and amphiphilic characteristics of the wild-type sequence are retained [13, 45, 86, 107]. These results suggest that the plasma membrane-targeting sequences of these proteins may not be recognized with the specificity normally anticipated for a protein-receptor interaction. Second, measurements of the lateral diffusion of GFP/H-Ras and GFP/K-Ras4B chimeric proteins in intact cells suggest that these species interact only transiently with integral membrane proteins, particularly at high expression levels, yet are nonetheless correctly targeted to the plasma membrane [71, 72]. Such findings suggest that the Ras proteins are not anchored to the plasma membrane by long-lived, highly specific associations with resident plasma membrane proteins. These findings, however, do not exclude the possibility that resident proteins of the plasma membrane could act in a catalytic manner to promote Ras targeting to this membrane compartment (e.g., by facilitating [re]palmitoylation of H- or N-Ras at the plasma membrane).

If the requirement for plasma membrane targeting receptors for Ras proteins remains an open question, it is legitimate to ask whether alternative models could explain how these proteins become targeted specifically to this membrane compartment. Palmitoylation of H-Ras, N-Ras and K-Ras4A at the ER, Golgi and/or plasma membranes, and the demonstrated transport of these Ras proteins to the plasma membrane by vesicular transport, could in fact explain the predominant plasma membrane association of these species through a 'kinetic trapping' mechanism [2, 19, 95]. In the case of K-Ras4B, as we have noted elsewhere [61, 86] relatively nonspecific electrostatic interactions could confer preferential localization to the plasma membrane if the inner surface of this membrane (or particular microdomains within it) exhibits a significantly higher local surface charge than do the cytoplasmic surfaces of other cellular membranes. This latter postulate, however, remains to be confirmed directly, though it is generally consistent with known properties of the plasma membrane [61, 86].

Plasma Membrane Association of Ras Proteins Is Dynamic

Recent studies have provided direct evidence that plasma membrane-targeting of different Ras proteins can be dynamic in character. First, and as discussed in the next section, at least some Ras proteins appear to be endocytosed as part of their normal function in transducing signals from activated cell-surface receptors. The activated GTP-bound form of H-Ras can be endocytosed and in fact accumulates in the enlarged endocytic compartment of BHK cells expressing a mutant form of Rab5 that stimulates en-

docytosis but not endosomal recycling [88]. Interestingly, enhanced endocytosis without net endosomal accumulation of Ras is observed in cells overexpressing wild-type Rab5 (which stimulates both endocytosis and endosomal recycling), suggesting that H-Ras can be efficiently recycled from endosomes to the plasma membrane.

Kenworthy and colleagues (57; A. Kenworthy, M. Philips and J. Lippincott-Schwartz, unpublished results) have used photobleaching experiments to demonstrate substantial transfer of N-Ras from the plasma membrane to the Golgi on a time scale of minutes. Since the half-life of the protein is of the order of hours [65], and since N–Ras does not accumulate indefinitely in the Golgi, these findings appear to reflect cycling of N-Ras between the Golgi and the plasma membrane. Unlike the endocytic uptake of H-Ras, it is not clear that transfer of N-Ras to the Golgi occurs by a vesicular pathway. A potential alternative mechanism for this process is suggested by the earlier observation of Magee et al. [65] that the palmitoyl modification of N-Ras is highly dynamic, with a half-life of roughly 20 minutes. Periodic depalmitoylation could allow N-Ras to transfer from the plasma membrane to the Golgi, either via simple spontaneous diffusion [93] or potentially with the assistance of a soluble 'chaperone' [70, 103]. Whether depalmitoylation plays any role in permitting intermembrane transfer of H-Ras (whose bound palmitoyl group turns over with a half-life of the order of 1–3 hr [3, 64, 80]) remains to be determined.

Biophysical and biochemical studies have shown that the carboxy-terminal targeting sequence of K-Ras4B binds strongly but reversibly to negatively charged membranes [30, 61, 86]. Yokoe and Mayer [114] have reported that a GFP/K-Ras4B fusion protein undergoes rapid (time scale of seconds) dissociation from and reassociation with the plasma membrane in intact mammalian cells, suggesting that the plasma membrane targeting of this protein may indeed be highly dynamic. Niv et al. [71, 72] have by contrast reported that fusion proteins linking GFP to wild-type or constitutively activated forms of K-Ras4B remain stably bound to the plasma membrane on a time scale of at least several seconds. Given that K-Ras4B appears to associate only transiently with integral proteins of the plasma membrane [72], it would be of considerable interest to assess whether the protein can dissociate from the plasma membrane on longer, but still biologically relevant time scales, as in vitro experiments have suggested [61, 86].

Do Ras Proteins Signal from Intracellular Membranes?

As discussed above, nascent Ras proteins pass through the endoplasmic reticulum (and, for H- and N-Ras, the Golgi) during their postranslational maturation, and in steady-state, significant amounts of H- and N-Ras are found associated with a perinuclear membrane compartment [2, 19, 112]. Several studies have reported that Ras proteins can be taken up in association with endocytic vesicles, and that such uptake may be markedly stimulated by ligands that activate the nerve growth factor (TrkA) and epidermal growth factor receptors [23, 42, 43, 48, 60, 84]. Activated Ras can in fact itself stimulate endocytosis by activating the guanine nucleotide exchange-promoting activity of RIN1 toward Rab5 [99]. Such observations raise the question whether Ras proteins can signal from intracellular compartments, and if so, whether such signaling may be functionally distinct from signaling mediated by these proteins at the plasma membrane. Recent studies suggest that the answer to both of these questions may be affirmative.

Roy et al. [88] have recently shown that inhibition of endocytosis impairs activation of Raf by H-Ras, though not by K-Ras4B. In the same study, overexpression of Rab5, which stimulates both endocytosis and endosomal recycling, was found to enhance activation of Raf by H-Ras. Interestingly, a GTPase-deficient mutant of Rab5, which promotes endocytosis but not recycling of endocytosed material to the cell surface, did not similarly enhance Raf activation by H-Ras. These experiments clearly suggest that H-Ras fulfills its signaling functions in part by (transiently) translocating to endosomal (and other?) compartments.

Further evidence that Ras proteins may not signal exclusively from the plasma membrane has recently been reported by Chiu et al. [18]. These workers used a reporter construct fusing fluorescent proteins to the Ras-binding domain (RBD) of Raf, which binds specifically to activated Ras, to localize activated forms of Ras proteins in living mammalian fibroblasts. H- and N-Ras, activated either by mutation or by addition of epidermal growth factor to serum-starved cells, were observed both at the plasma membrane and in a perinuclear compartment also labeled by a coexpressed Golgi marker. Jiang and Sorkin [48], using a similar approach, reported that activated H-Ras could be found associated with endosomes as well as with the plasma membrane. These findings contrast with a previous report that activated H-Ras is found specifically at the plasma membrane [69]. However, the latter study used a fluorescent reporter protein, in which two protein modules (the Raf RBD and cyan fluorescent protein) were inserted between the conserved and hypervariable regions of H-Ras, potentially altering the localization and/or the signaling properties of this construct compared to those of native H-Ras. Interestingly, Chiu et al. [18] found that mutant forms of H-Ras localized to the ER or Golgi can become activated in response to stimulation of cell-surface growth factor receptors, in a manner that requires the activity

of src-family tyrosine kinases but not the operation of the endocytic pathway.

Similarities in Targeting of Other Ras-Superfamily Proteins

Current evidence suggests that various singly-prenylated proteins of the Ras superfamily may be targeted to their subcellular destinations in part by mechanisms similar to those exploited by the Ras proteins themselves. As illustrated in Fig. 1, a number of Ras-superfamily proteins carry carboxy-terminal motifs that, as in the Ras proteins, combine a prenylated carboxy-terminal cysteine residue with either a polybasic sequence or one or more sites of palmitoylation ([68] and reference therein). In isolation these motifs can moreover frequently serve as plasma membrane targeting signals [68, 86]. However, for some Ras-superfamily members (in contrast to the Ras proteins themselves) the intracellular distributions of the full-length proteins differ markedly from those observed for reporter constructs incorporating the carboxy-terminal sequences alone [5, 68]. Proteins of the Rho family, for example, are partly localized to the cytoplasm in a complex with RhoGDI, which binds to both the conserved domains of these G-proteins and their prenylated carboxy-termini [31, 62, 75]. RhoGDI plays an important role in the functioning of Rho-family proteins by mediating their delivery to sites of remodeling of the juxtamembrane cytoskeleton [12, 90, 98]. It is not known at present whether soluble Ras-binding proteins may similarly participate in recruitment of Ras proteins to interact with particular regulatory or effector proteins.

Conclusions

In one sense it may appear surprising that we understand so imperfectly the mechanisms of subcellular targeting of the Ras proteins, given that both the sequences and the posttranslational modifications required for this targeting have been known for over a decade. However, elucidating the mechanisms of Ras targeting requires that we address some fundamental and challenging questions concerning the cellular 'management' of key signaling proteins, the complexity of the membrane milieu, with its highly spatially differentiated yet dynamic nature, and the diverse pathways by which molecules can transfer between different subcellular locations. These complications pose many experimental and, at times, conceptual challenges. However, they also make the Ras proteins a rich source for new paradigms to aid our understanding of the dynamic interplay of molecules, domains and compartments that underlies the richness of cellular signaling.

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