

Thematic review series: Lipid Posttranslational Modifications

CAAX modification and membrane targeting of Ras

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Abstract Proteins that terminate with a consensus sequence known as CAAX undergo a series of posttranslational modifications that include polyisoprenylation, endoproteolysis, and carboxyl methylation. These modifications render otherwise hydrophilic proteins hydrophobic at their C termini such that they associate with membranes. Whereas prenylation occurs in the cytosol, postprenylation processing is accomplished on the cytoplasmic surface of the endoplasmic reticulum and Golgi apparatus. Among the numerous CAAX proteins encoded in mammalian genomes are many signaling molecules such as monomeric GTPases, including the Ras proteins that play an important role in cancer. In the course of their processing, nascent Ras proteins traffic from their site of synthesis in the cytosol to the endomembrane and then out to the plasma membrane (PM) by at least two pathways. Recently, retrograde pathways have been discovered that deliver mature Ras from the PM back to the Golgi. The Golgi has been identified as a platform upon which Ras can signal. Thus, the subcellular trafficking of Ras proteins has the potential to increase the complexity of Ras signaling by adding a spatial dimension. The complexity of Ras trafficking also affords a wider array of potential targets for the discovery of drugs that might inhibit tumors by interfering with Ras trafficking.—Wright, L. P., and M. R. Philips. CAAX modification and membrane targeting of Ras. *J. Lipid Res.* 2006. 47: 883–891.

Supplementary key words farnesylation • trafficking • carboxyl methylation

Ras proteins have been studied intensively for more than a quarter century because of their role in human cancer. Indeed, Ras was one of the first oncogenes to be identified. At the biochemical level, Ras is the founding member of a large superfamily of monomeric GTPases that function as molecular switches. Ras is also the founding member of a class of peripheral membrane proteins known as CAAX proteins, where C stands for cysteine, A for an aliphatic amino acid, and X for any amino acid. The primary translation product of CAAX protein genes end with

a CAAX sequence that serves as a substrate for three enzymes that sequentially modify the sequence to create a lipidated, hydrophobic domain that mediates the association with cellular membranes. These enzymes include two prenyltransferases, farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase), Ras-converting enzyme 1 (Rce1), and isoprenylcysteine carboxyl methyltransferase (Icmt). Plasma membrane (PM) association of Ras was originally understood as the simple and direct consequence of CAAX processing by these enzymes. We now understand that CAAX modification is a highly orchestrated process that takes proteins on a journey through various subcellular compartments and can terminate on compartments other than the PM. An in-depth look at prenyltransferases, the first and best understood of the CAAX processing enzymes, was the focus of the review by Beese in this series (1), and Basso, Kirschmeier, and Bishop (2) described efforts to develop drugs to block farnesylation. The membrane trafficking of Ras and other CAAX proteins will be the focus of this review.

CAAX PROCESSING

By the late 1980s, it was recognized that the C terminus of Ras was responsible for its membrane targeting and was required for cellular transformation (3). Around the same time, it was discovered that both yeast mating factors and Ras2p of *Saccharomyces cerevisiae* terminated in a farnesylcysteine methyl ester (4). Clarke and colleagues (5) also appreciated that an invariant cysteine occurred as the fourth to last residue in a number of Ras family proteins, fungal mating factors, nuclear lamins, and cGMP phosphodiesterase, giving rise to the idea that a consensus sequence designated CAAX directed a series of posttranslational modifications that included lipidation with a polyisoprene, endoproteolysis, and carboxyl methylation. Some confusion persisted because, in 1986, Buss and Sefton (6) had shown that Ras proteins are palmitoylated.

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The picture was clarified when Hancock et al. (7) showed that all Ras proteins are polyisoprenylated but only some are palmitoylated.

We now know that when the primary translation product of a gene terminates with a CAAX sequence, it can, and most often does, become a substrate for a pair of cytosolic prenyltransferases that attach a polyisoprene lipid, via a stable thioether linkage, to the cysteine (reviewed by Beese in this series). Prenylated CAAX sequences then become substrates for Rce1, an endoprotease that removes the AAX amino acids (8). The endoproteolytic activity of Rce1 requires prenylation. Finally, the newly C-terminal prenylcysteine becomes a substrate for a specific protein carboxyl methyltransferase, Icmt, that methyl esterifies the α carboxyl group of the prenylcysteine (5, 9, 10). Unlike prenylation and proteolysis, carboxyl methylation is reversible under physiologic conditions (11), although a specific esterase that hydrolyzes the prenylcysteine methyl ester has not been identified. When a CAAX sequence ends in an amino acid other than leucine or phenylalanine (typically serine, methionine, or glutamine), it is modified with a 15 carbon farnesyl isoprenoid through the activity of FTase, whereas when the terminal amino acid is leucine or, in some cases, phenylalanine, the modification is with a 20 carbon geranylgeranyl isoprenoid through the action of GGTase (12). The end result of these modifications to the CAAX sequence is to render a previously hydrophilic protein domain into one that is hydrophobic and capable of interacting with phospholipid bilayers (Fig. 1).

Mammalian genomes encode three *ras* genes that give rise to four gene products: N-Ras, H-Ras, K-Ras4A, and the common splice variant of the *kras* gene, K-Ras4B (13). In 1990, Hancock, Paterson, and Marshall (14) showed that CAAX processing in the manner described above is necessary but not sufficient to target Ras proteins to the PM. Also required was a second signal that was found immediately upstream of the CAAX sequence within the C-terminal region of Ras proteins known as the hypervariable domain. There are two types of second signals. In the case of N-Ras, H-Ras, and K-Ras4A, the second signal

consists of one or two cysteine residues that are sites of palmitoylation. The Ras palmitoyltransferase was somewhat elusive but was eventually characterized at the molecular level in yeast by Deschenes and colleagues (15), a discovery that led to the identification of the mammalian ortholog, DHHC9/GCP16 (16). In the case of K-Ras4B, henceforth referred to as K-Ras, the second signal is a polybasic sequence rich in lysine residues (14).

Like monomeric Ras family GTPases, heterotrimeric G proteins are also targeted to membranes via the processing of a CAAX sequence that occurs on the γ subunit (17). As is the case with other CAAX proteins, the targeting of G proteins to the PM requires a second signal (18). Interestingly, whereas the second signal on Ras proteins occurs in *cis* on the same polypeptide, the second signal for heterotrimeric G proteins consists of either myristoylation or palmitoylation at the N terminus of the α subunit and therefore is in *trans* with respect to the CAAX sequence (18, 19). The placement of the two required signals on different subunits may have evolved to ensure that only the nascent holoenzyme reaches the PM, because dissociation of α from $\beta\gamma$ subunits is the mechanism whereby G proteins signal.

CAAX TRAFFICKING I: ANTEROGRADE

The trafficking of nascent Ras proteins from their site of synthesis on free polysomes in the cytosol to the PM was originally conceived of as a direct process mediated by the posttranslational processing of the CAAX motif described above. The discovery that postprenylation modifying enzymes are intrinsic membrane proteins that are restricted to the endomembrane called this view into question. Both mammalian Icmt (10) and the yeast ortholog, Ste14 (20), were found to be restricted to the endoplasmic reticulum (ER). The same year, Rce1 was also found to be restricted to the ER (21). Recently, the mammalian Ras palmitoyltransferase, DHHC9/GCP16, was found to reside in the membranes of the Golgi apparatus (16).

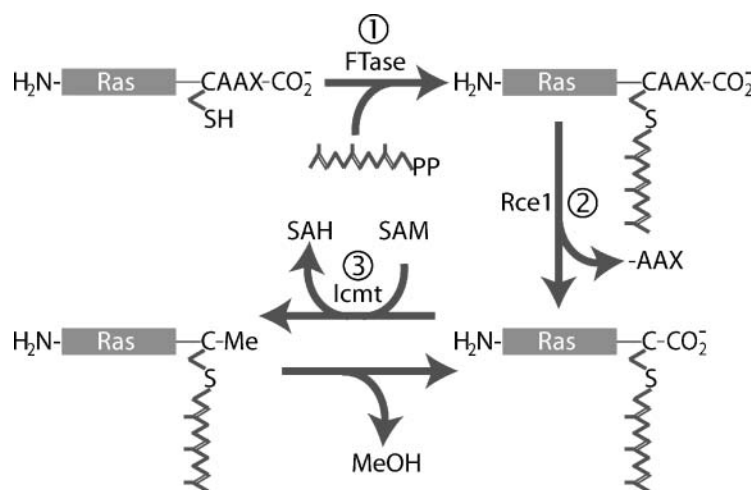


Fig. 1. CAAX processing. Proteins that end with a CAAX sequence (where C stands for cysteine, A for an aliphatic amino acid, and X for any amino acid) are processed sequentially by three enzymes. Unless the X amino acid is leucine or phenylalanine, the first step is the addition of a 15 carbon farnesyl isoprenoid via a thioether linkage by farnesyltransferase (FTase). The farnesylated CAAX sequence then becomes a substrate for Ras-converting enzyme 1 (Rce1), a specific endoprotease that removes the AAX amino acids. Finally, the newly C-terminal prenylcysteine becomes a substrate for isoprenylcysteine carboxyl methyltransferase (Icmt), which methyl esterifies the α carboxyl group. The methyl donor for this reaction is S-adenosyl-L-methionine (SAM), which is converted to S-adenosyl homocysteine (SAH). This last step is reversible under physiologic conditions, although a specific esterase has not been identified. The end result is to convert a hydrophilic C terminus into a hydrophobic domain that has affinity for membranes. MeOH, methanol.

Using live cell imaging and green fluorescent protein (GFP)-tagged Ras proteins, Choy et al. (22) showed that, consistent with the localization of the postprenylation processing enzymes, nascent Ras proteins trafficked via the cytosolic face of the ER and Golgi en route to the PM. Similar results were obtained using fixed cells expressing GFP-tagged Ras proteins (23). Importantly, these studies established that the pathway taken from the endomembrane to the PM was different for palmitoylated Ras proteins than it was for K-Ras. Whereas N-Ras and H-Ras trafficked through a vesicular pathway (22) that has characteristics of the classical secretory pathway (23), K-Ras, with its polybasic region, took an alternative route that remains uncharacterized. Interestingly, in *S. cerevisiae*, delivery of palmitoylated Ras proteins to the PM was not affected by mutations that disrupt the classical secretory pathway, although in strains that lacked one of the two subunits of the palmitoyltransferase, the same mutants did block the trafficking of Ras, suggesting that both classical and nonclassical vesicular pathways deliver Ras to the PM (24).

GFP-tagged Ras proteins that lack a second signal accumulate on the ER (22, 23). This observation suggests that prenylation of CAAX sequences is not a nonspecific signal for the association with all cellular membranes but rather specifies the cytoplasmic leaflet of the ER. The basis of this selectivity remains obscure. One possibility is that it is the affinity of prenyl-CAAX proteins for ER-associated Rce1 that accounts for the partition into the ER membrane. There is evidence for increased affinity for membranes once CAAX proteins are methylated by Icmt (25, 26). Thus, if Rce1 and Icmt act in a coordinated way, the sequential modifications they catalyze may serve to “trap” the prenylated proteins on ER membranes until they enter the pathways that carry them to the PM. Several Rho family Ras-related GTPases, such as Cdc42 and Rac2, lack functional second signals and, when dissociated from their cytosolic chaperone, RhoGDI, localize constitutively on the endomembrane (27, 28), supporting the idea that CAAX processing in isolation is a means to target proteins to the ER.

To fully understand Ras trafficking, one must consider the nature of prenylated Ras in the cytosol. Rho family GTPases have CAAX sequences but differ from Ras in two ways. First, most are geranylgeranylated, giving them intrinsically more hydrophobicity than their farnesylated cousins. Second, the bulk of fully processed, endogenous Rho proteins is found soluble in the cytosol in a 1:1 complex with RhoGDI (27) that acts as a chaperone and sequesters the hydrophobic C terminus of the Rho protein in a binding pocket (29). Significant amounts of Ras have been found in the cytosol (22), including farnesylated forms (30). Does farnesylated cytosolic Ras exist uncomplexed in an aqueous environment, or are there chaperones analogous to RhoGDI? Several candidate farnesyl Ras binding proteins have been identified, including PDE δ (31), PRA1 (32), and galectin (33). Does FTase act on Ras in such proximity to the ER that it is transferred directly to this compartment, or does one have to consider transit

through the aqueous environment from FTase to the ER? One interesting possibility is that the prenyltransferase itself acts as a chaperone for Ras and Rho. These enzymes have an unusual reaction mechanism wherein the product remains associated with the transferase until a new cycle of substrate binding causes it to be dislodged. Thus, if substrate binding were somehow linked to docking at the ER, the prenyltransferase itself could serve as the chaperone for nascent Ras and Rho. The Ras trafficking pathway to the PM, as it is currently understood, is summarized in **Fig. 2**. Once nascent Ras proteins reach the PM their initial journey is not over, because it is now clear that the PM is not a homogeneous structure but rather a patchwork of lipid microdomains such as cholesterol-rich rafts for which various Ras proteins have different affinities (34, 35). Not only are there isoform differences in how Ras proteins partition into these microdomains, but the GTP binding state of the Ras proteins also influences the partitioning (36).

CAAX TRAFFICKING II: RETROGRADE

Until recently, mature, fully processed Ras proteins that reached the PM were believed to remain on this compartment until degraded. Several studies have overturned this view by demonstrating retrograde trafficking. Unlike the stable thioether bonds that link isoprenoid groups to Ras, the palmitoyl lipids are linked via a labile thioester bond. At physiologic pH, these are subject to both enzymatic and nonenzymatic hydrolysis. It has been appreciated for two decades that the half-lives of N-Ras and H-Ras proteins exceed those of the associated palmitoyl groups (37–39). This observation led to the idea of a palmitoylation/depalmitoylation cycle (37). Goodwin et al. (40) used fluorescence recovery after photobleaching to show that N-Ras and H-Ras trafficked in a retrograde manner from the PM to the Golgi. When the entire Golgi was bleached in cells expressing GFP-N-Ras that were treated with cycloheximide to block new protein synthesis, 75% of the fluorescence was recovered with a half-life of <1 min (**Fig. 3**). This was considerably faster than the recovery time for glycosylphosphatidylinositol-linked proteins, suggesting that recovery occurred via a nonvesicular route. N-Ras and H-Ras that could not be palmitoylated was found by fluorescence recovery after photobleaching to recover on Golgi and ER membranes with a speed consistent with fluid phase transfer, and this conclusion was verified with fluorescence correlation spectroscopy. Rocks et al. (41) reported similar findings and went one step further. Using N-Ras and H-Ras tagged with photoactivatable GFP, these investigators showed unambiguously that the Ras on the PM traffics to the Golgi. Pools of N-Ras on the Golgi were replenished more rapidly than pools of H-Ras, consistent with the requirement for depalmitoylation of only one versus two cysteines.

Thus, a palmitoylation/depalmitoylation cycle regulates the trafficking of N-Ras and H-Ras between the PM and the Golgi. In this model, palmitoylation serves as a trap-

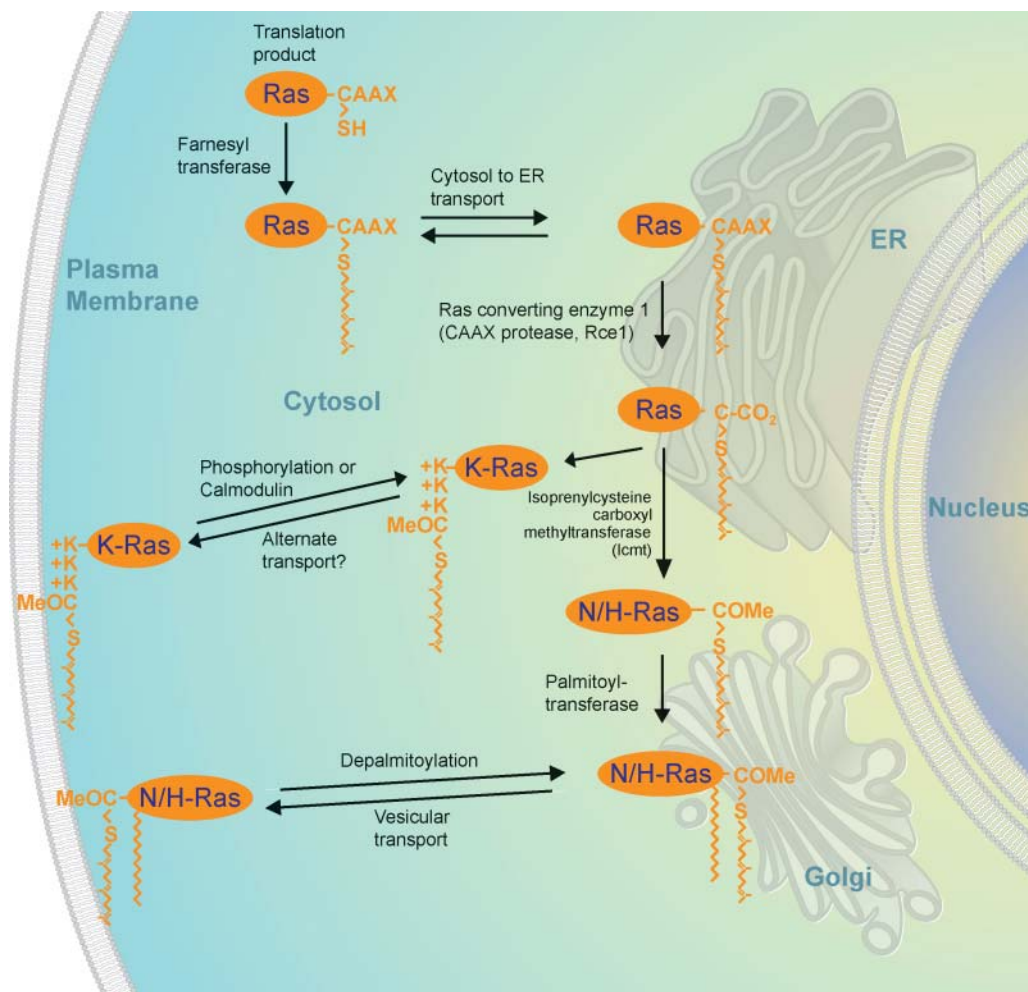


Fig. 2. Ras trafficking. Ras and other CAAX proteins are translated in the cytosol on free polysomes. Immediately posttranslationally, they become substrates for one of two cytosolic prenyltransferases. Prenylation targets the proteins to the endoplasmic reticulum (ER), where they encounter the subsequent processing enzymes, Rce1 and Icmt. Once CAAX processing is complete, the pathways used by the various isoforms diverge. K-Ras is sent to the plasma membrane (PM) via an uncharacterized pathway and can be returned to endomembrane after phosphorylation of the hypervariable region or through the action of calcium/calmodulin. In contrast, N-Ras and H-Ras are further processed on the Golgi by a palmitoyltransferase and then sent to the PM via vesicular transport. Retrograde traffic of N-Ras and H-Ras back to the Golgi occurs after depalmitoylation. [From Mor, A. and M. R. Philips. Compartmentalized Ras/MAPK signaling. *Annu Rev Immunol.* Epub ahead of print. January 16, 2006; doi: 10.1146/annurev.immunol.24.021605.090723.]

ping mechanism to retain in Golgi membranes farnesylated Ras proteins that would otherwise have relatively weak affinities for this and other membranes. The localization of the Ras palmitoyltransferase, DHHC9/GCP16, on the Golgi (16) supports such a model. In contrast to the classical secretory pathway, the Golgi, rather than the ER, serves as the entry point for vesicular traffic of palmitoylated Ras isoforms to the PM. In support of this idea are results of photobleaching and fluorescence correlation spectroscopy that revealed that nonpalmitoylated Ras proteins exchange rapidly between the ER and the cytosol, such that anterograde trafficking from the ER to the Golgi can be accounted for by nonvesicular traffic (40). Like ER-to-Golgi anterograde trafficking, retrograde traffic after depalmitoylation at the PM appears to be via the fluid phase (40, 41). Whether cytosolic chaperones anal-

ogous to RhoGDI are required for this branch of the Ras trafficking pathway remains to be determined.

Recently, K-Ras has joined the palmitoylated Ras isoforms in being found to traffic off of the PM. Fivaz and Meyers (42) showed that K-Ras was discharged from the PM of rat hippocampal neurons upon stimulation with glutamate. The GFP-K-Ras released from the PM of these cells accumulated on the Golgi apparatus. Calcium/calmodulin had previously been shown to associate with (43) and extract (44) K-Ras from membranes *in vitro*. Fivaz and Meyers (42) presented data to implicate calcium/calmodulin in the glutamate-stimulated release of K-Ras. Recently, we reported the release of K-Ras from the PM and elucidated an alternative mechanism (45). We discovered that the association of K-Ras with the PM is controlled by a farnesyl-electrostatic switch regulated by

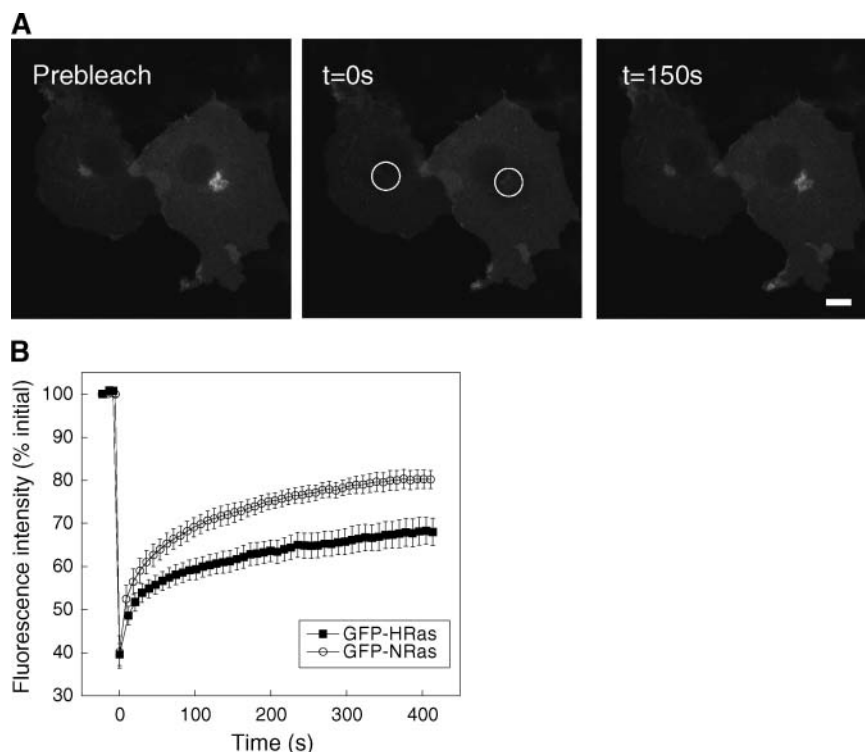


Fig. 3. Retrograde trafficking of palmitoylated Ras isoforms. A: Green fluorescent protein (GFP)-N-Ras was expressed in COS-1 cells such that at steady state expression is clearly seen on both the Golgi apparatus and the PM. The cells were treated with cycloheximide to block new protein synthesis, and then the entire Golgi was photobleached. The rapid recovery of GFP-N-Ras fluorescence on the Golgi at 150 s demonstrates that GFP-N-Ras derived from the PM can reconstitute the pool of Ras on that compartment. Further studies suggest that this retrograde movement follows depalmitoylation at the PM. B: Fluorescence recovery after photobleaching on the Golgi of GFP-N-Ras is more rapid than for GFP-H-Ras, consistent with the fact that N-Ras has only one palmitoyl modification, whereas H-Ras is modified by two. Points are plotted as mean \pm SEM. [From ref. 40.]

protein kinase C in a manner analogous to the myristoyl-electrostatic switch that regulates the membrane association of the MARKCS protein (46). Protein kinase C phosphorylates K-Ras on serine 181 within the polybasic region and thereby partially neutralizes the charge of the polylysine sequence. K-Ras discharged from the PM via the farnesyl-electrostatic switch accumulates on the ER, the Golgi, and, surprisingly, the outer mitochondrial membrane (Fig. 4). Even more surprising, phosphorylated K-Ras discharged from the PM stimulates apoptosis in a Bcl-X_L-dependent manner (45). Thus, the K-Ras farnesyl-electrostatic switch might be exploited to reverse K-Ras-driven cancer.

The sufficiency of a single phosphorylation event to destabilize the association of K-Ras with the inner leaflet of the PM suggests that the association of K-Ras with the PM is relatively weak to begin with. This idea is supported by several lines of investigation, including the observation that K-Ras can be readily extracted *in vitro* with high salt from cellular membranes (14) or even with isotonic cytosol (45). In very recent work, Silvius and colleagues (47) used an elegant technique to reveal *in vivo* the dynamic nature of the K-Ras association with membranes. Rapamycin is a cell-permeant fungal product that is capable of

binding, via different faces of the molecule, to two cellular proteins such that dimerization of these proteins can be induced in a controlled manner (48). Silvius and colleagues (47) engineered a rapamycin binding protein, FKBP, such that it is expressed on the outer mitochondrial membrane. They then expressed this construct along with K-Ras fused at its N-terminus with FRB, a protein domain that binds the other face of rapamycin. FRB-K-Ras was distributed normally in the PM of untreated cells. However, when rapamycin was added, FRB-K-Ras was seen to accumulate on mitochondria within minutes, demonstrating definitively that K-Ras is in a dynamic equilibrium with the inner leaflet of the PM, and when presented with a platform for which it has higher affinity, it will accumulate on that compartment.

ROLE OF POSTPRENYLATION PROCESSING

Most studies of the role of CAAX processing in protein trafficking and function have been performed by mutating the CAAX cysteine to another amino acid so that prenylation is not possible. Because prenylation is a prerequisite for the activities of Rce1 and Icmt, these studies

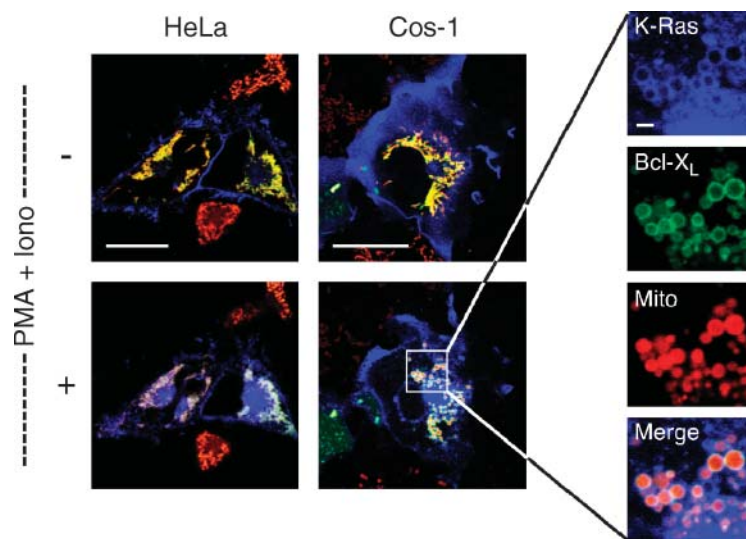


Fig. 4. K-Ras colocalizes with Bcl-X_L on the outer mitochondrial membrane after activation of protein kinase C. HeLa (left) and COS-1 (right) cells expressing cyan fluorescent protein (CFP)-K-Ras (blue) and yellow fluorescent protein (YFP)-Bcl-X_L (green) and stained with a red fluorescent mitochondrial dye are shown before (upper panels) and after (lower panels) treatment with PMA plus ionomycin (Iono). In the upper panel, CFP-K-Ras is seen on PM sheets and ruffles, and mitochondria appear yellow in transfected cells because of the overlap of YFP-Bcl-X_L and the mitochondrial dye. After activation of protein kinase C, the fragmented mitochondria appear rounded and reveal a white pseudocolor of triple overlap on their outer membranes, indicating a translocation of CFP-K-Ras to the mitochondrial surface. Bars represent 10 μ m. [From ref. 45.]

are not informative regarding the role of the individual modifications that constitute CAAX processing. The embryonic lethality of the disruption of the genes encoding either Rce1 or Icmt argues for the significant physiologic importance of postprenylation CAAX processing (49–51). The availability of cells deficient in Rce1 or Icmt, derived either from null embryos (49, 52) or from cells homozygous for floxed alleles and then infected with AdenoCre (51, 53), has permitted the study of the role of postprenylation processing. Whereas farnesylated Ras proteins were mislocalized in cells deficient in either Rce1 or Icmt, geranylgeranylated Rho proteins were not (26) (**Fig. 5**). A similar situation was observed when the localization of heterotrimer G protein γ subunits were studied; whereas farnesylated $G\gamma_1$ (found in transducin) was mislocalized in Icmt-deficient cells, geranylgeranylated $G\gamma_2$ was not (18). The results of functional studies of Ras and Rho proteins paralleled the studies of subcellular localization. Whereas either Rce1 or Icmt deficiency abrogated the transforming capacity of oncogenic K-Ras (51, 53), the geranylgeranylated Rho family proteins Rac1 and Cdc42 were capable of performing their function in regulating the actin cytoskeleton in cells deficient in Rce1 or Icmt (26). Importantly, when the farnesyl

and geranylgeranyl signals were swapped on Ras and Rho proteins, they reversed their sensitivities to Rce1 and Icmt deficiency (26).

These observations are consistent with the idea that whereas the 20 carbon geranylgeranyl modification, in and of itself, confers sufficient hydrophobicity to Rho proteins to allow them to associate with membranes and regulate effectors, the 15 carbon farnesyl modification is below the threshold for promoting membrane association and requires AAX proteolysis and carboxyl methylation. This hypothesis is consistent with *in vitro* liposome binding data that revealed that whereas methyl esterification of the α carboxyl group of a prenylated CAAX peptide affords only minimal added binding affinity when a geranylgeranyl modification is used, the increase is 20-fold for a farnesyl modification (25). Because carboxyl methylation is reversible under physiologic conditions (11), this modification may specifically regulate the affinity of farnesylated proteins for membranes. These observations suggest a reason for the evolution of two alternative forms of protein prenylation: farnesylation is a modification for proteins that require a relatively low and reversible affinity for membranes, whereas geranylgeranylation is for pro-

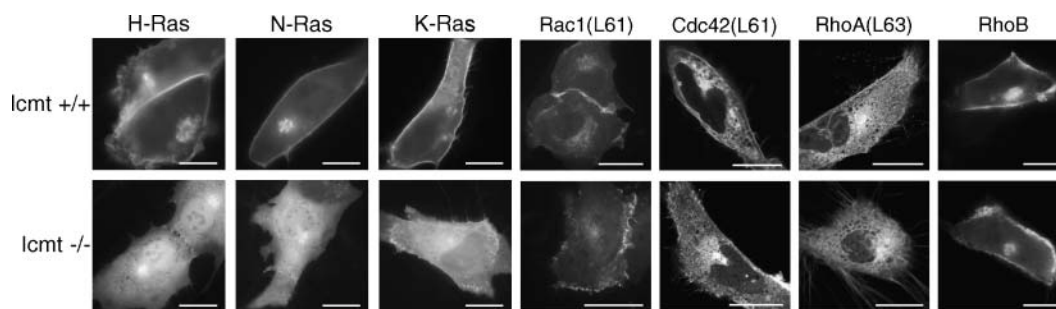


Fig. 5. Carboxyl methylation is required for the normal membrane targeting of farnesylated Ras but not geranylgeranylated Rho proteins. The Ras and Rho GTPases indicated were tagged at their N termini with GFP and expressed in murine embryonic fibroblasts derived from embryos that were either wild type (+/+) or null (-/-) at the Icmt locus. Live cells were imaged with a Zeiss 510 laser scanning microscope. Whereas farnesylated Ras proteins were mislocalized to the cytosol in Icmt-deficient cells, geranylgeranylated Rho proteins retained their normal subcellular localization in the deficient cells. Bars represent 10 μ m. [Modified from ref. 26.]

teins that require higher affinity. The recent discovery of the retrograde trafficking of Ras proteins from the PM to endomembranes (40–42, 45) described above is entirely consistent with this model. The large pool of soluble transducin in rod outer segments (54) but not seen for geranylgeranylated G proteins (55) and the effects of carboxyl methylation on transducin function (56) are also consistent with a model wherein farnesylation affords relatively weak membrane association that is regulated by reversible carboxyl methylation.

SIGNALING PLATFORMS

Until recently, the only compartment upon which Ras proteins were thought to function was the PM. The discovery that nascent Ras proteins traffic via the ER and the Golgi and that, at steady state, a significant pool of Ras is present on the Golgi raised the question of whether Ras could signal from intracellular membranes. We addressed this question by developing a genetically encoded fluorescent probe that could report when and where in living cells Ras became active (57). The probe consists of the Ras binding domain of Raf-1 fused to GFP. This probe revealed that whereas H-Ras activation was rapid and transient on the PM of fibroblasts stimulated with growth factors, there was a delayed and sustained activation on the Golgi. Surprisingly, in T-lymphocytes activated by cross-linking of the antigen receptor, activation of N-Ras and H-Ras was observed only on the Golgi and within minutes of stimulation (58, 59). The pathway upstream of Ras responsible for activation on the Golgi was mapped to Phospholipase $C\gamma/Ca^{2+}$ + Diacylglycerol/RasGRP1 and therefore is distinct from the Grb2/SOS pathway that activates Ras at the PM (58, 60). Another calcium-sensitive Ras exchange factor, RasGRF, was found to activate Ras on the ER (61). Rocks et al. (41) implicated retrograde trafficking of H-Ras as an alternative source of GTP-bound Ras on the Golgi. Downstream events in Ras signaling were also demonstrated on internal membranes. Ras stringently restricted to internal membranes with transmembrane tethers that substitute for CAAX targeting were capable of transforming cells (57). Moreover, Ras tethered to different compartments differentially regulated various downstream pathways (57, 62). Thus, Ras signaling is not restricted to the PM. Indeed, the complexities of Ras trafficking may affect signaling and help explain how a single regulatory molecule that, at the biochemical level, is a simple binary switch can have such pleiotropic effects on signaling.

TARGETS FOR ANTI-RAS DRUGS

The elucidation of CAAX processing in the late 1980s and early 1990s led to a high degree of enthusiasm for developing inhibitors of FTase as anticancer drugs. Of the three CAAX processing enzymes, FTase was chosen as the first target for several reasons. It catalyzes the first and rate-limiting step in CAAX processing. It is soluble and could

be purified. Indeed, as ER-restricted, polytopic membrane proteins that require folding within a membrane for enzymatic activity, Rce1 and Icmt pose a much greater challenge for drug discovery. Whereas Icmt acts on most Ras, Rho, and Rab proteins as well as on non-GTPase substrates and Rce1 acts on Ras and Rho proteins, the repertoire of substrates for FTase is narrower, so that one might expect less toxicity from an inhibitor. Finally, the observation that yeast lacking the Icmt ortholog, Ste14, did not have a significant growth defect diverted attention from this enzyme (9). However, the early neglect of Icmt based on yeast biology may have been a mistake, considering how different are the signaling pathways downstream of Ras in yeast versus higher metazoans. Indeed, targeted deletion of Icmt in mice proved to be embryonic lethal (49, 50), highlighting the biological differences in a dramatic manner. Nevertheless, FTase was the first CAAX processing enzyme targeted by pharmaceutical companies. FTase inhibitors have indeed been developed and have entered clinical trials [reviewed in this series by Basso, Kirschmeier, and Bishop (2)]. Although some clinical efficacy has been observed, the results are overall disappointing. From a mechanistic point of view, the biggest disappointment was the unexpected finding that, under conditions of FTase inhibition, N-Ras and K-Ras, the isoforms most often involved in human cancer, can be alternatively prenylated with a geranylgeranyl isoprenoid and retain full biological activity (63). Recognition of alternative prenylation has led to the development of GGTase inhibitors that are currently in preclinical testing and have shown promising results (64).

The surprising complexity of Ras trafficking elucidated during the past 7 years (Fig. 2) has revealed a rich landscape for therapeutic strategies that could be used instead of, or in addition to, FTase inhibition. The most obvious such strategy is to inhibit Rce1 and/or Icmt (65). Preclinical work in cells deficient in these enzymes has given some impetus to this approach. When cells from mice homozygous for a floxed Rce1 allele were transformed with K-Ras and then infected with AdenoCre to excise the Rce1 gene, the cells grew more slowly and formed fewer colonies in soft agar (51). A similar experiment revealed an even stronger inhibition of transformation when the Icmt gene was ablated (53). In support of the idea that Icmt may serve as a good target for anti-Ras therapy, Casey and colleagues (66) recently reported evidence that the mechanism of action of methotrexate, one of the oldest anticancer drugs on the market, may be in part inhibition of Icmt. Finally, these same investigators recently reported a small-molecule Icmt inhibitor identified in a high-throughput screen that has anti-Ras activity in tumor cells (67).

Our observation that farnesylated Ras proteins are sensitive to Rce1 or Icmt deficiency and geranylgeranylated Rho proteins are not (26) suggests that the anticipated enhanced toxicity of agents that inhibit postprenylation CAAX processing may not be as much of a concern as originally feared. Finally, the recent observation that Ras associated with internal membranes is capable of signaling

for transformation must be factored into the thinking about targeting the Ras trafficking pathway. Delivery to the PM can no longer be considered the only goal of an experimental therapeutic agent. Rather, inhibition of association with all cellular membranes may be required to block the biological activity of Ras.

CONCLUSIONS

CAAX processing, understood as a simple series of post-translational modifications, does not fully describe the complex processes required to deliver Ras and related proteins to the membrane compartments upon which they function. The subcellular localization of the processing events and the mechanisms whereby the biosynthetic intermediates are transported from compartment to compartment must be considered. When trafficking is taken into consideration along with the enzymatic modifications, a pathway emerges that is more complex than was previously imagined. The details of the CAAX trafficking pathway are still unfolding and should shed light on the reasons why a relatively complex pathway evolved to deliver CAAX proteins to their target membranes. Because complexity affords greater opportunity for regulation, we can expect to learn more about this aspect of Ras trafficking in the future. One practical benefit gained from the complexity of the trafficking system is that it affords a greater number of steps that can be interfered with to block the delivery of oncogenic Ras to membranes. ■

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