Research Capsule

Protein Prenylation: Key to Ras Function and Cancer Intervention?1

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The frequent association of mutated, oncogenic forms of cellular ras proteins with a broad spectrum of human malignancies has prompted intensive investigations into identifying their role in normal cellular physiology and into establishing the contribution of aberrant ras function to human tumorgenesis (1-4). Despite considerable knowledge of the structural and biochemical properties of ras proteins, we remain ignorant of the function of ras proteins. The recent discovery that ras transforming activity is critically dependent on modification (prenylation) by a farnesyl isoprenoid, an essential intermediate in cholesterol biosynthesis, has unveiled potentially important clues to ras function and identified novel prospects for cancer therapy (5-8). In this research capsule, we will summarize our present understanding of the role of protein prenylation in ras biological activity, and we will assess the promises and problems of pharmacological approaches to antagonizing prenylation. Finally, understanding the role of protein prenylation in ras function will provide a foundation for defining the significance of this previously obscure protein modification for the functions of a very diverse and growing number of isoprenylated proteins (9, 10).

CXXX-signaled Processing and Membrane Association Are Critical for ras Transforming Activity

The three human ras genes (H-, K-, and N-ras) encode four structurally related proteins of 188-189 amino acids whose functions as molecular switches are dictated by interaction with guanine nucleotides (11) (Fig. 1). The normal protein cycles between an active, GTP-complexed state, and an inactive, GDP-complexed state. Regulatory factors have been identified which stimulate guanine nucleotide dissociation (GDSs) 5 to promote ras-GTP formation (12-15) or which act as GTPase-activating proteins (ras GAP, NF1) to negatively mediate ras activity (16-21). Oncogenic ras proteins contain single amino acid substitutions at residues 12, 13, or 61 (1-4), are defective in GDP/GTP cycling, persist constitutively in the active, GTP-bound form, and chronically stimulate an as yet unidentified growth-stimulatory pathway(s) to promote the uncontrolled growth of the tumor cell (22, 23).

In addition to guanine nucleotide interactions, the second critical requirement for ras function is association with the inner face of the plasma membrane (6-8). ras proteins lack the conventional transmembrane or hydrophobic sequences associated with other membrane-associated proteins and are initially synthesized as soluble, cytoplasmic proteins (24). Their membrane association (25) is triggered by a series of closely linked posttranslational processing steps that are signaled by the conserved COOH-terminal CXXX motif present in all ras proteins (26-28) (Fig. 2). The first modification is the addition, via a thiether bond to the cysteine residue of the CXXX, of a C5, farnesyl isoprenoid moiety (29-31). The farnesyl moiety is a product of mevalonate (Fig. 3), the essential precursor of all cellular isoprenoids, including cholesterol (32). The other modifications to ras proteins are proteolytic removal of the three terminal XXX residues (33, 34) and, finally, carboxyl methylation of the now terminal farnesyl-cysteine residue (27, 33) (Fig. 2).

The critical contributions of CXXX-signaled processing to ras function have been demonstrated by two complementary lines of experimental evidence. First, COOH-terminal structural mutants of ras proteins that lack either the cysteine or XXX residues of the CXXX motif do not undergo any of the CXXX-signaled processing steps, do not associate with the plasma membrane, and are completely nontransforming (29, 35, 36). Second, inhibitors of ras isoprenylation (e.g., lovastatin) result in the accumulation of unprocessed, cytosolic proteins and also prevent ras biological activity (30, 37). Thus the CXXX-signaled modifications are clearly critical in the trafficking of ras proteins to the plasma membrane, and membrane association is apparently essential to trigger ras transformation.

Farnesyl Addition Is the Critical Modification for ras Membrane Association and Transforming Activity

Although the three linked CXXX-signaled modifications impart a hydrophobic nature to the protein and are critical for ras function, the precise contribution and functional role of each modification are presently not clear. Isoprenylation provides the greatest contribution to increased protein hydrophobicity; the proteolytic removal of the XXX residues may then enhance membrane association by allowing a better interaction of the prenyl group either with the lipid bilayer or with a possible membrane receptor(s). Carboxyl methylation may provide further enhancement to the hydrophobicity, and/or alter the conformation or ionic charge, of ras proteins. Whereas farnesylation is apparently a stable addition (31, 38), carboxyl methylation is reversible (27, 39, 40) and may have an additional function in regulating association.

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5 The abbreviations used are: GDS, guanine nucleotide dissociation stimulator; PDE, phosphodiesterase; HMG, hydroxymethylglutaryl.
with the plasma membrane. The observations that the degree of carboxyl methylation of 21–29-kilodalton proteins varied with cellular growth state (41), exhibited rapid methylation/demethylation by separate enzymatic steps (40), and exhibited a guanine nucleotide dependence (42) are supportive of this possibility. However, there is presently no evidence for regulated methylation/demethylation of ras proteins in particular (33).

Since blocking ras isoprenylation also prevents the subsequent two CXXX-signaled modifications (Fig. 2), the exact contribution of the individual CXXX-signaled modifications to membrane association and transforming activity has been difficult to assess. However, recent in vitro and in vivo studies have provided some preliminary clues. First, reconstitution of each CXXX-signaled modification in vitro using a reticulocyte lysate assay determined that the isoprenoid modification alone promoted a limited membrane association of K-ras4B, which was increased both by proteolytic removal of the XXX residues and by carboxyl methylation (43). Second, a mutant K-ras4B protein (Tyr-187), which undergoes isoprenylation but not proteolysis or carboxyl methylation in vivo, nevertheless exhibited significant membrane association (50%) and transforming activity (44). Third, a yeast mutant (ste14) that lacked ras methyl transferase activity exhibited no impairment of ras function or cell viability, and expressed a partially membrane-associated RAS protein (45). Since RAS function is essential for yeast viability, these results suggest that a nonmethylated RAS protein retains at least some degree of normal function. Altogether, these results suggest that, although all three CXXX-signaled modifications are required for optimal membrane association, isoprenylation alone is sufficient to promote the biological activity of both normal and oncogenic ras proteins.

The suggestion that isoprenylation is the critical modification for ras function contrasts sharply with studies of the yeast a-factor mating hormone. a-factor undergoes the same three CXXX-signaled modifications as ras, and, like unprocessed ras proteins, unprocessed a-factor peptides are completely inactive for biological activity (46, 47). However, since both nonfarnesylated variants and nonmethylated variants of a-factor are drastically impaired in biological activity, both modifications are critical for a-factor function (46, 48, 49). Thus, the specific contribution of each CXXX-signaled modification to protein function may well differ among different isoprenylated proteins. Furthermore, although carboxyl methylation may be dispensable for ras transforming activity, it is possible that this modification may still play an integral role in the function of normal ras proteins in mammalian cells.

CXXX-signaled Modifications Require Complementation by Other COOH-terminal Lipid Modifications and Sequences to Promote Full ras Function

Although the CXXX-signaled modifications are clearly critical for the plasma membrane association of ras proteins, observations from the studies of ras mutants and of other similarly modified proteins suggest that these modifications alone can confer neither optimal membrane affinity nor specific association of ras proteins with the plasma membrane. First, the lamin B protein undergoes the same three CXXX-signaled modifications as ras (50), yet it localizes to the nuclear envelope (51, 52). Second, a significant number of prenylated proteins are cytosolic rather than membrane associated (38, 53, 54). Instead, observations from several studies (29, 55, 56) suggest that sequences directly upstream of the CXXX motif (cysteines or lysine-rich sequences) provide secondary membrane-targeting signals that complement the CXXX-signaled modifications to promote both the avidity and specificity of the plasma membrane interactions.
required for ras function.

In the case of the H-, N-, and K-ras4A proteins, a subsequent modification by the fatty acid palmitate is signaled by cysteine residue(s) upstream of the CXXX motif (Fig. 1). Mutant ras proteins that lack these cysteine residues retain CXXX-signaled modifications but do not undergo palmitylation, no longer display a plasma membrane localization, and are predominantly cytosolic proteins (29, 55). Thus, palmitate complements the CXXX-signaled modifications to promote full association with the plasma membrane. Additionally, since palmitate has a very high turnover rate (>70-fold higher than the half-life of ras proteins), palmitylation may potentially play an additional regulatory role in ras biological activity (57).

The K-ras4B protein, on the other hand, lacks any upstream cysteines; instead, it contains stretches of lysine residues (Fig. 1). When the positively charged lysine residues were replaced with neutral glutamines, the protein was rendered cytosolic (55). Replacement of the lysines with similarly charged arginine residues retained membrane association, suggesting that the lysine residues provide a positively charged domain that may promote interaction with negatively charged phospholipid head groups (56). Thus, these lysine residues serve a function analogous to that of palmitylation. However, since these residues, in contrast to palmitate, are stable elements of K-ras4B, the K-ras4B protein may associate with the plasma membrane in a manner distinct from its palmitylated ras counterparts.

The importance of the palmitate- and lysine-rich components for the plasma membrane localization of ras proteins has also been demonstrated by characterizing the consequences of introducing different ras COOH-terminal sequences to heterologous, cytosolic proteins. For example, addition of tetrapeptide sequences representing the ras CXXX sequences to the COOH-terminus of Protein A promoted the isoprenylation of these chimeric proteins, but localization to the plasma membrane was not observed (29). However, the addition of the COOH-terminal 10 residues of H-ras (including both palmitylated cysteines) or 17 residues of K-ras4B (including the lysine-rich sequences) promoted efficient association of Protein A with the plasma membrane (56). Thus, the plasma membrane targeting information is apparently found in the ras sequences directly adjacent to the CXXX motif. An analogous result was observed in studies of the ras-related rab/YPT proteins, in which their localization to specific membranes of the endocytic pathway was dictated by COOH-terminal sequences immediately upstream of the isoprenylated terminal cysteine residues (58).

An interesting, and somewhat unexpected, observation has been that, although the presence of palmitylation or lysine-rich sequences is critical for ras association with the plasma membrane, oncogenic ras proteins that lack these elements but retain the CXXX-signaled modifications also retain strong transforming activity in NIH 3T3 cells (29, 55). These results suggest that an association with the plasma membrane may not be a critical requirement for ras transforming function. They also suggest that the critical requirement for CXXX-signaled modifications in transforming activity may involve a role unrelated to promoting plasma membrane interactions. One possible role may be to facilitate the interaction of ras proteins with critical regulatory proteins. For example, GDP/GTP exchange on K-ras4B is dependent on the presence of the CXXX-signaled modifications (15). Alternatively, since these transformation analyses have been performed in the rather permissive NIH 3T3 cell line, it is still possible that membrane association may be required for the biological activity of oncogenic ras in tumor cells. Finally, since oncogenic ras proteins are chronically activated, and therefore presumably independent of stimulation via upstream mitogenic signals, a plasma membrane association may no longer be important for oncogenic ras function but may remain essential
Isopentenyl (FARNESYLATED)

Fig. 3. Mevalonate and isoprenoid biosynthetic pathway. Mevalonate is the essential precursor for all isoprenoids, including cholesterol and the C15 farnesyl and C20 geranylgeranyl moieties that are added to proteins by farnesyl (FTase) or geranylgeranyl (GGTase) transferase. HMG CoA reductase inhibitors (e.g., lovastatin) block mevalonate biosynthesis and prevent all isoprenoid biosynthesis.

for normal ras function. Thus, the significance of membrane association for ras function remains to be clarified.

The ras proteins display their greatest divergence in the COOH-terminal 19–20 residues directly adjacent to the CXXX motif (designated the hypervariable domain; see Fig. 1). Thus, in addition to cysteine and lysine residues, other sequences unique to each ras protein may also contribute to the avidity and specificity of plasma membrane interactions, and possibly to other roles important for ras function. Since the membrane association of ras proteins is presumably required for promoting interaction with membrane-associated upstream and downstream components of their signal transduction pathways, the fact that each of the four human ras proteins possesses distinct COOH-terminal modifications and sequences (Fig. 1) may therefore reflect their association with distinct pathways.

Normal and Oncogenic ras Proteins May Have Different Membrane Association Requirements for Biological Activity

Although the majority of studies have concentrated on the role of posttranslational modifications on ras transforming activity, several observations suggest that the membrane association requirements of normal versus oncogenic ras proteins may be distinct. First, studies have shown that ras modification by the fatty acid myristate can functionally replace the CXXX-signaled modifications to promote the membrane association required for oncogenic ras transforming activity (59, 60). These results suggested that the CXXX-signaled modifications are not required for the intrinsic biological activity of ras proteins and that these modifications merely provide a nonspecific membrane targeting signal that can be replaced with myristate. However, the unexpected activation of transforming activity when normal ras is modified by myristate (60) suggests that myristate cannot correctly facilitate normal ras function, which instead may require the precise contributions of CXXX-signaled modifications.

A second distinction between the membrane association requirements of normal and oncogenic ras proteins was observed with studies addressing the importance of specific isoprenoid modification in ras function (56, 61). Whereas ras proteins are modified by a C15 farnesyl isoprenoid moiety, the majority of mammalian proteins are modified instead by a C20 geranylgeranyl isoprenoid group (54, 62, 63). In particular, nearly all of the other members of the ras superfamily are modified by geranylgeranyl, and to date, none of these related proteins has demonstrated oncogenic activity (8). Therefore, two recent studies have addressed whether addition of either isoprenoid can promote ras biological activity. Interestingly, CXXX mutants of oncogenic ras proteins which are specifically modified by a geranylgeranyl, rather than farnesyl, isoprenoid retain full transforming activity (56, 61). In contrast, geranylgeranyl-modified normal ras proteins were observed to possess growth-inhibitory activity in transfected NIH 3T3 mouse fibroblasts (61). Similarly, studies in yeast suggested that only farnesylated, but not geranylgeranylated, RAS protein is capable of stimulating adenylate cyclase activation (64). These observations indicate that oncogenic ras protein requires only a generalized membrane association which can be equally facilitated by different lipid additions, whereas normal ras may require specific modification by a farnesyl isoprenoid for its function.

The basis for specific isoprenoid modification for normal ras function is presently unclear. One possibility is that there are specific receptors that interact with either farnesylated or geranylgeranylated proteins. Although such prenylation-specific receptors have not been identified, the recent observation that geranylgeranyl-modified forms of K-ras4B (56) or H-ras* proteins exhibit different intracellular localizations from their authentic farnesylated counterparts is consistent with this possibility. Furthermore, there are indications from other studies that specific receptors for lipid-modified proteins (e.g., lamin B p60v-src) do exist (65, 66). Thus, the identification of a membrane component that specifically interacts with farnesylated ras proteins may provide a clue for identifying the elusive biochemical function of these proteins.

Isoprenylation Is a Common Modification of Other Proteins

Although modification of proteins by prenylation has been known for some time (67), and was first associated with yeast mating factors (46), only recently have the identity of isoprenylated proteins in mammalian cells

* A. D. Cox and C. J. Der, unpublished observations.
been established (6–10). Although the nuclear lamins and ras proteins represent the first characterized isoprenylated mammalian proteins, it is becoming apparent that other proteins that undergo this lipid modification represent critical components involved in the regulation of a range of diverse cellular functions.

The majority of recently identified isoprenylated proteins are additional members of the ras protein superfamily, whose specific members have been implicated in the regulation of intracellular vesicular transport (rab/YPT1) (68), cytoskeletal organization (rho) (69), the oxidative burst of phagocytic cells (rac) (70, 71), and negative growth (rap/Krev-1) (72). In those cases that have been studied, isoprenoid modification has been determined to be important for the involvement of ras-related proteins in these activities. For example, rab/YPT protein isoprenylation is apparently important for the association of these proteins with specific intracellular compartments (73) and in their regulation of intracellular transport processes (74, 75). rab/YPT proteins are believed to cycle between distinct membrane subcompartments and between membrane-associated and cytosolic states to facilitate transport processes (68, 76), and isoprenylation is therefore expected to play a key role in rab function.

A number of isoprenylated proteins other than ras also represent critical elements in signal transduction pathways. Isoprenylation is a critical modification for the γ subunits of the heterotrimeric G proteins, and this modification is important for their function in receptor-mediated signal transduction pathways in mammalian (77) and yeast (78) cells. In addition to the γ subunit of transducin, several other components of the retinal signal transduction pathway are also modified by isoprenylation. Rhodopsin kinase is modified by a farnesyl moiety, and its serine/threonine phosphorylation activity is markedly reduced in the absence of isoprenylation (79). A third component of this pathway, retinal cyclic GMP PDE, is composed of four subunits, of which two are modified by farnesyl (γ subunit) or geranylgeranyl (β subunit) moieties (80). Prenylation of PDE is believed to be important for its membrane attachment. Finally, several additional signal transduction pathways are blocked by inhibitors of protein prenylation, implicating the involvement of isoprenylated proteins in these processes (81, 82).

In summary, determining the role of isoprenylation in ras function will provide insights into the contributions of isoprenoid modification to the function of other isoprenylated proteins that are involved in signal transduction, intracellular vesicular transport, and other diverse cellular processes. Present evidence suggests that, like ras proteins, the biological activities of these proteins are also dependent on isoprenoid addition. Generally, isoprenoid addition is believed to influence membrane and protein-protein interactions, to contribute to specific intracellular localizations, and to regulate biological activity. However, it is likely that the precise role for protein prenylation will be distinct and unique for each isoprenylated protein.

Protein Prenyl Transferases: Targets for Cancer Treatment?

The discovery that the function of oncogenic ras protein is critically dependent on its modification by an essential intermediate in cholesterol biosynthesis has attracted significant interest in blocking ras isoprenylation as a means of cancer chemotherapy (5). This possibility seemed promising when early studies demonstrated the ability of drugs such as lovastatin and compactin (32) to induce cellular growth arrest of cell lines in vitro (83–86) and of tumors in vivo (87). Since these fungal compounds inhibit mevalonate synthesis (Fig. 3), and consequently all isoprenoid biosynthesis, one possible basis for growth inhibition was thought to be via prevention of ras isoprenylation. However, it has been shown that this growth arrest is not a consequence of antagonizing ras-transforming activity by blocking ras processing (88) but is presumably due to perturbations of other mevalonate-dependent processes.

Although general inhibitors of mevalonate biosynthesis (e.g., lovastatin and compactin) have been useful clinically for lowering cholesterol levels, their use in cancer chemotherapy has several significant drawbacks. First, since mevalonate is the precursor for all isoprenoid biosynthesis, including cholesterol (Fig. 3), lovastatin will clearly affect other isoprenylation pathways. Second, it has been shown that protein prenylation is much more sensitive than cholesterol biosynthesis to the effects of lovastatin (89). Finally, ras proteins represent but a small fraction of isoprenylated proteins. Thus, the relatively nonspecific action of these mevalonate inhibitors may limit their usefulness for blocking oncogenic ras function in tumors.

A more effective approach to pharmacological intervention in oncogenic ras activity is therefore the design of drugs that block ras isoprenylation farther down the mevalonate pathway (Fig. 3). The identification and isolation of a protein:prenyl transferase that catalyzes the isoprenoid addition to ras proteins (90–92) has provided a promising target for such intervention. Furthermore, although protein prenylation is a relatively common modification, recent studies demonstrate that there is a family of protein:prenyl transferases, each of which may modify only a subset of isoprenylated proteins (Table 1). For example, isoprenylated proteins that terminate in CXXX motifs are specifically modified by either a farnesyl or a geranylgeranyl transferase (61, 93–96), and present evidence indicates that geranylgeranyl-modified proteins that terminate in either CC, CXC, or CXX motifs are processed by different geranylgeranyl transferases (73, 95, 97, 98). Therefore, it is possible that specific blockage of ras farnesyl transferase will not perturb the processing and function of the majority of other isoprenylated proteins.

However, despite the promise of blocking oncogenic ras function via antagonism of ras farnesyl transferase activity, there are still several potential areas of nonspecific complications to this approach. First, other farnesylated cellular proteins (e.g., nuclear lamins, transducin γ) apparently are substrates for the same farnesyl transferase as ras proteins (90), so perturbation of their functions in cell division and visual signal transduction might be nonspecific consequences of such attempts to interfere with the function of oncogenic ras. Second, present evidence suggests that isoprenylation is critical for the biological activity of normal, as well as oncogenic, ras proteins. Since ras proteins are expressed in virtually all tissues (99, 100), and since normal ras function is critical for cellular proliferation (101), antagonizing normal ras function may result in serious consequences for the viability of normal cells. Nevertheless, if these compli-
cations can be overcome, inhibitors of ras farnesyl transferase activity may one day provide effective and selective inhibition of tumor cells harboring oncogenic ras proteins.

Synthetic peptides corresponding to the CXXX sequence of ras proteins have been demonstrated to be potent inhibitors of ras prenylation in vitro (90–92) and ras biological activity in vivo (37). Although peptides provide the necessary specificity to target ras isoprenylation, principal limitations for the use of such peptides in cancer treatment will be in their introduction into cells and in delivery of these inhibitors to their intracellular targets. Nevertheless, such peptide inhibitors have been very useful for characterizing ras farnesyl transferase specificity, and improvements in the design and delivery of peptides may hold promise for this approach to cancer chemotherapeutics.

The enzymes that catalyze the remaining two CXXX-signaled modifications and palmitylation might provide additional targets for blocking ras function. Membrane-associated enzymatic activities for proteolysis (43, 102), carboxyl methylation (43, 45, 103–105), and palmitate addition (106) are only now beginning to be isolated and characterized. However, these enzymatic activities are apparently less specific in their substrate utilization than is the ras farnesyl transferase. For example, the carboxyl methyl transferase responsible for modification of ras proteins apparently also methylates geranylgeranyl-modified CXXX proteins (39, 40, 45, 103, 104). Additionally, since farnesylation alone is sufficient for ras transforming activity (44), there may be only a limited benefit in blocking these modification steps to prevent oncogenic ras function in tumors.

Future Directions

The finding that ras proteins are isoprenylated by a farnesyl isoprenoid moiety has stimulated studies addressing several important questions. First, it is clear that isoprenoid modification is the key addition that triggers ras biological activity, and it is likely that isoprenylation promotes the interaction of ras proteins with targets necessary for transformation. Therefore, isoprenylation may provide a convenient handle on identifying the key components for ras biochemical function, and an important aim for future studies will be to determine whether receptors for farnesylated proteins exist. Second, since protein prenylation is emerging as a modification important for the function of a diverse group of proteins, understanding the contribution of isoprenylation to ras biological activity may establish important principles for understanding the contributions of prenylation to other proteins. Finally, further studies on the enzymology and substrate specificities of protein:prenyl transferases will provide important information for the identification and design of pharmacological agents that preferentially block ras isoprenylation. The identification of such drugs will then allow determination of whether specifically blocking ras function will be useful for cancer chemotherapy and whether oncogenic ras contributes clinically to the aberrant growth properties of the malignant cell.

Acknowledgments

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References


Table I Summary of isoprenylated proteins and protein prenyl transferases

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<th>Sequence</th>
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* Farnesylation.
? Carboxylmethylation.
• Carboxyl methyl transferase.
• 2',3'-Cyclic nucleotide phosphodiesterase.


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