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Biochemistry

Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation

(isoprenylation/palmitoylation/carboxyl-terminal processing/membrane association)

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ABSTRACT **Oncogenic forms of ras proteins are synthesized in the cytosol and must become membrane associated to cause malignant transformation. Palmitic acid and an isoprenoid (farnesol) intermediate in cholesterol biosynthesis are attached to separate cysteine residues near the C termini of H-ras, N-ras, and Kirsten-*ras* (K-*ras*) exon 4A-encoded proteins. These lipid modifications have been suggested to promote or stabilize the association of ras proteins with membranes. Because preventing isoprenylation also prevents palmitoylation, examining the importance of isoprenylation alone has not been possible. However, the oncogenic human [Val^{12}]K-ras 4B protein is not palmitoylated but is isoprenylated, membrane associated, and fully transforming. We therefore constructed mutant [Val^{12}]K-ras 4B proteins that were not isoprenylated to examine the effects of isoprenylation in the absence of palmitoylation. The nonisoprenylated mutant proteins both failed to associate with membranes and did not transform NIH 3T3 cells. In addition, inhibition of isoprenoid and cholesterol synthesis with the drug compactin also decreased [Val^{12}]K-ras 4B protein isoprenylation and membrane association. These results unequivocally demonstrate that isoprenylation, rather than palmitoylation, is essential for ras membrane binding and ras transforming activity. These findings clearly indicate the biological significance of ras protein modification by farnesol and suggest that this modification may be important for facilitating the processing, trafficking, and biological activity of other isoprenylated proteins. Because K-*ras* is the most frequently activated oncogene in a wide spectrum of human malignancies, study of this pathway could lead to important therapeutic treatments.**

The three cellular *ras* genes (H-, N-, and K-*ras*) encode related 21-kDa guanine nucleotide-binding proteins (GDP and GTP) (1). The biologic function of normal ras proteins is unknown. However, activated ras proteins, containing substitutions at residues 12, 13, or 61, can malignantly transform cells and are frequently detected in a wide spectrum of human neoplasms (1). Alterations in GTPase or GTP-binding properties of oncogenic ras proteins due to these activating substitutions favor formation of the active, GTP–ras complex (2–4).

The ras proteins are synthesized in the cytosol as inactive precursors and must undergo a series of posttranslational modifications to become membrane-associated and biologically active (5–9). The four C-terminal amino acids of ras proteins comprise a consensus sequence, CAAX, in which C represents cysteine, A represents any aliphatic amino acid, and X represents any amino acid; this motif is believed to signal the posttranslational modifications of ras proteins. Specifically, these posttranslational modifications include (*i*) removal of the three C-terminal amino acid residues, (*ii*) carboxyl methylation of the C-terminal cysteine, (*iii*) attachment of palmitic acid to a cysteine residue(s) near the C terminus, and (*iv*) attachment of an isoprenoid intermediate in cholesterol biosynthesis (farnesol) to the cysteine of the CAAX motif (6–12).

Previous studies demonstrated that mutant H- and N-ras proteins, which contained an amino acid substitution for Cys-186 (the cysteine of the CAAX motif), were not palmitoylated, membrane associated, or biologically active (5). This suggested that Cys-186 might be the site of palmitoylation and that palmitoylation was the primary posttranslational modification responsible for promoting or stabilizing ras protein membrane association. However, more recent studies have demonstrated that palmitoylation occurs at Cys-181 and -184, or Cys-181 in H- and N-ras proteins, respectively (6) and that Cys-186 may be the site of isoprenylation (6, 7). Because substitutions for Cys-186 also prevent isoprenylation (6), these recent studies suggest that isoprenylation, rather than palmitoylation, may be necessary or sufficient for ras protein membrane association and biologic activity. Unfortunately, because preventing isoprenylation also prevents palmitoylation, it has been difficult to directly determine the specific role of isoprenylation in ras protein membrane association and biologic activity. To circumvent this problem, we studied the effect of isoprenylation on the membrane association and biologic activity of oncogenic human Kirsten-ras exon 4B (K-ras 4B) proteins because these native proteins are not palmitoylated but are isoprenylated, membrane associated, and fully capable of malignantly transforming cells. Because the CAAX motif is believed to signal the posttranslational modifications of ras proteins (6, 7, 9–13), we used oligonucleotide-directed mutagenesis to introduce substitutions in the K-ras 4B CAAX consensus sequence that might prevent isoprenylation. In addition, we also studied the effect of compactin, a drug that inhibits isoprenoid and cholesterol synthesis, on K-ras 4B isoprenylation and membrane association. The results reported here clearly indicate that isoprenylation, rather than palmitoylation, is essential for the membrane association and transforming activity of K-ras 4B proteins.

MATERIALS AND METHODS

Construction of K-ras 4B Mutants. Oligonucleotide-directed mutagenesis and construction of DNA vectors were done as described (14). Two K-ras 4B mutants were generated: KSER, in which Cys-185 of the CAAX sequence was

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Abbreviations: K-*ras*, Kirsten *ras*; CAAX, protein sequence in which C = cysteine, A = aliphatic, and X = any amino acid; MVA, mevalonic acid.

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changed from cysteine (TGT) to serine (AGT), and KTER, in which the codon immediately following Cys-185 was changed to a termination codon (TGA). These mutations were introduced into a transforming human K-ras 4B cDNA (KVAL12) that encodes an additional non-ras 10-amino acid N-terminal leader (15). KVAL12 was isolated from the human colon carcinoma cell line SW480 and encodes a protein activated by a codon 12 mutation that changes glycine to valine (15). The nucleotide sequences of the mutant clones were verified by dideoxynucleotide chain-termination sequencing (14). KVAL12, KSER, KTER, and normal human K-ras 4B sequences (WT), which has glycine at position 12, were introduced into the pZIP-NeoSV(X)1 retrovirus expression vector (14).

Cell Culture and NIH 3T3 Transfection Assay. NIH 3T3 cells were transfected by the calcium phosphate precipitation technique with 10 ng of KVAL12, 100 ng of WT, and either 100 ng or 5 μg of KSER or KTER plasmid DNA per 60-mm dish. For focus-forming transformation assays, transfected cells were maintained in Dulbecco's modified Eagle's medium/10% calf serum, and transformed foci were quantified after 14 (KVAL12) or 21 (KSER, KTER, and WT) days (14). For soft agar growth or protein analyses, transfected cells expressing the different K-ras 4B proteins were established by selection in growth medium containing G418 at 400 μg/ml (Geneticin, GIBCO). To assay for growth in soft agar, G418-selected cells were suspended in 0.3% agar growth medium at a final density of 2×10^4 per 60-mm dish. Colony formation was monitored for up to 14 days (14).

Assessment of K-ras 4B Isoprenylation. To determine whether KSER and KTER mutant proteins were isoprenylated, G418-selected cells were labeled overnight with *R,S* [5-^{3}H]mevalonolactone (New England Nuclear) at 100 μCi/ml in the presence of 50 μM compactin (a gift of M. S. Brown and J. L. Goldstein, University of Texas Southwestern Medical Center, Dallas) (7, 16). [5-^{3}H]Mevalonolactone is converted to [^{3}H]mevalonic acid (MVA), and MVA is a precursor of the isoprenoid farnesol (17–21). Inhibition of endogenous MVA synthesis with compactin enhances the degree of labeling with exogenous [^{3}H]mevalonolactone (6, 7). Cells were lysed in high SDS/Tris/RIPA buffer (0.15 M NaCl/50 mM Tris·HCl, pH 7.0/1% Trasylol/1% sodium deoxycholate/1% Nonidet P-40/0.5% SDS/0.1 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol), centrifuged at 16,000 rpm for 60 min to remove insoluble material (7, 16), and ras proteins were immunoprecipitated with anti-ras monoclonal antibody Y13-259 (7, 16), and analyzed by SDS/PAGE (7, 16) and fluorography.

Subcellular Localization of K-ras 4B Proteins. G418-selected NIH 3T3 cells transfected with the appropriate plasmid DNA were labeled overnight with [^{35}S]methionine/cysteine (Tran^{35}S-label, ICN) at 200 μCi/ml (1 Ci = 37 GBq), and crude membrane-containing fractions were separated from cytosol by hypotonic lysis and centrifugation at 100,000 $\times$ *g* (7, 16). Separated fractions were clarified by centrifugation at 16,000 rpm in a Sorvall SM-24 rotor for 30 min, and ras proteins were immunoprecipitated from the separated fractions and analyzed by SDS/PAGE and fluorography.

Subcellular Localization of K-ras 4B Proteins in Compactin-Treated Cells. G418-selected NIH 3T3 cells transfected with KVAL12 were labeled overnight with [^{35}S]methionine/cysteine at 200 μCi/ml with or without 50 μM compactin and/or 2 mM mevalonolactone. Cells were hypotonically lysed, an aliquot of the lysate was removed, and the remaining lysate was separated into crude membrane- and cytosol-containing fractions. KVAL12 proteins from the total (nonfractionated) cell lysate, and the cytosolic and membrane-containing fractions were then immunoprecipitated and analyzed as described above.

RESULTS

Isoprenylation of K-ras 4B Proteins. Because the CAAX motif is believed to signal one or more of the posttranslational modifications of ras proteins (6, 7, 9–13), we used oligonucleotide-directed mutagenesis to introduce substitutions in the CAAX sequence that might prevent ras protein isoprenylation. We constructed a KSER mutant, in which a serine was substituted for the cysteine of the CAAX sequence, and a KTER mutant, in which a termination codon was introduced immediately after this cysteine residue. All [Val^{12}] K-ras 4B constructs encoded ras proteins with an N-terminal leader sequence that produced proteins with electrophoretic mobilities distinct from endogenous ras proteins. A normal human K-ras 4B (containing glycine at position 12) construct (designated WT), which lacks the N-terminal leader sequence, was included in all experiments to exclude the possibility that the Val-12 mutation or the leader sequence affected K-ras 4B isoprenylation or membrane association. To determine whether KSER and KTER mutant proteins were isoprenylated, transfected cells were labeled with [^{3}H]MVA and ras proteins in these cells were immunoprecipitated and analyzed by SDS/PAGE and fluorography (7, 16). No [^{3}H]MVA-derived label could be detected in KSER or KTER protein, although label was apparent in KVAL12, WT, and ras proteins endogenous to NIH 3T3 cells (Fig. 1). Parallel experiments with [^{35}S]methionine/cysteine-labeled cells demonstrated that KSER and KTER proteins were expressed at significantly greater levels than the quantity of endogenous ras proteins in these cells (see Fig. 2). Therefore, the inability to detect [^{3}H]MVA-derived label in KSER or KTER protein did not result from low levels of protein expression or protein instability.

Subcellular Location of K-ras 4B Proteins. To examine the role of isoprenylation in K-ras 4B membrane binding, cells were separated into crude membrane- and cytosol-containing fractions, and the subcellular location of KSER and KTER proteins was compared with that of KVAL12, WT, and endogenous ras proteins. The nonisoprenylated KSER and KTER proteins were cytosolic, whereas the majority of KVAL12, WT, or endogenous ras proteins were associated with membrane fractions (Fig. 2). In addition, analysis of cells by immunofluorescence also revealed that KVAL12 and

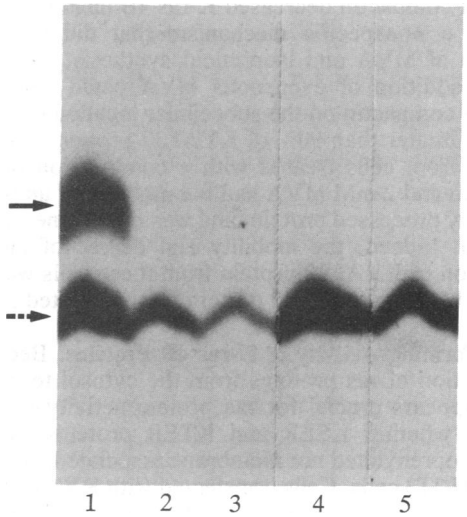

FIG. 1. Detection of label derived from [^{3}H]MVA in K-ras 4B proteins: KSER and KTER proteins are not isoprenylated. Lanes: 1, KVAL12; 2, KTER; 3, KSER; 4, WT; 5, endogenous ras proteins from NIH 3T3 cells. Solid arrow, Position of KVAL12 protein; dashed arrow, position of WT and endogenous ras proteins.

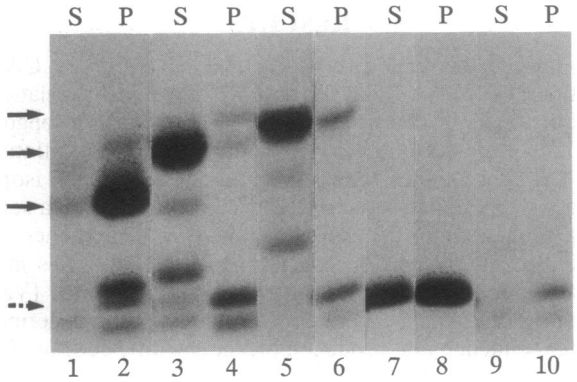

FIG. 2. Intracellular location of [^{35}S]methionine/cysteine-labeled K-ras 4B proteins: KSER and KTER proteins are associated with cytosolic rather than membrane fractions. Lanes: 1 and 2, KVAL12; 3 and 4, KTER; 5 and 6, KSER; 7 and 8, WT; 9 and 10, endogenous ras proteins from NIH 3T3 cells. Solid arrows, Positions of K-ras 4B proteins; dashed arrow, position of WT or endogenous ras proteins. Crude cytosolic and membrane-containing fractions are designated by S and P, respectively. The different mobilities observed with KSER, KTER, and KVAL12 proteins result from their different lengths, different amino acid sequences, or their lack of other posttranslational modifications.

WT, but not KSER or KTER proteins, were associated with plasma membranes (data not shown).

Effect of Compactin on Subcellular Location of K-ras 4B Proteins. To assess the contribution of isoprenylation to membrane association in a second way (6, 9), we determined whether inhibition of MVA and isoprenoid synthesis with the hydroxymethylglutaryl-CoA reductase inhibitor, compactin (22), would prevent membrane association of KVAL12 proteins. To avoid cell toxicity, we used a concentration of compactin (50 μM) that did not prevent all ras protein isoprenylation (see Fig. 1). As a result, KVAL12 proteins immunoprecipitated from compactin-treated, [^{35}S]methionine/cysteine-labeled cells existed in two forms. Approximately 50% had the mobility of an isoprenylated, fully processed protein and was associated with membranes (Fig. 3*A*, dashed arrow); the other 50% (Fig. 3*A*, solid arrow) had the mobility of nonisoprenylated, precursor KVAL12 protein detected after a 10-min labeling period (data not shown) and remained associated with the cytosol. To exclude the possibility that compactin decreased K-ras 4B membrane association by a nonspecific mechanism that did not involve inhibition of MVA and isoprenoid synthesis, we assessed whether addition of exogenous MVA could override the effects of compactin on the subcellular location of KVAL12 protein. Greater than 90% of KVAL12 protein immunoprecipitated from cells treated with a combination of 50 μM compactin and 2 mM MVA had the mobility of an isoprenylated, fully processed protein, and was membrane associated (Fig. 3*B*). Indeed, the mobility and degree of membrane association of KVAL12 protein from these cells were indistinguishable from KVAL12 protein from untreated cells (Fig. 3*C*).

Transforming Activity of K-ras 4B Proteins. Because the translocation of ras proteins from the cytosol to cell membranes appears crucial for ras protein activity (5, 16), we assessed whether KSER and KTER proteins, which are neither isoprenylated nor membrane associated, could transform NIH 3T3 cells. Cells transfected with KVAL12 DNA at 10 ng/dish exhibited strong focus-forming activity (Table 1). In contrast, cells transfected with up to 500-fold greater concentrations of KSER or KTER DNA failed to develop transformed foci or to grow in soft agar. The nontransforming properties of these two mutant proteins show that isoprenylation is essential for the transforming activity of K-ras 4B.

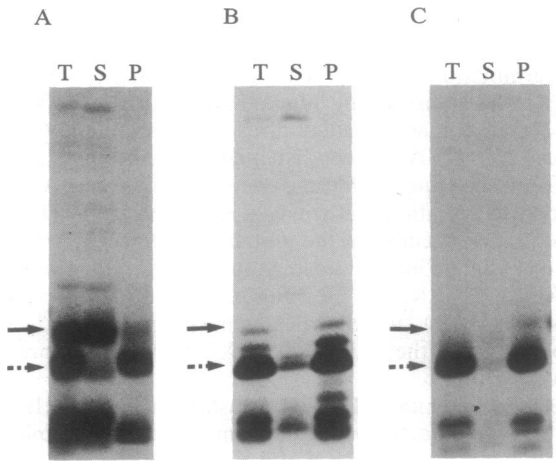

FIG. 3. Effect of compactin and MVA on subcellular localization of [^{35}S]methionine/cysteine-labeled KVAL12 protein. (*A*) Effect of compactin (50 μM) on intracellular location: compactin decreases the membrane association of KVAL12 protein. (*B*) Effect of the combination of exogenous MVA (2 mM) and compactin (50 μM) on intracellular location: MVA reverses the effects of compactin. (*C*) Intracellular location of KVAL12 protein from untreated cells. Solid arrow, Position of nonisoprenylated, precursor KVAL12 protein; dashed arrow, position of isoprenylated, fully processed KVAL12 protein. Total (nonfractionated) cell lysate, crude cytosolic fractions, and crude membrane-containing fractions are designated by T, S, and P, respectively.

Because the [Val^{12}]-activating mutation causes ras proteins to exist primarily in the active, GTP-bound form (2–4), we examined the possibility that the inability of KSER or KTER protein to cause transformation might have resulted from a shift of the nucleotide bound to these proteins from GTP to GDP. Two-dimensional thin-layer chromatography of radioactive guanine nucleotides released from ras proteins isolated from cells labeled *in vivo* with [^{32}P]orthophosphate (16) showed no significant differences in the nucleotide bound to KSER, KTER, or KVAL12, with GTP representing >75% of the bound nucleotide (data not shown). The inability of KSER or KTER protein to transform cells thus appears to result from its cytosolic location, rather than from alterations in its intrinsic biochemical properties.

Because genetic mutations that prevent K-ras 4B isoprenylation abolish transforming activity, we assessed whether compactin, which also inhibits isoprenylation, could reverse cellular transformation. KVAL12-transformed NIH 3T3 cells treated with 50 μM compactin did not revert to a normal flat

Table 1. Biochemical and biological properties of K-ras 4B proteins

K-ras 4B protein	Isoprenylation	Membrane association	Foci/μg of DNA*	Soft agar growth†
KVAL12	+	+	4160	+
KSER	−	−	0	−
KTER	−	−	0	−
WT	+	+	0	ND

*NIH 3T3 cells were transfected with 10 ng of KVAL12, 100 ng of WT, and either 100 ng or 5 μg of KSER or KTER plasmid DNA per 60-mm dish. Transformed foci were quantified after 14 (KVAL12) or 21 (KSER, KTER, and WT) days. Cells transfected with up to 5 μg per dish of KSER and KTER DNA were negative for the appearance of transformed foci for up to 30 days.

†NIH 3T3 cells were transfected with 5 μg of each plasmid DNA and selected in G418-containing growth medium. Cells (2×10^4) were plated in soft agar, and colony formation was monitored for up to 14 days. +, Colonies of 8–>200 cells; −, no colonies of >2 cells. ND, not determined.

morphology but instead underwent a dramatic rounding (data not shown).

DISCUSSION

Recent studies have demonstrated that a product derived from MVA is covalently attached to ras proteins (6, 7), nuclear lamins (17–19), and fungal mating factors (20, 21); this product has been identified by HPLC (7), mass spectrometry (19, 21), and proton NMR (21) to be the isoprenoid farnesol. Because an additional number of unidentified mammalian proteins are also modified by isoprenylation, the significance of this particular modification for protein function will be important to determine.

To assess the role of isoprenylation in ras membrane association and transforming activity, we have studied the effect of isoprenylation on the membrane localization and biological activity of nonpalmitoylated, oncogenic human K-ras 4B protein. Neither of two K-ras 4B mutants, KSER and KTER, with substitutions in their CAAX sequences was isoprenylated. A previous report using peptide mapping has suggested that the cysteine of the CAAX motif is the amino acid residue to which the farnesol group is attached (6). The observation that KSER or other ras proteins with a serine residue substituted for the CAAX cysteine is not isoprenylated (6, 7) is consistent with this finding. KTER does, however, retain this C-terminal cysteine, and the lack of isoprenylation of this mutant indicates that both the C and AAX components of the CAAX motif must be present for isoprenylation of the K-ras 4B protein to occur.

Isoprenylation appears to play a crucial role in the membrane association of K-ras 4B protein. (*i*) The nonisoprenylated KSER and KTER proteins remained in the cytosol and did not associate with cell membranes. (*ii*) Inhibition of MVA and isoprenoid synthesis with the hydroxymethylglutaryl-CoA reductase inhibitor, compactin (22), prevented both the isoprenylation and membrane association of KVAL12 proteins. Because exogenous MVA was able to override the effects of compactin, it is unlikely that compactin prevented KVAL12 isoprenylation and membrane association by a nonspecific mechanism that did not involve inhibition of MVA and isoprenoid synthesis. Similar consequences of hydroxymethylglutaryl-CoA reductase inhibition on ras membrane association have been described in two other recent studies (6, 9). Thus, either genetic mutations or pharmacological treatments that prevent K-ras 4B isoprenylation can prevent this protein from becoming associated with membranes. While palmitoylation may still contribute to H-, N-, and K-ras 4A membrane interaction, isoprenylation is probably the critical modification responsible for the association with membranes of these ras proteins as well.

The K-*ras* gene has two alternative fourth exons, 4A and 4B (23, 24). Exon 4A is contained in the viral K-*ras* oncogene, and exon 4B is preferentially transcribed in human cells (24). The C-terminal region of K-ras 4B protein differs considerably from H-ras, N-ras, K-ras 4A, or yeast RAS proteins. Not only is it the only human ras protein that lacks palmitate (6, 7), it also possesses a large number of positively charged residues, including a stretch of six contiguous lysines, in close proximity to its CAAX sequence (25). The function of these lysine residues is unknown. However, it has been suggested that this positively charged domain might functionally substitute for palmitate and might promote or enhance the binding of K-ras 4B proteins to membranes through ionic interactions with negatively charged membrane lipid head groups (6). Although these lysine-rich sequences could enhance the ability of isoprenylated K-ras 4B proteins to bind to membranes, our results clearly indicate that, in the absence of isoprenylation, this domain contributes little to K-ras 4B membrane binding.

Previous studies have demonstrated that translocation of ras proteins from the cytosol to cell membranes appears crucial for transforming activity (5, 16). Because these studies used ras proteins (H-ras, N-ras, and K-ras 4A) that were both palmitoylated and isoprenylated, the precise contribution of each of these posttranslational modifications to ras biological activity was not clearly established. Our results show that isoprenylation, rather than palmitoylation, is both essential and sufficient for expression of K-ras 4B transforming activity. However, it should be noted that neither of these posttranslational modifications is essential for the intrinsic transforming activity of ras because unprocessed ras proteins are transforming when brought to the cell membrane by an alternate pathway, such as myristoylation (16). The importance of farnesol modification to transformation is, therefore, probably related to its ability to facilitate ras trafficking to and association with the plasma membrane.

Recent studies have demonstrated that an isoprenoid, farnesol, is covalently attached to ras proteins (6, 7), as well as to other unrelated proteins that possess the CAAX C-terminal consensus sequence. As mentioned above, these include the human nuclear lamin γ subunits (17–19) and fungal mating factors (20, 21). In addition, other proteins that possess the C-terminal CAAX motif, such as the ras-related proteins rap, ral, and rho (6, 7, 9, 26, 27) or G protein (28) may be isoprenylated and this modification may also be essential for their biologic activity. Given the many proteins that may be isoprenylated, it may not be possible to use drugs such as compactin to specifically inhibit the biological effects of oncogenic ras proteins on cells. Our observation that [Val^{12}]K-ras 4B-transformed cells treated with compactin became rounded and did not revert to a normal morphology is consistent with this possibility. Nevertheless, these drugs may be useful for delineating the mechanism of action of ras and assisting in the development of more specific agents.

In summary, our results indicate that isoprenylation, in the absence of palmitoylation, is a major determinant of K-ras 4B membrane association and is essential for expression of K-ras 4B transforming activity. This particular protein modification may represent a general mechanism to facilitate the processing, trafficking, and biological activity of other isoprenylated proteins. Because K-*ras* is the most frequently identified oncogene in human malignancies (1), development of treatments that specifically prevent ras protein isoprenylation may be useful in either elucidating the role or inhibiting the effects of K-ras proteins in human carcinogenesis.

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