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Ras (*CXXX*) and Rab (CC/*CXC*) Prenylation Signal Sequences Are Unique and Functionally Distinct*

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Roya Khosravi-Far‡§, Geoffrey J. Clark¶, Karon Abe¶, Adrienne D. Cox¶, Teri McLain‖, Robert J. Lutz‖, Michael Sinensky‖, and Channing J. Der¶‡‡**

From The ¶Department of Pharmacology, Lineberger Cancer Center, ‡Genetics Curriculum, University of North Carolina, Chapel Hill, North Carolina 27599 and the ‖Eleanor Roosevelt Institute for Cancer Research, Denver, Colorado 80206

Rab proteins typically lack the consensus carboxyl-terminal *CXXX* motif that signals isoprenoid modification of Ras and other isoprenylated proteins and, instead, terminate in either CC or *CXC* sequences (C = cysteine, *X* = any amino acid). To compare the functional relationship between the Ras *CXXX* and the Rab CC/*CXC* motifs, we have generated chimeric Ras proteins terminating in Rab carboxyl-terminal CC or *CXC* sequences. These mutant Ras proteins were not isoprenylated *in vitro* or *in vivo*, demonstrating that the CC and *CXC* sequences alone are not sufficient to replace a *CXXX* sequence to signal Ras isoprenoid modification. Surprisingly, chimeric Ras/Rab proteins terminating in significant lengths of carboxyl-terminal sequences from Rab1b (7–139 residues), Rab2 (5–151 residues), or Rab3a (12 residues) were also not isoprenylated. These results demonstrate that the sequence requirements for isoprenoid modification of Rab proteins are more complex than the simple tetrapeptide *CXXX* sequence for isoprenoid modification of Ras proteins and suggest that the Rab geranylgeranyl transferase(s) requires recognition of protein conformation to signal the addition of geranylgeranyl groups. Finally, competition studies demonstrate that a common geranylgeranyl transferase activity is responsible for the modification of Rab proteins terminating in CC or *CXC* motifs.

Recent studies have established that a diverse class of yeast and mammalian proteins undergoes covalent modification by isoprenoid addition and that the functions of these proteins are likely to be critically dependent on these modifications (1-3). The Ras oncogene proteins are the best characterized isoprenylated proteins and provide an excellent prototype for understanding the role of prenylation in protein function (3-5). All Ras proteins terminate in a consensus carboxyl-terminal *CXXX* (C = cysteine, *X* = any amino acid) motif which signals the addition of a 15 carbon farnesyl isoprenoid, as well as two other closely linked posttranslational modifications (proteolytic removal of the *XXX* residues and carboxyl methylation of the farnesylated cysteine). These *CXXX*-signaled modifications are critical for Ras association with the plasma membrane and for Ras transforming activity (4-6).

The importance of the *CXXX* motif for isoprenoid modification of Ras proteins has been demonstrated by several lines of study. First, mutant Ras proteins lacking either the cysteine or the *XXX* residues of the *CXXX* motif are completely blocked in isoprenoid modification (7-9). Second, synthetic tetrapeptides corresponding to the *CXXX* sequences of the Ras proteins are both efficient competitors of, and substrates for, Ras farnesyl transferase activity (10-12). Third, the introduction of the H-Ras *CXXX* tetrapeptide sequence to heterologous proteins such as protein A or the α subunit of the heterotrimeric G_i protein results in the prenylation of these normally nonprenylated proteins (8, 13). Therefore, the *CXXX* tetrapeptide sequence is not only required but is also sufficient to signal for isoprenoid modification of Ras proteins and other isoprenylated proteins. Finally, the last residue of the *CXXX* sequence dictates the specific modification of the *CXXX*-containing proteins by different prenyl transferases which specifically promote the addition of either farnesyl or geranylgeranyl moieties (11, 14-16).

Although the Rab/YPT family of Ras-related proteins represents a major group of isoprenylated proteins (1, 17-20), most lack carboxyl-terminal *CXXX* sequences, and instead, typically terminate in either CC or *CXC* motifs (21-23). Mutant Rab proteins lacking their carboxyl-terminal CC or *CXC* sequences are not modified by prenylation (11, 18, 24-26), are cytosolic and lack biological activity. Thus the CC and *CXC* motifs are functionally analogous to the Ras *CXXX* motif and are critical for the posttranslational processing and subcellular localization of Rab proteins.

Biochemical and genetic evidence indicates that proteins terminating in CC/*CXC* sequences are modified by a geranylgeranyl transferase(s) that is distinct from the activities that modify proteins terminating in *CXXX* sequences (11, 27-29). Furthermore, since the terminal 3 residues of the *CXXX* motif are critical to signal isoprenoid modification of Ras proteins (11, 30), it is also clear that the sequence requirements for Rab isoprenoid modification are distinctly different from the sequences recognized by the *CXXX* prenyl transferases. Finally, the CC and *CXC* motifs signal modifications distinct from those signaled by *CXXX* sequences. The *CXC* motif of Rab3a has been shown to be modified by two geranylgeranyl moieties (17) and by carboxyl methylation (17, 31). The CC motif is modified by at least one, or possibly two, geranylgeranyl isoprenoids (18, 20, 32) but is not carboxylmethylated (20, 31). Thus the CC and *CXC* sequences contrast with the *CXXX* sequences, and with each other, signaling structurally distinct carboxyl-terminal modifications.

Recent evidence implicates mammalian Rab proteins as critical regulatory elements in intracellular trafficking (26,

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** Recipient of Tobacco Related Disease Research Program Fellowship 2FT0039.

‡‡ Recipient of an American Cancer Society faculty research award. To whom correspondence should be addressed. Tel.: 919-966-5634; Fax: 919-966-5640.

33). The localization of different Rab proteins at distinct intracellular membrane compartments of the endocytic and exocytic pathways (3, 34) is consistent with this function. For example, the Rab1b protein has been localized to the endoplasmic reticulum and Golgi (33), while Rab3a protein is associated with secretory vesicles (35, 36). Thus, current models suggest that each Rab protein interacts with two distinct membrane subcompartments and reversibly cycles between cytosolic and membrane fractions (34, 37). While the functional distinction between the *CXXX*, CC, and *CXC* motifs is presently not known, it has been demonstrated that the CC/*CXC* carboxyl-terminal sequences are critical for Rab function in regulating vesicular trafficking in endocytosis and exocytosis (24–26), and it is possible that this function may not be replaced by the carboxyl-terminal *CXXX*-signaled modifications.

Since proteins terminating in *CXXX versus* CC/*CXC* sequences undergo different modifications that are catalyzed by different enzymes, we have initiated studies to determine the Rab sequences required to signal isoprenoid modification and to determine whether *CXXX*, CC, and *CXC* motifs have different functional roles. Our results demonstrate that the CC/*CXC* sequences cannot functionally replace the Ras *CXXX* sequence to promote Ras processing and membrane association, that additional upstream sequences as well as protein conformation seem to play a critical role in prenylation of the Rab proteins, and finally, that proteins terminating in CC or *CXC* sequences are modified by a common geranylgeranyl transferase activity.

EXPERIMENTAL PROCEDURES

Generation of Ras and Rab Carboxyl-terminal Mutants and Ras/Rab Chimeric Proteins—Oncogenic H-Ras(61-Leu) or Rab1b mutant sequences that encode carboxyl-terminal mutants (Table I) were generated by oligonucleotide-directed mutagenesis using Taq polymerase chain reaction DNA amplification as described previously (18). H-*ras*/*rab*1b, H-*ras*/*rab*2, and H-*ras*/*rab*3a chimeric genes were constructed using polymerase chain reaction amplification to generate specific DNA fragments from H-*ras*, *rab*1b, *rab*2, and *rab*3a cDNA sequences using pairs of oligonucleotides to introduce the appropriate restriction sites at the 5′- and 3′-ends of the resulting DNA fragments (see Fig. 3). The resulting fragments were introduced into either pATRasH (Ras and Ras/Rab chimeras) or pET3a (Rab) bacterial expression vectors (38, 39). All sequences were verified by dideoxy sequencing (40). Ras, Rab, or Ras/Rab proteins were expressed and isolated from *Escherichia coli* strains PR13-Q or BL-21(DE3) as described previously (18). The mutant and chimeric genes were also introduced into the pZIP-NeoSV(X)1 retrovirus vector for expression in mammalian cells (41).

In Vitro Protein Prenylation Assays—Specific prenylation of bacterially expressed Rab1b carboxyl-terminal mutants, H-Ras carboxyl-terminal mutants, and H-Ras/Rab1b chimeras were determined using an *in vitro* rabbit reticulocyte lysate prenylation system as described previously (18). Purified proteins (1.2 μM) were incubated in a final volume of 50 μl with nuclease-treated reticulocyte lysate (Promega) in the presence of 5 μM [^{3}H]farnesyl pyrophosphate (FPP)[1] (2.5 Ci/mmol) (Du Pont-New England Nuclear), 5 μM [^{3}H]geranylgeranyl pyrophosphate (GGPP; 2.5 Ci/mmol) (University of South Florida), or 40 μM [^{3}H]MVA (lactone form; Du Pont-New England Nuclear) (24 Ci/mmol). Incubations were conducted at 30 °C for 1 h, and the reactions were stopped by addition of an equal volume of 2 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by SDS-PAGE and fluorography. The Rab1b carboxyl-terminal mutants were also incubated with partially purified YPT1 geranylgeranyl transferase activity (designated GGTase II; provided by J. Gibbs, Merck Sharp and Dohme Laboratories, West Point, PA) (11) in the presence of [^{3}H]GGPP, and [^{3}H]GG incorporation was visualized by SDS-PAGE and fluorography.

TABLE I
H-ras and rab1b carboxyl-terminal mutants

Protein	Carboxyl-terminal sequence	^{3}H incorporation		
		MVA	GGPP	FFPP
H-ras WT	SGPGCMSCKCVLS	+	−	+
H-ras(ΔVLS)	SGPGCMSCKC- - -	−	−	−
H-ras(CC)	SGPGSMSSKCC- -	−	−	−
H-ras(CSC)	SGPGSMSSKCSC- -	−	−	−
rab1b WT	TPVKSASGGCC	+	+	−
rab1b(ΔCC)	TPVKSASGG- -	−	−	−
rab1b(Δ7) CC	TP- - - - - - - CC	−	−	−
rab1b(CS)	TPVKSASGGCS	+	±	−
rab1b(SC)	TPVKSASGGSC	+	±	−
rab1b(CKC)	TPVKSASGGCKC	+	+	−
Rab1b(CVIM)	TPVKSASGGCVIM	+	+	+

Guanine Nucleotide Binding Assays—Two approaches were used to establish the ability of each Ras/Rab chimeric protein to bind GTP efficiently. First, 10 μl of purified bacterially expressed proteins (50 μg/ml) were incubated in a 50-μl reaction with 25 mM HEPES (pH 8.0) and 1 mM DTT, in the presence of 0.1 μM [γ-^{35}S]GTP (800 Ci/mmol; Du Pont-New England Nuclear) for 10 min at 30 °C. Each reaction was then stopped by adding 1 ml of 25 mM Tris (pH 8.0), 100 mM NaCl, 30 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mg/ml bovine serum albumin. The amount of bound [γ-^{35}S]GTP was measured by vacuum filtration and liquid scintillation counting (42). Second, bacterially expressed proteins were separated by SDS-PAGE and transferred to nitrocellulose filters, then incubated with 1 μM [γ-^{35}S]GTP in 50 mM Tris, 0.3% Tween, 0.5 mM EDTA, 0.5 mM $MgCl_2$, and 10 μM of GTP (43). The bound [^{35}S]GTP was then visualized by fluorography.

Characterization of Isoprenylation of Ras/Rab Chimeric Proteins in Vivo—NIH 3T3 mouse fibroblasts were transfected by the calcium phosphate precipitation technique (44), and transfected cells were isolated by selection in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 400 μg/ml G418 (Geneticin, GIBCO). NIH 3T3 cells expressing H-Ras/Rab chimeric proteins were established by cotransfection with an oncogenic c-*raf* cDNA plasmid construct. Details of the establishment of these cells will be described elsewhere.[2] NIH 3T3 cells expressing each mutant H-Ras and H-Ras/Rab protein were metabolically labeled for 16–18 h at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed calf serum and [^{3}H]MVA at 100 μCi/ml or [^{35}S]methionine/cysteine (Met/Cys; Translabel, ICN Pharmaceuticals) at 100 μCi/ml. Fractionation into crude membrane (P100) and cytosolic (S100) fractions and partitioning into Triton X-114 were done as described previously (18). H-Ras and chimeric Ras/Rab proteins were then immunoprecipitated with the Y13-259 (45) and Ras11 (46) anti-Ras monoclonal antibodies and analyzed by SDS-PAGE and fluorography.

Selective Inhibition of Protein Prenylation by Proteins with Different Carboxyl-terminal Motifs—To determine if proteins with distinct carboxyl-terminal motifs are modified by shared prenyl transferase activities, 0.75–1.0 μM of bacterially expressed H-Ras, G25K, Rab1b, and Rab3a were each incubated with nuclease-treated rabbit reticulocyte lysate and [^{3}H]FPP (for H-Ras) or [^{3}H]GGPP (for G25K, Rab1b, and Rab3a) alone, or in the presence of a >10-fold molar excess (10 μM) of the competing protein or peptide substrate. Reactions were done for 1 h at 30 °C, and the proteins were then resolved on SDS-PAGE, and fluorographed after treatment with Amplify (Amersham). The gel slices containing the labeled proteins were also treated with H_2O_2 to release the labeled material which was quantified by liquid scintillation as described previously (10, 18). The G25K bacterial expression construct was provided by R. Cerione (Cornell University, Ithaca, NY) (47).

RESULTS

Rab CC or CXC Sequences Are Necessary but Not Sufficient for Rab Isoprenylation—In order to determine the carboxyl-terminal sequence requirements for Rab isoprenylation, we generated Ras or Rab1b mutant proteins with altered carboxyl-terminal sequences (Table I). Bacterially expressed mutant proteins were then incubated with either rabbit reticu-

[1] The abbreviations used are: FPP, farnesyl pyrophosphate; ER, endoplasmic reticulum; GGPP, geranylgeranyl pyrophosphate; MVA, mevalonate; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

[2] G. J. Clark, R. Khosravi-Far, and C. J. Der, manuscript in preparation.

locyte lysate or partially purified geranylgeranyl transferase (designated GGTase II) and [^{3}H]MVA to determine prenylation efficiency *in vitro*. In contrast to the wild type H-Ras protein, mutant H-Ras proteins that terminate in either CC or CSC sequences were not prenylated *in vitro* (Table I). Furthermore, analysis of NIH 3T3 cells expressing the mutant proteins also failed to demonstrate incorporation of [^{3}H]MVA *in vivo* (data not shown). Consistent with the absence of isoprenylation, fractionation analysis demonstrated that, like the nonprenylated H-Ras(ΔVLS) mutant, the CC and CSC mutant proteins were located exclusively in the cytosolic (S100) fraction (Fig. 1). Thus the Rab CC or C*XC* sequences alone cannot replace the Ras C*XXX* sequence to promote Ras isoprenylation and membrane association *in vivo*.

While a Rab1b mutant protein lacking both carboxyl-terminal cysteine residues (Rab1b(ΔCC)) was not a substrate for isoprenylation *in vitro*, mutant Rab1b proteins retaining either cysteine residue (Rab1b(CS) and Rab1b(SC)) were radiolabeled with both [^{3}H]MVA and [^{3}H]GGPP (Fig. 2). These results suggested that both cysteines may be modified by isoprenoid addition and that the CC motif, like the C*XC* motif, may also signal modification by two geranylgeranyl

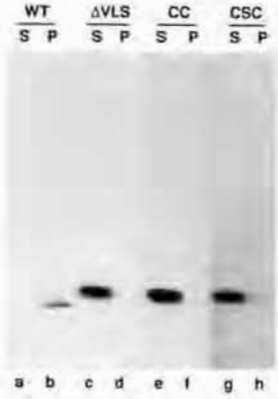

FIG. 1. **Localization of mutant Ras proteins terminating with CC or C*XC* sequences.** NIH 3T3 cells expressing H-Ras wild type (*lanes a* and *b*) or mutant proteins, either lacking the terminal 3 residues (designated ΔVLS; *lanes c* and *d*) or terminating in CC (*lanes e* and *f*) or CSC (*lanes g* and *h*) residues, were metabolically labeled with [^{35}S]Met/Cys for 16 h. The overexpressed proteins were then fractionated into crude cytosolic (*S*) and particulate (*P*) fractions and immunoprecipitated with anti-Ras Y13–259 monoclonal antibody. Proteins were then separated by SDS-PAGE and visualized by fluorography.

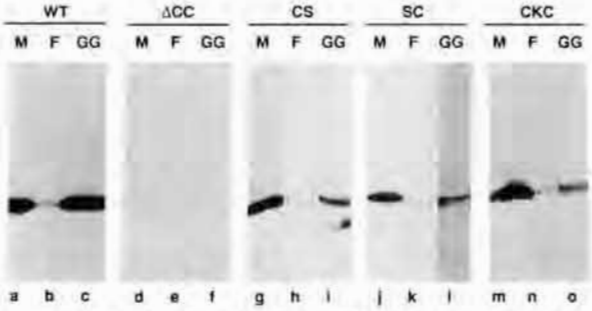

FIG. 2. ***In vitro* prenylation analysis of Rab1b carboxyl-terminal mutant proteins.** *In vitro* isoprenoid modification of bacterially expressed carboxyl-terminal mutant proteins of Rab1b was analyzed in an *in vitro* rabbit reticulocyte lysate prenylation system in the presence of [^{3}H]MVA (*M*), [^{3}H]FPP (*F*), or [^{3}H]GGPP (*GG*) as described under "Experimental Procedures." Lysates incubated with wild type Rab1b (*lanes a–c*), Rab1b(ΔCC) (*lanes d–f*), Rab1b(CS) (*lanes g–i*), Rab1b(SC) (*lanes j–l*), or Rab1b(CKC) (*lanes m–o*) were resolved on SDS-PAGE and analyzed by fluorography. We have previously shown that *in vitro* [^{3}H]MVA-labeled Rab1b proteins are modified by geranylgeranyl moieties (18).

moieties. In addition, the reduced incorporation of [^{3}H] into these two mutant proteins, when compared to the wild type protein, demonstrates that both cysteine residues are required for optimal prenylation of Rab1b. A Rab1b mutant protein that retains the double cysteines, but lacks 7 amino acids upstream from the cysteine residues (Rab1b(Δ7)CC), was not isoprenylated *in vitro* (Table I). Thus, Rab1b isoprenylation is dependent on both the CC motif and sequences upstream of these cysteine residues. Finally, Rab1b mutant protein terminating in either C*XC* (CKC or CSC) (Table I and Fig. 2, *lanes m–o*) or C*XXX* (CVLS) (Table I) sequences were also efficiently isoprenylated *in vitro*, indicating that Rab1b carboxyl-terminal sequences upstream of the CC motif are compatible with these two different prenylation signal sequences.

Rab Geranylgeranyl Transferases May Require Recognition of Protein Conformation for Isoprenylation—To determine the carboxyl-terminal sequence requirements for isoprenoid modification of Rab proteins, we generated a series of Ras/Rab chimeric proteins that contain the amino-terminal sequences of H-Ras but in which varying lengths of Rab1b (7–139 residues), Rab2 (5–151 residues), or Rab3a (12 residues) replaced the carboxyl-terminal region of H-Ras (Fig. 3). Surprisingly, none of these chimeric proteins was isoprenylated *in vitro* when incubated with reticulocyte lysate (representative examples are shown in Fig. 4, *lanes d*, *f*, and *i*) or partially purified GGTaseII (data not shown). Analysis of these chimeric Ras/Rab proteins in transfected NIH 3T3 cells demonstrated that these mutant proteins, in contrast to H-Ras(61L), were not metabolically labeled with [^{3}H]MVA, were present exclusively in the crude cytosolic (S100) fraction (Fig. 5*A*), and did not partition into the detergent phase after Triton X-114 extraction (Fig. 5*B*). Thus, despite possessing significant carboxyl-terminal sequences from Rab proteins, these chimeric proteins were still not substrates for isoprenylation *in vitro* or *in vivo*.

The lack of isoprenoid modification of the Ras/Rab chimeric proteins suggested that Rab carboxyl-terminal sequences alone are not sufficient for Rab geranylgeranyl transferase modification. Alternatively, the improper folding of the combined amino-terminal Ras and carboxyl-terminal Rab sequences, or the inaccessibility of the carboxyl termini to the prenyl transferase, may account for the lack of efficient isoprenoid modification of these chimeric proteins. To address the first possibility, we analyzed these chimeric proteins for their ability to bind [^{35}S]GTPγS. Bacterially expressed chimeric Ras/Rab proteins were analyzed by incubation with 10^{-7} M [^{35}S]GTPγS, followed by vacuum filtration through nitrocellulose filters, or by separation on SDS-PAGE and transfer to nitrocellulose followed by incubation with [^{35}S]GTPγS. Like authentic H-Ras, Rab1b, and Rab2 proteins, all chimeric Ras/Rab proteins efficiently bound [^{35}S]GTPγS (data not shown), indicating that they were properly folded to produce a functional conformation. To address the possibility that the carboxyl-terminal sequences were not available for isoprenylation, we generated chimeric Ras/Rab1b proteins which terminated in CVIM instead of in CC residues. In contrast to their non-C*XXX* containing counterparts, both the Ras/Rab1b.15(CVIM) and Ras/Rab1b.33(CVIM) chimeric proteins were good substrates for isoprenylation (Fig. 4, *lanes g* and *h*), indicating that their carboxyl-terminal regions were accessible for isoprenoid addition. Furthermore, both Ras/Rab1b.15(CVIM) and Ras/Rab1b.33(CVIM) displayed potent transforming activity when expressed in NIH 3T3 cells (data not shown), which provides further indication that these chimeric proteins are properly folded.

Crystal structure determination of the H-Ras protein demonstrated that the amino and carboxyl-terminal sequences reside on the same face of the protein (48, 49). Therefore, we

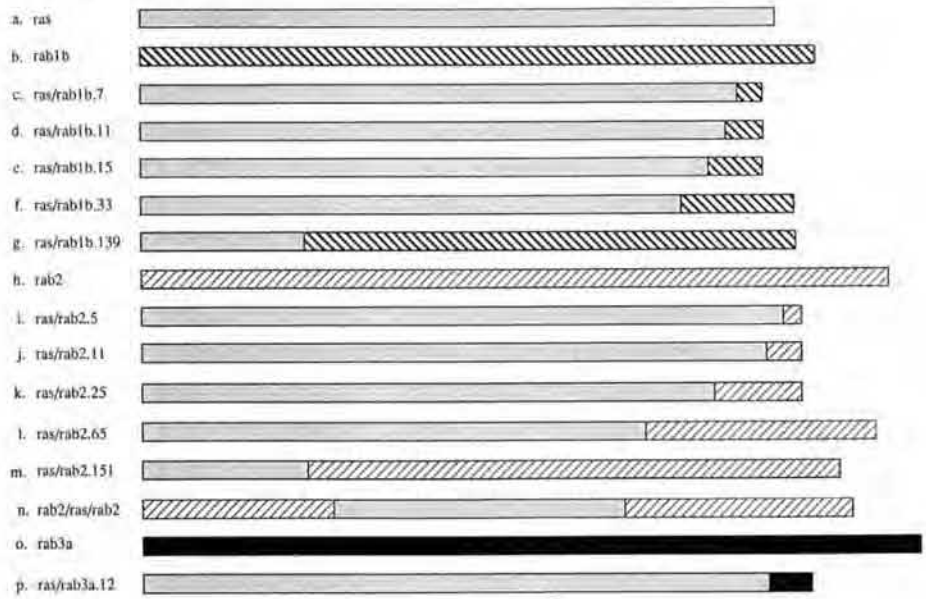

FIG. 3. **Structure of chimeric Ras/Rab proteins.** Proteins encoded by molecular constructs containing H-Ras (*shaded boxes*), Rab1b (*bold hatched boxes*), Rab2 (*light hatched boxes*), and Rab3a (*black boxes*) sequences are shown. The carboxyl-terminal residues of H-Ras were replaced by 7 to 139 carboxyl-terminal residues from Rab1b (*c–g*), 5 to 151 carboxyl-terminal residues from Rab2 (*i–m*), or 12 carboxyl-terminal residues from Rab3a (*p*). The *numbers* after each chimera indicate the number of amino acids from carboxyl-terminal of Rab proteins that replace analogous amino acids from H-Ras. In addition, a Rab2/Ras/Rab2 chimera (*n*) was generated which contains the first 60 amino-terminal residues of Rab2 followed by H-Ras residues 61–160 and terminates with the 65 carboxyl-terminal amino acids of Rab2.

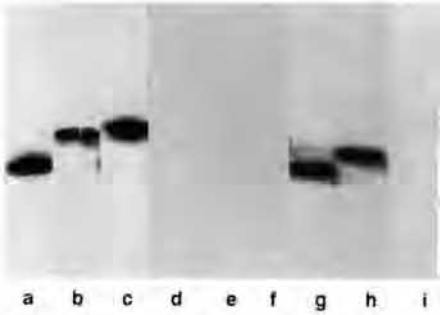

FIG. 4. ***In vitro* prenylation of Ras/Rab chimeras.** The isoprenoid modification of Ras/Rab recombinant proteins was analyzed in the rabbit reticulocyte lysate system in the presence of [^{3}H]MVA followed by SDS-PAGE analysis as described under "Experimental Procedures." *Lane a*, H-Ras (WT); *lane b*, Rab1b; *lane c*, Rab3a; *lane d*, Ras/Rab1b.33; *lane e*, Ras/Rab2.11; *lane f*, Rab2/Ras/Rab2; *lane g*, Ras/Rab1b.15 CVIM; *lane h*, Ras/Rab1b.33 CVIM; and *lane i*, Ras/Rab3a.12.

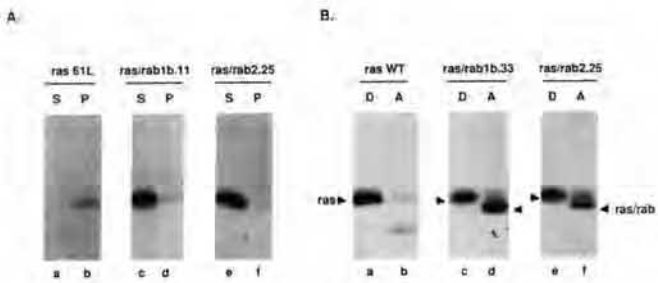

FIG. 5. ***In vivo* prenylation of Ras/Rab chimeras.** NIH 3T3 cells expressing each Ras/Rab chimeric protein (in the presence of oncogenic *c*-raf) were labeled with [^{35}S]Met/Cys. Cells expressing Ras(61L or WT) (*lanes a* and *b*), Ras/Rab1b.11 (*lanes c* and *d*), or Ras/Rab2.25 (*lanes e* and *f*) were ruptured and fractionated (*Panel A*) into crude cytosolic (*S*) and membrane-containing particulate (*P*) fractions, or lysed (*Panel B*) in 1% Triton X-114 and partitioned into detergent-depleted, aqueous (*A*), and detergent-enriched (*D*) phases. The recombinant proteins were then immunoprecipitated by a pool of Y13-259 and Ras11 anti-ras monoclonal antibodies. Membrane association and prenylation were analyzed by SDS-PAGE and visualized by fluorography. *Arrows* to the left show H-Ras (*WT*), and *arrows* to the right show the chimeric Ras/Rab proteins.

addressed the possibility that both amino- and carboxyl-terminal Rab sequences were required for recognition and modification by geranylgeranylation. A chimeric Ras/Rab (Rab2/Ras/Rab2) protein with both amino- and carboxyl-terminal Rab2 sequences was constructed (Fig. 3), and the bacterially expressed protein was analyzed in the rabbit reticulocyte lysate prenylation assay. The absence of [^{3}H]MVA incorporation into the Rab2/Ras/Rab2 chimeric protein (Fig. 4, *lane f*) indicated that additional Rab2 sequences, or proper protein conformation, were required to signal Rab prenylation.

A Common Prenyl Transferase(s) Activity Is Responsible for Modification of Rab Proteins Terminating with CC or CXC Sequences—To further characterize the prenyl transferase activities responsible for the isoprenylation of proteins terminating in C*XXX*, CC, or C*X*C sequences, we performed competition experiments to measure the ability of heterologous peptides or proteins to block isoprenylation of bacterially expressed H-Ras (carboxyl-terminal C*XXX* motif for farnesylation), G25K (carboxyl-terminal C*XXX* motif for geranylgeranylation), Rab1b (CC), or Rab3a (C*X*C) proteins using the reticulocyte lysate assay (Fig. 6). For each assay 1 μM of each substrate protein was incubated either alone, or in the presence of a 10-fold molar excess of the competing peptide or protein, and the incorporation of either [^{3}H]FPP or [^{3}H]GGPP into the substrate protein was determined.

H-Ras isoprenylation was selectively inhibited by a synthetic peptide corresponding to the carboxyl terminus of the farnesylated yeast **a**-factor (CVIA) (50), but not by full length G25K (CVIF), Rab1b (CC), or Rab3a (CSC) protein (Fig. 6*A*). Similarly, G25K isoprenylation was inhibited by a synthetic heptapeptide corresponding to the carboxyl terminus of the geranylgeranylated γ-$_6$ subunit of heterotrimeric G proteins (KFFCAIL) (16, 51, 52), but not by full length H-Ras, Rab1b, or Rab3a protein (Fig. 6*B*). In contrast to the ability of C*XXX*-containing peptides to efficiently inhibit the isoprenylation of Ras or G25K proteins, Rab1b and Rab3a peptides corresponding to the last 8 carboxyl-terminal amino acids of these proteins did not inhibit the isoprenylation of either Rab1b or Rab3a when used at peptide concentrations of up to 50 μM (data not shown). These data, together with the inability of the chimeric Ras/Rab proteins to serve as efficient substrates for isoprenylation, provide further evidence that Rab prenyl transferase(s) requires recognition of Rab sequences distant from the carboxyl termini.

To determine if Rab1b and Rab3a are modified by distinct

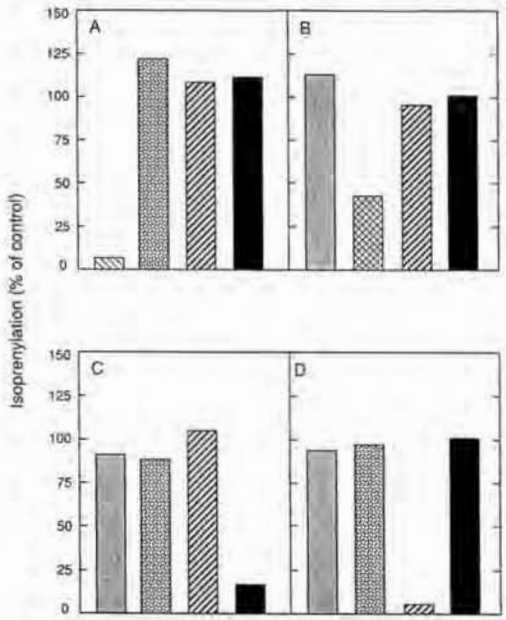

FIG. 6. **Selective inhibition of Ras and Rab isoprenylation *in vitro*.** Substrate proteins were incubated at a concentration of 1 μM with reticulocyte lysate and [^{3}H]FPP (for H-Ras) or [^{3}H]GGPP (for G25K, Rab1b, and Rab3a) in the presence of 10 μM inhibitor protein. Following incubation, proteins were resolved on SDS-PAGE, and incorporation of activity was determined as described under "Experimental Procedures." Results are expressed as means of duplicate determinations. Substrate (1 μM) is as follows: *A*, H-Ras; *B*, G25K; *C*, Rab1b; *D*, Rab3a. Inhibitor (10 μM): H-Ras (*shaded box*), G25K (*speckled box*), Rab1b (*bold hatched box*), Rab3a (*black box*), a-factor peptide (*light hatched box*), and G protein γ-$_6$ subunit carboxyl-terminal hepapeptide (*double hatched box*).

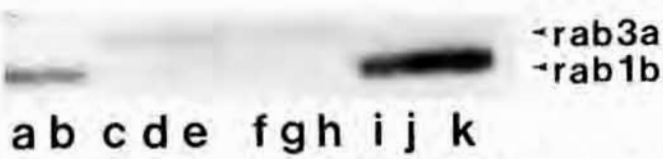

FIG. 7. ***In vitro* competition of Rab1b and Rab3a prenylation.** Substrate proteins (0.75 μM) were incubated with reticulocyte lysate and [^{3}H]GGPP alone or in the presence of excess (10 μM) competing protein. Following incubation, proteins were resolved by SDS-PAGE and fluorography. *Lanes a* and *b*, Rab1b alone; *lanes c–e*, Rab1b with excess Rab3a; *lanes f–h*, Rab3a alone; and *lanes i–k*, Rab3a with excess Rab1b.

or common geranylgeranyl transferase activities, the ability of excess full-length Rab proteins to block isoprenylation of the corresponding Rab protein was determined. While Rab1b isoprenylation was not inhibited by G25K or H-Ras proteins, geranylgeranylation of Rab1b was inhibited (by 84%) when assayed in the presence of a 10-fold molar excess of Rab3a (Fig. 6*C* and Fig. 7, *lanes c–e*). Similarly, Rab3a geranylgeranylation was inhibited (by 93%) when assayed in the presence of a 10-fold molar excess of Rab1b, but not H-Ras or G25K proteins (Fig. 6*D* and Fig. 7, *lanes i–k*). This level of inhibition correlated well with that predicted by the respective K_m values for these substrates (K_m for Rab1b = 1.0 μM; K_m for Rab3a = 2.3 μM) if their prenylation were catalyzed by the same enzyme(s). The predicted inhibition of Rab1b prenylation by Rab3a under these conditions is 71% and that for Rab3a by Rab1b is 88%. Thus these data suggest that the CC and C*X*C proteins are recognized by the same geranylgeranyl transferase(s).

DISCUSSION

While the carboxyl-terminal CC and C*X*C sequences of Rab proteins are functionally analogous to the Ras C*XXX* sequence in that they signal isoprenoid modification and promote membrane association, three previous observations have suggested that the sequence requirements for prenyl transferase recognition and isoprenoid modification of Rab proteins may be different from those of Ras and other C*XXX*-containing proteins. First, in contrast to the ability of C*XXX*-containing peptides to inhibit Ras isoprenylation, synthetic peptides containing the Rab CC or C*X*C sequences were not effective inhibitors of, nor substrates for, isoprenylation (11, 28, 29). Second, it has been shown that the noncysteine *XXX* residues of the C*XXX* motif are the critical residues for efficient recognition and modification by the C*XXX* prenyl transferases (11, 30, 53). Third, a mutant yeast Ras1 protein that terminated with the 6 carboxyl-terminal residues of yeast YPT1 was not a substrate for GGTase II activity (11). Despite these indications, our observations that chimeric Ras/Rab proteins terminating in significant carboxyl-terminal sequences from Rab1b, Rab2, or Rab3a were not isoprenylated were unexpected. These results clearly demonstrate that the sequence requirements for Rab isoprenylation are strikingly different from those for isoprenylated proteins that terminate in C*XXX* sequences, and suggest that Rab geranylgeranyl transferase(s) requires recognition of sequences outside the Rab carboxyl termini, and possibly protein conformation, for isoprenoid modification. Whereas C*XXX* carboxyl-terminal sequences are both necessary and sufficient to signal isoprenoid modification, our results indicate that multiple regions of the CC- and C*X*C-terminating Rab proteins combine to act as structural determinants of the isoprenylation signal.

Synthetic peptides corresponding to the C*XXX* sequences of Ras proteins have been very useful reagents for the identification and analysis of C*XXX* farnesyl and geranylgeranyl transferases (10, 14, 16) and have allowed a rapid characterization of the structural requirements for prenylation of C*XXX*-terminating proteins (11, 53, 16). Ras C*XXX* tetrapeptides are sufficient to both inhibit Ras isoprenylation and to serve as substrates for Ras farnesyl transferase activity (11, 12, 53). Furthermore, C*XXX* tetrapeptides have also greatly facilitated the affinity purification of Ras prenyl transferases and have provided a basis for the design of potential inhibitors of oncogenic Ras activity in human tumors (10). By contrast, since prenylation of Rab proteins requires recognition of primary or higher order structure besides the carboxyl-terminal prenylation domain, such carboxyl-terminal peptides will be less useful for the characterization of Rab prenyl transferases. In particular, assays of Rab prenylation and determination of specific sequence requirements for reactivity of the Rab substrates will require intact Rab proteins. Recent studies have identified geranylgeranyl transferase activities (designated GGTase II or Rab GGTase) for Rab proteins (11, 28, 29) which are distinct from the activity that geranylgeranylates proteins that terminate with C*XXX* sequences (GGTase I). The results from competition experiments described in this study demonstrated that Rab1b (CC motif) and Rab3a (C*X*C motif) were competitors of the isoprenylation of the reciprocal protein in a reticulocyte lysate assay. These results provide evidence that geranylgeranyl additions to CC or C*X*C terminating proteins are catalyzed by a common geranylgeranyl transferase activity(ies). Additionally, the successful isoprenylation of mutant Rab1b proteins in which C*X*C sequences replaces the authentic CC sequence suggests that the CC and C*X*C motifs require common upstream sequences, or protein conformation, for signaling geranylgeranyl addition. Finally, the recent demonstration that a partially purified Rab3a geranylgeranyl transferase activity also efficiently modified Rab1a is also consistent with a common prenyl transferase for these two motifs (29). Our observation that Rab1b mutants terminating in either SC or CS

are still geranylgeranylated are in agreement with results described by Kinsella and Maltese (32) and suggests that the prenylation of the 2 cysteines are independent, nonsequential reactions. Whether these two geranylgeranyl modifications are catalyzed by a single enzymatic activity remains to be determined.

While it is clear that the Ras *CXXX* and Rab CC or *CXC* motifs serve analogous roles in triggering isoprenoid modification and membrane association (6), the distinct processing signaled by each of these motifs suggests the possibility of distinct roles in regulating protein function. Proteins terminating in *CXXX* sequences are modified by either single farnesyl or geranylgeranyl moieties, and the isoprenylated cysteine also undergoes carboxyl methylation. In contrast, Rab proteins terminating in CC or *CXC* sequences apparently undergo modification by geranylgeranyl groups on both cysteines, but only the *CXC* proteins are further modified by carboxyl methylation. One aim of our studies has been to determine whether Ras function can be promoted by the modifications signaled by the CC or *CXC* motifs. The nonprenylated and cytosolic nature of mutant Ras proteins that terminate with CC or *CXC* sequences clearly indicate that these motifs alone do not functionally replace the *CXXX* motif for promoting Ras function. However, since these mutant proteins are not processed, how the function of Ras proteins in regulating signal transduction pathways in the cell is facilitated by Rab carboxyl-terminal modifications remains to be answered.

At present, there is limited information available addressing whether there is a functional basis for the distinct prenylation motifs. Our recent observation that normal Ras proteins that terminate in mutant *CXXX* sequences signaling for geranylgeranyl, rather than farnesyl, addition are potent inhibitors of cell proliferation suggests a distinct role for distinct prenylation motifs (54). In contrast, the observation that yeast YPT1, which is essential for cell viability (43) and facilitates endoplasmic reticulum (ER) to Golgi transport (55), could still support yeast viability when the CC motif is substituted with the H-Ras *CXXX* sequence (CVLS) might argue against a unique role for the different motifs in protein function. However, it was not determined whether this carboxyl-terminal mutant was correctly processed or whether it retained wild type function in transport. Therefore, at present, it is not known whether the *CXXX*, CC, and *CXC* motifs possess distinct roles in promoting protein function. Clearly, further analysis of this question will be required to establish the functional significance of different carboxyl-terminal sequences that signal protein prenylation. We have recently demonstrated the contribution of Rab1b protein in ER to Golgi transport (33), and we are presently determining whether mutant Rab1b proteins that terminate in either *CXC* or *CXXX* sequences also function in ER to Golgi transport.

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REFERENCES

1. Maltese, W. A. (1990) *FASEB J.* **4,** 3319-3328
2. Glomset, J. A., Gelb, M. H., and Farnsworth, C. C. (1990) *Trends Biochem. Sci.* **15,** 139-142
3. Cox, A. D., and Der, C. J. (1992) *Crit. Rev. Oncogenesis* **3,** 365-400
4. Rine, J., and Kim, S.-H. (1990) *The New Biologist* **2,** 219-226
5. Der, C. J., and Cox, A. D. (1991) *Cancer Cells* **3,** 331-340
6. Khosravi-Far, R., Cox, A. D., Kato, K., and Der, C. J. (1992) *Cell Growth Diff.* **3,** 461-469
7. Willumsen, B. M., Christensen, A., Hubbert, N. L., Papageorge, A. G., and Lowy, D. R. (1984) *Nature* **310,** 583-586
8. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) *Cell* **57,** 1167-1177
9. Jackson, J. H., Cochrane, C. G., Bourne, J. R., Solski, P. A., Buss, J. E., and Der, C. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3042-3046
10. Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., and Brown, M. S. (1990) *Cell* **62,** 81-88
11. Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O'Hara, M. B., Garsky, V. M., Marshall, M. S., Pompliano, D. L., and Gibbs, J. B. (1991) *J. Biol. Chem.* **266,** 14603-14610
12. Goldstein, J. L., Brown, M. S., Stradley, S. J., Reiss, Y., and Gierasch, L. M. (1991) *J. Biol. Chem.* **266,** 15575-15578
13. Jones, T. L. Z., and Spiegel, A. M. (1990) *J. Biol. Chem.* **265,** 19389-19392
14. Seabra, M. C., Reiss, Y., Casey, P. J., Brown, M. S., and Goldstein, J. L. (1991) *Cell* **65,** 429-434
15. Yamane, H. K., Farnsworth, C. C., Xie, H., Evans, T., Howald, W. N., Gelb, M. H., Glomset, J. A., and Clarke, S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 286-290
16. Yokoyama, K., Goodwin, G. W., Ghomashichi, F., Glomset, J. A., and Gelb, M. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5302-5306
17. Farnsworth, C. C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M. H., and Glomset, J. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6196-6200
18. Khosravi-Far, R., Lutz, R. J., Cox, A. D., Conroy, L., Bourne, J. R., Sinensky, M., Balch, W. E., Buss, J. E., and Der, C. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6264-6268
19. Johnston, P. A., Archer, B. T., III, Robinson, K., Mignery, G. A., Jahn, R., and Sudhof, T. C. (1991) *Neuron* **7,** 101-109
20. Wei, C., Lutz, R., Sinensky, M., and Macara, I. G. (1992) *Oncogene* **7,** 467-473
21. Hall, A. (1990) *Science* **249,** 635-249
22. Valencia, A., Chardin, P., Wittinghofer, A., and Sander, C. (1991) *Biochemistry* **30,** 4637-4648
23. Chardin, P. (1991) *Cancer Cells* **3,** 117-126
24. Molenaar, C. M. T., Prange, R., and Gallwitz, D. (1988) *EMBO J.* **7,** 971-976
25. Walworth, N. C., Goud, B., Kabcenell, A. K., and Novick, P. J. (1989) *EMBO J.* **8,** 1685-1693
26. Gorvel, J.-P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) *Cell* **64,** 915-925
27. Rossi, G., Jiang, Y., Newman, A. P., and Ferro-Novick, S. (1991) *Nature* **351,** 158-161
28. Horiuchi, H., Kawata, M., Katayama, M., Yoshida, Y., Musha, T., Ando, S., and Takai, Y. (1991) *J. Biol. Chem.* **266,** 16981-16984
29. Seabra, M. C., Goldstein, J. L., Sudhof, T. C., and Brown, M. S. (1992) *J. Biol. Chem.* **267,** 14497-14503
30. Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., and Der, C. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6403-6407
31. Newman, C. M. H., Giannakouros, T., Hancock, J. F., Fawell, E. H, Armstrong, J., and Magee, A. I. (1992) *J. Biol. Chem.* **267,** 11329-11336
32. Kinsella, B. T., and Maltese, W. A. (1991) *J. Biol. Chem.* **266,** 8540-8544
33. Plutner, H., Cox, A. D., Pind, S., Khosravi-Far, R., Bourne, J. R., Schwaninger, R., Der, C. J., and Balch, W. E. (1991) *J. Cell Biol.* **115,** 31-43
34. Balch, W. E. (1990) *Trends Biochem. Sci.* **15,** 469-472
35. Fischer von Mollard, G., Mignery, G. A., Baumert, M., Perin, M. S., Hanson, T. J., Burger, P. M., Jahn, R., and Südhof, T. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1988-1992
36. Darchen, F., Zahraoui, A., Hammel, F., Monteils, M.-P., Tavitian, A., and Scherman, D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5692-5696
37. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) *Nature* **348,** 125-132
38. Der, C. J., Pan, B.-T., and Cooper, G. M. (1986) *Mol. Cell. Biol.* **6,** 3291-3294
39. Studier, F. W. (1991) *J. Mol. Biol.* **219,** 37-44
40. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463-5467
41. Cepko, C. L., Roberts, B., and Mulligan, R. C. (1984) *Cell* **37,** 1053-1062
42. Quilliam, L. A., Der, C. J., Clark, R., O'Rourke, E. C., Zhang, K., McCormick, F., and Bokoch, G. M. (1990) *Mol. Cell. Biol.* **10,** 2901-2908
43. Schmitt, H. D., Wagner, P., Pfaff, E., and Gallwitz, D. (1986) *Cell* **47,** 401-412
44. Der, C. J., Weissman, B., and MacDonald, M. J. (1988) *Oncogene* **3,** 105-112
45. Furth, M. E., Davis, L. J., Fleurdelys, B., and Scolnick, E. M. (1982) *J. Virol.* **43,** 294-304
46. Hamer, P. J., Trimpe, K. L., Pullano, T., Ng, S., LaVecchio, J. A., Petit, D. A., Delellis, R., Wolfe, H., and Carney, W. P. (1990) *Hybridoma* **9,** 573-587
47. Shinjo, K., Koland, J. G., Hart, M. J., Narasimhan, V., Johnson, D. I., Evans, T., and Cerione, R. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9853-9857
48. de Vos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E. S., and Kim, S.-H. (1988) *Science* **239,** 888-893
49. Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) *EMBO J.* **9,** 2351-2359
50. Brake, A. J., Brenner, C., Najarian, R., Laybourn, P., and Merryweather, J. (1984) in *Protein Transport and Secretion* (Gething, M. J., ed) pp. 103-108, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
51. Mumby, S. M., Casey, P. J., Gilman, A. G., and Gutowski, S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5873-5877
52. Maltese, W. A., and Robishaw, J. D. (1990) *J. Biol. Chem.* **265,** 18071-18074
53. Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S., and Goldstein, J. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 732-736
54. Cox, A. D., Hisaka, M. M., Buss, J. E., and Der, C. J. (1992) *Mol. Cell. Biol.* **12,** 2606-2615
55. Segev, N., Mulholland, J., and Botstein, D. (1988) *Cell* **52,** 915-924