Differential Isoprenylation of Carboxy-Terminal Mutants of an Inhibitory G-Protein α-Subunit: Neither Farnesylation nor Geranylgeranylation Is Sufficient for Membrane Attachment

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ABSTRACT: To determine the effect of protein isoprenylation with farnesyl vs geranylgeranyl groups on membrane association in vivo, COS cells were transfected with cDNAs encoding the wild-type G-protein α_i1 (WT) subunit, the soluble nonmyristoylated G-protein α_i1 glycine to alanine mutant (GA), a double mutant in which the carboxy-terminal residues CGLF of GA were mutated to CVLS (GA-CVLS), and a double mutant in which the carboxy terminus of GA was mutated to CALL (GA-CALL). As opposed to the WT and GA proteins, the GA-CVLS and GA-CALL proteins were not pertussis toxin substrates nor were they recognized by antibodies that recognize the nonmutated α_i1 carboxy terminus. Only the GA-CVLS and GA-CALL proteins incorporated [^{3}H]mevalonate in the form of a farnesyl and a geranylgeranyl moiety, respectively. Subcellular localization, as assessed by immunoblotting and immunoprecipitation, revealed that the WT protein localizes almost exclusively to the membrane fraction, whereas the GA, GA-CVLS, and GA-CALL proteins localize predominantly to the soluble fraction. The soluble GA-CVLS and GA-CALL proteins were not carboxyl methylated, but the small amount localized to the membrane was partially carboxyl methylated. These results indicate that neither farnesylation nor geranylgeranylation is sufficient alone to lead to membrane association.

Isoprenylation is the first step of a multistep posttranslational modification which has been shown to occur on certain proteins including a yeast mating factor, nuclear lamins, p21^{ras}, and G-protein[1] γ-subunits (Glomset et al., 1990; Kamiya et al., 1979; Farnsworth et al., 1989; Yamane et al., 1990; Casey et al., 1989). Specific carboxy-terminal amino acid sequences are recognized by protein prenyl transferases, which add an isoprenyl group to a cysteine near the carboxy terminus in thioether linkage. For proteins whose extreme carboxy terminus contains a CAAX motif (C represents a conserved cysteine, A represents an aliphatic amino acid), a farnesyl group is added when X is a serine or methionine and a geranylgeranyl group is added when X is a leucine (Seabra et al., 1991; Kinsella et al., 1991; Moores et al., 1991). Following the addition of the isoprenyl group, the AAX residues are proteolytically removed and the free cysteinyl carboxylic acid is methylesterified. In vitro studies have shown that the carboxyl methylation requires a membrane-bound methyltransferase and that the methylation is reversible by hydrolysis of the methyl ester (Stephenson & Clarke, 1990; Perez-Sala et al., 1991).

Isoprenylation is crucial for the membrane attachment of certain proteins. For example, when site-directed mutagenesis was performed on the G-protein γ_2-subunit or on K-ras 4B to prevent isoprenylation, the proteins changed their localization from a particulate to soluble fraction (Simonds et al., 1991; Jackson et al., 1990). In regard to tightness of membrane attachment, studies on G-protein βγ-subunits have shown that βγ-subunits derived from rod photoreceptor cells can be released from membranes with aqueous, hypotonic buffers, while βγ-subunits derived from brain require detergent in order to achieve membrane release (Sternweis, 1986). The rod photoreceptor γ-subunit is modified by a farnesyl moiety, and the brain γ-subunit is modified by a geranylgeranyl moiety (Fukada et al., 1990; Yamane et al., 1990). These studies suggest that the hydrophobicity of the different isoprenyl groups attached to the γ-subunit of the βγ-subunit complex could determine the tightness of the membrane attachment. However, the heterogeneity of the γ-subunits and the membrane compositions cannot be excluded as an explanation for this differential membrane affinity.

Heterotrimeric G-proteins are associated with the cytoplasmic side of the plasma membrane where they act as signal transducers between receptors and intracellular effectors (Simon et al., 1991; Spiegel, 1992). They consist of separate α-subunits, which bind GTP and interact with receptors, effectors, and βγ-subunits. While the amino acid sequence of these subunits lacks potential hydrophobic membrane-spanning domains, functionally, the subunits behave as integral membrane proteins. The membrane attachment of the βγ-subunits is due at least in part to the isoprenylation of the γ-subunit (Simonds et al., 1991). Certain α-subunits, including α_i1, undergo myristoylation, the addition of a myris-

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[1] Abbreviations: G-protein, guanine nucleotide-binding regulatory protein; α_i1, the α-subunit of one subtype of the group of G-proteins associated with inhibition of adenylyl cyclase; α_t, the α-subunit of the photoreceptor G-protein transducin; β_1, an isoform of G-protein β-subunits; Y_2, an isoform of G-protein γ-subunits first isolated from brain tissue; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; C_{15}, farnesyl moiety; C_{20}, geranylgeranyl moiety; DMEM, Dulbecco's minimum essential medium.

tic acid to an amino-terminal glycine (via amide linkage following methionine cleavage), which is important in their membrane attachment (Buss et al., 1987; Jones et al., 1990). Site-directed mutagenesis of α_i1-subunit resulting in a glycine-2 to an alanine substitution prevents myristoylation and impairs membrane attachment and interaction with $\beta\gamma$-subunits (Jones et al., 1990; Mumby et al., 1990b).

Since some G-protein α-subunits have a carboxy-terminal sequence similar to the isoprenylation CAAX sequence, the possibility that these α-subunits were isoprenylated was investigated. Analysis of purified proteins and radiolabeling with [^{3}H]mevalonic acid showed that these proteins are not isoprenylated (Jones & Spiegel, 1990; Mumby et al., 1990a). When site-directed mutagenesis was performed to change the carboxy-terminal sequence of a myristoylation-defective α_i1-subunit to CVLS, the protein was isoprenylated but not membrane associated (Jones & Spiegel, 1990). However the identity of the isoprenyl group was not determined. Studies on the specificity of the prenyl transferases suggest that proteins ending in the sequence CVLS should undergo farnesylation (Reiss et al., 1991). Yamane et al. (1991) have suggested that the longer and more hydrophobic geranylgeranyl moiety may promote tighter membrane attachment than the farnesyl group. Failure of the CVLS mutant of the α_i1-subunit to associate with the membrane thus left open the possibility that a carboxy-terminal mutant undergoing geranylgeranylation would become membrane associated. To test the functional significance, in terms of in vivo membrane association, of isoprenylation with farnesyl vs geranylgeranyl groups, we transfected cells with myristoylation-defective α_i1 cDNAs endoding CAAX sequences that should engender differential isoprenylation and studied the nature of the isoprenyl groups as well as the subcellular localization of the resultant proteins.

MATERIALS AND METHODS

Materials. Full-length cDNAs encoding rat $G\alpha_i1$ (Jones & Reed, 1987), β_1 (Fong et al., 1986), and γ_2 (Gautam et al., 1989) were kindly provided by R. Reed and D. Jones (The Johns Hopkins Uniersity), J. Hurley (University of Washington), and N. Gautam (Washington University), respectively. Compactin (Endo et al., 1976) was a gift of A. Endo (Tokyo Noko University). *all-trans*-Geranylgeraniol was generously provided by R. Coates (University of Illinois). *trans*-Geraniol, *trans,trans*-farnesol, and solanesol standards were obtained from Aldrich Chemical. COS-7 cells were purchased from American Type Culture Collection. Dulbecco's minimum essential medium (DMEM) (high glucose) and media supplements were obtained from Biofluids, Inc. Radiochemicals were obtained from NEN-Dupont.

Site-Directed Mutagenesis and Vector Construction. The pCD expression vector (PCD), the insertion of the wild-type α_i1 cDNA (WT), the mutagenesis of the α_i1 cDNA to substitute an alanine for a glycine at amino acid position number 2 (GA), and the mutagenesis of the GA cDNA to code for a carboxy-terminal sequence of CVLS (GA-CVLS) have been previously described (Jones et al., 1990; Jones & Spiegel, 1990). Mutagenesis of the GA cDNA to code for a carboxy-terminal sequence of CALL (GA-CALL) was performed in a similar fashion as for GA-CVLS using a different 3′ oligonucleotide, GCGCCTCTAGATTACAA-GAGAGCACAGTCTTT, which encodes the new mutation and new *Hgi*AI and *Xba*I restriction sites. The presence of the mutation was screened by digestion with *Hgi*AI restriction enzyme and confirmed by direct sequencing. The pCDM8.1 expression vector and insertion of the β_1- and γ_2-subunits have been described (Simonds et al., 1991). Recombinant plasmids were all purified by CsCl gradient centrifugation (Sambrook et al., 1989).

Transfection and Metabolic Labeling. COS-7 cells grown in complete DMEM supplemented with 10% fetal bovine serum were seeded into 75-cm^2 culture flasks less than 24 h prior to beginning transfection. Subconfluent COS-7 cells were then transfected by the DEAE-dextran method (Cullen, 1987) with 2 pM of expression vector PCD alone or with cDNA corresponding to WT, GA, GA-CVLS, or GA-CALL. For expression of the $\beta\gamma$-subunits, COS cells were transfected with 2 pM of both the β- and γ-expression vectors. Total DNA used for transfections was kept constant by adding pCD vector without insert as necessary. COS cells, 24 h after transfection, in two 75-cm^2 flasks were treated with trypsin-EDTA and then pooled and split into three 100-mm Petri dishes. At 48 h, the cells were either harvested by scraping with a rubber policeman after thoroughly removing the media and washing with phosphate buffered saline, pH 7.4, or prepared for metabolic labeling. In order to decrease the endogenous cellular pools of targeted unlabeled compounds and maximize the radiolabeling, cells to be radiolabeled were preincubated (starved) for 1 h in media identical to the radiolabeling media except that it lacked the radiolabeled compound. For radiolabeling with [^{3}H]mevalonolactone, the cells were incubated in complete DMEM supplemented with 5% fetal bovine serum (dialyzed against 150 mM NaCl), 20 μM compactin, and 200 μCi/mL (*RS*)-[5-^{3}H]mevalonolactone (specific activity 28 Ci/mmol) for 18 h. For metabolic labeling with radiolabeled methionine, the cells were incubated in complete DMEM without methionine supplemented with 5% dialyzed fetal bovine serum and 200 μCi/mL L-[*methyl*-^{3}H]methionine (specific activity 80 Ci/mmol) for 18 h or 225 μCi/mL L-[^{35}S]methionine (specific activity 1100 Ci/mmol) for 5 h. Cell pellets were obtained after washing with phosphate-buffered saline, pH 7.4, by scraping and centrifugation at 500*g* for 10 min at 4 °C and then stored at −70 °C.

Subcellular Fractionation. The cell pellets were homogenized in a hypotonic buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 μg/mL soybean trypsin inhibitor, 3 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μM pepstatin A, 0.1 mM phenylmethanesulfonyl fluoride) by trituration through a 25-gauge needle. Nuclei and unbroken cells were pelleted by centrifugation at 500*g* for 3 min in an Eppendorf 5415 microcentrifuge. The pellet was washed in the same buffer. The supernatants from both of these centrifugations were combined and centrifuged at 109000*g* for 30 min in a Beckman TLA45 rotor at 4 °C. The pellet (particulate) and the supernatant (soluble) fractions were separated, the pellet was resuspended in homogenization buffer, and both fractions were recentrifuged under identical conditions to minimize cross-contamination. Protein concentrations were determined by using the Bio-Rad protein assay with bovine serum albumin as the standard.

Immunoprecipitation. The preparation and characterization of antisera raised to synthetic decapeptides corresponding to amino acid residues 159–168 of α_i1 (LD), the carboxy-terminal decapeptide of α_t (AS), and residues 55–64 of γ_2 (SE) have been described (Goldsmith et al., 1987, 1988; Simonds et al., 1991). Immunoprecipitation was carried out on equal amounts of total radiolabeled proteins which were solubilized in 50 mM TrisHCl, pH 7.4 (25 °C), 150 mM NaCl, 0.1% (w/v) Triton X-100, 0.02% (w/v) SDS, and 5 mM EDTA and precleared by incubating with rabbit IgG and then protein-

A–agarose. The affinity-purified antibodies or rabbit IgG as control were added to the resulting supernatant after clearing and being incubated overnight at 4 °C. Antigen–antibody complexes were recovered by incubating with protein-A–agarose. This complex was washed vigorously with the immunoprecipitation buffer followed by a final wash with the same buffer without detergent. Pellets were aspirated to dryness and prepared for either isoprenoid, methyl ester, or autoradiographic analysis.

Chemical Analysis of Isoprenoids. For HPLC analysis, [^{3}H]mevalonolactone-labeled proteins were immunoprecipitated with LD antibodies followed by protein-A–agarose. These pellets were then subjected to methyl iodide cleavage, and the isoprenyl alcohols were separated by HPLC according to the method of Mumby et al. (1990a).

Carboxyl Methylation Assay. Immunoprecipitated protein pellets from [*methyl*-^{3}H]methionine-labeled COS cell transfectants were dissolved, boiled for 3 min in denaturing buffer, and separated by SDS–PAGE. The region of the gel containing the immunoprecipitated protein was sliced, and carboxyl methyl ester analysis was carried out according to the procedure of Chelsky et al. (1984).

Miscellaneous Procedures. Protein samples and molecular weight standards were solubilized, denatured, and analyzed on 10% polyacrylamide gels by standard SDS–PAGE as previously described (Jones & Spiegel, 1990; Laemmli, 1970). Fluorography was accomplished on fixed gels that were treated with Amplify (Amersham) supplemented with 5% glycerol, dried, and exposed to XAR-2 film (Kodak) at −70 °C. Immunoblotting with the LD and AS antibodies and localization of antigen–antibody complexes with peroxidase-conjugated goat anti-rabbit antibodies were carried out as described (Goldsmith et al., 1987). [^{32}P]ADP-ribosylation with pertussis toxin was performed on 12 μg of the soluble fractions of PCD-, GA-, GA-CVLS-, and GA-CALL-transfected COS cells by the method of Ribeiro-Neto et al. (1985) with minor modifications (Jones & Spiegel, 1990). Purified transducin $\beta\gamma$-subunits (2.5 μg) (Shinozawa et al., 1980) were added to the reaction mixture.

RESULTS AND DISCUSSION

Transient Expression in COS Cells. COS cells were transfected with the pCD plasmid alone (PCD) or with plasmid and cDNA for mutated α_i1-proteins in which the amino-terminal glycine was changed to an alanine (GA) and double mutants in which the carboxy terminus was changed from CGLF to CVLS (GA-CVLS) or CALL (GA-CALL). After 48 h the cells were harvested and separated into particulate and soluble fractions by centrifugation. Immunoblots using the LD antibody which was generated to an internal decapeptide specific for α_i1 show a similar level of overexpression of the mutated α_i1-proteins as compared to the control PCD cells (Figure 1A). However, immunoblots using the AS antibody, which shows immunoreactivity to the carboxy terminus of α_i1, did not detect the double mutants (GA-CVLS, GA-CALL) while the endogenous proteins and the overexpressed GA protein were seen (data not shown).

After transfection, the COS cells were incubated with [^{35}S]-methionine for 5 h, separated into a particulate and soluble fraction, and then immunoprecipitated with the LD antibody specific for α_i1 or with rabbit IgG as control. The samples were analyzed by SDS–PAGE and fluorography. The GA, GA-CVLS, and GA-CALL proteins were specifically immunoprecipitated with LD antibodies as compared to nonspecific IgG (data not shown). The autoradiograph in Figure 1B shows that the GA, GA-CVLS, and GA-CALL proteins are primarily localized to the soluble fraction, whereas the small amount of endogenous α_i1 found in cells transfected with PCD is in the particulate fraction. The myristoylated wild-type α_i1-protein after transient expression is predominantly found in the particulate fraction (data not shown). The small amount of the GA, GA-CVLS, and GA-CALL proteins in the particulate fraction may be due to their interaction with the $\beta\gamma$-subunits for which they have a reduced affinity compared to the myristoylated α_i1-protein (Jones et al., 1990). Though the cells were incubated with the radiolabel for only 5 h, the distribution of the α-subunits to the particulate and soluble fractions is similar to that of the steady-state conditions as seen in the immunoblot (Figure 1A).

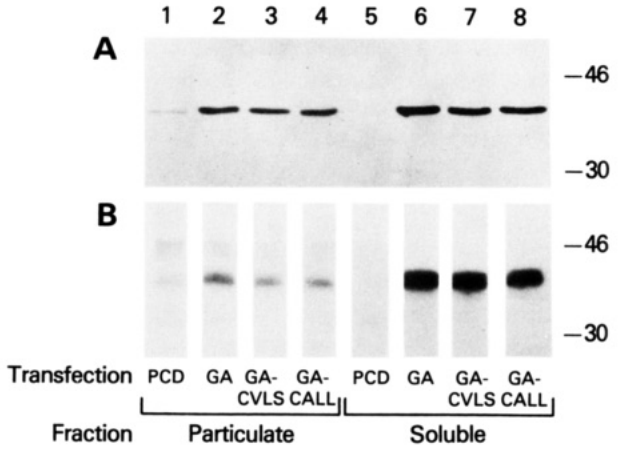

FIGURE 1: Expression and subcellular distribution of mutant α-subunit proteins. COS cells were transfected with either vector alone (PCD) or with cDNA of the α-subunit mutants (GA, GA-CVLS, or GA-CALL) and separated into particulate and soluble fractions by centrifugation at 109000*g* for 30 min. (A) Immunoblot of 40 μg of total particulate and soluble proteins with affinity-purified LD antibodies raised against an internal sequence of α_i1. (B) Cells were radiolabeled with [^{35}S]methionine, separated into particulate and soluble fractions, and then immunoprecipitated with affnity-purified LD antibody. Total protein from each fraction (15 μg) was immunoprecipitated as described in the Materials and Methods section and analyzed by SDS–PAGE and fluorography. Autoradiogram was exposed for 10 h at −70 °C.

Pertussis toxin catalyzes the ADP-ribosylation of certain α-subunits including α_i1 on the cysteine four residues from the carboxy terminus (Simon et al., 1991). Previously, it was shown that this modification does not occur in the CVLS mutants (Jones & Spiegel, 1990). Likewise in this study, we found that the GA-CALL mutant does not undergo ADP-ribosylation under conditions which led to the ADP-ribosylation of the GA protein (data not shown). Since pertussis toxin-catalyzed ADP-ribosylation occurs when the α-subunit is associated with the $\beta\gamma$-subunits (Spiegel 1987), the loss of ADP-ribosylation could be due to a decreased affinity for $\beta\gamma$. The amino acid substitution of CALL for CGLF could have changed the ability to undergo ADP-ribosylation by another mechanism or led to the loss of immunoreactivity with the AS antibody. However these differences could also be explained by isoprenylation of the cysteine and/or further processing of the carboxy terminus.

[^{3}H]Mevalonic Acid Radiolabeling. Incorporation of [^{3}H]-mevalonic acid has been used to demonstrate isoprenylation of particular proteins (Schmidt et al., 1984). Transfected COS cells were radiolabeled with [^{3}H]mevalonolactone in the presence of compactin to inhibit the endogenous production of mevalonic acid. The cells were separated into particulate and soluble fractions and analyzed by SDS–PAGE and flu-

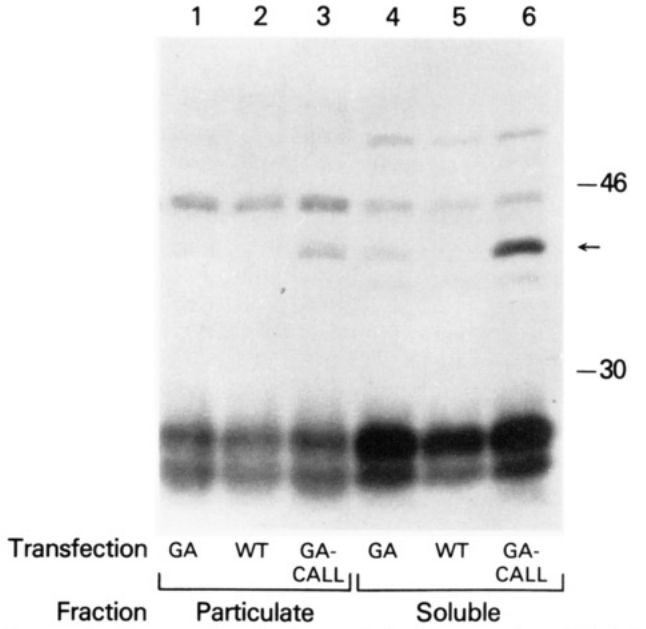

FIGURE 2: Expressed mutant α-proteins incorporate radiolabel derived from [^{3}H]mevalonolactone. COS cells were transfected with expression vectors encoding the wild-type α_i1-subunit (WT), nonmyristoylated α_i1 mutant subunit (GA), and nonmyristoylated carboxy-terminal α_i1 double mutant subunit (GA-CALL). Cells were radiolabeled with 200 μCi/mL [^{3}H]mevalonolactone for 18 h in the presence of 20 μM compactin and then centrifuged at 109000*g* for 30 min. Total protein from the particulate and soluble fractions (50 μg) was separated by SDS–PAGE and subjected to fluorography. The autoradiogram was exposed for 21 days at −70 °C. The arrow indicates a novel 41-kDa protein labeled with [^{3}H]mevalonolactone most strongly in lane 6 and less strongly in lane 4 of the soluble fraction and consistent with the location of α_i1-subunit.

orography (Figure 2). Radiolabel incorporation is seen in several endogenous proteins in all the transfected COS cell fractions. A unique radiolabeled 41-kDa protein is present in the GA-CALL transfected cells which is not present in the WT transfected cells. The distribution of this protein is similar to the distribution of the GA-CALL protein seen with immunoblotting; the majority found in the soluble fraction despite the fact that it is isoprenylated. A faint 41-kDa band is seen in the GA-protein which ends in the sequence CGLF. This may represent isoprenylation of a small amount of the overexpressed protein and is in agreement with recent work by Yokoyama et al. (1991), which suggested that in the context of overexpressing CAAX-containing proteins in eukaryotic cells, the prenyl transferase may have somewhat relaxed prenylation specificities probably due to the high protein/peptide acceptor concentrations. The reason that the WT does not incorporate [^{3}H]mevalonic acid despite the fact that it contains a CGLF carboxy terminus and is approximately equally overexpressed is not known at this time. The fact that WT is cotranslationally myristoylated at its amino terminus while GA is not may influence the events that occur at the carboxy terminus. By analogy with the ras three-dimensional structure, the carboxy and amino termini of the α-subunit may be in close proximity (Tong et al., 1989).

HPLC Analysis of Isoprenyl Groups. Based on in vitro studies of the prenyl transferases (Seabra et al., 1991; Kinsella et al., 1991; Reiss et al., 1991), we predicted that the GA-CVLS and GA-CALL proteins would be modified by a farnesyl and a geranylgeranyl moiety, respectively. To determine which isoprenyl group was modifying the mutant proteins, the proteins were immunoprecipitated and the isoprenyl group was analyzed by HPLC after cleavage from the protein with methyl iodide (Chelsky et al., 1984) (Figure 3).

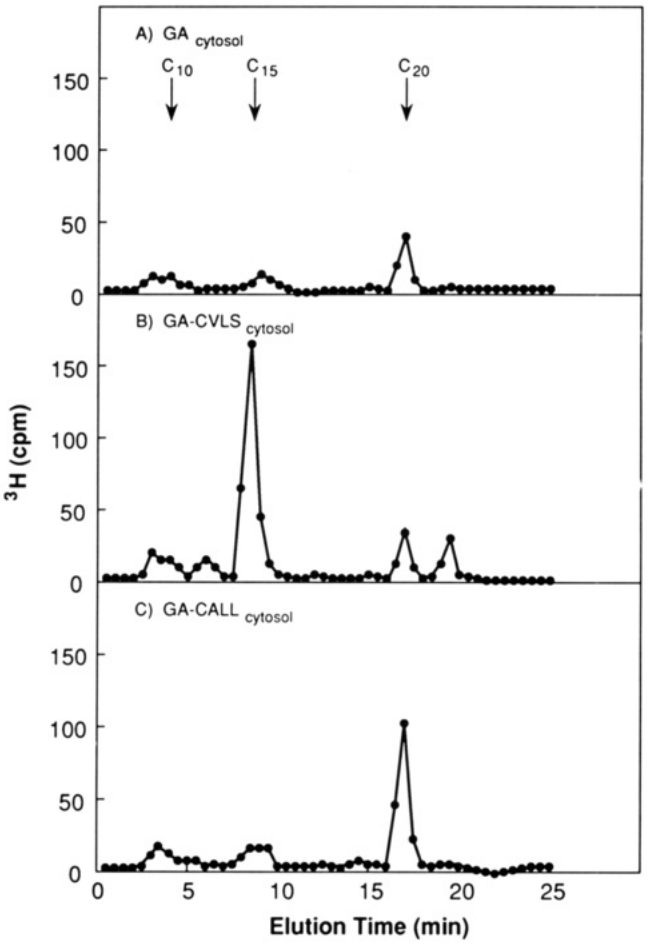

FIGURE 3: HPLC analysis of isoprenyl groups. G-protein α_i1 mutants were transfected into COS cells, and the cells were incubated with [^{3}H]mevalonolactone. The α_i1-proteins in the soluble fraction were immunoprecipated, and the isoprenyl group was released by cleavage with methyl iodide. The radioactive isoprenyl groups were then analyzed by HPLC. Isoprenyl groups from α_i1-proteins immunoprecipated from COS cells transfected with either (A) GA, (B) GA-CVLS, or (C) GA-CALL. The elution of authentic standards for geraniol (C10), *trans,trans*-farnesol (C15), and *all-trans*-geranylgeraniol (C20) are indicated.

Comparison with known standards showed that the GA-CVLS protein is modified with a farnesyl moiety and that the GA-CALL protein is modified with a geranylgeranyl moiety. Therefore, for the α_i-subunits, the mutations in the CAAX sequence were sufficient to determine the specificity of isoprenylation in vivo. The increased hydrophobicity of the isoprenylated proteins caused by the addition of either a C_{20} or C_{15} isoprenyl moiety, however, was not sufficient to target these proteins to the membrane (Figure 1).

Carboxyl Methylation. To characterize further the modifications of these α_i1 CAAX mutants in vivo, we studied whether these proteins underwent carboxyl methylation. Transfected COS cells were radiolabeled with [*methyl*-^{3}H]-methionine for 18 h, separated into particulate and soluble fractions, and immunoprecipitated with the LD antibody. Under these conditions, tritium is incorporated both as [*methyl*-^{3}H]methionine and as methyl groups from *S*-adenosyl-[*methyl*-^{3}H]methionine formed in vivo. The samples were divided and analyzed by SDS–PAGE. Gel slices were analyzed for total incorporation of methionine or for base-labile radioactivity, which is a measure of carboxyl methylation. Using similar methods, Gutierrez et al. (1989) demonstrated a stoichiometric methylation of p21$^{N\text{-}ras}$ in transfected COS cells. Here, as a positive control for carboxyl

Table I: Carboxyl Methylation Analysis[a]

	methionine,[b] dpm	base-labile MeOH, dpm	stoichiometry[c]
	Transfection 1		
soluble			
PCD	4 972	14	NC[d]
GA	52 969	314	0.05
GA-CVLS	45 933	389	0.07
GA-CALL	38 322	67	0.01
	Transfection 2		
soluble			
GA-CVLS	98 425	1247	0.10
GA-CALL	76 009	436	0.05
particulate			
GA-CVLS	11 174	371	0.27
GA-CALL	17 634	808	0.37
β-γ	6 969	1731	0.50

[a] In 2 separate experiments, COS cells transfected with vector alone (PCD) or with the vector containing the cDNA for mutant α_i1-subunits or β- and γ-subunits were radiolabeled with [*methyl*-^{3}H]methionine. Subcellular fractionation and immunoprecipitation with antibodies specific for the α- and γ-subunits were performed followed by SDS–PAGE. Gel slices containing the immunoprecipitated proteins were analyzed to determine total radioactivity (methionine anbd methyl ester incorporation) and base-labile radioactivity (methyl ester incorporation). [b] Base-labile radioactivity was subtracted from total radioactivity. [c] The methionine radioactivity was first divided by the number of methionines in the protein (8 for the α-subunit and 2 for the γ-subunits), and then the ratio of the methyl ester to the corrected methionine radioactivity was calculated. [d] Not calculated.

methylation, the methylation of the G-protein γ_2-subunit was determined in COS cells transfected with vectors containing the cDNAs for the β_1- and γ_2-subunits. The γ_2-subunit was analyzed for carboxyl methylation in the same way as the α_i1 mutants, except the SE antibody, specific for the γ_2-subunit, was used for the immunoprecipitation. The γ_2-subunit ends with the sequence CAIL and has been shown to undergo geranylgeranylation (Yamane et al., 1990; Mumby et al., 1990a). The carboxyl methylation assay showed a stoichiometry of 0.5 for the γ_2-subunit, indicating that 50% of the immunoprecipitated γ-proteins are carboxyl methylated (Table I). The actual methylation may be higher, due to some hydrolysis of the methyl ester during processing of the protein samples. Both α_i double mutants in the soluble fraction show only a minimal degre of carboxyl methylation (Table I), even though they had been isoprenylated. For the small fraction of the mutant proteins in the particulate fraction, a detectable level of methylation was observed.

These results in vivo are similar to results in vitro which indicate that the enzyme responsible for the carboxyl methylation is membrane bound. In vitro studies by Stephenson and Clarke (1990) suggest that methyl esterification of proteins containing a carboxy-terminal CAAX motif requires isoprenylation of the cysteinyl sulfhydryl group and removal of the AAX amino acids as well. As opposed to isoprenylation and carboxyl methylation, very little is known about the specificity and subcellular localization of the enzyme responsible for cleavage of the AAX amino acids. Although the carboxy-terminal mutants were isoprenylated, it was not determined what fraction, if any, of the isoprenylated proteins was further processed by cleavage of the AAX peptide, since there is no straightforward method for assessing this modification.

These in vivo studies indicate that isoprenylation can occur on appropriately mutated α_i1-proteins, but that neigher farnesylation nor geranylgeranylation are by themselves sufficient to promote membrane attachment. Studies on in vitro translated p21^{ras} show that methylation and proteolysis, in addition to farnesylation, are required for efficient membrane binding (Hancock et al., 1991a). Failure of the bulk of the mutated α_i1-proteins to undergo methylation may reflect the potential importance of the amino acids upstream from the CAAX motif which may act as determinants for the proteolytic and methyl esterification events following the initial isoprenylation. Alternatively, lack of methylation may reflect the inability of isoprenylation alone to promote membrane association sufficient to permit membrane-bound carboxyl methyltransferase to modify the mutant protein. Studies with isoprenylated p21^{ras} and the rab proteins show that carboxy-terminal domains in addition to the CAAX motif are crucial in proper targeting (Khosravi-Far et al., 1991; Chavrier et al., 1991). Membrane binding of a heterologous cytosolic protein required addition of a CAAX motif *plus* a second membrane-targeting signal upstream of the CAAX motif (Hancock et al., 1991b). Further studies are needed to investigate the role of the different isoprenyl groups in membrane and protein–protein interactions and to better understand the determinants necessary for complete CAAX protein processing.

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