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The γ subunit of transducin is farnesylated

(prenylation reaction/G protein)

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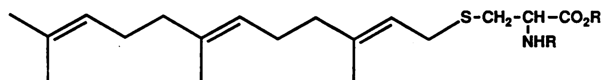
ABSTRACT **Protein prenylation with farnesyl or geranylgeranyl moieties is an important posttranslational modification that affects the activity of such diverse proteins as the nuclear lamins, the yeast mating factor *mata*, and the *ras* oncogene products. In this article, we show that whole retinal cultures incorporate radioactive mevalonic acid into proteins of 23–26 kDa and one of 8 kDa. The former proteins are probably the "small" guanine nucleotide-binding regulatory proteins (G proteins) and the 8-kDa protein is the γ subunit of the well-studied retinal heterotrimeric G protein (transducin). After deprenylating purified transducin and its subunits with Raney nickel or methyl iodide/base, the adducted prenyl group can be identified as an all-*trans*-farnesyl moiety covalently linked to a cysteine residue. Thus far, prenylation reactions have been found to occur at cysteine in a carboxyl-terminal consensus CAAX sequence, where C is the cysteine, A is an aliphatic amino acid, and X is undefined. Both the α and γ subunits of transducin have this consensus sequence, but only the γ subunit is prenylated. Therefore, the CAAX motif is not necessary and sufficient to direct prenylation. Finally, since transducin is the best understood G protein, both structurally and mechanistically, the discovery that it is farnesylated should allow for a quantitative understanding of this posttranslational modification.**

The posttranslational modifications of enzymes and regulatory proteins can radically alter their activities. A recently discovered modification of this type involves the adduction of an isoprene moiety to a terminal cysteine residue of proteins (1–4). Since the first report of farnesylation of proteins in the yeast mating factor *mata* (5) and the *ras* encoded proteins (6, 7), substantial interest has been focused on prenylated proteins. The structure of a farnesylated cysteine residue is shown in Scheme I.



Scheme I

Recent reports that proteins in HeLa cells (8, 9) and a protein from Chinese hamster ovary cells (10) were modified by a C-20 isoprenoid—geranylgeranyl—rather than the C-15 isoprenoid farnesyl raises the possibility of other types of prenylation of proteins. These modifications are thought to help anchor the protein to membranes by either hydrophobic or, perhaps, specific receptor-mediated events (6, 8). This anchoring process appears to be important to the functioning of the modified proteins. For example, the *ras* oncogene product is prenylated, and site-directed mutations in this protein that prevent its prenylation block the oncogenic activity of *ras* (6). Similar results are obtained by using mevinolin, a potent inhibitor of isoprenoid biosynthesis (11).

The nature of the covalent linkage between the isoprene unit and the protein remains somewhat obscure, but the release of the isoprene unit with Raney nickel strongly implicates a thioether bond to a cysteine residue (8–10). It appears that the prenylation reaction is directed at cysteine residues that are part of a CAAX motif (C, cysteine; A, aliphatic amino acid; X, undefined) located near the carboxyl-terminal end of the protein. In addition to the prenylation reaction, further enzymatic processing may occur at the terminal CAAX sequence. The AAX sequence can be removed and the cysteine residue, which is thereby exposed, is methylated (6, 12). Neither the timing of these modification reactions nor their relationship to each other has been established. Of substantial interest is whether the CAAX motif alone is both necessary and sufficient to direct the prenylation reaction or whether other elements of the proteins' structure are also important.

Although it is assumed that the prenylation reaction helps to anchor a protein to the membrane, clear biochemical evidence for this assumption has not been forthcoming. Moreover, the functional consequences of this putative anchoring process have not yet been demonstrated since none of the reported prenylated proteins have known biological functions. One way to study the structural, functional, and mechanistic consequences of prenylation is to study its effect on a well-characterized protein. It is also of some interest to study the effects of prenylation on a guanine nucleotide-binding regulatory protein (G protein) involved in signal transduction since several prenylated proteins have so far been shown to be in the "small" G-protein class (12). The retinal heterotrimeric G-protein transducin is the best understood G protein from the standpoint of both structure and mechanism (13, 14). The molecule is composed of a heterotrimeric αβγ structure and is associated with the rod outer segment (ROS) disk membranes prior to its interaction with photoactivated rhodopsin (13, 14). Transducin contains CAAX motifs on both its α and its γ subunits (15–18). We demonstrate here that the γ subunit is prenylated and that it is modified with a farnesyl moiety. These findings demonstrate that the "large" heterotrimeric G proteins can be farnesylated and they also should make it possible to investigate the mechanistic roles of prenylation in a quantitative manner.

MATERIALS AND METHODS

Materials. Bovine eyes were obtained from a local slaughterhouse. Frozen bovine retinas were obtained from Wanda Lawson Company (Lincoln, NB). L-[4,5-^{3}H]Leucine (68 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. *R,S*-[5-^{3}H(N)]Mevalonolactone (40 Ci/mmol) was from New England Nuclear. Lovastatin (mevinolin) was a gift from Merck Sharp & Dohme. Tissue culture RPMI 1640 medium was

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Abbreviations: ROS, rod outer segment; G protein, guanine nucleotide-binding regulatory protein.

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from GIBCO. Hydrofluor and Filtron X were from National Diagnostics (Manville, NJ). Amplify was from Amersham. Nerolidol, all-*trans*-farnesol, Raney nickel (active catalyst), and methyl iodide were obtained from Aldrich. All-*trans*-2,6,10-Trimethyl-2,6,10-dodecatriene was synthesized from farnesyl bromide (19, 20). The major isomer was the all-*trans* isomer as demonstrated by ^{1}H and ^{13}C NMR spectroscopy (21). The isomeric mixture of 2,6,10,14-tetramethyl-2,6,10, 14-hexadecatetraenes was synthesized from farnesyl acetone (Bedoukian Research, Danbury, CT) as described (8). Farnesane (2,6,10-trimethyldodecane) was purchased from Wiley Organics (Columbus, OH). Blue Sepharose CL-6B column packing was purchased from Pharmacia LKB and DEAE-Toyopearl 650S column packing was purchased from TosoHaas (Philadelphia). Leupeptin, pepstatin, and aprotinin were obtained from Boehringer Mannheim. Sephadex G-25, dithiothreitol, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, and GTP were obtained from Sigma.

Methods. *Retina incubations*. Bovine retinas were removed in the dark and each one was incubated at 37°C in 2 ml of RPMI 1640 medium containing 30 mM Hepes (pH 7.4), 35 units of penicillin per ml, 35 μg of streptomycin per ml, and 125 μM mevinolin (22). The medium was equilibrated at timed intervals with a mixture of O_2/CO_2 (95:5). After 1 hr of preincubation, radioactive precursors (250 μCi of [^{3}H]leucine or [^{3}H]mevalonolactone per retina) were added and incubations were continued for 6–8 hr longer.

At the end of the incubation, retinas were exposed to light and 15 ml of ice-cold 45% (wt/wt) sucrose in 20 mM Tris·HCl (pH 7.4), containing 1 mM $CaCl_2$ (buffer A) was added to each incubation vial. ROSs were detached by shaking the retinas in this solution and were collected by flotation. For obtaining the transducin-enriched fractions, ROS membranes were disrupted by passing through a narrow needle; they were then washed by a series of centrifugation steps as described (23). Finally, transducin was eluted from the membranes with 100 μM GTP. The transducin-enriched supernatant was concentrated by using Centriprep 30 from Amicon.

Analysis and quantitation of the radioactivity incorporated into proteins. For SDS/PAGE, aliquots of ROS membranes in buffer A were precipitated in chloroform/methanol (1:2, vol/vol) and washed in methanol (24) to remove noncovalently bound lipids. The protein pellets were dissolved in sample buffer (25) containing 5% SDS and boiled for 5 min. Transducin-enriched supernatants were boiled in sample buffer directly. Samples were run in either 15% or 5–20% linear gradient gels. After electrophoresis, proteins were visualized by Coomassie staining, gels were treated with fluorographic enhancer (Amplify), and exposure was carried out at −70°C for 15–25 days. Alternatively, proteins were transferred onto nitrocellulose membranes and visualized by Ponceau S staining (26); the nitrocellulose membranes were sliced with a razor blade, the slices were dissolved in Filtron X, and the radioactivity was counted.

Preparation of transducin and its subunits. Transducin was prepared by the procedure of Wessling-Resnick *et al.* (23).

Transducin α and $\beta\gamma$ subunits were purified as described by Fukada *et al.* (27). Protein was determined by the method of Bradford (28). SDS/PAGE analysis of the subunit fractions showed them to be at least 95% pure.

Chemical extraction of the isoprenoids. Two methods were used to extract the isoprenoid from transducin. The methyl iodide method used was identical to the procedure described by Maltese *et al.* (29). In this procedure, 3 mg of transducin was used and the isoprenoid product was dissolved in a minute amount of methylene chloride and injected directly into the gas chromatograph/mass spectrometer for analysis. The second method used involved the treatment of transducin and subunits with Raney nickel. This method was described by Farnsworth *et al.* (8). In this case, 6 mg of transducin was treated with Raney nickel and extracted with pentane. The pentane solution was dried and concentrated, and an aliquot was then injected directly into the gas chromatograph/mass spectrometer for analysis. Raney nickel treatment was repeated with 1 mg of the purified transducin α subunit and 1 mg of the purified transducin $\beta\gamma$ subunit. The same procedure was also repeated with 10 mg of aprotinin as control and no isoprenoids were detected by gas chromatography/mass spectrometry (GC-MS) analysis.

GC-MS. GC was performed on a Hewlett–Packard 5890 gas chromatograph directly coupled to a Hewlett–Packard 5970B mass selective detector. A 5-μl sample was manually injected into a fused silica capillary column (Durabond DB-1701; 30 m × 0.2 μm; J & W Scientific, Rancho Cordova, CA). Mass spectra were obtained in the electron ionization mode at 70 eV using the 2600-V multiplier setting. Data were analyzed with the Hewlett–Packard 59970 ChemStation software.

RESULTS

Prenylation Reactions in the Retina. Initial experiments were aimed at determining whether transducin was prenylated. If prenylation occurred, it was of further interest to determine which subunit was prenylated. As the prenylation reaction is an irreversible posttranslational modification, its study requires a system that actively supports protein synthesis. The capacity of rod photoreceptor cells to synthesize protein in detectable amounts in an *in vitro* system was assessed by using [^{3}H]leucine as radioactive precursor. The incorporation of radioactive leucine into proteins was analyzed by SDS electrophoresis of whole ROS samples, followed by autoradiography. As can be appreciated from Fig. 1, there is substantial incorporation of [^{3}H]leucine into ROS proteins after 6 hr of incubation under the conditions described above. In this and in the following experiments, interference by rhodopsin with the detection of less abundant polypeptides was minimized by boiling the samples in SDS prior to electrophoresis, thus causing the rhodopsin to aggregate.

The ability of rod photoreceptor cells to incorporate an isoprenoid precursor into specific polypeptides was explored by incubating the retinas with [^{3}H]mevalonolactone (which is converted to [^{3}H]mevalonic acid upon uptake by the cells) in the presence of an inhibitor of the endogenous mevalonate synthesis (mevinolin) (11, 30) as shown in Fig. 1. Under these conditions, radiolabeled polypeptides of apparent molecular masses 23–26 kDa and 8 kDa were detectable by SDS electrophoresis in samples from whole ROS after 25 days of fluorographic exposure. The 23- to 26-kDa proteins are doubtless the prenylated small G proteins previously described in neuroblastoma cells (12). The γ subunit of transducin is an excellent candidate for the prenylated 8-kDa protein.

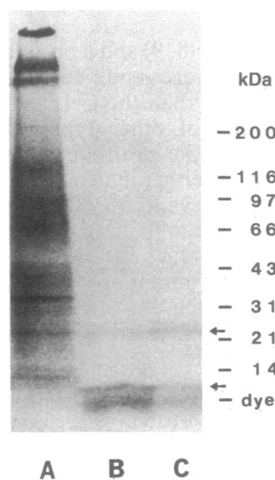

FIG. 1. *In vitro* incorporation of [^{3}H]leucine and [^{3}H]mevalonic acid into ROS proteins. Fresh bovine retinas were incubated for 6 hr with [^{3}H]leucine (lane A) or [^{3}H]mevalonic acid (lanes B and C) and in the presence of 125 μM mevinolin in all cases. Delipidated ROS samples were loaded on a 5–20% gradient gel. Fluorographic exposure was for 25 days. Arrows indicate the positions of the prenylated polypeptides.

To identify the labeled 8-kDa protein, transducin was partially purified from single retinas after incubation with [^{3}H]mevalonate and was analyzed by gel chromatography. Under these conditions, the fluorography of the transducin-enriched supernatant showed a single radiolabeled polypeptide whose apparent molecular mass is the same as that of purified transducin γ subunit (Fig. 2 *Upper*). This observation could be quantified by determining the amounts of radioactivity found in slices cut from a nitrocellulose blot of this supernatant (Fig. 2 *Lower*). These experiments suggest that only the γ subunit of transducin is prenylated.

Holotransducin Is Farnesylated. The experiments described above demonstrate that the γ subunit of transducin is prenylated in whole bovine retinas. The chemical nature of this prenyl group was investigated next. This problem was approached in two ways. In the first set of experiments, transducin was treated with Raney nickel to cleave the thioether bond to the protein, reductively transforming the prenyl moiety into the parent hydrocarbon. The GC-MS of the released hydrocarbons showed a set of five peaks, with retention times between 12.6 and 14 min, containing characteristic isoprenoid mass fragments (Fig. 3*A*). The mass spectrum of the major peak, with a retention time of 13.7 min, is identical within experimental limits to all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene standard (molecular mass ion, m/z = 206) both in terms of its GC retention time and in terms of its mass spectrum (Fig. 3 *B* and *C*). To further identify the prenyl group, the putative all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene was collected and further reduced with H_2 and Pt/C to yield farnesane as determined by GC-MS (data not shown).

The possibility that the protein is geranylgeranylated can be ruled out because 2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene and its fully reduced derivative phytane proved to have entirely different retention times on GC than 2,6,10-trimethyl-2,6,10-dodecatriene and farnesane, respectively. Moreover, the mass spectra of 2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene and phytane showed parent m/z

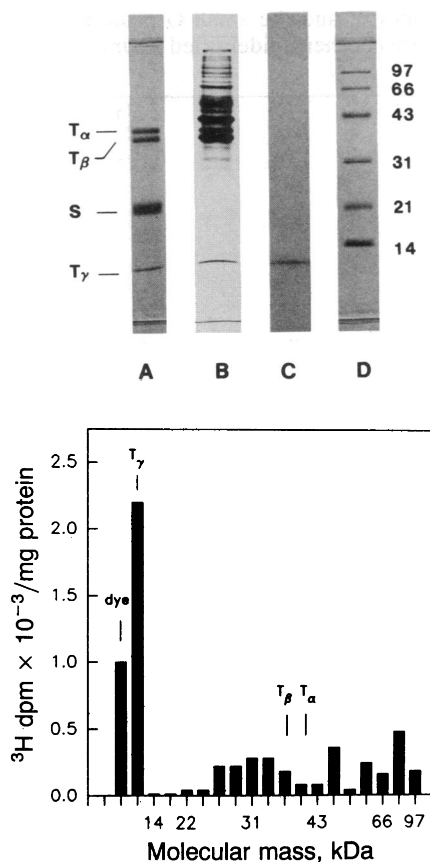

FIG. 2. *In vitro* incorporation of [^{3}H]mevalonic acid into the γ subunit of transducin. Fresh bovine retinas were incubated with [^{3}H]mevalonic acid in the presence of mevinolin for 8 hr. The transducin-enriched supernatant was obtained from ROS. (*Upper*) SDS/PAGE on 15% polyacrylamide gels. Lanes: A, standard of purified transducin (S, soybean trypsin inhibitor); B, 60 μl of transducin-enriched supernatant; C, autoradiography of lane B after 15 days of exposure; D, molecular mass markers (in kDa). (*Lower*) Approximately 50 μg of protein was loaded on a 5–20% linear gradient gel and transferred onto nitrocellulose after SDS/PAGE. The radioactivity present in 2-mm slices of the membrane was determined. The positions of the transducin γ, β, and α subunits are indicated by T_γ, T_β, and T_α, respectively.

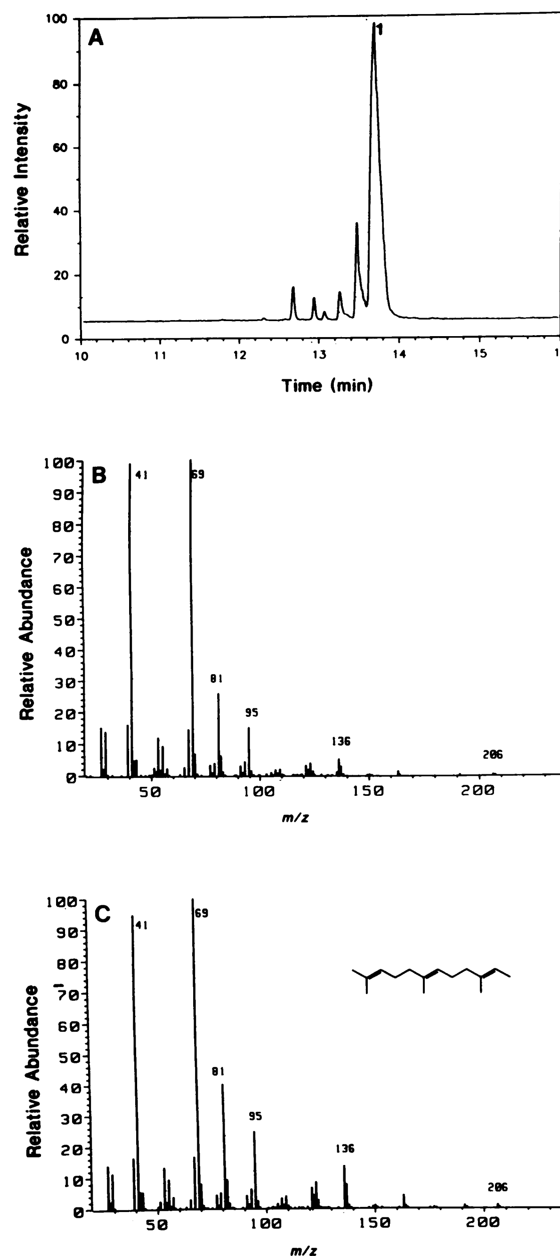

FIG. 3. Gas chromatography/mass spectrometry of pentane extracts of Raney nickel treatment of holotransducin. (*A*) Reconstructed ion chromatogram for the characteristic isoprene ion, m/z 69. Retention time of peak 1 is identical to that of the synthesized all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene. (*B*) Electron ionization mass spectrum of peak 1. (*C*) Electron ionization mass spectrum of synthesized all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene, whose structure is shown in the *Inset*.

peaks of 274 and 282, respectively. These peaks failed to occur in the mass spectra of samples from Raney nickel-treated transducin and its reduced product.

Finally, the methyl iodide deprenylation method was used to confirm that a farnesyl unit was adducted to transducin (5, 29). Various isoprenol products including farnesol, nerolidol, and α-bisabolol are formed due to rearrangement, and nerolidol tends to be the major product (31, 32). When purified transducin was processed with methyl iodide and the products were extracted, the GC-MS pattern shown in Fig. 4 was obtained. The peak at a retention time of 16.4 min had an identical retention time to nerolidol, as confirmed by coinjection with authentic material. Moreover, the mass spectra of the putative nerolidol and authentic nerolidol were also identical, as shown in Fig. 4 *B* and *C*.

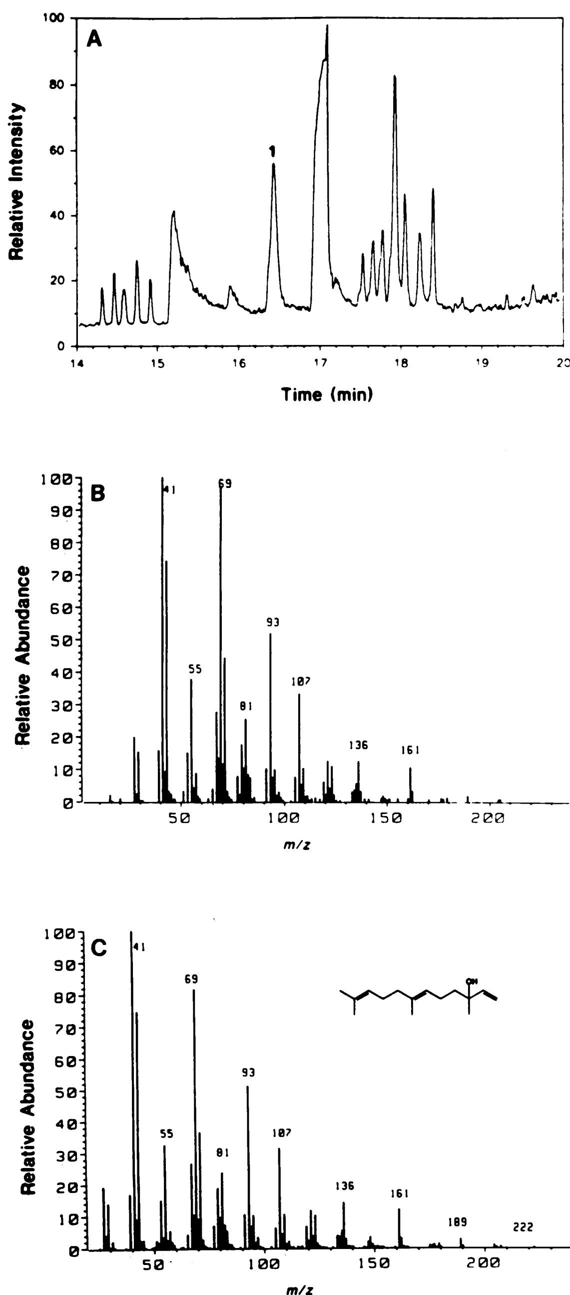

FIG. 4. Gas chromatography/mass spectrometry of pentane extracts of holotransducin after methyl iodide deprenylation. (*A*) Reconstructed ion chromatogram for the characteristic isoprene ion, m/z 69. The retention time of peak 1 is identical to that of nerolidol. (*B*) Electron ionization mass spectrum of peak 1. (*C*) Electron ionization mass spectrum of authentic nerolidol, whose structure is shown in the *Inset*.

The γ Subunit of Transducin Is Farnesylated. Both the α and γ subunits of transducin contain the carboxyl-terminal CAAX motif, and, hence, either or both subunits may be farnesylated. To address this issue, the transducin α and βγ subunits were separated and purified (27). The purified subunits were separately treated with Raney nickel catalyst to remove the putative farnesyl groups, and the released hydrocarbons were analyzed by GC-MS as shown in Fig. 5. Isoprenoid was not released from the α subunit, but all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene was released from the βγ subunit. Again, ion chromatograms for the characteristic mass ions, m/z 205 and 274, failed to demonstrate the presence of geranylgeranene and like species of the C20 class of isoprenoids. Since only the γ subunit of βγ contains a CAAX box, it is expected that only the γ subunit is farnesylated. This result is completely in accord with the retinal labeling studies described above.

DISCUSSION

Previous experiments on the prenylation reaction have shown that this reaction can involve either farnesylation or geranylgeranylation. For example, the lamins (8, 33), yeast mating factors (5), and the small G proteins (12) are farnesylated, whereas other unidentified mammalian proteins (9,

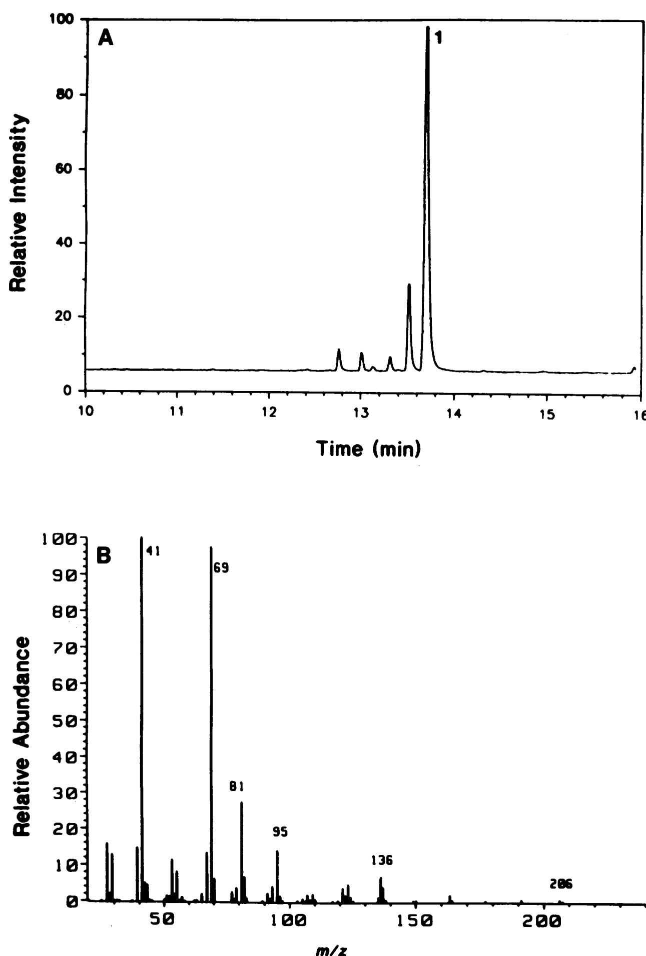

FIG. 5. Gas chromatography/mass spectrometry of pentane extracts of Raney nickel treatment of purified βγ subunits of transducin. (*A*) Reconstructed ion chromatogram for the characteristic isoprene ion, m/z 69. Retention time of peak 1 is identical to that of the synthesized all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene. (*B*) Electron ionization mass spectrum of peak 1.

10) are geranylgeranylated. In the experiments described here, it was first shown that isolated bovine retinas incorporate [^{3}H]mevalonic acid into proteins in the 23- to 26-kDa range and into an 8-kDa protein. This latter protein was shown to be the γ subunit of transducin. The 23- to 26-kDa proteins are probably the small G proteins described by others (12, 34). The chemical nature of the prenyl group adducted to transducin was further studied by GC-MS.

The methods used to demonstrate prenylated posttranslational modifications involve either methyl iodide/base treatment (29) or Raney nickel treatment (8). It should be mentioned that neither method provides unambiguous information concerning the precise chemical nature of the linkage between the sulfur atom of the cysteine and the isoprene moiety. Nevertheless, when these two degradative approaches were applied to transducin, it could readily be shown that a farnesyl group and not a geranylgeranyl group was attached to the γ subunit of this G protein. In a recent report (27), the possible presence of two distinct γ subunits in transducin has been suggested. It will be interesting to determine whether both forms are prenylated.

Neither the α nor the β subunit of transducin was prenylated, as determined by GC-MS analysis and radioactive labeling. Thus, the modification of transducin is in line with what is found in the small ≈23-kDa G proteins and in the lamins insofar as the chemical nature of the isoprenoid is concerned.

It has generally been assumed that the CAAX motif is the recognition site for the farnesylation reaction, and that farnesyl pyrophosphate is the donor molecule (2). After the farnesylation reaction is completed, a specific carboxypeptidase is thought to cleave off the AAX unit, and the modified and carboxyl-terminal cysteine is then carboxymethylated (6, 12). The farnesylation of transducin is interesting in these regards. Since the CAAX box on the α subunit has been found here not to be prenylated, the CAAX motif is not a necessary and sufficient precondition for prenylation. Clearly, other structural issues also need to be considered. That we have found no prenylation at the α-subunit CAAX box of transducin is consistent with the observation that the cysteine residue in this box is the site of ADP ribosylation reactions (35). It should be mentioned that transducin α-subunit CAAX is Cys-Gly-Leu-Phe and α-subunit CAAX is Cys-Val-Ile-Ser (16–18). It is possible that the farnesylating enzyme only recognizes the latter terminal sequence. Of further interest is the necessity of coupling the other modification reactions—cleavage and carboxymethylation—to farnesylation. Direct biochemical studies on the farnesylated γ subunit of transducin will establish its structural nature.

The studies described here show that the γ subunit of transducin is farnesylated and that this farnesylation reaction can be observed in retinal cultures. Since transducin is the best and most quantitatively understood of the G proteins, and is readily available in large quantities, it makes for an ideal system to study the functional consequences of farnesylation. These studies will be the focus of future investigations.

Note Added in Proof. Since this manuscript was accepted for publication, Fukada *et al.* (36) have shown by a different approach that the γ subunit of transducin is farnesylated.

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