Isoprenylation of the G Protein γ Subunit Is both Necessary and Sufficient for βγ Dimer-Mediated Stimulation of Phospholipase C †

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Isoprenylation of the G Protein γ Subunit Is both Necessary and Sufficient for $\beta\gamma$ Dimer-Mediated Stimulation of Phospholipase C†

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ABSTRACT: We have previously shown that isoprenylation and/or additional post-translational processing of the G protein γ_1 subunit carboxyl terminus is required for $\beta_1\gamma_1$ subunit stimulation of phospholipase C-β_2 (PLCβ_2) [Dietrich, A., Meister, M., Brazil, D., Camps, M., & Gierschik, P. (1994) *Eur. J. Biochem. 219*, 171−178]. To examine whether isoprenylation of the γ_1 subunit alone is sufficient for $\beta_1\gamma_1$-mediated PLCβ_2 stimulation or whether any of the two subsequent modifications, proteolytic removal of the carboxyl-terminal tripeptide and/or carboxylmethylation, is required for this effect, nonisoprenylated recombinant $\beta_1\gamma_1$ dimers were produced in baculovirus-infected insect cells, purified to near homogeneity, and then isoprenylated *in vitro* using purified recombinant protein farnesyltransferase. Analysis of the $\beta_1\gamma_1$ dimer after *in vitro* farnesylation by reversed phase high-performance liquid chromatography followed by delayed extraction matrix-assisted laser desorption/ionization mass spectrometry confirmed that the γ_1 subunit was carboxyl-terminally farnesylated but not proteolyzed and carboxylmethylated. Functional reconstitution of *in vitro*-farnesylated $\beta_1\gamma_1$ dimers with a recombinant PLCβ_2 isozyme revealed that farnesylation rendered recombinant nonisoprenylated $\beta_1\gamma_1$ dimers capable of stimulating PLCβ_2 and that the degree of this stimulation was only approximately 45% lower for *in vitro*-farnesylated $\beta_1\gamma_1$ dimers than for fully modified native $\beta_1\gamma_1$ purified from bovine retinal rod outer segments. Taken together, these results suggest that isoprenylation of the γ subunit is both necessary and sufficient for $\beta\gamma$ dimer-mediated stimulation of phospholipase C.

Heterotrimeric (α and $\beta\gamma$) guanine nucleotide-binding proteins (G proteins)[1] are essential elements of many transmembrane-signaling systems involved in coupling numerous receptors for extracellular mediators or sensory stimuli to the generation of intracellular signals by effector moieties such as adenylylcyclase, ion channels, or phospholipase C [reviewed in Gilman (1987) and Birnbaumer et al. (1990)]. G proteins may also be present at intracellular sites to regulate membrane traffic events [reviewed in Bomsel and Mostov (1992) and Nuoffer and Balch (1994)]. G protein activation is initiated by the receptor-catalyzed release of GDP from the heterotrimeric G protein, followed by the binding of GTP and the dissociation of the $\alpha\cdot\beta\gamma$ heterotrimer into the GTP-liganded α subunit and the free $\beta\gamma$ dimer. Although effector regulation was initially thought to be an exclusive property of GTP-liganded α subunits, it is now generally accepted that both α_{GTP} and $\beta\gamma$ are capable of mediating effector regulation [reviewed in Birnbaumer and Birnbaumer (1995) and Neer (1995)]. Thus, $\beta\gamma$ dimers have been reported to stimulate phospholipase A_2 (Axelrod, 1995), activate members of the β isozyme family of phospholipase C [reviewed in Noh et al. (1995)], open inwardly rectifying K^+ channels (Kunkel & Peralta, 1995; Krapivinsky et al., 1995), stimulate or inhibit certain types of adenylylcyclase [reviewed in Taussig and Gilman (1995)], stimulate phosphoinositide kinase (Stoyanov et al., 1995), and activate the Ras-Raf-MEK-MAPK pathway (Van Biesen et al., 1995; Touhara et al., 1995). In addition, $\beta\gamma$ dimers have been reported to interact with calmodulin (Katada et al., 1987), the heat shock protein hsp90 (Inanobe et al., 1994), phosducin (Hawes et al., 1994; Xu et al., 1995), c-Raf-1 (Pumiglia et al., 1995), the *Saccharomyces cerevisiae* MAPK scaffold Ste5p (Whiteway et al., 1995), and several proteins containing pleckstrin homology (PH) domains, including certain G protein-coupled receptor kinases [see Premont et al. (1995) for review] or Bruton tyrosine kinase (Tsukada et al., 1994; Langhans-Rajasekaran et al., 1995).

G protein α, β, and γ subunits are members of rapidly growing gene families [reviewed in Pennington (1994)]. To date, at least 16, 5, and 11 distinct genes encoding α, β, and γ subunits, respectively, are known in mammalian cells [reviewed in Pennington (1994); see also Watson et al. (1994) and Morishita et al. (1995)]. Additional structural diversity

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[1] Abbreviations: G protein, signal-transducing heterotrimeric guanine nucleotide-binding protein; $\beta_1\gamma_1^{CVIS}$, nonisoprenylated recombinant G protein $\beta_1\gamma_1$ dimer; $\beta\gamma_t$, $\beta\gamma$ dimer of retinal transducin; FTase, protein farnesyltransferase; FPP, farnesyl pyrophosphate; RP-HPLC, reversed phase high-performance liquid chromatography; PLC, phosphoinositide-specific phospholipase C; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate; Sf9 cells, *Spodoptera frugiperda* cells; DE-MALDI-MS, delayed extraction matrix-assisted laser desorption/ionization mass spectrometry.

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of G protein subunits is caused by alternative splicing of some α subunit mRNAs and by co- and/or post-translational modification of all three subunits [reviewed in Wedegaertner et al. (1995)] (Matsuda et al., 1994). G protein γ subunits are modified post-translationally at their carboxyl termini by isoprenylation, proteolytic cleavage, and methyl esterification [reviewed in Casey et al. (1994)]. The proteins are first either farnesylated (γ_1 and γ_{11}) (Ray et al., 1995) or geranylgeranylated (other γ subunits) at a cysteine at position −4 from the carboxyl terminus. Following S-isoprenylation, a membrane-bound protease(s) cleaves the three terminal amino acids (Ma & Rando, 1992; Ashby et al., 1992) and the resultant terminal carboxyl group is methyl esterified by a membranous methyltransferase (Stephenson & Clarke, 1992). The carboxyl-terminal γ subunit modifications are not required for the formation of the $\beta\gamma$ dimer but are essential for their interaction with membranes. Thus, $\beta\gamma$ dimers carrying a serine instead of cysteine at position −4 from the γ subunit carboxyl terminus are resistant to carboxyl-terminal processing and remain cytosolic when expressed in a variety of heterologous systems (Simonds et al., 1991; Muntz et al., 1992; Pronin & Gautam, 1992; Iñiguez-Lluhi et al., 1992; Dietrich et al., 1992). The carboxyl-terminal γ subunit modification(s) has also been suggested to be a critical determinant of receptor−G protein interaction (Kisselev et al., 1994; Scheer & Gierschik, 1995).

We have recently reported the expression of recombinant wild-type $\beta_1\gamma_1$ and isoprenylation-resistant mutant $\beta_1\gamma_1$C71S dimers in baculovirus-infected insect cells (Dietrich et al., 1994). The soluble wild-type $\beta_1\gamma_1$ preparation was found to contain both nonisoprenylated and isoprenylated $\beta_1\gamma_1$ dimers in approximately equal amounts. Isoprenylated wild-type $\beta_1\gamma_1$, but neither nonisoprenylated wild-type $\beta_1\gamma_1$ nor mutant $\beta_1\gamma_1$C71S dimers, was capable of stimulating phospholipase C-β_2 (PLCβ_2). Since baculovirus-infected insect cells are capable of performing isoprenylation, carboxyl-terminal proteolysis, and carboxylmethylation of a variety of proteins containing a carboxyl-terminal CXXX motif (Lowe et al., 1992), including G protein γ subunits (Robishaw et al., 1992), the contribution of the individual carboxyl-terminal modifications to the increased ability of isoprenylated $\beta_1\gamma_1$ dimers to stimulate phospholipase C remained unknown from our previous study. We have, therefore, established an *in vitro* system allowing the production of recombinant $\beta_1\gamma_1$ dimers which are isoprenylated but are neither proteolyzed nor methyl esterified at their carboxyl termini. Functional reconstitution of the *in vitro*-farnesylated $\beta_1\gamma_1$ dimers with a recombinant PLCβ_2 isozyme revealed that isoprenylation of the γ subunit carboxyl terminus is both necessary and sufficient for $\beta\gamma$ dimer-mediated stimulation of phospholipase C.

MATERIALS AND METHODS

Materials. Unlabeled farnesyl pyrophosphate, produced by American Radiolabeled Chemicals (St. Louis, MO), was obtained through Biotrend (Köln, Germany). [^{3}H]Farnesyl pyrophosphate was purchased from DuPont NEN (Dreieich, Germany). Carboxypeptidase A was from Calbiochem (Bad Soden, Germany). Microcon-10 and Centricon-10 centrifugal microconcentrators were from Amicon (Witten, Germany). All other materials were from standard vendors or sources previously described (Dietrich et al., 1992, 1994).

Purification of Nonisoprenylated Recombinant $\beta_1\gamma_1$ Dimers. *Trichoplusia ni* 5B1-4 cells (high five cells, Invitrogen) were grown in suspension culture, infected with baculovirus encoding $\beta_1\gamma_1$, homogenized, and fractionated into soluble and particulate constituents as described in Dietrich et al. (1994). The soluble fraction (20 mg of protein) was applied to a Mono Q HR 5/5 column, which had been equilibrated with buffer A containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and 100 μM phenylmethanesulfonyl fluoride. The flow rate was 0.5 mL/min. After application of the sample, the column was washed with 5 mL of buffer A and eluted with a linear gradient (10 mL) of NaCl (0 to 500 mM) in buffer A, followed by 5 mL of buffer A containing 500 mM NaCl. Fractions of 500 μL were collected and analyzed by SDS−PAGE and immunoblotting using antisera reactive against β_1 (SW) (Murakami et al., 1992) or β_1 and γ_1 (AS/4) (Gierschik et al., 1985). Nonisoprenylated and isoprenylated $\beta_1\gamma_1$ subunits were obtained at approximately 200 and 240 mM NaCl, respectively [*cf.* Figure 5 of Dietrich et al. (1994)]. Fractions containing nonisoprenylated $\beta_1\gamma_1$ obtained from five chromatographic runs on Mono Q were pooled (2 mg of protein), diluted with 3 volumes of buffer B containing 20 mM Tris/HCl (pH 7.5), 100 μM EDTA, 1 mM dithiothreitol, and 100 μM phenylmethanesulfonyl fluoride, and applied to a column (0.5 × 6 cm) of hydroxylapatite (Calbiochem, HPLC grade), which had been equilibrated with buffer B. The flow rate was 0.5 mL/min. The resin was washed with buffer B and eluted with a linear gradient (10 mL) of K_2HPO_4/H_3PO_4 at pH 7.5 (0 to 500 mM) in buffer B. Fractions of 500 μL were collected and analyzed by SDS−PAGE. A single peak containing $\beta_1\gamma_1$ eluted at approximately 35 mM K_2HPO_4/H_3PO_4. The peak fractions (1 mL; 0.4 mg of protein) were concentrated approximately 10-fold by ultrafiltration using Microcon-10 concentrators and applied to a Superdex 75 PC column (Pharmacia) equilibrated and run on a SMART micropurification chromatography system (Pharmacia) at a flow rate of 50 μL/min with buffer A containing 100 mM NaCl. Fractions of 25 μL were collected and analyzed by SDS−PAGE. The peak fractions, containing 160 μg of mostly homogeneous $\beta_1\gamma_1$, were pooled, diluted with 3 volumes of buffer C containing 20 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 6 mM $MgCl_2$, 100 μM phenylmethanesulfonyl fluoride, and 20% (v/v) glycerol, and subjected to two cycles of 5-fold concentration and dilution with buffer C using Microcon-10 concentrators. The samples were snap-frozen in liquid N_2 and stored at −80 °C.

Purification of Recombinant Protein Farnesyltransferase. *T. ni* cells were coinfected for 3 days with two recombinant baculoviruses encoding the α or the β subunit of rat protein farnesyltransferase (Chen et al., 1993). The multiplicity of infection (MOI) was 2 for either virus. The soluble fraction (40 mg of protein) was prepared from infected cells as described in Dietrich et al. (1994) and applied to a column (1 × 5 cm) of DEAE-Sephacel, which had been equilibrated with buffer A. The flow rate was 0.25 mL/min. The column was washed with 10 mL of buffer A and eluted with a linear gradient (20 mL) of NaCl (0 to 500 mM) in buffer A, followed by 5 mL of buffer A containing 500 mM NaCl. Fractions of 500 μL were collected and analyzed by SDS−PAGE and immunoblotting using a polyclonal antiserum reactive against both subunits of the recombinant protein. The protein farnesyltransferase eluted in a broad peak

between 175 and 350 mM NaCl. Peak fractions were pooled (24 mg of protein), subjected to two cycles of 5-fold concentration and dilution with buffer A using Centricon-10 concentrators, and applied to a Mono Q HR 5/5 column, which had been equilibrated with buffer A. The flow rate was 0.5 mL/min. The column was washed with 10 mL of buffer A and eluted with a linear gradient (10 mL) of NaCl (0 to 500 mM) in buffer A, followed by 5 mL of buffer A containing 500 mM NaCl. Fractions of 500 μL were collected and analyzed by SDS−PAGE. The protein farnesyltransferase eluted at 260 mM NaCl. The peak fractions containing 4 mg of protein farnesyltransferase at a purity of >90% were pooled, aliquoted, snap-frozen in liquid N_2, and stored at −80 °C.

In Vitro Isoprenylation of Purified Nonisoprenylated $\beta_1\gamma_1$ Dimers. Purified nonisoprenylated $\beta_1\gamma_1$ dimers were incubated for 1 h at 37 °C in a final volume of 15−20 μL containing 65 mM Tris/HCl (pH 7.5), 20 mM KCl, 1.8 mM dithiothreitol, 80 μM EDTA, 9 mM $MgCl_2$, 5 μM $ZnCl_2$, 80 μM phenylmethanesulfonyl fluoride, 18% (v/v) glycerol, 4 μg of purified recombinant protein farnesyltransferase, and either 115 μM unlabeled farnesyl pyrophosphate or [^{3}H]-farnesyl pyrophosphate (22.5 Ci/mmol) at concentrations specified in the legends of Figures 2 and 4.

Reversed Phase High-Performance Liquid Chromatography Analysis of the γ Subunit. Purified nonisoprenylated $\beta_1\gamma_1$, *in vitro*-isoprenylated $\beta_1\gamma_1$, native $\beta\gamma_t$, or buffer C (20 μL) was diluted with 30 μL of 5% (v/v) acetonitrile in H_2O with 10 mM trifluoroacetic acid (buffer D) and applied to a μRPC C2/C18 SC 2.1/10 column (Pharmacia) connected to a SMART system, which had been equilibrated with buffer D. The flow rate was 150 μL/min. After application of the sample, the column was washed with 1.5 mL of buffer D and eluted with a linear gradient (8.55 mL) of acetonitrile [5 to 95% (v/v)] in H_2O with 10 mM trifluoroacetic acid, followed by 1.5 mL of 95% (v/v) acetonitrile in H_2O with 10 mM trifluoroacetic acid (Parish & Rando, 1994).

Delayed Extraction Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Molecular weight determination of protein was performed by delayed extraction matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Lyophilized protein samples were redissolved in 10 μL of 5% formic acid. An aliquot (0.5 μL) of protein solution was mixed in a 1:1 ratio (v/v) with aqueous matrix solution [sinapinic acid in 1% formic acid and acetonitrile, 2:1 (v/v)] on the mass spectrometer probe tip. The protein/matrix solution was left to dry at ambient temperature, and the crystalline deposit was then rinsed with pure water prior to analysis. Weight analysis was performed in the reflector mode on a Bruker REFLEX time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with a gridless delayed extraction ion source. Spectra were acquired as the sum of ion signals generated by irradiation of the protein/matrix deposit by 50−100 pulses from a 337 nm N_2 laser. The singly and doubly charged ion signals from equine cytochrome *c* (M_r = 12 360.1) were used for internal mass calibration of all mass spectra. The mass accuracy was 10−25 ppm.

Carboxypeptidase A Treatment of in Vitro-Farnesylated $\beta_1\gamma_1$ Dimers. *In vitro*-farnesylated $\beta_1\gamma_1$ dimers (15 μL) were supplemented with 3 μL of carboxypeptidase A (3 units, in H_2O saturated with toluene) and 2 μL of 100 μM $ZnCl_2$. Aliquots of this sample (10 μL) were then incubated for the times indicated in the legend of Figure 4 at 30 °C. Following proteolysis, the samples were diluted with 40 μL of buffer D and then subjected to reversed phase high-performance liquid chromatography as described above.

Production of Recombinant Phospholipase C. PLC$\beta_2\Delta$, a deletion mutant of human PLCβ_2 lacking a carboxyl-terminal region necessary for stimulation by α_q subunits (F819−E1166) and carrying a serine to alanine replacement in position 2, was produced in baculovirus-infected *Spodoptera frugiperda* (Sf9) cells as described in Simoes et al. (1993). PLC$\beta_2\Delta$ is indistinguishable from wild-type recombinant PLCβ_2 in terms of its interaction with $PtdInsP_2$, Ca^{2+}, and G protein $\beta\gamma$ dimers.

Phospholipase C Assay. Phospholipase C activity was determined as described in Gierschik and Camps (1994). In brief, 25 μL of the *in vitro*-isoprenylated $\beta_1\gamma_1$ preparation was combined with 5 μL of the soluble fraction of PLC$\beta_2\Delta$-baculovirus-infected Sf9 cells [in 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 2 μg/mL soybean trypsin inhibitor, 3 mM benzamidine, 1 μM pepstatin, 1 μM leupeptin, and 100 μM phenylmethanesulfonyl fluoride], 35 μL of the lipid substrate [containing 56 μM [^{3}H]$PtdInsP_2$ (5 Ci/mol), 560 μM phosphatidylethanolamine, 100 mM Tris/maleate (pH 7.4), 6 mM EGTA, 20 mM LiCl, 20 mM 2,3-bisphosphoglycerate, and 1.8 mM deoxycholate], and 5 μL of $CaCl_2$ to adjust the concentration of free Ca^{2+} to 0.1 μM. The samples (70 μL) were incubated for 1 h at 25 °C and then analyzed for inositol phosphates as described (Gierschik & Camps, 1994).

Miscellaneous. The purification of $\beta\gamma_t$ from bovine rod outer segment membranes was done as described in Gierschik and Camps (1994). SDS−PAGE was performed using bipartite [17%/12% (w/v) acrylamide] resolving gels as described in Dietrich et al. (1992). Fluorography was performed using EN^{3}HANCE according to the protocol supplied by the manufacturer (DuPont NEN). The method used to stain the gels with silver is specified in Oakley et al. (1980). Protein concentrations were determined according to Bradford (1976) using bovine IgG as the standard.

RESULTS

We have previously shown that soluble fractions of $\beta_1\gamma_1$-baculovirus-infected insect cells contain similar amounts of nonisoprenylated and isoprenylated $\beta_1\gamma_1$ dimers and that these two forms of $\beta_1\gamma_1$ can be separated by anion exchange chromatography of this fraction on Mono Q (Dietrich et al., 1994). In the present study, we have purified the nonisoprenylated $\beta_1\gamma_1$ dimers ($\beta_1\gamma_1^{CVIS}$) to near homogeneity by subjecting the peak containing nonisoprenylated $\beta_1\gamma_1$ subunits obtained by Mono Q chromatography to sequential hydroxylapatite and gel permeation chromatography. The result of the SDS−PAGE analysis of the $\beta_1\gamma_1^{CVIS}$ preparation at various stages during the purification is shown in Figure 1. Typically, approximately 160 μg of mostly homogeneous $\beta_1\gamma_1$ was obtained from 100 mg of cytosolic protein from 270×10^6 infected insect cells.

The next experiments were designed to examine whether and to what extent purified $\beta_1\gamma_1^{CVIS}$ dimers served as a substrate for farnesylation by protein farnesyltransferase (FTase). To this end, FTase was produced in baculovirus-infected insect cells and purified to near homogeneity using a modification of a protocol developed by Casey et al. (1991). Purified $\beta_1\gamma_1^{CVIS}$ dimers were then incubated with purified

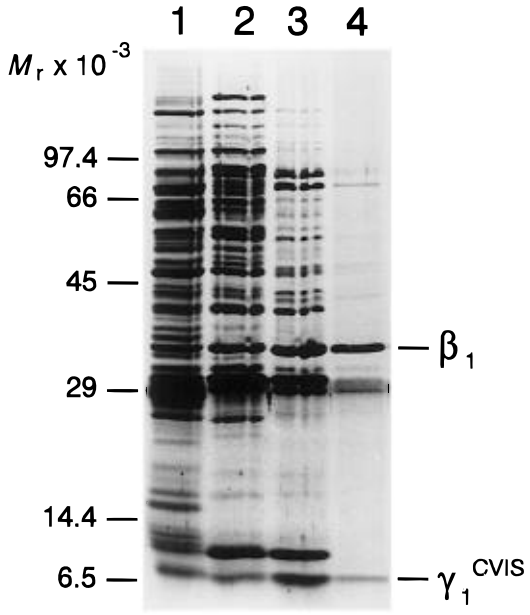

FIGURE 1: Purification of nonisoprenylated recombinant $\beta_1\gamma_1$ dimers. *T. ni* cells were infected for 3 days with $\beta_1\gamma_1$-baculovirus, homogenized, and fractionated into soluble and particulate constituents as described in Materials and Methods. Nonisoprenylated $\beta_1\gamma_1$ dimers ($\beta_1\gamma_1^{CVIS}$) were purified from the soluble fraction by sequential ion exchange, hydroxylapatite, and gel permeation chromatography. Aliquots of the soluble fraction (10 μg of protein) (lane 1) and of the peak fractions obtained by chromatography on Mono Q (10 μg of protein) (lane 2), hydroxylapatite (5 μg of protein) (lane 3), and Superdex 75 (0.3 μg of protein) (lane 4) were subjected to SDS−PAGE. Proteins were visualized by staining with silver. The positions of β_1, γ_1^{CVIS}, and the molecular mass standards are indicated.

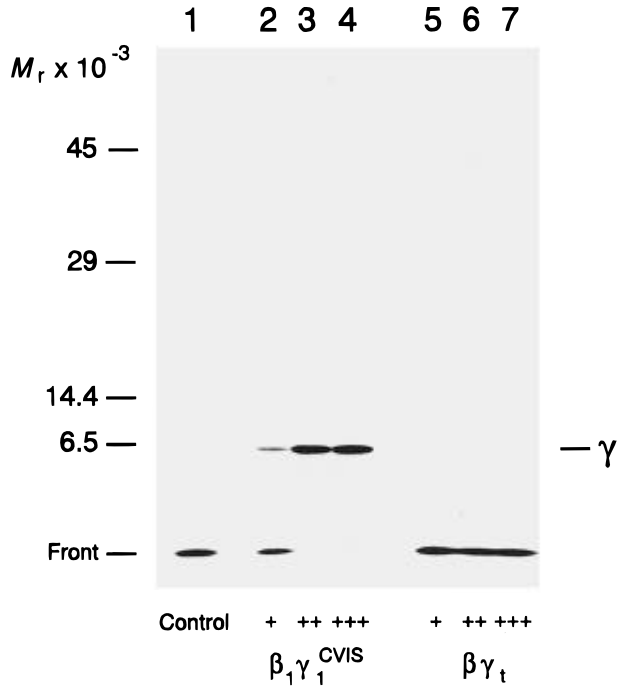

FIGURE 2: *In vitro* farnesylation of recombinant nonisoprenylated $\beta_1\gamma_1$ dimers. Increasing amounts (+, 0.2 μg; ++, 0.4 μg; and +++, 0.8 μg) of purified nonisoprenylated recombinant $\beta_1\gamma_1$ dimers ($\beta_1\gamma_1^{CVIS}$) (lanes 2−4) or $\beta\gamma$ subunits purified from bovine retina ($\beta\gamma_t$) (lanes 5−7) were incubated with purified recombinant protein farnesyltransferase and [^{3}H]farnesyl pyrophosphate (0.1 μM) as described in Materials and Methods. A control sample (Control) was generated by incubating protein farnesyltransferase and [^{3}H]-farnesyl pyrophosphate in the absence of $\beta\gamma$ dimers (lane 1). The samples were subjected to SDS−PAGE, and radiolabeled proteins were visualized by fluorography. The positions of the γ subunit, the molecular mass standards, and the dye front of the gel are indicated.

FTase in the presence of [^{3}H]farnesyl pyrophosphate ([^{3}H]-FPP), and the reaction products were analyzed by SDS−PAGE followed by fluorography. Native $\beta_1\gamma_1$ subunits purified from bovine retinal rod outer segments ($\beta\gamma_t$) were analyzed for comparison. A representative fluorogram is shown in Figure 2. The results show that the radiolabeled farnesyl moiety, which migrated in the dye front of the SDS−polyacrylamide gel in the absence of $\beta_1\gamma_1^{CVIS}$, was incorporated into a protein with an apparent molecular mass of approximately 6.5 kDa, most likely γ_1, in the presence of $\beta_1\gamma_1^{CVIS}$. The amount of incorporated radiolabel increased with the amount of $\beta_1\gamma_1^{CVIS}$ as long as free [^{3}H]FPP was available as a substrate of the transferase. In contrast, no radiolabeled material was evident in the 6.5 kDa range of the SDS−polyacrylamide gel when $\beta\gamma_t$ was incubated with FTase and [^{3}H]FPP. In additional experiments (results not shown), we found that $\beta_1\gamma_1^{CVIS}$ dimers, which had been treated *in vitro* with FTase in the presence of [^{3}H]FPP, behaved like $\beta_1\gamma_1$ dimers isoprenylated in intact insect cells upon chromatography on Mono Q, i.e. eluted after nonisoprenylated $\beta_1\gamma_1^{CVIS}$ [*cf.* Dietrich et al. (1994)]. Taken together, these results strongly suggest that the γ_1^{CVIS} polypeptide contained in purified $\beta_1\gamma_1^{CVIS}$ serves as a substrate for *in vitro* farnesylation by purified protein farnesyltransferase.

To examine the extent of the post-translational modification of γ_1^{CVIS} catalyzed by FTase, $\beta_1\gamma_1$ dimers were analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) on a C18 matrix. This procedure, which is based on the observation that the β_1 polypeptide irreversibly adsorbs to reversed phase matrix so that only γ_1 is observed on the chromatogram (Ovchinnikov et al., 1985), has previously been used to examine the status of post-translational modification of γ_1 (Ohguro et al., 1991; Fukada et al., 1994; Parish & Rando, 1994). The results of the chromatographic analyses of purified native $\beta\gamma_t$, purified nonisoprenylated recombinant $\beta_1\gamma_1^{CVIS}$, and purified $\beta_1\gamma_1^{CVIS}$ pretreated with FTase in the presence of cold FPP are shown in Figure 3. The buffer used as a solvent of these proteins was analyzed for comparison. The fully modified γ_t polypeptide eluted as a major peak (>90%) at a retention time of 30.5 min. An additional minor peak (<10%) was observed at 26.7 min. Nonisoprenylated recombinant γ_1^{CVIS} eluted at a retention time of 26.8 min. Importantly, the majority of this peak (approximately 65%) was shifted to a new position at 30.2 min upon preincubation of $\beta_1\gamma_1^{CVIS}$ with FTase and FPP. The residual minor portion of the peak did not change its position in the gradient. In additional experiments (not shown), we found that the peak observed at 30.2 min was not due to any of the constituents present in the *in vitro* farnesylation reaction, e.g. FTase or FPP. Attempts to increase the proportion of the peak shifting to the new position were not successful for reasons which are currently unknown (not shown). We also found (results not shown) that the γ subunits of *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$ and native $\beta\gamma_t$ eluted as two clearly resolved peaks from the C18 resin upon coinjection of the two $\beta\gamma$ species. Taken together, these results and those shown in Figure 3 have two important implications. One is that purified $\beta_1\gamma_1^{CVIS}$ is apparently

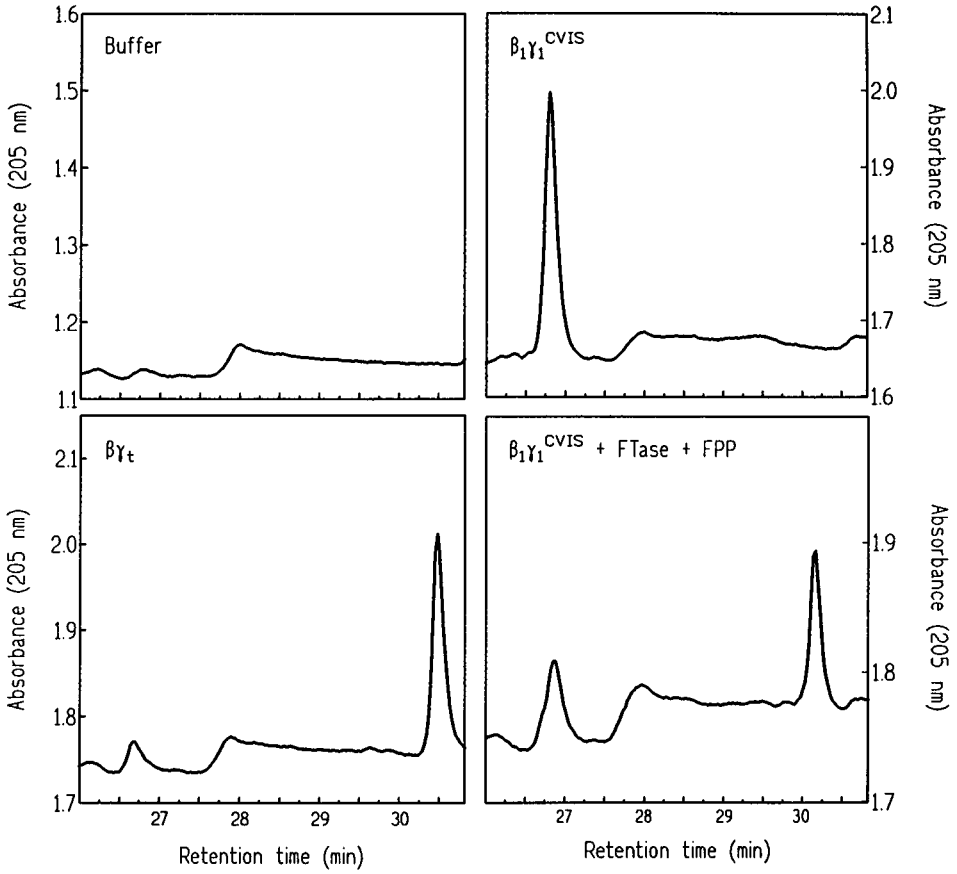

FIGURE 3: Analysis of native and recombinant $\beta\gamma$ dimers by reversed phase high-performance liquid chromatography. Samples containing control buffer (Buffer) (upper left panel), native $\beta\gamma_t$ (4 μg) ($\beta\gamma_t$) (lower left panel), nonisoprenylated recombinant $\beta_1\gamma_1$ (4 μg) ($\beta_1\gamma_1^{CVIS}$) (upper right panel), or nonisoprenylated recombinant $\beta_1\gamma_1$ pretreated with purified protein farnesyltransferase and farnesyl pyrophosphate (3 μg) ($\beta_1\gamma_1^{CVIS}$ + FTase + FPP) (lower right panel) were analyzed by reversed phase high-performance liquid chromatography on a μRPC C2/C18 SC 2.1/10 column. See Materials and Methods for experimental details. Note that the β_1 polypeptide is retained by the C18 reversed phase matrix, whereas most of the γ_1 subunit is recovered under the conditions used here (Fukada et al., 1994; Parish & Rando, 1994).

homogeneous with respect to the covalent structure of the γ subunit carboxyl terminus. Second, treatment of $\beta_1\gamma_1^{CVIS}$ with FTase and FPP leads to conversion of a major portion of the nonisoprenylated $\beta\gamma$ dimer to a new and apparently homogeneous $\beta\gamma$ species. The fact that two purified soluble proteins, $\beta_1\gamma_1^{CVIS}$ and FTase, were used in the *in vitro* farnesylation reaction strongly suggests that the new $\beta\gamma$ species contains γ subunits, which are farnesylated but are neither proteolyzed nor carboxylmethylated.

Delayed extraction matrix-assisted laser desorption/ionization mass spectrometry (DE-MALDI-MS) was used to directly assess the status of post-translational modification of the γ_1^{CVIS} carboxyl terminus after treatment of $\beta_1\gamma_1^{CVIS}$ with FTase and FPP. Samples of the fractions obtained at 26.8, 30.2, and 30.5 min upon RP-HPLC of purified nonisoprenylated recombinant $\beta_1\gamma_1^{CVIS}$, *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$, and native $\beta\gamma_t$, respectively, were analyzed. Table 1 shows that the measured molecular weights of the three γ_1 subunit species are in excellent agreement with the values predicted for nonmodified $\beta_1\gamma_1^{CVIS}$ (26.8 min), $\beta_1\gamma_1^{CVIS}$ farnesylated but not proteolyzed (30.2 min), and fully modified, i.e. farnesylated, proteolyzed, and carboxylmethylated, γ_1 (30.5 min). The results of these experiments thus confirm the identities of the three differentially modified γ_1 subunits.

Carboxypeptidase A has previously been shown to specifically remove the three carboxyl-terminal residues from isoprenylated γ subunits and to cease proteolysis at the then terminal prenylcysteine residue (Higgins & Casey, 1994). To characterize the interaction of farnesylated and proteolyzed but not yet carboxymethylated γ_1 with C18 reversed phase matrix and thus compare the hydrophobic properties of this γ_1 subunit to the hydrophobicities of the three γ_1 species analyzed in Figure 3 and Table 1, *in vitro* ^{3}H-farnesylated $\beta_1\gamma_1^{CVIS}$ dimers were treated with carboxypeptidase A and then analyzed by RP-HPLC. Figure 4 shows that ^{3}H-farnesylated γ_1 subunits eluted as a single peak with a retention time between 30 and 31 min at time zero of the incubation, which is consistent with the retention time observed for farnesylated γ_1^{CVIS} in Figure 3 (30.2 min). Treatment of ^{3}H-farnesylated γ_1^{CVIS} with carboxypeptidase A for 2 h led to an approximately 40% decrease of the peak observed for the nonproteolyzed material and to the appear-

Table 1: Delayed Extraction Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Recombinant γ_1 Subunits

retention time[a] (min)	measured M_r[b]	carboxyl-terminal modification	calculated M_r
26.8	8412.8 ± 0.1	γ_1^{CVIS}	8412.7
30.2	8616.9 ± 0.3	γ_1^{CVIS}, farnesylated	8617.1
30.5	8331.6 ± 0.1	γ_1^{C}, fully modified	8331.7

[a] Recombinant γ_1 subunits were purified by reversed phase high-performance liquid chromatography on a μRPC C2/C18 SC 2.1/10 column (*cf.* Figure 3). [b] Each value represents the mean ± SD of triplicate determinations.

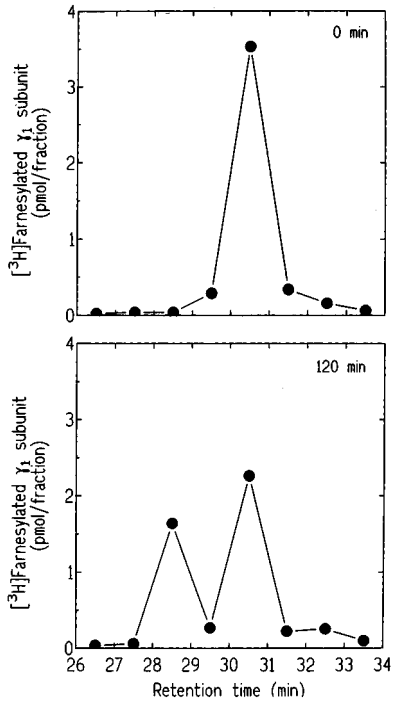

FIGURE 4: Carboxypeptidase A treatment of *in vitro*-farnesylated $\beta_1\gamma_1$ dimers. Purified nonisoprenylated recombinant $\beta_1\gamma_1$ dimers (1 μg) were treated with protein farnesyltransferase and [^{3}H]farnesyl pyrophosphate (1.5 μM) as described in Materials and Methods. The sample was then divided into two equal portions and incubated for 0 min (upper panel) or 120 min (lower panel) with carboxypeptidase A. Following proteolysis, the samples were subjected to reversed phase high-performance liquid chromatography on a μRPC C2/C18 SC 2.1/10 column. Fractions (1 min) were analyzed by liquid scintillation counting.

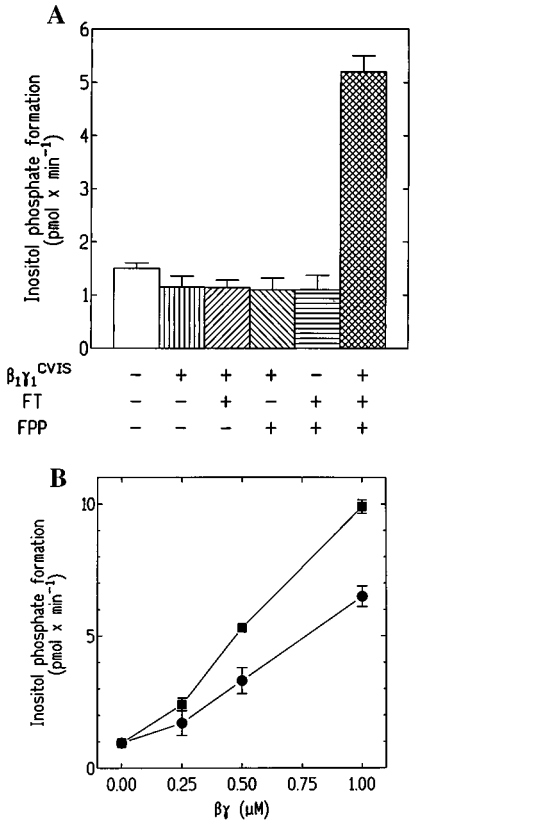

FIGURE 5: Stimulation of phospholipase C-$\beta_2\Delta$ (PLC$\beta_2\Delta$) by *in vitro*-farnesylated $\beta_1\gamma_1$ dimers. (A) Purified nonisoprenylated recombinant $\beta_1\gamma_1$ dimers ($\beta_1\gamma_1^{CVIS}$) were incubated as indicated at the abscissa in the absence (−) or presence (+) of protein farnesyltransferase (FTase) and farnesyl pyrophosphate (FPP) and then reconstituted with extracts of insect cells expressing PLC$\beta_2\Delta$ (0.3 μg of protein/sample) and phospholipid vesicles containing $PtdInsP_2$. The final concentration of $\beta_1\gamma_1^{CVIS}$ dimers in the phospholipase C assay was 1 μM. The reaction was terminated by addition of chloroform/methanol/HCl, and the samples were analyzed for inositol phosphates. See Materials and Methods for experimental details. (B) Increasing concentrations of *in vitro*-farnesylated $\beta_1\gamma_1$ dimers (closed circles) or native $\beta\gamma_t$ (closed squares) were reconstituted with extracts of insect cells expressing PLC$\beta_2\Delta$ (0.3 μg of protein/sample) and phospholipid vesicles containing $PtdInsP_2$. Each value represents the mean ± SD of triplicate determinations.

ance of a new peak corresponding in size to this decrease and eluting between 28 and 29 min. Assuming that the appearance of this peak reflects the removal of the three carboxyl-terminal residues V, I, and S from ^{3}H-farnesylated γ_1^{CVIS}, the results shown in Figures 3 and 4 establish the order of hydrophobicity of the various forms of γ_1 as γ_1^{CVIS} ≪ farnesylated γ_1^{C} ≪ farnesylated γ_1^{CVIS} < fully modified γ_1.

The effect of *in vitro* farnesylation on the ability of purified $\beta_1\gamma_1^{CVIS}$ to stimulate PLC$\beta_2\Delta$, a deletion mutant of PLCβ_2 indistinguishable from wild-type PLCβ_2 in terms of its susceptibility to stimulation by $\beta\gamma$ dimers, is shown in Figure 5A. Consistent with earlier results (Dietrich et al., 1994), purified nonisoprenylated $\beta_1\gamma_1^{CVIS}$ (1 μM) failed to stimulate PLC$\beta_2\Delta$. In marked contrast, *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$ led to a robust (3.4-fold) increase in the activity of PLC$\beta_2\Delta$ when tested at the same concentration. Note that this increase was not observed when either FPP or FTase was missing in the *in vitro* farnesylation reaction that preceded the phospholipase C assay. Results very similar to those shown in Figure 5A were obtained when full-length recombinant PLCβ_2 was used rather than PLC$\beta_2\Delta$ (not shown).

Figure 5B compares the effects of increasing concentrations of *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$ and native $\beta\gamma_t$ on inositol phosphate formation by PLC$\beta_2\Delta$. In this experiment, even $\beta\gamma_t$ was subjected to the *in vitro* isoprenylation protocol to control for a potential influence of any of the reagents used in the preincubation on $\beta\gamma$ dimer stimulation of PLC$\beta_2\Delta$. Furthermore, the concentrations of *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$ were adjusted to those of native $\beta\gamma_t$ according to the areas under the peaks corresponding to farnesylated γ subunits on analytical RP-HPLC chromatograms of the two samples (*cf.* Figure 3). Figure 5B shows that both recombinant $\beta_1\gamma_1^{CVIS}$ farnesylated *in vitro* and native $\beta\gamma_t$ stimulated PLC$\beta_2\Delta$ in a concentration-dependent manner. Most interestingly, however, the degree of this stimulation was only approximately 45% lower for *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$ than for native $\beta\gamma_t$ at all $\beta\gamma$ dimer concentrations tested. In additional experiments (results not shown), we found that the lower stimulatory effect of the *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$ preparation was not explained by a putative inhibitory effect of the residual nonisoprenylated $\beta\gamma$ dimers (*cf.* Figure 3) present in this preparation, since nonisoprenylated $\beta_1\gamma_1^{CVIS}$ (1 μM) did not inhibit the ability of native $\beta\gamma_t$ (0.5 μM) to stimulate PLC$\beta_2\Delta$.

DISCUSSION

The present study was initiated to determine the relative importance of the first of the three post-translational carboxyl-terminal γ subunit modifications with respect to the ability of G protein $\beta\gamma$ dimers to stimulate phospholipase

C. To this end, nonisoprenylated recombinant $\beta_1\gamma_1$ dimers were purified from soluble fractions of baculovirus-infected insect cells and used as a substrate for *in vitro* farnesylation by purified recombinant protein farnesyltransferase. *In vitro*-isoprenylated $\beta\gamma$ dimers were reconstituted with a recombinant phospholipase C-β_2 isozyme to specifically examine the functional significance of γ subunit isoprenylation. The results show that, among the three post-translational modifications of the γ subunit carboxyl terminus, isoprenylation is a major determinant of the ability of $\beta\gamma$ dimers to stimulate phospholipase C and is by itself sufficient to promote this stimulation.

Using a somewhat different experimental approach based on *in vitro* assembly of $\beta\gamma$ dimers from insect cell-expressed β subunits and bacterially expressed γ subunits, Higgins and Casey recently showed that only those $\beta\gamma$ dimers containing isoprenylated γ subunits were capable of supporting the pertussis toxin-mediated ADP ribosylation of α_o (Higgins & Casey, 1994). Although the γ subunit carboxyl termini of *in vitro* assembled $\beta\gamma$ dimers were not further analyzed in the latter study, it is likely that the active $\beta\gamma$ dimers contained γ subunits, which were isoprenylated but were neither proteolyzed nor carboxylmethylated, since none of the components necessary for the latter two modifications was present throughout the course of or subsequent to the *in vitro* assembly procedure. Thus, it is likely that γ subunit isoprenylation is a major determinant of $\alpha{\cdot}\beta\gamma$ association as well. Also pertinent to this issue is the previous observation that a mutant K-RasB protein capable of undergoing farnesylation, but not proteolysis or carboxylmethylation, nevertheless displayed efficient (approximately 50%) membrane association and transforming activity (Kato et al., 1992), indicating that farnesylation alone is sufficient for oncogenic Ras function. Furthermore, several lines of evidence suggest that carboxylmethylation may not be of major functional importance for several other prenylated proteins. Thus, *S. cerevisiae* cells lacking *STE14* carboxyl methyltransferase exhibited no detectable impairment of RAS function or cell viability (Hrycyna et al., 1991). Carboxylmethylation of Rab3A was not required for the interaction of the protein with membranes and Rab GDP dissociation inhibitor (Musha et al., 1992). In marked contrast, both proteolysis and carboxylmethylation appear to be of key importance for the interactions of fungal mating factors with the STE3 receptor and the STE6 transporter (Ishibashi et al., 1984; Anderegg et al., 1988; Marcus et al., 1991; Hrycyna et al., 1991; Sapperstein et al., 1994).

What, then, is the functional significance of proteolytic removal of the three last amino acids and carboxylmethylation of isoprenylated G protein γ subunits? Several observations pertinent to this issue need to be discussed at this point. Fukada et al. were able to demonstrate that the ability of $\beta\gamma_t$ to stimulate the rhodopsin-mediated binding of GTP[S] to α_t and the pertussis toxin-mediated ADP ribosylation of α_t was enhanced approximately 1.5- and 2-fold, respectively, by carboxylmethylation of γ_t (Fukada et al., 1994). Similar results were obtained by Parish and Rando (1994). A synthetic pentapeptide corresponding to the isoprenylated and proteolyzed carboxyl terminus of γ_t was shown to inhibit the pertussis toxin-mediated, $\beta\gamma_t$-stimulated ADP ribosylation of α_t (Matsuda et al., 1994). Interestingly, carboxylmethylation of the peptide caused an approximately 2.5-fold increase in its potency to elicit this inhibition. Carboxylmethylation of γ_t was also shown to enhance the interaction of $\beta\gamma_t$ with native and artificial lipid membranes (Fukada et al., 1994; Bigay et al., 1994). This effect was most striking when studied in the absence of α_t and was reduced in its presence or in the presence of α_t plus light-activated rhodopsin. Taken together, these findings led to the conclusion that methylation of the γ subunit plays a dual functional role by increasing the strength of both the $\alpha{\cdot}\beta\gamma$ association and the binding of the free $\beta\gamma$ dimer to the plasma membrane (Fukada et al., 1994).

Very recently, Rando and co-workers reported that enzymatic removal of the carboxyl-terminal methyl group of γ_t led to a dramatic, or even complete, loss of the ability of $\beta\gamma_t$ to activate a phosphoinositide 3-kinase from U937 cells and PLCβ_3 from rat brain (Parish et al., 1995). Taken together, the latter results and our observation that *in vitro*-farnesylated $\beta_1\gamma_1{}^{CVIS}$ is only approximately 45% less effective in stimulating PLC$\beta_2\Delta$ that native, i.e. fully modified, $\beta\gamma_t$ (*cf.* Figure 5B) clearly shed new light on the functional significance of the carboxyl-terminal proteolysis of the isoprenylated γ subunit. Thus, these findings not only imply that proteolysis causes a marked loss in the ability of the $\beta\gamma$ dimer to stimulate PLCβ but also raise the distinct possibility that carboxylmethylation of farnesylated proteins has evolved to compensate for a proteolysis-induced loss in effector activation. It is clear that a side by side comparison of isoprenylated but not yet proteolyzed γ subunits with isoprenylated, proteolyzed, but not yet carboxylmethylated γ subunits with respect to PLCβ activation is required to further explore this intriguing possibility.

Our findings raise important question about the molecular mechanisms by which farnesylation alone renders $\beta\gamma$ capable of stimulating phospholipase C. At first glance, it appears unlikely that isoprenylated $\beta_1\gamma_1{}^{CVIS}$ dimers stimulate PLC$\beta_2\Delta$ by directing the soluble enzyme to its lipid substrate $PtdInsP_2$, since nonmethylated $\beta\gamma_t$ was previously shown to interact with lipid bilayers and phospholipid−detergent micelles only very poorly, if at all (Fukada et al., 1994; Bigay et al., 1994). It is possible, however, that $\beta_1\gamma_1{}^{CVIS}$ cooperates with PLC$\beta_2\Delta$ with regard to binding to lipids, as shown in the latter two studies for nonmethylated $\beta\gamma_t$ and $\alpha_{t\cdot GDP}$. On the other hand, one should bear in mind that the $\beta\gamma$ dimers examined in those studies were both isoprenylated and proteolyzed at their γ subunit carboxyl termini, whereas the γ subunit of $\beta_1\gamma_1{}^{CVIS}$ is isoprenylated only. Importantly, two residues of the carboxyl-terminal γ subunit tripeptide VIS retained in farnesylated $\beta_1\gamma_1{}^{CVIS}$ are hydrophobic, and it is conceivable that these residues increase the hydrophobicity of the dimer by a considerable margin. Thus, analysis of the interaction of isoprenylated peptides with lipid bilayers has previously revealed that the free energy of interaction between a farnesylated nonmethylated carboxyl-terminal cysteine residue and the lipid bilayer, ΔG_p, is approximately −8 kcal mol^{-1} and that this energy increases (i.e. becomes more negative) by about 1.4−2.0 and by about 2.3−2.5 kcal mol^{-1} with carboxylmethylation of the cysteine and with replacement of the farnesyl by a geranylgeranyl group, respectively (Silvius & l'Hereux, 1994). The latter study also showed that the free energy of partitioning was about −800 cal mol^{-1} per methylene unit in peptides carrying simple *n*-alkyl groups rather than isoprenyl residues on their carboxyl termini. These findings raise the distinct possibility that the farnesylated nonproteolyzed $\beta_1\gamma_1{}^{CVIS}$ interacts with membranes

even better than fully processed $\beta\gamma_t$. Experiments designed to challenge this hypothesis are underway in this laboratory.

Another possibility to be considered at this point is that isoprenylation of the γ subunit carboxyl terminus is important for the interaction of $\beta\gamma$ with the PLCβ_2 polypeptide rather than the lipid bilayer. Thus, it seems possible that the *in vitro*-farnesylated $\beta_1\gamma_1^{CVIS}$ dimer interacts, *via* its modified carboxyl terminus, with a specific isoprenyl "docking site" on PLC$\beta_2\Delta$. Of interest, PLCβ_2 has previously been suggested to interact with $\beta\gamma$ dimers through a putative pleckstrin homology (PH) domain predicted for the amino terminus of PLCβ_2 (Parker et al., 1994). While the actual three-dimensional structure of this portion of PLCβ_2 is currently unknown, there is structural similarity between PH domains of other proteins, e.g. pleckstrin, and proteins that bind lipophilic molecules such as retinol-binding protein, β-lactoglobulin, bilin-binding protein, P2 myelin protein, and fatty acid-binding protein, with respect to the overall topology and the dimensions of the hydrophobic β-barrel core of these proteins (Yoon et al., 1994). Considering that PtdInsP_2, a known lipophilic PH domain ligand (Harlan et al., 1994; Lemmon et al., 1995), interacts with the domain through its inositol head group rather than through its acyl chains (Harlan et al., 1995; Hyvönen et al., 1995), interaction of the isoprenylated γ subunit carboxyl terminus with the hydrophobic PH domain β-barrel core remains an intriguing possibility.

On the other hand, it is also conceivable that isoprenylation alters the conformation of $\beta_1\gamma_1^{CVIS}$ so as to facilitate protein−protein rather than isoprenyl−protein interaction with PLC$\beta_2\Delta$. This view is supported by the apparent increase in the net negative charge of $\beta_1\gamma_1^{CVIS}$ that is caused by *in vitro* isoprenylation and is observed upon chromatography of the protein on Mono Q. This increase is not explained by the attachment of a farnesyl moiety to the γ subunit carboxyl terminus and may, therefore, reflect a conformational change of the β and/or the γ constituent of the $\beta\gamma$ dimer. Such a change is not without precedence in the literature. For example, geranylgeranylation of purified Rab3A led to a time-dependent loss in the ability of the protein to bind [^{35}S]-GTP[S] (Musha et al., 1992), and isoprenylation of the large hepatitis delta antigen altered the conformation of an epitope present at least 15 amino acids upstream of the isoprenylated cysteine residue (Hwang & Lai, 1993, 1994). It seems possible, therefore, that isoprenylation of the γ subunit carboxyl terminus causes a change in the overall conformation of the $\beta\gamma$ dimer and that this alteration promotes $\beta\gamma$ dimer stimulation of phospholipase C.

In conclusion, we have shown that isoprenylation of the γ subunit carboxyl terminus is both necessary and sufficient for $\beta\gamma$ dimer-mediated stimulation of phospholipase C. These observations notwithstanding, our results also point to important functional roles of the two subsequent carboxyl-terminal γ subunit modifications, proteolysis and methylation, with respect to phospholipase C regulation. Potential mechanisms for the isoprenylation-dependent increase in the ability of $\beta\gamma$ to stimulate phospholipase C include (i) targeting of the effector enzyme to its lipid substrate, (ii) a direct interaction of an isoprenyl moiety with a putative docking site on the effector enzyme, (iii) a conformational change of the $\beta\gamma$ dimer followed by direct protein−protein interaction with the effector, and (iv) any combination of these three mechanisms.

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SUPPORTING INFORMATION AVAILABLE

Two figures showing expression of full-length phospholipase C-β_2 in baculovirus-infected insect cells and stimulation of full-length phospholipase C-β_2 by *in vitro*-farnesylated $\beta_1\gamma_1$ dimers (3 pages). Ordering information is given on any current masthead page.

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