The Role of G Protein Methylation in the Function of a Geranylgeranylated $\beta\gamma$ Isoform†

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ABSTRACT: The γ subunit of heterotrimeric G proteins is isoprenylated and methylated on its carboxyl terminal cysteine residue. While retinal transducin is farnesylated, all other γ subunits are modified by geranylgeranylation. An immobilized form of pig liver esterase (iPLE) is able to hydrolyze the methyl ester of a geranylgeranylated $\beta\gamma$ isoform ($\beta_1\gamma_2$). Since methylation is the only reversible reaction in the isoprenylation pathway, it could be a site of regulation of G protein activity. With both the methylated and demethylated $\beta_1\gamma_2$ now available, the role of methylation for a geranylgeranylated heterotrimeric G protein may be addressed. Here, it is reported that methylation has no effect on the ability of $\beta\gamma$ to interact with an α subunit, as probed by ADP-ribosylation studies with pertussis toxin, and has a small effect (less than 2-fold) on the ability of geranylgeranylated $\beta\gamma$ to activate phosphatidylinositol-specific phospholipase C (PIPLC) and phosphoinositide 3 kinase (PI3K). In binding studies, demethylation only slightly decreased the ability of $\beta_1\gamma_2$ to adhere to azolectin vesicles. Therefore, methylation of heterotrimeric G proteins appears to have only a minor effect in signal transduction processes which can be correlated to a decrease in hydrophobicity of the $\beta\gamma$ subunit.

Many diverse proteins, including all of the G proteins,[1] are isoprenylated on their terminal cysteine residues which, in most cases, are also carboxymethylated (Clarke, 1992). Heterotrimeric G proteins are isoprenylated/methylated at the carboxyl terminus of their γ subunits (Yamane et al., 1990; Lai et al., 1990; Fukada et al., 1990; Mumby et al., 1990; Fung et al., 1990; Sanford et al., 1991). The vast majority of proteins are isoprenylated by geranylgeranylation (C20), rather than by farnesylation (C15) (Sinesky & Lutz, 1992; Schafer & Rine, 1992; Omer & Gibbs, 1994; Casey, 1995). In the biosynthetic pathway leading to isoprenylation/methylation, only methylation is reversible, and thus subject to metabolic control (Tan & Rando, 1992). In bacterial chemotaxis, protein methylation has been shown to play an important role in signal transduction (Kort et al., 1975; Shapiro et al., 1995).

The role of protein isoprenylation/methylation is understood to be a post-translational modification which enhances the binding of the modified protein to a target membrane (Muntz et al., 1992; Simonds et al., 1991). The increased hydrophobicity of the modified proteins, in and of itself, ensures that these proteins have a greater affinity for membranes than their unmodified counterparts. In addition, it is possible that the isoprenylated and methylated cysteine residues are ligands which mediate specific lipid−protein interactions. An approach to differentiating between these two mechanistic classes involves deleting the isoprenyl and/or methyl groups and determining the quantitative effects on function of these modifications.

Retinal transducin (T), a heterotrimeric G protein farnesylated and methylated on its γ subunit cysteine terminus (Lai et al., 1990; Fukada et al., 1990), is a particularly fruitful system to study, because it can be quantitatively studied with a known effector (rhodopsin), and because it is readily available in large quantities. Previous studies have demonstrated that removal of the farnesylated and methylated cysteine residue of T_γ produces a protein incapable of interacting with rhodopsin (Ohguro et al., 1991; Cheng et al., 1995). However, with native photoreceptor membranes containing rhodopsin, removal of the methyl group only affects transducin's GTP binding activity by a factor of approximately 2 (Parish & Rando, 1994; Fukada et al., 1994). These results roughly correlate with the difference in binding affinity of the methylated and demethylated transducin molecules in the presence of illuminated rhodopsin and T_α (Fukada et al., 1994) and would suggest that there is no specific effector role for the farnesylated/methylated cysteine residue. Further, membranes are required to see this effect since there is no difference between the ability of methylated and demethylated $T_{\beta\gamma}$ to stimulate GTP exchange on T_α in the presence of rhodopsin solubilized with detergent (Parish & Rando, 1994). On the other hand, a comparison of methylated and demethylated transducin with respect to their abilities to activate either PIPLC or PI3K revealed a more substantial role for methylation than that found in the interactions of transducin with rhodopsin (Parish et al., 1995). However, the naturally occurring $\beta\gamma$ subunits which activate

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[1] Abbreviations: T, transducin; iPLE, immobilized pig liver esterase; TFA, trifluoroacetic acid; NAD, nicotinamide adenine dinucleotide; G protein, GTP binding protein; PIPLC, phosphatidylinositol-specific phospholipase C; PI3K, phosphoinositide 3-kinase; BSA, bovine serum albumin; EIMS, electron ionization mass spectrometry; GDI, GDP dissociation inhibitor.

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these two important signal transducing enzymes are geranylgeranylated/methylated rather than being farnesylated/methylated (Smrcka & Sternweis, 1993; Stephens et al., 1994; Thomason et al., 1994; Dietrich et al., 1994; Ueda et al., 1994; Boyer et al., 1994; Wu et al., 1994). It was therefore of interest to investigate further the role of methylation in signal transduction, in order to determine whether the observed effects are better correlated with a change in protein hydrophobicity, or with a role involving specific lipid−protein interactions. Methylation is shown to have only a small quantitative role in the signal transduction processes investigated here. These effects are most likely related to the small decrease in hydrophobicity upon demethylation of the geranylgeranylated $\beta_1\gamma_2$ subunit.

MATERIALS AND METHODS

Materials

iPLE, azolectin, sodium cholate, and thymidine were from Sigma. Pertussis toxin was from List Biological Laboratories. [*adenylate*-^{32}P]NAD (2 mCi·mL^{-1}, 30 Ci·mmol^{-1}) was from NEN/Dupont. α_{i-1} was provided by Dr. E. Neer (Brigham & Women's Hospital).

Methods

Preparation of βγ Samples. Following a previously published procedure (Kozasa & Gilman, 1995), $\beta_1\gamma_2$ was prepared in a baculovirus expression system and purified. The final concentration of $\beta_1\gamma_2$ was 1.4 mg·mL^{-1} in 20 mM HEPES, pH 7.4/100 mM NaCl (buffer A) with 1% cholate. In order to hydrolyze the carboxyl-terminal methyl ester of γ_2, $\beta_1\gamma_2$ was diluted 30-fold with buffer A. After washing iPLE (60 μL) four times (1 mL) with buffer A, the diluted $\beta_1\gamma_2$ (600 μL) was added to each tube. Typically, a total of four or five hydrolyses were run simultaneously. After nutating each sample at room temperature for 24 h, buffer A containing 5% cholate (150 μL) was added to each tube to bring the cholate concentration back up to 1%. After nutating for 30 min at 4 °C, the supernatant of each hydrolysis was removed after centrifugation at 14 000 rpm (16000*g*) for 3 min. The remaining iPLE pellet was washed with buffer A containing 1% cholate (300 μL, two times). After 30 min at 4 °C, the supernatants were removed after centrifugation, as indicated above, and were combined with the original supernatants. The combined supernatants were concentrated with a Centricon 30 (Amicon) to a final volume of 100−150 μL.

A control sample of methylated $\beta_1\gamma_2$ was prepared by following a procedure identical to that used in the hydrolysis of $\beta_1\gamma_2$ except that the protein was not treated with iPLE. The control sample also was concentrated with a Centricon 30 to a final volume of 100−150 μL. Both methylated and demethylated $\beta_1\gamma_2$ were stored at −80 °C.

Transducin $\beta\gamma$ was prepared as described previously (Parish et al., 1995) and stored at 4 °C. The final concentration of the sample used here was 2 μM in 10 mM Tris, pH 7.4/100 mM NaCl/5 mM $MgCl_2$/1 mM DTT/0.1 mM EDTA.

HPLC Analysis of γ_2 Subunit. The extent of methylation of the γ_2 subunit was quantified by reverse phase HPLC. A C8 column (Dynamax 300 Å, Rainin) was used to separate the methylated and demethylated γ_2. $\beta_1\gamma_2$ samples were injected with guanidinium chloride (final concentration = 3 M). After running the column at 5% acetonitrile in water (10 mM TFA) for 10 min, a linear gradient was run from 5% to 95% acetonitrile in water (10 mM TFA) over 40 min. At that point, the solvent was held at 95% acetonitrile in water (10 mM TFA) for 10 min. The flow rate of the gradient was 0.75 mL·min^{-1}, and the absorbance of each injection was monitored at 205 nm. The retention times of methylated and demethylated γ_2 were approximately 46.5 and 45.0 min, respectively. A sample of each of these peaks was collected, concentrated to a small volume, and analyzed by electrospray ionization mass spectrometry to confirm their identities. The β_1 subunit did not elute from the HPLC column under these conditions.

ADP-Ribosylation of α_{i-1} by Pertussis Toxin. The procedure used here was based on that of Carty (1994). Pertussis toxin (10 μL, 50 μg·mL^{-1}) was activated by adding DTT (1.5 μL, 1 M) and SDS (1 μL, 2.5%) and incubating this sample for 20 min at 32 °C. To prepare the pertussis toxin working solution, the activated toxin was diluted with water (50 μL). The assay buffer contained 50 mM Tris, pH 7.4/2 mM EDTA/20 mM thymidine/20 mM DTT/10 μM NAD/40 μCi·mL^{-1} [*adenylate*-^{32}P]NAD. To each assay tube were added water (8 μL), α_{i-1} (1 μL, 12 nM in 50 mM Tris, pH 7.7/75 mM sucrose/6 mM $MgCl_2$/1 mM EDTA/1 mM DTT/0.6% Lubrol PX/10 mM NaF/10 μM $AlCl_3$/100 mM NaCl, isolated from bovine brain), and methylated or demethylated $\beta_1\gamma_2$ at varying concentrations (1 μL). To a control assay without $\beta_1\gamma_2$ was added buffer A containing 1% cholate (1 μL). Each assay tube was cooled to 0 °C, and the pertussis toxin working solution (2.5 μL) and the radioactive assay buffer (8 μL) were added. The final assay volume was 25 μL. After heating to 37 °C for 15 min, each assay tube was immediately cooled to 0 °C. Cold sample buffer (12.5 μL) was added to each assay, and an aliquot (10 μL) was run on a 12% SDS−PAGE gel. After Coomassie blue staining of the gel, the labeled α_{i-1} subunit was observed both by autoradiography (20−40 h) and phosphorimaging (3 h exposure, Molecular Dynamics phosphorimager). The intensity of each band was determined by integrating identical areas of the gel obtained from the phosphorimager.

$\beta_1\gamma_2$ Binding to Azolectin Vesicles. Large unilamellar vesicles were prepared as described by Bigay et al. (1994). The relative binding of methylated and demethylated $\beta_1\gamma_2$ subunits was based on a study which examined the relative affinities of methylated and demethylated $T_{\beta\gamma}$ for identical azolectin vesicles (Bigay et al., 1994). Each tube contained the appropriate $\beta\gamma$ subunit (approximately 1 μM final concentration) and the filtered azolectin vesicles (10 μL, 16 mg·mL^{-1}, as determined by dry weight). Each experiment with $\beta_1\gamma_2$ contained 5 μL of protein sample in 1% cholate. In order to compare the $\beta_1\gamma_2$ binding data with that of methylated $T_{\beta\gamma}$, 5 μL of 1% cholate was added to the experiment involving $T_{\beta\gamma}$. Each tube was supplemented with 20 mM Tris, pH 7.5/120 mM NaCl/1 mM $MgCl_2$ to bring the total assay volume to 50 μL. After incubating each tube at 30 °C for 30 min, the lipid-bound $\beta\gamma$ was separated from the soluble fraction by centrifugation (Beckman TL100, 98 000 rpm, 400000*g*) for 10 min at 4 °C. The supernatant was removed and the pellet was washed with 50 μL of 20 mM Tris, pH 7.5/120 mM NaCl/1 mM $MgCl_2$. After another centrifugation for 10 min at 4 °C, this wash was removed. The pellet and supernatant were dissolved in sample buffer

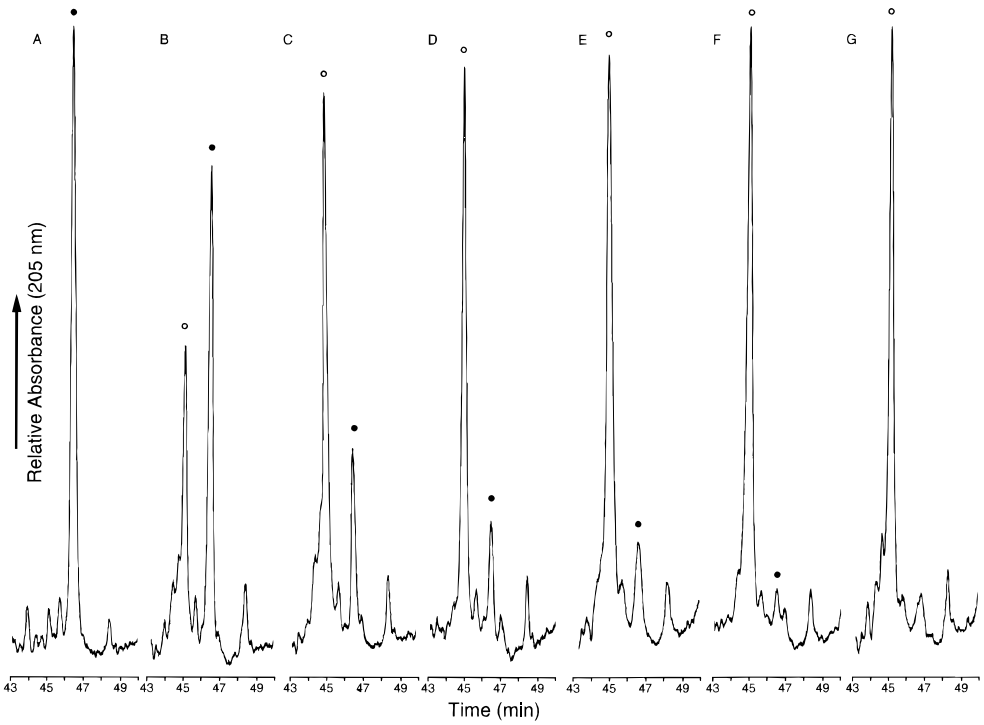

FIGURE 1: Time course of $\beta_1\gamma_2$ hydrolysis. The hydrolysis of methylated $\beta_1\gamma_2$ was accomplished as described in Materials and Methods. An aliquot (100 μL) was removed from a hydrolysis reaction at the indicated time, guandinium chloride (final concentration, 3 M) was added, and the sample was analyzed by reverse phase HPLC. Only the γ_2 subunit (● methylated, ○ demethylated) elutes from the column. A portion of each HPLC chromatogram is shown here. A, before iPLE addition; B, 3 h; C, 8 h; D, 13 h; E, 16 h; F, 20 h; G, 24 h.

and run on SDS−PAGE (12% gel) to determine the relative affinity of each $\beta\gamma$ for the azolectin vesicles. Each gel was stained with Coomassie blue and dried. While the γ subunit runs at the dye front under these conditions, the presence of the β subunit was used to indicate the location of the $\beta\gamma$ subunit. The percentage of each sample present in the pellet and supernatant fractions was quantified by densitometry.

Assay of PI3K and PIPLC Activity. The preparation and analysis of PI3K were described previously (Parish et al., 1995). PIPLC β2, histidine tagged at the amino terminus, was expressed in Sf9 insect cells and purified using nickel chelate chromatography (P. Sternweis, unpublished experiments). PIPLC activity was determined as described previously (Parish et al., 1995).

Miscellaneous Procedures. Protein concentrations were determined by the amido black method using BSA as a standard (Schaffner & Weissmann, 1973). Electrospray ionization mass spectra were obtained at the Harvard Microchemistry Facility.

RESULTS

Preparation of Demethylated $\beta_1\gamma_2$ Subunits. Previous studies in our laboratory demonstrated that an immobilized form of pig liver esterase (iPLE) was capable of hydrolyzing the carboxyl terminal methyl ester of farnesylated T_γ (Parish & Rando, 1994). It is shown here that methylated/geranylgeranylated $\beta_1\gamma_2$ subunits also can be hydrolyzed by iPLE. A time course for the hydrolysis is shown in Figure 1. After 24 h virtually complete hydrolysis of the methylated/geranylgeranylated $\beta_1\gamma_2$ subunits had occurred. The hydrolysis was found to be sensitive to detergent concentration and temperature. Complete hydrolysis was obtained at a cholate concentration of 0.03% and at 23 °C over a 24 h period. Higher detergent concentration or lower temperature markedly decreased the rate of hydrolysis (data not shown). Since $\beta_1\gamma_2$ is known to aggregate in the absence of detergent, the cholate concentration was immediately readjusted to 1% after completion of the hydrolysis. A control sample was treated in an identical manner except that it was not exposed to iPLE. This sample was indistinguishable from an untreated sample of $\beta_1\gamma_2$. Electrospray ionization mass spectrometry (EIMS) was used to confirm the identities of HPLC-purified methylated and demethylated geranylgeranylated γ_2 subunits. Molecular weights of 7750.0 and 7737.0 Da were obtained for methylated and demethylated γ_2, respectively. These values correspond well with the theoretical molecular weights of the proteins (methylated γ_2 = 7750 Da, demethylated γ_2 = 7736 Da) and are within the experimental accuracy of EIMS (approximately 0.01%). Therefore, the hydrolysis reaction provides the anticipated demethylated product. The molecular weights determined here correspond to γ_2 which lacks an amino terminal methionine, has been acetylated on the N-terminus, and is fully processed at the carboxyl terminus by geranylgeranylation/methylation. These data confirm that the protein utilized in these experiments, as obtained from the baculovirus expression system, is the desired γ_2 subunit.

ADP Ribosylation of α_{i-1} by Pertussis Toxin in the Presence of Methylated and Demethylated $\beta_1\gamma_2$ Subunits. One way to determine the effect of methylation on geranylgeranylated $\beta_1\gamma_2$ subunit function is to determine the effects the modified and unmodified subunits have on the pertussis toxin-mediated ADP-ribosylation of an α subunit (Ueda et al., 1994; Fukada et al., 1994). Pertussis toxin only ADP-ribosylates α·GDP in the presence of $\beta\gamma$ subunits (Neer et al., 1984). If methylation of $\beta\gamma$ is important in mediating specific lipid−protein interactions between α and $\beta\gamma$, then a large effect should be observed in the current experiments.

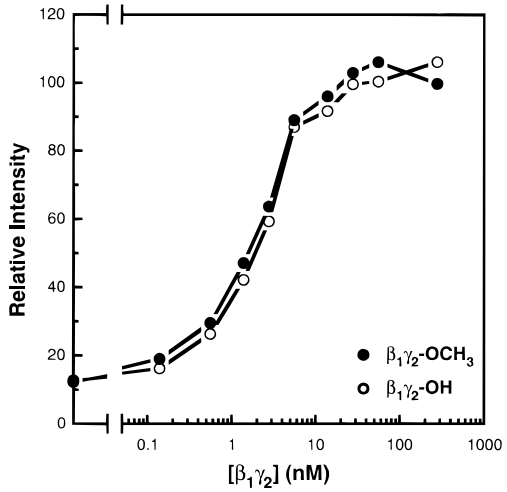

FIGURE 2: ADP-ribosylation of α_{i-1} by pertussis toxin in the presence of methylated and demethylated $\beta_1\gamma_2$. Each assay was performed as described in Materials and Methods. The final α_{i-1} concentration was 12 nM, and each assay contained 0.8 $\mu g \cdot mL^{-1}$ activated pertussis toxin. The incorporation of radioactivity was quantified from phosphorimager scanning of each SDS−PAGE gel (12%). Each band was integrated using an identical area (Imagequant, Molecular Dynamics). Each curve is the average of three experiments, and each individual experiment was run on three separate gels.

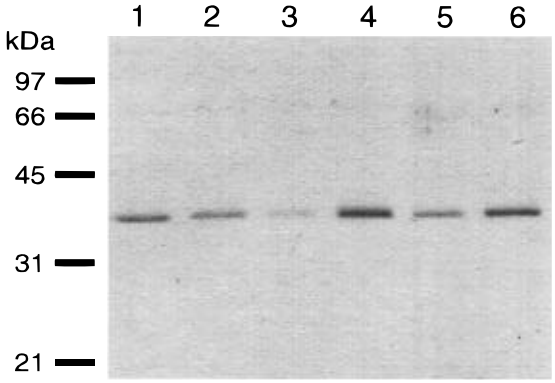

FIGURE 3: Binding of $\beta\gamma$ subunits to azolectin vesicles. $\beta\gamma$ subunits were incubated with azolectin vesicles, as described in Materials and Methods. The supernatant and pellet fractions were run on a 12% SDS−PAGE gel and stained with Coomassie blue. The observed band corresponds to the β subunit (35 kDa). Under these conditions, the γ subunit runs with the dye front and is not observed. Lanes 1 and 2: methylated $T_{\beta\gamma}$. Lanes 3 and 4: methylated $\beta_1\gamma_2$. Lanes 5 and 6: demethylated $\beta_1\gamma_2$. Lanes 1, 3, 5: supernatants. Lanes 2, 4, 6: pellets. This gel is representative of three separate experiments.

In fact, no difference was observed between methylated and demethylated $\beta_1\gamma_2$ subunits with respect to their abilities to facilitate ADP-ribosylation of α_{i-1} (Figure 2). No labeling was observed in the absence of pertussis toxin or α_{i-1} (data not shown). These experiments suggest little or no difference in the abilities of methylated and demethylated $\beta_1\gamma_2$ subunits to interact with α subunits.

Binding of Methylated and Demethylated $\beta_1\gamma_2$ Subunits to Azolectin-Based Vesicles. It was of further interest to determine whether methylation facilitates membrane association of $\beta_1\gamma_2$ subunits. In Figure 3, a series of experiments was performed to determine the relative abilities of methylated and demethylated $\beta_1\gamma_2$ subunits to associate with lipid vesicles. Methylated retinal transducin is shown to be distributed between the supernatant and vesicle fractions, with approximately 40% of the protein bound to the lipid pellet (Figure 3, lanes 1 and 2). Bigay et al. (1994) have demonstrated that demethylated retinal transducin $\beta\gamma$ associates less strongly (10−15% bound) with azolectin vesicles than its methylated counterpart (60−65% bound). Methylated $\beta_1\gamma_2$ subunits associate with membranes much more strongly (Figure 3, lanes 3 and 4) than was found for transducin. Approximately 90% of the methylated $\beta_1\gamma_2$ is present in the vesicle fraction. This observation is understood in terms of the increased hydrophobicity of a geranylgeranyl group relative to a farnesyl group. Demethylated $\beta_1\gamma_2$ subunits (Figure 3, lanes 5 and 6) still predominately associate with the membrane fraction (70% in pellet), but not to the same extent as the methylated form. It should be noted that the demethylated/geranylgeranylated $\beta\gamma$ is more hydrophobic than the methylated/farnesylated transducin $\beta\gamma$ (Figure 3, lane 2 versus lane 6). These data suggest that methylation would have only a small effect in enhancing membrane association of geranylgeranylated $\beta\gamma$ subunits to membranes.

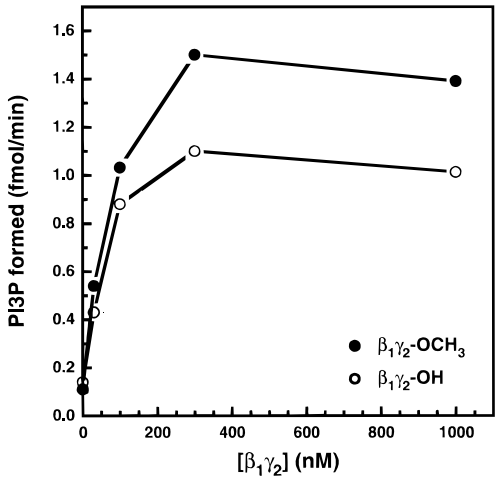

FIGURE 4: Activation of PI3K by methylated and demethylated $\beta_1\gamma_2$. Each assay contained 5 μL of partially purified PI3K that had been stored at −70 °C.

Activation of PI3K and PIPLC by Methylated and Demethylated $\beta_1\gamma_2$ Subunits. PI3K and PIPLC β2 are activated by a variety of geranylgeranylated $\beta\gamma$ subunit isoforms (Ueda et al., 1994; Stephens et al., 1994). Farnesylated $T_{\beta\gamma}$ has been shown to be approximately 10-fold less potent for activating these effectors as compared with geranylgeranylated $\beta\gamma$s (Ueda et al., 1994). It was of interest to determine whether the state of methylation of a geranylgeranylated $\beta\gamma$ isoform played any substantive role in the activation of PI3K and PIPLC. Enzyme activation profiles in the presence of methylated and demethylated $\beta_1\gamma_2$ subunits are shown for these enzymes in Figures 4 and 5. For both PI3K and PIPLC, only small effects of approximately 30% were observed on the efficacy, but not on the potency, of the methylated and demethylated subunits, with the methylated subunits being more effective.

DISCUSSION

One way to address the functional role of isoprenylation/methylation is to quantitatively probe the requirement for the methyl group in signal transduction processes. We had previously demonstrated that pig liver esterase is competent to hydrolyze the methyl ester of farnesylated/methylated proteins (Parish & Rando, 1994). It is shown here that this same esterase can also hydrolyze a geranylgeranylated/

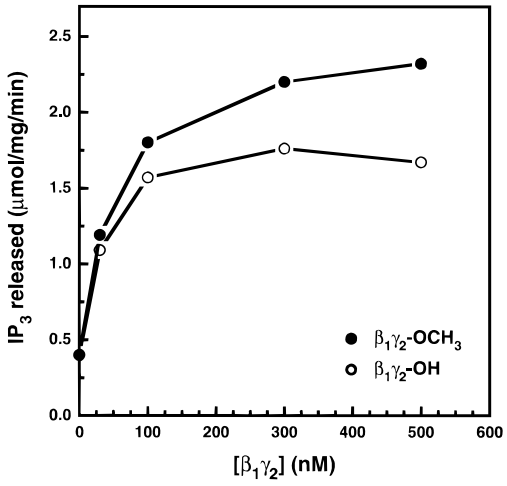

FIGURE 5: Activation of PIPLC β2 by methylated and demethylated $\beta_1\gamma_2$. Each assay contained 0.5 ng of purified PIPLC β2 from rat brain that had been stored at −70 °C.

methylated protein, namely, $\beta_1\gamma_2$. This allows us to probe the role of methylation in the functioning of geranylgeranylated/methylated proteins. While retinal transducin is farnesylated, geranylgeranylation is by far the predominant isoprenyl modification utilized in G protein biosynthesis.

If the isoprenyl/methyl modification is involved in specific lipid−protein recognition, then the methyl group would be expected to be important, since it neutralizes the potential negative charge on the carboxyl moiety. Alternatively, if methylation simply enhances the hydrophobicity of the modified protein, then its role should be relatively minor. If the latter scenario is correct, then the relative effect of the methyl group should be greater in farnesylated proteins than in geranylgeranylated proteins, due to the greater inherent hydrophobicity of the latter modification (Sinesky & Lutz, 1992). This would correlate with data on the hydrophobicity of small isoprenylated peptides (Silvius & l'Heureux, 1994). In that case, geranylgeranylated peptides which are not methylated have a greater affinity for membranes than their farnesylated/methylated counterparts. This can be further appreciated from Figure 3, where it is shown that methylation has only a small effect on the association of demethylated versus methylated $\beta_1\gamma_2$ subunits with azolectin-based vesicles. A larger relative effect was observed in the case of farnesylated transducin (Bigay et al., 1994). The far greater hydrophobicity of the geranylgeranyl group relative to farnesyl is demonstrated by the fact that the demethylated/geranylgeranylated $\beta_1\gamma_2$ binds more strongly to these vesicles than does methylated/farnesylated $T_{\beta\gamma}$.

As previously demonstrated, however, methylation plays only a minor quantitative role in the interactions of transducin with activated rhodopsin (Parish & Rando, 1994), suggesting that specific lipid−protein interactions are not mediated via the farnesylated/methylated cysteine residue. The small effect observed is probably due to the decreased hydrophobicity of demethylated $T_{\beta\gamma}$ (Fukada et al., 1994; Bigay et al., 1994). It must be remembered, however, that in the case of transducin, integral membrane-bound activated rhodopsin (R*) has a strong affinity for $T_{\alpha\beta\gamma}$ (Ohguro et al., 1991), and this affinity would have the effect of limiting the impact of the methyl group on membrane binding. This complicating factor would not apply in the instance where a $\beta\gamma$ subunit is required to activate an otherwise soluble enzyme at a membrane surface. Here, it is likely that the role of the $\beta\gamma$ subunit is to associate the enzyme with the membrane. The activation of PIPLC and PI3K by $\beta\gamma$ subunits may fall into this category.

In the studies reported here, the methylation state of $\beta_1\gamma_2$ proved to have a small but distinct effect on the ability of the subunits to activate either PIPLC or PI3K. A small quantitative effect of approximately 30% on the extent of activation of the enzymes was observed between the methylated and demethylated $\beta_1\gamma_2$ subunits. This is to be contrasted to a substantially larger effect observed when $T_{\beta\gamma}$ was studied (Parish et al., 1995). This difference can probably be attributed to the greater hydrophobicity of the geranylgeranylated $\beta_1\gamma_2$ subunit compared to $T_{\beta\gamma}$, which is farnesylated. The effects observed here can be attributed to the enhanced hydrophobicity due the methylation. Specific lipid−protein interactions would have been expected to generate a much larger effect.

The notion that lipid−protein interactions are not mediated in an important way by the state of isoprenylation/methylation could be further explored by studying the ADP-ribosylation of an α subunit in the presence of methylated and demethylated $\beta_1\gamma_2$. Pertussis toxin ADP-ribosylates G protein α subunits when α exists in the inactive heterotrimeric state. ADP-ribosylation requires the presence of $\beta\gamma$ subunits, but not membrane association (Neer et al., 1984). Here it is shown that the state of $\beta_1\gamma_2$ methylation had no measurable effect on the pertussis toxin-mediated ADP ribosylation of α_{i-1}. In essence, the small effects observed when comparing methylated and demethylated $\beta\gamma$ subunits are only seen when membranes are involved. This result should be compared with data on the ADP-ribosylation of T_α in the presence of methylated and demethylated $T_{\beta\gamma}$ (Fukada et al., 1994). In that case, a small quantitative effect of approximately 2-fold was observed.

The notion that methylation of isoprenylated proteins provides a small quantitative enhancement in G protein function through enhanced membrane binding is, of course, consistent with previously discussed observations on retinal transducin (Parish & Rando, 1994; Fukada et al., 1994). It is quite possible that these small effects are important in an efficient signal transduction system. However, these relatively small effects should be contrasted to the requirement for isoprenylation *per se*. In the case of retinal transducin, non-isoprenylated $T_{\beta\gamma}$ is inert with respect to interactions with activated rhodopsin, both in membranes and in detergent (Ohguro et al., 1991; Cheng et al., 1995). Similar all-or-nothing results have been observed in the case of *ras* proteins (Hancock et al., 1989; Porfiri et al., 1994). Moreover, methylation appears not to have a profound effect on *ras* function, at least with respect to membrane binding (Hancock et al., 1991).

From the experiments reported here, it is likely that the functional role of isoprenylation/methylation in heterotrimeric G proteins is to enhance the membrane binding of modified proteins by increasing their hydrophobicity, and that no lipid-specific protein receptors are involved. The hypothesis that methylation is not a primary controlling feature of isoprenylation/methylation is quite consistent with what is known about the enzymology of isoprenylated protein methylation. It is clear that a single carboxymethyltransferase enzyme methylates both farnesylated and geranylgeranylated proteins in mammals (Pérez-Sala et al., 1992). Greater enzyme

diversity would have been expected if methylation were a key regulatory event. In yeast, a single mutation (STE14) that renders the isoprenylated protein methyltransferase inert has only a quantitative effect on cell growth and viability, even though all or most methylation of isoprenylated proteins is apparently abolished (Hrycyna et al., 1991). Only mating is disrupted because the farnesylated/methylated undecapeptide mating factor is inert when demethylated (Anderegg et al., 1988). In addition, *rab3* in either its methylated or demethylated form interacts equally well with its GDI (Musha et al., 1992).

In the case of isoprenylation, a number of experiments in which the nature of the isoprenyl group (farnesyl vs geranylgeranyl) or the lipid structure (isoprenyl to myristoyl) has been altered indicate that a specific isoprenyl group is not required for function. In *ras* (Buss et al., 1989; Cox et al., 1992), rhodopsin kinase (Inglese et al., 1992), β-adrenergic receptor kinase (Inglese et al., 1992), and *rab6* (Beranger et al., 1994; Smeland et al., 1994), a specific isoprenylated carboxy terminus is not required for activity. These results would be particularly difficult to understand if specific lipid−protein interactions requiring the carboxyl terminal isoprenylated/methylated cysteine were involved.

The studies described here are, of course, directly relevant to heterotrimeric G protein function, but relevant to small G protein function only by analogy. It is clearly possible that in this latter class of G proteins an important effector role exists for isoprenylated/methylated cysteine moieties which involves specific lipid−protein interactions. These putative interactions have yet to be described, but it is interesting to note that farnesylcysteine analogs can have profound pharmacological effects on various types of cells (Scheer & Gierschik, 1993, 1995). For example, they can induce or inhibit superoxide generation in neutrophils (Ding et al., 1994; Philips et al., 1993), block pharmacologically induced platelet aggregation (Ma et al., 1994; Huzoor-Akbar et al., 1993), and inhibit capacitive Ca^{+2} entry into cells (Y. Xu, B. A. Gilbert, R. R. Rando, L. Chen, and A. H. Tashjian, submitted). Assuming that these effects are specific in nature, then it would be tempting to speculate that these farnesylcysteine analogs are interfering with as yet undescribed lipid−protein interactions mediated through isoprenylated/methylated moieties on appropriately modified proteins. The pharmacological targets of farnesylcysteine analogs will need to be identified before any firm conclusions can be drawn concerning putative effector roles of isoprenylated/methylated moieties.

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