Gqα is palmitoylated at residues Cys9 and Cys10. Removal of palmitate from purified Gqα, with palmitoyl-thioesterase in vitro failed to alter interactions of Gqα, with phospholipase C-β1, the G protein βγ subunit complex, or m1 muscarinic cholinergic receptors. Mutants C9A, C10A, C9A/C10A, C9S/C10S, and truncated Gqα, (removal of residues 1-6) were synthesized in SF9 cells and purified. Loss of both Cys residues or truncation prevented palmitoylation of Gqα. However, truncated Gqα, and the single Cys mutants activated phospholipase C-β1 normally, while the double Cys mutants were poor activators. Loss of both Cys residues impaired but did not abolish interaction of Gqα with m1 receptors. These Cys residues are thus important regardless of their state of palmitoylation. When expressed in HEK-293 or SF9 cells, all of the proteins studied associated entirely or predominantly with membranes, although a minor fraction of nonpalmitoylated Gqα, proteins accumulated in the cytosol of HEK-293 cells. When subjected to TX-114 phase partitioning, a significant fraction of all of the proteins, including those with no palmitate, was found in the detergent-rich phase. Removal of residues 1-34 of Gqα, caused a loss of surface hydrophobicity as evidenced by complete partitioning into the aqueous phase. The Cys residues at the amino terminus of Gqα, are thus important for its interactions with effector and receptor, and the amino terminus conveys a hydrophobic character to the protein distinct from that contributed by palmitate.

Heterotrimeric guanine nucleotide binding proteins (G proteins) link cell surface receptors with intracellular effectors (1-3). In the absence of activators, G protein α, β, and γ subunits are associated with each other and the inner surface of the plasma membrane. Although the G protein polypeptides are not intrinsically hydrophobic, they are membrane-bound at least in part because of covalent lipid modifications. G protein γ subunits are preylated and carboxymethylated at their carboxyl termini (4, 5). These modifications promote association of the βγ subunit complex with membranes and interactions of βγ with α and effectors (6). Members of the Gq subfamily of α subunits are myristoylated at their amino termini; this modification also promotes membrane anchorage and interactions of α with βγ and effectors (7-9). Although members of the Gq, Gs, and G12 subfamilies of α subunits are not myristoylated, they (and Gs family members, excepting transducin) are palmitoylated on one or more cysteine residues near their amino termini (10-12). Arachidonate may also be incorporated similarly (13). In contrast to myristoylation, palmitoylation of Gqα, subunits is a dynamic and regulated process. Activation of appropriate receptors appears to stimulate depalmitoylation of cognate Gqα, subunits (14-16).

The lipid modifications of Gqα, subunits are at or near the amino terminus, and this domain (upstream of the Gα, nucleotide binding region; roughly residues 1-35) has been implicated in a number of functions. The amino terminus is clearly important for interactions with βγ (17, 18), and it is implicated in interactions with receptors (19) and effectors (9, 18) as well. The amino and carboxyl termini of Gqα, form an associated subdomain of the GDP-bound form of the protein that becomes disordered upon activation (20, 21). The amino termini of Gqα, and G11α, differ from those of most other Gα, proteins. The site of initiation of translation is not clear (Met1 or Met7 in Gqα, Met1, Met3, or Met2 in G11α). The shorter form (assuming Met2 as initiator) is most similar to other Gα, subunits (22). It is not known if only one or both of these species is expressed naturally, although all currently identified Gqα, G11α, mRNAs (except that from Drosophila) encode the longer protein. Gqα, and G11α, also have two amino-terminal Cys residues (positions 9 and 10) that both serve as sites for palmitoylation (23-25).

The role of G protein palmitoylation is unclear, and several conflicting reports have appeared. In the case of Gqα, one study (23) suggests that palmitoylation is essential for membrane localization, while others (14, 15) indicate that nonpalmitoylated mutants of Gqα, remain associated with the plasma membrane. Wedegaertner et al. (23) also reported that palmitoylation is necessary for association of Gqα, with membranes and subsequent activation of its physiological effector, PLC-β1. In contrast, others have indicated that palmitoylation is not required for association of either Gq or G11, with membranes (24, 25), and Edgerton et al. (25) found that the C9A/C10A mutant of Gqα, failed to activate PLC-β1. Published data also suggest that palmitoylation is important for coupling of Gqα, with the NK2 receptor (25) but not the α2-adrenergic receptor (23). All of these studies have utilized transfected cells expressing G protein α subunits carrying mutations designed to prevent palmitoylation.

We have investigated the importance of amino-terminal domains of G proteins using the m1 muscarinic cholinergic recep-
tor Gq/PLC-β1 signaling pathway as a model system, and we have emphasized studies of purified and reconstituted proteins. We have found that the amino-terminal domain of Gq is clearly important for activation of PLC-β1. Although palmitoylation of Cys residues 9 and/or 10 is not necessary for interactions of Gq with PLC-β1 receptors, or βγ, the Cys residues themselves are important for interactions with PLC-β1 and, to a lesser extent, with receptor. The amino terminus of Gq also appears to confer a hydrophobic character on the protein, independent of its palmitoylation.

EXPERIMENTAL PROCEDURES

Materials—[3H]Palmitoyl-Ha-ras (26) and purified recombinant palmitoylthioesterase were prepared as previously described (27). Anti-Gq, sera W082 and ZB11 was supplied by P. C. Sternweis (University of Texas, Southwestern), and anti-Gq, serum 584 and common anti-Gq, serum P960 by S. M. Mumbery (University of Texas, Southwestern). Purified recombinant PLC-β1 was kindly provided by S. G. Rhee (National Institutes of Health).

Gq Mutants—Gq tagged with six histidine residues at the carboxyl terminus (GqCH6) and amino-terminal mutations of GqCH6 were generated by synthesizing oligonucleotide cassettes for substitution insertion as described elsewhere (28). The following complementary pair of oligonucleotides were synthesized to construct GqCH6: 5’-GCTGAAACTGAAAGGTACTGACTGACGATCCCCAATCAA-3’ and 3’-ACGTCGATTGTGATCTGATTGATTTCTCA-5’.

This camera encodes the extreme carboxyl terminus of Gq, the hexahistadine tag, and a stop codon, and it contains PstI and HindIII sites at its 5’ and 3’ ends, respectively. Following annealing, the cassette was cloned into the unique PstI site at the extreme carboxyl terminus of Gq, the coding region and the 3’ HindIII site of the Escherichia coli expression vector (Gq/NpTT-75). The resulting plasmid was linearizing with HindIII, blunt-ended with Klenow fragment, and partially digested with EcoRI to yield a 12 000 kilobase pair fragment encoding GqCH6. This DNA was cloned into baculovirus expression vector pVL1392 (EcoRI and Smal sites). This served as the parent for synthesis of amino-terminal mutants of GqCH6. These mutants (see Fig. 3) were synthesized by insertion of oligonucleotide cassettes using the unique NotI site of the pVL1392 polycloning region and the unique EcoRI site near the initiator codon of Gq. Recombinant baculoviruses were generated as described previously (29, 30). The nucleotide sequence for all Gq mutant DNA constructs was confirmed by dye terminator sequencing using an Applied Biosystems 373A automated sequencer (Perkin-Elmer).

Labeling and Isolation of [3H]Palmitate-labeled Gq Mutants—S19 cells (25 ml of culture; 1 × 109 cells/ml) were triply infected with viruses encoding hexahistadine-tagged wild-type or mutant Gq, together with viruses encoding Gβγ, and mixed for 1 h at 4°C. Resin was recovered by centrifugation of the resulting supernatant was combined with 100 ml of Ni-NTA resin (Qiagen) and mixed for 1 h at 4°C. Resin was washed by centrifugation and washed with 3 × 1 ml of buffer B (buffer A + 1% sodium cholate, 150 mM NaCl, 1 mM EDTA, 0.1% octyl-β-D-glucopyranoside). Highly enriched 3H-labeled recombinant GqCH6 was eluted by washing the resin five times with 100 ml of buffer C (buffer C containing 100 mM NaCl, 150 mM (imidazole, and 10% glycerol).

Recoveries of protein were monitored by silver staining and fluorography of the same gels as described previously (10).

Purification of Amino-terminal Mutants of Gq—GqCH6 and mutants were expressed in S19 cells and purified to near homogeneity by Ni-NTA affinity chromatography and Q-Sepharose anion exchange chromatography. S19 cells (2-4 liters; 1.5-2 × 109 cells/ml) were triply infected with the desired GqCH6 virus together with βγ and γ2 viruses for 48-60 h. Cells were pelleted, suspended in 400 ml of buffer A, and lysed by nitrogen cavitation at 4°C (500 p.s.i. for 3 h; Parr Instruments). Nuclei were removed from lysates by centrifugation, and supernatants were centrifuged (35,000 rpm, Beckman Ti-45) for 30 min at 4°C. The resulting membranes were suspended in 90 ml of buffer A (3-5 mg protein/ml) and extracted with 1% sodium cholate in buffer A for 1 h at 4°C. The resulting extract was collected by centrifugation (100,000 × g) and mixed with 5 ml of Ni-NTA resin for 1 h. After application to a 3 × 13-cm column, the resin was washed with 20 column volumes of buffer B and 3 volumes of buffer C. Protein was eluted with four 5 ml volumes of buffer D. After washing four 5 ml volumes of Ni-NTA, the elution was diluted 15-fold with buffer E (50 mM NaHepes, pH 7.4, 1 mM EDTA, 3 mM EGTA, 5 mM MgCl2, 2 mM dithiothreitol, 0.1 mM GDP, 10 mM NaF, 0.03 mM AlCl3) and applied to a Q-Sepharose column (5 ml) that had been equilibrated with buffer E. The column was washed with 5 column volumes of buffer F (50 mM NaHepes, pH 7.4, 1 mM EDTA, 3 mM EGTA, 5 mM MgCl2, 2 mM dithiothreitol, 100 mM GDP), and protein was eluted with a 40-ml linear gradient of NaCl (0–1 M) in buffer F.

Transfection of HEK-293 Cells—DNAs encoding Gq, Gqshort, and C9A/C10A Gq were donor into the mammalian expression vector pCMV-S (31); none of these constructs contained the hexahistadine tag. HEK-293 cells (150-mm dishes; 75% confluent) were transfected with 25 μg of purified DNA for 3 h using Lipofectamine (Life Technologies, Inc.) as described elsewhere (14). Cells were then harvested in lysis buffer (50 mM NaHepes, pH 7.4, 1 mM EDTA, 3 mM EGTA, 5 mM MgCl2, 150 mM NaCl, 100 μM GDP, and 0.2 mg/ml of phosphomethylsulfonetyl fluoride) and lysed by nitrogen cavitation. Intact nuclei were removed by centrifugation, and membranes and cytosol were separated (120,000 rpm; Beckman Ti-40). All fractions contained the same volume; membrane protein (5 μg for Gq alone and 25 μg for Gq+ βγ) and equal volumes of normalized samples from the cytosolic and nuclear fractions were analyzed by SDS-PAGE.

Amino-terminally Truncated Gq—The amino terminus of GTP-γS-activated GqCH6 (not hexahistadine-tagged) was sequenced by SDS-PAGE.

Measurement of Phospholipase C-β1 Activity—Activation of purified phospholipase C-β1 by purified subunits was performed as described elsewhere (29), as were assays of phospholipase C-β1 activity.

Reconstitution of m1 Muscarinic Receptors and G Proteins into Phospholipid Vesicles—The m1 muscarinic receptors, Gq (not hexahistadine-tagged), G protein βγ, subunits, and phospholipase C-β1 used for reconstitution assays were purified from baculovirus-infected S9 cells; these methods will be described elsewhere. Lipid-detergent micelles were prepared by rehydrating dried lipids (bovine liver phosphatidylethanolamine, bovine brain phosphatidylserine, and cholesteryl hemisuccinate) in buffer containing 20 mM NaHepes (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2 mM MgCl2, and 0.2% deoxycholate, followed by sonication under argon gas for 15 min at room temperature. The final concentration of lipids was 413 μM phosphatidylethanolamine, 245 μM phosphatidylserine, and 45 μM cholesteryl hemisuccinate. Micelles (25 μl) were then incubated with Gq (5 pmol of Gq, plus 10 pmol of β1γ2), for 10 min; m1 receptors (3 pmol) were added, and the volume was brought to 50 μl with 20 mM NaHepes, 1 mM EDTA, 0.1 mM GTPγS, 7.5, 100 mM NaCl, 1 mM EDTA, and 2 mM MgCl2. The mixture was applied to a 1-ml AlCa34 gel filtration column, and the protein-lipid vesicles were recovered in the void volume. BSA (0.1 mg/ml) was added, and the vesicles were treated with diethiothreitol (5 mM) for 1 h on ice before assay.

Assay of Reconstituted Proteins—GTP hydrolysis was measured essentially as previously described (33, 34). Briefly, receptor-Gq vesicles were mixed with 1 μM [γ−32P]GTP and either 1 μM carbachol or 10 μM atropine in a volume of 50 μl (final buffer: 20 mM NaHepes (pH 8.0), 100 mM NaCl, 1.1 mM EDTA, 0.2 mM GTPγS, 3.9 mg/totai MgCl2, and 0.26 mg/ml BSA). The mixture was incubated at 30°C for various times, and reactions were stopped by addition of 750 μl of NaCl (5% in 50 mM NaHepes, pH 7.5, 100 mM NaCl, 1.1 mM EDTA, and 2 mM MgCl2). The mixture was applied to a 1-ml AlCa34 gel filtration column, and the protein-lipid vesicles were recovered in the void volume. BSA (0.1 mg/ml) was added, and the vesicles were treated with diethiothreitol (5 mM) for 1 h on ice before assay.

m1 Receptor—catalyzed GTP-γS binding was measured at 30°C as described elsewhere (35), using 100 nM [35S]GTP-γS and 1 μM carbachol; the final buffer composition was the same as in GTP hydrolysis assays (see above). Data describing the time course of GTP-γS binding were fit to a two-component equation: y = A1 (1 − e−kt) + (nt + b), where A is the maximum amount of GTP-γS bound in response to carbachol, k is the rate constant for receptor-stimulated binding, m is the basal rate of binding (essentially constant over the assay interval), and b is the
amount of GTP\(\gamma\)S nonspecifically bound at zero time (t). For C9S/C10S
\(G_{\alpha,1}\), the linear component (mt + b) was omitted because there was no
observable binding of nucleotide in the absence of carbachol.

Trition X-114 Phase Partitioning of Proteins—Partitioning of protein
samples between aqueous and detergent-rich phases using TX-114 was
performed as previously described (36). Following appropriate treatments
(see "Results"), samples were prepared in 260 \(\mu\)L of a buffer
consisting of 50 \(\mu\)M Hepes, pH 8.0, 5 \(\mu\)M \(\beta\)-mercaptoethanol, 150 \(\mu\)M
NaCl, 1% Triton X-114, and 5 \(\mu\)M MgCl\(_2\). Of this, 200 \(\mu\)L were removed
and applied to the top of a 300- \(\mu\)L sucrose cushion (6%) prepared with
the same buffer containing 0.1% TX-114. The samples were then separated
into detergent and water phases as previously described (36). The result-
ing upper water and lower detergent phases were collected
and adjusted to contain equivalent total volumes (225 \(\mu\)L) of the original
buffer and equivalent amounts of TX-114. These were compared to the
original sample by SDS-PAGE and Western blot analysis.

Miscellaneous Procedures—Labeling of Sf9 cells with \([35S]\)methioni-
one and \([\mathrm{H}]\)palmitate and immunoprecipitation of radiolabeled
recombinant \(G_{\alpha,1}\) subunits were performed as described previously
(10). Fatty acids removed from \(G_{\alpha,1}\) by hydrolysis were analyzed by
HPLC (10).

RESULTS

When Sf9 cells are infected with baculovirus encoding \(G_{\alpha,1}\),
\(G_{\alpha,2}\) or \(G_{\alpha,1}\), along with viruses encoding \(\beta_2\) and \(\gamma_2\) subunits,
novely synthesized \(\alpha\) subunits accumulate in both membranes and
cytosol; however, only the membrane-associated \(\alpha\) sub-
units incorporate \([\mathrm{H}]\)palmitate (Fig. 1A).\(^3\) To study the func-
tional consequences of palmitoylation in greater detail, we
generated an affinity-tagged \(G_{\alpha,1}\) (hexahistidine at the carboxyl
terminus) using the baculovirus expression system. When syn-
thesized in Sf9 cells, \(G_{\alpha,1}\) incorporates \([\mathrm{H}]\)palmitate and
and can be purified rapidly from membrane extracts by affinity
chromatography with Ni-NTA resin (Fig. 1B).

Treatment with Palmitoylthioesterase—A palmitoylthioes-
terase capable of removing palmitate from c-Ha-ras and \(G_{\alpha,1}\)
have been puriﬁed from bovine brain, cloned, and expressed in
Sf9 cells (26, 27). Treatment of \([\mathrm{H}]\)palmitate-labeled \(G_{\alpha,1}\),
or c-Ha-ras with recombinant palmitoylthioesterase results in
almost total loss of label from the proteins (Fig. 2A). HPLC
analysis of the material so removed from \(G_{\alpha,1}\) reveals that
the label is in fact in palmitate (Fig. 2B). However, treatment of
purified \(G_{\alpha,1}\) with palmitoylthioesterase failed to alter the
protein’s capacity to activate PLC-\(\beta\)-1 (Fig. 2C). Similarly, such
treatment failed to alter interaction of \(G_{\alpha,1}\) with \(\beta_2\) as assessed by
the capacity of \(\beta_2\) to reverse activation of \(G_{\alpha,1}\) by AlF\(_3\) (Fig.
2D). Removal of palmitate from \(G_{\alpha,1}\) also failed to interfere with
the protein’s interactions with m1 muscarinic receptors as as-
sessed by the capacity of the purified receptor to stimulate
nucleotide exchange and GTP\(\gamma\)S binding to \(G_{\alpha,1}\) (in the presence
or absence of the receptor agonist carbachol).\(^4\) The capacity of
receptor and PLC-\(\beta\)-1 to stimulate the GTPase activity of \(G_{\alpha,1}\)
(i.e. GAP activity) was also unchanged by treatment with
palmitoylthioesterase (data not shown). The power of these
experiments to reveal functional effects of palmitoylation is
of course dependent on the stoichiometry of palmitoylation of
the purified \(G_{\alpha,1}\). Although this has not been determined precisely,
we believe that a significant amount of palmitate was present
(see below).

\(^3\) The label from \([\mathrm{H}]\)palmitate associated with cytosolic \(G_{\alpha,1}\) is
myristate, not palmitate (10). In the experiment shown in Fig. 1A, \(G_{\alpha,1}\)
is visualized as a pair of proteins with apparent molecular masses of 42
and 45 kDa. This is the result of unexpectedly efficient read-
ing of the altered polyhedron initiation codon contained upstream of the
inserted \(G_{\alpha,1}\) sequence in the original pVL1393 expression vector (29). This
altered initiation codon was placed out of frame with the \(G_{\alpha,1}\) sequence in
all subsequent experiments.

\(^4\) The m1 muscarinic receptor can stimulate nucleotide exchange on
\(G_{\alpha,1}\) in the absence of agonist, albeit at a slower rate than that observed
in the presence of an agonist such as carbachol (35).

![Fig. 1. Labeling and isolation of recombinant G protein \(\alpha\) sub-
units from Sf9 cells by immunoprecipitation or affinity chromato-
graphy. A, Sf9 cells were infected with viruses encoding G protein
\(\beta_2\) and \(\gamma_2\) subunits and either \(G_{\alpha,1}\), \(G_{\alpha,2}\), or \(G_{\alpha,3}\). Infected
cells were labeled with \([35S]\)methionine (left) or \([\mathrm{H}]\)palmitate (right)
fractionated into cytosol (C) and membranes (M), and subjected to
immunoprecipitation using specific antisera (Z811 for \(G_{\alpha,1}\),
\(G_{\alpha,2}\), or \(G_{\alpha,3}\), immunoprecitated, radiolabeled proteins were resolved
by SDS-PAGE and visualized by autoradiography or fluorography. In
this experiment \(G_{\alpha,1}\) is visualized as two bands; see footnote 3 for
explanation. B, Sf9 cells were infected with viruses encoding \(\beta_2\),
\(\gamma_2\), and hexahistidine-tagged \(G_{\alpha,1}\). Infected cells were incubated with
\([\mathrm{H}]\)palmitate and fractionated into cytosol and membranes. Cholate
extracts of membranes (L) were mixed with Ni-NTA resin, the flow
through (FT) was collected, and the resin was washed with high salt
(W1) and low salt (W2); bound protein was eluted using 150 mm imid-
azole (Bump). Fractions were resolved by SDS-PAGE and visualized by
silver staining (left) or fluorography (right).

Amino-terminal Mutations of \(G_{\alpha,1}\).—The likely sites of attach-
ment of palmitate to \(G_{\alpha,1}\) subunits are amino-terminal cysteine
residues (10–12, 15, 23); in the case of \(G_{\alpha,1}\), these are Cys\(^9\) and
Cys\(^10\). To define the role of Cys\(^9\) and Cys\(^10\) and the unique six
amino acid extension at the amino terminus of \(G_{\alpha,1}\), we gen-
erated single and double mutants of \(G_{\alpha,1}\) that substituted either
Ala or Ser for Cys\(^9\) and/or Cys\(^10\). In addition, we constructed
truncated forms of \(G_{\alpha,1}\) that were missing either the first 6 or
the first 10 amino acid residues (Fig. 3A). Some of these mu-
tants were coexpressed with \(\beta_2\) in Sf9 cells in the presence of
\([\mathrm{H}]\)palmitate and then isolated using Ni-NTA chromatography.
Consistent with previous reports (23, 25), C9A or C10A
\(G_{\alpha,1}\) failed to incorporate label. Unexpectedly, \(G_{\alpha,1}\) that is truncated by removal of resi-
dues 1–6 (and thus retains Cys\(^9\) and Cys\(^10\)) is not palmitoy-
Fig. 2. Removal of palmitate from Gqα by treatment with recombinant palmitoylthioesterase (rPTE). A, [3H]Palmitate-labeled rGqα, or c-Ha-ras purified by Ni-NTA affinity chromatography was incubated in the absence or presence of palmitoylthioesterase. Treated samples were resolved by SDS-PAGE, and labeled protein was visualized by fluorography. B, HPLC analysis of a [3H]-labeled mixture of standards of myristate, palmitate, and stearate top, or [3H]-labeled products removed from rGqα synthesized in the presence of [3H]palmitate; incubation was performed without (middle) or with (bottom) palmitoylthioesterase C. Purified rGqα was incubated with (●) or without (○) palmitoylthioesterase for 90 min at 30°C and then activated for 1 h with 1 mM GTPγS at 30°C. Treated rGqα was then mixed with purified phospholipase C-β1 and phosphatidylinositol(4,5)-bisphosphate vesicles and assayed for phospholipase C-β1 activity as described under “Experimental Procedures.” Enzymatic activity is expressed per ng of phospholipase C-β1 activity was measured as described.

We next studied the effects of amino-terminal mutations of Gqα on its interactions with m1 muscarinic receptors and PLC-β1 in reconstitution assays. Non-His-tagged Gqα was included in these experiments to assess the effects of the tag on receptor coupling. As shown in Fig. 5A, m1 receptors stimulate nucleotide exchange and GTPγS binding to Gqα, CH6, Gqα short, and C95/C105 Gqα. Although receptor-stimulated GTPγS binding was nearly identical for Gqα, CH6 and Gqα short, the rate of nucleotide exchange was significantly reduced (5-fold) for the double Cys → Ser mutant (Table I, Fig. 5A). In contrast, the nucleotide binding properties of the single Cys Ala mutants were largely unchanged (data not shown). Similarly, carbachol stimulates steady-state GTP hydrolysis by Gqα, CH6, Gqα short, and non-His-tagged Gqα, but the effect is reduced significantly with the double Cys → Ser mutant (Table I, Fig. 5B).

Phospholipase C-β1 is known to stimulate steady-state GTP hydrolysis by Gqα, particularly in the presence of receptor (34). Phospholipase C-β1 enhances receptor-stimulated GTP hydrolysis by greater than 11-fold for both Gqα, CH6 and Gqα short, but only 2-3-fold for C95/C105 Gqα (Table I; Fig. 5C). This is consistent with the relative inability of C95/C105 Gqα to activate phospholipase C-β1. The effects of phospholipase C-β1 on the single Cys → Ala mutants were similar to those on Gqα, CH6 (data not shown). A separate point is that carboxyl-terminal hexahistadine tagging of Gqα reduces the efficiency of receptor...

5 Much longer exposures reveal that a very small amount of label (<2% of wild type) is incorporated into truncated Gqα.

The Amino Terminus of Gqα

Results

Gqα, CH6 and five of the mutants were purified by a combination of Ni-NTA and Q-Sepharose chromatography for further characterization (Figs. 3C and 4). Yields of purified protein ranged between 0.1 and 1 mg/liter of Sf9 cell culture. Gqα(-10) could not be purified in reasonable amounts.

The capacity of each of these proteins to stimulate purified PLC-β1 is shown in Fig. 4. The nonpalmitoylated double Cys mutant (C9A/C10A) has a greatly reduced capacity to activate and apparent affinity for PLC-β1. In contrast, both of the single Cys → Ala mutants, which are palmitoylated, and the nonpalmitoylated truncation mutant, Gqα short, retain near full capacity to stimulate PLC-β1. The Cys → Ser double mutant (C95/C105) of Gqα was indistinguishable from C9A/C10A. Both C9A/C10A and C95/C105 Gqα could be activated, based on the capacity of bound GTPγS to protect the proteins from trypsin proteolysis (Fig. 4B, inset). To test the effect of removal of a larger portion of the amino terminus, we cleaved GTPγS-activated Gqα, CH6 with trypsin and recovered the product (NC-Gqα) by Ni-NTA chromatography; the hexahistidine-tagged carboxyl terminus was thus intact. Amino acid sequencing of NC-Gqα revealed that the first 34 residues were missing. The capacity of NC-Gqα to activate PLC-β1 closely resembles those of C9A/C10A and C95/C105 Gqα (Fig. 4C).
PHY.SF9 cells (6-liter culture) were infected with viruses encoding β1, β2, and β1/2 (C9A, C10A, and C9A/C10A) bound to Ni-NTA resin. Immuno blotting with a specific anti-Gq antibody resolved by SDS-PAGE and visualized by fluorography and by immunoblotting with a specific anti-Gq antibody resolved by SDS-PAGE and visualized by fluorography. Membrane extracts by Ni-NTA affinity chromatography. Samples were labeled with [3H]palmitate, and Gq binding was analyzed for their capacity to stimulate phospholipase C-β1 activity is expressed as pmol product/min/ng phospholipase C-β1.

Cellular Distribution of Gqα Mutations—HEK-293 cells were transiently transfected with DNA encoding wild type Gqα, C9A, C10A, and Gqα-short (Fig. 6). None of these proteins contained a hexahistadine or other tag. Cells were harvested 30 h after transfection, and nuclear, membrane, and cytosolic fractions were prepared. All of the wild type Gqα was in the low speed pellet or the membrane fraction; none was in the cytosol. By contrast, some portion of both nonpalmitoylated proteins was found in the cytosol. Nevertheless, of the material not found in the low speed pellet, more than half (roughly 60–70%) was associated with membranes.

Cellular distribution of hexahistidine-tagged wild type and mutant Gqα proteins was also examined in SF9 cells. In all cases, some portion of the expressed protein was found in all three cellular fractions (Fig. 6, B and C), but the majority was associated with membranes. Concurrent expression of Gqα with β1 and β2 subunits did not alter the cellular distribution of the proteins but did decrease (5-fold) the accumulation of Gqα. The majority (50–70%) of each wild type and mutant protein associated with membranes from the above samples could be extracted with sodium cholate when expressed with βγ1; a much smaller percentage of each Gqα subunit (<10%) was extracted when βγ was not present (data not shown).

TX-114 Detergent Partitioning—We also tested the capacity of wild type and mutant Gqα proteins to partition between water and detergent-rich (TX-114) phases, a crude measure of hydrophobicity (36). Bovine serum albumin is a water-soluble protein, and as expected, it partitioned exclusively into the water phase (Fig. 7A). The same was true of nonmyristoylated (E. coli-derived) Gqα. In contrast, the majority of myristoylated Gqα was found in the detergent phase (Fig. 7A).

Wild type and nonpalmitoylated Gqα proteins were synthesized in SF9 cells in the presence of [3H]palmitate, isolated by Ni-NTA chromatography, and subjected to TX-114 partitioning (Fig. 7B). As before, only the wild type protein incorporated [3H]palmitate. Wild type and nonpalmitoylated forms of Gqα partitioned roughly equally between the aqueous and detergent phases when analyzed by immunoblotting. However, nearly all of the [3H]palmitate-labeled wild type protein was found in the detergent phase. When compared with the properties of myristoylated and nonmyristoylated Gqα, these results suggest the existence of some factor(s) in addition to palmitate that confers hydrophobicity on Gqα. To test this hypothesis directly, we examined the behavior of purified Gqα treated with palmitoylthioesterase and amino-terminally truncated forms of both Gqα and Cys9,10Ser Gqα (Fig. 7C). The majority of wild type Gqα was found in the detergent phase. A distinct and reproducible shift was observed for both Cys9,10Ser Gqα and palmitoylthioesterase-treated wild type protein. Although the majority of either of these preparations was found in the aqueous phase, presumably due to loss of palmitate, a

Prior studies (G. H. Biddlecome, G. Berstein, and E. M. Ross, unpublished results) demonstrated that addition of a hexahistidine tag at the carboxyl terminus of Gqα decreased the capacity of phospholipase C-β1 to stimulate steady-state GTP hydrolysis (i.e., GAP effect) by impairing the interaction of Gqα with the m1 receptor that is necessary for rapid GDP/GTP exchange. Present studies revealed that nonmodified and hexahistidine-tagged Gqα shared similar rates of agonist-stimulated GTPase activity (Fig. 5; Table I). When subjected to TX-114 phase separation analysis, purified Gqα-short partitioned predominantly into the detergent phase. Treatment of Gqα-short with palmitoylthioesterase failed to alter this pattern. These results provide further evidence that Gqα-short is not palmitoylated.

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**Fig. 3.** Palmitoylation and purification of amino-terminal mutants of Gqα. A, amino-terminal sequences of wild type Gqα and mutants. All constructs were hexahistadine tagged at the carboxyl terminus. B, SF9 cells were infected with viruses encoding β1 and β2 subunits and either wild type Gqα (WT-Gqα), or amino-terminal mutants of Gqα (C9A, C10A, and C9A/C10A (C9,10A), or Gqα-short). Infected cells were labeled with [3H]palmitate, and Gqα subunits were recovered from membrane extracts by Ni-NTA affinity chromatography. Samples were resolved by SDS-PAGE and visualized by fluorography and by immunoblotting with a specific anti-Gqα serum (WO82). C, purification of Gqα. CH6 by affinity chromatography and anion exchange chromatography. SF9 cells (6-liter culture) were infected with viruses encoding β1, β2, and Gqα/CH6 subunits. Cholate extracts of cell membranes (L) were bound to Ni-NTA resin (top left) and eluted with a 150 mM imidazole buffer (B). The sample was then bound to and eluted from Q-Sepharose anion exchange resin (right, top and bottom); individual fractions were analyzed for their capacity to stimulate phospholipase C-β1 (bottom), and protein was visualized by Coomassie Blue staining (top). Phospholipase C-β1 activity is expressed as pmol product/min/ng phospholipase C-β1.
significant fraction remained associated with detergent. Of interest, however, the truncated forms of both Gqα and C9S/C10S Gqα were found exclusively in the aqueous phase, indicating a loss of surface hydrophobicity associated with removal of residues 1–34.

DISCUSSION

The amino-terminal domain of Gqα and, more specifically, residues Cys9 and Cys10 within this domain are important determinants of both the cellular localization of the protein and its interaction with phospholipase C-β1 and, to a lesser extent, the m1 muscarinic receptor. The cysteine residues are important per se. Their palmitoylation is not necessary to observe...
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Effect of Gqα mutations on m1 receptor- and phospholipase C-β1-stimulated activities of Gqα.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>GTP hydrolysis</th>
<th>Relative Ch stimulation -PLC-β1 (Cch/Atr)</th>
<th>Relative Ch stimulation + PLC-β1 (Cch/Atr)</th>
<th>Relative GAP effect (Cch-Atr+PLC)</th>
<th>GTPγS binding, k</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gqα</td>
<td></td>
<td>26 ± 0.1</td>
<td>48 ± 2</td>
<td>67 ± 6</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>GqαCH6</td>
<td></td>
<td>64 ± 0.2</td>
<td>49 ± 12</td>
<td>11 ± 0.7</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>Gqα_short</td>
<td></td>
<td>38 ± 0.3</td>
<td>46 ± 7</td>
<td>14 ± 0.9</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>C95/C105 Gqα</td>
<td></td>
<td>21 ± 0.2</td>
<td>40 ± 0.3</td>
<td>26 ± 0.2</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>

a Cch, carbachol; Atr, atropine; PLC, phospholipase C. Values are the mean (± S.D.) of three (GTP hydrolysis) or two (GTPγS binding) experiments, each of which consisted of duplicate determinations.

A HEK-293 (Gα alone)

B Sf9 (Gα alone)

C Sf9 (Gα + βγ)

FIG. 6. Cellular distribution of recombinant wild type Gqα and aminoterminal mutants of Gqα in HEK-293 and Sf9 cells. A, HEK-293 cells were transiently transfected with DNA (20 μg) encoding Gqα, Gqα_short, or Gqα C95/C105 for 30 h. None of these constructs was hexahistidine-tagged. Cells were harvested and fractionated as described under “Experimental Procedures” into a low speed nuclear pellet (NP), high speed membrane pellet (M), or cytosol (C). Equal volumes of normalized fractions were resolved by SDS-PAGE, and Gqα was visualized by immunoblotting with anti-Gqα serum W082. B, Sf9 cells were infected with a virus encoding GqαCH6 or each of the indicated mutants and fractionated as described under “Experimental Procedures” into nuclear pellet (NP), membrane pellet (M), or cytosol (C). Equal volumes of normalized fractions were resolved by SDS-PAGE, and Gqα was visualized by immunoblotting with anti-Gqα serum W082. C, same as in B, except Sf9 cells were infected with viruses encoding each indicated Gqα subunit together with G protein β2 and γ2 subunits.

characteristic interactions between Gqα and phospholipase C-β1, although we cannot rule out possible inhibitory effects of such modification. Both palmitoylation and some other feature of the amino terminus confer hydrophobicity on Gqα and influence its cellular distribution.

Two prior reports describe the failure of Cys9 and C10 mutants of Gqα to activate phospholipase C-β. In one case it was hypothesized that this was due to loss of palmitate (25), while in the other the defect was ascribed to loss of association of Gqα with the membrane (23). We ascribe this phenomenon to loss of the cysteine residues themselves. Two lines of evidence indicate that palmitate is not a major direct enhancer of the interactions between Gqα and phospholipase C-β1 or m1 receptors. First, removal of palmitate from Gqα with palmitoylthioesterase did not alter its observed interactions with the effector or receptor. Since we believe that the stoichiometry of palmitoylation of the purified protein is significant (but not 1 or greater, see below), we would have observed loss of a substantial stimulatory effect of palmitate (but not necessarily loss of an inhibitory one) upon removal of the fatty acid. Second, removal of the first six residues of Gqα effectively prevents palmitoylation of the protein without interfering with its interactions with phospholipase C-β1 or m1 receptors. However, mutation of the relevant Cys residues to either Ala or Ser impairs m1 receptor coupling and causes apparent loss of affinity of Gqα for phospholipase C-β1 and substantial loss of capacity to activate the enzyme as well. Cys residues rather than palmitoylated Cys residues are thus important. The role of palmitoylation of Gqα is in some ways distinctly different from that of myristoylation of members of the Gα subfamily of G proteins; myristate is an important determinant of the affinity of Gα proteins for both effectors and the βγ subunit complex (8, 9).

There are also similarities in the effects of myristoylation and palmitoylation, in that both modifications confer hydrophobic properties on the proteins involved and facilitate their interactions with membranes (10, 14, 37, 38). A significant fraction of both nonpalmitoylated mutants of Gqα, was found in the cytosol of transfected HEK-293 cells; in contrast, all of the wild type protein was membrane associated. Nearly all of purified Gqα, labeled with [3H]palmitate distributed to the detergent-rich phase in TX-114 partitioning experiments. We have suggested that amino-terminal acylation of G protein α subunits may not simply facilitate interactions with lipid bilayers but may regulate distribution of the proteins to specialized domains of the plasma membrane such as caveoli (39).

It has been difficult to determine the stoichiometry of palmitoylation of Gqα, We have been unable to observe any alteration of electrophoretic mobility attributable to palmitate; myristoylated Gqα subunits can be distinguished from their nonmyristoylated counterparts in this fashion. Mass spectrometric analysis has also been unsuccessful, apparently because of inhibitory effects of residual detergent. The best clue comes from TX-114 partitioning studies (Fig. 7). Palmitoylated Gqα is found almost exclusively in the detergent phase. Purified Gqα is distributed in both the aqueous and detergent-rich phases, and treatment of the protein with palmitoylthioesterase causes an observable (but not complete) shift of protein to the aqueous phase. Estimates based on the extent of this shift suggest a
stochiometry in the approximate range of 20–40%.

The precise role of Cys9 and/or Cys10 is unclear. Since the loss of either of these residues is well tolerated, they are not involved in formation of a critical disulfide bond with each other. They may be involved in direct intermolecular contacts with, for example, phospholipase C-β or receptors in intramolecular interactions that are important determinants of Gqα conformation. Higashijima and Ross (19) found that Cys3 in Gαq (the palmitoylated cysteine analogous to Cys9 or Cys10 in Gqα) interacts with Cys-substituted mastoparan, small amphipathic peptides that mimic the effects of receptors on G proteins. Similarly, Edgerton et al. (25) reported that Cys9 and Cys10 mutants of Gqα were unable to interact with the NK2 receptor. However, the same mutant Gαq protein had a relatively modest loss of capacity to interact with m1 receptors when tested in reconstituted systems (above). In all of these scenarios one might suspect that stoichiometric palmitoylation of both cysteine residues might inhibit the function in question. This may be true; alternatively, stoichiometric palmitoylation at both sites may not be possible.

The exact site of initiation of translation of Gqα (Met1 or Met2) is unknown. Direct amino-terminal sequencing of purified native protein was unsuccessful because of an uniden-

**REFERENCES**


8 P. Sternweis and C. Slaughter, personal communication.

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