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Cysteine 3 Is Not the Site of *in Vitro* Palmitoylation on $G_{s\alpha}$

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Several studies have examined the role of palmitoylation of G protein α subunits by nonenzymatic *in vitro* acylation using palmitoyl-CoA. Here, we investigated nonenzymatic palmitoylation of purified $G_{s\alpha}$ *in vitro*. GDP-bound $G_{s\alpha}$ was stoichiometrically autoacylated on cysteine residue(s) with micromolar concentrations of palmitoyl-CoA. The acylation led to a complete loss of steady-state GTPase activity and GTPγS binding to $G_{s\alpha}$. Mutation of Cys 3 to Ala in $G_{s\alpha}$ did not prevent either palmitoylation or its consequent functional alterations. However, stoichiometric palmitoylation of His_6-$G_{s\alpha}$ did not alter its GTPase activity or GTPγS binding. Isoelectric focusing of tryptic peptides from autoacylated wild type, His_6-tagged, and C3A mutant of $G_{s\alpha}$ showed that Cys 160 is the site of *in vitro* palmitoylation. Therefore, we conclude that *in vitro* palmitoylation of $G_{s\alpha}$ occurs on Cys 160 and this modification decreases the ability of the protein to exchange GTP for GDP; N-terminus elongation of $G_{s\alpha}$ prevents this latter effect without altering palmitoylation. © 2000 Academic Press

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Palmitoylation of G protein α subunits is a reversible and dynamic process, which is regulated *in vivo* by the activation of G-protein coupled receptors (reviewed in 1). The α subunits of members of the G_i, G_q and G_s subfamilies of heterotrimeric G-proteins are palmitoylated on a cysteine close to the amino terminus (reviewed in 1). Inactive heterotrimeric G-proteins are likely to be membrane bound with the G_α subunit palmitoylated (1, 2). Receptor activation enhances the rate of GTP-GDP exchange on the α-subunit of G-proteins and leads to dissociation of the GTP-bound, active, G_α from its $\beta\gamma$ subunits. In the activated state G_α-GTP becomes depalmitoylated (3). The intrinsic GTPase activity of the G_α hydrolyzes the bound GTP to GDP and the GDP-bound, inactive, G_α gets palmitoylated and re-associates with the $\beta\gamma$ subunits to form the heterotrimeric G-protein, thereby completing the activation-inactivation cycle (reviewed in 1, 2). To date, little is known about the role and regulation of palmitoylation in this cycle. It seems that palmitoylation increases the affinity of G_α for its $\beta\gamma$ subunits (4) and may interfere with binding of RGS-proteins (5). Recently an acyl-protein thioesterase was purified which is able to remove palmitate from different G_α subunits (6). However, to date no enzyme has been identified or isolated which palmitoylates G-proteins. Duncan and Gilman (7) have shown that palmitoyl-CoA can stoichiometrically palmitoylate $G_{i\alpha1}$ *in vitro* in the absence of an acyltransferase and that the kinetics for this reaction resembles those described for palmitoylation *in vivo*. These findings led to speculations that nonenzymatic acylation may also occur *in vivo* (1, 2, 7, 8). Regardless of how the palmitoylation *in vivo* occurs, nonenzymatic palmitoylation of $G_{i\alpha1}$ provides a convenient and powerful tool for the *in vitro* investigation of the role of this lipid modification (5, 6, 9).

It is well documented that the α subunit of the stimulatory G-protein ($G_{s\alpha}$) also undergoes a cycle of palmitoylation and depalmitoylation upon stimulation by its activated receptor (3, 10, 11). Recent approaches to test nonenzymatic palmitoylation have been limited by the low stoichiometry with which $G_{s\alpha}$ gets palmitoylated and the lack of evidence showing that the amino acid residue on $G_{s\alpha}$ which is palmitoylated *in vitro* is the same as the cysteine residue (Cys 3) palmitoylated *in vivo* (6, 7, 12). Therefore, the aims of our study were to achieve stoichiometric palmitoylation of $G_{s\alpha}$ *in vitro*, to determine the functional significance of this acylation, and to determine whether the cysteine residue, which is autoacylated *in vitro* is the same as that palmitoylated *in vivo*.

MATERIALS AND METHODS

Construction of the C3A mutant of $G_{s\alpha}$. Employing the full length $G_{s\alpha}$ cDNA (gift from Dr. A. G. Gilman) as template and primers

Abbreviations used: G_s, stimulatory GTP-binding protein of adenylyl cyclase; $G_{s\alpha}$, α subunit of G_s; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; IEF, isoelectric focusing; His_6, hexahistydyl; G$\beta\gamma$, β and γ subunits of heterotrimeric G proteins; IPG, immobilized pH gradient gel.

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corresponding to nucleotides 1–18 (sequence: 5′ TATATACCATGGGCGCTCTCGGAAAC 3′; (nucleotide substitutions underlined) and 1129–1145 (sequence: 5′ TATATAAGCTTTTAGAGCAGCTCATAC 3′) the C3A mutation was generated by PCR. The NcoI and HindIII sites in the 5′ and 3′ primers, respectively, facilitated the directional cloning of the cDNA into the plasmid pQE60. A similar approach was pursued for cloning the His_6-$G_{s\alpha}$ in the bacterial expression vector pRSET B.

Purification of G protein βγ-subunits and recombinant $G_{s\alpha}$. The expression and purification of the recombinant $G_{s\alpha}$ was performed as described by Graziano *et al.* (13). $G\beta\gamma$ subunits were purified as described by Neer *et al.* (14) with modifications of Mumby *et al.* (15). Heterotrimeric G_s was reconstituted by mixing $G_{s\alpha}$ with purified bovine brain $G\beta\gamma$ subunits ($G_{s\alpha}$:$\beta\gamma$ molar ratio of 1:5) and incubated for 1 h at 4°C (16). The His-tagged $G_{s\alpha}$ was purified as described (17). All G-protein α subunits were tested for functional activity in adenylyl cyclase activity assays with soluble recombinant adenylyl cyclase as described (17).

Steady state GTPase activity and GTPγ^{35}S binding assay. Monomeric $G_{s\alpha}$ or the reconstituted G proteins were incubated at 25°C in 25 mM Hepes·NaOH pH 8.0, 0.1 mM EDTA, 1 mM DTT and 150 μM $MgCl_2$. Hydrolysis of [γ-^{32}P] GTP which was linear for 30 min was measured in triplicate samples after 20 min by charcoal precipitation as described previously (18). The GTPγ^{35}S binding was monitored with 0.1 μM GTPγ^{35}S in 25 mM Hepes · NaOH pH 8.0, 0.1 mM EDTA, 1 mM DTT and 150 μM $MgCl_2$ as described previously (18).

In vitro palmitoylation of $G_{s\alpha}$. Purified $G_{s\alpha}$ (250 nM) was incubated for different times with varying concentrations of palmitoyl-CoA (Sigma) in a buffer containing 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 12.2 μM CHAPS and 1 μM GDP at 30°C. For GTPγ^{35}S binding and steady-state GTPase activity assays, portions of the palmitoylation reaction were diluted 10-fold in the final assay mix. As control, $G_{s\alpha}$ was incubated under identical conditions without adding any palmitoyl-CoA. The amount of active $G_{s\alpha}$ at the different time points was determined by stoichiometric binding of GTPγ^{35}S (16). To measure the amount of palmitoyl-CoA bound to $G_{s\alpha}$, [1-^{14}C] labeled palmitoyl-CoA (Sigma) was employed. Palmitoylated G-protein was mixed with Laemmli sample buffer lacking β-mercaptoethanol, heated for 15 min at 65°C and loaded on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie, then treated with En^{3}Hance (DuPont) for 30 min and rinsed with water for another 30 min. The gel was then exposed for 2–3 days at −80°C. Bands representing $G_{s\alpha}$ were excised and incubated in 1 ml NCS-II tissue solubilizer (Amersham) for 16 h at 37°C before counting.

Trypsin digestion and Isoelectric focusing (IEF) of $G_{s\alpha}$. $G_{s\alpha}$ was palmitoylated in presence of 5 μM palmitoyl-CoA for 1 h at 30°C as described above. Free palmitoyl-CoA was removed by exchanging the buffer against 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 12.2 μM CHAPS using Microcon 30 spin columns (Amicon). The eluted protein was then incubated with trypsin (0.2 mg/ml) at 37°C. After 15 h, fresh trypsin was added to a final concentration of 0.4 mg/ml and incubated for another 8 h at 37°C. The digested protein was analyzed using the Protean IEF Cell (BioRad) with 17 cm immobilized pH gradient gel (IPG) strips (pH range of 3–10) according to the instructions of the manufacturer (BioRad). The gel-strips were then exposed for 2–8 days at −80°C.

Trypsin protection assay. $G_{s\alpha}$ was palmitoylated in presence of 5 μM palmitoyl-CoA for 1 h at 30°C as described above. Thereafter, palmitoylated and untreated $G_{s\alpha}$ were incubated with or without 1 μM GTPγS in a buffer containing in 25 mM Hepes · NaOH pH 8.0, 0.1 mM EDTA, 1 mM DTT and 25 mM $MgCl_2$ for 45 min at room temperature. TPCK-Trypsin (Pierce) was added to a final concentration of 0.1 mg/ml and the incubation was continued for another 5 min at room temperature. The reactions were terminated by adding Laemmli sample buffer lacking β-mercaptoethanol, heated for 15 min at 65°C and loaded on a 10% SDS-polyacrylamide gel.

RESULTS AND DISCUSSION

Recent attempts to investigate the nonenzymatic palmitoylation of $G_{s\alpha}$ (6, 7) were suboptimal because stoichiometric palmitoylation of $G_{s\alpha}$ could not be achieved. To determine if $G_{s\alpha}$ can be autoacylated stoichiometrically, recombinant $G_{s\alpha}$ was purified to homogeneity following the protocol of Graziano *et al.* (13). GDP-$G_{s\alpha}$ was incubated with increasing concentrations of [^{14}C]palmitoyl-CoA and the amount of [^{14}C]palmitoyl-CoA bound to $G_{s\alpha}$ was determined (Fig. 1A). Increasing the concentration of palmitoyl-CoA during the 1 h of incubation at 30°C, increased the total incorporation of palmitate such that at palmitoyl-CoA concentrations of 5 and 10 μM, 0.9 mol palmitoyl-CoA was incorporated per mol of $G_{s\alpha}$; 50% of maximal palmitoylation of $G_{s\alpha}$ was achieved by 1 μM palmitoyl-CoA. The palmitoylation was time dependent and reached apparent stoichiometry at 60 min (Fig. 1A, inset). Increasing the incubation temperature to 37°C lowered the palmitoyl-CoA concentrations required for stoichiometric palmitoylation by nearly 10-fold (Fig. 1B). Hence, the concentration of palmitoyl-CoA required to observe 50% palmitoylation was 0.15 μM at 37° C as compared with 1 μM at 30° C (c.f. Figs. 1A and 1B). This concentration (0.15 μM) lies within the concentrations reported for cytosolic acyl-CoAs, which range from 0.5 μM to 3 μM (19, 20). Notably, under similar conditions the palmitoyl-CoA concentrations needed for the palmitoylation of $G_{s\alpha}$ are 4 times lower than the concentrations required for autoacylation of $G_{i\alpha1}$ (c.f. Fig. 1A and ref. 7).

Duncan and Gilman (7) reported that only a small part of the total protein was palmitoylated when heterotrimeric G_s or monomeric GTPγS-bound $G_{s\alpha}$ was employed in the autoacylation assay $G_{s\alpha}$ (20% and 5%, respectively). However, these authors found that the rate of palmitoylation for heterotrimeric G_i was four times higher than that for the monomeric GDP-$G_{i\alpha1}$ or GTPγS activated $G_{i\alpha1}$ (7). Therefore, we investigated the influence of the conformation of $G_{s\alpha}$ on nonenzymatic palmitoylation by monitoring autoacylation of heterotrimeric G_s, GDP-bound monomeric $G_{s\alpha}$, GTPγS activated $G_{s\alpha}$ and $Al_3F_4^-$ activated $G_{s\alpha}$ (Fig. 2A). Monomeric $G_{s\alpha}$ bound to either GDP or $Al_3F_4^-$ was palmitoylated to a similar extent. On the other hand, as compared with GDP-$G_{s\alpha}$ only 3% of GTPγS-$G_{s\alpha}$ and 30% of heterotrimeric $G_{s\alpha}$ was palmitoylated (Fig. 2A). These results indicate that inactive (GDP-bound) monomeric $G_{s\alpha}$ and its transition state ($Al_3F_4^-$-bound) are the preferred conformations for palmitoylation. These findings are consistent with the model for G-protein palmitoylation (1, 3). Additionally, our data (Fig. 2A) show that subtle conformational changes between the transition state ($Al_3F_4^-$-bound) and active (GTPγS-bound) $G_{s\alpha}$ dramatically alters the autoacylation by palmitoyl-CoA.

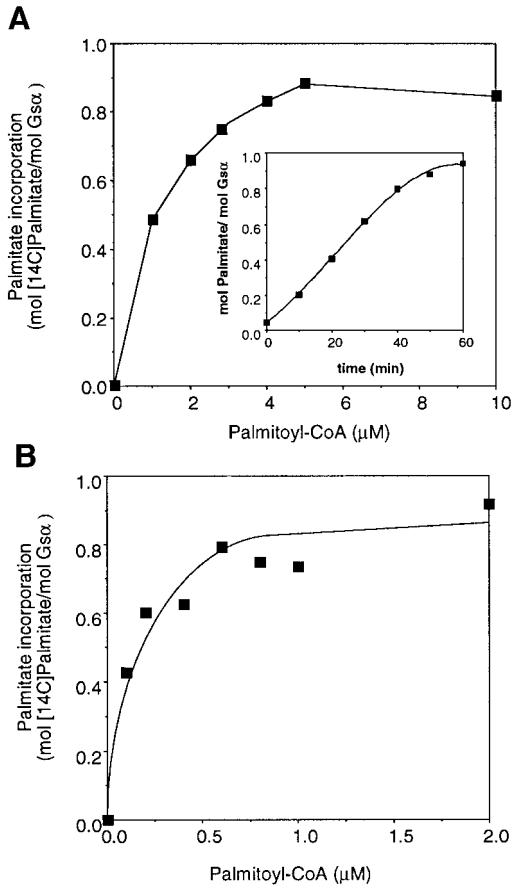

FIG. 1. Stoichiometric, nonenzymatic palmitoylation of $G_{s\alpha}$. (A) Palmitoylation of $G_{s\alpha}$ with varying concentrations of [^{14}C]palmitoyl-CoA at 30°C. GDP-bound $G_{s\alpha}$ (250 nM) was incubated as described under Materials and Methods with increasing concentrations of [1-^{14}C]palmitoyl-CoA. After 60 min at 30°C the reactions were stopped with Laemmli sample medium devoid of reducing agents and loaded on a 10% SDS-polyacrylamide gel. Bands corresponding to $G_{s\alpha}$ were excised and the incorporated radioactivity was counted. To monitor the time course of the reaction (inset), aliquots were withdrawn from the reaction mixture at different times, mixed with Laemmli sample medium, and stored on ice until loading on a 10% SDS Gel. A representative of four experiments is shown. (B) Same as A except that the reactions were performed for 60 min at 37°C.

Neutral hydroxylamine specifically cleaves thioester bonds which are generated by the reaction of palmitoyl-CoA with a cysteine residue (21). To determine whether the *in vitro* palmitoylation involved cysteine residues, we treated [^{14}C]-palmitate labeled $G_{s\alpha}$ with neutral hydroxylamine. Hydroxylamine treatment removed the radioactive label from $G_{s\alpha}$ and His_6-$G_{s\alpha}$ (Fig. 2B). Furthermore, palmitoylation was partly inhibited in the presence of 30 mM DTT (data not shown). These findings indicate that the palmitoylation of $G_{s\alpha}$ involves thioester bonds on cysteine residue(s).

To study the effects of palmitoylation on GDP-GTP exchange and GTPγS binding to $G_{s\alpha}$, we palmitoylated GDP-$G_{s\alpha}$ and measured steady-state GTPase activity and GTPγS binding. Steady-state GTPase activity of $G_{s\alpha}$ is limited by the GDP off-rate (22) and is a measure of GTP-GDP exchange. Palmitoylation of wild-type $G_{s\alpha}$ inhibited the steady-state GTPase activity of $G_{s\alpha}$ as well as its ability to bind GTPγS (Fig. 3). Notably, the

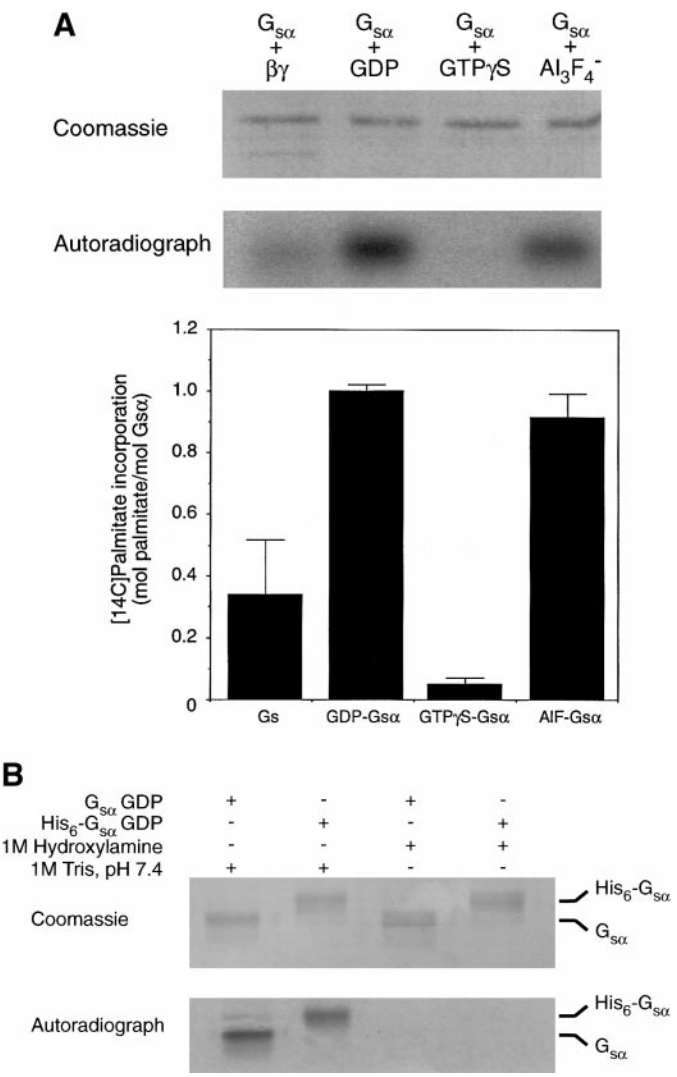

FIG. 2. Nonenzymatic palmitoylation of $G_{s\alpha}$ is determined by its conformation. (A) Reconstituted heterotrimeric G_s, GDP-bound $G_{s\alpha}$, GTPγS-bound $G_{s\alpha}$ and $Al_3F_4^-$-bound $G_{s\alpha}$ (250 nM each) were palmitoylated in presence of 5 μM [1-^{14}C]palmitoyl-CoA for 45 min at 30°C. The samples were then loaded on a 10% SDS-Gel and analyzed as described in the legend to Fig. 1. Top of panel shows a representative experiment in which $G_{s\alpha}$ on the gel was visualized by Coomassie staining and the same gel was then subjected to autoradiography to visualize the [^{14}C]palmitate incorporation. Bands from the gels were excised and counted for [^{14}C] content. The lower part of the panel shows these values presented as the mean ± SEM of three determinations. (B) Treatment of autoacylated $G_{s\alpha}$ or His_6-$G_{s\alpha}$ with 1 M hydroxylamine removes the [^{14}C]palmitate from the protein. Palmitoylation was performed as described above for 1 h at 30°C, then 1 M hydroxylamine or 1 M Tris, pH 7.4, was added and the reactions were incubated for additional 15 min at 30°C. Sample analysis was performed as described under (A). Note: The His_6-$G_{s\alpha}$ migrates as a higher molecular weight protein as compared to the wild-type $G_{s\alpha}$.

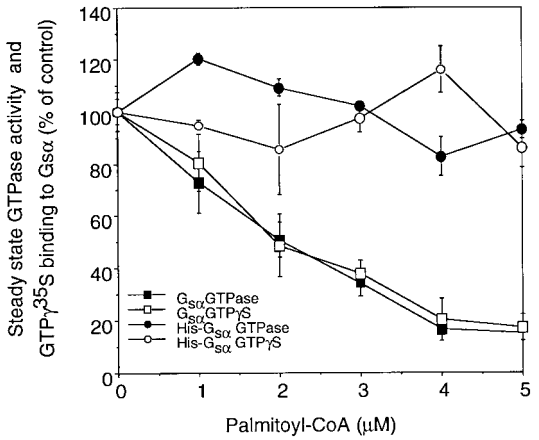

FIG. 3. Palmitoylation of $G_{s\alpha}$, but not His_6-$G_{s\alpha}$, inhibits the steady-state GTPase activity as well as its ability to bind GTPγS. Wild type $G_{s\alpha}$ or His_6-$G_{s\alpha}$ were incubated as described with increasing concentrations of palmitoyl-CoA. After 60 min at 30°C, the reactions were divided in two aliquots and each incubated at 25°C in 25 mM Hepes · NaOH pH 8.0, 0.1 mM EDTA, 1 mM DTT and 150 μM $MgCl_2$ with 100 nM [γ-^{32}P]GTP. ^{32}P-*ortho*-phosphate released from hydrolysis of [γ-^{32}P]GTP was measured after 20 min as described previously (18). GTPγ^{35}S binding was monitored in presence of 1 μM GTPγ^{35}S in 25 mM Hepes · NaOH pH 8.0, 0.1 mM EDTA, 1 mM DTT and 25 mM $MgCl_2$. Values presented are the mean ± variance. Data are representatives of at least 2 experiments done in duplicates.

percentage of inhibition correlated with the percentage of $G_{s\alpha}$, which was palmitoylated (c.f. Fig. 3 and 1A). Incubation of $G_{s\alpha}$ with CoA alone or adding palmitoyl-CoA directly to the GTPase activity assay (i.e. no pre-incubation of palmitoyl-CoA and $G_{s\alpha}$) did not interfere with enzyme activity (data not shown). Interestingly, the steady-state GTPase activity and GTPγS binding to His_6-$G_{s\alpha}$ which had been palmitoylated were not inhibited (Fig. 3) suggesting that different cysteine residues on $G_{s\alpha}$ and His_6-$G_{s\alpha}$ are palmitoylated. This suggestion was tested as described later.

Studies with mutated $G_{s\alpha}$ have shown that the N-terminal cysteine (Cys 3) is the site of palmitoylation *in vivo* (10, 23). Employing a mutant form of purified $G_{s\alpha}$ in which this cysteine residue is substituted by alanine (C3A), we tested if the same cysteine residue is palmitoylated *in vitro.* The sequences of mutant and wild type $G_{s\alpha}$ were each confirmed by DNA-sequencing of the respective cDNAs as well as matrix-assisted laser desorption ionization–mass spectrometry and sequencing of the purified proteins. Interestingly, we were not able to detect any differences in nonenzymatic palmitoylation between wild-type and C3A mutant $G_{s\alpha}$ (c.f. Figs. 2A and 4A). The inhibition of steady-state GTPase activity and GTPγS-binding occurred at EC_{50} values similar to those required to inhibit wild-type $G_{s\alpha}$ (c.f. Fig. 3 and 4B). Treatment with hydroxylamine was able to remove the radioactive labeled palmitate from the C3A mutant of $G_{s\alpha}$, indicating that the modification involves cysteine residue(s) (Fig. 4A). These findings demonstrate that the alterations in steady-state GTPase activity and GTPγS binding which are observed upon palmitoylation of $G_{s\alpha}$ *in vitro* are not related to the palmitoylation of Cys 3 on the protein but are due to palmitoylation of cysteine residue(s) other than the *in vivo* palmitoylation site.

As described above, in contrast to wild-type $G_{s\alpha}$ the steady-state GTPase activity and GTPγS binding to His_6-$G_{s\alpha}$ was not inhibited by palmitoylation (Fig. 3). Therefore, we investigated if the His_6-$G_{s\alpha}$ is palmitoylated at Cys 3. Digestion of GTPγS-bound $G_{s\alpha}$ by trypsin removes the N-terminal 20 amino acids in the protein (20); among these, the only cysteine residue is Cys 3. Notably, the GDP-bound $G_{s\alpha}$ is completely digested by trypsin treatment (20). Therefore, we reasoned that comparison between trypsin digested GTPγS-bound and GDP-bound palmitoylated His_6-$G_{s\alpha}$ would reveal

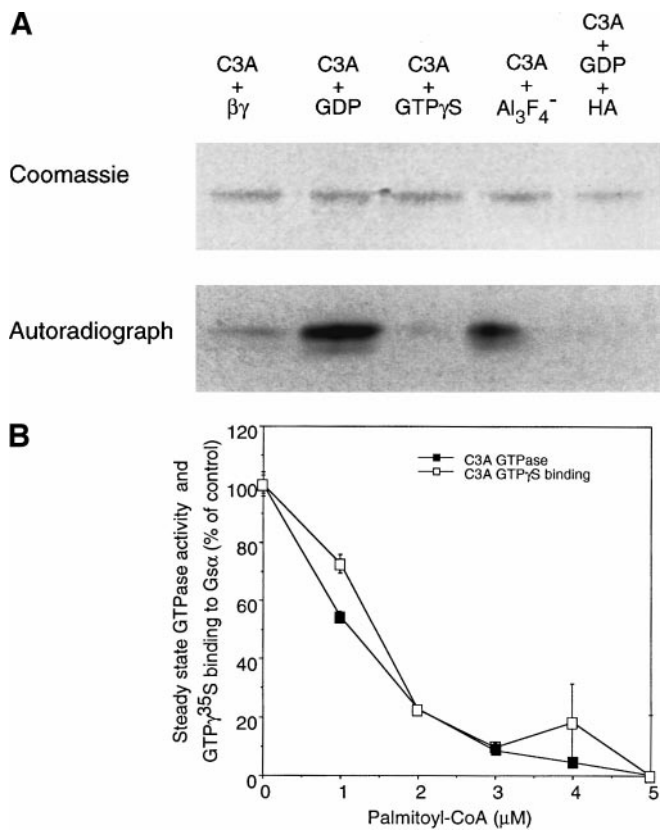

FIG. 4. Nonenzymatic palmitoylation of the C3A mutant of $G_{s\alpha}$ is not different from the wild-type $G_{s\alpha}$. (A) The C3A mutant of $G_{s\alpha}$ (250 nM) in its reconstituted heterotrimeric (+βγ), or monomeric GDP-bound, GTPγS-bound and $Al_3F_4^-$-bound forms were palmitoylated in presence of 5 μM [1-^{14}C]palmitoyl-CoA for 1 h at 30°C and analyzed on a 10% SDS-Gel. In the last lane the GDP bound C3A mutant was treated with 1 M hydroxylamine (HA) as described in Fig. 2A. (B) Autoacylation inhibits the steady-state GTPase activity of, and GTPγS binding to, the C3A mutant of $G_{s\alpha}$. The C3A mutant of $G_{s\alpha}$ was incubated as described above with increasing concentrations of palmitoyl-CoA for 60 min at 30°C prior to measurements of steady-state GTPase activity and GTPγS binding. Values presented are the mean ± variance from 2 experiments done in duplicates.

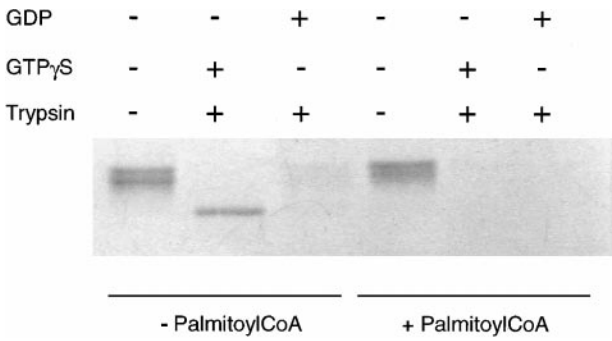

FIG. 5. The GTPγS-bound form of palmitoylated His_6-$G_{s\alpha}$ is not protected against trypsin cleavage. His_6-$G_{s\alpha}$ (250 nM) was palmitoylated in presence of 5 μM palmitoyl-CoA (Pal-CoA) for 1 h at 30°C followed by a 45 min incubation with 1 μM GTPγS or GDP under maximum guanine nucleotide binding conditions described under Materials and Methods. Controls were performed in parallel under the same conditions without palmitoyl-CoA. The His_6-$G_{s\alpha}$ was then treated with 0.1 mg/ml trypsin for 5 min and then analyzed on a 10% SDS-Gel. A Coomassie-stained gel is shown.

whether Cys 3 was the site of palmitoylation. Interestingly, while non-palmitoylated His_6-$G_{s\alpha}$ was partially protected from trypsin digestion (Fig. 5), the GTPγS-bound form of palmitoylated His_6-$G_{s\alpha}$ was not protected against trypsin cleavage (Fig. 5). Moreover, the GDP bound forms of both palmitoylated and non-palmitoylated His_6-$G_{s\alpha}$ were completely digested by trypsin (Fig. 5). These data suggest that palmitoylation of the His_6-$G_{s\alpha}$ does not allow its GTPγS bound form to assume a conformation which is partially protected from trypsin digestion. However, this palmitoylation-elicited change is subtle enough so that neither the GTP-GDP exchange nor the GTPγS binding to the His_6-$G_{s\alpha}$ are altered. Importantly, however, this lack of protection precluded us from drawing any inferences about the identity of the Cys residue(s) which is (are) palmitoylated on His_6-$G_{s\alpha}$.

To identify the cysteine residue(s) involved in the nonenzymatic palmitoylation of the wild-type, C3A mutant, and His_6-$G_{s\alpha}$, we analyzed the tryptic peptides derived from ^{14}C palmitoylated forms of the three proteins by isoelectric focusing. For all three forms of $G_{s\alpha}$, a tryptic peptide with an isoelectric point of approximately 4 (Fig. 6) was observed to be labeled with ^{14}C palmitoyl-CoA. This suggested that all three forms of $G_{s\alpha}$ are palmitoylated on the same cysteine residue. Notably, tryptic cleavage of $G_{s\alpha}$ produces only one peptide (aa 152–167) with an isoelectric point of 3.74 which contains a Cys residue (Cys 160). Therefore, it would appear that all three forms of $G_{s\alpha}$ are palmitoylated on Cys 160. Although Cys 160 is not involved in catalytic activity or GTP binding to $G_{s\alpha}$ (25, 26), Cys 160 is part of the catalytic pocket (25) and, therefore, palmitoylation of this residue may interfere with GDP/GTP exchange as well as GTP hydrolysis.

Given our observations that the wild-type and C3A mutant of $G_{s\alpha}$ are similarly palmitoylated *in vitro* and that although the His_6-$G_{s\alpha}$ is palmitoylated, the steady-state GTPase activity of this latter form as well as its ability to bind GTPγS are not altered by palmitoylation, permit us to draw the following inferences. First, palmitoylation of $G_{s\alpha}$ *in vitro* occurs on cysteine residues other than the Cys 3 which is palmitoylated *in vivo.* The data derived from the isoelectric focussing studies would suggest Cys 160 is the acylated residue. Second, our data indicate that the transition state ($Al_3F_4^-$-bound $G_{s\alpha}$) shares structural features with the inactive (GDP-bound) form of $G_{s\alpha}$ and this permits its autoacylation. However, because GTPγS-bound $G_{s\alpha}$ is not palmitoylated, its structure must be subtly different to that of the $Al_3F_4^-$-bound $G_{s\alpha}$. Most importantly, despite being palmitoylated on the same Cys residue (Cys 160) only the His_6-$G_{s\alpha}$, but not wild-type $G_{s\alpha}$ or its C3A mutant, are capable of exchanging GTP for GDP and binding GTPγS. Therefore, the N-terminal hexahistidyl-tag on $G_{s\alpha}$ somehow protects against inactivation of the G protein function due to palmitoylation of Cys 160. However, the structural change afforded by the N-terminal hexahistidyl-tag is not

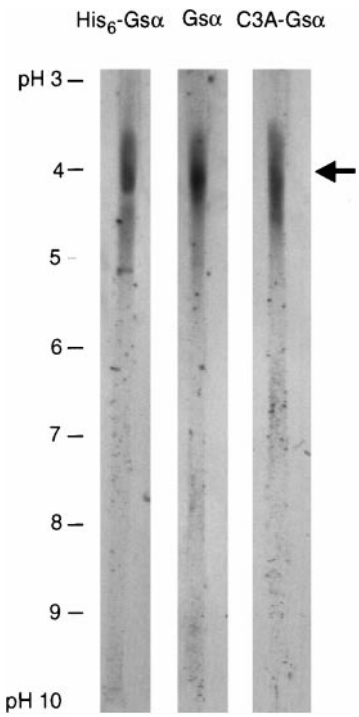

FIG. 6. Wild-type $G_{s\alpha}$, the C3A mutant of $G_{s\alpha}$, and His_6-$G_{s\alpha}$ are palmitoylated on the same cysteine residue. One μg each of the wild-type, C3A mutant, and His_6-$G_{s\alpha}$ was palmitoylated in the presence of 5 μM palmitoyl-CoA for 1 h at 30°C as described above and then digested with trypsin. The digests were analyzed using the protein isoelectric focusing cell (BioRad) IPG strips (BioRad; pH range of 3–10). Migration of the labeled tryptic peptide is shown by the arrow.

sufficient to protect the palmitoylated, GTPγS bound, $G_{s\alpha}$ against trypsin digestion.

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