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$G\alpha_s$ is palmitoylated at the N-terminal glycine

Christiane Kleuss[1] and Eberhard Krause[2]

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67–73, 14195 Berlin and [2]Forschungsinstitut für Molekulare Pharmakologie, Campus Berlin Buch, Robert-Rössle-Strasse 10, 13125 Berlin-Buch, Germany

[1]Corresponding author
e-mail: ckleuss@zedat.fu-berlin.de

Covalent lipid attachments are essential co- and post-translational modifications for signalling proteins. $G\alpha_s$, the α-subunit of the heterotrimeric G protein that activates adenylyl cyclase, is known to be palmitoylated at the third N-terminal amino acid, a cysteine. Palmitoylation is involved in anchoring $G\alpha_s$ to the membrane by increasing its intrinsic hydrophobicity. We identified by mass spectrometry a second, functionally even more important, covalent modification. It consists of another palmitoyl residue attached to the preceding glycine (Gly^2). Palmitoylation at this position has profound consequences for levels of signal transduction. It sensitizes the cell up to 200-fold for adenylyl cyclase-stimulating agents. The inhibitory inputs mediated by $G\alpha_i$ are downregulated to <10%. Thereby, Gly^2-palmitoylation of $G\alpha_s$ relieves cellular stimulation at the level of adenylyl cyclase whereas it renders the inhibitory modulation via $G\alpha_i$ more difficult.

Keywords: adenylyl cyclase/Gly2-palmitoylation/lipid modification/stimulatory G protein

Introduction

Heterotrimeric G proteins play a pivotal role in cellular signalling. They act as molecular switches that couple diverse receptors in the plasma membrane to a variety of intracellular effectors or signal generators. Efficiency of signalling depends on individual expression levels of the signalling partners and, even more importantly, their correct localization in the signalling compartment. Not until recently did it become evident that lipid modifications are an important determinant for localizing a protein into membranes and microdomains therein (Casey, 1995; Simons and Toomre, 2000).

G proteins are richly modified by lipids, which confer functional attributes such as protein–protein interactions (Linder *et al*., 1990; Casey, 1994; Tu *et al*., 1997; Chen and Manning, 2001) and a propensity for these otherwise soluble proteins to associate with membranes. The α-subunits of members of the G_i subfamily contain amide-linked myristate, and all Gα subunits (except $G\alpha_t$) contain palmitate in thioester linkage on one or more nearby cysteine residues.

Myristoylation occurs during protein translation, and the saturated C_{14}-lipid is permanently attached to the G protein on the N-terminal glycine residue (Gly^2) that constitutes the N-terminus after removal of the initiating methionine Met^1. Myristoylated G proteins adopt substantial functional features for cell signalling (Mumby *et al*., 1990), with targeting Gα to membranes being the most important.

In contrast, palmitoylation usually occurs at cysteine residues, is a dynamic protein modification (Cys-palmitoylation; Degtyarev *et al*., 1993; Mumby *et al*., 1994; Chen and Manning, 2000), and imparts pronounced hydrophobicity to the Gα subunit (Peitzsch and McLaughlin, 1993). Differences in opinion exist concerning the general functional consequences of G protein palmitoylation: be it membrane anchorage (Wedegaertner *et al*., 1993; Grassie *et al*., 1994) and/or specific interaction with regulators (Tu *et al*., 1997) and/or the selective targeting into membrane subdomains (Moffett *et al*., 2000; Waheed and Jones, 2002). For proteins that are acylated by both fatty acids, myristoylation usually precedes palmitoylation. So far, the functional consequences of myristoylation for the proteins role in signal transduction are thought to be dominant over potential effects of Cys-palmitoylation.

$G\alpha_s$, discovered for its ability to stimulate production of the second messenger cAMP, is Cys^3-palmitoylated like members of the $G\alpha_i$ subfamily, but it is not myristoylated, despite the presence of an N-terminal glycine residue. However, it was consistently observed that $G\alpha_s$ purified from sources such as mammalian liver or brain (we refer to it as 'native $G\alpha_s$') has a higher apparent affinity for its effector adenylyl cyclase than does the mammalian protein that had been expressed in and purified from bacteria (we refer to it as 'recombinant $G\alpha_s$'). Previous experiments excluded the possibility that the Cys^3-attached palmitoyl moiety of $G\alpha_s$ was responsible for this characteristic (Kleuss and Gilman, 1997). However, we reported the presence of an additional, at the time unidentified, lipophilic modification at or near the N-terminus of native $G\alpha_s$. Here, we determined this modification to be a second palmitoyl residue that is linked to the G protein at the extreme N-terminal glycine by an unusual amide linkage. The functional consequences of this modification for the interaction of $G\alpha_s$ with adenylyl cyclase and plasma membrane localization are profound and are discussed in the context of signal transduction.

Results

Mass spectrometric analysis

To identify the lipophilic modification at the N-terminus of $G\alpha_s$, we analysed both the short and long splice variants of native $G\alpha_s$ and the recombinant protein by peptide mass

Table I. MS/MS fragment ions generated from the N-terminal tryptic peptide of recombinant $G\alpha_s$ $[M + H]^+ = 735.3$

Sequence	Fragment ion	m/z_{calc}	m/z_{found}
GC	B_2	218.1	218.3
GCLG	B_4	388.2	388.4
K	$Y"_1$	147.1	147.3
SK	$Y"_2$	234.1	234.2
NSK	$Y"_3$	348.2	348.3
NSK	$Y*_3$	331.2	331.2
GNSK	$Y*_4$	388.2	388.4

Based on the single-charge precursor ion the MS/MS spectrum showed N-terminal and C-terminal fragment ions. The common nomenclature for fragment ions was used, i.e. B for N-terminal, Y" for C-terminal ions. (*) indicates the neutral loss of 17 mass units. Cysteine was modified by carbamidomethylation.

fingerprinting. Analysis of mass spectra of recombinant $G\alpha_s$ with regard to the presence of the N-terminal sequence revealed a mass peak at *m/z* 735.3, corresponding to the unmodified fragment amino acid 2–8 (GCLGNSK) after trypsin digestion. The evidence for an unmodified N-terminus is based on the B- and Y-ions of the CID spectrum (retention time 12.5 min) of on-line liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS; Table I). Mass peaks corresponding to unmodified N-terminal sequences were not present when native, purified $G\alpha_s$ protein was analysed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or LC-MS/MS. However, we found distinct mass peaks at *m/z* 973.5 in MALDI spectra of Lys-C and tryptic digests of native $G\alpha_s$ that were not present in recombinant $G\alpha_s$ spectra (Figure 1A). We concluded that the native protein carried an additional group at the N-terminus with a mass of 238, corresponding to the size of a palmitoyl moiety.

The site of modification was determined by LC-MS/MS as the N-terminal glycine (Figure 1B; Table II). Additional MS/MS experiments (data not shown) on the native $G\alpha_s$ N-terminal peptide prepared by tryptic in-gel digestion using 4-vinylpyridine instead of iodoacetamide for cysteine alkylation, and MALDI-MS of cyanogen bromide digested native $G\alpha_s$ confirmed these results.

To prove the chemical nature of the attached lipid, we prepared N-terminal peptides by solid-phase synthesis and chemically attached a palmitoyl group at the N-terminal glycine. As shown in mass chromatograms (*m/z* 973.5), the N-terminus generated by in-gel digestion of native $G\alpha_s$ and the reference peptide, palm-GCLGNSK, exhibited identical masses and retention times in LC analysis (Figure 2). These data corroborated that the hitherto unknown modification is a palmitoyl residue that is attached to the N-terminal glycine of $G\alpha_s$ via a covalent amide bond.

In vitro palmitoylation

In a previous study, we showed that the lipophilic modification present on native $G\alpha_s$ (i.e. the Gly^2-attached palmitoyl group) increased the apparent affinity of $G\alpha_s$ for adenylyl cyclase and the $G\beta\gamma$ complex (Kleuss and Gilman, 1997). Analogously, myristoylation at Gly^2 enabled high affinity interactions of $G\alpha_i$ with the effector adenylyl cyclase (Taussig *et al*., 1993) and the $G\beta\gamma$ complex (Linder *et al*., 1991). Therefore, we speculated

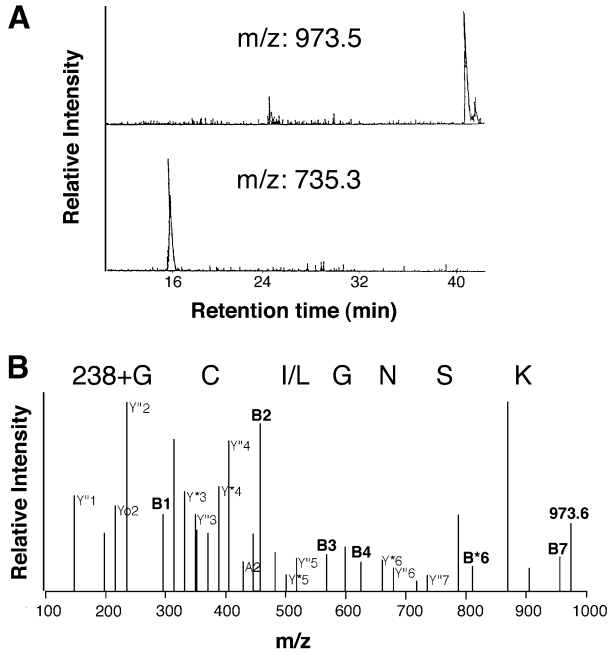

Fig. 1. Mass spectrometric analysis of $G\alpha_s$. Native or recombinant $G\alpha_s$ was in-gel digested and the resulting peptides analysed by MALDI-MS. Mass peaks with *m/z* 973.5 and 735.3 corresponded to the palmitoylated and non-palmitoylated N-terminal sequence $G^2CLGNSK$ of $G\alpha_s$, respectively. (**A**) LC chromatogram recorded for the native protein at *m/z* 973.5 and the recombinant protein at *m/z* 735.3. (**B**) MS/MS spectrum of *m/z* = 973.58 of native $G\alpha_s$. Relevant ions were labelled according to the accepted nomenclature and listed in Table II.

Table II. MS/MS fragment ions generated from the N-terminal Lys-C peptide of native $G\alpha_s$ (*m/z* 973.58)

Sequence	Fragment ion	m/z_{calc}	m/z_{found}
Palm-G	B_1	296.3	296.1
Palm-GC	B_2	456.3	456.2
Palm-GCL	B_3	569.4	569.0
Palm-GCLG	B_4	626.4	626.3
Palm-GCLGNS	B_6*	810.4	810.8
K	Y_1"	147.1	146.8
SK	Y_{o2}	216.1	215.6
SK	Y_2"	234.1	233.6
NSK	Y_3*	331.2	330.8
NSK	Y_3"	348.2	348.6
GNSK	Y_4*	388.2	388.0
GNSK	Y_4"	405.2	404.5
LGNSK	Y_5*	501.3	501.4
LGNSK	Y_5"	518.3	518.0
CLGNSK	Y_6*	661.3	660.9
CLGNSK	Y_6"	678.3	678.4

Based on the single-charge precursor ion the MS/MS spectrum showed N-terminal and C-terminal fragment ion series B_1–B_6 and Y_1–Y_6, respectively. (*) and ($_o$) indicate the neutral loss of 17 and 18 mass units, respectively. Cysteine was modified by carbamidomethylation.

that other features may be shared by $G\alpha_i$ and $G\alpha_s$, which are caused by the individual amide-linked acyl modification. Palmitoylation of $G\alpha_i$ and $G\alpha_o$ occurs spontaneously when the purified protein is incubated with palmitoyl–coenzyme A (Duncan and Gilman, 1996). These authors and others (Mollner *et al*., 1998) also showed that autopalmitoylation of $G\alpha$ specifically occurred at Cys^3. Incorporation of palmitate into $G\alpha_i$ is facilitated by the presence of amide-linked myristate at Gly^2. Indeed, Gly^2-

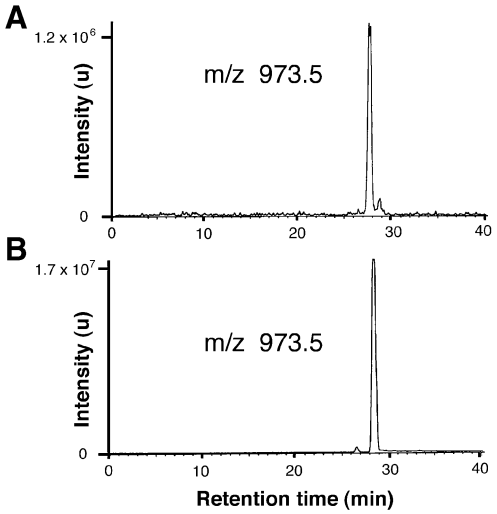

Fig. 2. LC/MS analysis. Mass chromatogram (*m/z* 973.5) of the peptide mixture generated by in-gel digestion of native $G\alpha_s$ with Lys-C (**A**) and of the peptide palm-GCLGNSK prepared by solid-phase peptide synthesis (**B**). Samples were separated by HPLC and detected on-line by electrospray mass spectrometry. Indicated are signal intensities in arbitrary units (u).

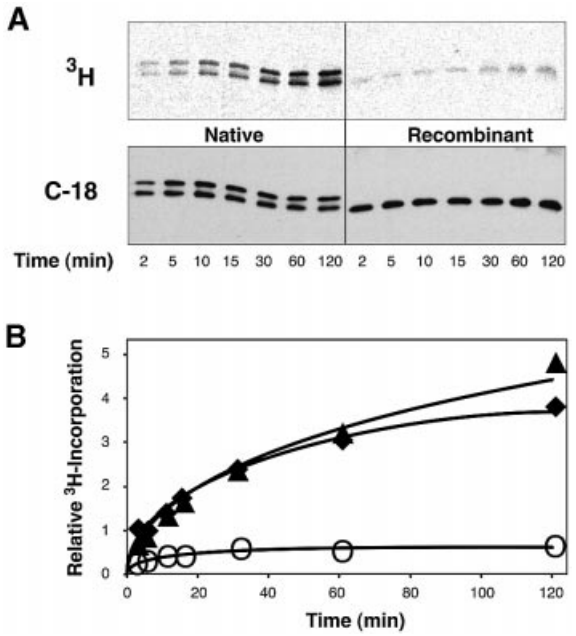

Fig. 3. Autopalmitoylation of $G\alpha_s$ at Cys^3. $G\alpha_s$ (2.5 μM) purified from recombinant bacteria (open circle) or native source (filled triangle for $G\alpha_{s\text{-short}}$, filled diamond for $G\alpha_{s\text{-long}}$) were incubated with 25 μM [9,10–^{3}H]palmitoyl–coenzyme A (20 000 c.p.m./pmol) for the indicated times at 20°C. (**A**) Shown are ^{3}H-images of reaction products after electrophoretic separation and transfer onto nitrocellulose membranes (upper panel), and immunostains with $G\alpha_s$-specific antiserum C-18 (lower panel). (**B**) Relative ^{3}H-incorporation into each protein was calculated from band intensities of the ^{3}H-imaging normalized to the intensity of the corresponding immunostain. Recombinant $G\alpha_s$ was palmitoylated with a stoichiometry of ~0.03 as determined by the filter binding assay described previously (Duncan and Gilman, 1996), implying >40% of native $G\alpha_s$ being palmitoylated after the 2 h incubation.

palmitoylation of $G\alpha_s$ enhanced the spontaneous incorporation of radioactivity from tritiated palmitoyl–coenzyme A into the protein (Figure 3). This was corroborated by the *in vitro* palmitoylation of a synthetic peptide representing the seven N-terminal amino acids of $G\alpha_s$ under similar reaction conditions. The reaction was more efficient using a peptide that was already palmitoylated at Gly^2 (Figure 4, lane P versus O).

Stimulation of adenylyl cyclase

Native, Gly^2-palmitoylated $G\alpha_s$ activated membrane-bound type I adenylyl cyclase with ~60-fold higher apparent affinity than recombinant, unmodified $G\alpha_s$ (Figure 5, top). Similar observations were made on adenylyl cyclase type V, a mixture of several adenylyl cyclase subtypes (mainly type V and VI) or a bisected adenylyl cyclase ($M_1C_1 + M_2C_2$; Graziano *et al.*, 1989; Kleuss and Gilman, 1997). According to the subtype of adenylyl cyclase used, the apparent K_d value of native $G\alpha_s$ was 0.1–0.3 nM, whereas recombinant $G\alpha_s$ stimulated the same adenylyl cyclases with an apparent K_d of 10–50 nM. For adenylyl cyclase type V, this resulted in an ~200-fold higher affinity stimulation by Gly^2-palmitoylated $G\alpha_s$ compared with recombinant $G\alpha_s$. The effect of Gly^2-palmitoylation on $G\alpha_s$ was not apparent with a soluble adenylyl cyclase (Figure 5, bottom). [Please note that 'soluble adenylyl cyclase' is an artificial construct of mammalian particulate adenylyl cyclase engineered to be soluble according to Tang *et al.* (Tang and Gilman, 1995) and does not refer to the mammalian soluble adenylyl cyclase expressed in testes.] In contrast, recombinant $G\alpha_s$ developed similar apparent affinities for all forms of adenylyl cyclase under the experimental conditions applied. There existed two major differences between the adenylyl cyclases used: (i) soluble adenylyl cyclase is devoid of amino acids representing the two hydrophobic clusters of mammalian particulate adenylyl cyclase; and

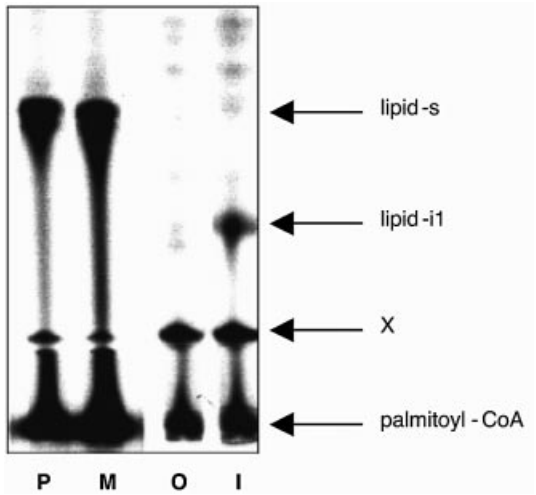

Fig. 4. Autopalmitoylation of peptides at Cys. Synthetic peptides representing the N-terminus of $G\alpha_s$ (P, M, O: GCLGNSK) or $G\alpha_i$ (I: GCTLSAEDK) were chemically modified at the N-terminal glycine by palmitate (P), myristate (M and I) or nothing (O). Peptides were subjected to autopalmitoylation and separated by thin-layer chromatography. Radioactivity was detected by fluorography. Shown is the fluorogram of a representative developed TLC plate. Arrows indicate the position of the bi-acylated N-terminal peptide of $G\alpha_s$ (lipid-s), bi-acylated N-terminal peptide of $G\alpha_i$ (lipid-i1), and the substrate palmitoyl–coenzyme A near to the origin. X denotes a non-identified spot that is already present in the substrate palmitoyl–coenzyme A and is located just above the preadsorbent/silica gel interface of the plate.

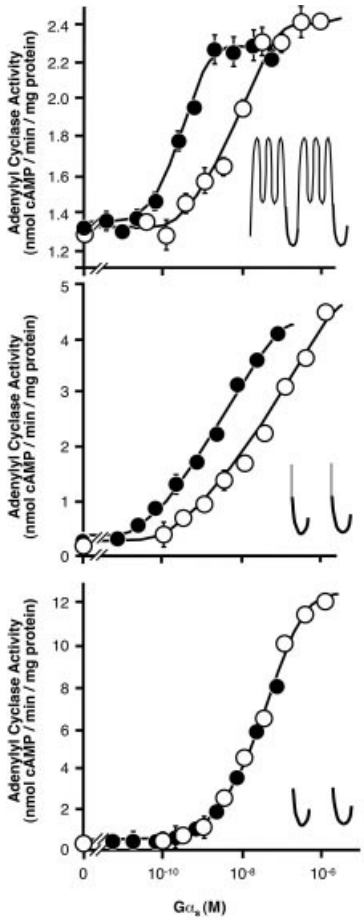

Fig. 5. Stimulation of adenylyl cyclase constructs by $G\alpha_s$. Membranes (2 μg protein) containing particulate adenylyl cyclase or soluble protein (20 μg, partially purified by Ni^{2+}–NTA agarose) were used. Increasing amounts of GTPγS-activated $G\alpha_s$ purified from bovine brain (native; closed circle) or bacterially expressed protein (recombinant; open circles) were added. Shown are adenylyl cyclase activities of full-length particulate adenylyl cyclase type I (top), soluble adenylyl cyclase halves anchored to the plasma membrane by fusion to the transmembrane span of the CD8-receptor (CD8-C_1 + CD8-C_2; center), and soluble adenylyl cyclase (bottom). The cartoons depict the putative topology of expressed adenylyl cyclase constructs; the CD8-span of adenylyl cyclase fusion construct is coloured grey. Values are representative of one experiment out of three similar assays performed in duplicates, SD is indicated.

(ii) soluble adenylyl cyclase is not localized at the membrane where particulate adenylyl cyclase naturally resides. To test whether the membrane localization of adenylyl cyclase was necessary for native $G\alpha_s$ to stimulate with high affinity, we anchored the soluble, catalytic parts of adenylyl cyclase into the plasma membrane by fusion to the heterologeous transmembrane span of the CD8-receptor (see Figure 5, centre). Concomitant expression of fusion constructs of both catalytic parts of adenylyl cyclase, CD8-C_1 and CD8-C_2, resulted in a particulate cAMP-generating enzyme. Gly^2-palmitoylated $G\alpha_s$ stimulated this membrane-anchored adenylyl cyclase construct more efficiently than non-modified $G\alpha_s$, indicating that high affinity stimulation was principally mediated by the membrane localization of the effector, not by topological or sequence-specific features of full-length adenylyl cyclase.

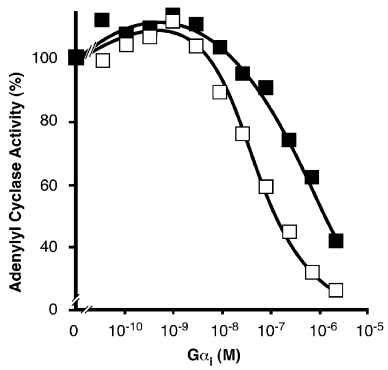

Fig. 6. Inhibition of adenylyl cyclase. Recombinant adenylyl cyclase type V in insect cell membranes (10 μg protein) was reconstituted with GTPγS-activated myristoylated $G\alpha_i$ to give the indicated final concentrations in the presence of 0.1 nM GTPγS-bound native $G\alpha_s$ (filled squares) or 20 nM GTPγS-bound recombinant $G\alpha_s$ (open squares). Please note, we determined the inhibitory action of myristoylated $G\alpha_i$ at comparable levels of adenylyl cyclase pre-stimulated activity by using only 1/200 the amount of Gly^2-palmitoylated than non-palmitoylated $G\alpha_s$. Pre-stimulated adenylyl cyclase activity in the absence of $G\alpha_i$ (100% activity) was 3.8 nmol/min/mg protein for recombinant $G\alpha_s$ and 4.2 nmol/min/mg protein for native $G\alpha_s$. Values are representative of one experiment out of three similar assays, mean SEM of duplicates was below 1%.

Inhibition of adenylyl cyclase

The increased affinity of Gly^2-palmitoylated $G\alpha_s$ for particulate adenylyl cyclase not only enhanced the ability of $G\alpha_s$ to stimulate adenylyl cyclase, but also considerably attenuated the efficiency of $G\alpha_i$ to inhibit the same effector. $G\alpha_i$ reconstitution showed high affinity interaction and regulation of adenylyl cyclase type V and VI and, to a lesser extent, type I, depending on the status of $G\alpha_i$ lipid modification. Myristoylated $G\alpha_i$ inhibited adenylyl cyclase with an IC_{50} of ~0.1 μM (Taussig *et al.*, 1993), whereas non-myristoylated $G\alpha_i$ was inactive (Linder *et al.*, 1990). The presence of the palmitoyl modification at Cys^3 in addition to the Gly^2-myristoylation did not affect these regulatory features of $G\alpha_i$ (C.Kleuss, unpublished results). The inhibitory action of myristoylated $G\alpha_i$ is only evident if adenylyl cyclase had been pre-stimulated, either by forskolin or naturally by $G\alpha_s$. If recombinant $G\alpha_s$ was used for pre-stimulation, myristoylated $G\alpha_i$ efficiently inhibited adenylyl cyclase type V (Figure 6). If adenylyl cyclase had been pre-stimulated by native $G\alpha_s$, myristoylated $G\alpha_i$ was a less-efficient inhibitor of cAMP-generating activity with an IC_{50} of 1 μM.

Discussion

Native $G\alpha_s$ is modified at the N-terminal glycine (Gly^2 after removal of the starter methionine) by a palmitoyl residue, as shown by mass spectrometric analysis. The Gly^2-palmitoylation was determined on both native $G\alpha_s$ splice variants, and is presumed to be a permanent modification due to the chemical stability of the amide-linkage as evident by its stability during the lengthy purification procedure of native $G\alpha_s$.

Gly^2-palmitoylation and Cys^3-palmitoylation

Our mass spectrometric data revealed only one palmitoyl group at the N-terminus of $G\alpha_s$ (at Gly^2). Cys^3-palmitoylation is reversible under mild experimental

conditions, while Gly^2-palmitoylation is permanent and stable. Therefore, native $G\alpha_s$ most probably was already devoid of Cys^3-attached palmitate in the end of our purification procedure. Nevertheless, prior to mass spectrometric analysis, we modified all cysteines present in the protein to remove disulfide bonds so that any residual *S*-palmitoyl modification (at Cys^3) would also have been removed from $G\alpha_s$. For the biochemical characterization, the Cys^3-palmitoylation status of $G\alpha_s$ was irrelevant, as previously described (Kleuss and Gilman, 1997). Under cellular conditions, $G\alpha_s$ most probably is temporarily palmitoylated on both residues, Gly^2 and Cys^3. The two neighbouring palmitoyl groups in $G\alpha_s$ are not equivalent, in contrast to other dually palmitoylated signalling proteins known to date. The newly described palmitoyl group on Gly^2 is attached via an amide linkage, while the palmitoyl group at Cys^3 is attached via a thioester bond (Linder *et al*., 1993). So far, palmitoylation via an N-terminal glycine was unknown to occur on heterotrimeric G proteins. Furthermore, palmitoylation via an amide bond (N-terminal cysteine or internal lysine) seems to be rare. N-palmitoylation has been reported for the vertebrate Sonic hedgehog protein (Pepinsky *et al*., 1998) and for the adenylyl cyclase of *Bordetella pertussis* (Hackett *et al*., 1994). Palmitoylation of Sonic hedgehog, which occurs via an amide bond at the N-terminal cysteine, is required for its embryonic patterning activities. The adenylyl cyclase of *B.pertussis*, consisting of 1706 amino acids, is internally palmitoylated at Lys^{983}. Toxicity of this adenylyl cyclase (i.e. the cell-invasive activity) is directly dependent on the palmitoylation at Lys^{983}. This exclusive group of molecules that gain their signalling abilities by N-palmitoylation is now extended to $G\alpha_s$. The distinct chemical nature of the two acyl anchors of $G\alpha_s$ implies different mechanisms of palmitoylation and potentially indicates divergent functional consequences for the protein. In particular, the functional consequences of Gly^2-palmitoylation of $G\alpha_s$ resemble those of myristoylation of $G\alpha_i$ (at Gly^2; both proteins can be additionally acylated by palmitate at their Cys^3) rather than the doubly palmitoylated $G\alpha_q$ (palmitoylation at Cys^9 and Cys^{10}).

Palmitoyl transfer reaction

The existence of a palmitoylated Gly^2 in $G\alpha_s$ adds a new dimension to the discussion about palmitoyltransferases. Although palmitoylation was first described over thirty years ago, the molecular mechanism of palmitate addition has been a matter of controversy. An activity capable of palmitoylating $G\alpha$ has been detected in mammalian plasma membranes (Dunphy *et al*., 1996). Unfortunately, the proteins responsible for this activity defied purification till now (Berthiaume and Resh, 1995; Dunphy *et al*., 2001). On the other hand, myristoylated $G\alpha$ subunits can be palmitoylated at the same sites that become palmitoylated *in vivo* in the absence of a specific acyltransferase using palmitoyl–coenzyme A as the acyl donor (Duncan and Gilman, 1996), raising the question of the requirement for an exogenous enzyme. Skinny hedgehog/sightless was identified as a palmitoyltransferase for *Drosophila* hedgehog (Chamoun *et al*., 2001). Hedgehog is palmitoylated through an atypical cysteine amide linkage at the N-terminus. However, it seems unlikely that skinny hedgehog can palmitoylate $G\alpha_s$ at Gly^2, as both proteins are not homologous in their N-termini and, moreover, the target amino acid in hedgehog is a cysteine residue.

Recently, a palmitoyltransferase that acylates through a conventional thioester linkage, was identified in yeast (Lobo *et al*., 2002). Erf2p/Erf4p can directly mediate palmitate transfer to yeast Ras using palmitoyl–coenzyme A as a donor. It remains to be shown that mammalian homologues of Erf2p/Erf4p act as palmitoyltransferases on mammalian Ras and/or $G\alpha$ proteins (Cys^3). Nevertheless, a putative Erf2p/Erf4p homologue is most likely not the catalyst for the Gly^2 palmitoylation of $G\alpha_s$. However, it is conceivable that a transacylation reaction from Cys^3 onto Gly^2 takes place. A similar reaction mechanism was already described for Sonic hedgehog N-terminal palmitoylation where palmitate is transferred from the thioester-bonded cysteine onto the amino group of the same cysteine after removal of the N-terminal signal peptide. The palmitate-transacylation reaction is compatible with preliminary data on Cys^3 mutants that prevent Gly^2-palmitoylation (data not shown).

Gly^2-palmitoylation impacts Cys^3-palmitoylation of $G\alpha_s$

In vivo, the exchange of palmitate at Cys^3 in $G\alpha_s$ and $G\alpha_i$ is enhanced upon stimulation of the coupling receptor through the combined processes of depalmitoylation and palmitoylation (Mumby *et al*., 1994; Chen and Manning, 2000). Wang *et al*. (1999) described that, beyond membrane anchorage, myristoylation of $G\alpha_o$ (at Gly^2) is important for the subsequent Cys^3-palmitoylation. Similarly, for $G\alpha_s$ we show here that Gly^2-palmitoylation facilitated the incorporation of the additional, reversibly attached palmitoyl residue at Cys^3 thereby enabling $G\alpha_s$ to switch between a lower (Gly^2-palmitoylated $G\alpha_s$) and higher hydrophobicity (Gly^2- plus Cys^3-palmitoylated protein). We observed increased Cys^3-autopalmitoylation upon Gly^2-palmitoylation in both, the short and long, splice variants of native $G\alpha_s$. Autoacylation was also enhanced in short synthetic peptides used in model reactions to represent the N-termini of $G\alpha_s$ and $G\alpha_i$. Apparently, the exact chemical nature of the lipid attached to Gly^2 was less important. A synthetic peptide representing the N-terminus of $G\alpha_s$ modified with myristate at the N-terminal glycine residue, i.e. a non-physiological modification for $G\alpha_s$, incorporated radioactivity as well as the palmitoylated peptide (see Figure 4, lane M). The underlying mechanism may be the preferred distribution of native $G\alpha_s$ or the corresponding Gly-acylated peptide into detergent micelles that harbour the substrate, palmitoyl–coenzyme A, thereby increasing the local concentration of the reaction partners and the mass-driven reaction.

Gly^2-palmitoylation of $G\alpha_s$ impacts regulation of adenylyl cyclase

A similar distribution phenomenon may contribute to the mechanism that renders Gly^2-palmitoylated $G\alpha_s$ more efficient to stimulate particulate adenylyl cyclase: Gly^2-palmitoylation directs $G\alpha_s$ into the plasma membrane to locally increase the protein concentration required for an efficient stimulatory coupling to the membrane-bound adenylyl cyclase. It is still unknown whether Gly^2-palmitoylation of $G\alpha_s$ also enables a lipid–protein inter-

action with specific transmembrane regions of adenylyl cyclase. Such a lipid–protein contact would require a second binding site of $G\alpha_s$ on adenylyl cyclase, i.e. an additional one to the protein–protein interaction domains shown in the crystal structure of adenylyl cyclase complexed with recombinant $G\alpha_s$ (Sunahara *et al*., 1997). This possibility seems, however, to be somewhat remote because the mere attachment of the adenylyl cyclase catalyst via the CD8 anchor already was sufficient to observe the functional consequences of Gly^2-palmitoylation at $G\alpha_s$ on adenylyl cyclase activity. However, we are aware of the different shaped concentration–activity curves of full-length adenylyl cyclase and the membrane-anchored constructs indicating some, although not a major, function of transmembrane spans of adenylyl cyclase in $G\alpha_s$ activation. We also observed subtle differences of native $G\alpha_s$ affinity for particulate adenylyl cyclase chimeras consisting of transmembrane spans derived from different adenylyl cyclase subtypes (mixed from adenylyl cyclase type I and II; data not shown). Taken together, we cannot exclude a specific lipid contact with adenylyl cyclase transmembrane domains formed by both clusters in an isoform-specific manner.

A lipophilic binding site in adenylyl cyclase transmembrane domains could also explain the different efficiencies of myristoylated $G\alpha_i$ to inhibit adenylyl cyclase type V according to the type of $G\alpha_s$ protein used for prestimulation. From three-dimensional structure and mutational analysis, it is evident that $G\alpha_s$ and $G\alpha_i$ interact with diagonally separated sites of distinct cytoplasmic halves of adenylyl cyclase. Therefore, the likely mechanism exerted by both $G\alpha$ subunits is an allosteric adenylyl cyclase regulation rather than a direct competition at those effector domains (Taussig *et al*., 1993). Surprisingly, myristoylated $G\alpha_i$ was a less efficient inhibitor of adenylyl cyclase if prestimulation was performed with 0.1 nM Gly^2-palmitoylated $G\alpha_s$ compared with 20 nM non-palmitoylated $G\alpha_s$. Therefore, an additional contact between the transmembrane domains of adenylyl cyclase and the acyl modification of one or the other $G\alpha$ subunit seemed conceivable. In such a scenario, the different acyl chains attached to $G\alpha$ might compete for a similar lipophilic binding site in adenylyl cyclase transmembrane domains, thereby superimposing the allosteric regulation mediated by adenylyl cyclase cytosolic domains and the membrane-delimited competition.

Irrespectively of the underlying mechanism, Gly^2-palmitoylation of $G\alpha_s$ has profound functional consequences for G_s-mediated signal transduction at the level of adenylyl cyclase regulation. Gly^2-palmitoylated $G\alpha_s$ (in contrast to non-palmitoylated or Cys^3-palmitoylated $G\alpha_s$) renders the cell more receptive for stimulatory inputs and less sensitive for inhibitory stimuli that are transmitted by G protein α-subunits. This process adds a new layer of regulatory possibilities to this exceedingly important signal transduction system.

Materials and methods

In-gel digestion

Native and recombinant $G\alpha_s$ were subjected to polyacrylamide gel electrophoresis. After visualization and destaining, $G\alpha_s$ spots were excised, washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile and dried in a vacuum centrifuge. Disulfide bonds were reduced by incubation in 30 µl of 10 mM DTT/100 mM ammonium bicarbonate in water (45 min at 55°C). Alkylation was performed by replacing the DTT solution with 55 mM iodoacetamide or 90 mM 4-vinylpyridine in 25 mM ammonium bicarbonate. After 20 min incubation at 25°C in the dark, the gel pieces were washed with 50–100 µl of 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile and dried in a vacuum centrifuge. The gel pieces were re-swollen in 10 µl of 5 mM ammonium bicarbonate, containing 500 ng endoproteinase Lys-C or trypsin (sequencing grade; Roche Diagnostics, Mannheim, Germany). After 15 min, 5 µl of 5 mM ammonium bicarbonate was added to keep the gel pieces wet during cleavage (37°C, overnight). For extraction, 15 µl of 0.5% (v/v) trifluoroacetic acid (TFA) in acetonitrile was added, followed by sonication for 5 min.

MALDI-MS

Measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA). Briefly, samples were prepared with α-cyano-4-hydroxycinnamic acid matrix and measured in the reflection mode at an acceleration voltage of 20 kV and a delay of 200 ns. Peptides from the tryptic and Lys-C digested proteins covered 71% (281 out of 394 amino acids) of the protein (SwissProt accession No. P04896).

LC-MS/MS

For on-line LC-MS measurements, 10 µl of the peptide mixture from in-gel digestion was dried under vacuum, redissolved in 5 µl of 20% (v/v) acetonitrile/0.1% (v/v) TFA in water, and subjected to reversed-phase HPLC separation on a C_{18} column (Vydac 218TP5115, 150 × 1 mm i.d., 5 µm, Promochem, Wesel, Germany). Elution was performed at a flow rate of 30 µl/min generated by a model 140B Applied Biosystems solvent delivery system. Mobile phase A was 0.06 % TFA in water and B was 0.05% TFA in acetonitrile–water (8:2, v/v). Runs were performed using a linear gradient of 25–95% B and 50–95% B in 40 min for recombinant and native $G\alpha_s$, respectively. Mass spectrometry was performed on-line on a triple quadrupole instrument (TSQ 700, Finnigan MAT, Bremen, Germany) equipped with the standard electrospray ion source operating in the positive mode with a capillary temperature of 200°C and a voltage of 4.5 kV. Nitrogen was introduced as sheath gas at a pressure of 3.4 bar and auxiliary gas (flow 960 ml/min). A sheath liquid of isopropanol–propionic acid (25:75, v/v) was applied at a flow rate of 4 µl/min. Total ion chromatograms and mass spectra (scan range 300–1600 *m/z*) were acquired. Fragment ion spectra were generated by collision-induced dissociation (CID) using argon as collision gas (3–4 mTorr) and collision energies ranging from 20 to 40 eV.

Peptide synthesis

Synthetic peptides representing the N-terminus of $G\alpha_s$ (GCLGNSK) were synthesized by solid-phase methods using Fmoc (Nα-9-fluorenylmethoxycarbonyl) chemistry in the continuous flow mode (Beyermann *et al*., 1996). The N-terminal glycine was acylated with palmitic acid or myristic acid using the amino acid coupling chemistry. After cleavage from the resin support, cysteine alkylation was performed by 55 mM iodoacetamide in 100 mM ammonium bicarbonate. Peptides were purified by preparative high-performance liquid chromatography to give final products of >95% purity.

Autopalmitoylation

Autopalmitoylation was performed according to Duncan and Gilman (1996). Recombinant $G\alpha_s$ protein was used as the C-terminal H_6-tagged protein as described previously (Kleuss and Gilman, 1997). Native $G\alpha_s$ was purified from bovine brain. Due to the lengthy purification procedure in the presence of 1–3 mM DTT, the native Gly^2-palmitoylated $G\alpha_s$ used throughout this study was not supposed to contain Cys^3-attached palmitate. Briefly, 2.5 µM $G\alpha_s$ was incubated with 25 µM [9,10–^{3}H]palmitoyl–coenzyme A (20 000 c.p.m./pmol) for 90 min if not otherwise indicated. Peptides were solubilized in DMSO and autopalmitoylated under similar conditions [final concentration 1% (w/v) DMSO]. Proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Dried membranes were exposed to ^{3}H-imaging screens before proteins were immunostained with $G\alpha_s$-specific antiserum C-18. Autoacylated peptides were applied to thin layer chromatography on discontinuous silica plates (Whatman LK6D Silica Gel, 60 Å) and developed in *n*-butanol: pyridine:acetic acid:water (60:30:5:15) for 3 h. Radioactivity was detected by fluorography.

Adenylyl cyclase

Adenylyl cyclases and mutants thereof were expressed in Sf9 cells using recombinant baculoviruses (Weitmann *et al*., 2001). Viruses encoding adenylyl cyclase type I and V were kindly provided by the laboratory of A.G.Gilman. Generation of viruses encoding bisected adenylyl cyclases type I and II was described previously (Weitmann *et al*., 1999), as well as generation of viruses encoding the soluble His_6-tagged adenylyl cyclase (Weitmann *et al*., 2001). Fusion constructs of CD8 (coding for amino acids 1–217 of the CD8 receptor; GI number 22902133) with sequences coding for domain C_1 of adenylyl cyclase type I and domain C_2 of adenylyl cyclase type II, were generated by PCR using *Sal*I recognition site as junction. The extracellular portion of the CD8 receptor was proposed to dimerize. Consequently, dimerization of the membrane anchored catalytic parts of adenylyl cyclase was alleviated. All adenylyl cyclases were expressed for 48–55 h in insect cells; cells were harvested and plasma membranes or cytosol were prepared by nitrogen cavitation. Membranes were washed and flash frozen as well as cytosolic fractions. Adenylyl cyclase activity was measured for 10 min at 30°C in the presence of 2 μM forskolin and/or $G\alpha_s$ as indicated (Kleuss and Gilman, 1997).

Miscellaneous

Immunoblotting was performed with commercially available antibodies (Santa Cruz). Labelled samples on nitrocellulose membranes or silica plates were visualized by ^{3}H-imaging system (BAS-1500, Fuji Film) or exposure to Biomax MS films (Kodak); quantification was performed with IPLab Gel (Signal Analytics, Vienna, VA).

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