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The α-subunits of G-proteins G_{12} and G_{13} are palmitoylated, but not amidically myristoylated

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Abstract

The α-subunits of the G-proteins G_{12} and G_{13} were expressed with a baculovirus system in insect cells and analysed for acylation. Both proteins incorporated tritiated palmitic and to a lesser extent also tritiated myristic acid. Radiolabel from both fatty acids was sensitive to treatment with neutral hydroxylamine. This result supports a thioester-type fatty acid bond and argues against amidical *N*-myristoylation. Fatty acid analysis after labeling with [^{3}H]palmitic acid showed that palmitate represents the predominant fatty acid linked to $G\alpha_{12}$ and $G\alpha_{13}$. Separation of cells into cytosolic and membranous fractions revealed that palmitoylated α-subunits of G_{12} were exclusively membrane-bound, whereas [^{35}S]methionine-labeled proteins were detected in soluble and particulate fractions. Inhibition of protein synthesis with cycloheximide did not block palmitoylation of the α-subunits, which indicates that palmitoylation occurs independently of protein synthesis.

Key words: Palmitoylation; G-protein; G_{12}; G_{13}; Acylation; Baculovirus expression

1. Introduction

Heterotrimeric regulatory guanine nucleotide-binding proteins (G-proteins) functionally couple membrane bound receptors to various intracellular effectors. In their inactive state, G-proteins form an oligomer composed of α-, β- and γ-subunits. Based on amino acid sequence homology of the α-subunits, G-proteins are grouped into four subfamilies, G_s, G_i, G_q and G_{12}. The specificity of signal transduction pathways is determined by different α-subtypes associated with βγ-dimers (for review see [1–3]).

In order to function as signal transducers, G-proteins have to be associated with the cytoplasmic face of the plasma membrane. Because membrane spanning domains are not present in α-subunits, hydrophobic protein modifications have been claimed to anchor these proteins to the plasma membrane [4]. Acylation of proteins belonging to three of the four subfamilies of α-subunits has been reported. The α-subunits G_i, G_o, and G_z, but not G_s and G_q are modified at an N-terminal glycine residue with myristic acid (C 14:0) via an amide linkage. Myristic acid is thought to increase the affinity of α_o for the βγ-complex and thus attaches α_o to the plasma membrane [5–8]. Heterogeneity of lipid modification was detected for transducin which is acylated at its N-terminal glycine via an amide bond, predominantly with lauric acid (C 12:0) and two unsaturated derivatives of myristic acid (C 14:1, C 14:2) [9,10]. Recently palmitoylation of $G\alpha_o$, $G\alpha_i$, $G\alpha_s$, $G\alpha_z$ and $G\alpha_q$ was reported. Palmitic acid (C 16:0) is attached to $G\alpha_o$ and $G\alpha_s$ via a thioester-type linkage to a cysteine residue located at position 3 [11–13].

In this study we have investigated potential acylation of the recently identified fourth subfamily of α-subunits, G_{12} and G_{13}. The genes coding for $G\alpha_{12}$ and $G\alpha_{13}$ have been cloned from a mouse brain cDNA library. $G\alpha_{12}$ and $G\alpha_{13}$ share 67% amino acid identity with each other and less than 48% identity with all other α-subunits. Especially the amino termini of $G\alpha_{12}$ and $G\alpha_{13}$, which are potential subjects for hydrophobic modifications, are quite distinct when compared to other α-subunits [14]. We show here that both $G\alpha_{12}$ and $G\alpha_{13}$ are palmitoylated in ester-type linkages, and that the acylated forms of these proteins are exclusively found associated with membranes.

2. Materials and Methods

2.1 Construction of recombinant baculovirus and Sf21 cell culture

cDNAs encoding the $G\alpha_{12}$ and $G\alpha_{13}$ subunit of heterotrimeric G-proteins were transferred to the baculovirus expression vector (pVL1392) as follows. To construct the expression vector for $G\alpha_{12}$ the 1.7 kb fragment cut with *Not*I and *Eco*RI was ligated to the respective sites of pVL1392. To construct the expression vector for $G\alpha_{13}$ the $G\alpha_{13}$ cDNA was cleaved with *Bgl*II and *Stu*I to yield a 1.5 kb fragment containing the entire coding sequence. The fragment was ligated to the

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*Bgl*II and *Sma*I sites of pVL1392. The resulting plasmids and linearized BaculoGOLD (Dianova) viral DNA were transfected into *Spodoptera frugiperda* insect cells (Sf-21) by lipofectin. Recombinant viruses were plaque-purified and amplified [15]. The positive viral clones for further experiments were tested by their ability to direct the expression of $G\alpha_{12}$ and $G\alpha_{13}$ in Sf21 cells, which was detected by immunoprecipitation with anti-$G\alpha_{12}$ (AS 233)- and anti-$G\alpha_{13}$ (AS 343) antiserum. Sf21 cells were grown in TC-100 medium (Gibco, Berlin, Germany) supplemented with 5% (v/v) calf serum.

2.2 Antibodies

Antibodies AS 233 (anti-α_{12}) and AS 343 (anti-α_{13}) were raised against synthetic C-terminal peptides corresponding to deduced amino acid sequences of $G\alpha_{12}$ and $G\alpha_{13}$ cDNA clones (amino acids 370–379 and 367–377, respectively) [14]. The antibodies were shown to be specific for recombinant protein expressed in *E. coli* and native $G\alpha_{12}$ and $G\alpha_{13}$ as described elsewhere (Offermanns, S., Langwitz, K.L. Spicher, K. and Schultz, G. (1994) Proc. Natl. Acad. Sci. USA, in press)

2 3. Metabolic labeling and immunoprecipitation

Sf-21 cells (1 × 106) in 3.5 cm diameter culture dishes were infected with recombinant baculovirus at a multiplicity of infection of 10 virus particles per cell. Two days later, cells were labeled with [^{35}S]methionine (50 μCi/ml in TC-100 medium without methionine; 1200 Ci/mmol, 'Express-[^{35}S]-protein labeling mix', DuPont, Bad Homburg, Germany), [^{3}H]myristic acid (1 mCi/ml in TC-100 medium; 33 Ci/mmol, DuPont) or [^{3}H]palmitic acid (1 mCi/ml in TC-100 medium; 47 Ci/mmol, DuPont) for the time periods indicated in the figure legends. In some experiments cycloheximide, was added to a final concentration of 50 μg/ml simultaneously with [^{3}H]palmitic acid. After labeling, cells were scraped into Eppendorf tubes, pelleted (3 min, 3000 × *g*), washed with ice-cold phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.4) and lysed with 600 μl of RIPA-buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, 20 mM Tris, 10 mM EDTA, 10 mM iodoacetamide). Insoluble material was pelleted (5 min, 20,000 × *g*) after three cycles of freezing and thawing. Antisera AS 233 and AS 343 were added to the resulting supernatant at a dilution of 1:50, and samples were agitated overnight at 4°C. After an additional incubation with 30 μl of Protein A-Sepharose Cl-4B (1:1 in RIPA; Sigma, St. Louis, USA) for 2 h, antigen–antibody complexes were washed three times with RIPA-buffer and once with PBS. The antigen was solubilized with gel-loading-buffer containing 2% SDS and 5% mercaptoethanol for 30 min at 37°C and subjected to SDS-PAGE in 10% gels followed by fluorography using salicylate [16].

2.4. Hydroxylamine-treatment

SDS-gels containing [^{3}H]myristic or [^{3}H]palmitic acid-labeled $G\alpha_{12}$ and $G\alpha_{13}$ were fixed (10% acetic acid, 10% methanol) and water-soaked for 30 min. They were then treated overnight under gentle agitation with 1 M hydroxylamine (pH 6.5; Merck, Darmstadt, Germany) or 1 M Tris (pH 6.5). The gels were water-soaked again and incubated twice for 30 min with dimethylsulfoxide (DMSO) to wash out cleaved fatty acids. The gels were again water-soaked to remove DMSO and then processed for fluorography.

2.5. Fatty acid analysis

[^{3}H]Palmitic acid labeled $G\alpha_{12}$ and $G\alpha_{13}$ were purified by immunoprecipitation and SDS-PAGE. After localizing α-subunits by fluorography, the bands were excised from the gel. The scintillator was removed by washing with water, and gel pieces were dried in a desiccator. Fatty acids were liberated by treatment with 6 N HCl for 16 h at 110°C and extracted 3 times with hexane. Pooled extracts were concentrated and separated by thin-layer chromatography (tlc) on RP 18 plates (Merck) with acetonitrile/acetic acid (1:1) as solvent system. Radioactivity on tlc-plates was measured with a radiochromatogram-scanner (Berthold, Wildbad, Germany). The identity of the peaks was confirmed by comparsion of R_f values with authentic radiolabeled reference substances run in parallel.

2.6. Cell fractionation

Sf-21 cells were scraped into Eppendorf cups, pelleted, washed once in PBS and resuspended in 600 μl of phosphate-buffer (50 mM, pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol. After three cycles of freezing in liquid nitrogen and thawing at 37°C, cells were separated into cytosolic and membrane fractions by centrifugation at 125,000 × *g* at 4°C for 30 min. The membrane pellet was solubilized in RIPA-buffer. 600 μl of 2 × RIPA-buffer was added to the cytosolic supernatant prior to immunoprecipitation.

3. Results and Discussion

High titer baculovirus stocks containing the cDNA of Gα12 or Gα13 in the viral genome were prepared (see section 2) and used to infect *Spodoptera frugiperda* insect cells (Sf-21). To monitor expression of the α-subunits, cells were labeled at 2 days after infection with [^{35}S]methionine. Cell extracts were subjected to immunoprecipitation using $G\alpha_{12}$- and $G\alpha_{13}$-specific peptide antisera, and precipitates were analysed by SDS-PAGE and fluorography (Fig. 1, left panel). Both baculovirus clones expressed proteins with molecular weights of roughly 44 kDa, which corresponds to the molecular mass of $G\alpha_{12}$ and $G\alpha_{13}$ as predicted from their deduced amino acid sequences. Controls failed to detect similar proteins in cells infected with non-recombinant baculovirus (see Fig. 1, left panel lanes marked wt). This provides additional evidence that the immunoprecipitated proteins shown in Fig. 1 (left panel) are indeed $G\alpha_{12}$ and $G\alpha_{13}$.

Insect cells are known to possess the enzymes to hydrophobically modify proteins [17,18]. In order to test for possible acylation of $G\alpha_{12}$ and $G\alpha_{13}$, recombinant baculovirus-infected cells were labeled with both [^{3}H]myristic and [^{3}H]palmitic acid. The resulting fluorogram (see Fig. 1, center and right panels) demonstrates that both proteins incorporated tritiated myristic as well as palmitic acid, although labeling with [^{3}H]myristic acid was barely detectable (see also below and Fig. 3). Inter-

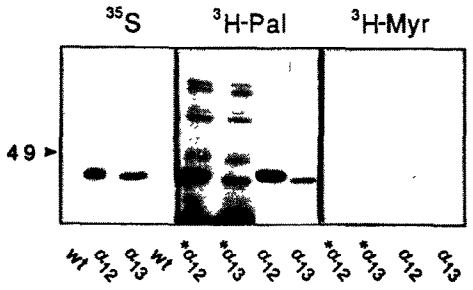

Fig. 1. Acylation of G_{12} and G_{13} α-subunits. Recombinant baculovirus infected Sf-21 cells expressing either $G\alpha_{12}$ (α_{12}) or $G\alpha_{13}$ (α_{13}) were labeled for 4 h with [^{35}S]methionine, [^{3}H]palmitic acid or [^{3}H]myristic acid as indicated. Using antibodies specific for $G\alpha_{12}$ (designated AS 233) or $G\alpha_{13}$ (designated AS 343), proteins were immunoprecipitated from cell extracts and subjected to SDS-PAGE and fluorography, or cell extracts were applied without prior immunoprecipitation (lanes marked with asterisks). Control insect cells were also infected with non-recombinant baculovirus (designated wt) and labeled with [^{35}S]methionine. Lysates were immunoprecipitated with the above-mentioned antibodies specific for $G\alpha_{12}$ (left panel, left lane) or $G\alpha_{13}$ (left panel, right lane). The position of a 49 kDa molecular weight marker protein is indicated.

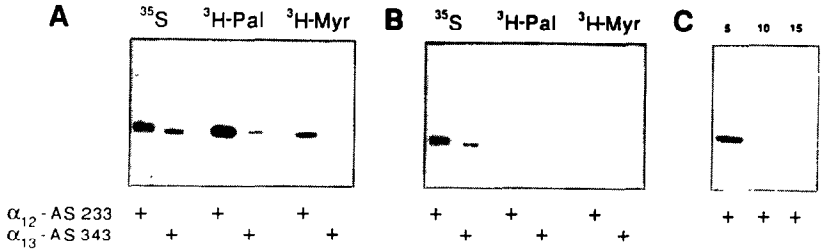

Fig. 2. Sensitivity of the fatty acid bond to hydroxylamine and 2-mercaptoethanol. (A and B) Sf-21 cells expressing $G\alpha_{12}$ (α_{12}) or $G\alpha_{13}$ (α_{13}) were labeled for 4 h with [^{35}S]methionine, [^{3}H]palmitic acid or [^{3}H]myristic acid as indicated. α-Subunits were immunoprecipitated as in Fig. 1 and run on an SDS-gel. The gel was treated with 1 M Tris (A) or 1 M hydroxylamine (B) prior to fluorography, as described in section 2. (C) [^{3}H]Palmitic acid-labeled $G\alpha_{12}$ was immunoprecipitated with antibody AS 233 and treated with gel-loading buffer containing 5%, 10% or 15% 2-mercaptoethanol for 15 min at 80°C prior to SDS-PAGE and fluorography. The percent concentration of 2-mercaptoethanol is indicated above the respective lanes.

estingly, this baculovirus-based expression system yielded sufficient $G\alpha_{12}$ and $G\alpha_{13}$, to allow detection of the palmitoylated proteins in unprocessed cell lysates, i.e. without immunoprecipitation (see Fig. 1, lanes marked with asterisks).

Next we determined the chemical nature of the fatty acid bond in $G\alpha_{12}$ and $G\alpha_{13}$, in order to distinguish between amide and ester-type fatty acid linkages and to exclude any possible metabolic interconversion of [^{3}H]myristic and [^{3}H]palmitic acid into [^{3}H]amino acids before their incorporation into proteins. Gels containing fatty acid labeled α-subunits expressed by Sf-21 cells and immunoprecipitated with antipeptide antibody AS 233 (specific for $G\alpha_{12}$) or with antibody AS 343 (specific for $G\alpha_{13}$) were treated with neutral hydroxylamine (Fig. 2B) or with Tris-buffer prior to fluorography. The results from this experiment show that hydroxylamine cleaved the [^{3}H]myristic and [^{3}H]palmitic acid derived label both from $G\alpha_{12}$ and $G\alpha_{13}$. This suggests that neither $G\alpha_{12}$ nor $G\alpha_{13}$ contained fatty acids linked via an amide-bond, which would be resistant to such treatment [19]. Therefore, the [^{3}H]myristic acid-derived labeling cannot represent the typical *N*-amidical myristoylation [20]. This is also consistent with the lack of a myristoylation motif at the N-termini of these proteins [14]. Furthermore, the [^{3}H]palmitic acid-derived radioactivity was sensitive to heating with a buffer supplemented with increasing concentrations of 2-mercaptoethanol (see Fig. 2C). This sensitivity to neutral hydroxylamine and reducing agents is a typical feature of thioester-type fatty acid linkages.

The site of palmitate modification has been identified for two α-subunits, $G\alpha_{s}$ and $G\alpha_{o}$ [12,13]. Either protein contains the carbon chain attached to a cysteine residue at position 3, which is located next to a glycine at position 2. Identical sequence motifs (Met-Gly-Cys) are present at the N-terminus of most other α-subunits [21]. However, the predicted amino acid sequences of $G\alpha_{12}$ and $G\alpha_{13}$ do not contain an N-terminal Met-Gly-Cys motif [14]. Assuming that both proteins are nevertheless palmitoylated near their N-termini, cysteine residues in position 11 in $G\alpha_{12}$ and in positions 14 and 18 in $G\alpha_{13}$ are likely candidates as acylation sites. Deletion of these cysteine residues by site-specific mutagenesis will help to clarify this.

It has been described that exogenously added fatty acids are often converted into other fatty acid species of different chain lenght before their attachment to acylated proteins [22,23]. In order to prove the identity of the actual protein-bound fatty acids in $G\alpha_{12}$ and $G\alpha_{13}$ after labeling with [^{3}H]palmitic acid, both proteins were subjected to fatty acid analysis. Fatty acids were hydrolyzed from gel-purified G-protein α-subunits and separated into the individual fatty acid species by tlc. Radiochromatogram scanning of the tlc plates (Fig. 3) revealed that both proteins are acylated predominatly with palmitic acid. Traces of stearic acid were also detected, but no myristic acid was found in these samples, which finding provides further evidence that G_{12} and G_{13} α-subunits are not myristoylated. Unfortunately, the faint labeling with [^{3}H]myristic acid (see Fig. 1, right panel) precluded chemical analysis of its protein-bound acyl chains.

To determine the intracellular location of $G\alpha_{12}$, recombinant baculovirus infected Sf-21 cells were labeled with [^{35}S]methionine for subcellular fractionation. Cells were then disintegrated and separated into soluble and particulate fractions by highspeed centrifugation before immunoprecipitation and SDS-PAGE. As apparent from the first two lanes of the fluorogram shown in Fig. 4A, amino acid labeled $G\alpha_{12}$ was found both in soluble and particulate fractions (designated ^{35}S). Liquid scintillation counting of the bands showed that approximately 60% of $G\alpha_{12}$ is membrane-bound and 40% is cytosolic. This is consistent with results reported for baculovirus-expressed $G\alpha_{o}$, $G\alpha_{i}$, $G\alpha_{q}$ and $G\alpha_{s}$ [11].

In order to investigate the possibility of different pools of α-subunits in the cell, e.g. for acylated and unacylated forms, we also analysed the subcellular distribution of fatty acid labeled $G\alpha_{12}$. As seen from the lanes marked [^{3}H]Pal in Fig. 4A, [^{3}H]palmitic acid labeled $G\alpha_{12}$ was found exclusively in the particulate fraction. This result suggests that there are indeed distinct pools of $G\alpha_{12}$, the palmitoylated form being membrane-bound and the pro-

tein without detectable palmitic acid-incorporation in the cytosolic fraction. Similar results were obtained for the $G\alpha_{13}$ subunit (not shown).

This suggests, as has been previously claimed for $G\alpha_o$ [13], that palmitoylation would anchor α-subunits to the plasma membrane. Alternatively, one could argue that the soluble pool of $G\alpha_{12}$ is not palmitoylated because these polypeptides in the first place fail to reach the membrane, the only site where palmitoylating activity has been detected [24]. From suggestive evidence with viral acylproteins [25] another possible function of covalently-linked palmitate may be their involvement in protein–protein interactions which are crucial for signal transduction. The N-terminus of the α-subunits does mainly participate in interactions with the βγ-complex, but also with an activated receptor [26]. If the N-terminus of $G\alpha_{12}$ and $G\alpha_{13}$ is indeed the attachment site for palmitate, covalently linked fatty acids may increase the affinity of the α-subunit for these proteins.

Palmitoylation can be either a stable modification, which persists throughout the life span of a given polypeptide, or the protein-bound fatty acids are subject to cycles of de- and re-acylation [27]. In the first case, palmitoylation would depend on ongoing protein-synthesis, whereas in the second case attachment of fatty acids would continue even after blocking protein synthesis. To distinguish between these options, $G\alpha_{12}$- and $G\alpha_{13}$-expressing Sf-21 cells were labeled with [^{3}H]palmitic acid in the absence or presence of cycloheximide. The resulting fluorogram (Fig. 4B) shows that despite of the block in protein synthesis labeling of both α-subunits still incorporated palmitic acid, albeit to a lesser extent than in the absence of cycloheximide. This emphasizes the post-translational nature of palmitoylation and indicates that previously synthesized G-protein α-subunit was made available for a second round of palmitoylation by deacylation. With identical results reported for Gαo and Gαs [12,13], the dynamic nature of palmitoylation may thus be a common feature of Gα-subunits and instrumental to the regulation of G-protein linked signal transduction [28,29].

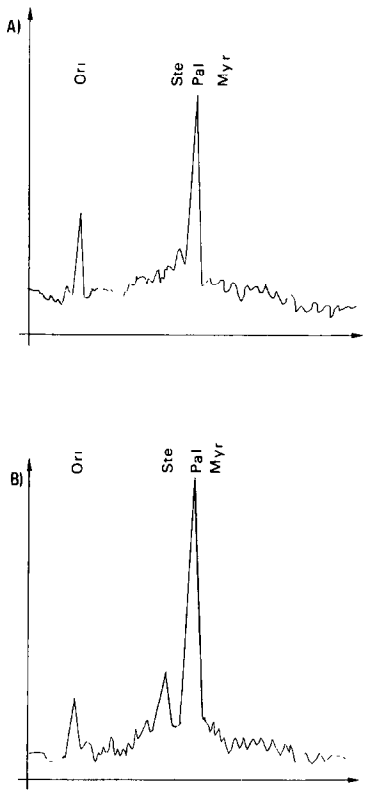

Fig. 3. Thin-layer chromatographic detection of cleaved fatty acids. [^{3}H]Palmitic acid-labeled $G\alpha_{12}$ (A) and $G\alpha_{13}$ (B) were purified by immunoprecipitation using antibodies AS 233 and AS 343, respectively. After SDS-PAGE and fluorography, labeled bands were excised and fatty acids hydrolyzed, extracted and separated by thin-layer chromatography. The relative distribution of radioactivity on the tlc-plate was determined with a radiochromatogram-scanner. Myr, Pal, Ste = position of reference substances [^{3}H]myristic, [^{3}H]palmitic and [^{3}H]stearic acid after run. Ori = point of sample application. Radioactivity (vertical axis) is plotted against migration (horizontal axis).

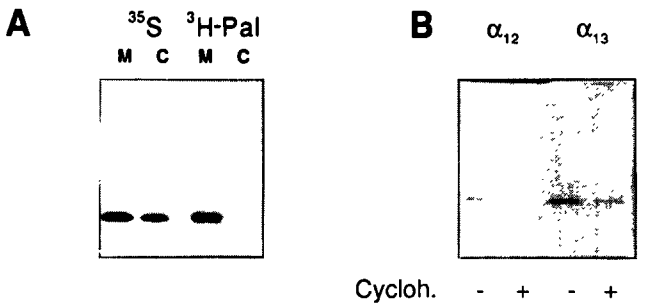

Fig. 4. Subcellular distribution of $G\alpha_{12}$ and palmitate labeling after block of protein synthesis. (A) Sf-21 cells expressing $G\alpha_{12}$ were labeled for 4 h with [^{35}S]methionine (^{35}S) or with [^{3}H]palmitic acid ([^{3}H]Pal). After disruption, cells were separated into cytosol (C) and membranes (M), and $G\alpha_{12}$ was immunoprecipitated from each fraction prior to SDS-PAGE and fluorography. (B) Sf-21 cells expressing $G\alpha_{12}$ or $G\alpha_{13}$ were labeled for 4 h with [^{3}H]palmitic acid in the absence (−) or presence (+) of cycloheximide (50 μg/ml) and processed as described for Fig. 1.

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