RESEARCH COMMUNICATION

Chemical inhibition of myristoylation of the G-protein $G_{11}\alpha$ by 2-hydroxymyristate does not interfere with its palmitoylation or membrane association

Evidence that palmitoylation, but not myristoylation, regulates membrane attachment

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The α -subunit of the G-protein $G_{11}\alpha$ is normally dually acylated at its N-terminus with the saturated fatty acids myristate and palmitate. Inhibition of protein myristoylation by treatment with 2-hydroxymyristate prevented neither the incorporation of [3 H]palmitate nor the membrane association of this protein when expressed in COS cells. Construction of a mutant of $G_{11}\alpha$ in which serine-6 was replaced by aspartic acid prevented both

myristoylation and palmitoylation, and the expressed protein was found primarily in the cytoplasmic fraction. These data indicate that myristoylation is not an absolute requirement for palmitoylation of $G_{11}\alpha$ and that palmitoylation, but not myristoylation, plays a key role in membrane association of this G-protein α -subunit.

INTRODUCTION

The widely expressed pertussis toxin-sensitive G-proteins all contain the N-terminal motif MGC and have been shown to be dually modified at their N-terminus. The initial modification is a co-translational attachment of myristic acid at the mature N-terminal glycine residue exposed by removal of the initiator methionine, and this is followed by subsequent post-translational addition of palmitic acid to cysteine-3 [1–3]. Early studies on the role of myristate were performed by construction of mutants of G-proteins in which the glycine residue was converted into alanine. This modification prevented myristoylation and was noted to result in poor membrane association of the expressed protein [4,5]. Such data led to the conclusion that myristoylation was a requirement for membrane association, as G-protein α -subunits do not contain a transmembrane spanning element [6]. Subsequent studies have indicated, however, that mutation of the N-terminal glycine can also result in prevention of palmitoylation of the protein [7–9] and this has clouded the issue as to the relative roles of the two acylations in membrane association. The other family of proteins which have an N-terminal MGC motif are the src-related non-receptor tyrosine kinases [1]. Analyses of N-terminal mutants of these kinases have produced similar data to the G-protein α -subunits [10–12].

In an extension of these studies, an inhibitor of myristoyl-CoA:protein N-myristoyltransferase (NMT), 2-hydroxymyristate, has been used to prevent myristoylation of non-receptor tyrosine kinases without interfering with palmitoylation of the proteins [13]. In the present study we have treated COS cells, transfected to express wild-type $G_{11}\alpha$, with 2-hydroxymyristic acid and also examined the myristoylation and palmitoylation potential of an S6D mutation (serine-6 replaced by aspartic acid) of $G_{11}\alpha$, which compromises the high-affinity consensus recognition sequence for NMT [14]. We demonstrate

that treatment with 2-hydroxymyristate prevents myristoylation of newly synthesised $G_{11}\alpha$ but not its palmitoylation or membrane association, and that prevention of myristoylation by the introduction of the S6D mutation into $G_{11}\alpha$ also prevents palmitoylation and compromises membrane interaction. These results indicate that palmitoylation is quantitatively more important for the membrane association of $G_{11}\alpha$ than myristoylation, as has been suggested from studies with acylated peptides [15,16].

MATERIALS AND METHODS

Generation of an S6D mutant of $G_{11}\alpha$ and its expression in COS cells

A cDNA encoding murine $G_{11}\alpha$ was subcloned into the mammalian expression vector pcEXV-3 [7]. An S6D mutant of $G_{11}\alpha$ was generated where aspartic acid substituted for serine at codon 6. Site-directed mutagenesis was achieved by PCR amplification from the pcEXV- $G_{11}\alpha$ plasmid, using a sense (5') mutagenic primer spanning the N-terminus (where the initiator methionine codon is shown in italics and the mutated bases are underlined): S6D mutant 5'-ACCATGGGCTGCACACTGGACGCTGAG-GACAAG-3', and a 3' antisense primer located approximately 300 bases downstream within the coding sequence for $G_{11}\alpha$ (5'-TCTCTTTATGACGCCGCGAGCTC). The PCR reaction was carried out using a Hybaid thermal reactor with 30 ng of linearized pcEXV- $G_{11}\alpha$ plasmid as template DNA and 100 pmol of each primer, with 30 amplification cycles consisting of denaturation at 94 °C (2 min 30 s), annealing at 53 °C (1 min 40 s) and primer extension at 72 °C (2 min). The PCR product and the C-terminal fragment of $G_{11}\alpha$ were digested with suitable restriction endonucleases and ligated with the pcEXV-3 backbone; the ligation mixture was then used to transform *Escherichia coli* HB101 cells. The correctness of the base substitution and the

Abbreviation used: NMT, myristoyl-CoA:protein N-myristoyltransferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; S6D mutation, serine-6 replaced by aspartic acid; G2A mutation, glycine-2 replaced by alanine; C3S mutation, cysteine-3 replaced by serine.

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absence of unwanted mutations within the remainder of the PCR-derived sequence were verified by DNA sequencing of bulk preparations of plasmid DNA. COS-7 cells were transfected with the pcEXV- $G_{11}\alpha$ plasmids by calcium phosphate precipitation [7]. Routine DNA manipulations, including DNA ligations, bacterial transformations, double-stranded DNA sequencing and plasmid purifications were carried out using standard procedures.

Cell growth and metabolic labelling

COS-7 cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 units/ml penicillin. Cells were metabolically labelled overnight 48 h after transfection with 150 $\mu\text{Ci}/\text{ml}$ [9,10- $^3\text{H}(\text{N})$]palmitic acid (DuPont/NEN, 30–60 Ci/mmol) or [9,10- $^3\text{H}(\text{N})$]myristic acid (DuPont/NEN, 10–60 Ci/mmol) in DMEM containing 5% dialysed FCS, 5 mM pyruvate, antibiotics and L-glutamine as above. Overnight labelling with 50 $\mu\text{Ci}/\text{ml}$ Tran ^{35}S -label® (ICN; > 1000 Ci/mmol) was carried out in DMEM containing 5% of the normal content of L-methionine and L-cysteine, 5% dialysed FCS, antibiotics and L-glutamine as above.

Immunoprecipitation and analysis

The polyclonal rabbit antiserum, IIC [17], was raised against a synthetic peptide predicted to represent amino acids 160–169 of the α -subunit of G_{11} and recognized a single band of molecular mass approx. 42 kDa in immunoblotting experiments. Immunoprecipitation experiments were performed on cells lysed in 0.2 ml of 1% (w/v) SDS containing 0.067 unit/ml aprotinin and 0.2 mM PMSF. After breakage of DNA by repeated pipetting and boiling for 4 min, 0.8 ml of the following mixture (Mix I) was added to each sample: 1.2% (w/v) Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris/HCl (pH 7.5) and protease inhibitors as above. A 1:1 suspension of Protein A–Sepharose® (Pharmacia) beads (20 μl) pre-washed three times in Mix II (4 parts of Mix I plus 1 part of 1% SDS) were added to each sample and left for 2 h at 4 °C with continuous rotation. An aliquot of supernatant was then withdrawn to determine the total radioisotope incorporation into cell proteins. To the remaining samples, 20 μl of the IIC anti- $G_{11}\alpha$ serum or pre-immune rabbit serum were added and the samples were incubated overnight at 4 °C with continuous rotation. Immunoprecipitates were washed three times with Mix II, once with 50 mM Tris/HCl (pH 6.8) and then dissolved in Laemmli loading buffer [18] containing 20 mM dithiothreitol, followed by SDS/12.5% (w/v)-PAGE. Bands were detected by fluorography using pre-flashed Kodak XAR-5 film [19].

Immunoblotting

For immunoblotting, proteins were resolved on SDS/12.5% (w/v)-PAGE and transferred to nitrocellulose membranes [20]. After overnight incubation with a 1000-fold diluted IIC antiserum in 20 mM Tris/HCl buffer (pH 7.5), containing 150 mM NaCl, 5% (w/v) dry skimmed milk, 0.2% (v/v) Tween 20 and 0.02% (w/v) sodium azide, proteins were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG using bromochloroindolyl phosphate and Nitro Blue Tetrazolium as substrates.

Cell fractionation studies

For cell fractionation experiments, cell pellets were carefully resuspended in 0.5 ml of 'low buffer' [5 mM Tris/HCl, 1 mM

MgCl_2 , 1 mM EGTA, 0.1 mM EDTA and protease inhibitors as above (pH 7.5)], incubated on ice for 30 min and passed 20 times through a 1 ml syringe with a 21G needle. Nuclei and debris were removed by centrifugation at 800 g for 5 min in a bench centrifuge at 4 °C. The post-nuclear supernatants were centrifuged for 30 min at 100000 g in a TLA 100.1 rotor of a Beckman TL100 centrifuge at 4 °C. Particulate (P100) fractions were treated as described above for whole cell extracts, and soluble (S100) fractions were similarly adjusted; immunoprecipitation then followed as above.

Treatment of COS-7 cells with 2-hydroxymyristate

2-Hydroxymyristate (Affiniti) was delivered to cells as a complex with BSA. To prepare the complex, 2-hydroxymyristate was first coated on Celite (1 mmol of fatty acid/g of Celite) and then transferred from particles to fatty acid-free BSA (Sigma) as previously described [21,22].

Two days after transfection with relevant cDNA species, COS-7 cells were incubated for 24 h with 1 mM 2-hydroxymyristate complexed with BSA. Cells were metabolically labelled with ^3H -fatty acids or Tran ^{35}S -label for the last 4 h and harvested to be processed as described above.

Measurement of the turnover of $G_{11}\alpha$

In order to determine the half-life of expressed $G_{11}\alpha$, 24 h after transfection COS-7 cells were labelled for 1 h with 50 $\mu\text{Ci}/\text{ml}$ Tran ^{35}S -label® in methionine-free culture medium. Cells were collected at the end of the labelling period (time 0) and 2, 4, 6, 8, 10 and 16 h after replacement of the culture medium with fresh normal growth medium, and processed for immunoprecipitation as described above.

RESULTS AND DISCUSSION

In a previous report [7] we demonstrated that substitution of glycine-2 by alanine (G2A) of $G_{11}\alpha$ by site-directed mutagenesis prevented, as expected, the incorporation of [^3H]myristate into the mutant polypeptide upon expression in COS cells. Unexpectedly, however, this mutation also inhibited the incorporation of [^3H]palmitate. We thus concluded that co-translational addition of myristate to the protein was a prerequisite for subsequent palmitoylation, and suggested two possible explanations: (1) that myristoylation was needed to target $G_{11}\alpha$ to a proper cellular location(s) where palmitoylation could occur, and/or (2) that only the myristoylated form of the protein could act as the substrate for palmitoylation. The latter interpretation may be questioned by recent findings suggesting that palmitoylation may be a chemical rather than an enzymic reaction [23]; C. Bano and A. I. Magee, unpublished work]. However, a third mechanism could also be hypothesized, whereby the absence of incorporation of [^3H]palmitate into the G2A mutant of $G_{11}\alpha$ could be secondary to the G2A mutation rather than to the lack of myristate. Such a suggestion is not without precedent, as mutation of glycine-2 to alanine in the G-protein $G_{12}\alpha$, which is not a substrate for myristoylation as it does not have a serine at codon 6, has been reported to alter the effectiveness of interaction of this protein with the $\beta\gamma$ -complex [24]. This may reflect that the polypeptide can no longer be palmitoylated at cysteine-3, although this has not been examined directly.

To test this hypothesis, we have examined whether wild-type $G_{11}\alpha$ protein could still be palmitoylated after a chemical blockade of its co-translational myristoylation. COS cells, either mock transfected or transfected to express wild-type $G_{11}\alpha$, were treated with 2-hydroxymyristate (1 mM), an inhibitor of NMT [25]. 2-

Figure 1 Turnover of $G_{11}\alpha$ when expressed in COS cells

The turnover of $G_{11}\alpha$ following expression in COS-7 cells was assessed as described in the Materials and methods section. Either mock transfected (m) or cells transfected to express wild-type $G_{11}\alpha$ were metabolically labelled with Tran ^{35}S -label $^{\text{®}}$, and samples were immunoprecipitated with antiserum I1C following removal of the Tran ^{35}S -label $^{\text{®}}$ for various periods of time (0–16 h). Samples were resolved by SDS/PAGE and autoradiographed for 7 days (A). Data from such experiments were quantified by densitometry (B) to allow estimation of the half-life of $G_{11}\alpha$.

Hydroxymyristate is metabolically activated in cells to the CoA-thioester, which can then competitively inhibit NMT activity in cells. Treatment with 2-hydroxymyristate was for 24 h, a period roughly equal to two half-lives of the expressed $G_{11}\alpha$, which was estimated to be 11.4 h, following metabolic labelling of the transfected cells with Tran ^{35}S -label $^{\text{®}}$, subsequent chase following removal of the radiolabel and immunoprecipitation of $G_{11}\alpha$ with antiserum I1C (Figures 1A and 1B). Thus, the bulk of the cellular $G_{11}\alpha$ present at the termination of the experiment would have been synthesized during the period of exposure to 2-hydroxymyristate. A small amount of immunoprecipitated ^{35}S -labelled $G_{11}\alpha$ was detected in cells which had been mock transfected (Figure 1A). This is entirely consistent with our previous studies which have demonstrated a low level of endogenous expression of this G-protein in COS cells [7].

Table 1 and Figure 2 (top), show that 2-hydroxymyristate treatment effectively reduced (by $75 \pm 7\%$; mean $\pm$ S.E.M., $n = 4$) the incorporation of [^3H]myristate into expressed wild-type $G_{11}\alpha$. As noted above, COS cells endogenously express a low level of $G_{11}\alpha$. The incorporation of [^3H]myristate into the endogenously expressed $G_{11}\alpha$ protein was also diminished to approximately the same extent by exposure to 2-hydroxymyristate (Table 1). By contrast, the incorporation of [^3H]palmitate into the expressed wild-type $G_{11}\alpha$ was not decreased ($126 \pm 19\%$; mean $\pm$ S.E.M., $n = 4$) by treatment with 2-hydroxymyristate (Table 1 and Figure 2, top). Thus, the absence of myristate on $G_{11}\alpha$ does not preclude subsequent palmitoylation of the expressed polypeptide.

As a serine at position 6 is required to provide the high-affinity consensus sequence for NMT [14], we generated a mutant (S6D) of $G_{11}\alpha$ in which serine-6 was replaced by aspartic acid. Transfection of this construct into COS cells resulted in expression of a polypeptide which had little ability to incorporate [^3H]myristic acid ($7 \pm 4\%$; mean $\pm$ S.E.M., $n = 3$) compared with incorporation of [^3H]myristic acid into the wild-type protein (Figure 2,

Table 1 Effect of 2-hydroxymyristate on incorporation of [^3H]myristate and [^3H]palmitate into transiently expressed forms of $G_{11}\alpha$

Fluorographs such as those of Figure 2, top, were scanned. The incorporation of the ^3H -labelled fatty acids into wild-type $G_{11}\alpha$ was taken to represent 100% and the other data are presented relative to this value. Data are presented as means $\pm$ S.E.M., $n = 4$ in the case of [^3H]palmitate and $n = 3$ for [^3H]myristate.

Transfection/treatment	[^3H]Myristate	[^3H]Palmitate
Mock	23 ± 7	2 ± 1
Wild-type $G_{11}\alpha$	100	100
Wild-type $G_{11}\alpha$ + 2-hydroxymyristate	25 ± 7	126 ± 19
S6D $G_{11}\alpha$	7 ± 4	5 ± 2

Figure 2 Palmitoylation defines membrane association of $G_{11}\alpha$

Top: 2-hydroxymyristate inhibits myristoylation but not palmitoylation of wild-type $G_{11}\alpha$ whereas S6D $G_{11}\alpha$ is a substrate for neither myristoylation nor palmitoylation. COS-7 cells were either mock transfected (lanes 1 and 2) or transfected with the plasmids pcEXV- $G_{11}\alpha$ (lanes 3 and 4) or pcEXV-S6D $G_{11}\alpha$ (lane 5) as described in the Materials and methods section. For the last 4 h cells were also labelled with [^3H]myristate (left hand panel) or [^3H]palmitate (right hand panel) in the absence (lanes 1, 3 and 5) or presence (lanes 2 and 4) of 2-hydroxymyristate (1 mM). Samples were subsequently immunoprecipitated with antiserum I1C, resolved by SDS/PAGE and fluorographed for 1 month. Bottom: the myristoylation status of $G_{11}\alpha$ does not determine its cellular distribution. COS-7 cells transfected as in the top panel, were subsequently metabolically labelled with Tran ^{35}S -label $^{\text{®}}$ (^{35}S -met) as described in the Materials and methods section. The cells were fractionated into particulate (p) and cytosolic (c) fractions as described in the Materials and methods section and immunoprecipitated with antiserum I1C, resolved by SDS/PAGE and autoradiographed for 7 days. A representative autoradiograph is displayed. Lanes are numbered as for the top panel.

top, and Table 1). This polypeptide also, however, failed completely to incorporate [^3H]palmitate (Table 1 and Figure 2, top).

Transfection of COS cells to express wild-type $G_{11}\alpha$ and metabolic labelling with Tran ^{35}S -label, followed by fractionation into cytosolic and particulate fractions, revealed that the bulk of the newly synthesized polypeptide was associated with the particulate fraction. Treatment of the cells with 2-hydroxymyristate did not affect partitioning of the ^{35}S -labelled $G_{11}\alpha$ between these fractions (Table 2 and Figure 2, bottom). By contrast, the cellular distribution of the expressed S6D mutant of $G_{11}\alpha$ was altered compared with the wild-type protein, with the majority of the expressed polypeptide now being found in the cytoplasmic fraction (Table 2 and Figure 2, bottom).

Studies using acylated peptides have indicated that the presence of myristic acid provides barely sufficient energy to anchor a peptide or polypeptide to a biological membrane. By contrast, palmitate has sufficient binding energy to anchor a protein stably [15]. Early studies on the G_i -family of G-proteins, involving mutagenesis of the glycine at codon 2, demonstrated that this prevented myristoylation of the polypeptide and also eliminated its membrane association [4–5]. Such data were taken to indicate

Table 2 Cellular distribution of transiently expressed wild-type and S6DG₁₁α following hypotonic lysis

Fluorographs such as those of Figure 2, bottom, were scanned. The cellular distribution pattern (P100 = particulate, C100 = cytoplasmic) of both endogenously expressed G₁₁α (mock transfected) and the transfected forms of G₁₁α are quantified whereby the total cellular content of the ³⁵S-labelled G-protein polypeptide is presented as 100%. Data are presented as means ± S.E.M., *n* = 3.

Transfection/treatment	Tran ³⁵ S-label	
	P100	C100
Mock	87 ± 7	13 ± 7
Wild-type G ₁₁ α	84 ± 5	16 ± 5
Wild-type G ₁₁ α + 2-hydroxymyristate	86 ± 8	14 ± 8
S6DG ₁₁ α	37 ± 13	63 ± 13

that myristoylation played a crucial role in this process [4–5]. However, these studies were performed before the appreciation that these G-proteins were also targets for palmitoylation [1–3], and a number of studies have indicated that generation of glycine-2 mutations severely compromises the ability of the expressed polypeptides to act as substrates for palmitoylation [7–9]. This however, does not seem to be an absolute, as recent data from Degtyarev et al. have demonstrated that a transiently expressed G2A mutant of G₁₁α is targeted to the membrane compartment and can be palmitoylated provided that G-protein βγ dimers are co-expressed [26]. The possibility that co-translational attachment of myristate to G₁₁α is not inherently required to allow palmitoylation of the wild-type G₁₁α sequence has been explored herein, as has the question of the relative importance of attachment of myristate and palmitate to G₁₁α for membrane association. The results demonstrate clearly that the myristoylation status of G₁₁α does not define the membrane association of this polypeptide, because the cellular distribution profile of recently synthesized copies of this polypeptide is unaltered in cells treated with 2-hydroxymyristate, which caused substantial inhibition of myristoylation of the polypeptide without inhibiting the incorporation of [³H]palmitate. Prevention of both myristoylation and palmitoylation of G₁₁α by construction and expression of an S6D mutant resulted in the bulk of the expressed polypeptide being found in the cytoplasmic fraction of the cell. This pattern of cellular distribution of G₁₁α following expression in COS cells is very reminiscent of those we have previously recorded for C3S (cysteine-3 replaced by serine) G₁₁α, which prevents palmitoylation but not myristoylation, and for both a G2A and a double G2A/C3S mutant [7], which are neither myristoylated nor palmitoylated, following expression in COS-cells [7]. These data strongly support the hypothesis that the key lipid element in membrane association of G₁₁α is the presence of palmitate at cysteine-3. We do not yet know, however, what are the absolute structural requirements in this polypeptide to allow palmitoylation to occur. The presence of myristate on glycine-2 is not a prerequisite (Figure 2, top). However, simply the maintained presence of unmodified glycine at the N-terminus (following removal of the initiator methionine) is also insufficient, as S6DG₁₁α did not act as a substrate for palmitoylation (Figure 2, top). Alteration of serine-6 to aspartic acid is a non-conservative substitution, but the studies presented with this mutant indicate once more that interpretation of data following site-directed mutagenesis must be treated with caution.

Membrane association of G₁₁α and other G_i-like G-proteins

may not be defined simply by the lipid acylation status of the polypeptide. We demonstrate herein that a fraction of even the expressed non-acylated S6DG₁₁α was associated with the particulate fraction. This again is similar to our previous data with transient expression in COS cells of G2A and G2A/C3S mutants of this polypeptide [7]. Furthermore, stable expression of a palmitoylation-resistant C3S mutant of G₀₁α in Rat 1 fibroblasts results in a substantial fraction of the polypeptide being membrane associated following hypotonic lysis of the cells [27]. This may reflect the ability of the mutant polypeptide to interact with the βγ-complex [27]. The N-terminus of G-protein α-subunits is known to be involved in binding to the βγ-complex [28], but it is not currently known whether the G2A or S6D mutations may compromise this interaction and thus interfere with membrane localization through βγ binding, resulting in an indirect inhibition of palmitoylation. Thus, although palmitoylation plays a key role in membrane association of these polypeptides it may not be the only relevant factor.

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