The role of palmitoylation of the guanine nucleotide binding protein G₁₁α in defining interaction with the plasma membrane

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Mutations of Cys-9 to serine, Cys-10 to serine and a combination of both alterations were produced in a cDNA encoding murine G₁₁α to potentially interfere with the ability of the expressed polypeptides to act as substrates for post-translational palmitoylation. Each of these mutants and the wild-type protein were expressed in simian COS-1 cells. Mutation of either cysteine-9 or cysteine-10 decreased the degree of palmitoylation of the protein by some 80% compared with the wild-type, while the double mutant totally failed to incorporate [³H]palmitate. By contrast, in all transfections the endogenously expressed simian G₁₁α incorporated [³H]palmitate to similar levels. Particulate and cytoplasmic fractions from these cells were subjected to SDS/PAGE under conditions which allow resolution of primate and rodent forms of G₁₁α. Immunoblotting of these fractions demonstrated that in all cases the endogenously expressed simian G₁₁α was exclusively associated with the particulate fraction, as was the transfected and expressed wild-type murine G₁₁α. By contrast, each of the mutated forms of murine G₁₁α displayed a distribution in which approx. 70% of the expressed protein was present in the particulate fraction and 30% in the supernatant. To examine the conformation of the particulate expressed forms of murine G₁₁α, these fractions were treated with various concentrations of sodium cholate and immunoblots were subse-

quently performed on the solubilized and remaining particulate proteins. Whereas essentially all of the endogenous simian G₁₁α was solubilized by treatment with 1% (w/v) sodium cholate and some 50% with 0.32% cholate, expressed wild-type murine G₁₁α was more recalcitrant to solubilization. However, that fraction of wild-type murine G₁₁α which was solubilized behaved identically to the endogenous simian G₁₁α on Superose-12 gel-exclusion chromatography. The particulate fraction of the C9S/C10S double mutant of murine G₁₁α was highly resistant to solubilization by sodium cholate, whereas the particulate fractions of the two single cysteine to serine mutants were intermediate to the wild-type and double mutant in their ability to be solubilized by this detergent. These data demonstrate that the palmitoylation status of the cysteine residues at positions 9 and 10 in murine G₁₁α plays a central role in defining membrane association of this G-protein and indicate that much of the particulate fraction of the expressed palmitoylation-resistant mutants is likely to represent non-functional rather than correctly folded protein. The use of transient assays to assess the functionality of particulate, palmitoylation-resistant, mutant G-protein α subunits is shown to be inappropriate without strict controls.

INTRODUCTION

The role of lipidation of the N-terminal region of the α subunits of heterotrimeric G-proteins, particularly in relation to the membrane association of these polypeptides, has been an intensively examined question [1,2]. Members of the G_i family of pertussis toxin-sensitive G-proteins become co-translationally N-terminally modified by addition of myristate. As mutation of glycine-2 to alanine (G2A) prevents both myristoylation of such polypeptides and their association with the plasma membrane [3,4], it has generally been assumed that this acylation plays a key role. However, the α subunits of members of each of the G_s [5–8], G_q [5,7] and G₁₂ [9] families of G-proteins are not substrates for myristoylation but do become membrane-associated and have recently been shown to become palmitoylated. The observations that probably all of the widely expressed G-protein α subunits can be palmitoylated close to their N-terminus [5–8] and that this acylation may be dynamic [10–12], and the realization

that G2A mutations of the G_i family α subunits frequently prevents their palmitoylation [10,13,14] unless steps are taken to co-express G-protein β and γ subunits at the same time [15], have raised the possibility that the palmitoylation status of a G-protein α subunit may play a key role in membrane association and potentially in interactions of the G-protein with receptor and effector [7,16].

In studies to date on the role of palmitoylation in the membrane association of G-protein α subunits, particularly those examining G_sα and G_qα (which are not substrates for myristoylation), very different results have been obtained in individual reports [5–8,16], where the apparent cellular distribution of the mutant proteins has varied from fully particulate to totally cytoplasmic. Such discrepancies clearly indicate a requirement for more stringent controls to be performed. In the present study we examine the role of palmitoylation of murine G₁₁α in membrane association by expressing wild-type and palmitoylation-deficient mutant forms of this G-protein in COS-1 cells and analysing the

Abbreviations used: G₁₁α, G_qα, α subunits of two closely related G-proteins which have been shown to allow regulation of phospholipase C-β1 in a fashion insensitive to treatment of cells with pertussis toxin; TE, 10 mM Tris/HCl, 1 mM EDTA, pH 7.5; DMEM, Dulbecco's modified Eagle's medium.

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palmitoylation and fractionation of the expressed murine polypeptides concurrently with that of endogenously expressed simian $G_{11\alpha}$. We also examine the physical state of the particulate and cytoplasmic fractions of the polypeptides following transient expression in COS-1 cells.

MATERIALS AND METHODS

Materials

Ampicillin and ATP were from Sigma (Poole, Dorset, U.K.). Nitrocellulose was from Costar (Cambridge, MA, U.S.A.). T4 polynucleotide kinase and T4 DNA ligase were from Promega (Southampton, U.K.). Lipofectin and all cell culture reagents were from Life Technologies (Paisley, U.K.). Trans[35 S] label [L-[35 S]methionine; [35 S]cysteine (> 1000 Ci/mmol)] was from ICN, and [9,10- 3 H(n)]palmitic acid (30–60 Ci/mmol) was from DuPont/NEN. All other reagents were from Fisons and were of the highest grade commercially available.

Phosphorylation of oligonucleotides

Samples of 500 μ l of the oligonucleotides used for mutagenesis of $G_{11\alpha}$ (Table 1), in dilute ammonium hydroxide, were dried in a Jouan RC 10.22 centrifugal evaporator, resuspended in 20 μ l of sterile water and dried again under vacuum. The resulting pellet was resuspended in sterile water and phosphorylated in the presence of 1 mM ATP and 24 units of T4 polynucleotide kinase, in a total volume of 50 μ l, for 2 h. The phosphorylated oligonucleotide was then isolated by ethanol precipitation.

Generation of Cys 9→Ser (C9S), Cys-10→Ser (C10S) and C9S/C10S mutants of murine $G_{11\alpha}$

Murine $G_{11\alpha}$ in the mammalian expression vector pCMV was a gift from Dr. M. I. Simon (California Institute of Technology, Pasadena, CA, U.S.A.). The cDNA encoding murine $G_{11\alpha}$ was subcloned into the plasmid pSV Sport 1 (Life Technologies) to minimize the size of the overall construct. Deletion PCR mutagenesis was carried out in the presence of 250 ng of pSV Sport 1-murine $G_{11\alpha}$, 250 μ M dNTP and 25 pmol of each of the relevant pair of phosphorylated oligonucleotides, as shown in Table 1. The reactions were overlaid with one drop of mineral oil and heated to 94 °C for 10 min before 2.5 units of Pfu DNA polymerase was added. The entire plasmid containing the murine $G_{11\alpha}$ cDNA was amplified with cycles of denaturation (94 °C for 42 s), annealing (57 °C for 60 s) and extension (72 °C for 10 min) for 30 cycles.

Following mutagenesis, the reaction product was electrophoresed on a low-melting-point agarose gel and purified on a PCR Prep column (Promega). The purified fragment was blunt-ended by treatment with 5 units of Klenow fragment for 3 h at 37 °C, repurified on a PCR Prep column, self-ligated using T4

DNA ligase and transformed into JM109 *Escherichia coli*. Ampicillin-resistant colonies were selected and plasmid DNA was prepared from them. The integrity of the PCR mutagenesis was verified by sequencing this DNA on an ABI 373A automated DNA sequencer using the Taq Dyedexy Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, U.K.) according to the manufacturer's instructions. The resulting murine $G_{11\alpha}$ mutants were then subcloned back into the original pCMV vector prior to expression.

Maintenance, transfection and metabolic labelling of COS-1 cells

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin in an atmosphere of 5% CO_2 at 37 °C. Cells were transfected at approx. 40% confluency with pCMV containing wild-type, C9S, C10S or C9S/C10S murine $G_{11\alpha}$ using either Lipofectin reagent (Life Technologies) according to the manufacturer's instructions or DEAE-dextran. For transfections (in 100 mm plates) using DEAE-dextran, 5 μ g of DNA in 250 μ l of sterile TE buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA) was combined with 200 μ l of sterile DEAE-dextran (10 mg/ml in PBS) and incubated at room temperature for 30 min. After 30 min the medium of the COS cells to be transfected was replaced with DMEM supplemented with 5 ml of 10% NUserum (Strattech Scientific Ltd.), 100 mM chloroquine and the DNA/dextran mixture added for 3 h. The cells were then shocked for 2 min with 10% (v/v) DMSO in PBS, washed once with PBS then incubated in DMEM plus 10% newborn calf serum as before. Cells were harvested after 72 h and particulate (P200) and cytosolic (S200) fractions were prepared as previously described [5,17]. Protein concentrations were determined by the method of Lowry et al. [18] using BSA as standard. Cells were metabolically labelled overnight 48 h after transfection with 150 μ Ci/ml [9,10- 3 H(n)]palmitic acid (DuPont/NEN; 30–60 Ci/mmol) in DMEM containing 5% dialysed foetal calf serum, 5 mM pyruvate, antibiotics and L-glutamine as above. Overnight labelling with 25 μ Ci/ml Trans[35 S] label (L-[35 S]methionine; [35 S]cysteine) (ICN; > 1000 Ci/mmol) was carried out in DMEM containing one-twentieth of the normal content of L-methionine and L-cysteine, 5% dialysed foetal calf serum, antibiotics and L-glutamine as above.

Immunoprecipitation and analysis

The polyclonal rabbit antiserum CQ [19] was raised against a synthetic peptide predicted to represent the C-terminal decapeptide shared by $G_{11\alpha}$ and $G_q\alpha$. Immunoprecipitation experiments were performed on cells lysed in 0.2 ml of 1% (w/v) SDS containing 0.067 trypsin inhibitory units/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 20 μ l sample of a 1:1 (w/v) suspension of Protein A-Sepharose (Pharmacia) beads prewashed three times in Mix II (4 parts of Mix I plus 1 part of 1% SDS) was added to each sample and left for 2 h at 4 °C with continuous rotation. An aliquot of supernatant was then withdrawn to determine total radioisotope incorporation into cell proteins. To the remaining samples was added 20 μ l of the CQ antiserum or pre-immune rabbit serum, and the samples were incubated overnight at 4 °C with continuous rotation. Immunoprecipitates were washed three times with Mix II and once with 50 mM Tris/HCl (pH 6.8), and then dissolved in Laemmli loading buffer con-

Table 1 Oligonucleotides employed to mutate murine $G_{11\alpha}$

The mutagenic bases are underlined. PCR mutagenesis was performed as described in the Materials and methods section using Pfu polymerase.

Oligonucleotide	Sense	Antisense
C9S α 11	5' TGCCCTGAGCGACGAGGTG 3'	5' AGACGCCATCATGGACTCCAG 3'
C10S α 11	5' TCCCTGAGCGACGAGGTG 3'	5' ACACGCCATCATGGACTCCAG 3'
C9S/C10S α 11	5' TCCCTGAGCGACGAGGTG 3'	5' AGACGCCATCATGGACTCCAG 3'

taining 20 mM dithiothreitol. Electrophoresis was accomplished as described below. Bands were detected by fluorography using pre-flashed Kodak XAR-5 film.

Sodium cholate extractions

Cells were harvested as normal, freeze/thawed, resuspended in 400 μ l of TE and homogenized with a Teflon/glass homogenizer. The samples were centrifuged at 200 000 *g* for 30 min at 4 °C and the soluble fraction (S200) was retained. The particulate fraction was resuspended in a final volume of 500 μ l of extraction buffer (10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl) with 0.1 %, 0.32 % or 1 % sodium cholate. The samples were incubated at 4 °C with stirring for 1 h and then centrifuged at 200 000 *g* for 30 min at 4 °C, generating a soluble supernatant and an insoluble pellet.

Superose gel-exclusion chromatography

A 200 μ l portion of soluble supernatant extracted with 1 % (w/v) sodium cholate as described above was applied to a Superose-12 gel filtration column (Pharmacia) which had been equilibrated with 2 column vol. of extraction buffer containing 1 % (w/v) sodium cholate. The sample was eluted by washing with extraction buffer; 500 μ l samples were collected, precipitated with trichloroacetic acid and then analysed by SDS/PAGE in conditions, as described below, suitable for the resolution of simian and murine G₁₁α. Molecular size standards [BSA (66.2 kDa), ovalbumin (42.7 kDa), myoglobin (16.7 kDa) and cytochrome *c* (12.3 kDa)] were chromatographed under identical conditions. Blue Dextran 200 was used to define the void volume.

Electrophoretic separation of species variants of G₁₁α

Membrane and cytosolic fractions were electrophoresed by SDS/PAGE in a 10 % (w/v) acrylamide gel containing 6 M urea. These conditions separate murine G₁₁α from simian G₁₁α and G_qα [20], as was confirmed in experiments using the G₁₁α-specific antiserum E976 [21] (a gift from Dr. J. H. Exton, Vanderbilt University, Nashville, TN, U.S.A.) (see the Results section). Proteins were transferred to nitrocellulose and probed with antiserum E976 or with antiserum CQ, which recognizes the C-terminal decapeptide of G₁₁α and G_qα, and visualized as described [22]. Following development, blots were scanned and quantified on a Bio-Rad GS670 imaging densitometer (Bio-Rad, Hemel Hempstead, U.K.) linked to an Apple Macintosh Quadra 800 computer.

RESULTS

COS-1 cells express endogenously simian G₁₁α (Figure 1). Transfection of these cells with a cDNA encoding G₁₁α (Figure 1) followed by resolution of membranes from these cells in 10 % (w/v) acrylamide SDS/PAGE supplemented with 6 M urea and immunoblotting with the G₁₁α-specific antiserum E976 [21] demonstrated considerable resolution of these species variants of this G-protein. This system thus allowed unambiguous concurrent detection of these two polypeptides.

A PCR-based strategy (see the Materials and methods section) was used to generate each of the C9S, C10S and C9S/C10S mutants of murine G₁₁α. Sequencing confirmed the presence of the anticipated base alterations without other mutations in the G-protein. Expression of the wild-type and each of the mutated forms of murine G₁₁α was achieved by transfection into COS-1 cells. Labelling of these cells with [³H]palmitate and resolution of membranes from these labelled cells demonstrated equivalent

Figure 1 Resolution of co-expressed simian and murine G₁₁α

Membranes (50 μ g) prepared from COS-1 cells which were either mock-transfected (lane 1) or transfected with a cDNA encoding wild-type murine G₁₁α (lane 2) were resolved in 6 M-urea-containing SDS/PAGE and immunoblotted using the G₁₁α-specific antiserum E976 [21]. The endogenous expression of simian G₁₁α-specific antiserum E976 [21]. The endogenous expression of murine G₁₁α is shown. The left-hand lane demonstrated the mobility of prestained lactic dehydrogenase. In this system this marker routinely migrates as a broad smear.

Figure 2 C9S, C10S and C9S/C10S variants of murine G₁₁α incorporate [³H]palmitate poorly compared with the wild-type polypeptide

COS-1 cells transfected as in Figure 1 were labelled with [³H]palmitate as described in the Materials and methods section and immunoprecipitated with antiserum CQ. Resolution of the immunoprecipitates in 6 M-urea-containing SDS/PAGE was followed by autoradiography for 28 days. The detected polypeptides which migrate more rapidly through the gel are the forms of murine G₁₁α. Similar levels of incorporation of [³H]palmitate were observed into the endogenous simian G₁₁α in each transfection (lanes 1–5). Incorporation of [³H]palmitate into both C9S (lane 3) and C10S (lane 4) murine G₁₁α was only some 20% (see Table 2) of that seen in the wild-type polypeptide (lane 2). No detectable incorporation of [³H]palmitate into C9S/C10S murine G₁₁α (lane 5) was noted. Lane 1 represents a mock transfection and thus only simian G₁₁α is present. Only the relevant section of the autoradiogram is displayed to allow observation of the separation of murine from simian versions of G₁₁α.

overall incorporation of [³H]palmitate into a range of polypeptides (results not shown). Immunoprecipitation using antiserum CQ [19] of both endogenous simian G₁₁α/G_qα and the murine forms of G₁₁α from such [³H]palmitate-labelled transfections and their separation in SDS/PAGE containing 6 M urea demonstrated that, in each of mock-transfected cells and in the cells transfected with the variants of murine G₁₁α, a polypeptide corresponding to simian G₁₁α/G_qα incorporated [³H]palmitate (Figure 2). Incorporation of [³H]palmitate was also observed into an immunoprecipitated polypeptide migrating more rapidly than simian G₁₁α in cells transfected with wild-type murine G₁₁α, but an equivalent radiolabelled polypeptide was not observed in the immunoprecipitates of mock-transfected cells (Figure 2). The degree of incorporation of [³H]palmitate into the expressed C9S and C10S forms of murine G₁₁α was markedly lower than in the wild-type protein, and the expressed C9S/C10S double mutant totally failed to incorporate [³H]palmitate (Figure 2). This was not a reflection of differences in the expression of the individual cDNA constructs, as levels of each of the variants of murine G₁₁α were similar when either analysis of immunoprecipitates of Trans[³⁵S]-labelled cells (results not shown) or specific detection by immunoblotting (see Figure 3) was examined. Quantification of incorporation of [³H]palmitate into these polypeptides (Table

Table 2 Quantification of [^3H]palmitate incorporation into wild-type and mutant variants of murine $\text{G}_{11}\alpha$

Experiments such as that of Figure 2 were analysed. COS-1 cells which were either mock-transfected or transfected with cDNAs encoding murine wild-type, C9S, C10S or C9S/C10S $\text{G}_{11}\alpha$ were resolved in 6 M-urea-containing SDS/PAGE. The absorbance of the signal from the incorporation of [^3H]palmitate into endogenous simian $\text{G}_{11}\alpha/\text{G}_q\alpha$ and the various forms of transfected murine $\text{G}_{11}\alpha$ was assessed. Data are presented as means $\pm$ half of the range.

Transfection	100 $\times$ Absorbance (units)	
	Endogenous simian $\text{G}_{11}\alpha/\text{G}_q\alpha$	Transfected murine $\text{G}_{11}\alpha$
Mock	20.0 $\pm$ 2.2	0
Wild-type	23.7 $\pm$ 5.9	22.6 $\pm$ 5.0
C9S	16.9 $\pm$ 8.0	5.1 $\pm$ 4.2
C10S	13.7 $\pm$ 1.6	2.7 $\pm$ 1.8
C9S/C10S	13.8 $\pm$ 0.8	0

Figure 3 Effect of palmitoylation status on membrane association of mutants of murine $\text{G}_{11}\alpha$

(a) Wild-type murine $\text{G}_{11}\alpha$ is entirely particulate. COS-1 cells which were either mock-transfected (lanes 1 and 2) or transfected with a cDNA encoding wild-type murine $\text{G}_{11}\alpha$ (lanes 3 and 4) were separated into particulate (lanes 1 and 3) and supernatant (lanes 2 and 4) fractions, resolved in 6 M-urea-containing SDS/PAGE and immunoblotted using antiserum CQ as primary reagent. Both the simian and murine $\text{G}_{11}\alpha$ were associated entirely with the particulate fractions. (b) Palmitoylation-resistant mutants of murine $\text{G}_{11}\alpha$ distribute between the particulate and supernatant fractions. COS-1 cells which had been either mock-transfected (lanes 1 and 2) or transfected with cDNAs encoding C9S (lanes 3 and 4), C10S (lanes 5 and 6) or C9S/C10S (lanes 7 and 8) variants of murine $\text{G}_{11}\alpha$ were separated into particulate (lanes 1, 3, 5 and 7) and supernatant (lanes 2, 4, 6 and 8) fractions, resolved in 6 M-urea-containing SDS/PAGE and immunoblotted using antiserum CQ as primary reagent. In all cases the endogenous simian $\text{G}_{11}\alpha$ was entirely associated with the particulate fraction, whereas each of the mutant forms of murine $\text{G}_{11}\alpha$ was distributed in both the particulate and supernatant fractions. No obvious differences were noted in the relative distributions of the three mutants.

2) demonstrated that the immunoprecipitated simian $\text{G}_{11}\alpha/\text{G}_q\alpha$ incorporated similar levels of [^3H]palmitate in each of the transfections, also indicating that similar immunoprecipitation efficiencies were obtained with each sample. Quantification of the incorporation of [^3H]palmitate into each of the C9S and C10S mutants of murine $\text{G}_{11}\alpha$ demonstrated this to be substantially less than 50% (C9S, mean = 22.7%; C10S, mean = 11.9%) of that observed in the wild-type polypeptide (Table 2).

Figure 4 Analysis of solubilized wild-type and C9S/C10S murine $\text{G}_{11}\alpha$ by Superose-12 gel-exclusion chromatography

The particulate fractions from COS cells which had been transfected with cDNAs encoding either wild-type (a) or C9S/C10S mutant (b) murine $\text{G}_{11}\alpha$ were solubilized with 1% (w/v) sodium cholate. A third of each of the samples was removed and the remainder was centrifuged at 200 000 g for 30 min at 4 °C. One half of the soluble fraction was retained, with the remainder being loaded on to a Superose-12 gel-exclusion column. The column was eluted, and 500 μl fractions were collected and precipitated with a final concentration of 6% (w/v) trichloroacetic acid. Samples were then resolved in 6 M-urea-containing SDS/PAGE and immunoblotted using antiserum CQ as primary reagent. In both panels the total starting material is shown in lane 1 and the solubilized protein is in lane 2, while lanes 3–12 represent individual fractions from the column ranging from $V_e = 10.5$ ml (lane 3) to $V_e = 15$ ml (lane 12). Panel (a) demonstrates that the solubilized wild-type murine $\text{G}_{11}\alpha$ behaved identically to the endogenous simian $\text{G}_{11}\alpha$. By contrast, (b) shows both the transfected C9S/C10S murine $\text{G}_{11}\alpha$ and the endogenous $\text{G}_{11}\alpha$ in the total protein sample (lane 1), but only the endogenous simian polypeptide in the soluble fraction (lane 2).

Transfection of COS-1 cells with wild-type murine $\text{G}_{11}\alpha$ followed by fractionation of the cells into particulate and supernatant samples prior to protein resolution and immunoblotting with antiserum CQ demonstrated that essentially the entire cellular complement of the endogenously expressed simian $\text{G}_{11}\alpha$ was particulate, as was the wild-type murine $\text{G}_{11}\alpha$ (Figure 3a). By contrast, expression of each of the C9S, C10S and C9S/C10S mutants of murine $\text{G}_{11}\alpha$ resulted in a different pattern of distribution. In each case some 30% of the expressed mutant forms of murine $\text{G}_{11}\alpha$ were found in the supernatant fraction and some 70% in the particulate fraction after cellular disruption (Figure 3b). In all cases this was not a reflection of particulate material being present in the supernatant fractions, as all of the endogenously expressed simian $\text{G}_{11}\alpha$ remained in the particulate fractions (Figure 3b). In each case, following expression of the murine $\text{G}_{11}\alpha$ forms, a weakly immunostained polypeptide which migrated more rapidly through the gel than the major band of murine $\text{G}_{11}\alpha$ was noted (Figures 3a and 3b). This polypeptide was distributed approximately equally between the particulate and supernatant fractions (Figure 3b).

Because of the observed mixture of particulate and cytoplasmic fractions of the murine $\text{G}_{11}\alpha$ mutants, we wished to ascertain whether murine $\text{G}_{11}\alpha$ which was present in the COS cell particulate fraction following transient expression was correctly folded or might represent some non-physiological aggregation. The particulate fraction of such cells following expression of wild-type murine $\text{G}_{11}\alpha$ was solubilized with 1% (w/v) sodium cholate, a condition widely used to solubilize G-proteins from membranes. While all of the endogenous simian $\text{G}_{11}\alpha$ was solubilized by such treatment, clearly only a proportion of the murine $\text{G}_{11}\alpha$ was solubilized (Figure 4a, compare lanes 1 and 2). Analysis of the solubilized samples by Superose-12 gel-exclusion chromatography followed by SDS/PAGE resolution of the

Figure 5 A palmitoylation-negative mutant of murine $G_{11}\alpha$ is resistant to sodium cholate solubilization

Particulate fractions prepared from COS cells which had been transfected with cDNAs encoding either wild-type (a) or C9S/C10S mutant (b) murine $G_{11}\alpha$ were solubilized with 0.1% (lanes 1 and 2), 0.32% (lanes 3 and 4) or 1% (lanes 5 and 6) (w/v) sodium cholate. The resulting soluble (lanes 2, 4 and 6) and non-soluble (lanes 1, 3 and 5) samples were then resolved in 6 M-urea-containing SDS/PAGE and immunoblotted using antiserum CQ as primary reagent. In both (a) and (b) the endogenous simian $G_{11}\alpha$ was fully solubilized by 1% (w/v) sodium cholate, with similar levels of the wild-type murine $G_{11}\alpha$ being solubilized. In contrast, (b) shows that the C9S/C10S mutant of murine $G_{11}\alpha$ was virtually resistant to sodium cholate extraction.

Figure 6 The C9S and C10S mutants of murine $G_{11}\alpha$ display an ability to be solubilized intermediate between the wild-type protein and the C9S/C10S double mutant

Particulate fractions prepared from COS cells which had been transfected with cDNAs encoding each of wild-type (lanes 1 and 2), C9S (3 and 4) C10S (5 and 6) or C9S/C10S (7 and 8) murine $G_{11}\alpha$ were solubilized with 1% (w/v) sodium cholate. The resulting soluble (lanes 2, 4, 6 and 8) and particulate (lanes 1, 3, 5 and 7) fractions were then resolved in 6 M-urea-containing SDS/PAGE and immunoblotted using antiserum CQ as primary reagent. In the experiment displayed the endogenous simian $G_{11}\alpha$ was largely solubilized in all samples, while some 21% of the wild-type murine $G_{11}\alpha$, 9% of both the C9S and C10S mutants and only 2% of the C9S/C10S mutant was found in the detergent-solubilized fraction.

fractions and immunoblotting as described above demonstrated that the solubilized wild-type murine $G_{11}\alpha$ behaved identically to the endogenous simian $G_{11}\alpha$ (Figure 4a). By contrast, when the same procedure was carried out with the particulate fraction derived from cells transiently transfected to express murine C9S/C10S $G_{11}\alpha$, only the endogenous simian polypeptide was detected in the subsequent immunoblots (Figure 4b). Such data suggested that the particulate mutant murine $G_{11}\alpha$ polypeptide might be resistant to solubilization by sodium cholate. To investigate this possibility, the particulate fraction of COS cells transfected to express wild-type murine $G_{11}\alpha$ was treated with concentrations of sodium cholate between 0.1 and 1% (w/v) (1 h, 4 °C) and the solubilized material and that which was not solubilized by this treatment was separated by SDS/PAGE to resolve simian and murine $G_{11}\alpha$ and immunoblotted as above. All of the immunodetectable endogenous simian $G_{11}\alpha$ was solubilized by treatment with 1% (w/v) sodium cholate, and some 50% was solubilized by treatment with 0.32% (w/v) cholate (Figure 5a). By contrast, the particulate wild-type murine

$G_{11}\alpha$ was more resistant to solubilization. Even with 1% (w/v) sodium cholate only some 30% was solubilized (Figure 5a). As anticipated from the results of Figure 4, sodium cholate treatment of the particulate fraction of COS cells transiently transfected to express the C9S/C10S double mutant of murine $G_{11}\alpha$ resulted in little solubilization of the mutant murine protein, while the endogenous simian polypeptide was effectively solubilized (Figure 5b). To further examine this question, the particulate fractions of COS cells transiently transfected to express wild-type or each of the C9S, C10S and C9S/C10S mutants of murine $G_{11}\alpha$ were treated with 1% (w/v) sodium cholate (1 h, 4 °C) and analysed as above. Both the C9S and C10S mutants displayed an ability to be solubilized that was intermediate between that of the wild-type protein and the C9S/C10S double mutant (Figure 6).

DISCUSSION

It is now apparent from a variety of studies that the palmitoylation status of the α subunits of a variety of widely expressed G-proteins can influence their association with cellular membranes [5–8,10–17]. These G-proteins are modified via thioester linkages of palmitate to one or more cysteine residues located in the extreme N-terminal region of the polypeptide. The degree of importance of such acylation has, however, not been easy to ascertain as results have varied widely between studies. For example, in studies on the α subunit of G_s , mutation of cysteine-3 has been reported either to render the expressed polypeptide largely cytoplasmic [7] or to have little effect on the ability of the polypeptide to be targeted to the particulate fraction [6,8]. By contrast, in the case of a C3S mutation of the α subunit of G_{11} , all reports [5,10,17] have indicated that the mutant is partially membrane-associated and partially present in the supernatant after cell fractionation. However, as $G_{11}\alpha$ is myristoylated on glycine-2 as well as being palmitoylated on cysteine-3, and since a glycine-2 to alanine mutation prevents both myristoylation and palmitoylation of the polypeptide [10] (as is also the case for other G-protein α subunits such as $G_{12}\alpha$ [14] and $G_{13}\alpha$ [13] with the initial sequence MGC), then it is difficult to determine the relative contributions of these two lipidations to membrane association.

The phosphoinositidase $C\beta$ family-linked G-proteins G_{11} and G_q are also substrates for palmitoylation [5,7] but, as in the case of the neuronal protein GAP 43, in these polypeptides dual palmitoylation occurs on adjacent cysteines [positions 9 and 10 in G_q [7] and G_{11} (the present study)]. Wedegaertner et al. [7] have reported the transiently expressed C9S/C10S double mutant of $G_q\alpha$ to be entirely cytosolic, with both the C9S and C10S single mutants being predominantly cytosolic. By contrast Edgerton et al. [16] have reported that expression in COS cells of a C9A/C10A palmitoylation-resistant mutant of $G_q\alpha$ resulted in a predominantly particulate distribution which was not different from that observed on expression of the wild-type protein.

Because of the variability of these reports over an issue which would seem to be relatively easy to assess, we wished to develop a strategy to determine the role of palmitoylation in membrane association which would have an internal control designed into every individual transfection. We further wished to examine a G-protein polypeptide which is not a substrate for co-translational myristoylation and part of the G_q family. We decided to examine $G_{11}\alpha$ in this study, as we have recently developed an SDS/PAGE system which allows separation of primate forms of $G_{11}\alpha$ from those produced in rodents [20], even though these species variant polypeptides have an overall sequence identity of some 97%. As the most commonly used cell lines for transient transfection

Table 3 N-terminal amino acid sequence alignments of some known acylated proteins

Residues known to be myristoylated are shown in *italics* and those known, or thought, to be palmitoylated are underlined.

Polypeptide	Sequence
G ₁₁ α	M <u>G</u> <u>C</u> L G N S K
G ₀ α	M <u>G</u> <u>C</u> T L S A E
G _q α	M T L E S I M A <u>C</u> <u>C</u> L S E E
G ₁₁ α	M T L E S M M A <u>C</u> <u>C</u> L S D E
GAP-43	M L <u>C</u> <u>C</u> M R R T
fyn p59	M <u>G</u> <u>C</u> V G C K D

assays are simian COS cell lines, we anticipated that we would be able to express wild-type and mutated forms of murine G₁₁α in COS cells and subsequently be able to identify unambiguously and provide relative quantification of these species variants by use of an antibody directed against an epitope in this G-protein that is entirely conserved between these species.

To demonstrate the validity of this concept, we initially demonstrated that COS-1 cells do express simian G₁₁α endogenously by immunoblotting membranes of these cells with an anti-peptide antiserum (E976) (Figure 1) which specifically identifies this polypeptide and not other members of the G_q family [21], and showed that transient expression of murine wild-type G₁₁α resulted in the ability to detect these proteins separately (Figure 1). We then developed conditions such that transient levels of expression of murine G₁₁α were similar to endogenous levels of expression of simian G₁₁α (Figures 3a and 3b). These experiments allowed demonstration that the level of incorporation of [³H]palmitate into either the C9S or C10S mutants of murine G₁₁α was considerably less than 50% of that obtained with the wild-type polypeptide. Such results indicate that the palmitoylation of these two adjacent cysteine residues does not occur independently of one another. A similar observation has been noted for the adjacent palmitoylated cysteine residues in G_qα [7]. In these and other experiments we used the antiserum CQ [19], even though it identifies both G₁₁ and G_q, as it has a much higher titre than antiserum E976, identifies an epitope which is entirely conserved in all species variants of G₁₁α which have yet been isolated as cDNAs [23–26], and is an effective antiserum in immunoprecipitation assays.

Separation of transfected COS-1 cells into particulate and supernatant fractions demonstrated that in all cases the immunodetected simian and wild-type murine G₁₁α was entirely particulate. However, in similar assays with C9S, C10S and C9S/C10S murine G₁₁α, each of these was distributed between the particulate and supernatant fractions, with some 30% of the detected expressed mutants present in the supernatant fraction. No obvious differences were observed between the two single mutants and the double C9S/C10S mutant (Figure 3b). Following transient expression of the wild-type and each of the mutants of murine G₁₁α, an extra polypeptide was determined immunologically which migrated further through the gel than the bulk of the murine G₁₁α (Figure 3b). The nature of this polypeptide is unclear, but it may represent either a proteolytically clipped form of the expressed murine G₁₁α or possibly a form of this polypeptide resulting from use of an alternate start codon (potentially methionine-7, as this is in a similar context to the initiation codon of other G-proteins) (Table 3).

The distribution ratio between the particulate and supernatant fractions of palmitoylated and non-palmitoylated forms of

murine G₁₁α recalls our previous studies [5,17] and those of others [8] with G₀1α. In those studies we noted that all of the cellular content of both wild-type and a C3S mutant of G₀1α in unbroken cells was capable of acting as a substrate for pertussis toxin-catalysed ADP-ribosylation, while following cell breakage only that in the particulate fraction remained so [17]. As the ability of G₀1α to act as a substrate for pertussis toxin is largely dependent upon interaction with the G-protein βγ complex [27], these results indicated that all of the non-palmitoylated G₀1α was capable of interacting with the βγ complex, and as this is restricted to the particulate fraction then either the non-palmitoylated C3S G₀1α was cycling between the membrane and the cytosol (a function which has been proposed for the palmitate group on G₀α [12]) or the avidity of the interaction of the C3S G₀1α with the membrane is lower than that of the wild-type protein and following cellular disruption in a hypotonic buffer, a significant fraction of the protein is released from the membrane. As G₁₁α is not a substrate for toxin-mediated modification it is not as easy to reach such conclusions for this G-protein, but clearly both of these possibilities exist. It was a concern to us, however, to attempt to explain why there were both membrane and cytoplasmic fractions of the mutant forms of G₁₁α. This was particularly the case (as noted above) since the two published studies on G_qα palmitoylation [7,16] had observed completely different cellular distributions for the palmitoylation-resistant forms of the polypeptide. One possibility was that the fraction associated with the particulate fraction following transient expression might represent incorrectly folded or aggregated protein. To examine this question we used the relative ability of the detergent sodium cholate to solubilize the endogenous and transfected versions of G₁₁α from the particulate fractions of the cells. In all cases the endogenous simian G₁₁α was effectively and virtually completely solubilized by treatment with 1% (w/v) sodium cholate for 1 h at 4 °C. By contrast, the particulate fraction of the C9S/C10S mutant of murine G₁₁α was virtually immune to solubilization with this concentration of sodium cholate (the standard condition used for G-protein solubilization) and remained in the non-solubilized particulate fraction (Figure 5b). Such data argue strongly that the particulate, mutant, transfected protein is not in the same conformational state as the endogenous simian protein. A very similar observation was noted for G_qα by Edgerton et al. [16]. While transfected wild-type murine G₁₁α was partially solubilized by treatment with 1% (w/v) sodium cholate under these conditions, even this was far from complete, and each of the two single mutants (C9S and C10S) was solubilized to a degree intermediate between that of the wild-type protein and the double mutant (Figures 5 and 6). A reduction in the levels of G₁₁α cDNA used for the transfection did not improve the ability of the C9S/C10S double mutant to be extracted from the particulate fraction by sodium cholate (results not shown). As such, the conclusions reached by Edgerton et al. [16] suggesting that palmitoylation of G_qα may define its ability to interact with a phospholipase C-coupled receptor [16] must be examined with caution. The difficulties noted herein as to the likely functionality of an expressed palmitoylation-resistant G-protein α subunit may only be a concern for such transient expression assays. We have previously stably expressed wild-type murine G₁₁α in human HEK293 cells and observed equivalent sodium cholate solubilization of both the endogenous human and the transfected murine G₁₁α in membranes of such cells (M. A. Grassie and G. Milligan, unpublished work). This may relate to the observations that a myristoylation-resistant G2A mutant of G₁α can reach the membrane and be palmitoylated when co-expressed with βγ subunits [15]. Thus it may be that heterotrimer formation is required in order to transport α

subunits to the membrane most effectively and to promote their correct association with the membrane.

The results provided in the present study indicate clearly that, following expression in COS-1 cells and cellular disruption, palmitoylation-resistant mutants of murine G₁₁α display a distribution pattern distinct from that of either wild-type murine G₁₁α or endogenously expressed (and presumably palmitoylated) simian G₁₁α. Such observations indicate that the palmitoylation status and potential of G₁₁α contributes to the cellular location of this polypeptide. However, the role of palmitoylation of this polypeptide in membrane association, which is clearly reduced, must be interpreted with care as the observed distribution also may reflect protein aggregation resulting from the mutations introduced into the polypeptide and/or from the levels of expression of the transiently expressed protein. These concerns suggest that considerable care must be exercised in relation to analysis of the relative functionality of different constructs which are used in such transient transfection assays. Without information on the fractions of expressed proteins which are in a functional conformation (rather than a simple assessment of levels of immunodetectable expressed protein), quantitative comparisons of functionality may be prone to error.

These studies were supported by a Medical Research Council (U.K.) project grant to G.M. and by a project grant from the Italian Ministry of University and Scientific and Technological Research to M.P.

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