RESEARCH COMMUNICATION

N-terminal fatty acylation of the α -subunit of the G-protein G_{i1} : only the myristoylated protein is a substrate for palmitoylation

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The α -subunit of the G-protein G_{i1} carries two fatty acyl moieties covalently bound to its N-terminal region: myristic acid is linked to glycine-2 and palmitic acid is linked to cysteine-3. Using site-directed mutagenesis on a cDNA construct of α_{i1} we have generated an α_{i1} -G2A mutant, carrying alanine instead of glycine at position 2, an α_{i1} -C3S mutant, in which serine replaced cysteine-3 and a double mutant with both substitutions (α_{i1} -G2A/C3S). These constructs were individually expressed by transfection in Cos-7 cells, and incorporation of fatty acids into the various mutants was compared with wild-type α_{i1} monitoring

metabolic labelling with [3 H]palmitate or [3 H]myristate. The disruption of the palmitoylation site in α_{i1} -C3S did not influence myristoylation, whereas prevention of myristoylation in α_{i1} -G2A also abolished palmitoylation. Co-translational myristoylation is thus an absolute requirement for α_{i1} to be post-translationally palmitoylated. The non-palmitoylated α_{i1} -C3S showed reduced membrane binding to the same extent as the non-myristoylated/non-palmitoylated α_{i1} -G2A and α_{i1} -G2A/C3S mutants, indicating that the attachment of palmitic acid is necessary for proper interaction with the membrane.

INTRODUCTION

The role of covalent fatty acylation in the anchorage of G-protein α -subunits to the cytoplasmic face of the plasma membrane has recently been an area of intense investigation [1]. G-proteins of the G_i -family are targets for co-translational attachment of myristic acid at glycine-2 [2,3]. Demonstration both that mutation of this amino acid to alanine [2,3] and that tryptic cleavage of the extreme N-terminal region [4] of such G-proteins prevents membrane association of the polypeptide have been interpreted to indicate a critical role for myristate in this process. Myristoylation of G_i -like G-proteins has also been reported to be essential for the signalling functions of these G-proteins [5,6]. However, G-proteins of other subfamilies are not substrates for this modification, but are membrane-associated. Recently a number of reports have demonstrated that members of each of the G_s [7–10], G_i [7,8,11], G_o [7,9] and G_{12} [12] families act as substrates for the post-translational addition of palmitic acid and, at least in the cases of G_o [7] and G_s [9,10], this has been shown to be prevented by mutation of cysteine-3 to serine (C3S). Furthermore, such mutation also reduced the interaction of these proteins with the plasma membrane [7–9].

In the present paper we demonstrate that expression of a C3S mutant of G_{i1} is able to result in the expressed protein being myristoylated, but not palmitoylated, but that both a G2A/C3S double mutant and a G2A mutant of this protein cannot be either myristoylated or palmitoylated. The myristoylated but non-palmitoylated mutant of this G-protein shows equivalent membrane/supernatant distribution as the non-myristoylated, non-palmitoylated mutants after expression of these forms in Cos-7 cells. These findings imply that palmitoylation plays a key role in G-protein α -subunit membrane association and that the previous interpretations of G-protein α -subunit G2A mutants that have been used to address the role of G-protein myristoylation [1–3] must be re-examined.

MATERIALS AND METHODS

Generation of α_{i1} mutants and their expression in Cos cells

The cDNA for α_{i1} was subcloned into the mammalian expression vector pcEXV-3. Three α_{i1} mutants were generated: the G2A mutant, where alanine replaced glycine at codon 2 of α_{i1} , the C3S mutant with serine instead of cysteine at codon 3 and the G2A/C3S mutant carrying both mutations. Site-directed mutagenesis was achieved by PCR amplification from the pcEXV- α_{i1} plasmid, using sense (5') mutagenic primers spanning the N-terminus (G2A: 5'-dGCCACCATGGCCTGCACACTGAGC; C3S: 5'-dGCCACCATGGGCTCCACACTGAGC; G2A/C3S: 5'-dGCCACCATGGCCTCCACACTGAGC; the initiator methionine codon is shown in italics and the mutated base(s) is/are underlined), and a 3' antisense primer located approx. 300 bases downstream within the coding sequence for α_{i1} (5'-TCTCTTTATGACGCCGCGAGCTC). PCR was carried out using a Hybaid thermal reactor with 30 ng of linearized pcEXV- α_{i1} plasmid as template DNA and 100 pmol of each primer, using 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 products and the C-terminal fragment of α_{i1} 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 base substitutions and the 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- α_{i1} plasmids by calcium phosphate precipitation [13]. Routine DNA manipulations, including DNA ligations, bacterial transformations, double-stranded DNA sequencing and plasmid purifications were carried out using standard procedures.

Figure 1 Expression and fatty acylation of mutants of the α -subunit of G_{i1}

(a) Expression of wild-type and each of G2A, C3S, G2A/C3S mutant α_1 subunits in cos-7 cells. Untransfected Cos-7 cells (Cos) or those transfected with either plasmid pcEXV alone (mock) or this plasmid incorporating a cDNA for wild-type α_1 (wt) or G2A, C3S and G2A/C3S α_1 mutants for 72 h were resolved by SDS/12.5% (w/v)-PAGE and immunoblotted for the presence of α_1 using antiserum I1C as primary reagent. Weak detection of this polypeptide was observed in both untransfected and mock-transfected Cos-7 cells, but this was increased greatly after transfection with the various constructs. (b) and (c) Fatty acylation of wild-type and each of G2A, C3S and G2A/C3S mutant α_1 subunits in Cos-7 cells. Cos-7 cells transfected as in (a) were labelled overnight 48 h later with [3 H]palmitate (lanes 1–5) or [3 H]myristate (lanes 6–10). Whole-cell lysates were resolved by SDS/12.5% (w/v)-PAGE before (b) or after (c) immunoprecipitation with antiserum I1C. For both panels: lanes 1 and 6, mock; lanes 2 and 7, wild-type; lanes 3 and 8, C3S; lanes 4 and 9, G2A; lanes 5 and 10, G2A/C3S. Molecular-mass (M) markers were provided by the Sigma MW-SDS-BLUE prestained kit. In (b) the arrowheads represent the approximate position of the $G_{i1}\alpha$ polypeptide. The examples shown are representative of four independent experiments.

Cell growth and metabolic labelling

Cos-7 cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C in DMEM supplemented with 10% (v/v) foetal-calf serum, 2 mM L-glutamine, 100 μ g/ml streptomycin and 100 i.u./ml penicillin. Cells were metabolically labelled overnight 48 h after transfection with 150 mCi/ml [9,10- 3 H(n)]palmitic acid (du Pont/NEN; 30–60 Ci/mmol) or [9,10- 3 H(n)]myristic acid (du Pont/NEN; 10–60 Ci/mmol) in Dulbecco's modified Eagle's medium (DMEM) containing 5% dialysed foetal-calf serum, 5 mM pyruvate, antibiotics and L-glutamine as described above. Overnight labelling with 25 mCi/ml Tran 35 S-Label (ICN; > 1000 Ci/mmol) was carried out in DMEM containing 1/20 of the normal content of L-methionine and L-cysteine, 5% dialysed foetal-calf serum, antibiotics and L-glutamine as described above.

Immunoprecipitation and analysis

The polyclonal rabbit antiserum I1C [14] was raised against a synthetic peptide predicted to represent amino acids 160–169 of the α -subunit of G_{i1} . This antiserum has been shown to be specific for $G_{i1}\alpha$ [15] and recognizes a single band of molecular mass approx. 41 kDa in immunoblotting experiments in a variety of tissues (see, e.g., [14]). 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 phenylmethanesulphonyl fluoride. 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 proteinase inhibitors as described above. A 20 ml portion of a 1:1 suspension of Protein A-Sepharose (Pharmacia) beads 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 examine the total radioisotope incorporation into cell proteins (see Figures 1a and 2a below). To the remaining samples 20 ml of the I1C anti- α_1 antiserum or preimmune rabbit serum were added and the samples 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 containing 20 mM dithiothreitol, followed by electrophoresis in an SDS/12.5% (w/v) PAGE gel [16]. Bands were detected by fluorography using preflashed Kodak XAR-5 film [17].

Immunoblotting

For immunoblotting proteins were resolved by SDS/12.5% (w/v) PAGE and transferred to nitrocellulose membranes [18]. After overnight incubation with a 1000-fold diluted I1C 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, 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 (produced following scraping of the cells from the plates) 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, pH 7.5) and proteinase inhibitors as described above, incubated on ice for 30 min and passed 20 times through 1 ml syringe/21G needles. Nuclei and debris were removed by centrifugation at 1000 g for 5 min in a bench centrifuge at 4 °C. The postnuclear supernatants were centrifuged for 30 min at 55000 rev./min 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 described above.

RESULTS

Site-directed mutagenesis of a cDNA encoding rat $G_{i1}\alpha$ [19], which was inserted into the eukaryotic expression vector pcEXV, was performed using a PCR-based strategy, as detailed in the Materials and methods section, to generate each of the G2A, C3S and G2A/C3S mutants of this G-protein α -subunit. Transfection of wild-type $G_{i1}\alpha$ or the three mutants into Cos-7 cells was performed using calcium phosphate precipitation. Successful transfection and similar levels of expression of the cDNA species was demonstrated by immunoblotting membranes of these transfected cells with the specific anti- $G_{i1}\alpha$ antiserum I1C (Figure 1a). Each of the mutant forms of $G_{i1}\alpha$ as well as the wild-type protein was successfully expressed at substantially higher levels than that of endogenously expressed $G_{i1}\alpha$, which could, however, be weakly detected in both untransfected Cos-7 cells and those which had been mock-transfected (Figure 1a).

Figure 2 Cellular localization of wild-type and G2A, C3S and G2A/C3S mutant α_1 subunits

Cos-7 cells were either mock-transfected (mock) or transfected with each of wild-type (wt) and the C3S, G2A or G2A/C3S mutations of $G_{i1}\alpha$ as described in the Materials and methods section and the legend to Figure 1(a) and labelled overnight 48 h later with Tran^{35}S -Label as described in the Materials and methods section. Cell homogenates were separated into S100 (s) and P100 (p) fractions (see the Materials and methods section) and resolved by SDS/12.5% (w/v)-PAGE before (a) or after (b) immunoprecipitation with antiserum I1C. The examples shown are representative of three independent experiments. Abbreviation: M, molecular mass.

At 48 h after transfection of Cos-7 cells with the various $G_{i1}\alpha$ constructs, the cells were labelled with either [^3H]myristate or [^3H]palmitate, and whole-cell lysates were resolved by SDS/PAGE before or after immunoprecipitation with antiserum I1C (Figures 1b and 1c). Both [^3H]myristate and [^3H]palmitate were incorporated into a wide range of cellular polypeptides (Figure 1b). Specific immunoprecipitation of $G_{i1}\alpha$ demonstrated that whereas incorporation of palmitate into this polypeptide was observed with expression of wild-type $G_{i1}\alpha$, it was not observed with either mutation which contained the C3S modification, in accordance with the conclusion that this cysteine acts as the acceptor site for thioester-linked palmitoylation [7]. More surprisingly, however, the G2A mutant of $G_{i1}\alpha$ also failed to incorporate [^3H]palmitate despite the maintenance of cysteine-3. Incorporation of [^3H]myristate was observed in immunoprecipitates of both wild-type $G_{i1}\alpha$ and the C3S mutant, but was not observed in either of the two forms in which the G2A mutation was present. In a number of cases weak incorporation of [^3H]myristate was detected in immunoprecipitations from mock-transfected cells (Figure 1c), presumably reflecting the low level endogenous expression of $G_{i1}\alpha$ (Figure 1a).

To assess the role of the fatty acylations in membrane association of $G_{i1}\alpha$, Cos-7 cells transfected as above with the various forms of $G_{i1}\alpha$ were labelled overnight with Tran^{35}S -Label. Cell homogenates were resolved into particulate and supernatant fractions, and these were separated by SDS/PAGE either before (Figure 2a) or after (Figure 2b) immunoprecipitation with antiserum I1C. Whereas some 90% of wild-type $G_{i1}\alpha$ was associated with the particulate fraction, in each of the C3S, G2A and G2A/C3S mutations this ratio was reversed, with some 80% of the ^{35}S -labelled G-proteins being present in the supernatant fractions (Figure 2b).

DISCUSSION

A key role for the N-terminal region of G-protein α -subunits in association of these polypeptides with the internal face of the plasma membrane was initially demonstrated by the observation that a limited proteolytic clip which removed some 2 kDa from this region rendered the remaining polypeptide soluble [4]. The subsequent demonstrations that G-proteins of the G_i -family were modified by the co-translational addition of the fatty acid myristic acid [1–3], that this addition was prevented by mutation of glycine-2 to alanine [1–3] and that such mutation limited membrane association of the expressed mutant [1–3] all provided strong evidence for a key role of this fatty acylation in this process. However, G-protein α -subunits of the G_s , G_q and G_{12} families have primary amino acid sequences inconsistent with them acting as substrates for N-terminal myristoylation and in many cases have been shown not to be modified by myristate [1,20]; they are, however, membrane-associated. Recent studies from a number of groups have demonstrated that all of these G-proteins are modified by post-translational acylation [7–12]. In the bulk of studies to date, the attachment of palmitate via a thioester linkage has been demonstrated, and in a number of cases mutation of likely target cysteine residues in the N-terminal region has been shown to prevent palmitoylation [7,9–10]. It is likely that a variety of fatty acids may become esterified to these sites, as after labelling of platelets with [^3H]arachidonic acid, covalent thioester-linked incorporation of this fatty acid into $G_{i2}\alpha$ has been recorded [21].

In the present study we have attempted to assess the relative importance of myristoylation of glycine-2 and palmitoylation of cysteine-3 in membrane association of the α -subunit of G_{i1} , a polypeptide known to be dually modified. To do so we con-

structed three mutant forms of this G-protein, a G2A mutant which we anticipated should be palmitoylated but not myristoylated, C3S, which we expected to be myristoylated but not palmitoylated, and the double G2A/C3S mutant which we reasoned would lack both acylations. To establish the verity of these hypotheses we transfected Cos-7 cells with cDNAs expressing each of these forms as well as with the wild-type $G_{i1\alpha}$. The expression of each form after transfection was measured by immunoblotting Cos-7 cell membranes with a specific $G_{i1\alpha}$ antiserum, I1C, which we have described previously [14]. $G_{i1\alpha}$ was expressed endogenously by Cos-7 cells at only very low levels, and the presence of the relevant polypeptide was recorded after transfection with each cDNA construct as a marked increase in immunological signal (Figure 1a). Immunoprecipitation of G_{i1} with antiserum I1C after labelling with either [3 H]myristate or [3 H]palmitate clearly demonstrated that wild-type G_{i1} was labelled with both fatty acids, that, as anticipated, the C3S mutant incorporated label from [3 H]myristate but not [3 H]palmitate, and that the G2A/C3S mutant was unable to incorporate either label. However, contrary to expectation, the G2A mutant also had the characteristics of the G2A/C3S mutant, failing to incorporate either myristate or palmitate. A similar observation has recently been noted for mutations of G_{α} [11] and for Fyn [22–23]. Yes and Lck, members of the Src-like family of non-receptor tyrosine kinases which are the only other protein family apart from the G_i -like-G-protein α -subunits to have been shown to be doubly acylated in their N-terminal region. The failure of the $G_{i1\alpha}$ G2A mutant to become palmitoylated may be due to a requirement for myristoylation of the protein in order to allow appropriate presentation to the membrane bound palmitoyltransferase [24,25].

Membrane association of the various mutant forms of $G_{i1\alpha}$ was analysed and compared with that of wild-type G_{i1} . Whereas transient expression of wild-type $G_{i1\alpha}$ in Cos-7 cells resulted in the vast majority of the polypeptide being associated with the particulate fraction, this was not true of any of the mutant constructs. In each case, the majority of the expressed protein was present in the supernatant fraction (Figure 2). A similar pattern of cellular distribution was observed for the form which was non-palmitoylated but myristoylated as for the forms which were both non-palmitoylated and non-myristoylated (Figure 2b). These results suggest that co-operation between myristate and palmitate is required for high-affinity membrane association of $G_{i1\alpha}$. In this context it should be noted that a combination of both palmitoylation and prenylation close to the C-terminus of the p21^{ras} proteins is required to provide high-avidity interaction of these proteins with the plasma membrane and to provide maximal transforming activity [26,27] and that GAP-43, which is doubly palmitoylated on adjacent cysteine residues close to the N-terminus, requires both sites to be acylated to produce maximal membrane association [28,29]. It is also interesting to note in this regard that while the N-terminal glycine of the α -subunit of rod transducin can be heterologously acylated by a variety of fatty acids, including myristate and dodecanoate [30,31], it is unlikely to be palmitoylated close to the N-terminus, as there are no cysteine residues within the N-terminal 60 amino acids, and that transducin is the one G-protein which can be eluted from the membrane to any significant extent without treatment with detergent. That palmitoylation may play a key role in membrane association of other proteins has been indicated by the observation that a tightly membrane-associated subpopulation of

spectrin is palmitoylated, whereas non-palmitoylated spectrin is easily extracted from red-cell membranes with low-salt buffer washes [32].

As such, previous studies which have constructed G2A mutants of G_i -like G-proteins and have interpreted the results (e.g. reduction in membrane association [1–3] or inability to regulate effector pathways such as adenyl cyclase or activation of MAP (mitogen-activated protein) kinase [5–6]) as defining a specific role for myristoylation will have to be re-evaluated in light of the observations reported herein.

This work was supported by the Italian National Research Council (C.N.R.; grant 93.00374.CT04) and by a project grant from the Medical Research Council (U.K.) to G.M.

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