RESEARCH COMMUNICATION

A novel N-terminal motif for palmitoylation of G-protein α subunits

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We have examined the post-translational processing of $G\alpha$ subunits expressed endogenously in rat PC12 and NG108-15 rat/mouse hybrid cells, and after transfection of cDNA expression constructs into COS cells. Thioester-linked palmitoylation of α_o , α_s , α_i/α_{11} and α_{12} has been detected by metabolic labelling with [3H]palmitate and immunoprecipitation. Palmitoylation of α_o occurs post-translationally in cells treated with protein-synthesis inhibitors, suggesting possible dynamic acyl-

ation. Palmitoylation of the C-terminal CAAX motif has been excluded. Site-directed mutagenesis of α_o has been used to implicate the site of modification as a cysteine residue next to the N-terminal myristoylated glycine, in a novel protein–lipid modification motif Met-Gly-Cys. The non-palmitoylated α_o mutant is still myristoylated but shows reduced membrane binding, suggesting that reversible palmitoylation may regulate $G\alpha$ localization and function.

INTRODUCTION

The classical heterotrimeric G-proteins mediate the response of second-messenger-generating systems to extracellular signals (Gilman, 1987). In order to do so they must associate with the cytoplasmic face of the plasma membrane. This association may be partly mediated by the $\beta\gamma$ subunits (Sternweis, 1986) but the N-terminus of the α subunits also seems to be involved (Eide et al., 1987; Neer et al., 1988). Indeed, the N-termini of several α subunits have been shown to be covalently modified by amide linkage of myristate (Buss et al., 1987; Schultz et al., 1987; Mumby et al., 1990), which is required for membrane binding (Jones et al., 1990). Recent evidence also suggests a role for a sequence near the C-terminus of α_s in membrane binding (Journot et al., 1991).

Another class of low-molecular-mass GTP-binding proteins, the ras proteins, has been shown to associate with membranes via a complex series of modifications involving the C-terminal CAAX motif (Hancock et al., 1989). These include prenylation (Casey et al., 1989; Hancock et al., 1989), proteolytic processing and carboxyl-methylation (Clarke et al., 1988; Gutierrez et al., 1989), and palmitoylation (Buss and Sefton, 1986; Hancock et al., 1989). $G\gamma$, and many $G\alpha$ subunits, display a C-terminal sequence resembling a CAAX motif, and the former has recently been shown to be carboxyl-methylated (Fung et al., 1990) and prenylated (Fukada et al., 1990). Using endogenously and exogenously expressed α subunits we have observed palmitoylation of $G\alpha_o$, α_s , α_i/α_{11} and α_{12} , in addition to myristoylation. For α_o this palmitoylation seems to occur on a cysteine residue adjacent to the N-terminus, in a novel motif for lipid modification of proteins, Met-Gly-Cys, and may be involved in regulating α -subunit function. A preliminary report of this work was presented at the 21st Annual Meeting of the Society for Neuroscience (Parenti et al., 1991).

MATERIALS AND METHODS

Rat PC12 cells were grown in RPMI 1640 plus 5% (v/v) foetal-calf serum and 10% (v/v) horse serum. NG108-15 mouse/rat hybrid cells were grown and differentiated with dibutyl cyclic

AMP as described previously (Mullaney et al., 1988). COS cells were grown as described in Newman et al. (1992). Labelling of cells with [$9,10\text{-}^3H$]palmitate (Amersham, U.K.; approx. 50 Ci/mmol) and [$9,10\text{-}^3H$]myristate (NEN, U.K.; approx. 22 Ci/mmol) was essentially as described before (Magee and Courtneidge, 1985; Magee et al., 1987; Hancock et al., 1989). Tran[^{35}S]label (ICN, U.K.; > 1000 Ci/mmol) was used to metabolically label cell proteins (Newman et al., 1992).

A polyclonal rabbit antiserum (G7) raised against a brain G_i/G_o preparation was used for some experiments (a gift from Dr. H. Ploegh, Netherlands Cancer Institute, Amsterdam). This serum immunoprecipitated two bands of molecular mass approx. 39 kDa and 36 kDa, as expected for the α - and β -subunits of a heterotrimeric G-protein (see the Results section). CS1 serum was raised to the C-terminal decapeptide of forms of $G\alpha_s$ (Milligan et al., 1989). CQ1 serum is against a C-terminal decapeptide common to α subunits of G_o and G_{11} (Mitchell et al., 1991). Serum SG1 was raised to the C-terminal decapeptide of rod transducin α and cross-reacts with $G\alpha_{11}$ and $G\alpha_{12}$ (Mitchell et al., 1989). In addition, we raised rabbit polyclonal antibodies against a trpE fusion protein with the C-terminal 93 amino acids of $G\alpha_o$. A cDNA fragment containing the corresponding coding sequence was subcloned in frame into the *Clal* site of the bacterial expression vector pATH3 (Dieckmann and Tzagoloff, 1985). The fusion protein was expressed in *Escherichia coli* strain TG1, purified by preparative SDS/PAGE, electroeluted, and used to immunize rabbits. The polyclonal rabbit antibody to cyclic AMP-dependent protein kinase was a gift from Dr. S. McKnight (University of Washington, Seattle, WA, U.S.A.).

Pertussis toxin was obtained from Porton Products, U.K. For ADP-ribosylation of G-proteins *in vivo*, cells were treated for 4–16 h with 1 μ g of toxin/ml in complete medium. Preparation of soluble (S100) and particulate (P100) fractions of cells and Triton X-114 partitioning were performed as described previously (Hancock et al., 1989; Gutierrez et al., 1989). Methods for immunoprecipitation, SDS/PAGE, fluorography, and hydroxylamine treatment of SDS-gel-resolved proteins have been described (Magee and Courtneidge, 1985; Magee et al., 1987). Chemicals were obtained from Sigma, U.K., or BDH, U.K., unless otherwise stated, and were of the highest purity available.

Expression of α_o in COS cells and generation of an α_o C3S mutant

The cDNA for α_o was subcloned into the mammalian expression vector pcEXV-3. COS7 cells were transfected with the pcEXV- α_o plasmid by electroporation (Newman et al., 1992). Site-directed mutagenesis to substitute serine for cysteine at codon 3 of α_o was achieved by PCR amplification from the pcEXV- α_o plasmid, using a sense (5') mutagenic primer spanning the N-terminus (5'-dGGGGCCACCATGGGATCTACTCTGAG; the initiator methionine codon is shown in *italics* and the mutated base is underlined), and a 3' antisense primer situated approx. 300 bases downstream within the coding sequence for α_o (5'-CACACCATCTTGGAGTC). The PCR reaction was carried out using a Hybaid Thermal Reactor with 20 ng of linearized pcEXV- α_o 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 product and the C-terminal fragment of α_o were digested with suitable restriction endonucleases and ligated with the pcEXV-3 backbone; the ligation mixture was then used to transform *E. coli* TG1 cells. Individual ampicillin-resistant colonies were screened for the cysteine to serine substitution, and for unwanted mutations within the remainder of the PCR-derived sequence, by DNA sequencing of miniprep DNA. One colony carrying the desired mutation was used for bulk preparation of plasmid DNA (pcEXV-C3S) for COS cell transfections. Routine DNA manipulations, including DNA ligations, bacterial transformations, double-stranded DNA sequencing and plasmid purifications were carried out using standard procedures.

RESULTS

The specificity of the G7 polyclonal anti-(G-protein) antiserum for $G\alpha_o$ was demonstrated by immunoblotting rat brain membranes with this antiserum alone and with mixtures of this antiserum and two anti-(peptide) antisera which identify specifically either ' α_i ' (antiserum AS7) or ' α_o ' (antiserum IM1) (Mullaney et al., 1988). Of the various G-protein α subunits, antiserum G7 was demonstrated to identify specifically α_o (Figure 1). As antiserum G7 was produced against holomeric G-proteins it also contains antibodies against the $G\beta$ subunit and resembles antiserum RV3, which we have characterized previously (Gierschik et al., 1986).

In order to test for acylation of $G\alpha$ subunits, PC12 cells were labelled with [3 H]palmitate or [3 H]myristate, separated into S100 and P100 fractions, and immunoprecipitated with antiserum G7. To control for non-specific labelling and for interconversion of fatty acids the same lysates were precipitated with a polyclonal antibody to cyclic AMP-dependent protein kinase which recognizes the catalytic (C-) subunit, a protein known to be myristoylated (Clegg et al., 1989). Figure 2 shows that G7 serum precipitates a band of molecular mass approx. 39 kDa, which incorporates label from both [3 H]palmitate and [3 H]myristate. No label was observed in the position of the $G\beta$ subunit, which is as least as abundant as the α subunit (Figure 3, lane 6). The 40 kDa cyclic AMP-dependent protein kinase C-subunit, however, incorporated label only from [3 H]myristate. Subcellular fractionation of the cell lysates demonstrated that the approx. 39 kDa $G\alpha$ subunit was found in the membrane fraction, while the cyclic AMP-dependent protein kinase C-subunit was predominantly soluble. These results suggest that this $G\alpha$ subunit carries two different fatty acid modifications.

NG108-15 cells were chosen for further experiments due to

Figure 1 Characterization of G-protein antisera

Rat brain membranes (25 μ g) were resolved by SDS/PAGE and immunoblotted with (a) a mixture of antisera G7 (1:2000 dilution) and AS7 (1:200 dilution); (b) a mixture of antisera G7 (1:2000 dilution) and IM1 (1:200 dilution); (c) a mixture of antisera AS7 and IM1 (each 1:200 dilution); (d) antiserum G7 (1:2000 dilution); (e) antiserum AS7 (1:200 dilution); and (f) antiserum IM1 (1:200 dilution). Antisera AS7 and IM1 have previously been demonstrated to identify specifically α_i and α_o respectively (Mullaney et al., 1988). The mixtures of antisera G7 and AS7 (a) and IM1 and AS7 (c) identified two distinct polypeptides of molecular mass approx. 40 kDa. By contrast, the mixture of antisera G7 and IM1 (b) identified only a single polypeptide in this region.

Figure 2 Acylation of α_o in PC12 cells

PC12 cells in 150 mm-diam. dishes were labelled overnight with 500 μ Ci Tran[35 S]label in medium containing 10% of the normal level of methionine or for 4 h with 1 mCi of [3 H]palmitate or [3 H]myristate. The P100 (Mem.) and S100 (Cyt.) fractions were prepared (see the Materials and methods section) and immunoprecipitated with the antisera to α_o (G7) or to cyclic AMP-dependent protein kinase C-subunit (cAK). The immunoprecipitates were resolved on a 12.5% SDS/polyacrylamide gel followed by fluorography. The position of the kinase C-subunit (C) and α_o are shown. Markers were: 43 kDa, ovalbumin; 25 kDa, α -chymotrypsinogen; 18 kDa, β -lactoglobulin; 14 kDa, lysozyme. Abbreviations: i, immunoprecipitation with G7 serum; n, non-immune precipitation with normal rabbit serum. Exposure time was 90 days.

their higher level of expression of α subunits. We have previously shown, using specific anti-(peptide) sera, that dibutyryl cyclic AMP treatment results in differentiation accompanied by down-regulation of α_i and up-regulation of α_o (Mullaney et al., 1988). The palmitoylated α subunit recognized by G7 serum was shown

Figure 3 Pertussis toxin, cycloheximide and hydroxylamine sensitivity of α_o palmitoylation

(a) NG108/15 cells in 50 mm-diam. dishes were left undifferentiated (lane 1) or differentiated with dibutyryl cyclic AMP (lanes 2–8). One dish was pretreated with 50 μ g/ml cycloheximide (lane 3) and two others with 1 μ g/ml pertussis toxin (lanes 4, 7 and 8). Cells were labelled with 200 μ Ci of [3 H]palmitate/ml (lanes 1–4) or 30 μ Ci of Tran[35 S]label/ml (lanes 5–8) for 4 h, then lysed, immunoprecipitated and analysed. Only the relevant region of the gel is shown (the 43 kDa marker was ovalbumin). Exposure was for 59 days (lanes 1–4) or 30 days (lanes 5–8). Lanes 1–4, 6, and 7 were exposed to G7 serum, and lanes 5 and 8, to non-immune serum. (b) Differentiated NG108/15 cells in 50 mm-diam. dishes were labelled for 4 h with 200 μ Ci/ml of [3 H]palmitate (lanes 3, 4 and 6) or [3 H]myristate (lanes 1, 2 and 5), then lysed, immunoprecipitated (lanes 2, 3, 5 and 6, G7 serum; lanes 1 and 4, non-immune serum) and resolved in duplicate on a 15% (w/v) acrylamide gel. Lanes were treated with 1 M hydroxylamine, pH 7.4 (lanes 5 and 6) or 1 M Tris/HCl, pH 7.4 (lanes 1–4) for 1 h at 25 °C, followed by fluorography. Exposure was for 75 days. The dark band above the ovalbumin marker was seen in some analyses but was non-specifically precipitated by both immune (lanes 2, 3, 5 and 6) and non-immune (lanes 1 and 4) sera.

Figure 4 Palmitoylation of α_o , α_q , α_{11} and α_{12}

(a) NG108/15 cells in two 90 mm-diam. dishes were labelled for 4 h with 100 μ Ci/ml [3 H]palmitate, lysed in RIPA buffer (Newman et al., 1992), precleared with 20 μ l of normal rabbit serum and Protein A–Sepharose and immunoprecipitated overnight with 5 μ l of sera CQ1 (lane 1), SG1 (lane 2) or CS1 (lane 3), followed by resolution on an SDS/10% (w/v) acrylamide gel. Exposure time was 28 days. (b) A 90 mm-diam. dish of NG108/15 cells was labelled as above, precipitated with normal rabbit serum or α_s antiserum (CS1) and resolved by SDS/12.5% PAGE in duplicate. One half of the gel was treated with hydroxylamine (H) and the other with Tris (T), followed by fluorography for 56 days. Molecular-mass markers were from the Sigma SDS-7B kit. The arrowhead indicates the α_o band.

to be up-regulated after such treatment of NG108-15 cells, which is consistent with its identification as α_o (Figure 3a, lanes 1 and 2).

These two fatty acid modifications could also be distinguished by their susceptibility to inhibition by protein-synthesis inhibitors which abolished incorporation of [3 H]myristate (results not

shown), consistent with co-translational N-myristoylation (Buss et al., 1987), but had little or no effect on incorporation of [3 H]palmitate (Figure 3a, lanes 2 and 3). The nature of the linkage of palmitate to the α_o subunit was tested by incubation of SDS/PAGE-resolved protein with hydroxylamine under conditions which cause cleavage of thioester-linked fatty acid (Magee and Courtneidge, 1985). Figure 3(b) shows that neutral hydroxylamine removed [3 H]palmitate label (lanes 3 and 6) from α_o , but not [3 H]myristate label (lanes 2 and 5). The efficiency of incorporation of [3 H]palmitate was too low to allow recovery of cleaved fatty-acyl hydroxamate and analysis to confirm the identity of the label. However, the results shown in Figure 2 argue against non-specific incorporation or interconversion.

Since palmitoylation occurs in thioester linkage we attempted to identify the acylation site. α_o and several other α subunits, as well as γ subunits, have a C-terminal sequence resembling the CAAX motif with a cysteine residue four amino acids from the C-terminus. This is believed to be the site of pertussis-toxin-catalysed ADP-ribosylation. In order to test whether this could be the palmitoylation site we pretreated differentiated NG108-15 cells with toxin, followed by metabolic labelling with [3 H]palmitate or Tran[35 S]label. Figure 3(a) shows that pertussis toxin caused a distinct increase in apparent molecular mass of α_o (lanes 6 and 7) as previously noted (Goldsmith et al., 1987), consistent with efficient ADP-ribosylation, but had no effect on [3 H]palmitate incorporation (Figure 3a, lanes 2 and 4).

Antisera specific for the other α subunits, α_s (serum CS1), α_q/α_{11} (serum CQ1) and $\alpha_{11,2}$ (serum SG1), were used to immunoprecipitate lysates of [3 H]palmitate-labelled undifferentiated NG108/15 cells (Figure 4a). The band immunoprecipitated by antiserum SG1 is definitely α_{12} rather than α_{11} , as NG108/15 cells do not express α_{11} (McKenzie and Milligan, 1990). All these subunits were found to incorporate [3 H]palmitate. The doublet seen with antiserum CQ1 (lane 1) may represent α_q and α_{11} , both of which are expressed in NG108/15 cells (G. Milligan, unpublished work). Neutral hydroxylamine sensitivity was also demonstrated for the palmitate label incorporated into the α_s subunit (Figure 4b) and the α_i subunit (results not shown) precipitated by the CS1 and SG1 sera respectively, indicating a thioester linkage.

A survey of known palmitoylated proteins does not reveal any consensus amino acid sequence or conserved position within the polypeptide chain for the modification. Indeed, the only constant feature seems to be that the palmitoylation site is close to the cytoplasmic face of the membrane (Clarke et al., 1988) and this may be required for it to be available to a membrane-bound acyltransferase. Several α subunits, including those found here to incorporate [3 H]palmitate, have a cysteine residue adjacent to the N-terminus, which is involved in membrane anchoring and is often myristoylated (Lochrie and Simon, 1988). This cysteine residue was therefore tested as a possible site for palmitoylation. A cDNA construct of α_o was mutagenized to replace cysteine-3 with serine (α_o -C3S) and then expressed by transfection in COS cells, in parallel with the wild-type protein (α_o -wt). Figure 5 (upper panel) shows that both α subunits were well expressed, as judged by labelling with Tran[35 S]label and immunoprecipitation with a polyclonal serum to the trpE- α_o fusion protein described in the Materials and methods section. Figure 5 (lower panel) shows that only the α_o -wt protein, and not the α_o -C3S mutant, was labelled with [3 H]palmitate, consistent with cysteine 3 being the palmitoylation site. In separate experiments we confirmed that both α_o -wt and α_o -C3S were myristoylated to a similar extent (results not shown).

The hydrophobic properties of α_o -wt and α_o -C3S were tested in two ways. Triton X-114 partitioning revealed that both

Figure 5 Mutation of Cys-3 in α_o abolishes palmitoylation

COS cells were transfected with expression constructs of α_o -wt (wt) and α_o -C3S (C3S) in the pEXV plasmid, or were mock-transfected (m). Three days later cells were labelled with Tran[³⁵S]label (³⁵S-labelled, top panel) or [³H]palmitate (³H-labelled, bottom panel) and immunoprecipitated with normal rabbit serum (n) or trpE- α_o antiserum (i). Immunoprecipitates were resolved by SDS/12.5% (w/v)-PAGE and fluorographed for 70 days. Molecular-mass markers were from the Sigma SDS-7B kit. The arrowhead indicates the α_o band.

proteins partitioned largely into the detergent phase (Figure 6a), demonstrating that the addition of myristate alone was sufficient to cause the proteins to become hydrophobic. Interestingly, whereas the α_o -wt protein was quantitatively associated with the P100 membrane fraction of the transfected cells, the α_o -C3S mutant protein showed a significant portion (20–30%) in the S100 soluble fraction (Figure 6b). The doublet seen in Figure 6(b) (wt, P) may be due to resolution of alternatively spliced forms of α_o or to partial proteolysis.

DISCUSSION

We have used antisera reactive with a range of G-protein α subunits to study their post-translational modification in mammalian cells. Other authors have detected stoichiometric amounts of myristate, but only low amounts of palmitate, in several α subunits by metabolic labelling or analysis of purified proteins (Buss et al., 1987; Schultz et al., 1987); the amount of palmitate was regarded as insignificant. We detected thioester-linked palmitate in endogenous α_o in amounts comparable with myristate, by metabolic labelling, in both PC12 and NG108/15 cells; α_o was also palmitoylated in transfected COS cells. Endogenous α_o , α_{12} and α_q/α_{11} were also found to be palmitoylated in NG108/15 cells. The discrepancy between previous data and ours could be due to differences in the steady-state level of palmitoylation due to turnover (see below and Magee et al., 1987), or due to loss of thioester-linked palmitate during purification. The specificity of labelling with [³H]palmitate was confirmed by: (i) lack of incorporation into the more abundant β -subunit; (ii) incorporation of [³H]myristate, but not [³H]palmitate, into the catalytic subunit of cyclic AMP-dependent protein kinase; and (iii) cleavage of [³H]palmitate (from α_o and α_s), but not [³H]myristate (from α_o), by neutral hydroxylamine. Inhibition of protein synthesis had little effect on metabolic incorporation of [³H]palmitate into α_o , indicating that continuous turnover of the palmitate moiety may be occurring, as in the case of p21^{ras} (Magee et al., 1987). Pulse-chase experiments were not feasible because of the low endogenous level of expression. It has been postulated that

Figure 6 Palmitoylation affects hydrophobicity and localization of α_o

(a) COS cells were transfected and labelled with Tran[³⁵S]label as described for Figure 5, lysed in 1% (w/v) Triton X-114 and partitioned. The detergent (D) and aqueous (A) phases were immunoprecipitated with the trpE- α_o antiserum. Exposure time was 2 days. Abbreviations: m, mock; wt, α_o wild type; C3S, α_o C3S mutant. (b) COS cells transfected and labelled as in (a) were homogenized and resolved into S100 (S) and P100 (P) fractions, followed by immunoprecipitation with trpE- α_o antiserum. Molecular-mass markers were from the Sigma SDS-7B kit. The arrowhead indicates the α_o band.

reversible palmitoylation of ras proteins may regulate their activity (Hancock et al., 1989). Pertussis toxin, which modifies the cysteine residue of the CAAX-like motif of some α subunits (West et al., 1985), had no effect on palmitoylation of α_o .

The observation that several of the α subunits found to be palmitoylated contained a cysteine residue next to their N-terminal glycine residue, with the exception of α_q/α_{11} , which have cysteines at positions 9 and 10, prompted us to test this amino acid as the acylation site. The failure of the α_o -C3S mutant to be palmitoylated is consistent with this hypothesis. Although we have not tested the other α subunits by mutagenesis it is likely that cysteine-3 is the palmitoylation site for all of them with the exception of α_q/α_{11} where the cysteines at positions 9 and/or 10 may be the site(s). A search of the latest release of the Swiss Prot database for the N-terminal sequence Met-Gly-Cys revealed two major types of protein: G α subunits and several members of the src family of non-receptor tyrosine kinases. Members of the src family are known to be N-terminally myristoylated and we have obtained evidence for palmitoylation of c-yes and c-fyn (A. I. Magee and S. A. Courtneidge, unpublished work). Thus N-terminal Met-Gly-Cys may constitute a motif for double modification by myristate and palmitate. The case of α_q/α_{11} , where putative palmitoylation sites are at positions 9 and 10 (Strath-

mann and Simon, 1990), suggests that the distance between the N-terminus and palmitoylation sites may be variable.

Substitution of cysteine-3 with serine in α_o did not affect myristoylation at the adjacent N-terminal glycine, as would be expected from the known properties of the N-myristoyl-transferase (Gordon et al., 1991). However, myristoylation cannot be absolutely required for palmitoylation to occur, since α_s is palmitoylated but not myristoylated, despite having a glycine in position 2 (Jones et al., 1990). In contrast, the α subunit of transducin, α_t , is N-terminally modified with myristate and other related fatty acids (Casey, 1992), but has no adjacent cysteine residue. It is probable, therefore, that α_o will not be palmitoylated, which may explain the unique property of this α subunit of being released from membranes by non-hydrolysable GTP analogues (Spiegel, 1990).

There are conflicting data as to the regions of α subunits required for interaction with $\beta\gamma$ and for membrane association; both the N- and C-terminal regions have been implicated. Juhn et al. (1992) found that α_s subunits with a deletion of amino acids 3–28 or 1–59 were still membrane-associated, suggesting that the N-terminal region might be involved in a more subtle function than simple membrane association. Higashima and Ross (1991) showed that cysteine-3 of α_o could be oxidatively cross-linked to a mastoparan analogue containing a cysteine residue, suggesting that this region of α_o could be involved in receptor coupling. Thus reversible palmitoylation at this site could regulate this coupling, and vice versa.

Double acylation with myristate and palmitate may, in some cases, be necessary for strong membrane binding. Indeed, many myristoylated proteins are found in the cytosol (Magee and Courtneidge, 1985; McIlhinney et al., 1985; Olson et al., 1985). Theoretical calculations show that myristate is much less hydrophobic than palmitate (Black and Mould, 1991) and may not in itself be sufficient for membrane binding. This is supported by our observation that the α_o -C3S mutant is less well associated with the membrane fraction than the α_o -wt protein, and is consistent with a role for regulated reversible palmitoylation in modulating α -subunit membrane association and function. Fatty acyl chains need not necessarily interact directly with the lipid bilayer, but rather may be involved in protein interactions, as is the case for the polio virus VP4 protein (Moscufo and Chow, 1992). Interestingly, the GAP43 protein, which acts as a guanine-nucleotide-release protein for G_o , is doubly palmitoylated on cysteine residues at positions 3 and 4. Dynamic palmitoylation of GAP43 *in vivo* regulates its ability to interact with G_o and hence its nucleotide-exchange activity (Sudo et al., 1992).

Palmitoylation alone may be sufficient for membrane binding, since α_s and α_q/α_{11} are palmitoylated but not myristoylated and yet are tightly bound to membranes (Jones et al., 1990). Abolition of the palmitoylation sites of these α subunits would be expected to have a dramatic effect on their localization. The relative contributions of myristate and palmitate to localization, function and regulation will be complex and require detailed study. This double modification pathway is somewhat analogous to that for ras proteins, where prenylation and carboxyl-methylation of the C-terminus co-operates with nearby palmitoylation sites or a polybasic region to specify membrane binding and intracellular targeting (Hancock et al., 1989).

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