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Receptor regulation of G-protein palmitoylation

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ABSTRACT **Many α subunits of heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) are palmitoylated. Exposure of cells to the β-adrenergic agonist isoproterenol increased incorporation of [^{3}H]palmitate specifically into α_s, the α subunit that mediates stimulation of adenylyl cyclase. Pulse–chase experiments suggested that isoproterenol increased turnover of α_s-bound palmitate. Mutagenesis of Cys-3 in α_s or α_o (a homologous α subunit) prevented palmitoylation of these proteins. Differing results were obtained when mutations of Cys-3 in α_s or α_o were expressed in cells and assayed for their distribution between soluble and membrane fractions. Some α subunits, including α_o, are myristoylated at the amino-terminal glycine residue. Mutation of this glycine prevented both myristoylation and palmitoylation of α_o, indicating that myristoylation precedes palmitoylation of dually acylated α subunits. The amino-terminal sequences and fatty acylation properties of dually acylated α subunits are strikingly similar to those of some members of the Src family of protein-tyrosine kinases. The amino-terminal sequence Met-Gly-Cys-Xaa-Xaa-Ser/Cys shared by these proteins may represent a motif for cotranslational and posttranslational processing that includes myristoylation of the glycine residue and reversible palmitoylation of the cysteine residue.**

A family of guanine nucleotide-binding regulatory proteins (G proteins) serves in signal transduction systems to link receptors exposed at the cell surface to intracellular effectors. Upon activation by receptor, a heterotrimeric G protein dissociates into a GTP-bound α subunit and a $\beta\gamma$ subunit complex, which are then able to modulate effector activity. The structurally homologous α subunits of the G_i subfamily (α_i, α_o, and α_z) are modified by myristate (C14:0), bound in amide linkage at their amino-terminal glycine residues (1, 2). Retinal transducin α subunit (α_t) is heterogeneously modified at the same site by myristate and by C14:1, C14:2, and C12:0 fatty acids (3, 4). Myristoylation increases the apparent affinity of modified α subunits for $\beta\gamma$ and for effector and appears to facilitate association of α with cellular membranes (2, 5–7). Some G protein α subunits, including G_s and G_q, are not myristoylated. However, members of the G_s and G_q subfamilies of α subunits are palmitoylated (C16:0) (8). In addition, members of the G_i subfamily (with the exception of α_t) are both palmitoylated and myristoylated at separate sites (8). We have proposed that Cys-3 is the site of palmitoylation of members of the G_s and G_i subfamilies.

Palmitoylation can be a dynamic posttranslational modification of proteins, unlike myristoylation, which is usually an irreversible cotranslational modification (9). We have speculated that a cycle of acylation and deacylation could regulate the activity and membrane association of G-protein α subunits (8). As initial steps to test this hypothesis, we have examined receptor-dependent changes in the palmitoylation of α_s, and we have tested the capacity of mutant α subunits that cannot be palmitoylated to associate with membranes.

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METHODS

Construction of Expression Vectors. Mutagenesis was performed on bacteriophage clones harboring the complete antisense strand of a rat α_o cDNA (10) (in M13mp19) or the sense strand of a bovine α_s (long) cDNA (11) (in M13mp18). The oligonucleotide primer sequences for generating the Cys-3 → Ala (C3A) mutations were 5′-GCCACCATGGGC-GCCACTCTGAGCGC-3′ for α_o and 5′-TGTTTCCGAGGG-CGCCCATGGCG-3′ for α_s. The mutations were verified by sequencing. Subcloning of inserts into the pCMV5 expression vector (2, 12) was accomplished with *Bgl* II and *Hin*dIII for C3A α_o and with *Kpn* I and *Hin*dIII for C3A α_s. The C3A α_s insert was removed from pCMV5 with *Nco* I and *Acc* I and shuttled through NpT7-5 to simplify subcloning into the baculovirus expression vector pVL1392. Methods for construction of wild-type α_s sequences in pCMV1 and pVL1392 and wild-type and G2A α_o sequences in pCMV1 have been published (2, 8).

Infection, Transfection, Biosynthetic Radiolabeling, and Harvesting of Cells. Culture and infection of Sf9 cells with recombinant baculoviruses have been described (8). Nearly confluent COS-M6 cells were transfected in six-well tissue culture plates with Lipofectin according to the manufacturer's instructions (GIBCO/BRL, Gaithersburg, MD). Biosynthetic labeling and extraction of proteins for immunoprecipitation were performed as described (8). For fractionation, Sf9 cells were disrupted by nitrogen cavitation and COS cells by homogenization in hypotonic buffer; homogenates from each cell type were subjected to centrifugation as described (13).

Immunotechniques. For immunoprecipitation, extracts of whole cell fractions were incubated on ice overnight with affinity-purified G protein-specific antibodies (14) or with mouse monoclonal antibody H68.4, which is specific for the transferrin receptor (generously provided by I. Trowbridge, The Salk Institute, La Jolla, CA). The next morning rabbit anti-mouse heavy and light chain IgG (affinity-purified; Pierce) was added to samples containing mouse antibodies, followed 10 min later by the addition of fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem) to all samples to precipitate the immunoglobulin. The immunoprecipitates were washed and resolved by SDS/PAGE in 10% or 11% polyacrylamide gels. Western immunoblotting was performed as described (15).

RESULTS

Structural Requirements for Palmitoylation of α Subunits. The alkaline conditions under which palmitate can be cleaved from α subunits are consistent with acylation of a cysteine residue (8). Limited proteolysis indicated that the proteins are modified by palmitate within ≈30 amino acid residues of the amino terminus (8). We proposed that the site of palmitoylation of α subunits is located at position three (Cys-3) of members of the α_s and α_i subfamilies (α_i, α_o, α_z), since this is the only cysteine in the first 65 amino-terminal residues of these proteins. Consistent with this hypothesis is that α_t does

not have a cysteine residue at position 3 and is not palmitoylated (3, 4).

A role for Cys-3 in the palmitoylation of α_o and α_s was tested by mutagenesis of the codon for cysteine to one for alanine (C3A). Alanine is the residue that is present in the analogous position of nonpalmitoylated α_t. Expression of the wild-type and C3A mutant α subunits was detected by immunoprecipitation of extracts of transfected COS cells that had been incubated with [^{35}S]methionine (Fig. 1 *A* and *B*). [^{3}H]Palmitate was incorporated into the wild-type proteins but not the C3A mutants, demonstrating a role for this cysteine residue in palmitoylation of α_o and α_s (Fig. 1 *A* and *B*). These results are in agreement with data published during the course of this work (16, 17). Myristoylation of α_o, however, was not affected by the C3A mutation, since [^{3}H]myristate was incorporated into the wild-type and mutant proteins (Fig. 1*B*).

It was previously demonstrated by mutagenesis that glycine at position two (G2) is necessary for myristoylation of α_o and α_i (2, 7). Here, we tested the effect of the G2A mutation on the incorporation of [^{3}H]palmitate into α_o and α_{i2}. Neither G2A mutant protein expressed in COS cells was palmitoylated (Fig. 1*C*). These results suggest that myristoylation (or membrane association) must occur before α_o or α_i can be palmitoylated.

Role of Palmitoylation in Anchorage of α Subunits to Membranes. Cellular distribution of C3A α_o (nonpalmitoylated) was compared with that of the wild-type protein by transfection and crude fractionation of COS cells. Expression was detected by immunoprecipitation of α from fractions isolated from cells incubated with [^{35}S]methionine. We were not successful in detecting the mutant protein by Western immunoblotting (data not shown). Wild-type α_o protein was found largely in the P1 (1000 × *g* pellet) and P2 (200,000 × *g* pellet) fractions (Fig. 2*A*). By contrast, a considerable amount of the nonpalmitoylated C3A mutant was found in the cytoplasmic S2 fraction (200,000 × *g* supernatant), consistent with palmitoylation contributing to anchorage of α_o to the membrane (Fig. 2*A*). In contrast, no difference was observed between the distribution of wild-type and C3A mutant α_s; both proteins expressed in COS cells were found in the P1 and P2 fractions (Fig. 2*A*). The majority of the overexpressed [^{35}S]methionine-labeled proteins could be extracted from the pellets with 1% (wt/vol) sodium cholate, indicating that the proteins are not simply denatured and aggregated (data not shown). The similarity of the distribution of wild-type and C3A α_s was confirmed by expression of the proteins in Sf9 cells infected with recombinant baculoviruses. Wild-type and C3A α_s proteins were distributed similarly among the P1, P2, and S2 fractions.

Modulation of Palmitoylation of α_s by β-Adrenergic Agonist. Since palmitoylation can be a dynamic modification of proteins, we examined the time course of incorporation of [^{3}H]palmitate into α subunits over 5 min to 16 hr. The greatest radiolabeling of endogenous α_s and α_i in COS cells was achieved within 30–60 min; maximal labeling of the transferrin receptor was achieved after 16 hr (data not shown).

To learn whether receptor stimulation could regulate palmitoylation of α_s, COS cells were incubated with [^{3}H]palmitate in the presence or absence of the β-adrenergic receptor agonist isoproterenol and/or the antagonist propranolol. In these experiments the COS cells were *not* transfected, and endogenous α_s was examined. Incorporation of [^{3}H]palmitate into endogenous α_s was increased when cells were treated with isoproterenol, but not when propranolol was included (Fig. 3). Isoproterenol did not affect synthesis of α_s; the agonist caused no change in the incorporation of [^{35}S]methionine into the protein (data not shown). The agonist-induced increase in the incorporation of [^{3}H]palmitate was specific for α_s, since the incorporation of radiolabel into endogenous α_i and the transferrin receptor was unchanged by isoproterenol (Fig. 3 *B* and *C*). The increase in the incorporation of [^{3}H]palmitate into α_s caused by isoproterenol was most pronounced at early time points (Fig. 4*A*). The difference between isoproterenol-treated and control cells was diminished at later times (30 min). To determine whether the effect of isoproterenol was a result of increased cyclic AMP synthesis, forskolin was used to stimulate adenylyl cyclase directly or dibutyryl cyclic AMP was added. Incubation of cells with either agent (10 μM for 20 min) had no effect on the incorporation of [^{3}H]palmitate into α_s.

Pulse–chase experiments were conducted to attempt to determine whether the effect of isoproterenol was to increase the incorporation of palmitate into α_s or its turnover. Cells were incubated for 15 min with [^{3}H]palmitate, after which the radioactive fatty acid was removed and medium containing unlabeled palmitate, with or without isoproterenol, was added. Radioactivity in α_s was lost more readily in cells incubated with isoproterenol, suggesting that the agonist increased the turnover of palmitate in α_s (Fig. 4*B*).

DISCUSSION

Structural Requirements for Palmitoylation of α Subunits. Several lines of evidence (outlined under *Results*) point to Cys-3 as a site of α-subunit acylation by palmitate. Importantly, when C3 is mutated, [^{3}H]palmitate is not incorporated into α_o or α_s (Fig. 1; refs. 16 and 17). However, short of isolation and analysis of a palmitoylated peptide, caution must be taken in this assignment, since it is possible that this cysteine residue could be required in some ancillary fashion. For example, mutation of Gly-2 in $G_{i\alpha}$ and $G_{o\alpha}$ also prevents palmitoylation (Fig. 1); mutation of a carboxyl-terminal cys-

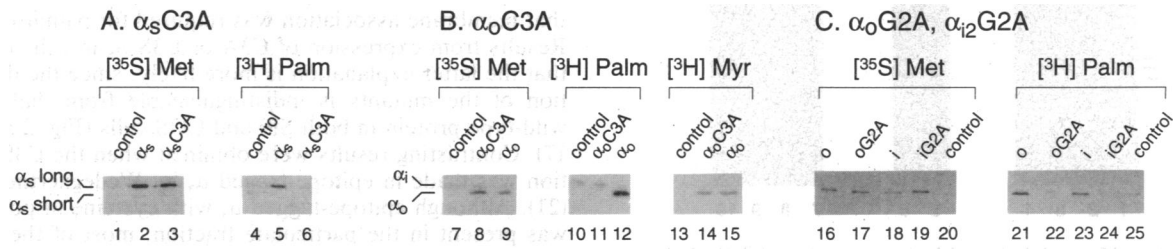

FIG. 1. Requirement for Cys-3 and Gly-2 for palmitoylation of α subunits. Expression and fatty acylation of α subunits were assayed in transfected COS cells by metabolic radiolabeling and immunoprecipitation. (*A*) C3A mutation of α_s. The long form of wild-type α_s and the C3A mutant were expressed at similar levels in transfected cells as indicated by incorporation of [^{35}S]methionine (lanes 2 and 3). The antibodies utilized (no. 584) recognize both the long and short forms of endogenous α_s. [^{3}H]Palmitate was incorporated only into wild-type α_s (lane 5 versus 6). (*B*) C3A mutation of α_o. The A569 antibodies utilized recognize both endogenous α_i and expressed α_o. Both wild-type and C3A mutant α_o were labeled with [^{3}H]myristate (lanes 14 and 15), but only wild-type α_o was labeled with [^{3}H]palmitate (lane 12 versus 11). (*C*) G2A mutation of α_o and α_{i2} (previously shown to prevent incorporation of [^{3}H]myristate). [^{3}H]Palmitate was incorporated into the wild-type (lanes 21 and 23) but not the G2A mutant proteins (lanes 22 and 24). Film exposure to fluorograms ranged from 3 to 10 days.

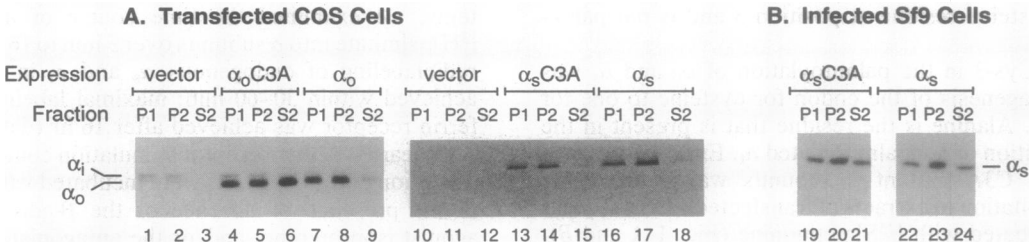

FIG. 2. Distribution of wild-type and nonpalmitoylated C3A mutant α subunits in fractionated cells. Fraction P1 is the pellet from a 1000 × *g* centrifugation, P2 is the 200,000 × *g* pellet, and S2 is the 200,000 × *g* supernatant. (*A*) Immunoprecipitation of α subunits from transfected COS cells that had been incubated with [^{35}S]methionine (100 μCi/ml for 60 min; 1 μCi = 37 kBq). Control cells were transfected with the pCMV5 vector. The A569 antibodies recognize endogenous α_i and expressed wild-type and C3A mutant α_o. Wild-type α_o was largely confined to the membrane-containing pellets (lanes 7 and 8), whereas a significant amount of the nonpalmitoylated mutant C3A protein was present in the cytosolic S2 fraction (lane 6). Both wild-type and mutant C3A α_s proteins were confined to the pellets (lanes 13, 14, 16, and 17), which were extracted and immunoprecipitated with 584 antibodies. (*B*) Western immunoblotting of Sf9 cells infected with recombinant baculoviruses to express wild-type or C3A mutant α_s together with G-protein β_1 and γ_2 subunits. No discernible effect of the mutation was observed on distribution of α_s.

teine in p21^{ras} blocks not only prenylation of that cysteine but also palmitoylation of an upstream cysteine (18).

The amino-terminal sequences and fatty acylation properties of most members of the α_i subfamily are similar to those of some members of the Src-related family of tyrosine kinases (16). These proteins (α_i, α_o, α_z, p56^{lck}, and p59^{fyn}) are both myristoylated and palmitoylated (8, 19, 20). The two kinases that are dually acylated have a cysteine residue at position 3, as do members of the α_i subfamily (Fig. 5). When Cys-3 of p56^{lck}, p59^{fyn}, or α_o is altered, the expressed proteins do not incorporate [^{3}H]palmitate (ref. 20; D. Lublin, personal communication). p60^{src}, which has a serine residue at position 3, is not palmitoylated but is myristoylated at Gly-2 (Fig. 5). The amino-terminal sequence Met-Gly-Cys (shared by myristoylated and palmitoylated α subunits, p56^{lck}, and p59^{fyn}) may represent a motif for cotranslational and posttranslational processing that would include cleavage of the initiator methionine, myristoylation of the glycine, and palmitoylation of the cysteine (particularly when serine or cysteine is present at position 6). These considerations suggest that the Src family members Lsk, Lyn, Fgr, Syn, Hck, and Yes may also be dually acylated.

Surprisingly, mutation of Gly-2 blocked not only myristoylation of α_o and α_i [as described previously (2, 7)] but also palmitoylation (Fig. 1*C*). A similar requirement for myristoylation to precede palmitoylation has been observed for p59^{fyn} and p56^{lck} (D. Lublin, personal communication). Myristoylation of G_α subunits and the kinases may be necessary for substrate recognition by a protein palmitoyltransferase; alternatively, myristoylation may be necessary for association of substrates with membranes, where protein palmitoyltransferase activities have been detected (9).

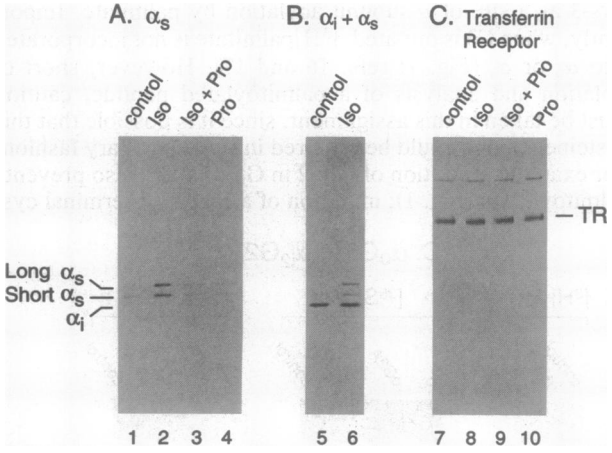

FIG. 3. Specific modulation of the incorporation of [^{3}H]palmitate into endogenous α_s by isoproterenol. COS cells (*not* transfected) were incubated for 20 min in [^{3}H]palmitate-containing medium in the presence or absence (control) of 1 μM isoproterenol (Iso) and/or 5 μM propranolol (Pro). Cell extracts were immunoprecipitated with α_s-specific 584 antibodies (*A*), with common α antibodies (A569) to immunoprecipitate both α_i and α_s (*B*), or with monoclonal antibody H68.4 to immunoprecipitate the transferrin receptor (TR) (*C*). Film was exposed for 6 weeks.

Fatty Acylation and Membrane Association of α Subunits. We and others concluded previously that myristoylation facilitates association of G_α subunits with membranes because nonmyristoylated G2A α_o or α_i, when expressed in COS cells, was found in the cytoplasmic fraction (2, 7). However, in light of the more recent experiments demonstrating a lack of palmitoylation as well as myristoylation of the G2A mutants (Fig. 1*C*), it became unclear which fatty acid (or both) contributes to membrane anchorage of α. We tested the effect of a loss of a single fatty acid (palmitate) with the C3A mutant proteins. A significant amount of C3A α_o was found in the cytoplasmic fraction (Fig. 2*A*), whereas G2A α_o is almost entirely cytoplasmic (2, 7). Thus, it appears that both acylations contribute to the association of α_o with membranes.

In contrast to α_o, the gross distribution of α_s between particulate and soluble fractions was unaffected by the C3A mutation (Fig. 2) or by the C3S mutation studied by Degtyarev *et al.* (17). However, there could be subtle differences between the subcellular distribution of wild-type and mutant α_s that are not detected by crude fractionation. In our previous experiments in which α_s was expressed in Sf9 cells, the protein was found in both the pellet and soluble fractions (8); [^{3}H]palmitate was incorporated only into particulate α_s. We could not distinguish whether palmitoylation was necessary for association of α_s with membranes or the converse—that membrane association was required for palmitoylation. Results from expression of C3A or C3S α_s in cells indicate that the latter explanation is more likely, since the distribution of the mutants is indistinguishable from that of the wild-type protein in both Sf9 and COS cells (Fig. 2 and ref. 17). Contrasting results were obtained when the C3S mutation was made in epitope-tagged α_s by Wedegaertner *et al.* (21). Although epitope-tagged α_s with cysteine at position 3 was present in the particulate fraction, most of the tagged C3S mutant was present in the soluble fraction from human embryonic kidney 293 cells. The epitope tag on C3S α_s is the most obvious reason for the discrepancy between the results of Wedegaertner *et al.* (21) and those of Degtyarev *et al.* (17) and our own. Mutation of Arg-201 in epitope-tagged α_s also causes a change in distribution of the protein (22). The tagged mutant protein was found in the soluble fraction; however, in another study the untagged protein was present in the par-

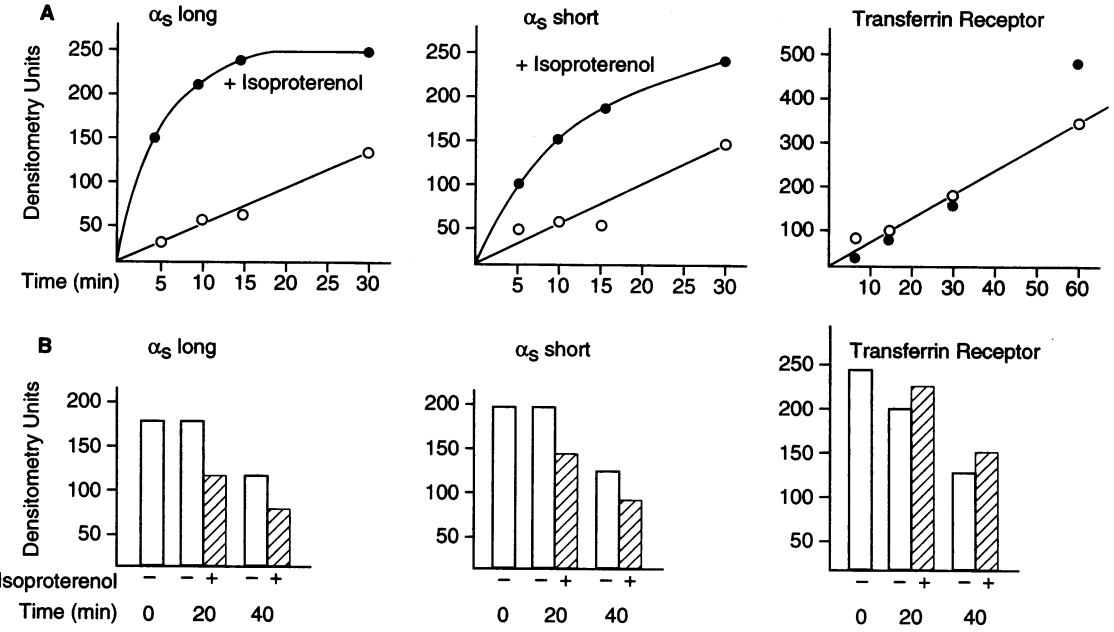

FIG. 4. Time course and pulse–chase of [^{3}H]palmitate incorporation into endogenous α_s, and modulation by isoproterenol. COS cells (*not* transfected) were assayed by immunoprecipitation, SDS/PAGE, fluorography, and densitometry. (*A*) Time course of incorporation of [^{3}H]palmitate into endogenous proteins in the presence of 1 μM isoproterenol (●) or its absence (○). (*B*) Pulse–chase. After a 15-min incubation with [^{3}H]palmitate, cells were rinsed and incubated with medium containing 30 μM unlabeled palmitate and 5% serum with (+) or without (−) 1 μM isoproterenol for 20 or 40 min. Film was exposed for 2.5 months.

ticulate fraction (17). Epitope-tagged and untagged mutant α_s (mutations at Cys-3 or Arg-201) should be compared directly to determine whether the tag causes differential distribution of the protein.

Association of acylated or nonacylated α subunits with cellular membranes may be correlated with their affinity for the $\beta\gamma$ subunit complex. Nonacylated α_o has a reduced affinity (relative to acylated protein) for purified $\beta\gamma$ or membranes (2, 5, 7). In contrast, α_s has an intrinsically higher affinity for $\beta\gamma$ than does α_o or α_i (23), and its distribution is not detectably affected in cell fractionation assays when palmitoylation is prevented by mutation of Cys-3 (Fig. 2 and ref. 17). *In vitro* experiments with purified proteins and phospholipid vesicles have demonstrated that $\beta\gamma$ can function as an anchor for otherwise soluble α_o (23). In summary, our results indicate that palmitoylation alone does not appear to dictate membrane localization of α subunits but can (at least) enhance membrane association promoted by myristoylation.

Protein	Myristate	Palmitate	Amino Terminal Sequences
αs	-	+	M G **C** L G N S K T
αi1	+	+	M G **C** T L S A E D
αi2	+	+	M G **C** T V S A E D
αi3	+	+	M G **C** T L S A E D
αo	+	+	M G **C** T L S A E E
αt1	+	-	M G A G A S A E E
αz	+	+	M G **C** R Q S S E E
αq	-	+	M T L E S I M A **C** C L S E E A
lck	+	+	M G **C** V C S S N P
fyn	+	+	M G **C** V Q C K D K
src	+	-	M G S S K S K P K

FIG. 5. Amino-terminal sequences (single-letter amino acid code) and fatty acylation of G-protein α subunits and Src-related tyrosine kinases. The proteins that are palmitoylated have a cysteine residue at position 3 (boxed). The site of acylation by myristate is glycine at position 2. The amino-terminal sequence Met-Gly-Cys-Xaa-Xaa-Ser/Cys shared by the dually acylated proteins may represent a motif for processing that would include myristoylation of glycine and reversible palmitoylation of cysteine.

We hypothesize that tandem acylation of G-protein α subunits and certain members of the Src family may promote localization of these proteins in plasma membrane specializations termed caveolae and/or their association with glycosylphosphatidylinositol-anchored membrane proteins [which are concentrated in caveolae (24, 25)]. Localization of G-protein α subunits in caveolae has been detected with immunogold (S.M.M., A.G.G., and K. Muntz, unpublished data) or by immunoblotting of purified membrane fractions (ref. 24; W. Chang, S.M.M., A.G.G., and R. G. W. Anderson, unpublished data). Dually acylated members of the Src family are associated with glycosylphosphatidylinositol-anchored proteins, and such association is dependent on both myristoylation and palmitoylation (ref. 20; D. Lublin, personal communication). The tyrosine kinase c-Yes is also enriched in caveolar preparations (24).

Regulation of Palmitoylation by Receptor. Palmitoylation of α_s is a dynamic modification. [^{3}H]Palmitate is incorporated into the protein with a time course and characteristics that reflect turnover, rather than protein synthesis, and such incorporation is modulated by isoproterenol, a β-adrenergic agonist. Similarly, receptor agonists have been shown to cause an increase in the incorporation of [^{3}H]palmitate into the β_2-adrenergic receptor (26). The pulse–chase experiments described above suggest that isoproterenol causes an increase in the turnover of palmitate in α_s. The most obvious mechanistic hypothesis is that the activated α subunit is the preferred substrate for a palmitoylthioesterase. If receptor (or a downstream mediator) were to activate such an enzyme, one would not anticipate specificity for a given receptor-activated G_α subunit, which was observed. Although this mechanism would suggest a net decrease in the level of palmitoylation of α_s, conclusions cannot be drawn since stoichiometries have not been measured. It will be of interest to learn whether receptor-mediated changes in the palmitoylation of α subunits will influence their intrinsic capacities to interact with receptors and/or effectors or their distribution in the plasma membrane.

It is intriguing to speculate that palmitoylation of α could influence its interactions with receptor, since the amino terminus of α_o interacts with a mastoparan derivative. Mastoparan (a wasp venom peptide) activates G proteins by promoting GDP/GTP exchange in a manner that closely resembles that of receptor (27). [^{125}I-Tyr^3,Cys^{11}]Mastoparan, when covalently bound to nonpalmitoylated α_o, is cross-linked to Cys-3 (28). If mastoparans interact with α subunits at a site similar to that utilized by activated receptors, it seems likely that palmitoylation of Cys-3 of α would influence its interaction with receptor.

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