$G\alpha_{12}$ Requires Acylation for Its Transforming Activity

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ABSTRACT: The α subunit of the heterotrimeric G protein G_{12}, harboring a mutation in the GTP binding domain (Q229L), behaves as a potent oncogene in NIH 3T3 cells. This α subunit, like most other G protein α subunits, undergoes palmitoylation, the reversible posttranslational addition of palmitate to cysteine residues. We investigated the role of palmitoylation of α_{12} in membrane localization and transformation efficiency and whether another lipid modification, myristoylation, could substitute for palmitoylation. NIH 3T3 cells were stably transfected with plasmids that expressed the wild-type α_{12}, the constitutively active Q229L (QL) mutant, and mutants in which C11 was changed to S (C11S) and S2 and R6 were changed to G and S, respectively (S2G). Incorporation of [^{3}H]palmitate was found in the endogenous and expressed α_{12} but not in the C11S mutants. Incorporation of [^{3}H]myristate was found only in the S2G mutants. The wild type, QL mutant, and all the acylation mutants were found in the particulate fraction. Cells expressing the nonpalmitoylated C11S,QL mutant did not undergo transformation. The S2G mutation in the nonpalmitoylated C11S,QL mutant restored the transformation efficiency to a greater level than that of the palmitoylated QL mutant as measured by foci formation, growth in soft agar, and growth rate. Palmitoylation was critical for the transformation efficiency of α_{12} but not specifically required because myristoylation could substitute for these functions.

Heterotrimeric G proteins[1] that transduce signals from cell surface receptors to intracellular effectors are composed of α, *β*, and *γ* subunits. This family of proteins can be divided into four groups, G_s, G_i, G_q, and $G_{12/13}$, based on sequence homology of the α subunits (*1*). G_{12} and G_{13} were discovered using a homology-based PCR strategy on a mouse brain cDNA library and are ubiquitously expressed (*2, 3*). An understanding of their physiologic function is emerging but is still relatively unknown. A role in the regulation of cell growth has been suggested by studies showing that a mutation in the GTP binding domain of α_{12} or α_{13} resulting in constitutive activation leads to transformation of transfected NIH 3T3 cells (*4*−*8*) and Rat-1 cells (*8*). In addition, microinjection of antibodies to G_{12} inhibit thrombin-stimulated DNA synthesis (*9*). The proximal downstream effectors of these proteins are not known but studies have shown G_{12}/G_{13} effects on phospholipase A2 (6), the Na/H exchanger (*10, 11*), the Jun kinase/stress-activated protein kinase pathway (*12*), EGF-stimulated MAP kinase activity (*8*), stress fiber formation and focal adhesion assembly (*13*), and AP-1-mediated transcriptional activity (*9*). Receptor coupling to G_{12}/G_{13} has been shown for the thrombin, thromboxane A2 (*14*), bradykinin (*15*), and TSH receptors (*16*), with other receptors yet to be identified.

G protein α subunits including α_{12} and α_{13} undergo posttranslational lipid modifications. Myristoylation, which occurs on α_i, α_o, α_z, and α_{td} (*17*−*20*), is the irreversible attachment of 14-carbon myristate to amino-terminal glycine residues through an amide bond (*21*). Myristoylation increases the affinity of the α subunit for *βγ* and is critical for the membrane attachment of these proteins (*18, 22, 23*). Palmitoylation has been found on α_o, α_i, α_s, α_q, α_z, α_{11}, α_{12}, and α_{13} (*24*−*30*). It is the reversible posttranslational addition of the 16-carbon fatty acid palmitate, through thioester bonds to cysteine residues (*31*). The site of the modification on α_{12} is not known, but mutation of amino-terminal cysteine residues on other α subunits prevents the modification (*24, 26*−*29, 32*).

Possible functions for α subunit palmitoylation are regulation of signal transduction and localization of the α subunit to the membrane (*33*). Activation of α_s by the *β*-adrenergic receptor or cholera toxin leads to turnover of palmitate, suggesting a role in signaling (*32, 34, 35*). Another function is membrane attachment since the myristoylated α subunits require palmitoylation for membrane attachment (*32, 36*). For the nonmyristoylated α subunits, α_s, α_q, and α_{11}, palmitoylation has been reported to have a major (*27*), intermediate (*29*), and minor (*26, 32*) effect on membrane attachment, possibly due to differences in expression systems. The function of palmitate in mediating α subunit−effector interactions has been investigated for only α_s and α_q. Palmitoylation is not required but enhances the stimulation of adenylyl cylcase by α_s (*27*). For α_q, mutation of the

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[1] Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagles medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MARCKS protein, myristoylated, alanine-rich protein kinase C substrate.

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amino-terminal cysteine residues blocks palmitoylation and activation of phospholipase Cβ (*27*, *37*, *38*).

In this study we addressed the function of palmitoylation of α_{12} on its membrane localization and transforming activity. We found that a nonpalmitoylated mutant localized to the membrane fraction but lacked transforming ability. A myristoylated, nonpalmitoylated mutant transformed NIH 3T3 cells better than the palmitoylated protein.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The Q229 to L mutation in the murine α_{12} (a gift from Melvin I. Simon, California Institute of Technology) (*2*) had been prepared previously (*4*). We created the amino-terminal mutants by a PCR method using oligonucleotides that contain the mutation and anneal to the 5′ end of the cDNA and nonmutated oligonucleotides that anneal 385 base pairs downstream (*39*). The oligonucleotides with the mutations and new *Bgl*II or *Sal*I restriction sites are as follows: GCGGGGCGCGGCCGCGGCCTGAGGGCGGCCATGTCCGGGGTGGTGCGGACCCTTAGCAGATCTTTGCTGCCGGC for mutation of C11 to S and GCGGGGCGCGGCCGCGGCCTGAGGGCGGCCATGGGCGGGGTGGTGTCGACCCTTAGC for mutation of S2 to G and S6 to R. The PCR fragments were digested and ligated into *Sac*II and *Afl*II sites in the pSP72-α_{12} and pSP72-α_{12} Q229L plasmids. The complete cDNA was then cloned into a *Not*I site of a modified pZipNeoSv(x) (pZVN) vector (*40*). This vector contains *neo*, a dominant selectable marker that confers resistance to the antibiotic geneticin (G418). The mutation and the entire sequence of the PCR-amplified fragment was confirmed by dideoxy sequencing. The plasmids were purified using Qiagen Maxi-prep columns.

Transfection. NIH 3T3 mouse fibroblasts were maintained in complete DMEM containing 10% calf serum, 100 units of penicillin/mL, and 100 μg/mL streptomycin (Biofluids, Rockville, MD) (*41*). Transfection of the NIH 3T3 cells in 100 mm tissue culture dishes with 1 μg of plasmid DNA/dish was performed by the calcium phosphate technique (*42*). Mass populations of transfected cells were selected by their growth in the presence of 400 μg/mL geneticin (Gibco/BRL). The number of transformed foci was determined 2−3 weeks after transfection.

Metabolic Labeling and Cell Fractionation. Transfected cells were incubated in 75 cm^2 flasks in complete DMEM and geneticin until they were nearly confluent. After incubation in serum-free DMEM for 2 h, cells were incubated in 5 mL of serum-free DMEM containing 1% DMSO and 500 μCi/mL of either [^{3}H]myristate or [^{3}H]palmitate (American Radiolabeled Chemicals, specific activity 40 and 60 Ci/mmol, respectively) for 1 h. Cycloheximide (50 μg/mL) was present for 30 min of the preincubation and during the labeling with [^{3}H]palmitate to prevent incorporation of [^{3}H]-myristate from intracellular conversion of [^{3}H]palmitate. For [^{35}S]methionine labeling, the cells were incubated for 5 h in DMEM with 20 μM methionine and 75 μCi of [^{35}S]-methionine/mL (American Radiolabeled Chemicals, specific activity 1175 Ci/mM). The cells were scraped in ice-cold phosphate-buffered saline and centrifuged at 2000*g* for 10 min. The cell pellet was stored at −70 °C. For fractionation, the cells were homogenized and separated into particulate and soluble fractions by centrifugation at 125000*g* for 1 h as described (*36*).

Immunotechniques. The QE antibody, specific for α_{12}, was prepared by immunizing rabbits with a decapetide of the carboxy-terminal sequence of α_{12} (QENLKDIMLQ) followed by affinity purification against this decapeptide (*43*, *44*). For immunoprecipitation, equal amounts of protein from the particulate fractions were solubilized in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% (w/v) SDS, and 1% (v/v) Triton X-100 and then incubated overnight at 4 °C with 10 μg/mL QE antibody. Protein A−Sepharose (Pharmacia) was added and the samples were washed, separated on SDS−PAGE, and prepared for fluorography as previously described (*22*). Immunoblotting was performed with the QE antibody (1 μg/mL), a peroxidase-labeled secondary antibody (Amersham) diluted 1/4000, and the enhanced chemiluminescence kit (Amersham) for detection.

Transformation Assays. For determination of anchorage-independent growth, suspensions of 5×10^3 cells in 0.57% agar (Difco) in complete DMEM were added to each well of 6-well plates that contained 1% agar in complete DMEM. Colonies >0.15 mm in diameter were counted after 15 days of culture. Determination of growth in low serum conditions was performed by incubating 2×10^4 cells/well in 24-well plates overnight in complete DMEM and then incubating for 6 days in DMEM containing 0.1% calf serum. Cell counts were obtained in triplicate every other day. The growth rate of cells was determined by incubating 1×10^4 cells/well in triplicate in 24-well plates in complete DMEM and performing cell counts on day 2, 4, 5, and 6.

Detergent Solubilization. Particulate fractions (80 μg of protein) of transfected cells were centrifuged at 430000*g* for 4 min in a TL100.1 rotor (Beckman) and the supernatant was removed. The pellet was resuspended in 40 μL of a detergent solution with either 1% (w/v) Triton X-100 or 1% (w/v) sodium cholate in a buffer of 150 mM NaCl, 5 mM Hepes, pH 7.4, 50 mM mannitol, and protease inhibitors (*36*). The samples were incubated on ice for 30 min with occasional vortexing. A 20 μL portion of the sample was removed and mixed with SDS sample buffer, and the remaining 20 μL was centrifuged at 430000*g* for 4 min. The soluble and pellet fractions were separated and prepared for SDS−PAGE and immunoblotting.

Limited Trypsin Digestion. Particulate fractions (50 μg) of the transfected cells were incubated with or without 0.25 μg of L-1-(tosylamimo)-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) in a buffer of 50 mM Hepes, pH 7.4, 1 mM EDTA, 10 mM $MgCl_2$, 100 μM GTP, 3 mM dithiothreitol, and 0.05% (w/v) Lubrol PX for 2 min at 30 °C. The reaction was stopped by the addition of 20 μg of soybean trypsin inhibitor (Boehringer-Mannheim) and SDS sample buffer. The proteins were separated on SDS−PAGE and analyzed by immunoblotting.

Miscellaneous. The protein concentration was determined using the Bio−Rad protein assay reagent. SDS−PAGE was performed on 10% polyacrylamide Tris−glycine gels (Novex).

RESULTS

Mutagenesis and Metabolic Labeling. We made mutations in the amino terminus of the wild-type α_{12} and an α_{12} mutant

Table 1: Wild-Type and Mutant α_{12} Subunits

α_{12} subunit	amino terminal sequence[a]	palmitoyl.	myristoyl.
WT	M S G V V R T L S R C L L	+	-
C11S	M S G V V R T L S R **S** L L	-	-
S2G	M G G V V **S** T L S R C L L	+	+
S2G,C11S	M G G V V **S** T L S R **S** L L	-	+

[a] Mutated residues are in boldface type; probable site of palmitoylation is underlined; site of myristoylation is double-underlined.

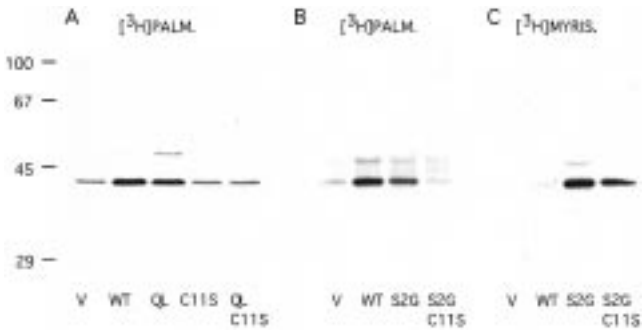

FIGURE 1: Incorporation of [^{3}H]palmitate and [^{3}H]myristate into α_{12} and its mutants. NIH 3T3 cells were transfected with the pZipNeoSV(x) vector alone or containing the cDNAs of the wild-type or mutant α_{12} proteins, followed by selection with the antibiotic G418. Proteins were radiolabeled by incubating cells in either 500 μCi/mL [^{3}H]palmitate (A, B) or 500 μCi/mL [^{3}H]myristate (C) for 1 h. The cells were homogenized and fractionated by centrifugation into particulate and soluble fractions. α_{12} was immunoprecipitated from the particulate fractions with an affinity-purified antibody raised against the carboxy-terminal decapeptide of α_{12} and then analyzed by SDS−PAGE and fluorography. The exposure time was 6 weeks at −70 °C. The molecular mass markers in kilodaltons are shown to the left.

in which Q229 was changed to L (Table 1). The Q229L mutation blocks GTPase activity so that the α subunit binds GTP and is constitutively active (*4*, *5*, *8*). Mutation of S2 and R6 to G and S, respectively, created a consensus sequence for myristoylation (*21*).

NIH 3T3 cells were stably transfected with the pZipNeoSV(x) vector alone or with the cDNAs for the wild-type or mutant α_{12} subunits and metabolically labeled with [^{3}H]-palmitate or [^{3}H]myristate. Immunoprecipitation of α_{12} showed incorporation of [^{3}H]palmitate into a 43 kDa band, the endogenous α_{12}, in all the cell lines and the overexpressed WT α_{12} and the QL and S2G mutants (Figure 1A,B). Mutation of C11 to S prevented [^{3}H]palmitate incorporation into α_{12}. [^{3}H]Myristate incorporation into α_{12} only occurred in the S2G mutants (Figure 1C). Both [^{3}H]palmitate and [^{3}H]myristate were incorporated into the S2G mutant. The α_{12} protein and mutants were expressed to similar levels as seen by immunoprecipitation from [^{35}S]methionine-labeled cells and immunoblotting (Figure 2).

A band migrating at 49 kDa was detected with immunoprecipitation of the expressed α_{12} subunits. This band could represent an α_{12} protein with an earlier translational start or additional covalent modifications. Coimmunoprecipitation of an α_{12} binding protein is unlikely because the band was only detected after [^{3}H]palmitate or [^{3}H]myristate labeling in the cells expresssing the α_{12} proteins that incorporated [^{3}H]palmitate or [^{3}H]myristate, respectively.

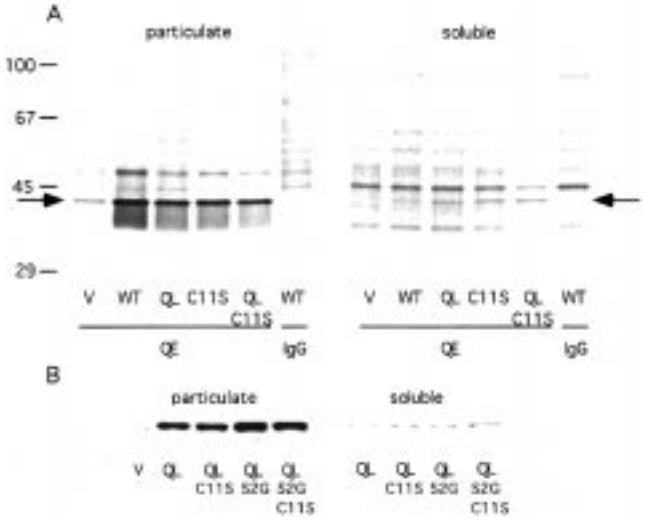

FIGURE 2: Membrane localization of α_{12} and its mutants. NIH 3T3 cells expressing α_{12} and its mutants were incubated with 75 μCi/mL [^{35}S]methionine for 5 h and then homogenized and separated into particulate and soluble fractions by centrifugation. (A) α_{12} was immunoprecipitated with the specific QE antibody or rabbit IgG and then analyzed by SDS−PAGE and fluorography. (B) Protein (40 μg) from each fraction was used for immunoblot analysis using the QE antibody, peroxidase-linked second antibody, and enhanced chemiluminescence for detection.

Membrane Localization. We determined the localization of α_{12} in NIH 3T3 cells stably transfected with α_{12} and the acylation mutants by immunoprecipitating α_{12} from the particulate and soluble fractions of [^{35}S]methionine-labeled cells (Figure 2A). The endogenous α_{12} in the vector-transfected cells and the overexpressed wild-type α_{12} was found exclusively in the particulate fraction. About 10% of the QL, C11S, and C11S,QL mutant proteins were localized to the soluble fraction. Immunoblots of the particulate and soluble fractions also showed most of the nonpalmitoylated, C11S mutant protein and the myristoylated mutants in the particulate fraction (Figure 2B).

Transformation Activity. Cells expressing the GTPase-deficient Q229L mutant of α_{12} have potent transforming activity (*4*, *5*, *8*). In this study, the cells expressing the palmitoylated QL mutant showed properties of transformation—formation of foci, non-anchorage-dependent growth, serum-independent growth, and a rapid doubling time (Figure 3 and Table 2). All of these characteristics of transformation were absent in the cells transfected with the nonpalmitoylated C11S,QL mutant. Myristoylation alone could restore the transformation ability of α_{12}. The cells expressing the myristoylated S2G,C11S,QL mutant underwent transformation with a higher efficiency than the palmitoylated QL mutant (Figure 3 and Table 2). Their doubling time was faster and the number of foci was greater. The cells expressing the myristoylated, S2G,QL and S2G,C11S,QL mutants also appeared different than both the transformed, QL-expressing and nontransformed C11S,QL-expressing cells—the colonies were smaller, the cells attached poorly to the plates, and most cells were round or had short extensions (Figure 4).

Overexpression of the wild-type α_{12} also leads to cell transformation but at a lower efficiency (*4*, *45*). The cells transfected with the WT α_{12} and the myristoylated S2G and S2G,C11S mutants all transformed cells to a similar degree

Table 2: Transforming Activity of NIH 3T3 Cells Expressing Wild-Type and Mutant α_{12} Subunits

cell line	lipid	foci/μg of DNA	growth in soft agar	growth in 0.1% serum	doubling time (h)
pZVN		<1	−	−	42
α_{12} WT	palm.	41 ± 16	−	−	51
α_{12} QL	palm.	432 ± 30	+	+	31
α_{12} C11S,QL		<1	−	−	46
α_{12} S2G,C11S,QL	myr.	680 ± 62	+	+	26
α_{12} S2G,QL	myr./palm.	586 ± 97	ND[a]	ND	ND

[a] Not determined.

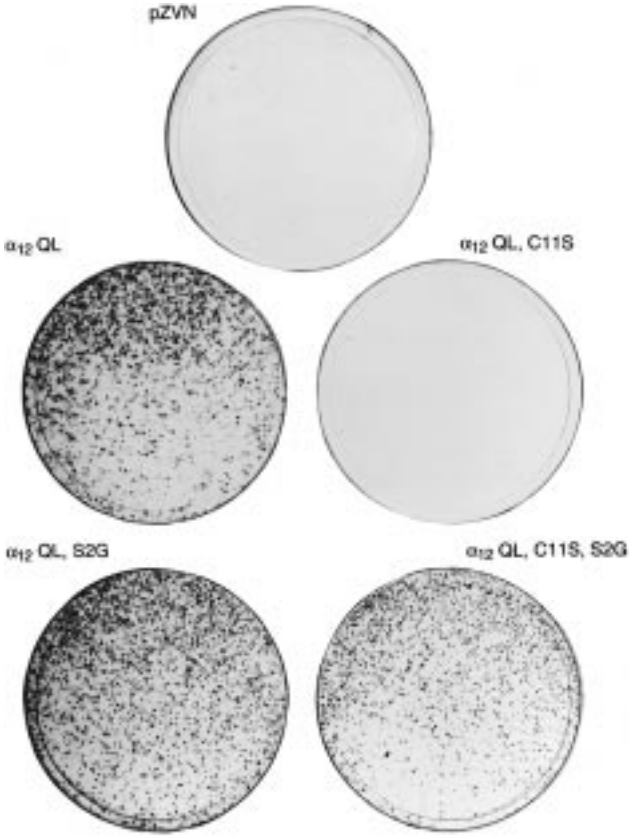

FIGURE 3: Foci formation in NIH 3T3 cells expressing constitutively active α_{12} and its acylation mutants. NIH 3T3 cells were transfected with 1 μg of vector alone (pZVN) or with cDNAs of mutant α_{12} as indicated. Cultures were maintained in DMEM with 10% calf serum. Plates were stained with methylene blue 3 weeks after transfection.

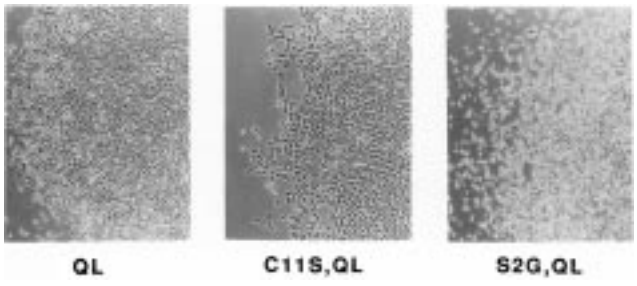

FIGURE 4: Colonies of transfected NIH 3T3 cells. NIH 3T3 cells transfected with the α_{12} mutants QL, C11S,QL, and S2G,QL were photographed with a Zeiss Axiovert 405M microscope.

as detected by foci formation. The cells expressing the nonpalmitoylated C11S mutant did not form foci (data not shown).

We tested whether the nonpalmitoylated C11S α_{12} mutant was in a functional conformation by assessing its detergent solubility and resistance to trypsin cleavage. The C11S,QL mutant was solubilized by both Triton X-100 and cholate to a similar degree as the QL mutant (Figure 5 A,B). Cholate solubilized about 80% and Triton X-100 about 40% of the overexpressed α_{12} mutants. α Subunits are significantly protected from trypsin proteolysis, except for cleavage at an amino-terminal site, when they are in their active, GTP-bound form (*46*). Incubation of the QL mutant, but not the wild-type α_{12}, with GTP and trypsin resulted in the generation of a stable, 38 kDa fragment because the QL mutation hinders GTP hydrolysis (Figure 5C). Trypsin digestion of the C11S,QL mutant also generated a 38 kDa protein. These results indicate that the expressed C11S,QL mutant was in a functional conformation. Neither improper folding nor aggregation was therefore responsible for the loss of its transforming activity.

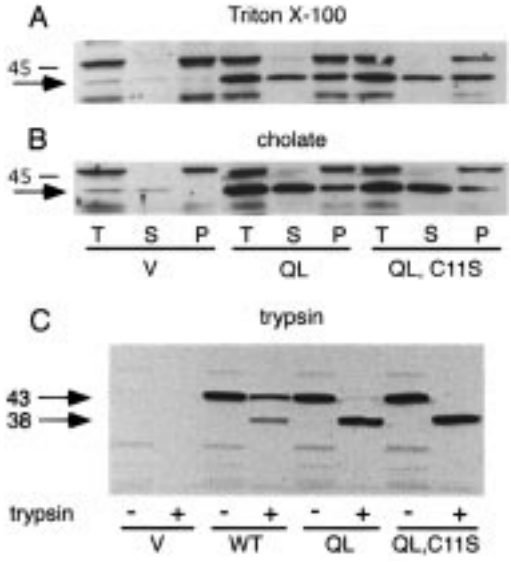

FIGURE 5: Detergent solubility and trypsin digestion of α_{12} mutants. (A, B) Particulate fractions of NIH 3T3 cells transfected with the vector alone (V) or the α_{12} mutants QL and C11S,QL were treated with either 1% Triton X-100 (A) or 1% cholate (B) for 30 min on ice. Half of the sample was removed (T) and the rest was centrifuged at 430000*g* for 4 min. The pellet (P) was separated from the supernatant (S) and the samples were analyzed by SDS−PAGE and immunoblotting. The arrow points to the 43 kDa α_{12} band. (C) Particulate fractions of NIH 3T3 cells transfected with the vector (V), α_{12} wild type (WT), and QL mutants were treated with 0.25 μg of trypsin for 2 min at 30 °C. The reaction was stopped by soybean trypsin inhibitor and SDS sample buffer. The samples were analyzed by SDS−PAGE and immunoblotting. The arrows point to the 43 kDa α_{12} band and the 38 kDa digestion product.

DISCUSSION

α_{12}, unlike many other G proteins, does not have a well-defined signaling cascade but activation of this pathway

Table 3: Amino-Terminal Sequence of α Subunits

subunit	sequence[a]
α_{12}	M S G V V R T L S R **C** L L P A E A G A R E R R A G A A R D A E R E A R R R S[b]
α_{13}	M A D F L P S R S V L S V **C** F P G **C** V L T N G E A E Q Q R K S
α_{i1}	M G **C** T L S A E D K A A V E R S
α_{td}	M G A G A S A E E K H S
α_{z}	M G **C** R Q S S E E K E A A R R S
α_{q}	M T L E S I M A **C** **C** L S E E A K E A R R I N
α_{s}	M G **C** L G N S K T E D Q R N E E K A Q R E A N

[a] Probable sites of palmitoylation are in boldface type; positively charges residues are underlined; possible sites of phosphorylation on α_{12} are double-underlined. [b] Start of $\beta\gamma$ contact in box.

causes cellular transformation. In this study, we found that palmitoylation or myristoylation of α_{12} was required for the transforming activity. Acylation was not critical for membrane attachment because a nonacylated mutant was found in the membrane fraction. Mutants that underwent myristoylation differed from the palmitoylated wild-type α_{12} because the cells expressing the myristoylated mutant had a higher transformation efficiency and a different phenotype.

The role of palmitoylation in effector interactions has been studied previously for only α_s and α_q with no consistent function found. For α_s, a nonpalmitoylated, constitutively active mutant could activate adenylyl cyclase but not as well as the palmitoylated form (*27*). The intracellular location rather than the palmitate per se may have been responsible for the lower level of activation because most of the nonpalmitoylated mutant was in the cytosol. For α_q, mutation of C9 and C10 inhibits palmitoylation and the ability of α_q to activate phospholipase Cβ (*27*, *37*, *38*). The cysteine residues rather than palmitate may be critical for the effector interactions because purified α_q, treated with protein palmitoylthioesterase to remove palmitate, retained its ability to activate phospholipase Cβ (*37*). However, myristoylation of a nonpalmitoylated α_q mutant lacking C9, C10 restored some of the phospholipase Cβ activity, suggesting that either the cysteine residues or acylation is necessary for effector interactions (*27*).

For α_{12}, the immediate downstream effector is not known (*4*, *47*). α_{12} can activate the Na/H exchanger and kinase pathways but probably not directly (*10*, *12*, *48*). Expression of either the wild-type or constitutively active α_{12} causes neoplastic transformation that is a sensitive end point for activation of an α_{12}-mediated cascade (*45*, *49*). Mutation of C11 to S on α_{12} blocked all transformation activity. Unlike α_q, the cysteine residue in α_{12} does not appear itself to be critical for effector interactions, since the S2G,C11S mutant was fully transforming. The nonpalmitoylated α_{12} may be similar to α_s in that it can partially activate its downstream effector but this is inadequate to activate the cascade. Instead, acylation and the amino terminus of α_{12} may play a unique role in its protein function (discussed below).

The subtle differences we found between the cells expressing the palmitoylated wild-type and the myristoylated mutants may be due to the wild-type undergoing cycles of palmitoylation/depalmitoylation and resulting changes in membrane affinity compared to the irreversibility of myristoylation and constant membrane affinity. A mutant of α_s that undergoes myristoylation instead of palmitoylation has a basal adenylyl cyclase activity severalfold greater than the wild type (*27*). The round cell shape and small colony size seen in the cells expressing the myristoylated mutants may have resulted from greater stimulation of an α_{12} signaling pathway. The Rho family of GTP-binding proteins may be involved because they control phenotypic responses and α_{12} can regulate some of these Rho-dependent effects (*13*).

Palmitoylation was not critical for the membrane attachment of α_{12} because most of the nonpalmitoylated C11S α_{12} mutant was found in the membrane fraction. The membrane localization is unlikely to be due to aggregation that occurs for nonpalmitoylated α_{i1} and α_{11} in transfected Cos cells (*29*, *36*) because the C11S protein could be solubilized by Triton X-100 and cholate. Stable transfection of α subunits leads to an increase in $\beta\gamma$ expression (*50*) that may explain the membrane attachment of C11S α_{12}. Palmitoylation is not required for heterotrimer association though it increases the affinity of α subunits for $\beta\gamma$ (*36*, *51*).

The site of palmitate binding to α subunits has not been confirmed by chemical analysis but cysteine residues near the amino terminus are the probable sites. Tryptic cleavage studies first showed that palmitoylation occurs near the amino terminus of α subunits (*25*). All α subunits (except transducin and gustducin) have one or two cysteine residues at their amino termini and mutagenesis of these residues prevents palmitoylation. The lack of [^{3}H]palmitate incorporation into the C11S α_{12} mutant indicated that this residue is critical for the modification and is probably the site of palmitate binding because it is the only cysteine residue at the amino terminus. Lipid modification of cysteine residues is heterogeneous, with palmitate being the primary species but myristate, stearate, and oleate can also be incorporated (*52*). The functional difference, if any, between these groups has not been determined. In a previous study, using similar [^{3}H]palmitate labeling conditions and reagents, we determined that the incorporated tritium migrated with palmitate on thin-layer chromatography (*26*).

The amino terminus of α_{12} is distinctive compared to other α subunits. This region is the most divergent between α_{12}

and α_{13}, which overall share 67% amino acid sequence identity (*2*). It extends further from the $\beta\gamma$ contact area, which starts at S38, than any other α subunit (Table 3). The cysteine that undergoes palmitoylation in α_{12} is 27 residues from the conserved $\beta\gamma$ contact area compared to 13 residues for the other α subunits (except α_s). The region has 10 arginine residues and a net positive charge of +5. α_{12} undergoes phosphorylation in platelets after receptor activation and in cell lines overexpressing α_{12} after phorbol ester treatment (*53*, *54*). The site of phosphorylation by protein kinase C in vitro is on the amino terminus of α_{12} (*53*) (Table 3). The combination of acylation, positively charged residues, and phosphorylation is also found on the MARCKS protein and the amino terminus of Src (*55*). For these proteins, the positively charged residues bind to negatively charged phospholipids in the membrane and in conjunction with the hydrophobic myristoyl group cause membrane attachment that is reversible with phosphorylation (*56*, *57*).

The amino terminus of the α subunit lies on the membrane-facing surface of the crystal structures of the α_{td} and α_{i1} heterotrimer (*58*, *59*). Near the amino terminus, the α subunit forms a helix and contacts the side of the β subunit structure. For α_{12}, the longer amino terminus may extend along the surface of the membrane through attachment by palmitate and the positive residues to aid membrane attachment and locate α_{12} to specific domains enriched in acidic lipids. The concentration of α_{12} is too low to create domains, but α_{12} may join domains formed by other membrane proteins with basic residues such as MARCKS that are present in higher concentrations. Establishment of the domains can have functional consequences for signaling. The MARCKS protein, at physiologic concentrations, inhibits phospholipase C activity (*60*). The basic residues on MARCKS create domains that sequester PIP_2, the lipid substrate for phospholipase C. Phosphorylation releases MARCKS from the membrane and generates a burst of phospholipase C activity. The interactions between α_{12} and membrane domains could be an important regulatory step in α_{12} signaling.

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