Acylation of $G\alpha_{13}$ is important for its interaction with thrombin receptor, transforming activity and actin stress fiber formation

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Abstract Palmitoylation of α-subunits in heterotrimeric G proteins has become a research object of growing attention. Following our recent report on the acylation of the monopalmitoylated $G\alpha_{12}$ [Ponimaskin et al., FEBS Lett. 429 (1998) 370–374], we report here on the identification of three palmitoylation sites in the second member of the G_{12} family, $G\alpha_{13}$, and on the biological significance of fatty acids on the particular sites. Using mutants of α_{13} in which the potentially palmitoylated cysteine residues (Cys) were replaced by serine residues, we find that Cys-14, Cys-18 and Cys-37 all serve as palmitoylation sites, and that the mutants lacking fatty acids are functionally defective. The following biological functions of $G\alpha_{13}$ were found to be inhibited: coupling to the PAR1 thrombin receptor, cell transformation and actin stress fiber formation. Results from established assays for the above functions with a series of mutants, including derivatives of the constitutively active mutant $G\alpha_{13}$Q226L, revealed a graded inhibitory response on the above mentioned parameters. As a rule, it appears that palmitoylation of the N-proximal sites (e.g. Cys-14 and Cys-18) contributes more effectively to biological function than of the acylation site located more internally (Cys-37). However, the mutant with Cys-37 replaced by serine is more severely inhibited in stress fiber formation (80%) than in cell transformation (50%), pointing to the possibility of a differential involvement of the three palmitoylation sites in $G\alpha_{13}$.

Key words: Palmitoylation; Thrombin receptor; Actin stress; Fiber; $G\alpha_{12}$ protein

1. Introduction

Heterotrimeric G proteins, composed of Gα- and Gβγ-subunits, couple activated heptahelical receptors to effector molecules such as adenylyl cyclases, phosphatidylinositol-specific phospholipase C, cGMP, phosphodiesterase and ion channels [1–4]. Receptor activation causes release of GDP from Gα, allowing GTP to bind. Gα and Gβγ dissociate, and both subunits remain activated until the intrinsic GTPase activity of Gα hydrolyses GTP to GDP. In order to function as signal transducer, G proteins have to be localized at the cytoplasmic face of the plasma membrane. Covalent lipid modifications on the α-subunits and on the heterodimeric βγ-subunit are one of the important factors mediating such membrane association. Isoprenylation at the N-terminus of the γ-polypeptide provides a strong membrane anchor for βγ-subunits [5,6]. In addition, all G protein α-subunits so far examined are modified at or near their N-termini by covalent attachment of myristic and/or palmitic acids [7–9]. Unlike myristate, which is stably bound to the protein in an amide linkage, palmitate is attached via a labile thioester linkage to a cysteine residue. With the finding that palmitoylation is a dynamic process, it is now accepted that repeated cycles of palmitoylation and depalmitoylation could be involved in regulating the signalling activity and function of G protein α-subunits [10]. Another possible function of palmitoylation discussed more recently is the targeting of α-subunits to subdomains of the plasma membrane as rafts or caveolae [11–13].

The more recently identified G_{12} family of heterotrimeric G proteins is comprised of the pertussis toxin insensitive $G\alpha_{12}$ and $G\alpha_{13}$ subunits which are expressed ubiquitously [14] and show a relatively slow rate of guanine nucleotide exchange and hydrolysis [15,16]. $G_{12/13}$ are activated through various receptors, including those for thrombin, thromboxane A_2, angiotensin AT_{1A} and lysophosphatidic acid (LPA) [15,17,18]. Studies with constitutively activating mutants have resulted in the identification of several novel functions of $G\alpha_{12}$ and $G\alpha_{13}$, including transforming activity, activation of c-Jun N-terminal kinase cascade [19,20], regulation of Na^+/H^+ exchanger [21], induction of apoptosis [22] and stimulation of the Rho-dependent stress fiber formation and focal adhesion assembly [23]. Whereas there are clear similarities in the effects induced by $G\alpha_{12}$ and $G\alpha_{13}$, differences in the signalling mechanisms have also been reported [24]. Recently it was shown that a nucleotide exchange factor (GEF) for Rho, GEF115, could be directly activated by $G\alpha_{13}$ but not by $G\alpha_{12}$ [25]. On the other hand, GEF115 functions as a GTPase-activating protein for both $G\alpha_{12}$ and $G\alpha_{13}$.

Here we report experiments designed to examine the effect of acylation on the signalling function of the $G\alpha_{13}$ subunit. By site-directed mutagenesis, we show that $G\alpha_{13}$ is palmitoylated on three cysteine residues Cys-14, Cys-18 and Cys-37, two of which (Cys-14 and Cys-18) represent predominant binding sites for palmitate. We present evidence that palmitoylation of $G\alpha_{13}$ is required for Rho-dependent stress fiber formation and that transforming activity of constitutively active $G\alpha_{13}$ is drastically impaired when fatty acid binding sites on the $G\alpha_{13}$

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are mutated. Furthermore, we show that acylation is not required for heterotrimer binding to the membrane and also present evidence that a lack of palmitoylation in the $G\alpha_{13}$ subunit results in significant inhibition of coupling to the PAR1 thrombin receptor.

2. Materials and methods

2.1. Recombinant DNA and site-directed mutagenesis

The construction of pVL1392 vector encoding the $G\alpha_{13}$ wild type ($G\alpha_{13}$wt) was described previously [26]. The $G\alpha_{13}$ mutants ($G\alpha_{13}$M1; M2; M3; M1-2; M1-2-3) with the substitution of serine for cysteine 14; 18; 37; 14 and 18; 14, 18 and 37, respectively, were performed by 'Chameleon™' double-stranded, site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol or by overlap-extension PCR mutagenesis using an oligonucleotide containing the mutation corresponding to the above substitutions. For expression in mammalian cells, cysteine mutants were introduced into constitutively active $G\alpha_{13}$ mutant (Q226L; $G\alpha_{13}$QL) cloned in pCDNA3 plasmid (Invitrogen). The final mutants were verified by DNA sequencing. Recombinant baculoviruses encoding β_1- and γ_2-subunits were kindly provided by P. Gierschik (Ulm, Germany), and those encoding the human thrombin receptor and $G\alpha_{13}$ were recently described [26,27].

2.2. Metabolic labeling and immunoprecipitation

Spodoptera frugiperda (Sf9) cells in 3.5 mm dishes were infected with recombinant baculoviruses at a multiplicity of infection of at least one for each type of virus. After 48 h, Sf9 cells were labeled with Tran[^{35}S]-label (>1000 Ci/mmol, ICN) or [^{3}H]palmitic acid (30–60 Ci/mmol, DuPont NEN) and immunoprecipitated as described previously [28]. Antibodies AS343 raised against the C-terminal domain of $G\alpha_{13}$ (amino acids 367–377, LHDNLKQLMLQ) were used for immunoprecipitation [14,29]. Antibodies were kindly provided from the laboratory of G. Schultz (Berlin, Germany).

2.3. Cell fractionation

Infected and methionine-labeled Sf9 cells were washed once with phosphate-buffered saline, resuspended in extraction buffer (10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol) and homogenized by sonification. The homogenate was centrifuged at $5000\times g$ for 5 min, and the resulting supernatant fraction was centrifuged at $125\,000\times g$ at 4°C for 30 min. The final fractions were diluted in 2×RIPA buffer (2% Triton X-100, 2% deoxycholate, 0.2% sodium dodecyl sulfate (SDS), 300 mM NaCl, 40 mM Tris–HCl (pH 7.4), 20 mM EDTA, 20 mM iodoacetamide) prior to immunoprecipitation.

2.4. Assay for [^{35}S]GTPγS binding

Agonist-promoted binding of [^{35}S]guanosine 5′-(3-*O*-thio)triphosphate to $G\alpha_{13}$ wild type as well as the different fatty acid-deficient mutants was performed according to the method described by Ponimaskin et al. [28]. Briefly, membranes from Sf9 cells expressing the thrombin receptor (PAR1) and/or G protein subunits were resuspended in 55 μl of 50 mM Tris–HCl (pH 7.4) containing 2 mM EDTA, 100 mM NaCl, 3 mM $MgCl_2$ and 1 μM GDP. After adding [^{35}S]GTPγS (1300 Ci/mmol, DuPont NEN) to a final concentration of 30 nM, samples were incubated for 5 min at 30°C in the absence or presence of thrombin receptor activator SFLLRNPNDKYEPF (Sigma). The reaction was terminated by adding 600 μl of 50 mM Tris–HCl (pH 7.5) containing 20 mM $MgCl_2$, 150 mM NaCl, 0.5% NP-40, 200 μg/ml aprotinin, 100 μM GDP and 100 μM GTP for 30 min on ice. The samples were incubated for 20 min with 150 μl of a 10% suspension of Pansorbin cells (Calbiochem) to remove non-specifically bound proteins followed by 1 h at 4°C with 10 μl of antiserum AS343 directed against $G\alpha_{13}$ subunits, which had been preincubated with 100 μl of 10% suspension of protein A-Sepharose. Immunoprecipitates were washed three times, boiled in 0.5 ml of 0.5% SDS, before 4 ml of Ecolite+(ICN) was added and radioactivity measured by scintillation spectrometry.

2.5. Focus formation assay

Focus formation assays were performed as described previously [30]. Briefly, Rat-1 cells (60 mm dishes in triplicate) were transfected with cDNA constructs and cultured for 14 days in the presence of 5% fetal calf serum (FCS). Foci were stained with Giemsa and counted. Three independent experiments were performed.

2.6. Stress fiber formation assay

Swiss 3T3 cells were seeded on glass coverslips and grown overnight. To obtain quiescent and serum-starved cells, cultures were incubated with DMEM supplemented with 0.1% FCS for 24 h, followed by 48 h incubation in DMEM without serum. Plasmids were injected into the nucleus together with Texas Red dextran (5 mg/ml) to visualize injected cells. Injected cells were incubated for 12 h followed by fixation with 4% paraformaldehyde for 20 min and permeabilization with 0.2% Triton X-100 for 5 min. To visualize the polymerized actin, cells were stained with 0.5 μg/ml fluorescein isothiocyanate-phalloidin for 40 min and examined using an inverted microscope (Zeiss Axiovert 100). To determine the percentage of stress fiber positive cells, only cells detected by their Texas Red fluorescence were counted. A minimum of 100 cells were investigated in each case and experiments were repeated at least twice.

3. Results and discussion

3.1. Palmitoylation sites on $G\alpha_{13}$

We had previously shown that $G\alpha_{13}$ is acylated and the fatty acid bond is sensitive to neutral hydroxylamine and reducing agents, demonstrating a thioester-type fatty acid linkage [26]. Inspection of the amino acid sequence of $G\alpha_{13}$ revealed three potential acylation sites located near the N-terminus: cysteine residues 14, 18 and 37. A role for these Cys residues in palmitoylation was tested by site-directed mutagenesis. Cysteine residues were mutated to serine either individually to produce mutants M1 (C14S), M2 (C18S) or M3 (C37S), double-mutated (M1-2) or all three cysteines were replaced (M1-2-3). Results of labeling experiments with [^{35}S]methionine followed by immunoprecipitation with $G\alpha_{13}$ antibodies shown in Fig. 1 (left panel) indicate that $G\alpha_{13}$ Cys

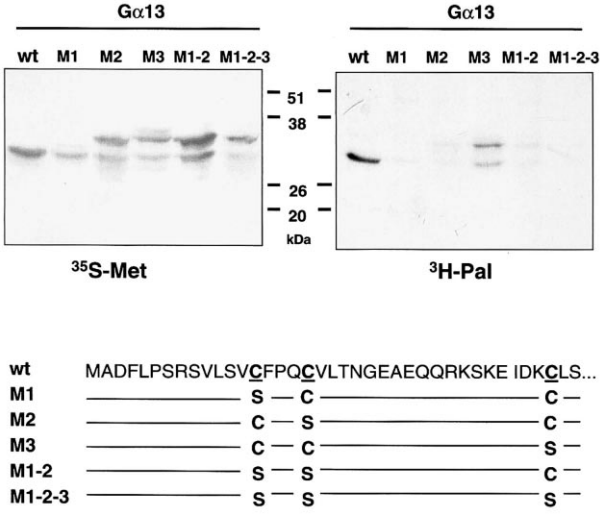

Fig. 1. $G\alpha_{13}$ is palmitoylated on three cysteine residues (Cys-14, Cys-18 and Cys-37) but mainly on Cys-14 and Cys-18. $G\alpha_{13}$ wild type ($G\alpha_{13}$wt) and different $G\alpha_{13}$ mutants ($G\alpha_{13}$M1; M2; M3; M1-2; M1-2-3) with the substitution of serine for cysteine 14; 18; 37; 14 and 18; 14, 18 and 37, respectively, were expressed in Sf9 cells, labeled either with [^{35}S]methionine/cysteine (left panel) or [^{3}H]palmitic acid (right panel) and subjected to immunoprecipitation with antibodies AS343 raised against $G\alpha_{13}$, SDS–PAGE and fluorography. Exposure time is 1 day for labeling with [^{35}S]methionine/cysteine and 3 weeks for labeling with [^{3}H]palmitate. In the lower panel, the N-terminal sequences of $G\alpha_{13}$wt and their Cys mutants are given in single-letter code.

mutants were as efficiently expressed as $G\alpha_{13}$wt. The reason for the more slowly migrating bands of $G\alpha_{13}$ in the gel with all mutants except M1 is unknown at present. One possibility is the presence of differently folded protein populations, because the gel shown in Fig. 1 was run under non-reducing conditions (in the absence of mercaptoethanol). After labeling with [^{3}H]palmitate, acylation levels differed for all mutants investigated (Fig. 1, right panel). Importantly, the replacement of either single Cys-14 (M1) and Cys-18 (M2) or both these residues (M1-2) resulted in a similarly strong reduction of palmitoylation, whereas mutation of cysteine residue 37 (M3) produced only a minor decrease in palmitoylation. Mutant M1-2-3 with all three cysteine residues replaced to serine failed to incorporate any detectable levels of radiolabeled palmitate even after prolonged exposure time. These results demonstrate the involvement of all three cysteine residues Cys-14, Cys-18, Cys-37 in palmitoylation of $G\alpha_{13}$ and suggest that cysteine residues 14 and 18 represent the main linkage sites for covalent attachment of palmitate.

$G\alpha_{13}$ shares a 67% sequence identity to $G\alpha_{12}$ and both proteins demonstrate clear similarities in their signalling functions. In spite of this fact, the amino-terminus of $G\alpha_{13}$ is quite distinct when compared to $G\alpha_{12}$. The N-terminus of $G\alpha_{13}$ is shorter and possesses three apparent acylation sites, whereas $G\alpha_{12}$ has only one palmitoylated cysteine residue at position 12 [28,31]. The distinct differences between $G\alpha_{12}$ and $G\alpha_{13}$ signalling reported recently for activation of GEF115 [24,25] could potentially be caused by the distinctive palmitoylation of these two subunits.

3.2. Palmitoylation influences the coupling of G_{12} to the PAR1 thrombin receptor

In order to determine whether palmitoylation of $G\alpha_{13}$ is required for signalling, we studied a number of signalling events accessible to quantitative assays. Firstly, we compared the interaction of the thrombin receptor with wild type $G\alpha_{13}$ as well as with their fatty acid-deficient mutants in membrane preparations from infected Sf9 insect cells. PAR1 receptor, the Gα- and Gβγ-subunits were co-expressed from recombinant baculoviruses and then agonist-promoted binding of [^{35}S]GTPγS was measured in the membranes from transfected cells. Using this system, we found for $G\alpha_{13}$wt a 5.5-fold increase in the binding of [^{35}S]GTPγS after co-expression of thrombin receptor in response to the agonist (Fig. 2). When $G\alpha_{13}$M1, M2, M1-2 or M1-2-3 instead of the $G\alpha_{13}$wt were expressed beside the thrombin receptor and βγ-subunits, incorporation of [^{35}S]GTPγS after stimulation with agonist was severely reduced. In contrast, after expression of $G\alpha_{13}$M3 agonist stimulated [^{35}S]GTPγS binding was only moderately impaired when compared to the control values obtained with $G\alpha_{13}$wt (Fig. 2). These differences in the incorporation efficiency were not due to a different subcellular distribution between $G\alpha_{13}$wt and its acylation-deficient mutants. On the one hand, subcellular fractionation of Sf9 cells transfected with $G\alpha_{13}$ revealed that in the absence of βγ-subunits about one half of $G\alpha_{13}$wt and more than two thirds of $G\alpha_{13}$M1-2-3 are present in the soluble fraction, indicating that palmitoylation contributes to membrane binding. However, co-expression of βγ targeted more than half of $G\alpha_{13}$M1-2-3 to the membrane, making the intracellular distribution of the non-palmitoylated mutants similar to that of wild type (not shown). Thus, membrane binding of $G\alpha_{13}$ seems to require

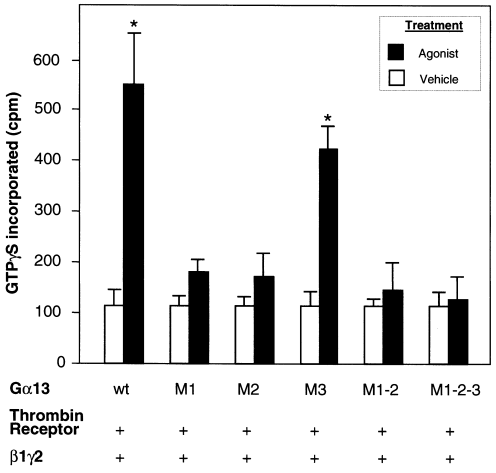

Fig. 2. Receptor-promoted binding of [^{35}S]GTPγS to $G\alpha_{13}$wt and its different acylation-deficient mutants. Membranes were prepared from Sf9 cells expressing proteins as indicated below. [^{35}S]GTPγS binding was assessed after incubation with or without 30 μM thrombin receptor activator (SFLLRNPNDKYEPF) followed by immunoprecipitation using $G\alpha_{13}$-specific antiserum. Data points represent the mean ± S.E.M. from three independent experiments. Statistically significant increases from values obtained with $G\alpha_{13}$wt or with $G\alpha_{13}$M3 are noted (*$P < 0.01$).

acylation only when it is solitary, while protein–protein interactions and the prenyl group present in the γ-subunit predominate in membrane attachment of the full heterotrimer.

These data are consistent with our recent observation that the thrombin receptor PAR1 couples to G proteins of the G_{12} family [28]. They also suggest that acylation of cysteine residues 14 and 18, but not of cysteine 37 is essential for normal communication with the thrombin receptor and indicate that the ability of $G\alpha_{13}$ to be activated trough the receptor correlates with its level of palmitoylation.

3.3. $G\alpha_{13}$ requires acylation for its transforming activity

We have shown previously that a GTPase-deficient constitutively activated mutant of $G\alpha_{13}$ (Q226L; $G\alpha_{13}$QL) induces neoplastic transformation of NIH3T3 and Rat-1 cells, which is a cellular end point for activation of a $G\alpha_{13}$-mediated cascade [30]. To determine if lipid modification is required for $G\alpha_{13}$-dependent neoplastic transformation, we have compared focus-forming activity of $G\alpha_{13}$QL with that of $G\alpha_{13}$QL/M1, $G\alpha_{13}$QL/M2, $G\alpha_{13}$QL/M3 or $G\alpha_{13}$QL/M1-2-3 (Fig. 3A,B). Control experiments demonstrated that in Rat-1 cells, $G\alpha_{13}$QL induced foci formation, whereas $G\alpha_{13}$wt did not lead to cell transformation (Fig. 3B). Inspecting the fatty acid-deficient mutants resulted in a differential picture. While $G\alpha_{13}$QL/M1, $G\alpha_{13}$QL/M2 and $G\alpha_{13}$QL/M1-2-3 mutants lost their ability to induce foci formation (Fig. 3), $G\alpha_{13}$QL/M3 mutant with cysteine 37 replaced to serine was still able to induce focus formation in about 50% of the cell population when compared to the control cells transformed with $G\alpha_{13}$QL (Fig. 3). These results suggest that the palmitoylation sites or presence of Cys-14 and Cys-18 are required for $G\alpha_{13}$-induced focus formation while Cys-37 seems to be less important for this biological activity. These results are in line with those of Wedegaertner et al. [32] who reported that an acylation-deficient mutant of Ga_s can partially activate its downstream effector but this is inadequate to activate the cascade. How-

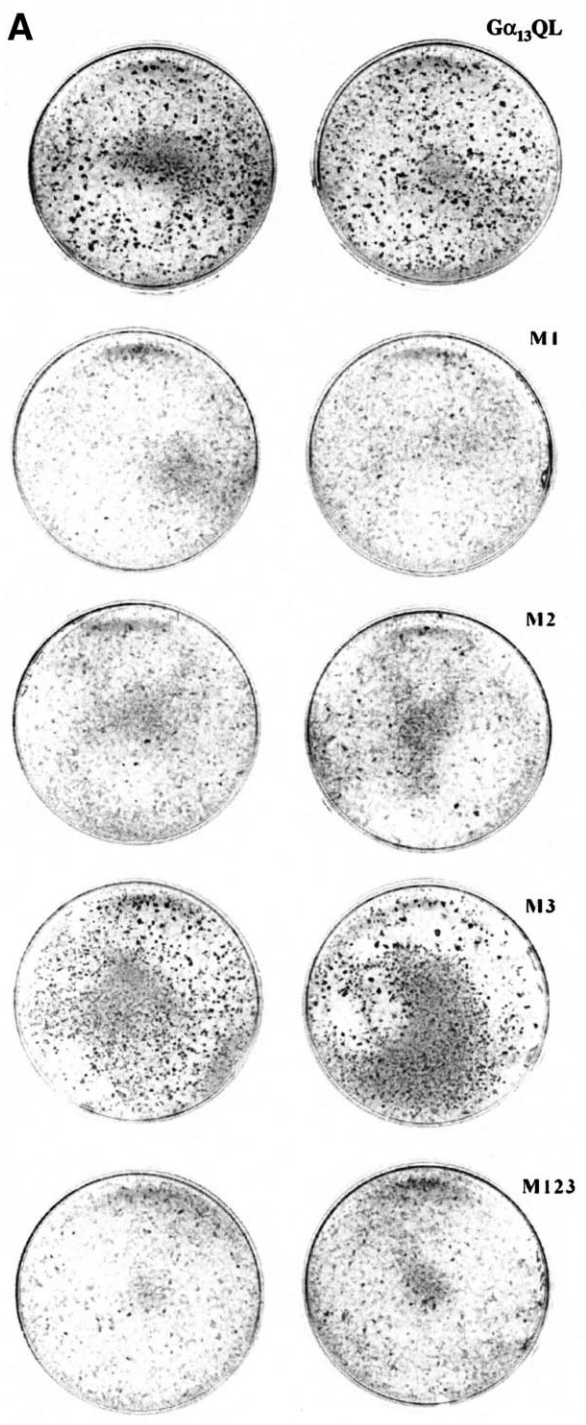

Fig. 3. Focus formation in Rat-1 cells expressing constitutively active $G\alpha_{13}$ and its acylation-deficient mutants. A: Rat-1 cells were transiently transfected with cDNA as indicated using LipofectAMINE 2000 (Gibco), empty vector was added to each transfection to keep the total amount of DNA at equivalent levels. Cells were plated on 60 mm dishes and after 14 days incubation stained with Giemsa. B: For quantification, foci on each dish were counted. The mean values ± S.D. from three separate experiments are shown. One set of duplicates is shown.

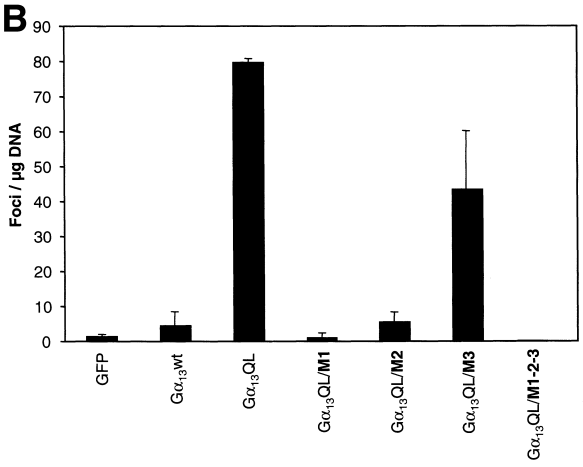

Fig. 3 (*continued*).

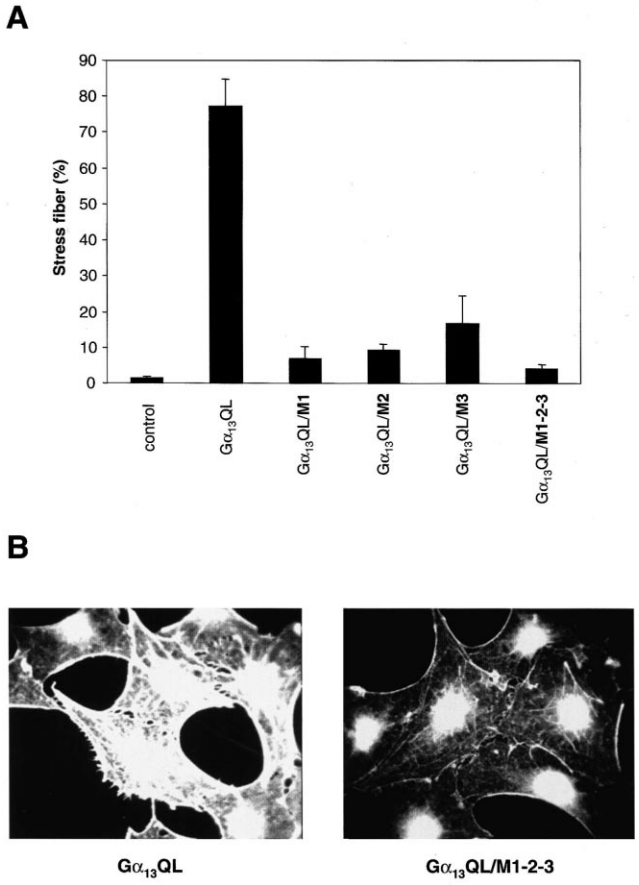

Fig. 4. Palmitoylation is involved in stress fiber formation induced by $G\alpha_{13}$QL. Quiescent Swiss 3T3 cells were nuclear microinjected with pCDNA3-$G\alpha_{13}$QL or pCDNA3-$G\alpha_{13}$QL/Cys mutants in the presence of Texas Red. A: For quantification of stress fiber formation, microinjected cells were first identified by their Texas Red fluorescence, and then the fraction of stress fiber positive cells was counted. The mean value of triplicates ± S.D. is shown. B: Stress fiber assembly induced by $G\alpha_{13}$QL (left panel) or by $G\alpha_{13}$QL/M1-2-3 (right panel) was monitored using rhodamine-phalloidin. Typical examples of stress fiber in individual cells are shown.

ever, $G\alpha_{13}$ has three palmitoylation sites as compared to one in many other α-subunits [10,32,33]. This allows to test for the biological significance of fatty acids located in different places along the N-terminal domain. From the data in Fig. 3, it appears that palmitoylation of α_{13} contributes to cell transformation most effectively when it occurs most proximally to the N-terminus.

3.4. Acylation of $G\alpha_{13}$ is involved in stress fiber formation

It has recently been reported that $G\alpha_{13}$ mediates LPA-induced actin polymerization in a Rho-dependent manner [17,34]. Similarly, expression of a GTPase-deficient constitutively activated mutant of $G\alpha_{13}$ (Q226L; $G\alpha_{13}$QL) triggers actin polymerization in a receptor-independent manner [23]. In order to characterize the functional role of palmitoylation of $G\alpha_{13}$ in the Rho-dependent signalling, we compared the ability of $G\alpha_{13}$QL and a series of $G\alpha_{13}$QL/Cys mutants to form actin stress fibers. We first expressed the constitutively activated $G\alpha_{13}$QL mutant in Swiss 3T3 fibroblasts by microinjecting expression plasmids into quiescent cells. Fig. 4A demonstrates that $G\alpha_{13}$QL stimulates the formation of actin stress fibers in about 80% of the cells, whereas the injection of $G\alpha_{13}$wt displayed the same low basal level of stress fibers as in non-injected cells (not shown). When $G\alpha_{13}$QL/M1, $G\alpha_{13}$QL/M2 or $G\alpha_{13}$QL/M1-2-3 were expressed, the stimulation of stress fiber formation was drastically reduced (Fig. 3A). Interestingly, expression of the GTPase-deficient form of $G\alpha_{13}$ with cysteine 37 replaced by serine ($G\alpha_{13}$QL/M3) reduced stress fiber induction to less than 20% of the control level. As with cell transformation, principally stress fiber formation through $G\alpha_{13}$ is most severely affected when fatty acids are deleted from an N-proximal location. However, in this case the innermost acylation site may have a more prominent role as with the induction of cell transformation.

Taken together, our results with recombinant $G\alpha_{13}$ are consistent with the current notion that palmitoylation is a common feature of Gα-subunits (e.g. $G\alpha_s$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_z$ and $G\alpha_{12}$ ([28], for review see [33])) which significantly contributes to the regulation of signalling by G proteins. Extending this general conclusion, we suggest that the position of palmitoylation sites is of functional importance. Cysteine residues closer to the N-terminus may contribute more effectively to signalling than those located more internally. Mutational shifting of palmitoylation sites along the N-terminal domain of α-subunits provides a promising tool for testing this hypothesis in the future.

4. Note added in proof

In the process of publishing our data, a paper appeared on the same subject matter and with results very similar to ours: Bhattacharyya, R. and Wedegaertner, P.B. (2000) J. Biol. Chem. 275, 14992–14999.

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