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A cysteine-11 to serine mutant of $G\alpha_{12}$ impairs activation through the thrombin receptor

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Abstract We have recently reported that $G\alpha_{12}$ is acylated with palmitic acid [Veit et al., FEBS Lett. 339 (1994) 160–164]. Here we identify cysteine 11 as the sole palmitoylation site and assess the function of $G\alpha_{12}$ palmitoylation after expression of wild type and acylation-deficient mutant in insect cells. Our experimental approach yielded the following results. (1) Palmitoylation of $G\alpha_{12}$ has no influence on the subunit interactions. (2) Palmitoylation promotes membrane binding of $G\alpha_{12}$ when this protein is expressed alone. Membrane attachment of the heterotrimer occurs independent of the presence of fatty acids in $G\alpha_{12}$. (3) Assays for agonist-stimulated binding of [^{35}S]GTPγS after expression of the human thrombin receptor (PAR1) along with $G\alpha_{12}$ and the βγ subunits revealed a 70% inhibition with the palmitoyl-deficient mutant.

Key words: Palmitoylation; G-protein; $G\alpha_{12}$; Thrombin receptor

1. Introduction

Heterotrimeric regulatory guanine nucleotide binding proteins (G-proteins) are central in the transducing of signals from activated receptors to appropriate intracellular effectors such as enzymes and ion channels [1–4]. Each heterotrimer consists of a relatively hydrophilic α subunit, which exchanges GDP for GTP upon activation by a receptor, and the relatively hydrophobic βγ complex. In order to function as signal transducers, G-proteins have to be associated with the cytoplasmic face of the plasma membrane. This membrane association may be partly mediated by the highly lipophilic βγ subunits [5,6]. On the other hand, essentially all Gα subunits are modified by covalently attached myristic and/or palmitic acids [7–9]. It is now widely accepted that these lipid modifications (especially palmitoylation) greatly facilitate and are in some cases essential for anchoring Gα subunits to the membrane or specifying their membrane localization, or both [10–12]. Palmitoylation is a dynamic modification of proteins, unlike myristoylation, which is usually an irreversible cotranslational modification. Therefore it has been suggested that cycles of palmitoylation and depalmitoylation could also regulate the signaling activity and membrane association of G-protein α subunits [12–14]. In the case of $G\alpha_s$ and $G\alpha_q$ activation by agonist stimulation leads to increased turnover of palmitate [15–17]. Treatment with cholera toxin also promotes palmitate turnover of $G\alpha_s$ [16]. In contrast, turnover of bound palmitate on $G\alpha_i$ and $G\alpha_s$ is decreased by co-expression of Gβγ, which inhibits activation [6,18]. Accumulation of the depalmitoylated form of $G\alpha_s$ in chronically morphine-treated A431/μ13 cells increases intrinsic $G\alpha_s$ activity and promotes $G\alpha_s$/adenylyl cyclase interaction [19]. Palmitoylation of $G\alpha_z$ decreases the affinity of G_z GTPase activating protein for the GTP-bound form of $G\alpha_z$ thereby inhibiting the rate of GTP hydrolysis [20]. Preventing palmitoylation of $G\alpha_z$ enhances G_z-mediated inhibition of adenylyl cyclase [9].

Here we report experiments designed to examine the effects of acylation on membrane attachment and signaling function of a member of the more recently identified fourth subfamily of α subunits, $G\alpha_{12}$. The pertussis toxin-insensitive $G\alpha_{12}$ and $G\alpha_{13}$ subunits are expressed ubiquitously [21] and show relatively slow guanine nucleotide exchange and hydrolysis [22,23]. $G\alpha_{12/13}$ proteins are involved in mitogenesis and transformation probably through activation of c-Jun N-terminal kinase pathways [24,25] as well as in regulation of Na-H exchange [26]. Recent work on $G\alpha_{12/13}$ has focussed on their oncogenic potential, when overexpressed in cells [27,28]. The activated receptors of thromboxane A_2 and thrombin couple to $G\alpha_{12}$ and $G\alpha_{13}$ in humane platelet membranes [29], and $G\alpha_{13}$ interacts with the angiotensin AT_{1A} receptor in rat portal vein myocytes [30]. Recently we have demonstrated that both $G\alpha_{12/13}$ subunits are palmitoylated in ester-type linkage and that the acylated form of $G\alpha_{12}$ is largely membrane-bound [31].

In the present study we identify Cys residue 11 as the sole palmitoylation site on $G\alpha_{12}$ and show that acylation is not necessary for the heterotrimer formation and its binding to the membrane. We also investigate the selectivity in coupling of the thrombin receptor to $G\alpha_{12}$ wild type as well as its acylation-deficient mutant by agonist-promoted binding of [^{35}S]guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) [32] and present evidence that a lack of palmitate in the $G\alpha_{12}$ subunit results in drastically reduced receptor coupling.

2. Materials and methods

2.1. Baculoviruses and site-directed mutagenesis

The construction of pVL1392 vector encoding the $G\alpha_{12}$ wild type ($G\alpha_{12}$wt) was described previously [31]. The $G\alpha_{12}$ mutant ($G\alpha_{12}$mut) with the substitution of serine for cysteine 11 was performed with the 'Chameleon' double-stranded, site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol using an oligonucleotide containing the mutation corresponding to the above substitution. The final mutant was verified by DNA sequencing. Recombinant baculoviruses encoding β_1, γ_2 and γ_3 subunits were kindly provided by P. Gierschik (Ulm), and those encoding the human thrombin receptor and $G\alpha_{12}$ were recently described [31,33].

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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 [31]. Antibodies AS1905 raised against the N-terminal peptide corresponding to the deduced amino acid sequence of $G\alpha_{12}$ (amino acids 1–21), antibodies AS398 against β subunits (β_1–β_4) and AS292 against the γ subunits (γ_2+γ_3) were used for immunoprecipitation [34].

2.3. Cell fractionation

Infected and methionine labeled Sf9 cells were washed once with PBS, resuspended in extraction buffer (10 mM Tris-HCl (pH 7.4); 1 mM EDTA; 150 mM NaCl; 1 mM DTT) and homogenized by sonification. The homogenate was centrifuged at 5000×*g* for 5 min, and the resulting supernatant fraction was centrifuged at 125000×*g* at 4°C for 30 min. The membrane pellets were incubated in extraction buffer containing 1% (w/v) sodium cholate for 1 h at 4°C and centrifuged again at 125000×*g* for 30 min. The final fractions were diluted in 2×RIPA buffer (2% Triton X-100; 2% deoxycholate, 0.2% 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 $G\alpha_{12}$ wild type as well as the fatty acid-deficient mutant was performed according to the method described by Barr et al. [32]. 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 the 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 a $G\alpha_{12}$ subunit-directed antiserum, 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, then 4 ml of Ecolite+ (ICN) was added and radioactivity measured by scintillation spectrometry.

3. Results and discussion

3.1. Palmitoylation site on $G\alpha_{12}$

We had previously shown that $G\alpha_{12}$ is acylated and the fatty acid bond is sensitive to neutral hydroxylamine and reducing agents, demonstrating a thioester-type fatty acid linkage [31]. We proposed that the site of palmitoylation of $G\alpha_{12}$ subunit is located at position 11, since this is the only Cys residue in the first 240 N-terminal residues of this protein. A role for this Cys residue in the palmitoylation was tested by mutagenesis of the codon for cysteine to one for serine. Results of labeling experiments with [^{35}S]methionine shown in Fig. 1 (left panel) indicate that $G\alpha_{12}$ Cys mutant was as efficiently expressed as $G\alpha_{12}$wt. However, [^{3}H]palmitate was incorporated only into the wild type protein but not the mutant, demonstrating the essential involvement of this Cys residue 11 in palmitoylation of $G\alpha_{12}$ (Fig. 1, right panel) and confirming the very recent data by Jones and Gutkind [35]. The amino-terminus of $G\alpha_{12}$ is quite distinct when compared to other acylated α subunits and does not contain the Met-Gly-Cys motif, which has been proposed as acylation consensus sequence for most other acylated Gα subunits [8] and some members of the Src-related family of tyrosine kinases [36].

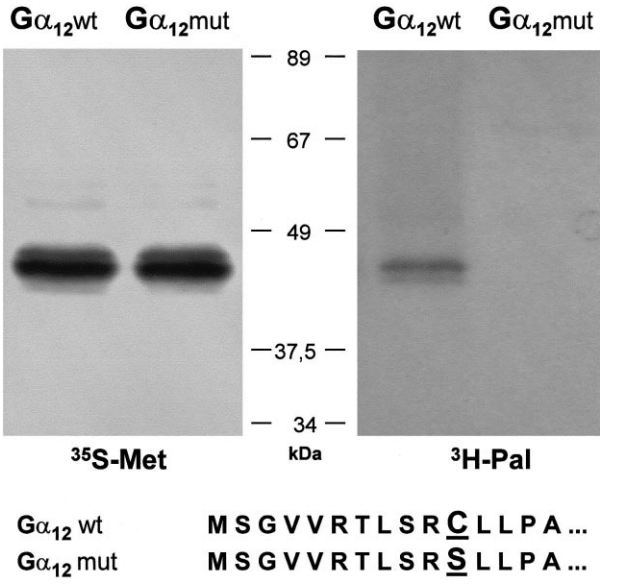

Fig. 1. Mutation of Cys-11 on $G\alpha_{12}$ abolishes palmitoylation. $G\alpha_{12}$ wild type ($G\alpha_{12}$wt) and $G\alpha_{12}$ with cysteine residue 11 replaced by serine ($G\alpha_{12}$mut) were expressed in Sf9 cells, labeled with either [^{35}S]methionine/cysteine (left panel) or [^{3}H]palmitic acid (right panel) and subjected to immunoprecipitation, SDS-PAGE and fluorography. Exposure time is 1 day for labeling with [^{35}S]methionine/cysteine and 4 weeks for labeling with [^{3}H]palmitate. The N-terminal sequences of $G\alpha_{12}$wt and $G\alpha_{12}$mut are given in single-letter code [42].

The Met-Gly-Cys amino-terminal sequence possibly represents a motif for dual modification with both myristic and palmitic acids (except for $G\alpha_s$, which is modified by palmitate [5]). The case of $G\alpha_{q/11}$, which is only palmitoylated and which contains putative palmitoylation sites at positions 9 and 10 [13,37,38], and the case of $G\alpha_{12}$ reported here indicate that the distance between the N-terminus and acylation sites in palmitoylated Gα subunits may be variable.

3.2. Role of palmitoylation for interaction with βγ subunits and membrane association

The role of palmitoylation of various α subunits for heterotrimer formation with βγ subunits has been subject of several studies some of which exclude the involvement of fatty acids with heterotrimerization [39], while others favor a causative link [6,16]. We extended such investigations to $G\alpha_{12}$ and analyzed the interaction of $G\alpha_{12}$wt or $G\alpha_{12}$mut with βγ by examining the capacity of antibodies directed against the individual subunits (α, β or γ) to immunoprecipitate all three proteins after co-expression in Sf9 cells. When the βγ subunits were expressed alone they could not be immunoprecipitated with anti-α antibodies (Fig. 2A, second and third lanes). However, after co-expression of $G\alpha_{12}$wt, both β and γ proteins were also detectable with Gα-directed antibodies (Fig. 2A, last two lanes). Immunoprecipitation of Sf9 cells expressed either α_{12}wt or βγ or all three subunits together with antibodies directed against the β or γ subunit yielded similar results (not shown). When $G\alpha_{12}$mut was expressed alone or co-expressed with βγ subunits the immunoprecipitation pattern with antibodies directed against either the α or β or γ subunit was quite similar to that for $G\alpha_{12}$wt. This is shown in Fig. 2B for the immunoprecipitates brought down

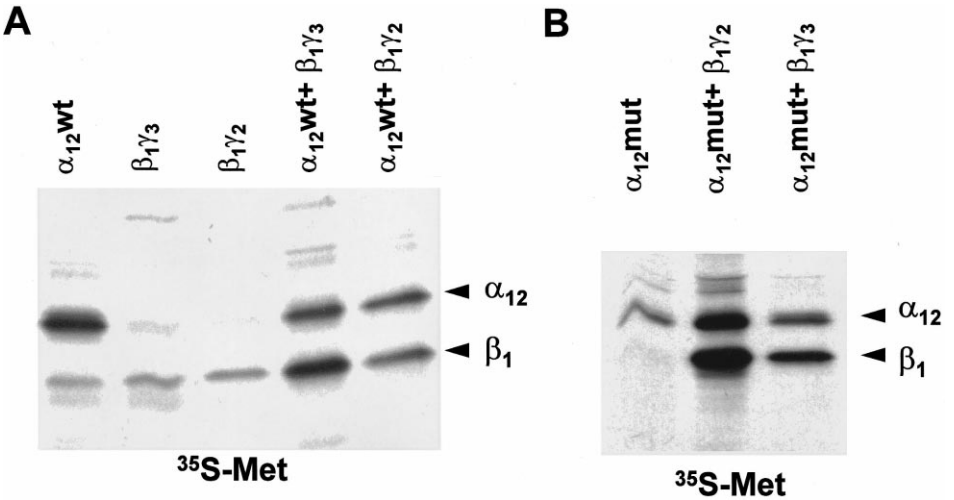

Fig. 2. Assay for palmitate involvement in heterotrimerization by subunit co-precipitation using anti-Gα_{12} antibodies. A: Insect cells expressing either Gα_{12}wt or G$\beta_1\gamma_2$, or G$\beta_1\gamma_3$ or all three subunits together were labeled with [^{35}S]methionine/cysteine. After lysis, recombinant proteins were immunoprecipitated with antibody directed against Gα_{12} subunits and subjected to SDS-PAGE and fluorography. B: Results of expressing Gα_{12}mut alone or together with either G$\beta_1\gamma_2$ or G$\beta_1\gamma_3$ after immunoprecipitation with anti-Gα_{12} antibodies.

with anti-Gα_{12} antibodies. These data indicate that Gα_{12}mut also forms a complex with $\beta\gamma$ subunits and suggests that palmitoylation of Cys residue 11 is not necessary for interaction of Gα_{12} with $\beta\gamma$.

We demonstrated previously that Gα_{12} expressed in Sf9 cells accumulates in both membranes and cytosol. However, only the membrane-associated α subunits had [^{3}H]palmitate incorporated [31] indicating the presence of at least two distinct populations of this protein at different intramolecular locations. In order to determine whether palmitoylation is critical for attaching Gα_{12} to membranes, we compared the subcellular distribution of Gα_{12}wt and its non-acylated mutant (Gα_{12}mut) after infection of Sf9 cells with the respective recombinant baculoviruses alone or during co-expression of both the β_1 and γ_2 subunits. To exclude the possibility that non-functional (denatured and aggregated) Gα_{12} protein may mimic a true membrane fraction, high speed membrane pellets were extracted with 1% sodium cholate before immunoprecipitation and SDS-PAGE. The same amount (30–40%) of each Gα_{12}wt and Gα_{12}mut protein associated with membranes was extractable with detergent. (Some part of recombinant Gα_{12} proteins was denatured and aggregated, as indicated by resistance to extraction with 1% sodium cholate.) Thus, the resulting protein bands shown in Fig. 3A (lines designated Mem) represent truly membrane-bound Gα_{12}. Densitometric analysis revealed that in the absence of $\beta\gamma$ subunits nearly one half of Gα_{12}wt and more than two thirds of Gα_{12}mut are present in the soluble fraction (cytosolic Gα_{12}), indicating the importance of palmitoylation for membrane binding (Fig. 3B). However, co-expression of $\beta\gamma$ targeted more than half of Gα_{12}mut to the membrane, making the intracellular distribution of the non-palmitoylated mutant similar to that of wild-type (Fig. 3A,B).

Taken together the above results demonstrate that the non-palmitoylated Gα_{12} subunit can form heterotrimers with the $\beta\gamma$ subunits and at the same time this interaction restores membrane localization of Gα_{12}. Thus, in line with reports on other types of α subunits [18], membrane association of Gα_{12} is a complex process. Membrane binding of Gα_{12} requires acylation only when it is solitary, while protein-protein interactions predominate in membrane attachment when it is trimerized with the $\beta\gamma$ subunits.

3.3. *Influence of palmitoylation on coupling of G_{12} to the thrombin receptor*

We compared the interaction of the thrombin receptor with normal and fatty acid-deficient Gα_{12} subunits in membrane preparations from infected cells. Co-expression of receptor and the α subunit (alone or together with $\beta\gamma$) in Sf9 cells and measurement of agonist-promoted binding of [^{35}S]GTPγS provides a useful experimental approach for assessing the selectivity of receptor-G-protein coupling [32]. Using this system we found for normal G_{12} (i.e. α_{12}wt, β_1 and γ_2) a 12.6-fold increase in the binding of [^{35}S]GTPγS after co-expression of thrombin receptor in response to the agonist (Fig. 4). Expression of Gα_{12}wt alone also demonstrated increased binding of [^{35}S]GTPγS after exposition to agonist, although at a much reduced level (Fig. 4). In this case the binding efficiency varied considerably, and we propose that variable amounts of endogenous G$\beta\gamma$ in different membrane preparations could be responsible for this variable stimulation. When Gα_{12}mut was expressed beside the thrombin receptor but without $\beta\gamma$ subunits, the agonist had no significant effect on binding. Co-expression of $\beta\gamma$ subunits reconstituted the capacity of Gα_{12}mut to bind [^{35}S]GTPγS significantly, but stimulation by agonist was reduced to less than 30% when compared to control values with Gα_{12}wt (Fig. 4). This reduction was not due to different expression level of Gα_{12} or retarded incorporation of [^{35}S]GTPγS into Gα_{12}mut, because the amount of Gα_{12}wt and Gα_{12}mut in membrane preparations was the same and both G_{12}wt as well as the acylation-deficient mutant showed similar kinetics (not shown).

Our results are consistent with the current notion that the thrombin receptor PAR1 couples to G-proteins of the G_{12} family [29,32], and they suggest that palmitoylation (or presence of Cys residue 11) significantly contributes to normal communication of the thrombin receptor with Gα_{12}. The precise role of the palmitoylated cysteine residue is unclear. It

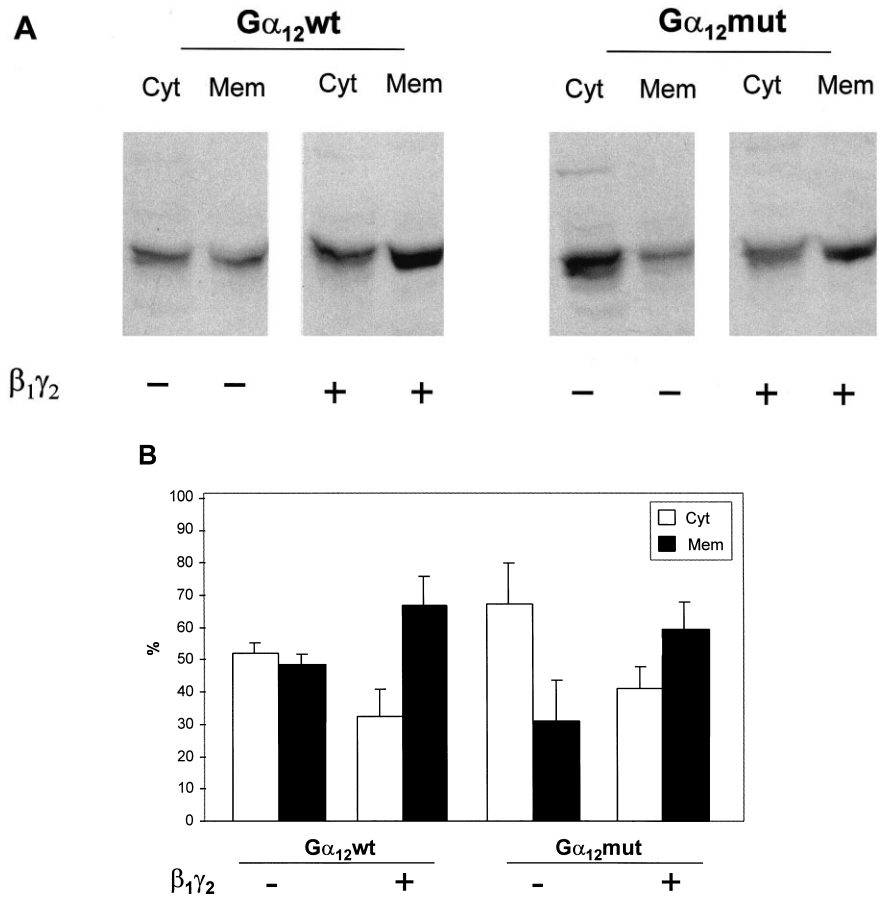

Fig. 3. Intracellular distribution of $G\alpha_{12}$wt and $G\alpha_{12}$mut. A: Insect cells expressing either $G\alpha_{12}$wt or $G\alpha_{12}$mut alone or together with β_1 and γ_2 subunits were labeled with [^{35}S]methionine/cysteine. After lysis and separation into cytosolic (Cyt) and membrane (Mem) fractions $G\alpha_{12}$ protein was immunoprecipitated from each fraction with antibodies directed against $G\alpha_{12}$ and subjected to SDS-PAGE and fluorography. Results are representative of four independent experiments. B: Result of densitometric analysis of the fluorogram shown in A and of additional fluorograms from three independent experiments ± S.E.M.

may be involved in formation of intramolecular contacts within $G\alpha_{12}$ or in intermolecular interactions with the receptor. It has been reported that Cys-3 of $G\alpha_o$ (palmitoylated cysteine analogous to Cys-11 in $G\alpha_{12}$) interacts directly with a mastoparan derivative, a small amphipathic peptide which activates G-proteins [40]. Likewise, the acylated Cys-11 of $G\alpha_{12}$ could also influence interactions with the thrombin receptor in an acylation-dependent manner.

Finally, palmitoylation has been shown to be a dynamic modification and cycles of acylation and deacylation may play a role in regulating the activity of some Gα proteins [17,41,42]. Supporting this view, we have also observed that palmitoylation of recombinant $G\alpha_{12}$ undergoes turnover although, unlike previous observation with $G\alpha_s$ [43], depalmitoylation of $G\alpha_{12}$ is relatively slow and is not increased by treatment with agonist (not shown). Our results presented here on $G\alpha_{12}$ are in line with the general understanding that the dynamic nature of palmitoylation is a common feature of Gα subunits which contributes to the mechanisms of regulating signaling by G-proteins.

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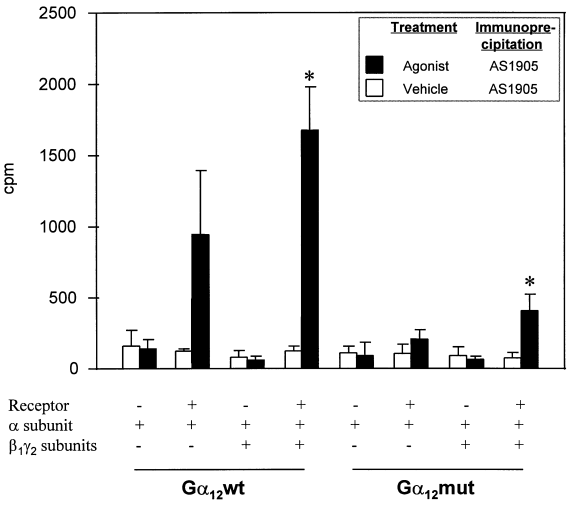

Fig. 4. Receptor-promoted binding of [^{35}S]GTPγS to $G\alpha_{12}$wt and $G\alpha_{12}$mut. Membranes were prepared from Sf9 cells expressing proteins as indicated below. [^{35}S]GTPγS binding was assessed after incubation with or without 30 μM thrombin receptor activator (SFLLRNPNDKYEPF) followed by immunoprecipitation using $G\alpha_{12}$-specific antiserum. Data points represent the mean ± S.E.M. from four independent experiments. Statistically significant increases from values obtained with α_{12}wt+βγ or with α_{12}mut+βγ without thrombin receptor are noted ($^*P < 0.01$).

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