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Stable changes in expression or activation of G protein α_i or α_q subunits affect the expression of both β_1 and β_2 subunits

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G proteins consist of three subunits: α, β and γ. Four β subunits have been cloned: β_1 and β_4 (36 kDa), and β_2 and β_3 (35 kDa). We studied endogenous β subunits in mouse NIH 3T3 fibroblasts stably expressing high levels of G protein α subunits after transfection with cDNAs encoding α_{i1}, α_{i2}, α_{i3} and α_q. Immunoblots showed that NIH 3T3 cells express β_{36} and β_{35} subunits; in these cells, β_{35} subunits are four times more abundant than β_{36} subunits. We could detect β_1 and β_2 mRNA, but neither β_3 nor β_4 mRNA. We found that a stable increase in expression of wild-type α_{i1}, α_{i2}, α_{i3} or α_q subunits is always accompanied by an increase in β_1 and β_2 mRNA and protein levels. There was no evidence of selectivity for an increase in β_1 rather than β_2 subunits depending on the type of α subunit overexpressed. However, constitutive activation or inactivation of α subunits induced specific changes in β subunits. Expression of constitutively inactivated α_{i2} subunits was accompanied by an increase in mRNA and protein levels of both β subunits. In contrast, cells expressing constitutively activated α_{i2} subunits did not show any change in the amount of β proteins expressed in membranes, despite a significant increase in β_1 and β_2 mRNA. We conclude that stable changes in the levels of expression or degree of activation of Gα subunits affect the level of expression, and possibly the turn-over, of β subunits, without selectivity among β_1 and β_2 subunits.

G protein; α and β subunit; Protein expression; mRNA expression

1. INTRODUCTION

Heterotrimeric G proteins couple the activation of specific seven-transmembrane domain receptors to the regulation of ion channels or intracellular enzymes [1–4]. At least fifteen distinct α genes, four β genes and six γ genes have been identified [1,5,6]. Until recently, only α subunits were considered critical for specificity of receptor–effector coupling. $\beta\gamma$ complexes were thought to be functionally interchangeable among all G proteins. $\beta\gamma$ complexes are now recognized to regulate various effectors [7–15] and to stimulate receptor kinases [16]. Two types of β subunits had previously been described: β_{35} and β_{36}, based on their apparent molecular mass [17]. The four β subunits cloned, β_1 and β_4 (36 kDa), and β_2 and β_3 (35 kDa) (Murakami et al., unpublished data), are highly homologous, yet can be distinguished by different associations with γ subunits [18–20]. There is also evidence that α subunits and $\beta\gamma$ complexes do not associate randomly [21,22] but it is not clear if α and $\beta\gamma$ subunit association is determined by specific affinities among the different subunits, tissue distribution of the various subunits, or a combination of both.

Changes in expression of one or several G protein subunits occur frequently in vivo, usually during cell differentiation or exposure to hormones. A change in expression of one subunit is not necessarily accompanied by a similar change in the levels of other subunits [23]. However, adipocytes of adrenalectomized animals show a decrease in mRNA and protein expression of both α_s and β subunits; corticosterone has the opposite effects [24]. Expression of both α_{i2} and β subunits is increased in adipocytes and myocardial cells of hypothyroid rats [25–28]. 3T3 L1 fibroblasts induced to differentiate into adipocytes show increased protein levels of α_s, α_i, α_o, and β_{36} and β_{35} subunits [29]. In almost all cases of stable increases in expression of G protein subunits, increased protein expression is explained by increased mRNA levels [28,30,31]. Thus, in vivo, a stable change in α subunit levels is often, but not always, associated with a similar change in $\beta\gamma$ complexes. Although we and others did not detect any change in β subunit levels in COS cells transiently overexpressing α subunits [32], we observed a significant increase in β subunits in NIH 3T3 cells stably overexpressing wild-type α_{i2} l[33].

In this study, we sought to determine: (1) whether stable increases of various α subunits generally affect the level of protein expression of β subunits; (2) whether a change in activation of α subunits affects $\beta\gamma$ com-

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plexes; and (3) whether, in a given cell type, there is any selectivity of distinct α subunits for particular subtypes of β proteins. In order to address these questions, we used NIH 3T3 cells stably expressing wild-type and mutated α subunits of the following G proteins: G_{i1}, G_{i2}, G_{i3} and G_q, and studied the expression of endogenous β subunits at the protein and mRNA levels.

2. MATERIALS AND METHODS

2.1. *Cell lines*

Geneticin-resistant NIH 3T3 cells stably expressing wild-type and mutated α_{i1}, α_{i2}, α_{i3} and α_q were described previously [33–35].

2.2. *Immunoblot analysis*

Confluent cultures of NIH 3T3 cells were scraped, pelleted and washed three times in 10 ml of PBS, pH 7.5. Membranes were prepared as described previously [33,34]. The protein concentration was determined by the Bradford method [36] with IgG (BioRad) used as a standard, and membrane suspensions were adjusted to 1 mg/ml. In order to improve resolution on acrylamide gels, membrane protein suspensions were heated at 85°C for 10 min with 0.75% SDS and 10 mM DTT, cooled on ice, then incubated for 60 min on ice in the presence of 40 mM *N*-ethylmaleimide (NEM) [37]. 20 to 50 μg of NEM-treated membrane proteins were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes (Applied Biosystems Inc.) and immunoblotted with the following affinity-purified anti-sera: AS 7, EC, QL, respectively selective for α_{i1} and α_{i2} (AS 7) [38], α_{i3} (EC) [39] and α_q (QL) [40]. To study β subunit expression, we used the antisera MS, directed against the amino-terminal sequence MSELDQLRQE of the β_1 subunit, and SW, directed against the carboxy-terminal sequence GSWDSFLKIWN of β subunits [41,42]. Antigen–antibody complexes were detected with ^{125}I-labeled protein A. Blots were exposed to Hyperfilms-MP (Amersham) for 2–4 h and scanned by PhosphorImager (Molecular Dynamics).

2.3. *mRNA analysis*

Cells were grown to half confluency, scraped, pelleted, washed three times with sterile PBS and kept frozen at −70°C. Poly(A)$^+$ mRNAs were prepared using Micro-FastTrack mRNA Isolation Kits (Invitrogen, San Diego, CA) according to manufacturer's instructions. Poly(A)$^+$ mRNAs were then separated on 1% agarose-formaldehyde gels (3 μg/lane), and transferred to nylon membranes (Nytran, Schleicher and Schuell). Membranes were wet in 5 × SSPE (1 × SSPE: 10 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4), prehybridized for 4 h at 42°C in 30 ml of 5 × Denhardt's solution, 50% formamide, 5 × SSPE, 0.1% SDS and 200 μg/ml salmon sperm DNA. Hybridization was performed overnight at 42°C with random-primed ^{32}P-labeled β cDNA probes (> 10^6 cpm/ml) in 30 ml of the same solution used for prehybridization. The probes used were the entire coding region of bovine β_1, human β_2 and β_3, mouse β_4 and β-actin cDNAs, kindly provided by, respectively, Drs. J. Hurley (University of Washington), N. Gautam (Washington University School of Medicine), M. Levine (Johns Hopkins University School of Medicine) and M. Simon (California Institute of Technology). After hybridization, the membranes were washed twice in 6 × SSPE + 0.2% SDS for 15 min, twice in 1 × SSPE + 0.2% SDS for 15 min at room temperature, and once in 1 × SSPE + 0.2% SDS for 30 min at 65°C. Blots were exposed to XAR-2 Kodak films for three days and later scanned by PhosphorImager (Molecular Dynamics). Probes were stripped from blots by incubation in 200 ml water at 65°C for 30 min, followed by incubation at 65°C for 45 min in 200 ml of a solution of formamide, 10 mM EDTA and 10 mM Tris, pH 7.8. The same blots were then reprobed with a different β probe. The intensity of β_1 and β_2 mRNA signals was analyzed using PhosphorImager counts and normalized based on the β-actin signal obtained by the hybridization of the same blot with a β-actin labeled probe. Sizes of mRNA were determined by comparison with the migration of a 0.25–9.5 kilobase RNA ladder standard (BRL, Gaithersburg, MD).

3. RESULTS

3.1. *β subunit expression in NIH 3T3 cells*

NIH 3T3 cells express the following G protein α subunits: α_s, α_{i2}, α_{i3}, α_q and α_{12}. α_{i1} and α_o are not expressed in these cells [34]. Using a β-common antibody (SW), we determined that NIH 3T3 cells express both β_{36} and β_{35} subunits (Fig. 1). β_{35} proteins are approximately four times more abundant than β_{36} proteins. β_1 and β_4 are 36 kDa proteins, β_2 and β_3 are 35 kDa proteins (Murakami et al., unpublished data). Since we do not have antibodies specific for each of the four β proteins, we determined which of the four β subunits are expressed in NIH 3T3 cells by studying β mRNA expression. β_1 and β_2 mRNAs were easily detected: in NIH 3T3 cells, β_2 mRNA is approximately three times more abundant than β_1 mRNA (Fig. 3). Repeated probing of four distinct mRNA preparations and Northern blots failed to detect the presence of β_3 and β_4 mRNAs in NIH 3T3 cells, although β_3 mRNA (≃ 3.0 kb) was detected in bovine retina RNA in the same experiment (data not shown). We conclude that NIH 3T3 cells express β_1 and β_2, but that β_3 and β_4 are not expressed, or that the level of expression of β_3 and β_4 is extremely low (below detection) compared to β_1 and β_2. Although we cannot exclude that a still unknown β subunit might be expressed, we assume that in NIH 3T3 cells, β_{36} is β_1, and that β_{35} is β_2. We have not studied γ subunits in this work because we do not have high-affinity γ antibodies.

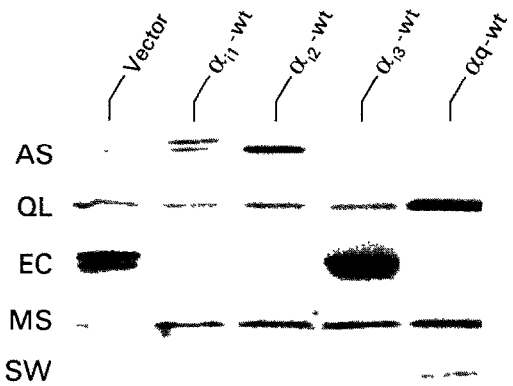

Fig. 1. Protein levels of α and β subunits in NIH 3T3 cells overexpressing wild-type $\alpha_{i1,2,3}$ and α_q. 30 μg of membrane proteins of cells transfected with vector alone or overexpressing wild-type α_i or α_q were separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed with different antisera. AS recognizes α_{i1} (41 kDa) and α_{i2} (40 kDa); QL recognizes α_q; EC recognizes α_{i3} (41 kDa) and, to a lesser degree, α_{i2}. MS recognizes only β_{36} in NIH 3T3 cells, thus reflects β_1 levels; SW recognizes β_{36} and β_{35} and has a stronger affinity for β_{35} than for β_{36}. Experiments were repeated three times

3.2. *Expression of β_{36} and β_{35} subunits is increased in NIH 3T3 cells overexpressing wild-type G protein α subunits*

Fig. 1 shows the levels of expression of both α and β subunits in NIH 3T3 cells stably transfected with various wild-type α subunits. In NIH 3T3 cells overexpressing α_{i1}-wt cells, the amount of transfected α_{i1} subunits was two to three times the level of endogeneous α_{i2}; there was a four-fold increase in α_{i2} in α_{i2}-wt cells; a three-fold increase in α_{i3} in α_{i3}-wt cells, and a five-fold increase in α_q in α_q-wt cells (Table I). We used SW and MS antisera to study endogenous β subunit expression in these cells. SW recognized two bands in all cells, corresponding to β_{36} and β_{35}. MS recognized only the upper band, β_{36}. In our hands, MS recognizes β_{36} better than SW. Since the mRNA studies showed only β_1 (36 kDa) and β_2 (35 kDa) mRNAs in NIH 3T3 cells, we suggest that in NIH 3T3 cells, proteins detected by the SW antiserum are β_1 and β_2, while the MS antiserum recognizes only β_1. In all NIH 3T3 cells overexpressing wild-type α subunits, expression of both β_{35} and β_{36} subunits was increased. The increase in β_1 (β_{36}) subunits appeared to be more pronounced (more than 2-fold) than the increase in β_2 (β_{35}) subunits (usually less than two-fold), even though β_1 is the least abundant β subunit in NIH 3T3 cells. This was particularly evident when Western blots were probed with the MS antiserum. α_q-wt cells showed the biggest increase in β subunits (3.6-fold increase in β_1 subunits over control). However, no obvious specificity pattern for β subunits was detected depending on the subtype of the overexpressed α subunit.

Table I

Changes in α and β protein expression and β_1 and β_2 mRNA levels after overexpression of α subunits

	mRNA expression (-fold increase)		Protein expression (-fold increase)		
	β_1	β_2	α	β_{36}	β_{35}
Vector	–	–	–	–	–
α_{i2}-wt	1.6	2.4	4	2.7	1.5
α_{i2}-Q205L	2.8	3.1	6	–	–
α_{i2}-G204A	1.4	2.6	3	3.0	2.5
α_{i1}-wt	1.7	2.0	2–3	2.4	1.8
α_{i3}-wt	1.9	2.4	3	2 3	1.5
α_q-wt	1.2	2.6	5	3.6	2.1

The Western blots shown in Figs. 1 and 2 and the Northern blot shown in Fig. 3 were analyzed by phosphoscreen imaging (Molecular Dynamics). The intensity of β_1 and β_2 mRNA signals was normalized against β-actin signal (β_1 counts/β-actin counts and β_2 counts/β-actin counts, respectively) and expressed as -fold-increase over β_1 and β_2 mRNA levels in vector-transfected cells. (–) means no change compared to vector-transfected cells. Increase in β_1 mRNA expression in α_q-wt cells was 1.2 in the Northern blot shown in Fig. 3 but was higher than 1.7 in three other Northern blots. Increases in protein expression were estimated using PhosphorImager counts of α and β subunit–antibody complexes labeled with [^{125}I]Protein A.

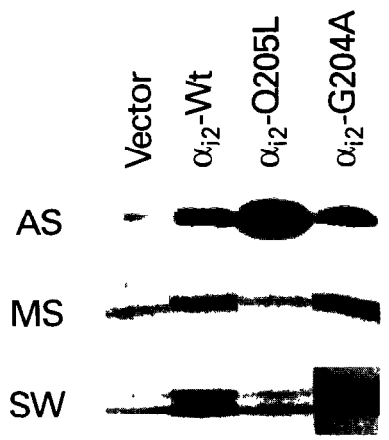

Fig. 2. Protein levels of α and β subunits in NIH 3T3 cells expressing wild-type and mutated forms of α_{i2}. 30 μg of membrane proteins of cells transfected with vector alone or expressing α_{i2}-wt, α_{i2}-Q205L or α_{i2}-G204A were separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed with AS, MS and SW. AS recognizes α_{i2} and MS, β_{36} (β_1). SW recognizes β_{36} and β_{35} (β_2). Experiments were repeated at least three times.

3.3. *Expression of constitutively activated and inactivated α_{i2} subunits has opposite effects on β subunit protein levels*

Unactivated G proteins are heterotrimers, but after activation of the protein, βγ complexes are released from α subunits. We tried to determine if a stable change in the state of activation of α subunits had any effects on the level of expression of β subunits. Fig. 2 shows the levels of protein expression of α_{i2} and β subunits in NIH 3T3 cells expressing wild-type and two mutated forms of α_{i2}. The Q205L mutation decreases GTPase activity and constitutively activates α_{i2} subunits; the G204A mutation prevents dissociation of βγ subunits from α subunits and constitutively inactivates α_{i2} subunits [33,43–45]. Increases in expression of α_{i2} are about four-fold in α_{i2}-wt cells, six-fold in α_{i2}-Q205L cells and three-fold in α_{i2}-G204A cells. There was no significant change in β_{36} and β_{35} protein levels in cells expressing α_{i2}-Q205L, even though these cells express the highest levels of α_{i2} proteins. In cells expressing α_{i2}-G204A, the levels of both β_{36} (β_1) and β_{35} (β_2) increased two to three times, slightly more than in cells overexpressing wild-type α_{i2}.

3.4. *βmRNA levels in NIH 3T3 cells overexpressing α subunits*

To determine whether the increase in β proteins was due to an increase in mRNA synthesis and/or stability, we measured mRNA levels of β_1 and β_2. Expression of β_1 and β_2 mRNA was easily detected in NIH 3T3 cells on four independent Northern blots. β_3 and β_4 mRNAs were never detected on any of the blots. The sizes of

mRNAs detected by the labeled probes were approximately 2.9–3.0 kb (β_1), 1.8 kb (β_2), and 2.0 kb (β-actin). We found that expression of both β_1 and β_2 mRNAs was increased whenever expression of an α subunit was increased (Fig. 3). The increase in β_1 and β_2 mRNAs varied between 1.4- and 3.1-fold (Table I), in agreement with the increases observed in β protein levels (usually less than three-fold). Unexpectedly, we found that cells expressing activated α_{12} (α_{12}-Q205L) also showed increased β_1 and β_2 mRNA levels (Fig. 3). These cells do not show any significant change in membrane β protein levels (Fig. 2).

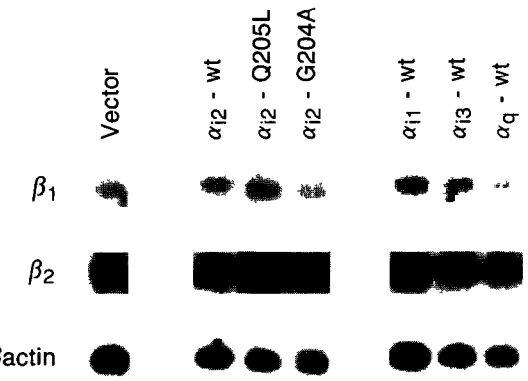

Fig. 3. β_1 and β_2 mRNA expression in NIH 3T3 cells overexpressing wild-type and mutated forms of α subunits. Poly(A)$^+$ mRNA extracted from NIH 3T3 cells transfected with vector alone or overexpressing wild-type and mutated forms of α subunits were separated on 1% agarose-formaldehyde gels (3 μg/lane) and transferred to nylon membranes. Four distinct mRNA preparations and Northern blots were probed with random-primed ^{32}P-labeled β_1, β_2, β_3, β_4 and β-actin cDNA probes (>10^6 cpm/ml). This figure shows one typical blot, with the exception that the increase in β_1 mRNA expression in α_q-wt cells was higher than 1.7-fold in all three other Northern blots. β-Actin mRNA levels were: for α_{i1}-wt cells, 100%; for α_{i3}-wt cells, 70%, for α_{i2}-wt cells, 60%; for α_{i2}-Q205L, α_{i2}-G204A and α_q-wt cells, 50%, of β-actin mRNA levels expressed in cells transfected with vector alone.

4. DISCUSSION

We have previously reported on effects of α subunit overexpression in fibroblasts [33,34,46]. Now, considering recent reports on functions for $\beta\gamma$ complexes, we have investigated in more detail the effects of α subunit overexpression on β subunits. We found that in fibroblasts stable increases in expression of α_i and α_q subunits were consistently associated with an increase in β subunits. These findings are in agreement with most in vivo studies of G_i expression [24–29]. α and β subunits increase simultaneously; however, the $\alpha/\beta\gamma$ ratio in any given cell type is still not well defined. Previous attempts to address this question did not take into account the diversity and relative expression of G protein subunits [47]. In our studies, there is no evident correlation between the extent of the increase in α subunit expression and the increase in β_1 and β_2 subunit expression. However, because the SW antiserum is directed against a highly conserved C-terminal sequence of β subunits, SW may be able to recognize β subunits that are still unknown. Thus, a two-fold increase in expression of β_{35} and β_{36} may well represent a large increase in the total amount of β subunits.

We found no evidence of selectivity of α_{i1}, α_{i2}, α_{i3} or α_q for β_1 or β_2 subunits. Although β_1 is four times less abundant than β_2 in fibroblasts, in all the cells tested, the increase in expression of β_1 is higher than the increase in β_2 proteins. The tissue distribution of β subunits varies. β_1 and β_2 are ubiquitous; β_3 seems to be expressed in most tissues [5], β_4 has been cloned from the brain; in most cell types, β_1 is usually the most abundant β subunit. β_1 (β_{36}) is the β subunit that has been found associated with plasma membrane, seven-transmembrane domain receptors [48–50], but the subcellular localization of β subunits is still unknown. We and others have shown that α_i subunits have distinct subcellular localizations: in fibroblasts, α_{i3} is located predominantly to the Golgi, not to the plasma membrane [46,51]. Despite the distinct targeting of α_{i3}, the increases in both β_1 and β_2 subunits observed in α_{i3} overexpressing cells are comparable to the β protein changes observed in cells overexpressing other α subtypes. Therefore, it seems unlikely that β_1 and β_2 subunits determine the subcellular localization of G proteins. Since β and γ subunits form a complex, it is reasonable to assume that γ subunit expression is also increased in cells overexpressing α subunits. Several groups have suggested that the specificity of both membrane targeting and signal transduction might be determined by γ subunits [52,53]. Specific associations between α subunits and $\beta\gamma$ complexes have been described in the brain and the retina [21,22], proving, as for α subunits, a selective tissue expression of certain β and γ subtypes. However, Graf et al. [54] found that in erythrocyte membranes, α subunits were coupled to any of the β and γ subunits expressed. Similarly, in NIH 3T3 cells, we did not find any evidence of selective association of transfected α subunits with β_1 or β_2. These apparently opposite findings are not mutually exclusive. In a given cell type, α subunits may associate indifferently with any of the β and γ subunits present, and $\alpha/\beta\gamma$ subunit association may be determined mostly by the selection of subtypes of G protein subunits expressed in the cell. Some $\alpha/\beta\gamma$ associations may be more favorable than others for coupling to one particular type of receptor or effector, thus determining the specificity of signal transduction and membrane targeting [49,53].

Our results suggest that the increases in β protein levels in NIH 3T3 cells result from increases in mRNA levels of β_1 and β_2 subunits. The increase in β_1 proteins is larger than the increase in β_2 proteins, yet the increase in mRNA is more pronounced for β_2 than for β_1. Levine et al. [28] have described similar findings in myocardial cells of hypothyroid rats. Possible explanations are a greater stability of mRNA for β_1 than β_2, or different

half-lives of β_1 and β_2 proteins. In the latter case, the half-life of β_2 should be shorter than the half-life of β_1. We also found that inactivated α_{i2}-G204A, even though it is almost always expressed at significantly lower levels than wild-type α_{i2}, induces increases in β_1 and β_2 mRNA and protein levels comparable to those found in α_{i2}-wt cells. This mutation (G226A in $G\alpha_s$ [45,55], G204A in $G\alpha_{i2}$) prevents the change of conformation necessary for GTP-induced dissociation of α from $\beta\gamma$ subunits and, presumably, the inactivated α subunit has a high affinity for $\beta\gamma$ complexes. In contrast, we found the highest increases in β_1 and β_2 mRNA, but no change in β protein levels in the membranes, in cells expressing activated α_{i2}. Attempts to study the turn-over of α_{i2}-Q205L and β subunits were unsuccessful, presumably because the AS and SW antibodies failed to precipitate sufficient amounts of [^{35}S]methionine-labeled proteins. Levis and Bourne [55] have recently shown that activated α_s is present in the cytosol, is degraded rapidly and has a very short half-life. We found that at least 30% of α_{i2}-Q205L subunits are present in the cytosol in NIH 3T3 cells, but detected only traces of β subunits in the cytosol (data not shown). Our hypothesis is that α_{i2}-Q205L, and $\beta\gamma$ complexes associated with α_{i2}-Q205L, are also degraded rapidly, inducing an increase in β mRNA and protein synthesis, but no change in β protein levels. Similar changes in β subunit protein and mRNA expression were observed in other cell types expressing α_{i2}-G204A and α_{i2}-Q205L (unpublished observations).

In conclusion, the degree of expression and activation of α subunits affects the synthesis and possibly the rate of degradation of β subunits. Therefore, possible effects on $\beta\gamma$ effectors should not be neglected. In fibroblasts, there is no specificity of α_i or α_q subunits for either β_1 or β_2 subunits, and β subunits do not seem to be responsible for the specificity of subcellular membrane targeting of G proteins. This does not exclude that specific associations of $\alpha/\beta/\gamma$ subunits may determine the selectivity for receptor or effector coupling.

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