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Proper processing of a G protein γ subunit depends on complex formation with a β subunit

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G protein β and γ subunits function as a tightly associated complex We show that complex formation with the β subunit is a critical step for post-translational processing of a γ subunit. When expressed alone in a cell line, the γ3 subunit type is isoprenylated but degraded; co-expression with the β1 subunit type stabilizes the γ3 protein. Furthermore, our experiments with partial cell fractionation indicate that the γ3 protein is localized differently in the cell depending on whether or not it is bound to the β subunit. Binding of the γ subunit to the β subunit is thus one of the prerequisites for the appropriate intracellular localization of the βγ complex and potentially, for normal G-protein function.

G protein; βγ complex formation

1. INTRODUCTION

G proteins are heterotrimers of α, β and γ subunits that are central components of the majority of signaling pathways. Each subunit is part of a large family of related proteins and several subtypes of each subunit may be expressed in the same cell [1]. Different α and β subunit types have been shown to be involved in the transduction of distinctly different signals in the same cell [2–4]. Thus G proteins composed of different subunit types may have different functional properties. Previously we have shown that different β and γ subunit types show selectivity of interaction when expressed in a cell line [5]. Similar results were obtained after expression of different β and γ subunit types in vitro [6]. Some reports indicate that different α subunits may also have different affinities for different βγ complexes [7,8]. Although some of the rules for the association of subunits have been elucidated, the mechanisms which assemble a particular G protein heterotrimer inside a cell and target it to specific receptors on the plasma membrane are unknown.

We have initiated studies to determine the specific processing steps involved in the formation and appropriate localization of the G protein βγ complex. At least eight different γ subunit types have been identified ([9–11] and Gallagher, C. and Gautam, N., unpublished). In this report we have examined the effect of co-expression of a β subunit type on the intracellular stability and localization of a γ subunit type, γ3. Results from this analysis demonstrate that, while the γ3 subunit can be expressed alone and isoprenylated, it does not form a stable product that is localized correctly. Only complex formation with the β1 protein makes γ3 stable and results in localization to appropriate membranes.

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2. EXPERIMENTAL

2.1. *Construction of expression vectors*

Expression vector pEV1 has been described previously, as well as the vector containing the cDNA for the β1 subunit [5] The cDNA for the γ3 subunit [9] was inserted into the *Hin*dIII and *Xba*I sites of pEV1.

2.2. *Cell culture, transfection, protein stability assay and labeling with [^{3}H]mevalonic acid*

QT6 quail fibroblast cells were cultured and transfected as described [5]. To study the effect of isoprenylation, cells were transfected 15 h after transfection to medium containing 25 μM compactin (gift from Dr. I. Udovichenko, Kansas State University) For immunoblot analysis, cells were usually harvested 48 h after transfection. To examine the stability of expressed proteins, transfectants were grown for 2 days and then a portion of the culture was harvested and frozen The remaining portion was incubated in medium containing 40 μg/ml cycloheximide for one more day and then harvested. It was shown that cycloheximide at this concentration inhibits most fresh protein synthesis in cells by comparing labeled proteins from cells grown in the absence of cycloheximide with proteins from cells grown in the presence of cycloheximide for a defined period of time. Beginning 15 h after transfection cells were labeled with 125 μCi/ml [^{3}H]mevalonate (American Radiolabeled Chemicals Inc., 50 Ci/mmol) for 24 h in the presence of 25 μM compactin As we have shown earlier [5], when labeled with [^{3}H]mevalonate control QT6 cells (i.e. untransfected cells or cells transfected with vector containing the β1 cDNA alone) did not show any labeled bands in the molecular weight range 6–12 kDa.

2.3 *Fractionation of cells*

Cells were harvested in Tris-buffered saline with 1 mM EDTA,

washed and lysed by freezing and thawing twice in hypotonic TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) with protease inhibitors (phenyl methyl sulfonyl fluoride and leupeptin). DNase I was added to the suspension After 5 min incubation, the suspension was centrifuged at room temperature in an Eppendorf microfuge (~14,000 × g) for 15 min and the supernatant (cytosolic fraction) was carefully removed from the membrane fraction. Protein concentrations were determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as the standard. Approximately 50% of the total cell protein was in the supernatant and the remaining in the membrane fraction

2.4. *Electrophoresis, immunoblotting and fluorography*

SDS-PAGE and immunoblotting were performed as described [5], except that the tank buffer was pH 8 6 for electrophoresis. 15 μg of protein were loaded in each lane. The BN-1 antiserum [12] was used to detect the β-subunit intrinsic to QT6 cells as described before [5]. Antiserum specific to the γ3-subunit was raised by using a synthetic peptide specific to the NH_2 terminal portion of the protein [9] The antibodies from this serum were purified using standard procedures and used for immunostaining at the concentration 0.45 μg/ml. No protein bands in the molecular weight range 3–12 kDa were detected in control QT6 cells with the γ3-specific antibody. Immunoblots were visualized using alkaline phosphatase-based staining (Promega) For fluorography gels were fixed, pretreated with Amplify (Amersham) and exposed at −80°C for different time periods, as mentioned in the appropriate figure legends. The autoradiograms were scanned using a laser scanning densitometer (Molecular Dynamics).

2.5. *[^{35}S]Methionine pulse-chase labeling*

The details for metabolic labeling of transfected QT6 cells with [^{35}S]methionine and immunoprecipitation of γ subunits have been described [13]. Briefly, 24 h after transfection, cells were preincubated in methionine-free DMEM (Sigma) with 5% dialysed fetal bovine serum and 1% dimethylsulfoxide for 1 h. Then cultures were labeled with 200–300 μCi/ml of [^{35}S]methionine (ICN, 1,000 Ci/mmol) in the same medium for 5 min (pulse) and a portion of the culture was harvested. The remaining portion of the culture was changed to fresh regular medium which contained approximately 20-fold more methionine in comparison to the medium containing labeled methionine Cells were incubated in this medium for different periods of time (chase). Cells were lysed as mentioned before, protein concentration was measured and samples with equal amounts of total protein were used for further analysis Total cell homogenates were fractionated into a cytosolic fraction and a Triton X-100 (0.2%)-extractable fraction from total membranes. Proteins were precipitated from these fractions with the γ3 antibody using protein A–Sepharose (Pharmacia), electrophoretically separated and visualized by autoradiography No proteins were precipitated with the γ3-specific antibody from control QT6 cells using the same procedure.

3. RESULTS AND DISCUSSION

A quail fibroblast cell line, QT6, was transiently transfected with cDNAs for the G protein β1 and γ3 proteins. Transfected cells were lysed, fractionated into crude membrane and cytoplasmic fractions, and the proteins in the fractions were examined by immunoblotting with γ3 antibody (see section 2 for details). Fig. 1A shows immunoblots of proteins from transfectants expressing γ3 alone or together with β1. Each sample was separated on two adjacent lanes. The cytoplasmic fraction is in the left lane and the membrane fraction in the right lane. The γ3 protein was predominantly localized to the membrane fraction when it was expressed alone or in the presence of β1. Two forms with different mobilities were synthesized in both cases. Co-expression with the β1 protein resulted in a significant increase in the slower mobility form of γ3. γ3 possesses on its COOH terminus the sequence, CALL, that is the signal for modification of cysteine by a geranylgeranyl moiety. Isoprenylation of a portion of the γ3 molecules could thus be a potential cause for the existence of two γ3 forms with different electrophoretic mobilities. This possibility was examined by growing transfectants in the presence of compactin. Compactin inhibits production of mevalonate and thus the isoprenylation of the γ subunits. In the presence of compactin almost all the synthesized γ3 protein was in the faster mobility form, even though it was co-expressed with β1 (Fig. 1A). The γ3 protein with the slower mobility is therefore the isoprenylated form.

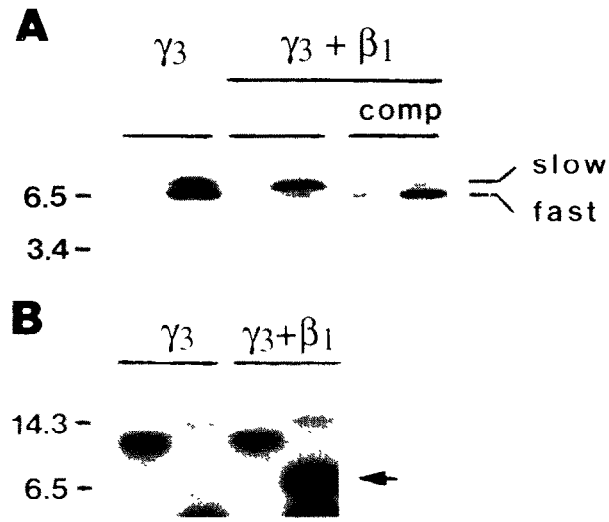

Fig. 1. Effect of β1 subunit on formation of isoprenylated γ3 protein QT6 cell transfectants containing cDNAs shown above the panels were examined as described in section 2. All electrophoresis was performed such that the membrane fraction from a sample was separated on the adjacent right lane to the cytosolic fraction from the same sample. (A) Immunoblot analysis with γ3-specific antibody Different transfectants were cultured with or without compactin (comp) and analysed. Two forms of the γ3 protein with different mobilities (slow and fast) were detected, as indicated. (B) Metabolic labeling of cells expressing γ3 with [^{3}H]mevalonate. The autoradiogram was exposed for 30 days. Arrow indicates expressed γ3 protein. Molecular weight standards (kDa) are shown to the left of the panel.

These results indicated that the β subunit has a significant effect on the amount of isoprenylated γ3 protein synthesized in these cells. Metabolic labeling experiments were used to determine more directly whether the γ3 protein was isoprenylated in the presence of the β subunit. Transfectants expressing γ3 alone or γ3 together with β1 were metabolically labeled with [^{3}H]mevalonate, the precursor for isoprenoids, and the labeled proteins examined as shown in Fig. 1B. In the presence of the β subunit the γ3 protein in the membrane fraction incorporated a significant amount of ^{3}H. Very little labeled γ3 protein was detected in the absence of β1. The

same samples that were analysed by autoradiography were also examined by immunoblotting. This showed that both in the presence and absence of the β subunit the $\gamma 3$ protein was expressed at the same level (data not shown). These results confirmed that co-expression of a β subunit dramatically increases the proportion of isoprenylated $\gamma 3$ protein made in the cell.

The potential causes for the effect of a β subunit on the modification of a γ subunit were as follows. One possibility was that the geranylgeranyl transferase was more active on the $\gamma 3$ protein in the $\beta\gamma$ complex compared to the γ subunit alone. This was unlikely since the transferase works well even on a tetrapeptide substrate [14]. Another possibility was that complex formation with a β subunit translocates the γ subunit to the appropriate cell compartment where the modifying enzyme is located. A third possibility was that $\gamma 3$ could get isoprenylated in the absence of the β subunit but the modified γ subunit was unstable when it was not present in a $\beta\gamma$ complex.

The following experiments were performed to examine whether the effect of the β subunit on the γ subunit was due to proteolytic removal of the modified γ subunits in the absence of the β subunit or due to a direct effect of the β subunit on isoprenylation. Cells were transfected with the $\gamma 3$ subunit alone or $\gamma 3$ together with $\beta 1$, and grown for 2 days. A portion of the cells was then harvested and frozen. The remaining portion was incubated with cycloheximide, an inhibitor of protein synthesis, for one more day. These cells were then harvested and examined by immunoblotting along with the portion of the cells that were frozen. Fig. 2 shows immunoblots of proteins from different transfectants. In the presence of cycloheximide, less $\gamma 3$ protein was present indicating that it was degraded at a significant rate. But when the β subunit was co-expressed with the $\gamma 3$ protein, the amount of $\gamma 3$ protein in cells after treatment with cycloheximide was not significantly less than in cells before the treatment. These results showed that the β subunit potentially protected the $\gamma 3$ protein from degradation.

To examine whether the stabilizing effect of the β subunit on $\gamma 3$ was due to complex formation between

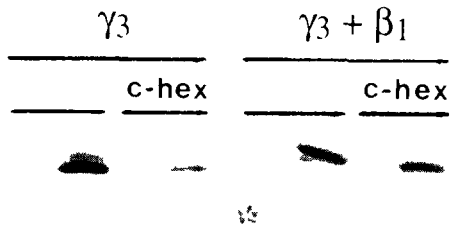

Fig. 2. Stability of $\gamma 3$ protein and its dependence on $\beta 1$ co-expression. Immunoblot of proteins from $\gamma 3$ or $\gamma 3 + \beta 1$ transfectants before and after treatment with (c-hex). Blots were probed with the $\gamma 3$ antibody. In each sample the left lane represents the cytosolic fraction and the right lane the total membrane fraction.

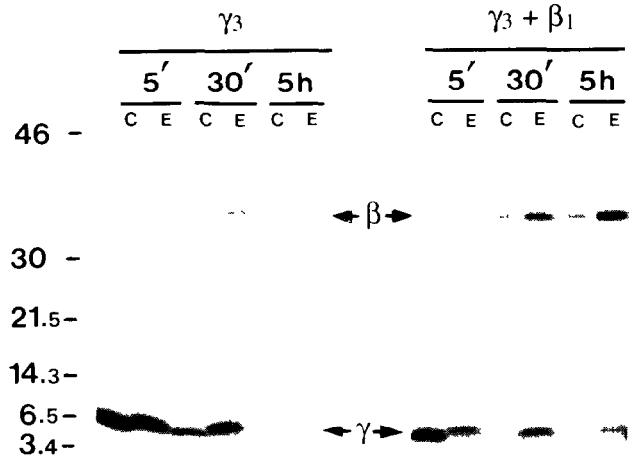

Fig. 3. Pulse chase [^{35}S]methione labeling of $\gamma 3$ protein in the absence and presence of the $\beta 1$ subunit. Transfectants containing $\gamma 3$ alone or $\gamma 3$ together with $\beta 1$ were metabolically labeled with [^{35}S]methionine for 5 min and part of the culture was harvested (5′). The remaining portion of the culture was transferred to fresh medium with an excess of unlabeled methionine and incubated for an additional 30 min (30′) or 5 h (5h). Cells were lysed and fractionated into a cytosolic fraction (C) and Triton X-100 (0.2%)-extractable fraction (E) from total membranes. Proteins were precipitated from these fractions with the $\gamma 3$ antibody. Immunoprecipitates were electrophoretically separated and visualized by autoradiography. Autoradiogram was exposed for 1.5 days. Positions of protein molecular weight standards (kDa) are shown at left.

the β and γ subunits the following pulse-chase experiments with [^{35}S]methionine were performed. Transfectants were labeled with a short pulse of [^{35}S]methionine followed by chases with an excess of unlabeled methionine for different periods of time (see section 2 and Fig. 3 legend for details). Cells were lysed and fractionated into a cytosolic fraction and a membrane fraction. Proteins in the membrane fraction were extracted with Triton X-100. Proteins were precipitated from these fractions with the $\gamma 3$ antibodies, electrophoretically separated and visualized by autoradiograhy. Fig. 3 shows the results of pulse–chase experiments using transfectants expressing $\gamma 3$ alone or $\gamma 3$ co-expressed with $\beta 1$. After exposing cells to a pulse of [^{35}S]methionine for 5 min the $\gamma 3$ protein was found in both the cytosolic and membrane extract fractions. The slower mobility of the $\gamma 3$ form present in the membrane extract fraction (Fig. 3, $\gamma 3$ alone, lane E of 5′ pulse) in comparison to the form found in the cytosol (same sample, lane C) indicated that a portion of the $\gamma 3$ synthesized during the pulse was isoprenylated. After exposure of cells to an excess of unlabeled methionine for 30 min or 5 h (chase), a labeled protein with the same mobility as the $\beta 1$ subunit was co-precipitated with $\gamma 3$ from these cells, indicating that the β subunit endogenous to QT6 cells forms a complex with the $\gamma 3$ protein. When expressed alone, the labeled $\gamma 3$ protein precipitated after a 5 h chase was much less than the amount precipitated after 30 min

(Fig. 3, compare in the $\gamma 3$ sample, the 30′ extract with the 5 h extract). Densitometry of the autoradiogram shows that the proportion of $\gamma 3$ protein after 5 h is 7% of the amount of protein after 30′. This indicated that most of the freshly synthesized $\gamma 3$ protein was degraded in 5 h even if it was isoprenylated; but if $\gamma 3$ was co-expressed with $\beta 1$, a significant proportion (~50% as determined by densitometry) of labeled $\gamma 3$ protein precipitated after a chase of 30 min was still present in precipitates after a chase of 5 h (Fig. 3, compare in the $\gamma 3 + \beta 1$ sample, the 30′ extract with the 5 h extract). These results show that $\gamma 3$ and $\beta 1$ indeed form a complex inside the cell since the $\beta 1$ protein is co-precipitated by an antibody against $\gamma 3$. The results also indicate that the $\gamma 3$ subunit is not protected from degradation by isoprenylation but through complex formation with the appropriate β subunit.

Although the γ subunit is a membrane-bound protein it is most likely that it is synthesized in the cytosol since it was predominantly present in the cytosolic fraction after a short pulse of 5 min (Fig. 3, $\gamma 3$ and $\gamma 3 + \beta 1$, 5′ pulse sample). The same lanes show that most of the $\gamma 3$ in the cytosolic fraction was the unmodified faster mobility form while most of the $\gamma 3$ extracted from membranes was the isoprenylated slower mobility form. The unmodified form was not detected in the membrane and the modified form was not detected in the cytosol. These results indicate that either isoprenylation of $\gamma 3$ takes place on the membranes or that $\gamma 3$ is translocated very rapidly to the membranes after isoprenylation. Also, the post-translationally modified γ subunit is degraded if it does not bind the appropriate β subunit.

Although the results of [^{35}S]methionine labeling indicated that both the modified and the unmodified forms of the $\gamma 3$ protein were degraded in the absence of the β subunit, results from immunochemical analysis (Fig. 1A) showed that, when expressed alone, a significant amount of unmodified $\gamma 3$ was present in the membrane fraction. In the pulse–chase experiment described above, only the Triton extracts from membrane fractions were examined (Fig. 3). Since unmodified $\gamma 3$ could potentially be localized in the membranes remaining after Triton extraction, these membranes were solubilized in 1% SDS and the proteins precipitated with the $\gamma 3$ antibody, as in [13]. When the immunoprecipitates were examined by autoradiography labeled $\gamma 3$ was detected in this fraction (data not shown). The amount of labeled $\gamma 3$ precipitated from this fraction after a 10 min pulse of [^{35}S]methionine was the same as the amount precipitated after 1 h chase with unlabeled methionine. This indicates that a portion of the $\gamma 3$ synthesized in the cell was present in a form that was not extractable with Triton X-100. More importantly, this form was more stable than the Triton-extractable protein. Although the experiment with cycloheximide indicated that the unmodified $\gamma 3$ in membranes was degraded at a significant rate (Fig. 2), the relative stability of this form in comparison to modified $\gamma 3$, as well as the high rate of synthesis when $\gamma 3$ was expressed alone (Fig. 3, $\gamma 3$ sample, 5′ pulse), would lead to an accumulation of the unmodified Triton-resistant form that is seen in Fig. 1A.

To obtain more detailed information about the localization of the γ subunit the following experiment was performed. All fractionation steps were at 4°C. Transfectant cells expressing $\gamma 3$ alone or $\gamma 3$ with $\beta 1$ were lysed and fractionated initially by low speed centrifugation at $1{,}000 \times g$ for 1 min. The pellet (heavy membrane fraction) was solubilized with Triton X-100 (0.2%) and centrifuged at $14{,}000 \times g$ for 15 min. The supernatant (Fig. 4A and B, samples E1) and the pellet after centrifugation (Fig. 4A and B, samples P1) were examined on an immunoblot. The supernatant from the initial $1{,}000 \times g$ centrifugation was centrifuged at $14{,}000 \times g$ for 15 min. The supernatant from this centrifugation, the cytosolic fraction, was analysed on an immunoblot (Fig. 4A and B, samples C). The pellet from the $14{,}000 \times g$ centrifugation (light membrane fraction) was solubilized with Triton X-100 (0.2%) and centrifuged for 15 min at $14{,}000 \times g$. The supernatant (Fig. 4A and B, samples E2) and the pellet after the centrifugation (Fig. 4A and B, samples P2) were also examined by immunoblotting. As expected, when $\gamma 3$ was expressed alone (Fig. 4A) most of the protein was unmodified and present in the P1 fraction which is the heavy membrane fraction after extraction with Triton. A relatively small amount of $\gamma 3$ was modified and Triton extractable. This protein could be the portion of $\gamma 3$ bound to the β subunit endogenous to QT6 cells. When the $\beta 1$ protein was co-expressed with $\gamma 3$, the localization of $\gamma 3$ was changed dramatically. A significant proportion of the $\gamma 3$ protein made in the presence of $\beta 1$ was present in the light membrane fraction (Fig. 4B, E2) and most of the protein was Triton-extractable from both membrane fractions. The intracellular distribution of the β subunit endogenous to QT6 cells was examined using a similar fractionation procedure. The β subunit native to the cells was present in the same Triton-soluble fractions in which the modified $\gamma 3$ protein was present when co-expressed with $\beta 1$ (Fig. 4C). This indicates that when $\gamma 3$ is complexed with $\beta 1$ it is localized to the appropriate cell membranes. Results from the analysis of the unmodified γ subunit indicate that it is not localized in an appropriate membrane and is potentially present in the form of aggregates in the cell or in intracellular structures from which it cannot be extracted with Triton.

The effect of co-expression of $\beta 1$ and $\gamma 2$ have been examined before [15,16]. One report showed that when expressed alone, the $\beta 1$ or $\gamma 2$ proteins could not be detected in COS cells but when the $\beta 1$ and $\gamma 2$ cDNAs were co-expressed, significant amounts of the corresponding proteins were detected in the same cell line [14]. Other reports have, however, shown that different β and γ subunit types can be detected at significant levels when expressed alone [5,16]. Results communi-

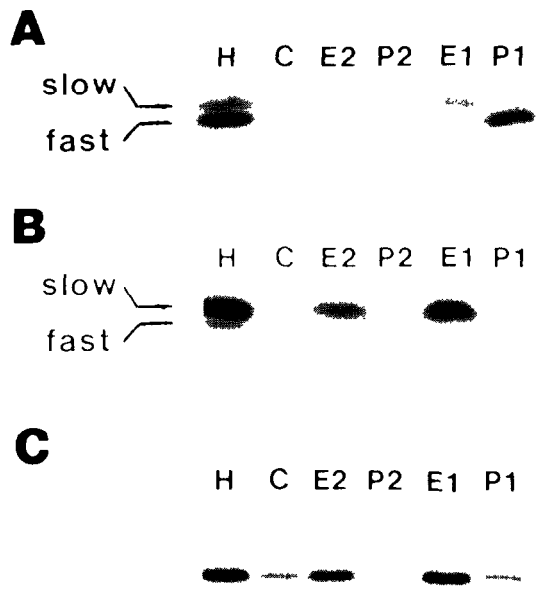

Fig 4. The subcellular distribution of the $\gamma 3$ protein depends on $\beta 1$ subunit co-expression. (A) Immunoblot of proteins from different fractions of transfectants expressing $\gamma 3$ alone. (B) Immunoblot of proteins from different fractions of transfectants expressing $\gamma 3$ and $\beta 1$. These immunoblots were probed with the $\gamma 3$ antibody. (C) Immunoblot of proteins from different fractions of untransfected QT6 cells probed with an antibody specific to the β subunit, BN-1. Lane H is the total homogenate (15 μg protein). Cultured cells were divided into two equal portions; one portion was homogenized (H), the other portion was fractionated The same proportion of each fraction, as well as the homogenate, were electrophoresed on the gel so that the proteins in each lane are from approximately the same number of cells. Lanes P1 and E1, pellet and supernatant after solubilization of a crude membrane fraction ($1{,}000 \times g$) with Triton and centrifugation. Lanes P2 and E2, pellet and supernatant after solubilization of a '$14{,}000 \times g$ light membrane fraction' with Triton and centrifugation. Lane C, supernatant (cytosolic fraction) after precipitation of the light membranes by centrifugation. Detailed description of the fractionation is in the text.

cated here provide an explanation for this discrepancy. In the absence of the β subunit the γ subunit is pelleted by slow speed centrifugation. This fraction was not analysed by Simonds et al. Since the $\gamma 2$ protein was not detected when expressed alone, the effect of the β subunit on the stability or localization of this protein was not examined in that work. The intracellular localization of $\gamma 2$ and $\beta 1$ subunit types have been examined immunocytochemically in transient transfectant COS cells [16]. In that study when expressed alone $\gamma 2$ and $\beta 1$ were located in intracellular vesicles in the perinuclear region. Immunocytochemical analysis performed by us on QT6 transfectants containing either $\beta 1$ or $\gamma 3$ alone shows these proteins aggregated or trapped in unknown organelles (to be published elsewhere).

The results reported above indicate that the formation of the G protein $\beta\gamma$ complex is essential for the appropriate post-translational processing of the $\gamma 3$ subunit. The following is a tentative scheme describing the pathway that this G protein γ subunit traverses inside the cell. The $\gamma 3$ protein is made in the cytosol. It is then rapidly translocated to the membrane. It is not clear where the geranylgeranylation, proteolysis and methylation of $\gamma 3$ occur, although there is evidence that the geranylgeranyl transferase is cytosolic and the enzymes that carry out subsequent processing are on membranes [17]. If $\gamma 3$ fails to bind an appropriate β subunit soon after synthesis, a significant portion of the $\gamma 3$ protein evades isoprenylation to form either aggregates or to get transported to unknown compartments in the cell. $\gamma 3$ that is modified in the absence of the β subunit degrades rapidly. Complex formation with the β subunit stabilises the γ subunit and makes it competent for translocation to appropriate membranes. Post-translational processing and intracellular targeting of G protein γ subunits is thus potentially complex and involves several discrete events.

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