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Biochemistry

Interaction between G-protein β and γ subunit types is selective

(signal transduction/subunit families)

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ABSTRACT **Signal-transducing guanine nucleotide-binding proteins (G proteins) are made up of three subunits, α, β, and γ. Each of these subunits comprises a family of proteins. The rules for association between members of one family with members of another to form a multimer are not known; it is not clear whether associations are specific or nonspecific. Other than transducin (G_t), the G protein in rod photoreceptors, most purified G proteins contain more than one subtype of β or γ subunits. The G_t α subunit is associated only with β_1 and γ_1. It is not known whether this specificity is due to the differential expression of these subunit types in a cell type or due to intrinsically different affinities between different β and γ subunit types. We have used a transfected cell assay system to examine the association of the β_1, β_2, and β_3 proteins with the γ_1 and γ_2 proteins. Results show that γ_1 does not associate with β_2 and that β_3 does not associate with γ_1 or γ_2. Differences in affinities between types of G protein subunits will impose restrictions on the formation of certain heterotrimers and determine which G protein will be active in a cell. A chimeric molecule of β_1 and β_2 was used to broadly map the regions on these subunits that determine specificity of association.**

G proteins are a family of signal-transducing molecules that couple an enormous variety of receptors to effectors (1, 2). G proteins are heterotrimeric, made up of α, β, and γ subunits. The α subunits are a large family of proteins (3). It is increasingly evident that the β and γ subunits are also families of proteins (3–6). It is not known whether subtypes from one subunit family have different affinities for subtypes from another family and, as a result, whether distinct heterotrimers arise through specific associations among certain α, β, and γ subtypes. Although G proteins purified from different mammalian tissues often contain more than one type of β or γ subunit, β and γ subtypes are not found associated at random with all α subtypes (ref. 6 and unpublished data). But it is not clear whether specificity of association is due to selective binding of some subunit types or due to the differential expression of certain subtypes in a particular cell. We have used an assay in cultured cells to examine the following questions. Do various β subtypes and γ subtypes possess different affinities for each other? Which amino acids in the β subunit are involved in interaction with the γ subunit?

The analysis is restricted to the β and γ subunits although it could potentially be extended to the α subunits. The function of the β and γ subunits is not as clear as the function of α subunits, which are GTPases capable of modulating effector function (1, 2). Important roles for the $\beta\gamma$ complex in effector modulation have been shown: recent evidence indicates that the complex can modulate the activity of adenylate cyclases (7) and there is evidence for the role of these subunits in regulating K^+ channel function (8).

The assay that has been used here to examine association of β and γ subtypes depends on the inability of G-protein γ subunits to associate with the cell membrane when they are not isoprenylated. The γ subunits of G proteins possess at their COOH terminus the sequence Cys-Ali-Ali-Xaa (Ali, aliphatic amino acid; Xaa, any amino acid) (4–6). This sequence is present in members of the Ras family also. The cysteine is modified by a lipid moiety (9–14). In cells cotransfected with the γ_2 and β_1 cDNAs, the proteins associate with the membrane fraction (15). When a mutant γ_2 with the cysteine in the sequence Cys-Ali-Ali-Xaa changed to serine was cotransfected with β_1, both the γ_2 and β_1 proteins were present in the cytosol. Since the β_1 protein was present in the cytosol along with the mutant γ_2 protein, we infer that β_1 can associate with γ_2. Mutant forms of the γ_1 and γ_2 proteins with the cysteine in the Cys-Ali-Ali-Xaa sequence altered have been cotransfected here with three different β subunits (β_1, β_2, and β_3) to examine whether there is similar specificity of association between these proteins. By examining the membrane and cytosolic fractions from the transfected cells it was possible to infer whether a particular β subunit was associated with a particular γ subunit. A chimeric form of β_1 and β_2 has been used to broadly define the region on the G-protein β subunits involved in interaction with the γ subunit.

MATERIALS AND METHODS

Expression Vectors, Mutants, and Chimeric Molecules. The cDNAs for the β and γ subunits were inserted into an expression vector, pEV1, containing the Rous sarcoma virus (RSV) promoter and the simian virus 40 poly(A) tail region (from J. Johnson, California Institute of Technology). The vector has several restriction sites downstream of the RSV promoter that are in the following sequence 5′ to 3′: *Hin*dIII, *Sph* I, *Pst* I, *Sal* I (*Acc* I, *Hin*cII), *Xba* I, *Bam*HI. The γ_1 and γ_2 cDNAs (4, 16) were inserted using the *Hin*dIII and *Xba* I sites. The β_1 and β_3 cDNAs (17, 18) were excised with *Eco*RI, end-filled with Klenow fragment of DNA polymerase, and inserted into the *Sal* I site of pEV1 after the ends were made blunt. The β_2 cDNA (19) was excised with *Eco*RI and *Eco*RV, end-filled, and inserted into the same site as the β_1 cDNA. Vectors containing the cDNAs in the appropriate orientation were used for transfections. The γ_1 and γ_2 cDNAs were mutated by the polymerase chain reaction (20). The oligodeoxynucleotides TG3 (5′-TATAAGCTTGGCAAACAGTT-TGCTTA-3′) and TG4 (5′-TATTCTAGATTCTTATGA-AATCACAGAGCCT-3′) were used so that the COOH-terminal amino acid sequence of γ_1 was changed from CVIS to SVIS, and the alteration was checked by determining the nucleotide sequence. The amplified mutant cDNA was inserted into the pEV1 vector by using the *Hin*dIII and *Xba* I sites. The mutant γ_2 cDNA with a change in the sequence from CAIL to SAIL was obtained from W. Simonds (National Institutes of Health) (15).

The β_2N56/β_1 chimera that encodes the NH_2-terminal 56 amino acids of β_2 and the rest of the amino acids from β_1 was

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Abbreviations: RSV, Rous sarcoma virus; Ali, aliphatic amino acid residue.

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constructed by cutting both the β_1 and β_2 cDNAs with *Bgl* II, a site that is conserved in both cDNAs, and splicing the portion of β_2 that is toward the 5′ end of the *Bgl* II site with the portion of the β_1 cDNA toward the 3′ end of the same site. The chimeric molecule was checked by restriction mapping.

Antibodies. Antisera specific to the different γ subunits were raised by using synthetic peptides specific for the NH_2-terminal portion of the γ subunits (6). The antibodies from these sera were purified by standard procedures and used in the experiments presented. The β_1 antiserum has been described (21). It was determined that this antiserum is specific for the β_1 protein at the dilutions used. The β_2 antiserum was raised against a synthetic peptide corresponding to amino acids 25–39 of the β_2 protein. The β_3 antiserum was raised against a synthetic peptide corresponding to amino acids 25–39 of the β_3 protein. The γ subunit-specific antibodies were unable to react with their antigens in the presence of the appropriate peptide. The β subunit-specific antibodies were not tested with the appropriate peptides.

Cell Culture, Transfection, and Labeling with [^{3}H]Mevalonate. QT6 quail fibroblast cells (22) obtained from J. H. Steinbach (Washington University Medical School) were grown in medium 199 with Earle's salts and L-glutamine, supplemented with 10% tryptose phosphate broth, 5% fetal bovine serum, 1% dimethyl sulfoxide, and penicillin/streptomycin. Subconfluent cells were transfected by the calcium phosphate precipitation method combined with glycerol shock. Five micrograms of the expression vector pEV1 containing the β subunit-specific cDNAs and 10 μg of the vector with the γ subunit-specific cDNAs were used for transfections. When the β or γ subunits were expressed alone the total amount of DNA was kept constant by adding pEV1 DNA containing cDNA for the bacterial β-galactosidase (*lacZ*). pEV1 containing *lacZ* was also used for determining the efficiency of transfection into these cells by staining the cells for β-galactosidase enzyme activity. Usually about 40–60% of the cells were transfected. For immunological analysis, cells were harvested 48 hr after transfection.

Twenty hours after transfection, cells were labeled with [^{3}H]mevalonolactone (DuPont/NEN; 40 Ci/mmol; 1 Ci = 37 GBq) at 50 μCi/ml for 24 hr in the presence of 25 μM lovastatin (obtained from A. Daugherty, Washington University Medical School).

Fractionation of Cells. Cells were harvested in Tris-buffered saline with 1 mM EDTA, washed, and lysed in hypotonic buffer [10 mM Tris·HCl, pH 8.0/1 mM EDTA with protease inhibitors (phenylmethylsulfonyl fluoride and leupeptin)] by freezing and thawing twice. DNase I was added to the suspension and incubated for 5 min. The suspension was then centrifuged in an Eppendorf 5415 microcentrifuge ($\approx$14,000 $\times$ g). 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.

Electrophoresis, Immunoblotting, and Fluorography. SDS/polyacrylamide gel electrophoresis was used to separate proteins. In all cases $\approx$20 μg of proteins was loaded in each lane. To improve resolution of the β and γ subunits of G proteins, gels were made such that the polyacrylamide concentration in the top half was 12% and in the bottom half, 17%. For immunoblotting, proteins from the gel were transferred to Immobilon P membranes (Millipore). The location of β or γ subunits on the blot was determined with the help of prestained molecular weight markers and amido black staining of the membrane. Blocking in 5% bovine serum albumin removed the amido black stain. Antibodies or antisera were suspended in 20 mM Tris·HCl, pH 7.5/250 mM NaCl/0.05% Tween 20. Antibodies or antisera were use at the following dilutions: β_1 (antiserum), 1:1,000,000; β_2 (antiserum), 1:800; β_3 (antibodies), 0.5 μg/ml; γ_1 (antibodies), 0.45 μg/ml; γ_2 (antibodies), 0.25 μg/ml. Immunoblots were visualized by alkaline phosphatase-based staining.

Immunoblots were scanned on an Ultroscan XL laser densitometer (2222-010; LKB). Only a section of the band and not the whole band was scanned and the quantitation is approximate. Peak areas of bands from cytosolic and membrane fractions were added and taken to be 100% of protein expressed in the cell. The amount of protein in cytosol relative to the total was expressed as a percentage from the ratio of the appropriate peak areas. These percentages are shown only in cases where differences in the distribution of the protein between cytosol and membrane fractions cannot be seen clearly.

For fluorography gels were fixed, pretreated with Autofluor (National Diagnostics, Manville, NJ), and exposed at −80°C for 32 days.

RESULTS

Expression of Various β Subunits Alone and with the Wild-Type and Mutant γ_1 Protein. cDNAs for three different β subunit types (β_1, β_2, and β_3) were placed under the control of the RSV promoter and transfected into QT6 cells. Cells were harvested after 48 hr, and membrane and cytosolic fractions were examined by immunoblotting with β subtype-specific antibodies. Based on the immunoreactivity to the β_1-specific antibody, untransfected QT6 cells possess a β_1-like subunit (Fig. 1*A*). In cells transfected with different β subtype cDNAs, proteins of the appropriate molecular weight that reacted with

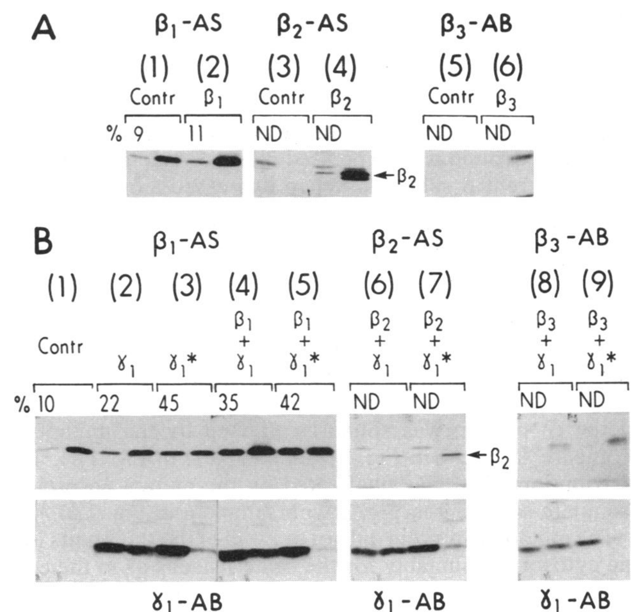

FIG. 1. Expression of β subunits alone (*A*) and together with wild-type and mutant γ_1 (*B*). Each sample was electrophoresed in two lanes: the left lane of each pair contained the cytosolic fraction and the right lane the membrane fraction. The percentage of the β subunit present in the cytosol relative to the total amount expressed is above lanes containing the cytosolic fraction (ND, not done). These figures are approximate (*Materials and Methods*). Cells were transfected with the expression vector pEV1 containing the cDNAs shown above the lanes. In all cases where a particular cDNA was transfected alone it was cotransfected with a *lacZ*-expressing plasmid. γ_1*, mutant (Cys→Ser). Antisera (AS)/antibodies (AB) used for a particular blot are shown above in the case of the β subunits and below in the case of the γ subunits. The β_1 antiserum reacts with a β subunit intrinsic to QT6 cells. The β_2 antiserum reacts with a protein in the cytosolic fraction of QT6 cells. Arrow indicates the β_2 subunit. Sometimes a band of $\approx$34 kDa specific for β_2 appeared on immunoblots. Contr, control (untransfected QT6 cells). Sample numbers in parentheses are those referred to in the text. Immunoblots above are representative of three or more experiments with all samples except β_3+γ_1 and β_3+γ_1*, which were examined twice.

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β subunit-specific antibodies were expressed. All three proteins—β_1, β_2, and β_3—were present in the membrane fraction. Results of expressing the γ_1 subunit alone or together with the three different β subunits are shown in Fig. 1*B*. When expressed alone a significant proportion of the wild-type γ_1 protein was unexpectedly present in the cytosol (this behavior is explained in the *Discussion*) (sample 2 in Fig. 1*B*). The mutant γ_1 in which the COOH-terminal cysteine has been changed to serine was present almost entirely in the cytosol (sample 3 in Fig. 1*B*). Coexpression of the wild-type or mutant γ_1 protein with the β_1 protein increased the proportion of β_1 present in the cytosol 3- or 4-fold in comparison to cells that expressed β_1 alone (compare sample 4 or 5 in Fig. 1*B* with sample 2 in Fig. 1*A*). γ_1 also associated with the β subunit endogenous to QT6 cells. QT6 cells expressing the γ_1 mutant contained 4-fold more β subunit in the cytosol than untransfected cells (compare sample 3 with sample 1 in Fig. 1*B*). The wild-type and mutant γ_1 proteins did not have any discernible effect on the distribution of β_2 or β_3 (compare sample 6 or 7 in Fig. 1*B* with sample 4 in Fig. 1*A* and samples 8 or 9 in Fig. 1*B* with sample 6 in Fig. 1*A*).

Even though the mutant γ_1 was expressed at high levels, not all the β_1 protein was drawn into the cytosol. This could be due to the following reasons. (*i*) Since only about 40–60% of the cells were transfected, many cells did not contain the introduced γ subunit. (*ii*) The expression of the introduced cDNA was transient and therefore its product was incapable of associating with β subunits present in preexisting $\beta\gamma$ complexes. (*iii*) Endogenous γ subunits were present that were capable of binding the β_1 protein.

Expression of Various β Subunits with the Wild-Type and Mutant γ_2 Proteins. In a similar experiment the wild-type or mutant γ_2 protein was expressed alone or together with the three different β subunits. When expressed alone the wild-type γ_2 protein was present predominantly in the membrane fraction (sample 2 in Fig. 2). In contrast, a significant proportion of the mutant γ_2 in which the COOH-terminal cysteine was changed to serine was present in the cytosol (sample 3 in Fig. 2). Coexpression of the mutant γ_2 with β_1 increased the proportion of the β_1 protein in cytosol almost 5-fold when compared with cells with β_1 alone (compare sample 5 in Fig. 2 with sample 2 in Fig. 1*A*). The distribution of the β_2 protein was similarly affected by the mutant γ_2 (compare sample 7 in Fig. 2 with sample 4 in Fig. 1*A*). The distribution of β_3 was unaffected by the mutant γ_2 protein (compare sample 9 in Fig. 2 with sample 6 in Fig. 1*A*).

The mutant γ_2 protein did not draw all of the β subunits into the cytosol, presumably for the same reasons as in the case of the γ_1 mutant.

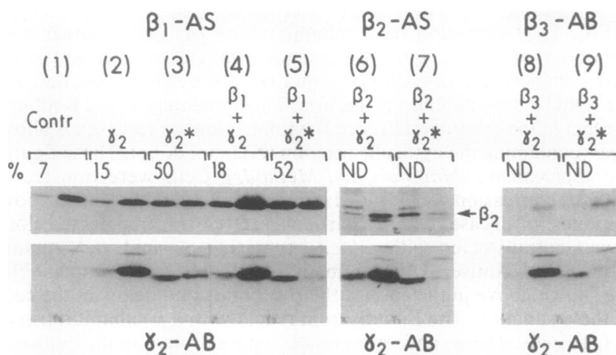

FIG. 2. Coexpression of wild-type γ_2 and mutant γ_2* with different β subunit types. Details are similar to those in Fig. 1. Upper panels show the different β subunits; lower panels show the γ_2 protein. Immunoblots above are representative of two or more experiments with all the samples.

Expression of a Chimeric Protein (β_2N56/β_1) Alone and with Wild-Type and Mutant γ Subunits. A cDNA specifying a chimeric molecule in which the NH_2-terminal 56 amino acids were from β_2 and the remaining amino acids were from β_1 (β_2N56/β_1) was constructed. This cDNA was coexpressed with the wild-type and mutant γ_1 and γ_2 proteins (Fig. 3). Coexpression of the mutant γ_1 protein with the β subunit chimera increased the proportion of β_2N56/β_1 in the cytosol 6-fold in comparison to cells expressing the β_2N56/β_1 chimera alone (compare sample 4 with sample 2 in Fig. 3). The γ_2 mutant had a similar effect on the β subunit chimera. Coexpression of the mutant γ_2 protein with β_2N56/β_1 increased the proportion of β_2N56/β_1 in the cytosol 6-fold in comparison to cells expressing the β_2N56/β_1 chimera alone (compare sample 6 with sample 2 in Fig. 3).

[^{3}H]Mevalonic Acid Labeling of Wild-Type and Mutant γ_1 and γ_2 Proteins. To examine whether the cysteine → serine mutation in the γ subunits had an effect on the ability of the proteins to be isoprenylated in QT6 cells, cells cotransfected with various γ subunit cDNAs and the β_1 subunit were labeled with [^{3}H]mevalonic acid. Mevalonic acid is a precursor for the synthesis of the isoprenoids that are covalently linked to the cysteine residue in the Cys-Ali-Ali-Xaa sequence of the γ subunits. Cells with appropriate DNA constructs were grown in the presence of [^{3}H]mevalonic acid, harvested, and fractionated into membrane and cytosolic fractions. These fractions were analyzed by SDS/polyacrylamide gel electrophoresis followed by fluorography (Fig. 4). The wild-type γ_1 subunit was labeled and found mostly in the membrane fraction. The wild-type γ_2 was also labeled and was detected only in the membrane fraction. The mutant forms of these subunits were not labeled in either the cytosolic fraction or the membrane fraction.

DISCUSSION

G-protein γ subunits with a mutated COOH-terminal cysteine are unable to associate with the membrane but retain their ability to associate with the β subunit (ref. 15 and results

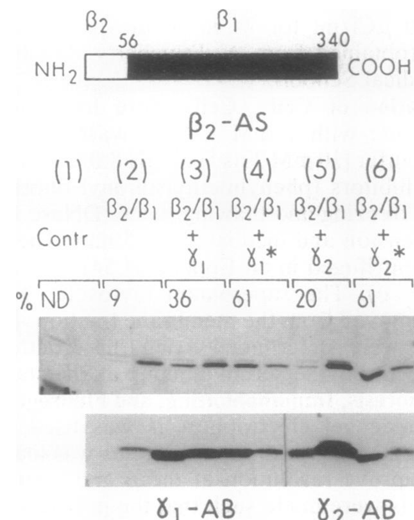

FIG. 3. Expression of a chimeric β subunit. (*Upper*) Structure of chimeric β_2N56/β_1 protein consisting of the NH_2-terminal 56 amino acids of β_2 and the COOH-terminal portion (amino acid residues 57–340) of the β_1 molecule. (*Lower*) Expression of β_2N56/β_1 (β_2/β_1) with the wild-type and mutant (*) γ_1 and γ_2 subunits. The percentage of β_2N56/β_1 protein in the cytosol relative to the total are shown above lanes containing the cytosol fraction. The antiserum (AS)/antibodies (AB) used are shown above and below the blots. Immunoblots with the γ_1 antibody sometimes show a band of the same size as γ_1 in QT6 cells. β_2/β_1 and β_2/β_1+γ_2* were examined twice and other samples, once.

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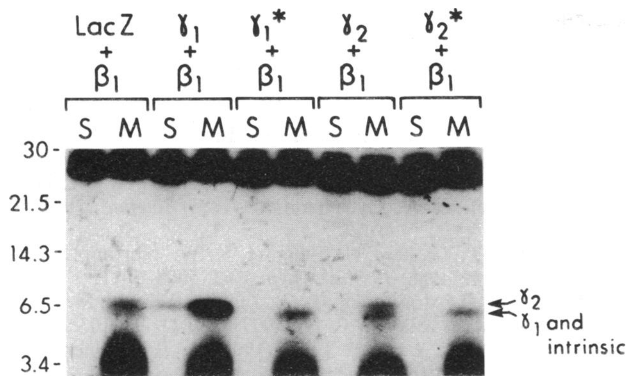

FIG. 4. Metabolic labeling of introduced γ subunits with [^{3}H]-mevalonic acid in QT6 cells. Thirty micrograms of protein was loaded in each lane. Autoradiogram was exposed for 32 days. Arrows at right indicate positions of introduced γ subunits and a protein intrinsic to QT6 cells that is of approximately the same mobility as γ_1. Positions of protein molecular size standards (kDa) are shown at left. S, soluble; M, membrane.

above). Mutant forms of two γ subunits were used to determine their relative abilities to associate with three different β subunits. There is considerable evidence from the analysis of purified G proteins that the β and γ subunits are tightly bound to each other. It was therefore inferred that colocalization of a β subunit type with a γ subunit type to the cytosolic fraction of transfected cells indicated that these two proteins associate with each other.

Expression of the γ_1 mutant in QT6 cells affects the distribution of the β subunit endogenous to QT6 cells. The γ_1 mutant also affects the distribution of coexpressed β_1. In both cases significant proportions of the β subunits are present in the cytosol along with the mutant γ subunit. The γ_1 mutant does not have any effect on the β_2 or β_3 protein. This implies that γ_1 binds β_1 (and the β subunit endogenous to QT6 cells, which is like β_1 since it reacts with an antibody specific for β_1). However, γ_1 does not associate with the β_2 or β_3 protein. In comparison, expression of the γ_2 mutant affects the distribution of β_1, β_2, and the endogenous β subunit but has no effect on the β_3 protein. Thus γ_2 is capable of associating with β_1 and β_2 but not β_3. Since neither γ_1 nor γ_2 associates with β_3, it is possible that the γ subunit with which it associates is any of three other γ subunits (γ_3, γ_4, or γ_5) that have been identified (ref. 6 and C. Gallagher and N.G., unpublished work) or one that has not been identified so far. It is unlikely that the behavior of β_3 is due to improper folding of the protein, since the β_1, β_2, γ_1, γ_2, and β_2N56/β_1 proteins expressed in QT6 cells associate to form $\beta\gamma$ complexes and also the γ subunits are modified posttranslationally in an appropriate way.

The γ_1 and γ_2 proteins have been shown to be isoprenylated at the COOH-terminal cysteine (9–12). The experiment involving mevalonate incorporation shows that the mutant γ subunits, as expected from previous work, are not isoprenylated like the wild-type proteins. The behavior of the mutant γ subunits is therefore due to the lack of a lipid moiety at the COOH terminus. Unexpectedly, a significant proportion of the wild-type γ_1 protein is present in the cytosolic fraction. Examination of the cytosolic fraction from cells transfected with γ_1(plus β_1) and labeled with [^{3}H]mevalonic acid (Fig. 4) indicates that very little γ_1 in cytosol is labeled. In comparison, when the γ_1 protein is examined in transfectants by immunoblotting, about half of the protein is found in the cytosol (Fig. 1*B*). Thus a portion of the γ_1 protein remains unassociated with membranes because it is not isoprenylated. Earlier work has shown that a portion of the native γ subunit in photoreceptor cells is not farnesylated, lacks the last four amino acids, and is incapable of activating the α subunit (9). The presence of two forms of the γ_1 subunit in transfectants of QT6, an avian fibroblast cell line, implies that the mechanism that generates two species may be general and not peculiar to photoreceptor cells. The results also show that the unfarnesylated species is capable of associating with the appropriate β subunit.

The β_1 protein translated *in vitro* has been shown to be capable of association in rabbit reticulocyte lysate with γ_1 and γ_2 proteins that were similarly translated (23) but there has been no evidence so far for specificity of association between β and γ subunit types. The $\beta\gamma$ complex associated with transducin (G_t), the G protein in mammalian rod outer segments, contains only the γ_1 and β_1 proteins. Since β_1 and γ_1 are the only β and γ subtypes expressed in the rod photoreceptors, it has not been clear whether complexes of specific subtypes such as $\beta_1\gamma_1$ occur because of differential expression in certain cell types or whether different members of the β and γ subunit families possess differential affinities for each other. Since there is now evidence that the β and γ subunits are families of proteins with distinct structures, like the α subunits, the ability of one subunit type to selectively bind one or more members of another subunit family implies that the formation of certain G-protein heterotrimers is favored over others even in situations where several members of each subunit family are expressed in the same cell. The behavior of the γ_1 protein indicates that selective binding of certain subunit types could be a mechanism by which the nature of G proteins, and thus the nature of signaling paths inside a cell, could be determined.

Although the β and γ subunits of purified G proteins are known to be bound to each other very tightly, little information exists regarding the specific regions of the two subunits that are involved in intersubunit interaction. Crosslinking studies have indicated that the cysteine at position 25 of the β_1 subunit interacts with cysteines at positions 35 and 36 in γ_1 (24).

When the amino acid sequences of the different β subunits are compared, the NH_2-terminal 40 amino acids in the β subunits are more divergent than the remaining portions of these proteins (25). It is reasonable to assume that in such closely related proteins the domains involved in intersubunit interaction are in the same regions of the different subtypes. A simple model would invoke a role for this NH_2-terminal portion of different β subunits in providing specificity of interaction between β and γ subunits. The NH_2-terminal 56 amino acids of β_1 were therefore replaced with the same portion of β_2. This β subunit chimera, β_2N56/β_1, associates with both γ_1 and γ_2. The ability of β_2N56/β_1 to bind γ_1 and γ_2 equally well implies that the portion of β_2 that helps discriminate between γ_1 and γ_2 is not in the NH_2-terminal portion of the protein.

Further analysis of chimeric molecules of β and γ subunits combined with mutagenesis will help identify the specific amino acids involved in interaction between the subunits.

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