Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 46-53

MEMBRANE ATTACHMENT OF RECOMBINANT G-PROTEIN α-SUBUNITS IN EXCESS OF βγ SUBUNITS IN A EUKARYOTIC EXPRESSION SYSTEM

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Received August 11, 1989

Summary Recombinant cDNAs encoding the α-subunits of G_{i1}, G_{i2}, G_{i3}, G_o and G_s were transfected into COS cells with the pCD-PS mammalian expression vector. Expression of each G_α was verified using subtype-specific peptide antisera on immunoblots. Quantitative immunoblotting of α and β subunits indicated: i) that there was no change in expression of endogenous β subunits, and ii) overexpression of α subunits could achieve a ratio of α:β > 25:1. Despite the excess of α over β, the G_α subunits were found predominantly in the membrane fraction. The results demonstrate that G_α subunits can attach to the membrane independently of βγ subunits.

A family of homologous guanine nucleotide binding proteins (G-proteins) has been identified which upon interaction with activated hormone or sensory receptors transmit information across the membrane to specific effector proteins in the course of a GTP-dependent activation-deactivation cycle (1-3). G-proteins are heterotrimeric in structure with $\alpha_1\beta_1\gamma_1$ subunit composition. Upon receptor activation the α subunit exchanges bound GDP for GTP, and the βγ complex dissociates, liberating the activated α-subunit to modulate target enzymes or ion channels.

G-proteins are positioned at the cytoplasmic face of the cell membrane but the mechanism of their attachment to the membrane is poorly understood. The deduced primary structures of G_α subunits lack significant hydrophobic domains (4). Highly purified G_α proteins can be isolated in the absence of detergent (5,6) and soluble, bioactive recombinant G_α proteins can be readily generated in prokaryotic expression systems (7,8). In contrast, βγ complexes purified from brain require detergent for solubility (5). The model of βγ as a hydrophobic anchor for G_α in

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the membrane was strengthened by the experiments of Sternweis showing that the stable incorporation of purified G_α into phospholipid vesicles required $\beta\gamma$ (5).

Several G_α species have been recently shown to be acylated by amide linkage with myristic acid (9,10), a modification which may facilitate the membrane attachment of certain proteins (11). The relative role of myristylation, $\beta\gamma$ binding or yet unrecognized factors in the membrane localization of G_α is unresolved. We describe here the use of COS cells for the transient overexpression of recombinant G_α protein. The newly expressed G_α species properly localized to the cell membrane fraction in spite of a relative deficiency of $\beta\gamma$ demonstrable by immunoblotting. The results point to factors other than or in addition to $\beta\gamma$ that account for G_α membrane localization.

Materials and Methods

Preparation of G_α cDNA expression plasmids. Rat olfactory cDNAs corresponding to the *alpha*-subunits of G_s, G_{i1}, G_{i2}, G_{i3}, and G_o (12) were kindly provided by Dr. Randall R. Reed (Johns Hopkins University). The pCD-PS plasmid (13) derived from the Okayama-Berg expression vector (14) was used for transfection of G_α cDNAs. Inserts in the pCD-PS polylinker site for expression of $G_s\alpha$ and $G_{i1}\alpha$ represented the entire cDNA clone as described (12). Restriction fragments representing the EcoR1-Xbal1/Spe1 portion of $G_{i2}\alpha$ cDNA, the EcoR1-BamH1 portion of the $G_{i3}\alpha$ clone and the EcoRV-EcoR1 portion of $G_o\alpha$ cDNA were used for the corresponding pCD-PS constructs.

Cell transfection. The calcium phosphate precipitation method of Chen and Okayama (15) was used for transfection of COS-7 cells as previously described (16). Transfected cells from four replicate 10 cm culture plates were combined, harvested and washed in PBS by centifugation and stored as pastes at -70° prior to fractionation and analysis.

Cell fractionation. Cell pastes were thawed and lysed in 2 ml of 20 mM Tris-HCl pH 7.5 on ice by trituration through an 18 g stainless steel needle. A crude membrane pellet was prepared by centrifugation at 31,000 X g for 30 min in an SS-34 rotor at 4°. The supernatant fraction (~ 2 ml) was transferred to an ultracentifuge tube and recentrifuged in a Beckman 70.1 Ti rotor at 165,000 X g for 60 min at 4°. The upper ~1.5 ml of high-speed supernatant was carefully removed and used as a cytosolic fraction for subsequent analysis by SDS-PAGE and immunoblotting. The crude membrane pellet from the first centrifugation was resuspended in 1 ml Tris buffer for use in blotting studies. Protein determinations were by the method of Bradford (17) using BSA as the standard.

Antisera and antibodies. Preparation and character-ization of antisera to synthetic peptides derived from G_α or $G\beta$ sequence including AS/7 (18), LD/1, LE/3, SQ/2, MS/1, GA/1 (19) and GO/1 (20) have been previously described (see references and text for description of respective specificities). Generation of antiserum RM to the $G_s\alpha$ carboxyl-terminal decapeptide RMHLRQYELL, and RM

antibody affinity purification on a peptide support followed previously established procedures (18-20) and is described elsewhere (21).

SDS-PAGE and immunoblotting were by the methods previously described (22,23).

Results and Discussion

Transfection of COS cells with the pCD-PS expression vector containing G_α cDNA inserts resulted in the transient expression of the corresponding protein after 72 to 96 hours (Figs. 1 and 2). The newly expressed G_α subunits could be identified on immunoblots of transfected COS membrane proteins by the appearance of novel bands or an increment in signal at the position of endogenous G_α bands using subtype-specific peptide antisera (18-20).

Transfection of cells with G_{i1} cDNA resulted in an increment in AS/7 reactivity at M_r 41 kDa and the appearance of reactivity to LD/1, a G_{i1}-specific antiserum (Fig. 1). An increment in AS/7 reactivity at M_r40 kDa and the appearance of a strong signal with LE/3, an antiserum generated against an internal peptide sequence specific for G_{i2}, documented the transient expression of G_{i2} in cells transfected with this cDNA. G_{i3} transfection resulted in the appearance of strong SQ reactivity in the 41 kDa band (Fig. 1). SQ antiserum was developed against a G_{i3}-specific internal sequence, and identifies the M_r 41 kD G_α in HL-60 cells (19).

Expression of $G_o\alpha$ was manifest by the appearance of a novel M_r 39 kDa species on immunoblots which reacted with GO/1 antiserum (20)(Fig. 1). Transfection with a cloned cDNA

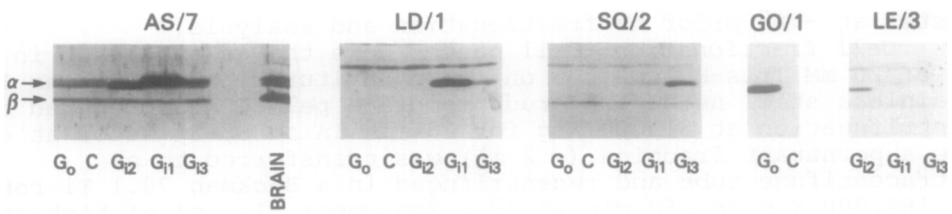

Fig. 1. Immunoblotting of G_α proteins expressed in COS cells. Samples of the membrane fraction from control or G_α-transfected COS cells containing 50 µg protein were analyzed by immunoblotting with the G_α-peptide antiserum indicated above each panel (final dilution 1:100; see text for description of antisera specificity). Below each lane the G_α-cDNA pCD-PS insert used for COS transfection is indicated (C, sham-transfected control). In the leftmost panel a standard lane containing 50 µg of protein from a 1% cholate extract of bovine brain membranes is included. The β-peptide antiserum MS/1 (19) was included in the AS/7 immunoblot (1:250 dilution) for reference. Positions of α and β subunits indicated in margin at left.

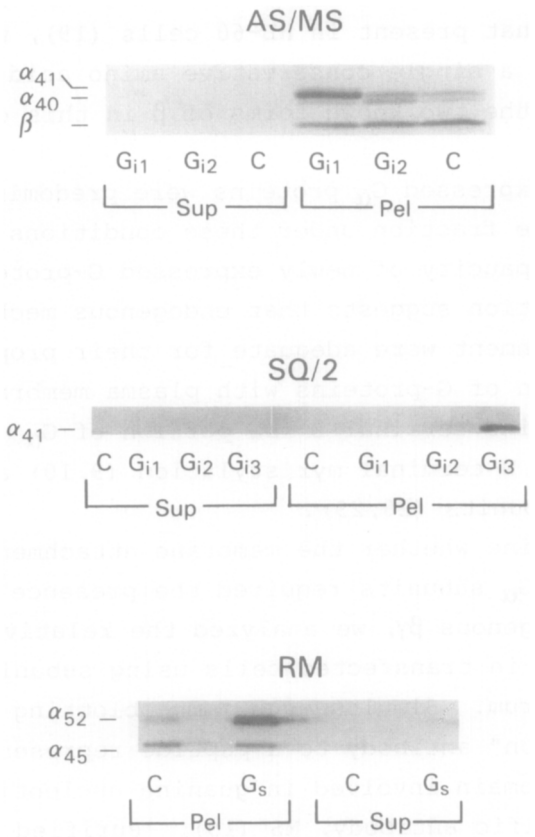

Fig. 2. Subcellular distribution of transiently expressed G_α proteins detected by immunoblotting. Samples of the high-speed supernatant (Sup) or crude membrane pellet (Pel) of COS cell lysates were analyzed by immunoblotting with the antisera or affinity-purified antibody indicated above each panel (AS/7, SQ/2 antisera at 1:100, MS/1 antiserum at 1:250 and RM affinity-purified antibody at 10 µg/ml final dilution; see text for description of antibody specificities). Below each lane the G_α-cDNA employed for transfection is shown (C, sham-transfected control). Positions of α (and in top panel, β) subunits shown at left with subscripts indicating approximate M_r in kD. SDS-PAGE in top panel performed with a low concentration of bis-acrylamide to improve resolution of M_r 40 and 41 kD species (19). The amount of protein applied per lane was 35 and 25 µg for supernatant and pellets respectively in the top panel, 50 µg for both fractions in the middle panel and 25 µg for all lanes in the bottom panel.

corresponding to the larger form of $G_s\alpha$ (12) resulted in the intensification of the M_r 52 kDa band reactive with the $G_s\alpha$ C-terminal antibody RM (21) , and reversal of the pattern of RM-reactivity in control cells in which the lower 45 kDa form predominates (see Fig. 2).

The complement of $\beta\gamma$ subunit remained unchanged by G_α cDNA transfection, as assessed by immunoblotting with an antibody to the amino-terminal decapeptide of β_{36}, MS (leftmost panel of Fig.

1; see also Fig. 2, upper panel). This antibody also recognizes β_{35}, such as that present in HL-60 cells (19), which would be expected since a single conservative amino acid substitution distinguishes the two known forms of β in this decapeptide region (24,25).

The overexpressed G_α proteins were predominantly localized to the membrane fraction under these conditions of transfection (Fig. 2). The paucity of newly expressed G-protein in the cytosolic fraction suggests that endogenous mechanisms for membrane attachment were adequate for their proper insertion. The association of G-proteins with plasma membranes is known to involve the amino-terminal 2 kDa portion of G_α (26,27), possibly as a result of N-terminal myristylation (9,10) and/or interaction with the $\beta\gamma$ subunits (28,29).

To determine whether the membrane attachment of the overexpressed G_α subunits required the presence of an equivalent amount of endogenous $\beta\gamma$, we analyzed the relative abundance of α and β subunits in transfected cells using subunit-specific antipeptide serum. Simultaneous immunoblotting was performed with a "G-common" antibody to a peptide representing a highly conserved G_α domain involved in guanine nucleotide binding, GA, and the β-specific antibody, MS (19). Purified holotransducin was used as a quantitative standard with which to compare the endogenous COS β and the transfected $G_{i3}\alpha$ immunoreactivities. GA antibody was generated to a G-α peptide sequence identical in transducin and G_{i3}, and should therefore react with the two α-subunits equipotently. While transducin β-subunits are exclusively β_{36}, the MS antibody is equally reactive with the β_{35} species found in conjunction with β_{36} in COS cells. The use of non-selective G_α and G_β antibodies allowed quantitative comparison of the total α:β ratio in G_α-transfected membranes with controls. Because of its higher titer, the MS antibody was deliberately diluted so as to give approximately equal strength of β signal with the α signal produced by a 1:100 dilution of GA antibody when tested on immunoblots against the subunits of holotransducin which include α and β in a ratio of 1:1(compare the β signal in Fig. 3 with that in the leftmost panel of Fig. 1, or the upper panel of Fig. 2). Immunoblots of COS membranes under conditions in which the intensity of transducin subunit staining was linear with added holotransducin showed that in control COS cells the α:β ratio was close to one since the intensities of α and β labeling were comparable, but demonstrated a large increase

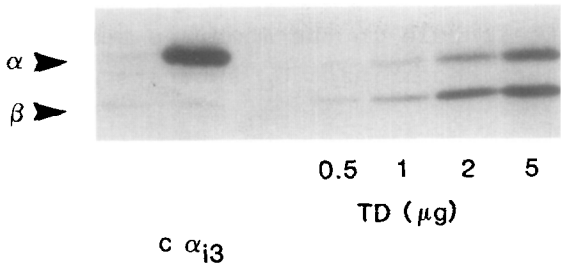

Fig. 3. Excess of G_α over $\beta\gamma$ in transfected COS cells estimated by relative immunoreactivity of G-protein subunits in G_α-transfects and holotransducin standards. Samples of COS membranes (50 μg protein) from control (C) or $G_{i3}\alpha$ (α_{i3}) transfected cells and increasing amounts of bovine holotransducin (amount in μg shown below each lane) were applied to the same gel and analyzed by immunoblotting with the G-common antiserum, GA (19), at 1:100 and the β antiserum MS/1 at 1:2000 final dilutions. Positions of α and β subunits indicated at left.

in the ratio of α to β subunit upon $G_{i3}\alpha$ transfection, estimated by densitometry to exceed 25-fold.

The above results demonstrate the successful expression in COS cells of a variety of genetically defined G-proteins, including G_s, G_{i1}, G_{i2}, G_{i3} and G_o. The pattern of immunoreactivity with specific peptide antisera and M_r on SDS-PAGE correlate well with results of *in vitro* translation of the corresponding mRNAs previously reported (19). The three transfected $G_i\alpha$ subunits demonstrate biologic viability and functional interaction with $\beta\gamma$ as assessed by pertussis toxin labeling (data not shown). Transfection of COS (30) and *cyc*$^-$ S49 lymphoma cells (31) with $G_s\alpha$ cDNA has been reported by others.

The expressed G_α proteins are processed and preferentially targeted to the cell membrane, rather than the cytosolic fraction. The data furthermore show that G_α subunits remain membrane associated even when expressed to a level greatly exceeding the amount of endogenous $\beta\gamma$. This argues against a model of G_α membrane attachment requiring stoichiometric amounts of $\beta\gamma$ subunit (5). This model was based on the observed inability of purified G_α subunits to attach to phospholipid vesicles independently of $\beta\gamma$ (5). These results, however, may reflect alterations in G_α occurring during purification and/or differences between native membranes and artificial vesicles in binding of G_α. Our data point to the potential role of factors in addition to $\beta\gamma$, such as G_α myristylation (9,10), in the proper membrane attachment and functioning of signal transducing G-proteins. The transient eukaryotic expression system described here should prove useful for analysis of myristylation and $\beta\gamma$

interaction and their role in the membrane targeting and function of normal and mutant recombinant G_α subunits.

Acknowledgments We thank Drs. Randall Reed and David Jones for providing the rat G_α cDNAs, Dr.Celia Unson for peptide synthesis, Dr. Paul Goldsmith for the generation and characterization of antisera, Dr. Tom Bonner and Alice Young for advice and help with construction of pCD-PS and G_α cDNA inserts, and Charles Woodard and Ruth Vinitsky for expert technical assistance.

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