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Association of the γ_{12} subunit of G proteins with actin filaments

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SUMMARY

Recent studies have suggested an association between heterotrimeric G proteins, which play a major role in transmembrane signal transduction, and intracellular components. We therefore examined the subcellular localization of isoforms of G protein γ subunits in Swiss 3T3 and C6 glioma cells, mainly containing the γ_5 and γ_{12} subunits. Immunocytochemical double staining with phalloidin showed co-localization of the γ_{12} subunit with actin filaments (F-actin), while the γ_5 co-localized with vinculin, suggesting an association with focal adhesion. Pretreatment of cells with Triton X-100 eliminated the γ_5 but not the γ_{12} staining. Co-localization of γ_{12} and F-actin was preserved when F-actin was disorganized with cytochalasin D or reorganized using fetal calf serum. Large amounts of γ_{12} were recovered in the vimentin- and tubulin-free F-actin-rich fraction prepared from crude cytoskeleton preparations by double depolymerization-repolymerization. Co-localization of $G_{i2}\alpha$, β and γ_{12} in the F-actin-rich fraction suggested the existence of γ_{12} as a βγ or heterotrimeric complex. Furthermore, purified $\beta\gamma_{12}$ was found to associate with F-actin in vitro more tightly than $\beta\gamma_5$. These results strongly suggest that the γ_{12} subunit associates with F-actin in cells. The observed differential distribution of γ_{12} and γ_5 implies functional differences for the two γ subunits.

Key words: G protein γ subunit, Actin filament, Focal adhesion

INTRODUCTION

Heterotrimeric G proteins, which are involved in signal transduction from cell surface receptors to cellular effectors (Neer, 1995), are composed of α, β and γ subunits, the latter two being tightly associated under physiological conditions. Upon receptor stimulation, the α subunits dissociate and directly act on various effectors in the plasma membranes. Recent studies have indicated that the remaining βγ complexes also play roles in regulating cellular activity, for example of the K^+ channel, phospholipase C-β, phospholipase A_2, receptor kinases and phosphoinositide kinases (Iñiguez-Lluhi et al., 1993; Neer, 1995).

Analysis of purified proteins and cloned cDNAs has revealed the existence of multiple forms of β and γ subunits in addition to many α subunit isoforms. At the amino acid level, the five mammalian β subunits exhibit strong conservation (Iñiguez-Lluhi et al., 1993; Neer, 1995), while the mammalian γ subunits show considerable divergence. At present, the γ subunit family consists of at least eleven members, γ_1 (Hurley et al., 1984; Yatsunami et al., 1985), γ_2 (Gautam et al., 1989; Robishaw et al., 1989), γ_3 (Gautam et al., 1990), γ_4 (Ray et al., 1995), γ_5 (Fisher and Aronson, 1992), γ_7 (Cali et al., 1992), two forms of γ_8 (Ryba and Tirindelli, 1995; Ong et al., 1995), γ_{10}, γ_{11} (Ray et al., 1995) and γ_{12} (Morishita et al., 1995). Functional differences among the various species of βγ complexes have been attributed to the γ rather than the β subunit (Iñiguez-Lluhi et al., 1992; Asano et al., 1993; Kisselev and Gautam, 1993; Ueda et al., 1994). Most previous results, however, have indicated that while the biological properties of $\beta\gamma_1$, the βγ complex of transducin, are appreciably different from those of the other βγ complexes, there is otherwise no pronounced variation.

The transcripts for β_1 through β_4 are expressed ubiquitously, while that for the β_5 subunit is expressed only in the brain (Iñiguez-Lluhi et al., 1993; Neer, 1995). In contrast, the mammalian γ subunits show more variation in their tissue-specific distribution. The γ_1 and one of the γ8 forms are specifically expressed in retinal rods and cones, respectively (Hurley et al., 1984; Yatsunami et al., 1985; Ong et al., 1995). The other γ_8 form is expressed only in olfactory and vomeronasal neuroepithelia (Ryba and Tirindelli, 1995), while γ_3 is localized only in the brain (Gautam et al., 1990; Cali et al., 1992; Morishita et al., 1992; Asano et al., 1995). The γ_2, γ_4, γ_5, γ_7, γ_{10}, γ_{11} and γ_{12} forms, however, are distributed in a variety of tissues (Gautam et al., 1990; Cali et al., 1992; Morishita et al., 1992, 1995; Asano et al., 1995; Ray et al., 1995). Analysis of various lines of cloned cells has shown the existence of multiple isoforms of γ subunit in a single cell (Asano et al., 1995; Morishita et al., 1995).

Several reports suggest that G proteins are associated not only with the plasma membrane, but also with intracellular components, in a variety of cells, though the physiological significance of this remains to be elucidated. Pertussis toxin-sensitive G proteins were detected on intracellular membranes such as those secretory granules (Toutant et al., 1987), endo-

plasmic reticulum (Audigier et al., 1988) and Golgi cisternae (Ercolani et al., 1990; Stow et al., 1991). In contrast, certain G protein subunits were suggested to be associated with the cytoskeleton. The $G_{i2}\alpha$, $G_s\alpha$ and $\beta\gamma$ complexes, for example, were found in Triton X-100-insoluble fractions of neutrophils (Särdahl et al., 1993) and S49 lymphoma cells (Carlson et al., 1986). Immunocytochemical studies have shown the $G_q\alpha/G_{11}\alpha$ subunits to co-localize with actin filaments (F-actin) in WRK_1 cells (Ibarrondo et al., 1995), and that the $\beta\gamma$ complex coexists with cytokeratin filaments in starfish oocytes (Chiba et al., 1995).

With respect to specific γ subunit isoforms, γ_5 has been localized to areas of focal adhesion in neonatal cardiac fibroblasts (Hansen et al., 1994), but data for other isoforms are limited. In the present study, we examined whether various isoforms of γ subunit were differentially localized in the cell. We found that large amounts of γ_{12} in Triton X-100-insoluble fractions of Swiss 3T3 cells and C6 glioma cells, and immunocytochemical studies indicated an association with F-actin. In contrast, γ_5 was solubilized by Triton X-100 treatment, suggesting a membrane link.

MATERIALS AND METHODS

Cell culture

Swiss 3T3 cells and C6 glioma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2, 95% air. For subcellular fractionation, cells were grown on 10 cm diameter dishes to subconfluency (80-90%). For FCS stimulation, cells were made quiescent by growing them to confluence and then maintaining them in 0.5% FCS in DMEM for 48 hours.

Antibodies against γ subunits

The antibodies against the γ subunits of G proteins have been previously described and characterized (Asano et al., 1995; Morishita et al., 1995). Briefly, peptides with sequences unique to each of the γ subunits were synthesized, conjugated to keyhole limpet hemocyanin and injected into rabbits. The antibodies were purified from antisera using antigen-coupled agarose columns.

Purified subunits of G proteins

Four forms of the $\beta\gamma$ complex, containing γ_2, γ_3, γ_7 and γ_{12} (designated $\beta\gamma_2$, $\beta\gamma_3$, $\beta\gamma_7$, and $\beta\gamma_{12}$, respectively) as well as mainly the β_1 subunit were purified from bovine brain and spleen (Asano et al., 1993). The $\beta\gamma_5$ complex was purified from bovine lung by a similar method and demonstrated to contain at least β_1 and β_2 in addition to γ_5 by immunoblotting and analysis of amino acid sequences (data not shown). $G_{i2}\alpha$ was purified from bovine lung (Morishita et al., 1988).

Immunocytochemistry

All steps were performed at room temperature. Swiss 3T3 and C6 glioma cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes, subsequently permeabilized with 0.2% Triton X-100 for 2 minutes, and blocked for nonspecific binding of antibody by treatment with 10% goat serum in Tris-buffered saline (TBS) for 1 hour. The cells were then incubated with primary antibody in TBS containing 1% goat serum for 1 hour, washed with TBS, and incubated with secondary antibody or tetramethylrhodamine isothiocyanate (TRITC)-phalloidin in TBS containing 1% goat serum for 1 hour. Antibodies against γ subunit were applied at 10 μg/ml and the monoclonal antibody against vinculin at 1:100 dilution. Phalloidin was used according to the manufacturer's instructions. The secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100) and TRITC-conjugated goat anti-mouse IgG (1:100). All samples were examined with a confocal laser scanning microscope (MRC-1024; Bio-Rad Laboratories, Hercules, CA).

Fractionation of cultured cells

Triton X-100-soluble and -insoluble fractions were prepared from Swiss 3T3 and C6 glioma cells essentially as described previously (Payrastre et al., 1991). Briefly, cells were washed with PBS, incubated for 10 minutes at room temperature with 2 mM EDTA in PBS, centrifuged and washed once with PBS. The washed cells (1×10^7 cells/ml) were incubated for 5 minutes at 0°C with 0.5% Triton X-100 in 20 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 2 μg/ml trypsin inhibitor and centrifuged at 12,000 ***g*** for 3 minutes. The supernatant was used as the Triton X-100-soluble fraction. The pellet was washed once with the same buffer and used as the Triton X-100-insoluble fraction. Polymerized actin and actin-binding protein-rich fractions were prepared as described previously (Payrastre et al., 1991). The Triton X-100-insoluble fraction obtained from Swiss 3T3 cells was suspended and incubated with depolymerizing buffer containing 0.1 M Pipes, pH 6.5, 0.6 M KI, 0.1 M KCl, 10 μg/ml leupeptin and 0.5 mM PMSF for 20 minutes at 4°C, and then centrifuged at 40,000 ***g*** for 20 minutes at 4°C. Soluble depolymerized material in the supernatant was repolymerized by dialysis two times for 3 hours at 4°C against 1 litre of a buffer containing 10 mM Pipes, pH 6.8, 0.5 mM EGTA, 2 mM $MgCl_2$ and 0.5 mM PMSF. The polymerized actin was recovered by centrifugation (12,000 ***g*** for 5 minutes at 4°C) and submitted to a second depolymerization-repolymerization cycle. The final polymerized actin fraction is referred to below as the F-actin-rich fraction.

Assay for association of various βγ complexes with F-actin in vitro

Association of $\beta\gamma$ complexes with F-actin in vitro was examined by the high-speed-centrifugation method described by Sobue et al. (1981). F-actin was prepared from 1 mg/ml globular actin (G-actin) by polymerization in polymerizing buffer containing 5 mM Tris-HCl, pH 7.5, 0.2 mM dithiothreitol, 0.2 mM ATP, 0.1 M KCl and 2 mM $MgCl_2$ at 25°C for 1 hour. F-actin (45 μg) and various amounts of $\beta\gamma$ complexes were incubated for 30 minutes at 30°C in polymerizing buffer containing 0.3% Triton X-100. The samples were centrifuged at 200,000 ***g*** for 20 minutes at 4°C and the sedimented proteins resuspended in the same buffer before again being centrifuged at 200,000 ***g*** for 20 minutes. The sediments thus obtained were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against γ subunits.

Immunoblotting

Tricine/SDS-PAGE was performed by the method of Schägger and von Jagow (1987), and SDS-PAGE (10% acrylamide) based on the method of Laemmli (1970). Immunoblotting was carried out as described previously (Asano et al., 1995) employing diaminobenzidine or a chemiluminescence reagent (Renaissance; DuPont NEN, Boston, MA). Proteins were quantitated with a Micro BCA protein assay kit (Pierce, Rockford, IL) using BSA as the standard.

Materials

Actin from rabbit muscle was obtained from Nacalai Tesque (Kyoto, Japan). Antibodies against $G_{i2}\alpha$ and β subunit of G proteins were previously generated with individual proteins and the antibody against β mainly reacted with β_1 (Morishita et al., 1988). The antibody against $G_q\alpha/G_{11}\alpha$ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against vinculin and the TRITC-conjugated goat anti-mouse IgG were from Sigma (St Louis, MO). TRITC-phalloidin was purchased from Molecular Probes (Eugene, OR).

FITC-conjugated goat anti-rabbit IgG was from Biosource International (Camarillo, CA).

RESULTS

Immunocytochemical localization of γ_{12} and γ_5 in Swiss 3T3 and C6 cells

Swiss 3T3 and C6 cells contain γ_5 and γ_{12} as major γ subunits, but do not contain detectable amounts of γ_2, γ_3 and γ_7 (Asano et al., 1995; Morishita et al., 1995). We examined the intracellular localization of γ_{12} and γ_5 in these cell lines by immunocytochemical methods. In Swiss 3T3 cells, the staining of γ_{12} was found associated with intracellular structures resembling stress fibers (Fig. 1A). This immunostaining pattern showed complete overlap with that of phalloidin which stains F-actin specifically, clearly indicating that γ_{12} co-localizes with actin fibers (Fig. 1B). The staining pattern of γ_5 essentially showed complete overlap with that of vinculin (Fig. 1D,E), indicating correspondence to focal adhesion plaques as reported previously (Hansen et al., 1994). In addition, γ_5 staining was often detected in the end portions of stress fibers terminating in focal adhesions, unlike vinculin staining (Fig. 1D), as reported previously (Hansen et al., 1994). The specificity of the immunostaining patterns of these γ subunits was examined by incubating both antibodies with the respective peptides used for their generation. As shown in Fig. 1C and F, preincubation with respective peptides completely abolished the immunostaining. However, these treatments did not affect the staining of F-actin and vinculin (data not shown). The data thus indicated that antibodies against γ_{12} and γ_5 specifically recognize proteins containing peptide sequences unique to γ_5 and γ_{12}, respectively, in the cells .

In C6 glioma cells, essentially the same staining patterns were observed (Fig. 2). The γ_{12} subunit co-localized with F-actin, though actin stress fibers were found to be relatively poorly developed (Fig. 2A,B). The γ_5 subunit associated with focal adhesions as observed in Swiss 3T3 cells (Fig. 2D,E). Fluorescence of nuclear components was also noted but this appeared to be non-specific, because this was not affected by preabsorbing the antibody with the antigen peptide, in contrast to the areas of focal adhesion (Fig. 2D,F).

To further investigate the association of γ_{12} with actin fibers, cell proteins were extracted with 0.5% Triton X-100 before paraformaldehyde fixation. Triton X-100 treatment did not affect the association of γ_{12} with F-actin in Swiss 3T3 cells (Fig. 3A,B, compared with control cells shown in Fig. 1A,B). In contrast, the immunostaining of γ_5 was completely eliminated by Triton X-100 treatment (Fig. 3C). The staining of vinculin, however, was not affected (Fig. 3D). These results suggested a cytoskeletal association of γ_{12} and a membrane localization for γ_5. An absence of interaction between γ_{12} and

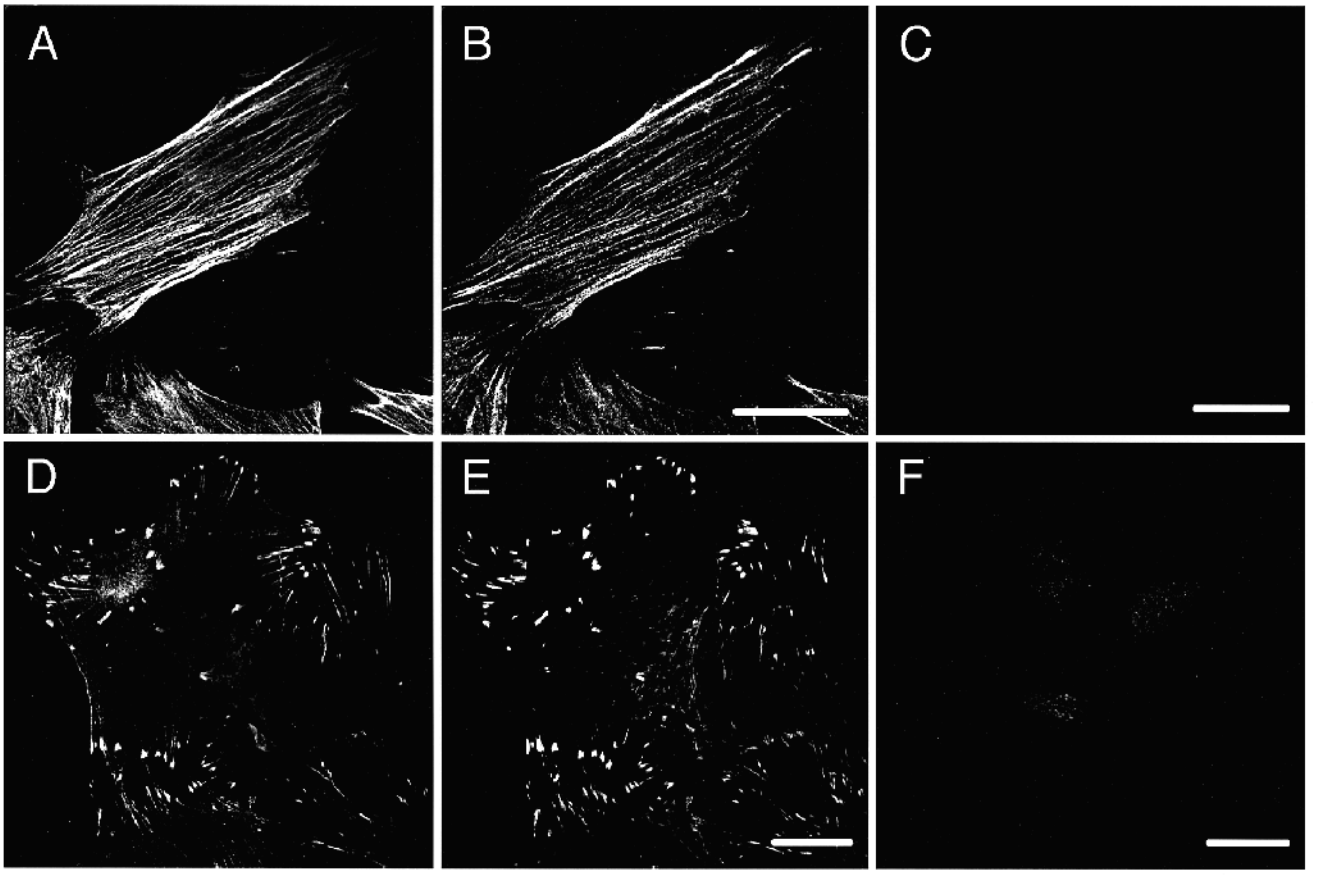

Fig. 1. Localization of γ_{12} and γ_5 in Swiss 3T3 cells. Swiss 3T3 cells were double stained for γ_{12} (A) and phalloidin (B), or for γ_5 (D) and vinculin (E). The complete overlap of γ_{12} staining with actin fibers detected using phalloidin indicated co-localization with F-actin. Preincubation of antibodies against γ_{12} and γ_5 with the respective peptides used for antigens completely abolished the γ_{12} (C) and γ_5 staining (F). Bars, 50 μm.

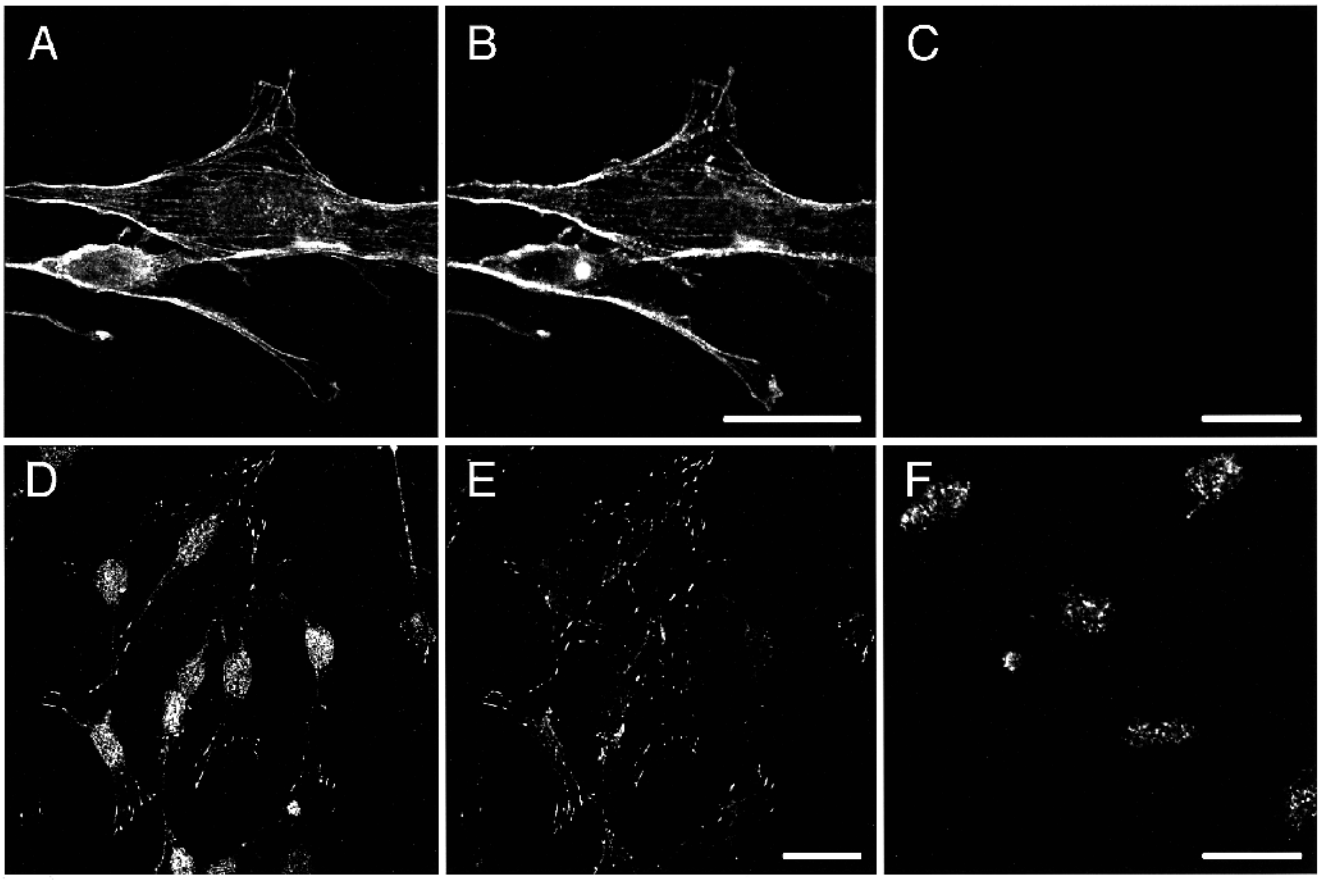

Fig. 2. Localization of γ_{12} and γ_5 in C6 glioma cells. C6 cells were double stained for γ_{12} (A) and phalloidin (B), or for γ_5 (D) and vinculin (E). Co-localization of γ_{12} with F-actin in these cells indicated that the association of γ_{12} with F-actin is independent of cell type. Preincubation of the antibody against γ_{12} with the antigen peptide completely abolished the γ_{12} staining (C), while preabsorbing the antibody against γ_5 with the antigen peptide eliminated the γ_5 staining except that in the nuclei (F), which thus appeared to be non-specific. Bars, 40 μm.

G-actin could be concluded from Fig. 3A, because cytosolic proteins are washed out with Triton X-100 treatment. This was also supported by the finding that the cytoplasmic fraction of Swiss 3T3 cells, which contains mostly G-actin, did not include a significant amount of γ_{12} (data not shown).

To confirm the specificity of the staining in the cells with the antibody against γ_{12}, the cell lysate and Triton X-100-insoluble fractions were analyzed by immunoblotting. The antibody against γ_{12} recognized a major γ_{12} protein band and two extra bands in whole cell lysates of both Swiss 3T3 and C6 cells (Fig. 4). However, these two bands disappeared in the Triton X-100-insoluble fractions, indicating that the immunostaining associated with F-actin could indeed be attributed to recognition of γ_{12}. In addition, the antibody against γ_{12} did not cross-react with actin molecules (data not shown).

Changes of the distribution of γ_{12} accompanying reorganization or disorganization of F-actin

It is well known that actin stress fibers are rapidly constructed when quiescent cells are activated by growth factors (Ridley and Hall, 1992). We therefore studied the distribution of γ_{12} in quiescent cells and after stimulation with FCS. In quiescent cells, F-actin was found to be dispersed throughout the cytoplasm with detectable organization near the plasma membrane (Fig. 5B). In these cells, the immunostaining pattern of γ_{12} overlapped the actin pattern (Fig. 5A). Stimulation with 20% FCS induced rapid reorganization of actin into stress fibers (Fig. 5D), again with γ_{12} co-localization (Fig. 5C). The time course of this rearrangement of γ_{12} upon FCS treatment paralleled that of actin (data not shown), with the formation of prominent stress fibers within 30 minutes.

When Swiss 3T3 cells were incubated with 1 μM cytochalasin D, the F-actin of the cells was degraded and some filaments became aggregated (Fig. 5F). However, the co-localization of γ_{12} with F-actin was well preserved (Fig. 5E).

Existence of the γ_{12} subunit in the Triton X-100-insoluble fraction of Swiss 3T3 and C6 cells

The subcellular localization of γ_{12} and γ_5 in Swiss 3T3 and C6 cells was examined by biochemical methods. After cell fractionation using Triton X-100, each fraction was analyzed by immunoblotting (Fig. 6). Incubation of cells with low concentrations of Triton X-100 solubilizes many membrane components, but poorly solubilizes cytoskeletal components. Large amounts of γ_{12} were present in the Triton X-100-insoluble as well as the Triton X-100-soluble fractions of both cell lines. In contrast, most γ_5 was observed in the Triton X-100-soluble fraction in the two cell lines (Fig. 6), consistent with the immunocytochemical results (Figs 1-3). The γ_{12} in the Triton X-100-insoluble fraction appears to correspond to the γ_{12} asso-

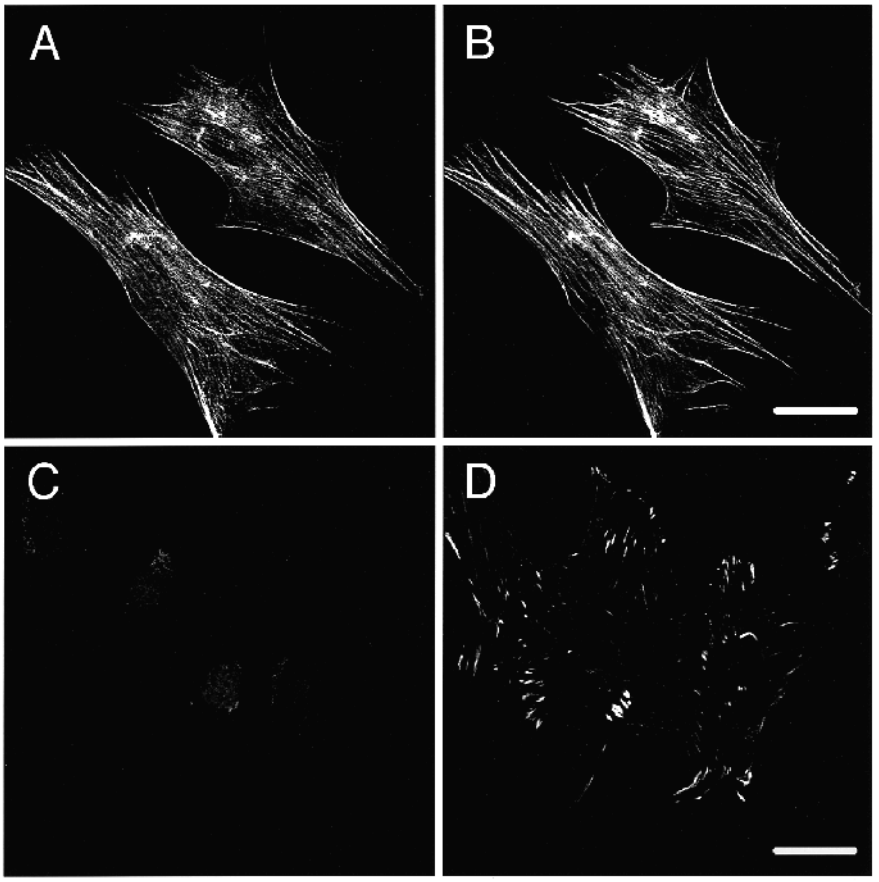

Fig. 3. Immunostaining of γ_{12} and γ_5 after Triton X-100 treatment of Swiss 3T3 cells. Before fixation, Swiss 3T3 cells were incubated on ice in PBS with 0.5% Triton X-100 for 10 minutes. The fixed cells were double stained for γ_{12} (A) and F-actin (B), or for γ_5 (C) and vinculin (D). Extraction of cells with Triton X-100 did not affect either γ_{12} or phalloidin immunofluorescence staining, but eliminated the γ_5 staining without solubilizing vinculin. Bars, 50 μm.

ciated with F-actin. The Triton X-100-soluble and -insoluble fractions were also established to contain β, $G_{i2}\alpha$ and $G_q\alpha/G_{11}\alpha$ subunits (Fig. 6), suggesting that γ_{12} is localized in cytoskeletal components as a βγ or heterotrimeric complex. The γ_{12} subunit from rat C6 glioma cells migrated slightly more slowly than the purified bovine $\beta\gamma_{12}$ or mouse Swiss 3T3 cell forms (Fig. 6), probably as a consequence of species-specific differences (Morishita et al., 1995).

Association of γ_{12} with F-actin-rich fraction

To confirm that the γ_{12} is indeed associated specifically with F-actin among the various cytoskeletal components, an F-actin-rich fraction lacking detectable amounts of vimentin and tubulin was prepared from the Triton X-100-insoluble fraction of Swiss 3T3 cells by a two-step polymerization-depolymerization procedure (Fig. 7). As expected, a large amount of γ_{12} was detected in the F-actin-rich fraction, along with β and $G_{i2}\alpha$ subunits (Fig. 7).

Association of $\beta\gamma_{12}$ complexes with F-actin in vitro

To study whether the $\beta\gamma_{12}$ complex directly and selectively binds to F-actin in vitro, we tested the association of purified βγ complexes containing different γ subunits with F-actin by the high-speed centrifugation method. Under the experimental conditions used, F-actin itself and F-actin associated proteins were precipitated and separated from the free βγ complexes that remained in the supernatant. The $\beta\gamma_{12}$ and $\beta\gamma_3$ complexes co-precipitated with F-actin in a dose-dependent manner (Fig. 8), indicating direct binding. However, the $\beta\gamma_2$, $\beta\gamma_5$ and $\beta\gamma_7$ complexes were scarcely co-precipitated with F-actin (Fig. 8). Negligible amounts of βγ complexes were precipitated when they were incubated without F-actin (data not shown). Because the purified βγ complexes used in this experiment mainly contain the β_1 subunit (Asano et al., 1993) except for the $\beta\gamma_5$

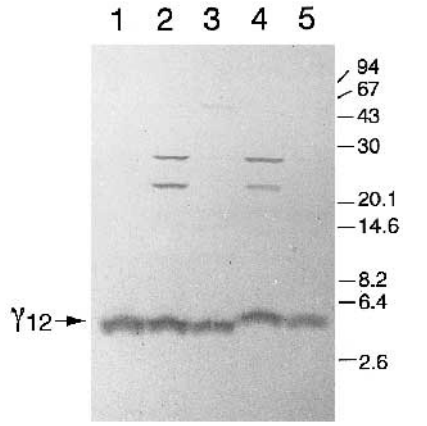

Fig. 4. Specificity of the antibody against γ_{12} in Swiss 3T3 and C6 cells. Cell lysates and Triton X-100-insoluble fractions of Swiss 3T3 and C6 cells (25 μg of protein) were subjected to Tricine/SDS-PAGE and immunoblotted with the antibody against γ_{12}. Lane 1, 5 ng of purified $\beta\gamma_{12}$ complex; lane 2, lysate of Swiss 3T3 cells; lane 3, Triton X-100-insoluble fraction of Swiss 3T3 cells; lane 4, lysate of C6 cells; lane 5, Triton X-100-insoluble fraction of C6 cells. Numbers on the right indicate molecular masses in kDa.

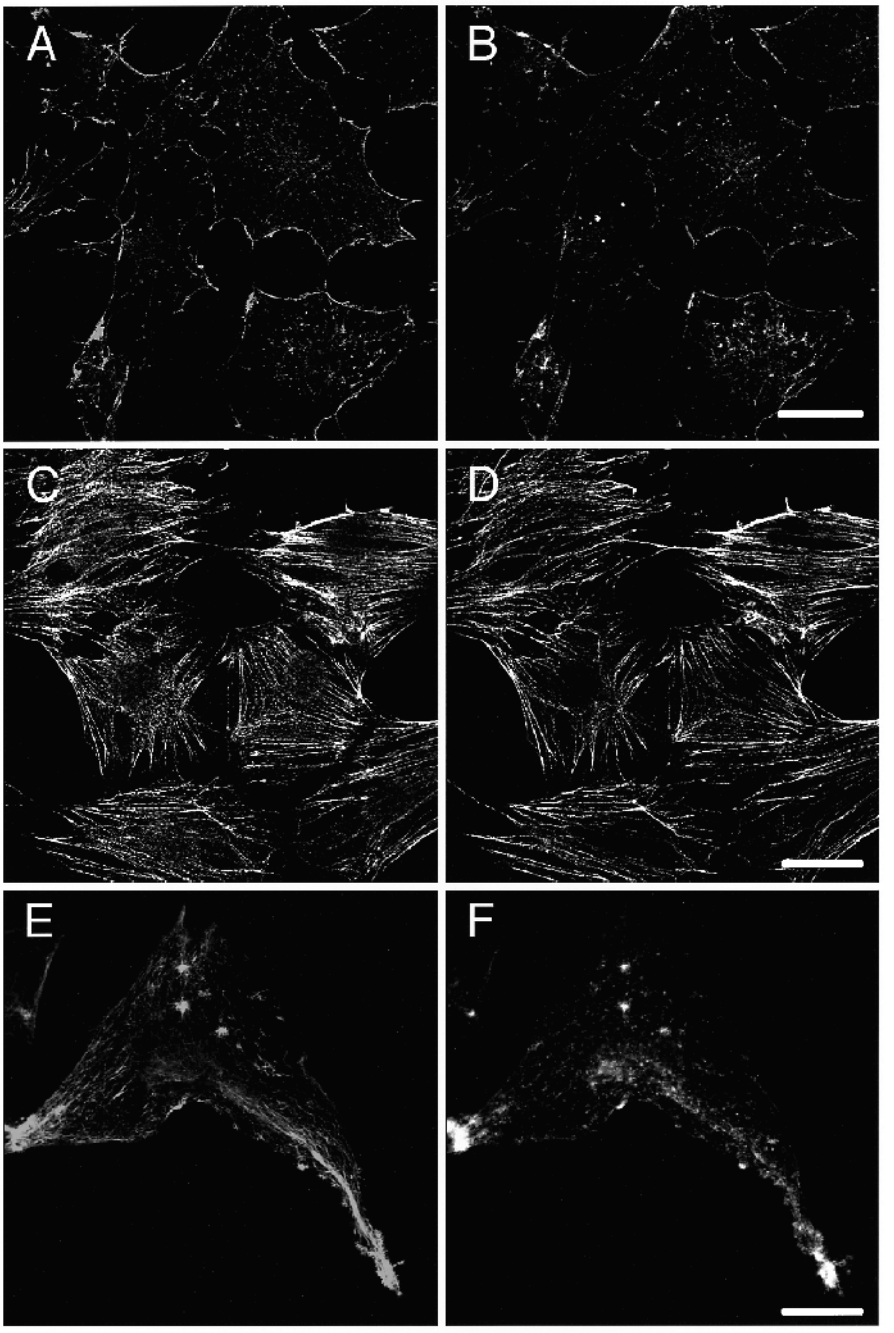

Fig. 5. Influence of FCS and cytochalasin D treatment on the localization of γ_{12} and F-actin in Swiss 3T3 cells. Quiescent Swiss 3T3 cells (A,B) were stimulated by 20% FCS for 30 minutes (C,D), and double stained for γ_{12} (A,C) and F-actin (B,D). Swiss 3T3 cells were treated with 1 μM cytochalasin D for 60 minutes and were double stained for γ_{12} (E) and F-actin (F). Under all conditions a complete overlap of the staining of γ_{12} and F-actin was observed. Bars, 50 μm.

complex, which has not been well characterized, the specific association with F-actin seems to be dependent on the subtype of γ subunit rather than on the β subunit type. These results are consistent with the observed subcellular distribution of γ_{12} and γ_5 in cultured cells.

DISCUSSION

It is generally accepted that heterotrimeric G proteins are associated with plasma membranes and mediate signals from cell-surface receptors to intracellular effectors. The α and γ subunits of G proteins are modified by lipids, and myristoylation and/or palmitoylation of α subunits and isoprenylation of γ subunits are important for membrane attachment (Iñiguez-Lluhi et al., 1993; Neer, 1995). However, the present study provides a body of experimental evidence indicating that the γ_{12} subunit is in fact associated with F-actin in Swiss 3T3 and C6 glioma cells. The argument for this are as follows: (1) the immunocytochemical study demonstrated γ_{12} to be co-localized with F-actin (Figs 1, 2), but not with vimentin or tubulin (data not shown); (2) Triton X-100 treatment of cells did not eliminate the γ_{12} staining; (3) co-localization of γ_{12} with F-actin was not affected by disorganization and reorganization of F-actin by cytochalasin D and FCS, respectively; (4) the subcellular fractionation with Triton X-100 showed γ_{12} to be present not only in the membrane but also the cytoskeletal fractions; (5) an association of γ_{12} with an F-actin-rich fraction was observed after

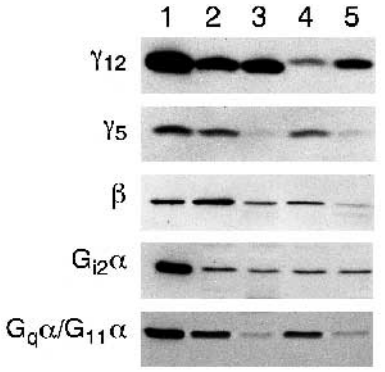

Fig. 6. Immunoblot analysis of subcellular fractions of Swiss 3T3 and C6 cells with antibodies against various subunits of G proteins. Swiss 3T3 and C6 cells were extracted with 0.5% Triton X-100 as described in Materials and Methods. Fractions were then subjected to Tricine/SDS-PAGE for the γ subunits or SDS-PAGE for the β, $G_{i2}\alpha$ and $G_q\alpha/G_{11}\alpha$ subunits and immunoblotted with the respective antibodies. The standards (lane 1, from the top to the bottom) were purified $\beta\gamma_{12}$, $\beta\gamma_5$, $\beta\gamma_{12}$ and $G_{i2}\alpha$ (5 ng each) and the membrane fraction of rat brain (15 μg of protein). Lane 2, Triton X-100-soluble fraction of Swiss 3T3 cells; lane 3, Triton X-100-insoluble fraction of Swiss 3T3 cells; lane 4, Triton X-100-soluble fraction of C6 glioma cells; lane 5, Triton X-100-insoluble fraction of C6 glioma cells. Lanes 2-5 represent the total proteins obtained from 1×10^5 cells.

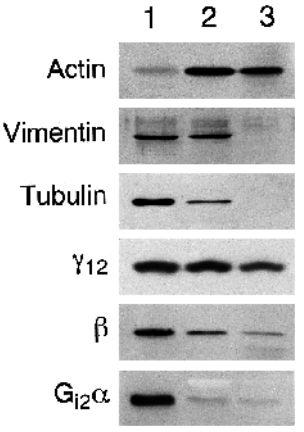

Fig. 7. Immunoblot analysis of an F-actin-rich fraction of Swiss 3T3 cells prepared from the Triton X-100-insoluble fraction using two-step depolymerization-repolymerization. The fractions were subjected to Tricine/SDS-PAGE for the γ subunits or SDS-PAGE for the actin, vimentin, tubulin, and β and $G_{i2}\alpha$ subunits and immunoblotted with the respective antibodies. Standards (lane 1, from the top to the bottom) were skeletal muscle actin (1 μg), vimentin (1 μg), tubulin (1 μg), $\beta\gamma_{12}$ (5 ng), $\beta\gamma_{12}$ (5 ng) and $G_{i2}\alpha$ (5 ng). (Lane 2) Triton X-100-insoluble fraction of Swiss 3T3 cells (5 μg of protein); (lane 3) F-actin-rich fraction of Swiss 3T3 cells (5 μg of protein).

two depolymerization-repolymerization cycles; (6) purified $\beta\gamma_{12}$ complexes directly associated with F-actin in vitro. The demonstrated existence of β and α subunits in the Triton X-100-insoluble and F-actin-rich fractions further suggested that γ_{12} forms βγ complexes or heterotrimeric complexes. The co-localization of $\beta\gamma_{12}$ complexes with F-actin implies a role of G proteins not only in transmembrane but also partly in intracellular signal transduction.

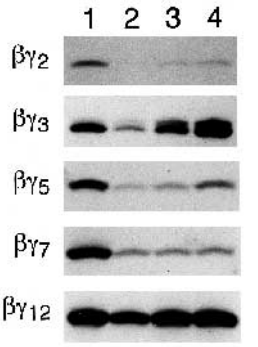

Fig. 8. Binding of various βγ complexes containing different γ subunits to F-actin in vitro. Samples (45 μg) of polymerized actin were incubated with 0.5 μg (lane 2), 1 μg (lane 3) or 2 μg (lane 4) of purified βγ complexes for 30 minutes at 30°C and then centrifuged at 200,000 ***g*** for 20 minutes. The sediments were analyzed by immunoblotting with antibodies against the respective γ subunit. For standards 5 ng samples of the purified βγ complexes were applied (lane 1).

Focal adhesions are sites where clusters of integrin receptors bind to extracellular matrix proteins such as fibronectin and collagen. A number of proteins are found in association at the intracellular face of the plasma membranes, including vinculin, talin and α-actinin (Burridge et al., 1988). Some of these proteins connect integrin to F-actin. In the present study the γ_5 subunit, another major γ species in Swiss 3T3 and C6 cells, was found to be present in the areas of focal adhesion and along stress fibers in the cells as reported previously (Hansen et al., 1994). In contrast to the γ_{12} case, however, Triton X-100 treatment of cells eliminated the immunofluorescence of γ_5 on both focal adhesions and associated stress fibers, while not affecting that of vinculin. The results suggested that γ_5 in the areas of focal adhesion are probably linked with membrane components, and that γ_5 associated with stress fibers might bind indirectly to F-actin or actin-binding proteins through lipids. Actually, phosphatidylinositol 4,5-bisphosphate was earlier found to bind to α-actinin, an actin-binding protein (Fukami et al., 1992).

The γ_5 and γ_{12} subunits are widely distributed in a variety of tissues and cells (Gautam et al., 1990; Cali et al., 1992; Morishita et al., 1992, 1995; Asano et al., 1995; Ray et al., 1995). We have observed a differential subcellular distribution for the two in PC12 pheochromocytoma cells (data not shown) as well as the Swiss 3T3 and C6 cells described here. In several rat tissues including brain, kidney, stomach, intestine and urinary bladder, γ_{12} was found in both Triton X-100-soluble and -insoluble fractions, while in most cases the γ_5 subunit was limited to the Triton X-100-soluble fraction (data not shown). These results suggest that the differential distribution of γ_{12} and γ_5 observed in the present study are typical of many kinds of cell.

The present study showed the specific association of $\beta\gamma_{12}$ to F-actin in both cultured cells and in vitro. Both Triton X-100-soluble and -insoluble fractions of Swiss 3T3 and C6 cells contained β subunits which were detected by our antibodies, suggesting the existence of β_1 (Morishita et al. 1988). An antibody against β_2 showed faint bands on immunoblots of

both fractions of these cell lines (data not shown) but the existence of other isoforms could not be clarified because specific and well-characterized antibodies are not available. However, the purified βγ complexes, including $\beta\gamma_2$, $\beta\gamma_3$, $\beta\gamma_7$ and $\beta\gamma_{12}$, contained mainly β_1, as shown previously (Asano et al., 1993). The additional βγ complex, $\beta\gamma_5$, which was purified by a similar method, contained at least β_1 and β_2 (data not shown). Among these purified βγ complexes, $\beta\gamma_{12}$ as well as $\beta\gamma_3$ associated with F-actin more strongly than $\beta\gamma_2$, $\beta\gamma_5$ and $\beta\gamma_7$, suggesting that the γ subunit is responsible for the selective association of βγ complexes with F-actin. Thus, one of the physiological roles of heterogeneity of γ subunits might be to determine the subcellular localization of G proteins formed by such complexes.

Although the purified $\beta\gamma_3$ also associated with F-actin in vitro, γ_3 is not detectable in Swiss 3T3 and C6 cells (Asano et al., 1995; Morishita et al., 1995). In PC12 cells, however, it is present but primarily in the Triton X-100-soluble fraction (data not shown). Since the actin level of PC12 cells is much less than in Swiss 3T3 and C6 cells, it is unlikely that significant amounts of $\beta\gamma_3$ are associated with F-actin in this case.

A previous paper demonstrated an association between $G_q\alpha/G_{11}\alpha$ and F-actin in WRK_1 cells (Ibarrondo et al., 1995). We have observed a similar co-localization immunohistochemically in Swiss 3T3 cells (data not shown). Various enzymes involved in signal transduction such as phospholipase C, phosphoinositide kinases, diacylglycerol kinase and protein kinase C are found associated with the cytoskeleton in a variety of cells (Grondin et al., 1991; Payrastre et al., 1991; Vaziri and Downes, 1992; Blobe et al., 1996). Thus, the present data are in line with the recent reports which suggests that the cytoskeleton provides a place where various molecules related to signal transduction assemble and interact with each other. Treatment of quiescent fibroblasts with various stimulators such as FCS and lysophosphatidic acid induces the formation of stress fibers and focal adhesions (Ridley and Hall, 1992). The present study showed that γ_{12} translocated to stress fibers when quiescent Swiss 3T3 cells were stimulated by FCS. In platelets, G protein subunits, such as $G_{i2}\alpha$, $G_s\alpha$, $G_q\alpha$ and βγ (Ozawa et al., 1996), and various enzymes, including phospholipase C, phosphatidylinositide kinases and diacylglycerol kinase (Grondin et al., 1991; Banno et al., 1996) are also known to translocate to the cytoskeleton on being stimulated by thrombin. Thus, these G proteins and enzymes might be involved in mediating integrin receptor signaling. The specific localization of γ_5 and γ_{12} in focal adhesions and F-actin, respectively, documented here implies the possibility that these two γ subunits play different roles in a pathway of signal transduction from integrin.

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