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Effect of Transient Overexpression of $G_{q\alpha}$ on Soluble and Polymerized Tubulin Pools in GH_3 and AtT-20 Cells

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Abstract In order to study G_q-tubulin interaction in the cytosol, GH_3 and AtT-20 cells (stably expressing TRH receptor) were transiently transfected with $G_{q\alpha}$ cDNA. Forty-eight hours after transfection, thyrotropin-releasing hormone (TRH)-stimulated prolactin (PRL) secretion by $G_{q\alpha}$-transfected GH_3 cells increased by 90% compared to mock-transfected cells. In addition, using immunocytochemistry it was observed that $G_{q\alpha}$-specific staining was much more prominent in $G_{q\alpha}$-transfected GH_3 and AtT-20 cells (also transfected with $G_{q\alpha}$) compared to mock-transfected cells. Thus, transfection resulted in successful overexpression of functional $G_{q\alpha}$. Forty-eight hours after transfection, cells were processed to obtain soluble and polymerized tubulin fractions. Tubulin levels were determined in these fractions by immunoblotting using polyclonal anti-tubulin antibodies. Compared to mock-transfected cells soluble tubulin levels decreased in $G_{q\alpha}$-transfected GH_3 and AtT-20 cells, by 33 and 52%, respectively. Moreover, compared to mock-transfected cells a 50% reduction in the ratio (an index of the flux between tubulin pools) of soluble and polymerized tubulin levels was observed in $G_{q\alpha}$-transfected GH_3 and AtT-20 cells. To determine whether these effects on tubulin were mediated by G_q directly, we examined the influence of purified G_q on tubulin polymerization. G_q (0.5 μM) inhibited polymerization of crude tubulin (present in GH_3 cell cytosol) by 53%. In contrast to its effects on GH_3 cell cytosol tubulin, G_q stimulated purified tubulin polymerization by 160%. These results suggest that G_q modulates the polymerization and depolymerization cycles of tubulin and that this modulation is in turn influenced by other unknown cellular components.

Key words: G proteins, cytoskeleton, pituitary cells, signal transduction, prolactin, thyrotropin-releasing hormone

Guanine nucleotide-binding regulatory proteins (G proteins) promote the coupling of their receptors to G proteins. G proteins are composed of a variable α subunit (39–46 kDa) and β and γ subunits (35–36 and 8 kDa, respectively). The regulation of a particular effector is determined by the type of G protein that is coupled to a specific hormone receptor. For example, in the pituitary, corticotropin-releasing hormone receptor coupling to $G_{s\alpha}$ results in stimulation of adenylyl cyclase, somatostatin receptor coupling to $G_{i\alpha}$ causes inhibition of adenylyl cyclase, and thyrotropin-releasing hormone (TRH) receptor coupling to $G_{q\alpha}$ results in the stimulation of phospholipase C [Birnbaumer et al., 1990; Burrin, 1994; Spiegel et al., 1992].

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Work from various laboratories suggests that an interaction of cytoskeletal proteins with G proteins may play a role in agonist-mediated signal transduction. For example, (1) studies with isolated cell membranes have established that compounds which are known to influence tubulin function (e.g., colchicine or taxol) altered receptor-G protein coupling in various systems, suggesting a role for membrane-associated tubulin in signal transduction [Ravindra and Aronstam, 1990, 1993; Leiber et al., 1993], (2) in COS 1 cells transfected with $G_{s\alpha}$, tubulin-Gpp(NH)p complex potentiated isoproterenol-stimulated adenylyl cyclase activity [Popova et al., 1994], and (3) Gephyrin, a 93 kDa microtubule-binding protein, appears to link the glycine receptor to tubulin in synaptic membranes [Kirsch et al., 1993]. In addition, actin cytoskeleton may play a role in the association of P_{2y}-purinergic receptor, $G_{q\alpha}$, and phospholipase C in the turkey erythrocytes [Vazir and Downes, 1992]. Furthermore, using immuno-gold elec-

tron microscopy, transferrin receptors were colocalized with the cytoskeleton in the human retinal pigment epithelial cells [Hunt et al., 1991]. Taken together, these studies suggest that an interaction of G proteins with cytoskeletal proteins at the cell membrane influences signal transduction.

In addition to an interaction at the plasma membrane, in vitro experiments with purified G proteins and tubulin suggest that an interaction between these proteins might also occur in the cytosol. The observation that purified G_i and G_o inhibited tubulin polymerization [Wang and Rasenick, 1991] suggests that G proteins may act as physiological regulators of tubulin function in vivo. To further delineate tubulin-G protein interaction, we examined the effect of transient overexpression of $G_{q\alpha}$ on the equilibrium between soluble (i.e., depolymerized) and polymerized tubulin pools in GH_3 and AtT-20 cells (stably expressing TRH receptor). We report here that overexpression of $G_{q\alpha}$ altered the dynamic equilibrium between the two tubulin pools. We also examined the effect of purified heterotrimeric G_q on the ability of crude tubulin (present in GH_3 cell cytosol) and purified tubulin to polymerize into microtubules. Heterotrimeric G_q exhibited divergent effects on tubulin polymerization in these two cell-free systems: it inhibited tubulin polymerization in GH_3 cell cytosol whereas it stimulated the polymerization of purified tubulin.

MATERIALS AND METHODS

Materials

Thyrotropin releasing hormone (TRH; catalog P-2161) and anti-rabbit IgG alkaline phosphatase conjugate (catalog A-3687) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{125}I]-goat anti-rabbit IgG F $(ab')_2$ fragment (8.2 μCi/μg), and [^{125}I]PRL (35.2 μCi/μg) were purchased from DuPont-NEN (Wilmington, DE). Polyclonal tubulin antibody (catalog AB935) was purchased from Chemicon International, Inc. (Temecula, CA). All other chemicals used in the present study were purchased from Sigma.

Purified heterotrimeric G_q [Blank et al., 1991] was generously provided by Dr. John Exton, Vanderbilt University, Nashville, TN. Tubulin, purified from bovine brains and free of microtubule-associated proteins [Hamel and Lin, 1984], was a generous gift of Dr. Ernest Hamel, NIH, Bethesda, MD. $G_{q\alpha}$ cDNA [Qian et al., 1993] was kindly provided by Dr. Gary Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO.

Production of $G_{q\alpha}$ Antibodies

$G_{q\alpha}$ antiserum used in this study was generated in rabbits. Antiserum F-8 was generated using the peptide CDEKVSAFENPYVD, synthesized commercially by Multiple Peptide Systems (San Diego, CA). Amino acid sequence for the peptide was chosen from the published sequence of cDNA clones considered to encode a portion of the α subunit of G_q. The peptide was synthesized with a cysteine residue at the amino terminus to facilitate coupling to keyhole limpet hemocyanin with M-maleimidobenzoyl-N-hydroxysuccinimide ester. Immunization of rabbits and collection of blood were according to conventional methods. Antiserum was characterized by immunoblotting analysis of purified bovine brain G protein preparations.

Transfection of GH_3 Cells With $G_{q\alpha}$ cDNA

GH_3 cells were maintained at a density of $0.17 \times 10^5/cm^2$ in T-150 flasks containing Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. Twenty-four hours before transfection, GH_3 cells were seeded to 6-well, flat bottom plates (Falcon, Catalog 3046, Beckton Dickinson, Lincoln Park, NJ) at a density of 10^6 per well. Cells were transiently transfected with either vector or $G_{q\alpha}$ cDNA according to previously described methods [Kunapuli et al., 1994]. One microgram of DNA was mixed with 25 μl of lipofectamine (Gibco-BRL, Gaithersburg, MD) in 175 μl of Opti-MEM (Gibco-BRL) and added to cells. After 7 h, Ham's F-10 medium supplemented with 30% horse serum and 5% fetal calf serum was added to the wells, and the reaction was allowed to proceed for 48, 54, or 60 h. After transfection, cells were washed twice with Opti-MEM and challenged with 100 nM TRH for 10 min at 37°C. The medium was collected and kept frozen at −70°C until further analysis.

PRL levels were estimated in the medium collected from individual wells. The concentration of prolactin (PRL) in the medium was determined by a double antibody radioimmunoassay using National Hormone and Pituitary Program reagents and procedures. Determinations were made in duplicate at two dilutions and all samples from an experiment were measured in

one assay. The antibody to prolactin was NIDDK-rPRL-I-6 and NIDDK-rPRL-RP-3 served as the reference standard. The intra and inter assay coefficients were 8 and 12%, respectively.

Transfection of AtT-20 Cells $G_{q\alpha}$ cDNA

AtT-20 cells stably transfected with TRH receptor [Nussenzveig et al., 1993] were a kind gift from Dr. Marvin Gershengorn, Cornell University Medical College, NY. These cells were maintained at a density of $0.17 \times 10^5/cm^2$ in T-150 flasks containing Dulbecco's Modified Eagle Medium supplemented with 15% horse serum and 2.5% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. AtT-20 cells were transfected for 48 h with $G_{q\alpha}$ cDNA as described above.

Immunocytochemistry of $G_{q\alpha}$

GH_3 and AtT-20 cells were transfected for 48 h with $G_{q\alpha}$ cDNA and challenged with 100 nM TRH as described above. The cells were then fixed at room temperature for 30 min in 2% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4 (PBS) and washed with PBS. Cells were permeabilized with 100% methanol, washed with PBS, and incubated at room temperature for 1 h with $G_{q\alpha}$ antibody (1:500 dilution). After washing with PBS, cells were incubated at room temperature for 1 h with anti-rabbit IgG alkaline phosphatase conjugate (1:400 dilution) and again washed with PBS. The enzyme reaction was initiated by the addition of nitro blue tetrazolium and X-phosphate (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's recommendations. Cells were kept in dark at room temperature and the development of blue color was monitored. Once the extent of color development was considered to be satisfactory, the reaction was stopped with the addition of 0.1M Tris-HCl buffer, pH 7.5. Cells were observed with a Nikon inverted microscope using standard brightfield optics and images were recorded on Kodak TMax negative film (ASA 100).

Isolation of Soluble and Polymerized Tubulin Fractions

Forty hours after transfection, cells were homogenized at room temperature in MES buffer (100 mM morpholinoethane sulfonic acid, 2 mM $MgCl_2$, 50 mM KCl, and 4 mM EGTA, pH 6.8) with a motor-driven Teflon pestle (Kontes, Vineland, NJ). The homogenate was spun at 100,000*g* for 60 min at 27°C. The supernatant contained monomeric tubulin and was designated the "soluble tubulin fraction," and stored at −70°C until further analysis. The pellet was resuspended in MES buffer (pH 6.8), and kept at 4°C for 60 min. Exposure to cold temperature results in the depolymerization of microtubules present in the pellet. The suspension was then spun at 100,000*g* for 60 min at 4°C, and the supernatant was designated the "polymerized tubulin fraction," and stored at −70°C until further analysis [Ravindra and Grosvenor, 1988]. Protein content was estimated in both the fractions using bovine serum albumin (BSA) as the standard [Bradford, 1976].

Preparation of Cytosol Fraction From GH_3 Cells

GH_3 cells were homogenized in MES-Ca^{2+} buffer (100 mM MES, 2 mM $MgCl_2$, 50 mM KCl, 4 mM $CaCl_2$, pH 6.8) and the homogenate was kept at 4°C for 60 min. Exposure to cold temperature and Ca^{2+} causes the depolymerization of "cold-labile" as well as "cold-stable" fractions of microtubules [Lieuvin et al., 1994]. The homogenate was spun at 100,000*g* for 60 min at 4°C and the supernatant containing tubulin was collected. The Ca^{2+} present in the supernatant fraction was chelated by the addition of 4 mM EGTA. The tubulin-containing fraction was dialyzed overnight against 0.2 M sucrose and concentrated using aquacide (Calbiochem, La Jolla, CA catalog 1785); dialysis tubing with a molecular weight cut off of 15 kDa was used. Protein content was estimated using bovine serum albumin (BSA) as the standard [Bradford, 1976].

In Vitro Tubulin Polymerization Using GH_3 Cell Cytosol Fraction

Tubulin polymerization in GH_3 cell cytosol was conducted as described previously [Ravindra and Grosvenor, 1987]. The reaction mixture consisted of 7.4 mg/ml of cytosol fraction, purified G_q or rabbit IgG (a control for G_q), and 1 mM GTP in MES buffer (100 mM MES, 2 mM $MgCl_2$, 50 mM KCl, 4 mM EGTA, pH 6.8) in a total volume of 100 μl. Samples were incubated for 30 min at 37°C, and then spun at 100,000*g* for 60 min at 37°C to sediment the polymers formed during incubation with GTP. An aliquot of the supernatant was removed, and the unpolymerized tubulin present therein was detected by immunoblotting and quantified by densitometric scanning of the immunoblots. The

amount of polymers formed was determined by subtracting the amount of monomers remaining in the supernatant after the incubation at 37°C from those present before the reaction.

Determination of Tubulin Levels

Proteins in the 100,000*g* supernatant (obtained after the polymerization reaction) and proteins in the soluble and polymerized tubulin fractions were separated by electrophoresis using 12% SDS-PAGE gels [Laemmli, 1970], and proteins were transferred to PVDF membranes (Bio-Rad Laboratories, Richmond, CA) using a Trans-blot apparatus (Bio-Rad). The membranes were incubated with polyclonal anti-tubulin antibodies (Chemicon) followed by incubation with [^{125}I]labeled second antibody. After washing, the membranes were exposed to X-ray films and the autoradiographs developed. Tubulin was quantified by scanning the autoradiographs with a laser densitometer.

In Vitro Tubulin Polymerization Using Purified Tubulin

Tubulin polymerization was monitored by turbidimetry with measurement of the change in absorbance at 350 nm in a Beckman DU 70 recording spectrophotometer [Gaskin et al., 1974]. The reaction mixture consisted of 31.3 μM tubulin (3.13 mg/ml), 0.14 μM G_q or 0.14 μM rabbit IgG (a control for G_q), 1.1 mM GTP, MES buffer at pH 6.8 in a total volume of 365 μl. The cuvettes containing the samples were placed in a recording spectrophotometer and polymerization monitored at 37°C.

Data Analysis

Statistical differences among the groups were determined by analysis of variance and Fisher tests using the Statview II program on a Macintosh IIci computer. Means were deemed significantly different at $P < 0.05$.

RESULTS

Determination of the Efficiency of Transfection of $G_{q\alpha}$

Since TRH utilizes $G_{q\alpha}$ in signal transduction [Hsieh and Martin, 1992] we reasoned that overexpression of $G_{q\alpha}$ in GH_3 cells would result in enhanced prolactin (PRL) secretion. Compared to mock-transfected cells, TRH-stimulated PRL secretion by $G_{q\alpha}$-transfected cells was 90, 70, and 58% greater at 48, 54, and 60 h, respectively (Fig. 1; $P < 0.05$). These results suggest that the optimal time for transfection of $G_{q\alpha}$ in GH_3 cells is 48 h.

Moreover, immunocytochemistry was used for a visual comparison of $G_{q\alpha}$ expression levels. We found that $G_{q\alpha}$-specific staining was much more prominent in $G_{q\alpha}$-transfected GH_3 and AtT-20 cells (also transfected with $G_{q\alpha}$) compared to mock-transfected cells, confirming the increased levels of $G_{q\alpha}$ expression and accumulation in $G_{q\alpha}$-transfected cells. Staining was negligible in control cells (i.e., non-transfected) incubated with anti-rabbit IgG alkaline phosphatase conjugate alone (Fig. 2A–F). In view of the fact that $G_{q\alpha}$ antibody was raised against a 14 amino acid peptide it must be noted here that the present data reflect an interaction between the antibody and the peptide (which is a part of $G_{q\alpha}$) present in the cells.

Effect of Overexpression of $G_{q\alpha}$ on Soluble and Polymerized Tubulin Levels in GH_3 and AtT-20 Cells

According to the generally accepted model of G protein function, the binding of an agonist to its receptor facilitates receptor coupling to G protein, leading to the dissociation of α subunit of G protein from βγ subunits [Spiegel et al., 1992]. In the present study, in order to dissociate $G_{q\alpha}$ from βγ subunits, cells overexpressing $G_{q\alpha}$ were exposed to TRH. Forty-eight hours

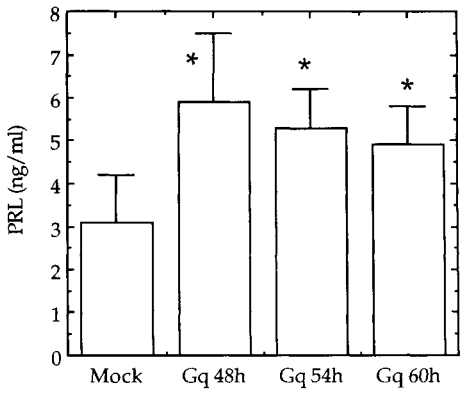

Fig. 1. Effect of TRH on prolactin secretion by mock and $G_{q\alpha}$ transfected GH_3 cells. After transfection cells were challenged with 100 nM TRH for 10 min at 37°C and prolactin in the medium was determined by radioimmunoassay. Each point represents the mean ± S.E.M. of six determinations, three determinations from each of two independent experiments. The value obtained at 48 h with mock-transfected cells is shown. Asterisk (*) indicates value significantly different from the mock value ($P < 0.05$).

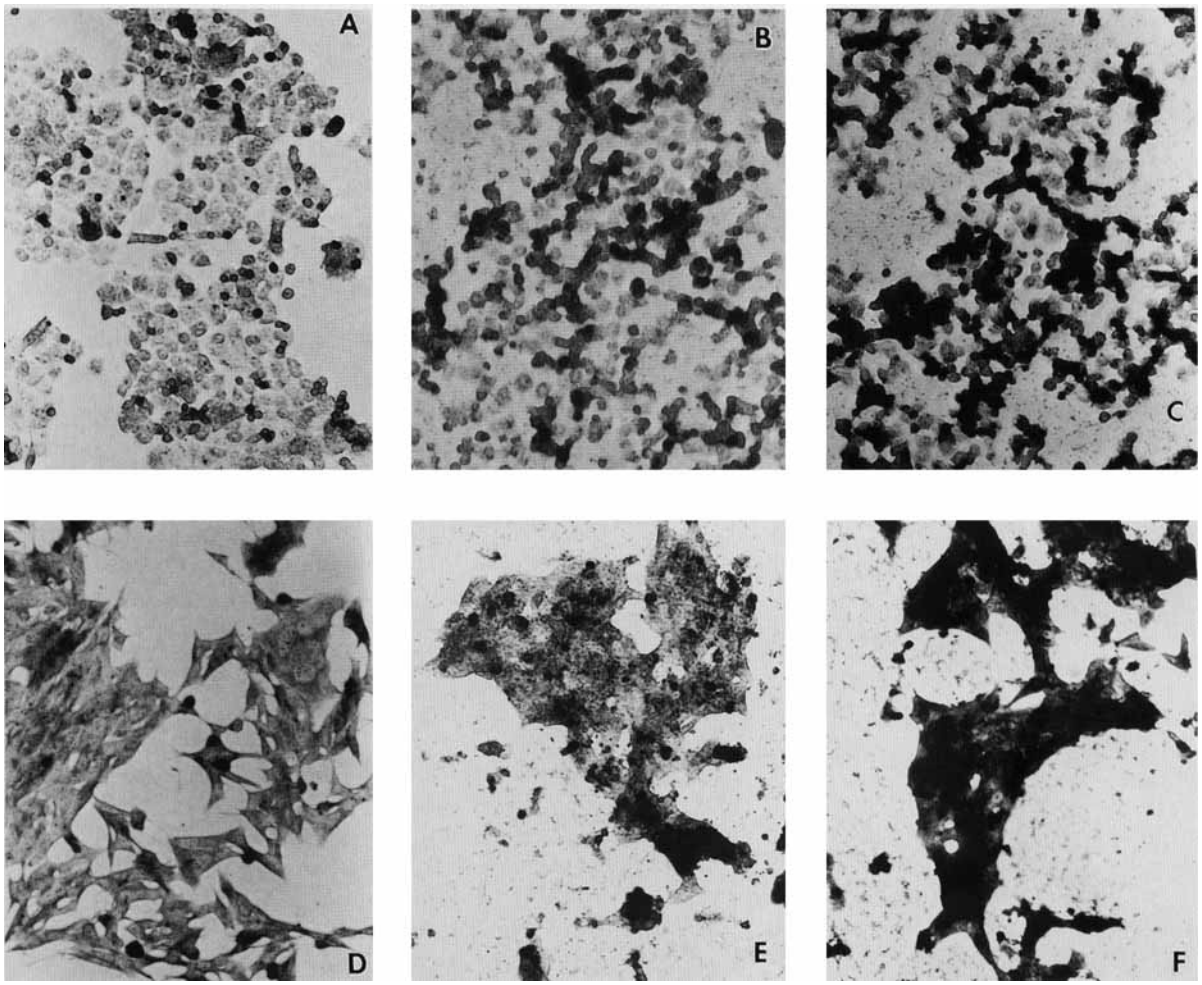

Fig. 2. Localization of $G_{q\alpha}$ by immunocytochemistry. GH_3 and AtT-20 cells were transfected for 48 h with $G_{q\alpha}$ cDNA and cells were incubated at room temperature for 1 h with $G_{q\alpha}$ antibody (1:500 dilution) followed by an incubation with anti-rabbit IgG alkaline phosphatase conjugate (1:400 dilution) for 1 h at room temperature. The enzyme reaction was conducted as described in Materials and Methods and cells were observed with a Nikon inverted microscope using standard brightfield optics. Photographs from one of two similar experiments are shown. **A:** GH_3 cells (non-transfected) incubated with anti-rabbit IgG alkaline phosphatase conjugate alone. **B:** Mock-transfected GH_3 cells incubated with $G_{q\alpha}$ antibody and anti-rabbit IgG alkaline phosphatase conjugate. **C:** $G_{q\alpha}$-transfected GH_3 cells incubated with $G_{q\alpha}$ antibody and anti-rabbit IgG alkaline phosphatase conjugate. **D:** At-T20 cells (non-transfected) incubated with anti-rabbit IgG alkaline phosphatase conjugate alone. **E:** Mock-transfected At-T20 cells incubated with $G_{q\alpha}$ antibody and anti-rabbit IgG alkaline phosphatase conjugate. **F:** $G_{q\alpha}$-transfected At-T20 cells incubated with $G_{q\alpha}$ antibody and anti-rabbit IgG alkaline phosphatase conjugate.

after transfection, GH_3 cells were challenged with 100 nM TRH for 10 min and soluble and polymerized tubulin fractions were prepared. Tubulin levels in these two fractions were detected by immunoblotting and quantified by densitometric scanning of the immunoblots. Compared to mock-transfected cells soluble tubulin levels decreased by 33% in $G_{q\alpha}$-transfected cells (Fig. 3A,B; $P < 0.05$). Although the polymerized tubulin levels increased by 54% in $G_{q\alpha}$-transfected cells, this increase was not statistically significant.

In order to determine if $G_{q\alpha}$-induced changes in tubulin levels occur in other pituitary cell types, AtT-20 cells (stably expressing TRH receptor) were used. Forty-eight hours after transfection, AtT-20 cells were challenged with 100 nM TRH for 10 min and soluble and polymerized tubulin fractions were prepared. Tubulin levels in these two fractions were detected by immunoblotting and quantified by densitometric scanning of the immunoblots. Compared to mock-transfected cells soluble tubulin levels decreased by 52% in $G_{q\alpha}$-transfected cells (Fig. 3C;

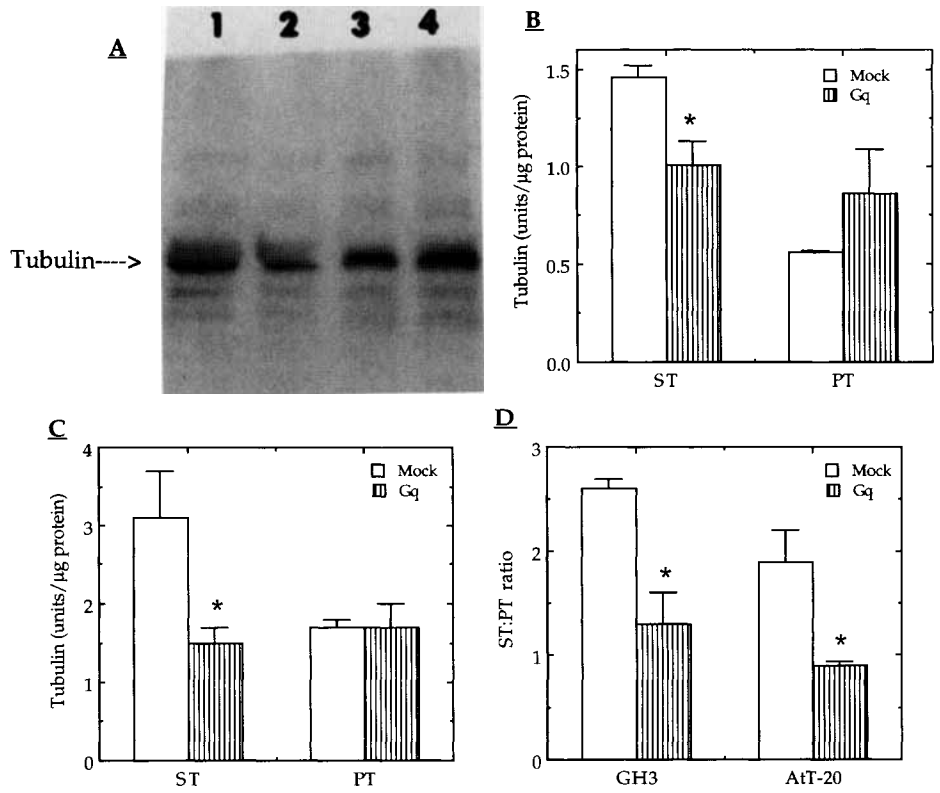

Fig. 3. Soluble and polymerized tubulin levels in mock and $G_{q\alpha}$ transfected GH_3 and At-T 20 cells. **A:** Autoradiograph of soluble (24 μg protein) and polymerized (42 μg protein) tubulin fractions obtained from GH_3 cells. *Lane 1:* soluble tubulin in mock transfected cells; *lane 2:* soluble tubulin in $G_{q\alpha}$ transfected cells; *lane 3:* polymerized tubulin in mock transfected cells; *lane 4:* polymerized tubulin in $G_{q\alpha}$ transfected cells. A representative autoradiograph is shown. **B:** Densitometric analysis of immunoblots obtained with GH_3 cells. Each point represents the mean ± SEM of data from three experiments. Asterisks indicate the value significantly different from the mock value ($P < 0.05$). ST, soluble tubulin; PT, polymerized tubulin. **C:** Densitometric analysis of immunoblots obtained with At-T20 cells. Each point represents the mean ± SEM of data from three experiments. Asterisks indicate the value significantly different from the mock value ($P < 0.05$). ST, soluble tubulin; PT, polymerized tubulin. **D:** Ratio of soluble and polymerized tubulin levels. Each point represents the mean ± SEM of data from three experiments. Asterisks indicate the value significantly different from the mock value ($P < 0.05$). ST, soluble tubulin; PT, polymerized tubulin.

$P < 0.05$). However, there was no change in polymerized tubulin concentration.

The ratio of soluble and polymerized tubulin levels, an index of the flux between tubulin pools, in mock- and $G_{q\alpha}$-transfected GH_3 cells was 2.6 ± 0.1 and 1.3 ± 0.3, respectively (Fig. 3D; $P < 0.05$). A similar reduction in the ratio was also observed with AtT-20 cells; the ratio of soluble and polymerized tubulin levels in mock- and $G_{q\alpha}$-transfected AtT-20 cells was 1.9 ± 0.3 and 0.9 ± 0.05, respectively (Fig. 3D; $P < 0.05$).

Effect of Purified Heterotrimeric G_q on Polymerization of Tubulin in GH_3 Cell Cytosol Preparation

Tubulin polymerization method using rat pituitary cell lysates was previously characterized; the extent of polymerization was linear as a function of protein concentration, was dependent upon GTP concentration present in the reaction mixture, and was inhibited by cold temperatures, Ca^{2+}, and tubulin antibody [Ravindra and Grosvenor, 1987]. Moreover, using GH_3 cell lysates as well as purified tubulin, it was established that the optical densities of the Western blots were in the linear range of detection (data not shown).

G_q inhibited tubulin polymerization in GH_3 cell cytosol preparation. G_q, at 0.25 and 0.5 μM, significantly ($P < 0.05$) inhibited polymerization of tubulin by 59 and 53%, respectively. IgG (used as a control for G_q) at similar concentrations did not affect the polymerization process (Fig. 4A,B); IgG neither enhanced nor inhibited

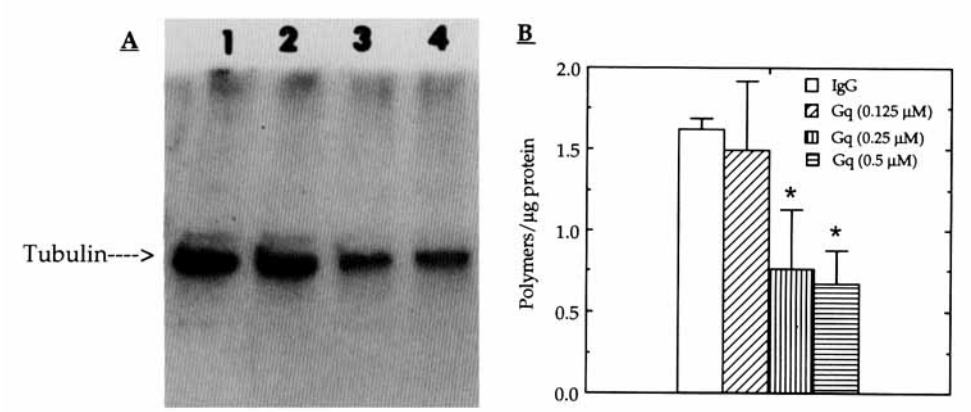

Fig. 4. Effect of heterotrimeric G_q on in vitro polymerization of tubulin in GH_3 cell cytosol. GH_3 cell cytosol (7.4 mg/ml) was incubated for 30 min at 37°C in MES buffer with 1 mM GTP. The samples were centrifuged at 100,000*g* to sediment the polymers. The amount of unpolymerized tubulin remaining in the supernatant fraction was determined by immunoblotting. **A:** Representative autoradiograph showing the amount of unpolymerized tubulin remaining in the supernatant fraction. Six micrograms protein were immunoblotted. *Lane 1:* 0.5 μM G_q; *lane 2:* 0.25 μM G_q; *lane 3:* 0.125 μM G_q; *lane 4:* 0.5 μM rabbit IgG. **B:** Densitometric analysis of the effect of G_q on tubulin polymerization. The amount of polymers formed was calculated by subtracting the amount of monomers remaining in the supernatant after the incubation at 37°C from those present before the reaction. Each point represents the mean ± SEM of data from three experiments. Asterisks indicate the value significantly different the control (IgG) value ($P < 0.05$).

the ability of tubulin to polymerize (data not shown).

Results from one experiment suggest that inhibition of polymerization by G_q is dose-dependent. The inhibition was 81, 64, and 59% at 0.125, 0.25, and 0.5 μM of G_q, respectively. However, dose-dependent inhibition was not evident when data from three independent experiments were combined to obtain mean ± SEM.

Effect of Purified Heterotrimeric G_q on Polymerization of Purified Tubulin

In order to examine the effect of purified heterotrimeric G_q on purified tubulin polymerization, 3.13 mg/ml (31.3 μM) tubulin was incubated with 0.14 μM G_q and GTP (1.1 mM), and the samples were placed in a recording spectrophotometer (to measure changes in turbidity) and polymerized at 37°C. Compared to IgG (a control for G_q), G_q stimulated tubulin polymerization (as indicated by increased turbidity) by 20% (Fig. 5A). Tubulin polymerization was neither enhanced nor inhibited by IgG (data not shown).

In an attempt to determine if incubation with G_q influenced the ability of microtubules (formed during the incubation at 37°C) to depolymerize when exposed to cold temperatures, the cuvettes containing the samples were placed on ice for 10 min. Samples exposed to either G_q or IgG depolymerized to the same extent (data not shown). Thus, in contrast to its influence on polymerization, G_q had no effect on depolymerization. Additional GTP (1.01 mM) was added to the reaction mixture and the samples were returned to the spectrophotometer and polymerized for the second time at 37°C. In the second cycle of polymerization, compared to IgG, G_q stimulated the tubulin polymerization by 160% (Fig. 5B). The samples were again exposed to cold temperature to depolymerize the microtubules formed during the second cycle of polymerization. Again, samples exposed to either G_q or IgG depolymerized to the same extent (data not shown). Here again, additional GTP (0.86 mM) was added to the reaction mixture and samples repolymerized at 37°C. In the third cycle, now presumably in the presence of excess GDP which renders G_q inactive by forcing heterotrimeric assembly, the ability of G_q to stimulate the polymerization reaction was not observed (Fig. 5C).

DISCUSSION

Overexpression of $G_{q\alpha}$ in GH_3 and AtT-20 cells resulted in a significant decrease in the monomer/polymer ratio, suggesting an interaction between G_q and the tubulin-microtubule system in cell cytosol. It is not clear whether the modest changes in the ratio are due to a direct interaction between these two protein families. It is noteworthy that the tubulin fractions were prepared using all of the cells irrespective of whether they took up foreign cDNA; changes in the level of the two tubulin pools obviously would have

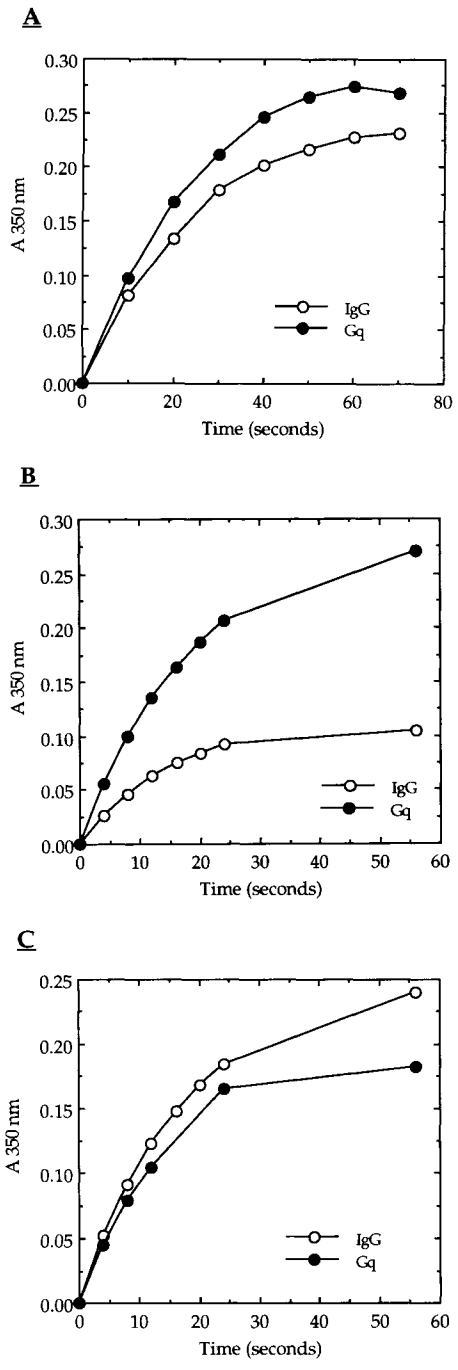

Fig. 5. Effect of heterotrimeric G_q on the polymerization of purified tubulin. One of two similar experiments is shown. **A:** First cycle of polymerization. Purified tubulin (3.13 mg/ml; 31.3 μM) was incubated with 1.1 mM GTP, G_q (0.14 μM) or rabbit IgG (0.14 μM) in MES buffer and polymerized as described in Methods. Total volume of the reaction mixture in each cuvette was 365 μl. **B:** Second cycle of polymerization. After the first cycle of polymerization, the cuvettes were placed on ice resulting in depolymerization of microtubules. GTP (1.01 mM) was added to the samples on ice and the cuvettes returned to the spectrophotometer and the samples polymerized at 37°C. With the addition of 40 μl of GTP, the total volume of the reaction mixture increased to 405 μl. The concentrations of tubulin, G_q, and IgG were 28.2 (2.82 mg/ml), 0.12, and 0.12 μM, respectively. **C:** Third cycle of polymerization. After the second cycle of polymerization, the cuvettes were placed on ice resulting in depolymerization of microtubules. GTP (0.86 mM) was added to the samples on ice and the cuvettes returned to the spectrophotometer and the samples polymerized at 37°C. With the addition of 60 μl of GTP, the total volume of the reaction mixture increased to 465 μl. The concentrations of tubulin, G_q, and IgG were 24.6 (2.46 mg/ml), 0.11, and 0.11 μM, respectively.

been more dramatic if only those cells overexpressing $G_{q\alpha}$ were analyzed. There are two factors which limit the use of transient transfection. First, it is relatively inefficient, only 30% of the cells expected to take up the foreign cDNA. Second, the integrated gene often undergoes substantial modifications and rearrangements, and mutations during the process of transfection are common. Therefore, it is necessary to determine whether cells are compromised as a result of the transfection process. That $G_{q\alpha}$-transfected GH_3 cells responded to TRH by producing more PRL than mock-transfected cells suggests that cells retained their functional properties.

GTP causes dissociation of α subunit of a G protein from βγ subunits and GDP promotes association of α subunit of a G protein with βγ subunits; GTP-bound G_α is active whereas GDP-bound G_α is inactive [Spiegel et al., 1992]. In the experiments using purified heterotrimeric G_q and purified tubulin, GTP added to the reaction mixture can activate the dissociation of $G_{q\alpha}$ from βγ subunits. The observations that G_q promoted tubulin polymerization in the first and second cycle (Fig. 5A,B), but not in the third cycle (Fig. 5C), lend credence to the hypothesis that excess GDP, formed as a result of GTP hydrolysis, causes the subunits of G_q to reassemble to the inactive state.

The in vitro effects of purified G_q on purified tubulin, may not reflect an in vivo interaction between these two protein families. Moreover, the observation that G_q inhibits tubulin polymerization in GH_3 cell cytosol preparation appears to contradict the finding that G_q promotes polymerization of purified tubulin. This may be explained in two ways. First, the dynamic properties of tubulin in the cytosol fraction may differ from those of highly purified tubulin. The microtubule-associated proteins (MAPs) present in the cytosol preparation may influence tubu-

lin-G_q interaction. Purified tubulin used in the present study is devoid of MAPs. Second, the relative concentrations of G_q and tubulin present in the reaction mixture might determine whether tubulin polymerization is promoted or inhibited. It was reported that 20 μM of G_i or G_o inhibited the polymerization of 15 μM purified tubulin [Wang and Rasenick, 1991]. In contrast, in the present study, we observed that 0.12 μM G_q promoted the polymerization of 28.2 μM purified tubulin. On the other hand, using GH_3 cell cytosol preparation we determined that 0.5 μM G_q inhibited the polymerization of 1.5 μM tubulin (tubulin concentration is an estimate based on the assumption that 2% of the total protein in GH_3 cell cytosol preparation is tubulin).

Local compartmental domains of G_q, GTP/GDP, and tubulin concentrations may influence the nature of this regulation, i.e., stimulation or inhibition. In response to agonist stimulation, α subunits of G proteins have been reported to translocate from cell membranes into the cytosol in S49 lymphoma cells [Ransnas et al., 1989] and rat adipocytes [Haraguchi and Rodbell, 1990]. Based on these previous observations as well as the data presented here, we propose that immediately after TRH receptor couples to G_q, the α subunit of G_q is translocated into the cytosol. The observation that $G_{q\alpha}$ is present in GH_3 cell cytosol supports this idea [Wilson et al., 1994]. Once in the cytosol, $G_{q\alpha}$ influences tubulin monomer-polymer equilibrium (Fig. 6). Although the actual mechanism is not clear, it is generally accepted that in the rat pituitary, microtubules are involved in the transport of PRL secretory granules from the Golgi to the cell membrane [reviewed in Ravindra and Grosvenor, 1990]. In this scheme, membrane-associated tubulin is also included because data from various systems suggest a role for membrane-associated tubulin in signal transduction [Ravindra and Aronstam, 1990, 1993; Leiber et al., 1993; Popova et al., 1994].

In summary, we have demonstrated that transient overexpression of $G_{q\alpha}$ resulted in a decrease in the ratio of soluble (i.e., depolymerized) and polymerized tubulin levels in GH_3 and AtT-20 cells. In addition, heterotrimeric G_q exhibited divergent effects on tubulin polymerization: it inhibited tubulin polymerization in GH_3 cell cytosol whereas it stimulated the polymerization of purified tubulin. These results suggest that G_q modulates the function of tubulin-

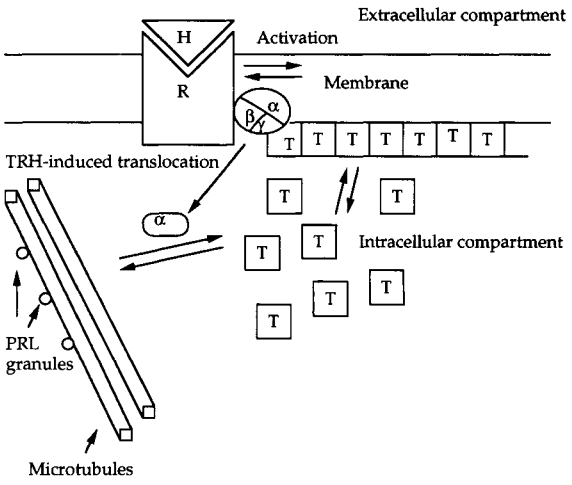

Fig. 6. Model depicting the role of $G_{q\alpha}$ in modulating tubulin function cytosol. In response to TRH stimulation, $G_{q\alpha}$ is translocated into the cytosol, and once in the cytosol, it interacts with tubulin pools to influence the monomer-polymer equilibrium. H, hormone; R, receptor; T, tubulin heterodimer; α, β, γ subunits of G proteins; PRL, prolactin.

microtubule system. Given the ubiquitous and highly conserved nature of G proteins and tubulin, similar interaction between these two protein families may take place in other cell types as well. In the future, with the availability of sufficient quantities of purified α, β, and γ subunits of G_q, it should be possible to delineate the role of the balance of factors that regulate tubulin polymerization.

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