G Protein β2 Subunit Antisense Oligonucleotides Inhibit Cell Proliferation and Disorganize Microtubule and Mitotic Spindle Organization

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Abstract   The association of G protein β2 subunit (Gβ2) with mitotic spindles in various mammalian cells has been demonstrated previously. Recently, we have identified the association of Gβ2 protein with microtubules (Wu et al., [1998] J. Cell. Biochem. 70: 552-562). In the present experiment we have demonstrated the possible functional role of Gβ2 in microtubule and mitotic spindle organization in mammalian cells. When Gβ2 antisense phosphorothioate oligonucleotides were transfected into mammalian cells, inhibition of cell proliferation with cell death after a 4-day treatment was observed. If the transfected cells were incubated for two days and their Gβ2 and microtubules were examined by Western blotting and immunofluorescence localization, marked reduction of the Gβ2 protein, fragmentation and disassembly of cytoplasmic microtubules, and disorganized mitotic spindles were found. We conclude that the Gβ2 protein is closely associated with microtubule assembly and may play a potential role in the regulation of cell proliferation and microtubule and mitotic spindle organization in mammalian cells. J. Cell. Biochem. 83: 136–146, 2001.   © 2001 Wiley-Liss, Inc.

Key words: Gβ2 protein; antisense oligonucleotide transfection; microtubule assembly; mitotic spindle organization; nasopharyngeal carcinoma cells

Signal-transducing GTP-binding proteins (G proteins) are present in all eukaryotic cells, and they regulate metabolic, humoral, neural, and developmental functions. G proteins are heterotrimers, the family is known to contain at least 21 genes that encode the alpha subunits, five genes that encode beta subunits, and multiple genes encoding gamma subunits [Gilman, 1987; Simon et al., 1991; Watson et al., 1994; Morishita et al., 1995; Neer, 1995]. The G proteins consist of two functional units, an α subunit that binds and hydrolyzed GTP and a βγ subunit that functions as a single entity. The β and γ subunits form a dimer that only

dissociates when it is denatured. Both α and β subunit can bind to the receptor and activate target effectors.

The β subunits consist of five subtypes and the β5 subunit has been identified in the vertebrate (mouse) retina cells [Watson et al., 1996]. The amino acid sequence of each has between 53 and 90% identity [Simon et al., 1991; Watson et al., 1994]. The β subunits are predicted to contain an N-terminal region thought to form an amphipathic α helix such as coiled-coils [Lupas et al., 1992], followed by seven repeating units of ∼43 amino acids each [Simon et al., 1991]. The repeating units are examples of a class of repeating sequences (WD repeats) found in a family of protein engaged not only in signal transduction, but also in control of cell division, transcription, processing of pre-mRNA, cytoskeletal assembly, and vesicle fusion [Neer et al., 1994, 1995]. Recent experiments have shown that βγ subunits can also direct downstream regulation [Clapham and Neer, 1993]. For example, Gβγ and Gα both activate the cardiac K^+ channel [Codina et al., 1987; Logothetis et al., 1987] and muscarinic potas-

sium channels [Reureny et al., 1994]. Gβγ also regulate the activity of adenylyl cyclase by activating type II and IV, inhibiting type I, and have no effect on type III, V, and VI [Tang and Gilman, 1991; Federman et al., 1992; Taussig et al., 1993] and activate the β-subfamily of phospholipase C (β3 > β2 > β1) [Camps et al., 1992; Katz et al., 1992; Smrcka and Sternweis, 1993]. Gβγ modulate receptor function by controlling receptor phosphorylation and subsequent desensitization [Haga and Haga, 1992; Inglese et al., 1992; Pitcher et al., 1992] and activate phospholipase A2 [Kim et al., 1989] and phosphoinositide 3 kinase [Stephens et al., 1994].

Antisense oligonucleotides with sequences complementary to a specific gene or its mRNA are useful tools in the study of gene functions [Wahlestedt et al., 1993; Schmid et al., 1996; Wu et al., 1997] and may be useful as therapeutic agents [Askari and McDonnell, 1996; Duchosal et al., 1996; Nyce and Metzger, 1997]. Recently, we have shown the association of Gβ2 protein with the mitotic spindles [Lin et al., 1992] and microtubules [Wu et al., 1998]. In this study, we have used a strategy employing antisense oligonucleotides to examine the function of the Gβ2 protein in vivo. We have shown that Gβ2 antisense oligonucleotide caused marked reduction of the Gβ2 protein expression, disassembly of cytoplasmic microtubules and disorganization of mitotic spindles. Our data suggests that Gβ2 may play an important role in regulation of microtubule assembly and stabilization of mitotic spindles.

MATERIALS AND METHODS

Preparation of Antibodies Against Gβ2

Polyclonal rabbit antibodies against the N terminus of β2 subunit of G protein was described previously [Lin et al., 1992]. Other antibodies, such as anti-α- and -β-tubulins were purchased from Sigma Chemical Co. (St Louis, MO); the fluorescence-labeled 2nd antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Antisense Oligonucleotides

Phosphorothioate oligonucleotides (PONs) corresponding to human G protein β2 subunit were synthesized by Oligos Etc., Inc. (Wilson-ville, OR). Two distinct positions of human G protein β2 subunit sequence [Gao et al., 1987] were chosen for the synthesis of antisense PONs: AS-1, (−5)–(+10) (5′-GCTCACT-CATGGCGC-3′), and AS-3, (−1)–(+15) (5′-CTCCAGCTCACTCATG-3′). The sense PONs: S-1, (−5)–(+10) (5′-GCGCCATGAGTGAGC-3′) and S-3, (−1)–(+15) (5′-CATGAGT-GAGCTGGAG-3′) were synthesized as controls. To select a specific antisense probe, one needs to consider the secondary structure, the loop position in the single strand region of mRNA. According to these conditions we found two specific sites good for Gβ2 antisense. These regions are only specific for Gβ2 but not for other subtype of Gβ subunits. One position of human G protein β1 subunit sequence [Codina et al., 1986] was also chosen for the synthesis of antisense B1-AS, (−5)–(+10) (5′-CTCACT-CATCTTCC-3′) and sense B1-S, (−5)–(+10) (5′-GGAAGATGAGTGAGC-3′) for the same studies.

Incorporation of Gβ2 Antisense PONs Into Mammalian Cells and Observation of Their Morphological Changes

For the present study we used nasopharyngeal carcinoma (NPC) cell line (NPC-TW 01) and fibroblasts as cell models. NPC-TW-01 cell line was established in our laboratory [Lin et al., 1993]. HFY1 (human normal fibroblast cell line) was obtained from American type culture collection. To determine the optimal concentration of antisense PONs, NPC cells (6×10^3 cells / well) or HFY1 cell (2×10^3 cells/well) were grown in 96-well plates overnight. They were then transfected with 5 μg/ml lipofectin reagent containing 0, 0.1, 1, and 10 μM PONs, respectively, for 4 h. Cells were further cultured in a regular culture medium containing various concentrations of PONs as mentioned above for five days. Surviving cell numbers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [Mosmann, 1983]. Briefly, cells were treated with 100 μl/well of MTT solution (2 mg/ml) for 4 h at 37°C. The MTT solution was removed and the blue formazan product was dissolved in 100 μl DMSO. The colorimetric determination of MTT reduction was made at an absorption wavelength of 540 nm.

For incorporation of PONs into the NPC cells, cells were cultured in growth medium to 60% confluence in six-well culture dishes. They were

then washed two times with 3 ml serum free medium. For the incorporation experiment, 100 μl of serum free medium containing 5 μg of lipofectin reagent (GIBCO/BRL) was mixed with 100 μl of serum free medium containing the appropriate amount of oligonucleotides, and incubated at room temperature for 15 min. Then, cells in each well were added with 0.8 ml serum free medium, and 0.2 ml of the above mentioned lipofectin–oligonucleotide mixture. The cells were incubated at 37°C for 4 h, washed twice with growth medium to remove the lipofectin solution, and cultured in 2 ml of growth medium containing 1 μM PONs for various times. The transfected NPC cells were observed under the inverted microscope after incubation for 1, 2, 3, 4, and 6 days, respectively. Some cultured cells, after incubation for four days, were reincubated in a fresh normal culture medium for another two days. For comparison, normal human fibroblasts (HFY1) (2×10^3 cells/well) were also transfected by the same sense and antisense PONs. Their morphological changes were examined.

Western Blot Analysis of Gβ2 Protein in NPC Cells After Transfecting Its Antisense PONs

NPC cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum (FCS) in a 10% CO_2 incubator at 37°C. Cells were then transfected with 5 μg/ml of lipofectin reagent containing 1 μM of sense and antisense PONs for 4 h, respectively. NPC cells were further cultured in growth medium (DMEM + 5% FCS) containing 1 μM PONs for another 48 h. The cytosolic extract obtained from the sense and antisense PONs-transfected NPC cells were subjected to Western blot analysis using 12% SDS–PAGE [Lin et al., 1992]. Proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) microporous membrane using a Hoefer Transblot apparatus (Hoefer Scientific Instruments, San Francisco, CA) overnight in transfer buffer (25 mM Tris base, 190 mM glycine, 0.01% SDS, and 20% methanol). The PVDF membranes were incubated with 5% skim milk in TBS solution (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) to block nonspecific binding sites on the blot, and then incubated with primary antibodies at 37°C for 1 h. For the negative control, the antibodies were pretreated with excess peptide antigen. Peroxidase-conjugated goat anti-rabbit IgG was used as the second antibody. The blot was added to the substrate solution (0.05% 3,3′diaminobenzidine·4HCl + 0.01% H_2O_2 in 50 mM Tris-HCl, pH 7.4) at room temperature with agitation until the bands appeared. In order to enhance the Gβ2 protein band from antisense PONs transfected NPC cells for better densitometric analysis, we have applied two fold more protein concentration from this cell extract for Western blotting.

Immunofluorescence Staining of Gβ2 and Tubulin

The localization of Gβ and tubulin by immunofluorescence staining was performed according to our previously published method [Wu and Lin, 1994]. Briefly, sense or antisense PONs transfected NPC cells were fixed in 3% paraformaldehyde for 10 min, then incubated overnight with affinity purified rabbit antibodies against Gβ2 protein [Lin et al., 1992] or monoclonal antibody against tubulin, respectively, followed by incubation with fluorescein or rhodamine-labeled second antibodies, and then mounted for fluorescent microscopic observation. The antibodies anti-Gβ2 protein and anti-α tubulin were used at a concentration of 2 and 1 μg/ml, respectively. For comparison, we also stained the actin molecules in the Gβ2 sense and antisense PON-transfected cells with rhodamine-labeled-phalloidin.

Identification of Apoptotic Cells

Apoptosis was assessed by the DNA terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) as described previously [Gavrieli et al., 1992] with some modification. In brief, NPC cells, after treatment with 1 μM of sense and antisense PONs for 48 h, were fixed for 20 min in a mixture of fixative containing 3% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, which contained 8.5% sucrose and 0.002% $CaCl_2$ (PBSC). After washing, cells were treated with 3% H_2O_2 + 0.1% NaN_3 in PBSC to inactivate endogenous peroxidase activity, followed by washing in PBSC and treated with 0.1% Triton-X-100 for 10 min to permeate the plasma membrane. The cells were rinsed in PBSC and immersed in TdT buffer (30 mM

Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 10 min, then transferred to a fresh TdT buffer containing 1 μM biotin-dUTP and 0.8 e.u./μl of terminal deoxynucleotidyl transferase (Promega, Madison, WI) and incubated at 37°C for 2 h. The cells were then washed in SSC buffer (300 mM NaCl, 30 mM sodium citrate) to stop the reaction. The cells were washed in PBSC, blocked with 2% human serum albumin in PBS for 10 min, rewashed, and incubated with avidin–biotin peroxidase complex (Vector Laboratories, Inc. Burlingame, CA). After 30 min, cells were stained with the chromogen 3,3′-diaminobenzidine tetrachloride.

RESULTS

Determination of the Optimal Concentration of PONs for Transfection Experiment

When different concentration of Gβ2 antisense PONs was transfected into NPC cells, a remarkable inhibition of cell growth was found when 1 μM was used (Fig. 1A). Ten micromolar treatment showed no further remarkable inhibition but increased the nonspecific cytotoxic effect. While 0.01 and 0.1 μM did not revealed any inhibition of cell growth. These findings were also seen in HFY1 cell lines (Fig. 1B). However, inhibition of fibroblasts by 1 and 10 μM antisense PONs (Fig. 1B) seemed slightly weaker than that of NPC cells (Fig. 1A), probably due to the antisense PONs block the cells in mitotic phase, and NPC cells have shorter doubling time (10.5–11 h) than the fibroblasts (>24 h). The sense PONs also showed no specific inhibition of cell growth (Fig. 1). Therefore, we

used 1 μM of PONs for all the following experiments.

Cell Growth After Gβ2 Antisense PONs Transfection

When NPC cells were transfected with control Gβ2 sense PONs, and incubated for various time periods, their cell morphology showed no specific change and cell numbers increased from Day 1 to 4 (Fig. 2A, S from 0 to 4), similar to the untreated NPC cells. However, when Gβ2 antisense PONs (AS-1) were transfected, on Day 1, cell numbers were similar to those on Day 0, but more rounded-up cells than attached cells were seen (Fig. 2A, AS-1, 1). On Day 2 (Fig. 2A, AS-1, 2), the total cell number including the rounded-up cells was slightly lower than that of Day 0 and some cells showed membrane blebbing. On Day 3 (Fig. 2A, AS-1, 3), the cell number was lower than that of Day 2, but some rounded-up and interphase cells were still visible. On Day 4 (Fig. 2A, AS-1, 4), the total cell number was markedly reduced. On Day 6 (data not shown), most tumor cells revealed degeneration and death. In the antisense PONs treated cells, we have found some tumor cell showing cellular blebbing, indicative of apoptosis. To further confirm antisense PONs induced cell death was through apoptosis, in situ TUNEL staining was performed. When the two day-antisense PONs treated cells were analyzed with TUNEL reaction, some attached cells revealed brown-yellow reaction product in their nuclei (Fig. 5F) but not in sense PONs treated cell (Fig. 5E). On Day 4, if normal growth culture medium was used to replace the medium containing antisense PONs, after 2 days (Fig. 2A, AS-1, 2′) the cell number started

NPC 039
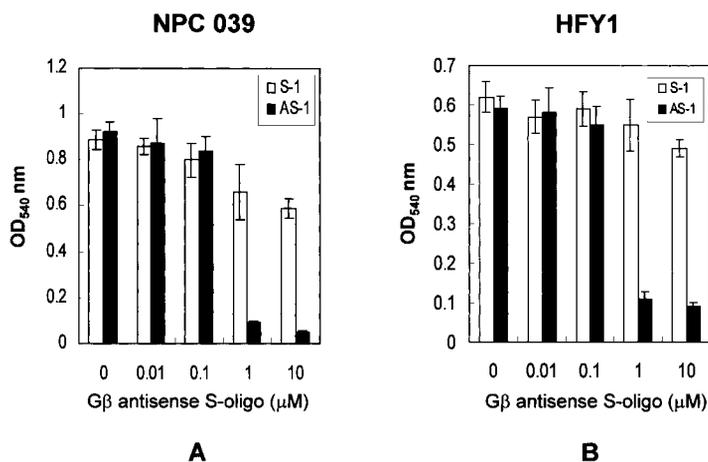
HFY1

Fig. 1. Effects of different doses of Gβ2-specific antisense oligonucleotides on proliferation of NPC and HFY1 cells. NPC-TW039 (**A**) and HFY1 (**B**) cells were seeded at 6×10^3 and 2×10^3/well in 96-well plates and allowed to grow for overnight in 5 and 10% FCS, respectively. The cells were incubated with different concentrations (0.01, 0.1, 1, and 10 μM) of Gβ2-specific sense or antisense oligonucleotides, respectively. Control cultures (0 μM) received no oligonucleotides. After five days of culture, the surviving cell numbers were determined by MTT assay. The optimal concentration of antisense PONs is 1 μM.

to increase; after 4 days (Fig. 2A, AS-1, 4′) the cell number and morphology had recovered to their original state. When a human fibroblast cell line (HFY1) was transfected with these sense (S-1) and antisense PONs (AS-1), the results were similar to those observed in NPC cells (Fig. 2B).

Effect of Gβ2 Protein Expression and Distribution in NPC Cells After Transfection of Its Antisense PONs

When Gβ2 antisense PON-transfected NPC cells were cultured for two days and collected, their proteins isolated and analyzed by Western
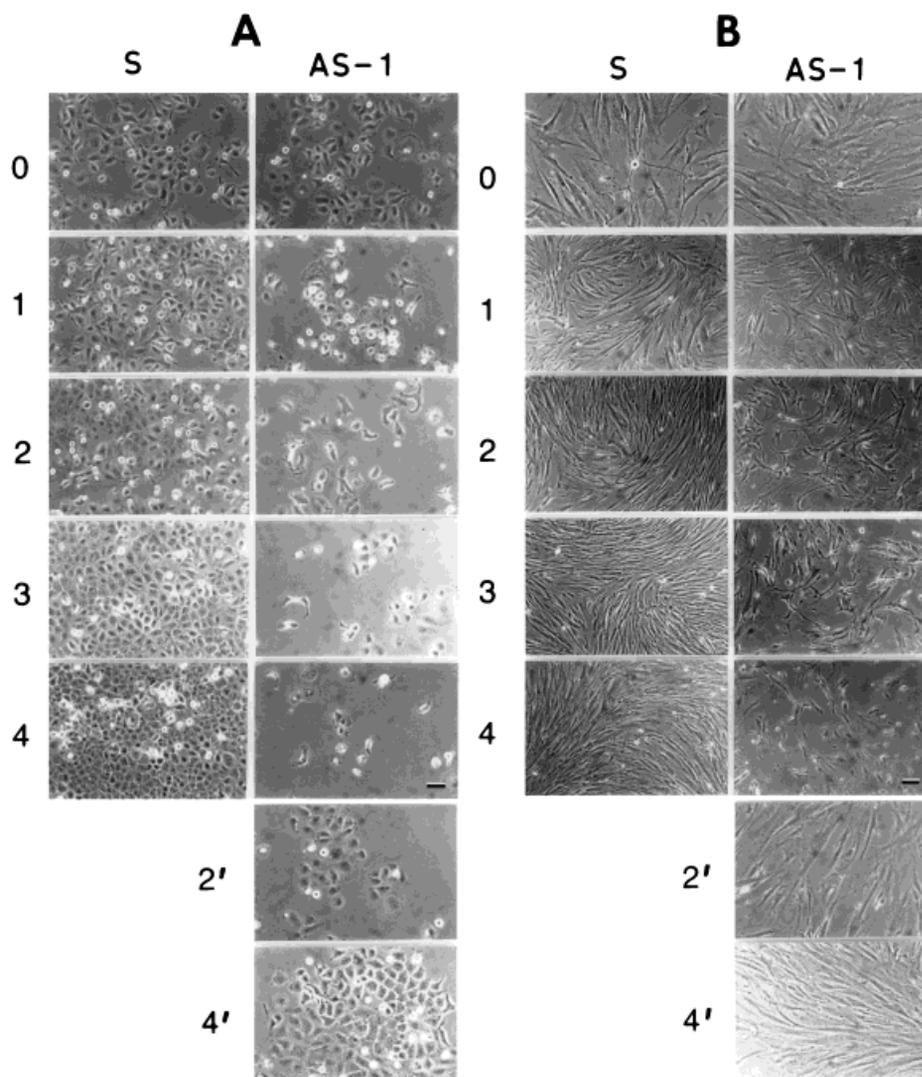
Fig. 2. Effects of Gβ2 antisense PONs on NPC cell proliferation. NPC cells were transfected with 1 μM Gβ2 sense (S) or antisense (AS-1) PONs for 4 h and incubated in culture medium containing 1 μM PONs for 1, 2, 3, and 4 days, respectively. On the fourth day some cells were washed and replaced with fresh normal culture medium and incubated for another 2 or 4 days. **A:** NPC cells. **B:** Human fibroblasts. In the Gβ2 sense PON-transfected cells from Day 0 to 4, the cell numbers increased daily with normal morphology (A: S, 0–4; B: S, 0–4). In the Gβ2 antisense PON-transfected cells (A: AS-1, 0–4, 2′–4′; B: AS-1, 0–4, 2′–4′), at Day 1, the number of rounded up cells is increased, but the total cell number is stable; at Day 2, both the rounded up cell number, and the total cell number are slightly decreased, with some cells showing membrane blebbing; at Day 3, the total cell number is moderately decreased and some cells are rounded up and degenerated. At Day 4, the total cell number is markedly reduced, leaving only a few cells attached. When the cells at Day 4 were washed and replaced with the fresh medium and incubated for another 2 days (2′), some remaining cells start to proliferate and increase the cell number; after 4 days incubation (4′), the cell number is markedly increased, showing a number similar to the original plated cells (Day 0). Both the NPC cells and human fibroblasts show a similar response to antisense PON-transfection. (Bar = 32 μm in A and B).

blotting using antibodies against Gβ and α-tubulin, a remarkable decrease of Gβ2 protein but not Gβ1 subunit or α-tubulin was observed (Fig. 3, lane 2), when they were compared with sense PON-transfected cells (Fig. 3, lane 1). This result shows an inhibitory effect of antisense oligo to Gβ2 protein expression. (Since Gβ1 and Gβ2 have a similar molecular weight with same number of amino acid residues but different post-translational modification, it is difficult to separate both Gβ1 and Gβ2 molecules into two bands). In order to enhance the Gβ2 signal in the antisense treated specimen and to quantitative easier by densitomentry, we have loaded two-fold more protein to lane 2 for the Western blotting. Therefore the internal control α tubulin band is stronger in lane 2 than in lane 1; similarly the β1 band is also stronger in lane 2 than in lane 1. For better understanding of the result of this Western blotting we have used densitometry to quantitate the signal intensities of the proteins in two lanes. The result showed 84.4% protein inhibition of Gβ2 by its antisense oligo transfection as shown in Table I. If NPC cells were transfected with the negative control Gβ2 sense PONs and cultured for two days, then fixed for immunostaining using antibodies anti- Gβ2 protein, the distribution of Gβ2 protein was localized on the plasma membrane, in the perinuclear and Golgi regions and in some nonspecific cytoplasmic areas (Fig. 4A). However, when the Gβ2 antisense PONs (AS-1) were used under the same conditions, most of the Gβ2 immunostaining disappeared, leaving only a few spots (Fig. 4B, arrowhead) in the cytoplasm of each cell. (The site of each Gβ-stained spot might be the centrosome.) A similar

Fig. 3. Western blot analysis of Gβ proteins and tubulin in NPC cells transfected with Gβ2 sense (**lane 1**) and antisense (**lane 2**) PONs for 48 h. Antibodies against Gβ2 (cross reacted with Gβ1), and α-tubulin were used to stain the total cell extract(s). Lane 1: Sense PON-transfected cells showing one Gβ1 (Mr 36 kD) band and one Gβ2 (Mr 35 kD) band. Lane 2: Antisense PON-transfected cells showing a normal Gβ1 band and a very weak Gβ2 band. The internal control α-tubulin (α-tub) band in lane 2 is stronger than in lane 1, because the protein concentration applied to lane 2 was two-fold higher than in lane 1.

result was observed when another Gβ2 antisense PONs (AS-3) was applied (data not shown).

Morphological Changes of Microtubules and Mitotic Spindles in NPC Cells and Fibroblasts After Gβ2 Antisense PON Transfection

When NPC cells well transfected with Gβ2 antisense PONs for two days, more rounded-up cells were observed. The increase of rounded-up cells may be due to an arrest of the transfected cells at metaphase from the alteration of microtubule and mitotic spindle organization. In order to investigate this phenomenon, we have observed the change of microtubule distribution in the cells transfected with Gβ2 antisense PONs by immunofluorescence staining using antibody against tubulin. When NPC cells were treated with Gβ2 sense PONs for two days and fixed for anti-tubulin staining, the distribution of microtubules in the interphase and mitotic cells was normal (Fig. 4C and G). If,

TABLE I. Effects of Gβ2 Antisense Oligonucleotide on Gβ2 Protein Expression in NPC Cells[a]

Treatment	Density ratio of Gβ2/Tubulin protein	Density ratio of Gβ1/Tubulin protein
Gβ2 sense	0.697	0.711
Gβ2 antisense	0.109	0.653

[a]NPC cells were treated with sense and antisense PONs for two days. The Gβ proteins and tubulin in PONs treated cells were analyzed by Western blotting and the relative protein signal intensities were determined by densitometric analysis. In Western blotting, two-fold more protein concentration from antisense treated cells than that of sense treated cells was loaded. The density ratio shown in the table was obtained after two-fold more protein value was adjusted. Tubulin ratio of sense: antisense = 1:1.109.

Fig. 4. Immunofluorescence localization of Gβ2 protein and α-tubulin in NPC cells and human fibroblasts transfected with Gβ2 sense and antisense phosphorothioate oligonucleotides (PONs). NPC cells were transfected with 1 μM of Gβ2 sense (**A**) and antisense (**B**) PONs, respectively, incubated for two days and fixed for immunofluorescence staining using anti-Gβ antibody. A: NPC cells transfected with Gβ2 sense PONs. The distribution of Gβ protein in NPC cells is mainly in the Golgi and perinuclear regions (arrowheads) and on the plasma membrane (solid arrows). B: NPC cells transfected with Gβ2 antisense PONs. The fluorescein-labeled Gβ protein is markedly reduced. Very weak staining on plasma membrane, in the perinuclear and Golgi regions is shown. In each cell Gβ staining is revealed only at one or two spots (arrowheads) (Bar = 20 μm in A and B). NPC cells (**C–I**) and fibroblasts (**J–L**) were transfected with Gβ2 sense (C, G, and J) and antisense (D–F, H, I, K, and L) PONs and incubated for 2 days. Cells were then fixed for immunostaining of α-tubulin. In the sense PON-transfected cells, normal distribution of microtubules in the interphase cell (C and J) and mitotic spindles in the M phase cell (G) is found. In the antisense PON-transfected cells, however, the microtubules in the interphase cells show a markedly disorganized and fragmented pattern (D, K, arrowhead) and disassembled feature (E, J, arrowhead). In some cells, marked reduction of the number of microtubules can be seen (F); in the mitotic cells, markedly disorganized mitotic spindles are shown (H, I, arrowheads). (Bar = 10 μm in C–F and J–L; Bar = 20 μm in G–I).

however, Gβ2 antisense PONs were used, most interphase cells (about 60–70%) revealed marked disorganization and fragmentation of microtubules (Fig. 4D and E, arrowheads). Some even showed severe reduction of microtubule number (Fig. 4E and F). The mitotic spindles also revealed marked disorganization in most mitotic cells (Fig. 4H and I). When human fibroblasts (HFY1) were transfected with Gβ2 antisense PONs, a remarkable fragmentation of microtubules (Fig. 4K, arrowhead) and reduction of their number (Fig. 4L) could also be identified, similar to the NPC cells. Fibroblasts transfected with Gβ2 sense PONs showed normal microtubule distribution (Fig. 4J). Similar findings, such as disorganization and fragmentation of microtubules, were also observed when the other Gβ2 antisense PONs (AS-3) was applied (Fig. 5B). When

fibroblasts were also treated with Gβ1 antisense PONs (B1-AS) for two days and fixed for anti-tubulin staining, the distribution of microtubules in the interphase cells was normal (Fig. 5A). If NPC cells (data not shown) or fibroblasts (Fig. 5C and D) were transfected with sense (S-1) or antisense (AS-1) PONs and the actin proteins were stained with rhodamine labeled phalloidin, the distribution of stress fibers in both sense and antisense transfected cells showed similar normal distribution.

DISCUSSION

For observation of the functional relationship between Gβ2 subunit and cell growth, NPC cells were transfected with Gβ2 antisense PONs. Result showed that the cell proliferation was markedly arrested. Some cells were rounded up
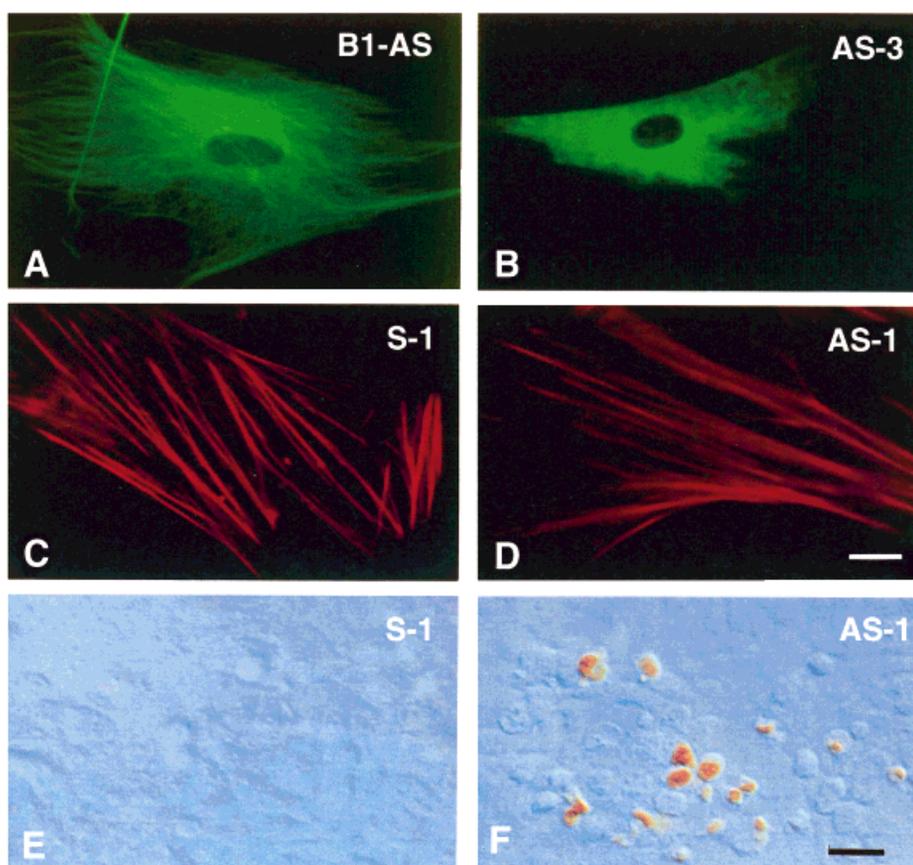
Fig. 5. **A–D:** Immunofluorescent localization of tubulin and actin in human fibroblasts, which have been transfected with sense and antisense PONs. Cells were transfected with 1 μM of Gβ1 antisense (B1-AS, A) and Gβ2 antisense (AS-3, B) PONs, respectively. Fibroblasts transfected with Gβ2 sense (S-1, C) and antisense (AS-1, D) PONs show a same immunofluorescent actin pattern, similar to the actin distribution in a normal fibroblast. **E** and **F:** Gβ2 antisense PONs induces apoptosis in NPC cells. The NPC cells were treated with sense (E, S-1) and antisense (F, AS-1) PONs two days and fixed for TUNEL assays. Apoptotic nuclei are identified by brown-yellow staining in antisense PONs treated cells (F) but not in sense PONs treated cells (E). (Bar = 20 μm in A–D; Bar = 40 μm in E and F).

gradually, and revealed degenerated and apoptotic changes, probably due to the loss of the functions of the Gβ2 protein or the effects of its downstream proteins, through some unidentified mechanisms. For investigation of the effect of antisense PONs transfection on the Gβ2 protein synthesis, the Gβ2 antisense PONs-transfected NPC cells were examined morphologically and biochemically. It was found that Gβ2 antisense PONs can suppress Gβ2 protein synthesis (84.4% protein inhibition) and cannot suppress Gβ1 or α-tubulin protein synthesis. Furthermore, we have also observed the distribution of actin in the antisense transfected cells. When Gβ2 antisense PON-transfected cells were stained with rhodamine labeled phalloidin, the actin distribution was found to be similar to the sense transfected cells (Fig. 5C and D). These results suggest that Gβ2 antisense PONs cannot inhibit other unrelated protein synthesis. In our other experiments, we have found that only Gβ2 antisense PONs inhibits the cell proliferation but not Gβ1, Gβ3, and Gβ4 antisense PONs. Furthermore, the Gβ1 antisense PONs could not cause microtubule disassembly too (Fig. 5A).

The findings that marked fragmentation and reduction of microtubules in the interphase cells and disorganization of mitotic spindles in the mitotic cells in the antisense PON-transfected NPC cells, clearly demonstrate that the Gβ2 subunit may play some role in regulation of microtubule and mitotic spindle organization. Although it may not be a direct cause-and-effect relation, we propose that it is an indirect effect through the regulation of other factors by the Gβ2 subunit, because our experimental data show that the concentration of Gβ2 protein in NPC cells is much weaker and less than that of tubulin (Fig. 3, lane 1). It is unlikely that the Gβ2 subunit is involved in regulation of microtubule disassembly because the microtubules were disassembled when the Gβ2 subunit was depleted. The same phenomenon is found similar in NPC tumor cells and normal human fibroblasts (Fig. 4A). These findings, together with the fact that no change of α-tubulin or actin molecules is found in the Gβ2 antisense PON-transfected cells (Fig. 3), indicate that the Gβ2 subunit is essential for regulating microtubule assembly. Lack of Gβ2 protein can result in disassembly of cytoplasmic microtubules and disorganization of mitotic spindles as shown in the present experiment.

It has been shown that the structure of β subunit contains seven repeating units (WD repeats) as described by Neer [1995]. This family of proteins is engaged not only in signal transduction, but also in control of cell division, cytoskeletal assembly, vesicle fusion, etc. as mentioned in the introduction. Our present experiment provides evidence to support the hypothesis that Gβ2 protein containing WD repeats may engage in microtubule assembly in NPC cells, a result similar to the recent reports that βγ subunit can enhance microtubule assembly in an in vitro cell free system [Roychowdhury and Rasenick, 1997; Wu et al., 1998].

It is well known that the extent of microtubule stabilization by the microtubule-associated proteins (MAPs) depends on the affinity of these proteins for the microtubules and this affinity is under cellular control. Phosphorylation of MAPs often suppresses microtubule assembly [Lindwall and Cole, 1984; Gelfand, 1991; Correas et al., 1992]. Several MAPs have been reported to be phosphorylated by protein kinases. For example, Tau protein can be phosphorylated by calcium/calmodulin dependent protein kinase [Baudier and Cole, 1987] and protein kinase C [Hoshi et al., 1987]. MAP2 is phosphoylated by cAMP-dependent protein kinase [Theurkauf and Vallee, 1983], protein kinase C [Tsuyama et al., 1986], insulin receptor kinase [Kadowaki et al., 1985], and mitogen-activated protein kinase [Hoshi et al., 1992]. Binding of MAPs such as MAP2 to microtubules and its ability to stimulate tubulin polymerization were reduced after MAPs are phosphorylated [Murthy and Flavin, 1983; Correas et al., 1992]. Shiina et al. [1992] have identified and purified a major MAP (p220) from Xenopus eggs. P220 protein purified from interphase cells bound tightly to microtubules and stimulated tubulin polymerization, whereas p220 purified from mitotic cells was phosphorylated and showed little or no such activity. Gliksmann et al. [1992] have found that addition of okadaic acid (an inhibitor of phosphatase 1 and 2A) to the extract of interphase sea urchin eggs induced rapid (<5 min) conversion to short, dynamic microtubules typical in mitosis. The major change was an elimination of rescue. Thus, modulation of the rescue frequency by phosphorylation-dependent mechanisms may be a major regulatory pathway for selectively controlling microtubule dynamics, without dra-

matically changing velocities of microtubule elongation and shortening [Gliksmann et al., 1992]. Since a depletion of Gβ2 subunit could decrease microtubule assembly in NPC and fibroblast cells as shown in the present experiment, this depletion of Gβ2 subunit may either have a function similar to that of okadaic acid which inhibits phosphatase activity, or have a function similar to enhance the protein kinase activity. This would result in increase phosphorylation of MAPs and thus lead to decreased affinity of MAPs for microtubules and decreased microtubule assembly. In order to clarify whether the Gβ2 protein can regulate dephosphorylation of MAPs as proposed by us, and whether or not this regulation of dephosphorylation of MAPs is due to increased phosphatase activity or decreased protein kinase activity, this hypothesis needs to be confirmed by further biochemical experiments.

In conclusion, our experiments indicate that Gβ2 protein is associated with microtubule and may play an important role in regulation of microtubule assembly and mitotic spindle organization.

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