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Activating and inactivating mutations of the α subunit of G_{i2} protein have opposite effects on proliferation of NIH 3T3 cells

(guanine nucleotide-binding proteins/site-directed mutagenesis/stable transfection/ADP-ribosylation)

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ABSTRACT **Previous studies have demonstrated that mutations of highly conserved residues in the α subunit of G_s (α_s) can inhibit either the intrinsic GTPase activity (glutamine-227 to leucine, Q227L) or the ability of the protein to be activated by GTP (glycine-226 to alanine, G226A). We stably transfected NIH 3T3 cells with cDNAs encoding G_{i2} α subunit (α_{i2}) containing either wild-type sequence or the homologous mutations Q205L and G204A. High expression of wild-type α_{i2}, Q205L α_{i2}, and G204A α_{i2} was confirmed in transfected cells by immunoblot analysis. The overexpression of all three α_{i2} proteins was accompanied by an increase in β-subunit expression. Q205L α_{i2} was a poor substrate for ADP-ribosylation by pertussis toxin as compared with wild-type α_{i2}. Expression of Q205L α_{i2} markedly decreased forskolin- or cholera toxin-stimulated intracellular cAMP levels in intact cells, confirming the constitutively activated state of the protein. In contrast, G204A α_{i2} increased intracellular cAMP and was resistant to guanosine 5′-[γ-thio]triphosphate-induced inhibition of ADP-ribosylation by pertussis toxin, as expected for an inactive α_{i2}. Transfection of wild-type, Q205L, or G204A α_{i2} cDNA did not induce focus formation of NIH 3T3 cells. However, overexpression of Q205L α_{i2} induced a decreased serum requirement, a reduced doubling time, and an 8- to 10-fold increase in [^{3}H]thymidine incorporation. Q205L α_{i2} cells formed small colonies in soft agar, demonstrating some degree of anchorage-independent proliferation. Expression of G204A α_{i2} slowed the growth of NIH 3T3 cells. We conclude that α_{i2} plays an important role in regulation of fibroblast growth.**

G proteins serve as intermediaries between activated membrane receptors and their effector enzymes and/or ion channels (1, 2). They are heterotrimeric proteins, composed of three subunits: α, β, and γ. The α subunit binds guanine nucleotide, possesses intrinsic GTPase activity, and in most cases is responsible for modulation of effector activity. The domains involved in binding GTP are highly conserved among diverse members of the G-protein family (3, 4). One such domain [G-3 region (3)], implicated in binding to the γ phosphate of GTP and changing conformation upon binding of GDP vs. GTP, has the consensus sequence DVGGQR (single-letter amino acid code) in most α subunits. Since function of GTP-binding proteins is regulated by the binding of GTP vs. GDP, mutations that alter hydrolysis of GTP or activation by GTP will have a critical effect on signal transduction. Mutation of glutamine-61 in the G-3 region of $p21^{ras}$ results in constitutive activation through a decreased GTPase activity. Mutation of the comparable amino acid, glutamine-227, in the α subunit of G_s (α_s), the G protein that mediates stimulation of adenylyl cyclase, leads to a constitutive increase in cAMP formation (5–8). Mutation of glycine-226 to alanine (G226A) in the G-3 region of α_s blocks activation of adenylyl cyclase, because α_s is unable to assume the GTP-bound active configuration (9, 10). The mutant protein is, however, capable of coupling normally with receptors. Such a mutant could function as a dominant inhibitor.

Initially, only a limited number of G proteins were recognized. G_s and G_i were originally identified functionally as the G proteins that stimulate and inhibit adenylyl cyclase activity, respectively. The latter response could be blocked by pertussis toxin. It is now clear that there is substantial diversity in the G-protein family (4). There are multiple pertussis toxin-sensitive G proteins encoded by three distinct α_i genes, α_{i1}, α_{i2}, α_{i3}, and by an α_o gene (1–4). Their specific functions are still mostly unknown, and which protein is responsible for adenylyl cyclase inhibition is not clear for most cell types. Inhibition by pertussis toxin implicates one or more of these G proteins in a variety of biological effects. An important example is control of cell proliferation, particularly in fibroblasts (11–13). Pertussis toxin partially inhibits fibroblast proliferation stimulated by serum, and this inhibition may be specific to certain growth factors or hormones (13–15). α_i is also involved in cell differentiation, particularly in myeloid cells (16, 17). Which of the three α_i genes is responsible for pertussis toxin action on cell proliferation and differentiation is unknown.

To begin to address this question, we made the mutations homologous to α_s Q227L and G226A in one of the three candidate α_i genes, α_{i2}. Given the conservation of the GTP-binding site, these mutations should produce constitutively active (Q205L) and inactive (G204A) forms of α_{i2}. Such mutants could stimulate (Q205L) or inhibit (G204A) α_{i2} signaling pathways. These α_{i2} mutants were stably transfected into NIH 3T3 mouse fibroblasts and tested for their degree of activation, their ability to decrease cAMP production, and their effect on NIH 3T3 cell growth.

MATERIALS AND METHODS

Cell Cultures. Cell culture reagents and Geneticin (G418) were purchased from Biofluids (Rockville, MD) and GIBCO/BRL. NIH 3T3 cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (18). Medium was changed twice a week.

Site-Directed Mutagenesis and Construction of Expression Vectors. The insert of plasmid DJG18, encoding a rat α_{i2} cDNA (19) (kindly provided by R. Reed, Johns Hopkins University), was subcloned into M13mp19 and site-directed mutagenesis was performed by a modification of the method of Nakamaye and Eckstein (20) (Amersham, catalogue no. RPN.2322). The mutagenic oligonucleotides had the following sequences: G204A α_{i2}, 5′-CCGCTCAGATCGC-

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Abbreviation: GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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TGGGCGCCCACATCAAACAT-3'; Q205L α_{i2}, 5'-CTTCCGCTCAGATCGTAGACCACCCACATC-3'. Mutations were confirmed by dideoxy sequencing. Wild-type and mutant α_{i2} cDNAs were ligated into the *Eco*RI site of shuttle vector pSP72NotI and then into the *Not* I site of a modified retroviral expression vector, pZipNeoSV(X) (21). In the latter constructs, the transcription of α_{i2} cDNAs and the G418-resistance gene is directed by the Moloney murine leukemia virus long terminal repeat.

Transfection of NIH 3T3 Cells. NIH 3T3 cells were transfected with 1 μg of linearized DNA per 10-cm plate by calcium phosphate precipitation (Stratagene mammalian transfection kit, catalogue no. 200285) (22). Twenty-four hours after transfection, cells were grown in selective medium containing G418 (0.75 mg/ml) for at least 2 weeks. G418-resistant cells were cloned by serial dilution. Twelve clones were selected for each construct, and the studies described in this article were performed with the clones demonstrating the highest level of α_{i2} expression (three or four clones for each construct). All results shown are of one clone that was representative of the three or four clones tested for each construct.

Immunoblot Analysis. Confluent cultures of NIH 3T3 clones were scraped, pelleted by centrifugation, and washed three times in 10 ml of phosphate-buffered isotonic saline (PBS) at pH 7.5. The cell pellets were frozen (−70°C) and thawed once, homogenized in a Dounce homogenizer, and centrifuged for 10 min at 1000 rpm at 4°C. The supernatant was centrifuged for 30 min at 14,000 rpm (11,600 × *g*) in a microcentrifuge at 4°C. Pellets (membranes) were resuspended and stored at −70°C in 10 mM Tris buffer, pH 7.4, containing 0.25 M sucrose and 0.10 mg of leupeptin per ml. The protein concentration was determined by the Bradford method (23) with IgG (Bio-Rad) used as a standard.

Membrane proteins (100 μg per lane) were resolved in an SDS/10% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the following affinity-purified antisera: AS7, specific for α_{i1} and α_{i2}; RM, specific for α_s; and MS, specific for β subunits (24–26).

ADP-Ribosylation by Pertussis Toxin. Membrane proteins (10 μg) were suspended in 50 μl of 100 mM Tris buffer (pH 7.5) containing 10 mM thymidine, 1 mM ATP, 0.1 mM GTP or 0.1 mM guanosine 5'-[γ-thio]triphosphate (GTP[γS]), 10 mM arginine, 0.1% Lubrol, and 5 μCi of [^{32}P]NAD (New England Nuclear, 800 Ci/mmol; 1 Ci = 37 GBq) and were preincubated for 15 min at 37°C. Preactivated pertussis toxin (List Biological Laboratories, Campbell, CA; 0.5 μg in 10 μl) was added and ADP-ribosylation carried out at 37°C for 20 min. The reaction was stopped with 50 μl of stopping buffer (0.02% bovine serum albumin/0.5% sodium deoxycholate/500 μM NAD) and proteins were precipitated with 1 ml of ice-cold 20% trichloroacetic acid. After centrifugation (10 min, 11,600 × *g*, 4°C), supernatants were discarded and pellets washed once with cold acetone. Pellets were resuspended and analyzed by SDS/10% PAGE followed by autoradiography.

cAMP Assay. NIH 3T3 cells were plated at 50,000 cells in 1 ml per well and grown to half-confluency in DMEM with 10% calf serum in 24-well plates (previously coated with fibronectin, 1 μg/ml in 1 ml of PBS, for 60 min at 37°C). The medium was replaced with DMEM (1 ml per well) containing 10 mM Na Hepes (pH 7.5), 1 mM 3-isobutyl-1-methylxanthine, and either 0.1 mM forskolin or 1 nM cholera toxin. The plates were incubated at 37°C for 30 min and then placed on ice. The incubation medium was replaced with 1 ml of 0.1 M HCl/0.1 mM $CaCl_2$ and the plates were stored at −20°C. After thawing, samples were acetylated by adding 20 μl of acetic anhydride/triethylamine (1:2.5, vol/vol). cAMP was measured by radioimmunoassay (27) and DNA content of each well was determined to normalize for differences in cell number.

Soft-Agar Colony and Transformation Assays. For assays of anchorage-independent growth, suspensions of 5×10^3 cells in a growth medium containing 0.33% agar (Difco) were overlaid onto 0.55% agar gel. Colonies over 0.10 mm in diameter were scored after a 15-day incubation at 37°C. For the transformation assays, NIH 3T3 cells were transfected with 1 or 5 μg (α_{i2} constructs) or 0.2 μg (v-*src*) of DNA per 10-cm plate. Transformed foci were scored after 2 or 3 weeks.

Doubling Time. Cells were plated at 10^3 cells per well in 24-well plates and grown in DMEM with 10% calf serum. Twenty-four hours later, cells were trypsinized and counted in triplicate in a hemocytometer. The cells were then grown in DMEM with 1% or 5% calf serum, with the medium replaced every other day. Cell counts were determined in triplicate every day from day 1 to day 6. The results obtained were analyzed for exponential doubling time.

[^{3}H]Thymidine Incorporation. Twenty-four-well culture plates were coated with fibronectin. Cells were then plated at 10,000 cells per well with 1 ml of medium and incubated for 48 hr at 37°C in DMEM with 10% calf serum. Cells were washed three times with PBS and then incubated with DMEM containing various concentrations of calf serum (0%, 1%, 5%) overnight at 37°C. One microcurie of [^{3}H]thymidine (Amersham, 28 Ci/mmol) was added to each well. Cells were incubated for 6, 12, 18, 24, or 48 hr and then washed twice with 1 ml of cold PBS and twice with 1 ml of cold 5% trichloroacetic acid and solubilized with 250 μl of 0.25 M NaOH. After 2 hr at 37°C, the 250-μl cell lysates were analyzed for ^{3}H cpm by scintillation counting. Experiments were carried out in triplicate and repeated at least three times.

RESULTS

Expression of α_{i2} in Transfected Cells. G418-resistant NIH 3T3 cells were obtained for all α_{i2} constructs. After serial dilution cloning, 12 clones were selected for each construct and screened for membrane expression of α_{i2} by immunoblotting with AS7 antiserum. Since NIH 3T3 cells express α_{i2} and α_{i3} but not α_{i1} (A.M.S., unpublished observation), AS7 immunoreactivity is a measure of α_{i2} expression. For each construct, we chose the 3 or 4 clones expressing the highest levels of α_{i2} for the experiments described below (Fig. 1). Nontransfected NIH 3T3 cells and NIH 3T3 cells transfected with vector alone expressed α_{i2} at a comparable level. The expression of wild-type α_{i2} and Q205L α_{i2} was similarly high, ≈10 times the level of control cells. G204A α_{i2} was expressed at a lower level (Fig. 1*A*), up to 5 times the level of

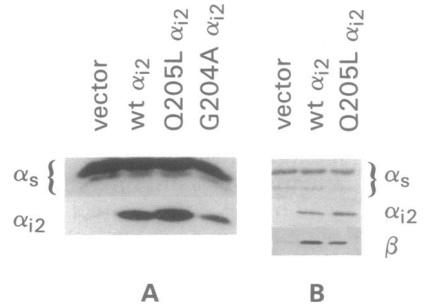

FIG. 1. Expression of α_{i2}, α_s, and β subunits in transfected NIH 3T3 cells. Membrane proteins (100 μg per lane) of NIH 3T3 cells transfected with vector alone or with wild-type (wt), Q205L, or G204A α_{i2} cDNA were resolved by SDS/10% PAGE and transferred to nitrocellulose. The blots were incubated with AS7 (α_{i2}) or RM (α_s) antiserum (*A*) or with AS7, RM, or MS (β) antiserum (*B*). Detection of the antibody–antigen complex was by ^{125}I-labeled protein A (*A*) or by peroxidase-labeled second antibody (*B*).

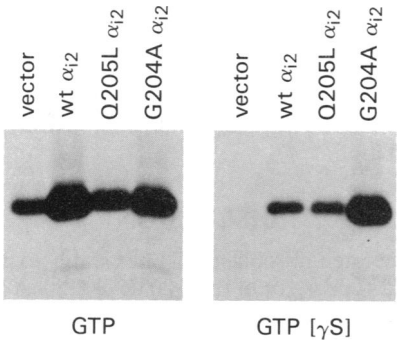

FIG. 2. ADP-ribosylation of transfected wild-type and mutant α_{i2} by pertussis toxin. Membranes (10 μg of protein) of NIH 3T3 cells transfected with vector alone or with wild-type (wt), Q205L, or G204A α_{i2} cDNA were preincubated for 15 min at 37°C in the presence of either 0.1 mM GTP or 0.1 mM GTP[γS] and then ADP-ribosylated by pertussis toxin for 30 min at 37°C. The autoradiogram was developed after 4 hr.

endogenous α_{i2} expression in control cells. Immunoblotting carried out on the same membrane preparations with RM antiserum showed some degree of variation in α_s expression in different NIH 3T3 clones, but only 2 clones transfected with α_{i2} cDNAs out of 16 tested showed a significant (≈50%) reduction in α_s expression compared with control cells. Changes in α_s expression were not correlated with changes in cAMP production and growth rate (see below) observed in clones transfected with α_{i2} cDNAs. Immunoblotting with MS antiserum showed at least a 3-fold increase in β-subunit expression in the cells transfected with wild-type α_{i2}. The increase in β subunits in Q205L α_{i2} cells appeared smaller (Fig. 1*B*).

The α Subunits Expressed by Transfected Wild-Type, Q205L, and G204A α_{i2} Constructs Interact with βγ and Are ADP-Ribosylated by Pertussis Toxin. Pertussis toxin ADP-ribosylates α_{i2} when the G_{i2} protein is in its heterotrimeric form (28). Membranes from transfected cells were treated with pertussis toxin in the presence of [^{32}P]NAD (Fig. 2). As expected, membranes containing wild-type α_{i2} showed a marked increase in labeling, confirming that transfected wild-type α_{i2} can interact and associate with βγ. The level of ADP-ribosylation was similar for wild-type and G204A α_{i2} cell membranes. However, Q205L α_{i2} cell membranes demonstrated a lower level of ADP-ribosylation despite somewhat higher levels of α_{i2} protein expression (see Fig. 1).

Table 1. cAMP accumulation in NIH 3T3 cells expressing wild-type and mutant α_{i2}

Cells	cAMP, pmol per μg of DNA per 30 min		
	Basal	Forskolin (0.1 mM)	Cholera toxin (1 nM)
Vector	6.6 ± 0.6	438 ± 22	84 ± 7
Wild-type α_{i2}	6.9 ± 1.6	465 ± 23	87 ± 17
Q205L α_{i2}	8.1 ± 0.6	182 ± 11	48 ± 1
G204A α_{i2}	15 ± 1.7	523 ± 96	121 ± 4

Values represent the mean (±SEM) of triplicates for one clone representative of three for each construct. Experiments have been repeated three times.

GTP[γS], a nonhydrolyzable GTP analogue, activates α subunits and causes irreversible dissociation of α from the βγ subunit complex, thus preventing ADP-ribosylation of α_i proteins by pertussis toxin (1–3, 28). GTP[γS] pretreatment eliminated labeling in control cell membranes, and decreased labeling significantly in wild-type α_{i2} and Q205L α_{i2} cell membranes but did not change the level of ADP-ribosylation in G204A α_{i2} cell membranes (Fig. 2).

Effects of Transfected α_{i2} on Intracellular cAMP Levels. Cells transfected with vector alone or with wild-type α_{i2} increased their intracellular cAMP 65-fold when stimulated by 0.1 mM forskolin and 13-fold when stimulated by 1 nM cholera toxin (Table 1). In contrast, cells transfected with Q205L α_{i2} showed only a 23-fold intracellular cAMP increase in the presence of forskolin and a 5-fold increase in the presence of cholera toxin. This is a 55% (forskolin) and 45% (cholera toxin) inhibition of stimulated adenylyl cyclase activity. In contrast, cells transfected with G204A α_{i2} demonstrated an increase in both basal and stimulated intracellular cAMP levels.

Effects of α_{i2} on NIH 3T3 Cell Proliferation. *Soft-agar colony and transformation assays.* The acquisition of anchorage-independent growth often correlates with tumorigenicity. Clones expressing α_{i2} were assayed for their ability to form colonies in soft agar. Cells (5×10^3) containing vector alone or the wild-type or G204A α_{i2} construct failed to grow in 0.3% agar, whereas cells containing Q205L α_{i2} construct formed small colonies (Fig. 3). The size and number of these colonies were considerably smaller than those observed with v-*src*-transfected cells (Table 2). However, transfection with Q205L, wild-type, or G204A α_{i2} cDNA did not induce focus formation in NIH 3T3 cells. Parallel transfections with v-*src* (0.2 μg of DNA per plate) (29) consistently yielded >100 foci

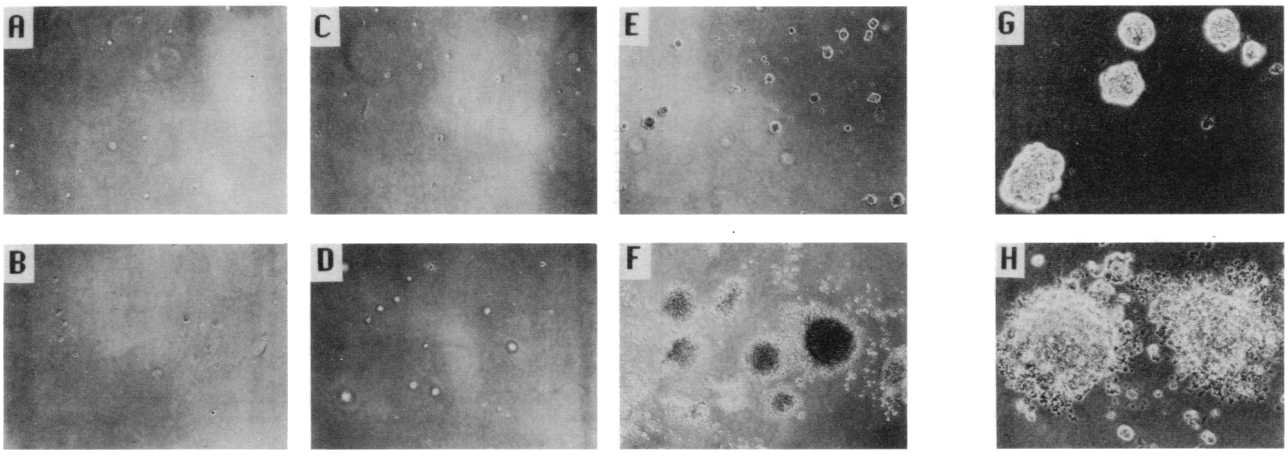

FIG. 3. Growth in soft agar. Nontransfected or transfected NIH 3T3 cells were plated in 0.3% agar containing DMEM plus 10% calf serum. Photographs were taken after 15 days of growth. (*Left*) Nontransfected cells (*A*) or cells transfected with vector [pZipNeoSV(X)] alone (*B*), wild-type α_{i2} (*C*), G204A α_{i2} (*D*), Q205L α_{i2} (*E*), or v-*src* (*F*) are shown. (×10.) (*Right*) Q205L α_{i2} colonies (*G*) and v-*src* colonies (*H*) are shown at a higher magnification. (×40.)

Table 2. Growth properties of NIH 3T3 cells expressing wild-type and mutant α_{i2}

Cells	Growth in monolayer culture: Doubling time, hr	Growth in monolayer culture: Saturation density, no. $\times 10^{-6}$	Growth in agar: Colony size, mm	Growth in agar: Efficiency, %
Control	18.0	8.4 ± 0.04	0.10	<0.5
Wild-type α_{i2}	17.2	15.1 ± 0.8	0.10	0.7
Q205L α_{i2}	16.3	20.7 ± 0.6	0.10	7.6
			0.15–0.30	38
G204A α_{i2}	20.0	4.8 ± 0.2	0.10	<0.5
v-*src*	15.8	27.6 ± 2.0	0.15–0.30	>90

The doubling times relate to the initial exponential phase of cell growth in 5% calf serum, and the saturation density represents the number of cells per 10-cm plate (grown in the presence of 10% calf serum) after 4 days of cell confluency. In soft agar, Q205L α_{i2} cells formed two types of colonies: small (<0.10 mm in diameter) and large (0.15–0.30 mm in diameter).

per plate. Thus, the overexpression of constitutively activated α_{i2} alters growth and anchorage dependency in NIH 3T3 cells, although it lacks focus-forming activity.

Growth characteristics. We studied the growth rate of α_{i2}-transfected cells in monolayer cultures. Detailed growth curves were established for cells incubated in 1% and 5% calf serum. The data obtained are summarized quantitatively in Table 2. Cells expressing Q205L α_{i2} had the shortest doubling time (16.3 hr) and the highest saturation density (20.7×10^6 cells per plate). Cells expressing wild-type α_{i2} also grew to a higher saturation density (15.1×10^6 cells per plate) than control cells (8.4×10^6 cells per plate). Doubling time for control and wild-type α_{i2} cells was 18.0 hr and 17.2 hr. In contrast, cells expressing G204A α_{i2} had the longest doubling time (20.0 hr) and the lowest saturation density (4.8×10^6 cells per plate).

Q205L α_{i2} cells showed a reduced serum requirement and a marked increase in DNA synthesis. [^{3}H]Thymidine incorporation in the absence of serum (Fig. 4 *Left*) was 8- to 12-fold higher for Q205L α_{i2} cells than for control cells. Interestingly, wild-type α_{i2} cells also showed a moderate (3-fold) increase in [^{3}H]thymidine incorporation. In contrast, G204A α_{i2} cell DNA synthesis was inhibited and the cells incorporated an average of 50% less [^{3}H]thymidine than control cells. Similar results were obtained in the presence of 1% serum (data not shown). In the presence of 5% serum (Fig. 4 *Right*), both Q205L α_{i2} and wild-type α_{i2} cells incorporated more [^{3}H]thymidine than control cells, but the difference was less striking. In contrast, G204A α_{i2} cells still demonstrated a marked growth inhibition.

DISCUSSION

We have generated fibroblast cell lines stably expressing high levels of wild-type α_{i2} or of the Q205L or G204A mutant form of α_{i2}. Stable overexpression of α_{i2} affects the level of endogeneous β subunits. Transient expression of α_{i1} and α_{i2} subunits in COS monkey kidney cells did not result in a change in β expression (30), consistent with the findings of Wong *et al.* (31) in human kidney cell line 293. However, the amount of β subunits in the membrane of stably transfected NIH 3T3 cells expressing α_{i2} showed a clear increase. Osawa *et al.* (32) also reported an increase in β expression in Chinese hamster ovary (CHO) cells stably transfected with α_{i2}. In NIH 3T3 cells, the increase in β expression is variable and seems to depend on the state of activation of the transfected α subunit, for Q205L α_{i2} cells show a smaller increase in β. These results suggest that both the amount and the state of activation (see below) of α subunits are important for the regulation of $\beta\gamma$ expression in the cell membrane. A variable reduction of α_s was observed in some clones, unrelated to the type of transfected α_{i2}.

The G204A and Q205L mutations in α_{i2} have effects comparable to their equivalent mutations in α_s. In contrast to wild-type α_{i2}, G204A α_{i2} is efficiently ADP-ribosylated by pertussis toxin upon binding to GTP[γS], suggesting that the mutant fails to enter the activated conformation and to dissociate from $\beta\gamma$ after binding to GTP. This behavior is characteristic of the "H21a" mutation (G226A) described in α_s (9). In contrast, Q205L α_{i2} is relatively poorly ADP-ribosylated by pertussis toxin when bound to either GTP or GTP[γS], suggesting a persistent state of activation of the protein and dissociation from $\beta\gamma$. This implies that the GTP-binding site is functionally identical in α_s and α_{i2} and that the Q205L mutation causes constitutive activation of the α_{i2} pathways, whereas the G204A mutant causes inactivation and could function as a dominant inhibitor.

Our data confirm in fibroblasts that α_{i2} inhibits adenylyl cyclase, as previously demonstrated in platelets (33). The

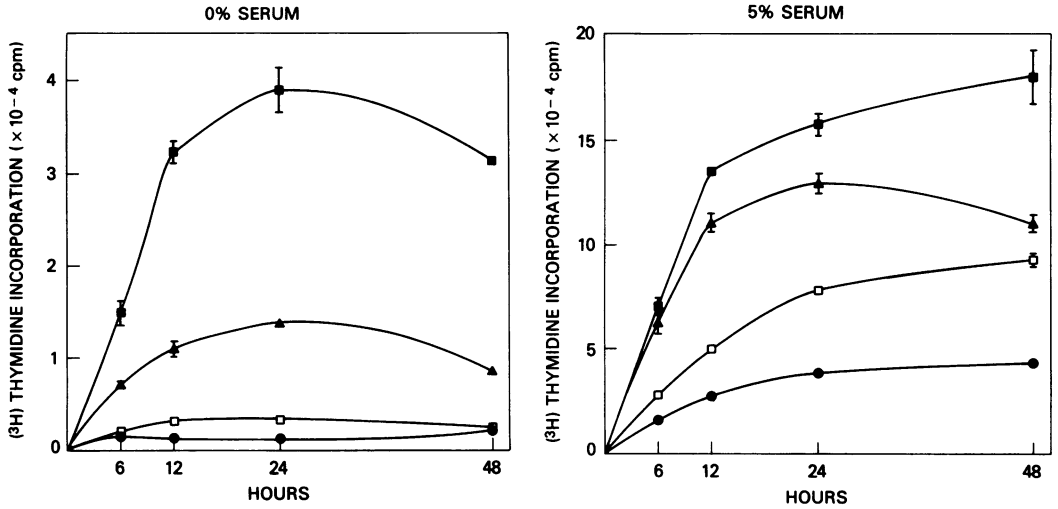

FIG. 4. DNA synthesis in NIH 3T3 cells expressing α_{i2}. Cells were plated at 10^3 cells per ml per well in 24-well plates and grown for 48 hr in DMEM plus 10% calf serum. After several washes, the cells were incubated overnight at 37°C with 0% (*Left*) or 5% (*Right*) serum. [^{3}H]Thymidine incorporation was assayed as described in *Materials and Methods*. Values represent the mean ± SEM of triplicates for clones transfected with vector alone (□) or with wild-type α_{i2} (▲), Q205L α_{i2} (■), or G204A α_{i2} (●) cDNAs.

constitutively active Q205A α_{i2} inhibits stimulated adenylyl cyclase, although basal levels of cAMP are unaffected. These findings are consistent with those of Wong *et al.* (31). In contrast, cells transfected with inactive G204A α_{i2} have higher basal and stimulated levels of cAMP than control cells. These results suggest that α_{i2} may function to inhibit adenylyl cyclase under both basal and stimulated conditions.

Pertussis toxin-sensitive G proteins are involved in regulating proliferation of fibroblasts (11–15), but which specific G proteins are involved is not clear. Transfection of α_o into Y-1 adrenal cells caused growth inhibition rather than stimulation (34). α_{i2} is ubiquitously expressed and has been shown to be activated by at least one type of growth factor, insulin-like growth factor II (35). Thus, G_{i2} is a likely candidate for the pertussis toxin-sensitive G protein mediating serum stimulation of fibroblast proliferation. Transfection of either wild-type or mutant forms of α_{i2} significantly alters growth of NIH 3T3 cells. Activated and inactivated α_{i2} mutants have opposite effects on cell proliferation. Cells transfected with activated Q205L α_{i2} have a doubling time significantly shorter and a saturation density significantly higher than those of control cells. Furthermore, these cells are able to form small colonies in soft agar, indicating abnormal, anchorage-independent growth. Similarly, overexpression of wild-type α_{i2} increases saturation density and reduces serum requirement, though to a lesser degree than with the activated mutant. In contrast, transfection of inactive G204A α_{i2} impairs NIH 3T3 cell growth, resulting in a longer doubling time and lower saturation density than control cells.

Recently, α_s activating mutations, including Q227R and R201C or R201H (which also reduce the intrinsic GTPase activity), have been found in human pituitary tumors (7, 36). Homologous mutations of α_{i2} (R179C and R179H) have been detected in human adrenal and ovarian tumors (37). Constitutive activation of proteins that transmit proliferative signals can promote cell transformation or tumor growth (38). Our data suggest that in fibroblasts the constitutively activated form of α_{i2} is not by itself transforming, but such a mutant could contribute to neoplastic proliferation.

Several issues raised by this study require further investigation. It is unclear whether all the effects of α_{i2} on cell proliferation result from changes in cAMP levels, or whether regulation of other pathways such as phospholipase C activation, arachidonic acid release, and increased calcium influx could be involved in α_{i2} regulation of NIH 3T3 growth. After this paper was submitted for review, a study by Lowndes *et al.* (39) appeared showing that expression of Q205L α_{i2} in CHO cells not only inhibited stimulation of cAMP formation but also altered receptor-stimulated phospholipase A_2 activity. The NIH 3T3 clones expressing mutant α_{i2} should be valuable tools to study interaction of α_{i2} and growth factor (or hormone) receptors involved in fibroblast proliferation. It is also not clear whether other pertussis toxin-sensitive G proteins such as G_{i1} and G_{i3} can regulate cell proliferation. Nonetheless, the present studies clearly establish that G_{i2} is an important regulator of cell proliferation. Given its ubiquitous expression, alterations in G_{i2} function could affect cell growth in a broad range of tissues and cell types.

Note Added in Proof. Recently, the R179C mutant of α_{i2} was shown to induce neoplastic transformation of Rat-1 cells (40).

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