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Analysis of the Fibroblast Transformation Potential of GTPase-Deficient *gip2* Oncogenes

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Expression of GTPase-deficient G_{i2} α subunit (α_{i2}) mutant polypeptides and overexpression of the wild-type α_{i2} polypeptide in Rat 1a, Swiss 3T3, and NIH 3T3 fibroblasts altered normal growth regulation and induced a loss of contact inhibition. In Rat 1a cells (but not in NIH 3T3 or Swiss 3T3 cells), expression of the GTPase-deficient α_{i2} mutant polypeptides allowed colony formation in soft agar, which correlated with a loss in anchorage dependence and a decreased serum requirement. The altered growth regulatory properties of Rat 1a cells induced by expression of α_{i2} mutant polypeptides was not significantly inhibited by cotransfection with a dominant negative Ha-*ras* mutant polypeptide (Asn-17*ras*^{H}), indicating that the activated G_{i2} membrane signal transduction protein is uniquely capable of altering the regulation of Rat 1a cell growth by a predominantly c-*ras*-independent mechanism. The results show that GTPase-deficient α_{i2} mutant polypeptides have the properties of an oncogene that can induce the phenotypic characteristics of transformation in Rat 1a cells but that only a subset of these changes is observed with NIH 3T3 and Swiss 3T3 cells.

The oncogenic potential of mutant G protein α subunit polypeptides has been implied from their presence in several neuroendocrine tumors (19, 23). Mutations that inhibit the GTPase turn-off function of α_s, the α chain polypeptide which stimulates adenylyl cyclase, have been identified to occur with fairly high frequency in both pituitary adenomas and thyroid tumors. The role of activated α_s mutants in the transformation of somatotrophs and thyrocytes is somewhat predictable, because cyclic AMP (cAMP) induces a proliferative response in both cell types (7). Lyons and coworkers also found GTPase-inhibiting mutations in the G_{i2} α subunit polypeptide, α_{i2}, in a subset of ovarian and adrenal cortical tumors (23). It is less apparent how constitutively active, GTPase-deficient α_{i2} mutant polypeptides could be involved in the transformation of these cell types, since no second messenger system previously characterized to be regulated by G_{i2} (adenylyl cyclase inhibition, K^+ channels, and PLA_2) has been shown to be mitogenic in ovarian or adrenal cortical cells. However, G_{i2}-like proteins have been implicated in the thrombin-, bombesin-, and lysophosphatidic acid-stimulated mitogenic responses of specific cell types, including Swiss 3T3, NIH 3T3, and Rat 1a fibroblasts (5, 21, 37, 42). The role of G_{i2}-like proteins was based largely on the ability of pertussis toxin treatment of the cells to inhibit these growth factors from stimulating DNA synthesis (pertussis toxin inhibits G_{i2} by catalyzing the ADP-ribosylation of the α_{i2} subunit polypeptide [5, 15, 21, 25]). More recently, cloning of the thrombin receptor cDNA has demonstrated that it has the predicted seven membrane-spanning structures and signature sequences characteristic of G protein-coupled receptors (38), substantiating the notion that thrombin receptor-stimulated mitogenic responses are G protein coupled.

Indeed, if G_{i2} is involved in the signalling of cell surface receptors like those for thrombin and bombesin, then constitutively active α_{i2} mutant polypeptides would be predicted to impart altered growth control when expressed in cells responding mitogenically to growth factors such as thrombin and lysophosphatidic acid. We have placed mutations that inhibit its intrinsic GTPase activity in the α_{i2} polypeptide, these are mutations the same as or similar to those present in the α_s and α_{i2} genes found in a subgroup of neuroendocrine tumors (22, 23). When expressed in three different fibroblast cell lines (Rat 1a, NIH 3T3, and Swiss 3T3), the GTPase-deficient α_{i2} mutant polypeptides were capable of altering the growth characteristics of all three cell types. Our findings indicate that expression of GTPase-deficient α_{i2} mutant polypeptides and overexpression of the wild-type α_{i2} polypeptide was capable of inducing a transformed phenotype in fibroblasts and that activated G_{i2} mutant polypeptides induce a loss of normal growth control similar to that induced by previously defined oncogenes.

MATERIALS AND METHODS

Cell lines. Rat 1a, NIH 3T3, and Swiss 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum and 5% calf serum. The GP+E-86 virus packaging line (24) was maintained in DMEM containing 10% fetal calf serum.

The virus-packaging GP+E-86 cells were transfected with 15 μg of pMV7 DNA (16) containing the appropriate α_{i2} insert by electroporation with a Bio-Rad Gene Pulser (500 V, 25 μF). Transfected GP+E-86 cells were selected by growth in the presence of 800 μg of G418 per ml for 2 weeks. Resistant colonies were expanded into cell lines, and GP+E-86 supernatants containing retrovirus encoding wild-type or mutant G protein α subunit cDNAs were collected. Virus titers were determined by infection of NIH 3T3 cells and quantitation of G418-resistant colonies. Virus titers were normalized to give similar numbers of NIH 3T3 G418-resistant colonies, and the viruses were used for infection.

Rat 1a, NIH 3T3, and Swiss 3T3 cells were infected with

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the appropriate virus by incubation overnight in the presence of 8 μg of Polybrene per ml. On the following day, the medium was changed, and 24 h later the cells were placed in the presence or absence of G418. For selection of stable clones, G418-resistant colonies were isolated and characterized for expression of the appropriate α_{i2} polypeptides or G protein β subunit (27). Focus formation in infected cells was determined 3 weeks after infection in the absence of G418 selection by Giemsa staining.

Analysis of α_{i2} expression. For immunoblotting, 50 μg of membrane protein from G418-resistant clones isolated from infected Rat 1a, NIH 3T3, and Swiss 3T3 cells was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% acrylamide), transferred to nitrocellulose, and probed with antibodies specific for α_i or βγ subunits (27, 41). The levels of expression of the virus-encoded α_{i2} polypeptides relative to expression in the appropriate control cells were analyzed with a Molecular Dynamics computing densitometer.

Measurement of cell growth. Clonal cell lines were derived from Rat 1a, NIH 3T3, or Swiss 3T3 cells infected either with virus overexpressing wild-type α_{i2} or expressing α_{i2}R179C or α_{i2}Q205L or with control virus lacking an α_{i2} cDNA insert. Expression of the appropriate virus-encoded α_{i2} mutant polypeptides and overexpression of the wild-type α_{i2} polypeptide were analyzed as described above. NIH 3T3 and Swiss 3T3 cells (5×10^4 each) and Rat 1a cells (7×10^5) were plated in 6-well dishes. On the next day, the medium was changed to DMEM with 0.1% bovine serum albumin (BSA) (Rat 1a) or 0.5% fetal calf serum (NIH 3T3 and Swiss 3T3), and the cells were incubated for 24 h to obtain quiescence. Cells were then stimulated with growth factors (20 ng of epidermal growth factor (EGF) per ml, 20 ng of platelet-derived growth factor per ml, 5 μg of insulin per ml, and 3 nM bombesin) or the indicated concentration of serum. Cells were harvested 96 h later by using 0.25% trypsin and 400 μM EDTA in phosphate-buffered saline and were quantitated with a hemocytometer. For measurement of DNA synthesis, 2×10^4 cells of the appropriate clone were plated in 24-well plates and cultured as described above to obtain quiescence. Cells were stimulated with different growth factors or fetal calf serum, and 16 h later, 0.5 μCi of [^{3}H]thymidine (ICN Radiochemicals) was added to each well, and the cells were incubated for an additional 4 h. Cells were then washed in Hanks' balanced salt solution and solubilized in 0.1% SDS, DNA was collected by filtration on GF/C Whatman glass fiber filters, and trichloroacetic acid-precipitable counts were determined by scintillation counting. Conditioned medium was collected both from the indicated clones of Rat 1a cells overexpressing α_{i2} and expressing α_{i2}Q205L and α_{i2}R179C and from two wild-type Rat 1a clones after incubation for 3 days in DMEM containing 10% fetal calf serum. The conditioned medium was diluted 1:2.5 with fresh DMEM containing 0.1% BSA and added to quiescent cultures of wild-type Rat 1a cells.

TGF-α stimulation of Rat 1a cell growth in soft agar. Wild-type Rat 1a cells in the presence of 250 pg of transforming growth factor α (TGF-α) per ml or Rat 1a clones expressing α_{i2}Q205L, α_{i2}, or v-*ras* were cloned in soft agar. Duplicate dishes of cells were cultured for 15 days in the presence or absence of 6 μg of anti-TGF-α antibody (Oncogene Science) per ml or anti-Ia antibody (gift from John Cambier) of the same isotype. For wild-type Rat 1a cells with or without TGF-α, α_{i2}Q205L and α_{i2} clones (5,000 cells per dish) were seeded in 5 ml of 0.3% agar in DMEM containing 10% fetal calf serum. The v-*ras*-expressing Rat 1a clone was seeded at 500 cells per dish. Wild-type cells cloned in the absence of TGF-α formed a small number of minute colonies that were 5 to 10 times smaller in diameter than colonies formed in the presence of TGF-α. The α_{i2}, α_{i2} Q205L, and v-*ras* colonies were of sizes similar to that of colonies of wild-type cells grown in the presence of TGF-α. For anti-Ia antibody experiments, 400 cells of each clone (wild type, α_{i2}, and α_{i2}Q205L) per dish were cultured in 0.3% agar in DMEM containing 10% fetal calf serum. Wild-type Rat 1a cells were cloned in the presence of 250 pg of TGF-α (Upstate Biotechnology, Inc.) per ml. In the absence of added TGF-α, the wild-type Rat 1a cells failed to form colonies in soft agar.

Cotransfection of α_{i2} mutant polypeptides and dominant negative *ras*. Rat 1a cells were transfected with 100 ng of Asn-17*ras*^{H} (a gift from Larry A. Feig, Tufts University School of Medicine) and 1 μg of α_{i2}, α_{i2}Q205L, or v-*ras* in either pZipneo or pMV7 by the calcium phosphate precipitation technique, as described by Ausubel et al. (1). For pMV7 α_{i2} and α_{i2}Q205L expression vectors, the G418 (neomycin) resistance gene was excised so that G418 resistance was contributed only by the pZipneo Asn-17*ras*^{H} construct. Three days after transfection, the cells were subcultured into medium containing G418 (800 μg/ml). Drug-resistant colonies appearing 3 weeks later were quantitated after Giemsa staining.

RESULTS

Overexpression of wild-type α_{i2} and expression of the GTPase-deficient α_{i2}R179C and α_{i2}Q205L mutant polypeptides in Rat 1a, Swiss 3T3, and NIH 3T3 cells caused a loss of normal-growth control in all three cell types. Figure 1 shows the loss of contact inhibition and the focus-forming potential of the different α_{i2} constructs in the three cell types. It is apparent that overexpression of the wild-type α_{i2} gene product caused a loss of contact inhibition to differing degrees in all three cell types and that the α_{i2}-overexpressing cells grew to higher densities than the control cells infected with virus not having an α_{i2} cDNA insert. The GTPase-deficient α_{i2}Q205L mutant polypeptide was also more effective in inducing a loss of contact inhibition, allowing a cell density greater than that observed with overexpression of the wild-type α_{i2} polypeptide to be obtained. This is particularly apparent with the Rat 1a and Swiss 3T3 cells, in which expression of the α_{i2}Q205L mutant polypeptide allowed a high cell density to be achieved. In contrast, the loss of contact inhibition in NIH 3T3 cells was fairly similar with overexpression of the wild-type α_{i2} and with expression of the GTPase-deficient α_{i2}Q205L mutant polypeptide.

It is also apparent in comparing Rat 1a cells expressing the α_{i2}R179C and α_{i2}Q205L mutant polypeptides that the α_{i2}Q205L mutation resulted in a greater loss of growth control than that observed with the α_{i2}R179C mutation. The greater transformation potential of α_{i2}Q205L versus the α_{i2}R179C mutant polypeptide was predicted on the basis of the fact that the GTP-activated conformation of the α_{i2}Q205L polypeptide has greater stability than the α_{i2}R179C polypeptide (14, 22, 40). Thus, the ability of α_{i2} mutant polypeptides to induce a transformedlike phenotype in Rat 1a cells is related to the stability of the GTP-activated α_{i2} conformation, a direct correlate with the ability of G protein α subunits to activate appropriate effector enzymes.

Expression of virus-encoded α_{i2} and GTPase-deficient α_{i2}R179C and α_{i2}Q205L mutants. Clonal cell lines were derived from focus-forming Rat 1a, Swiss 3T3, and NIH 3T3

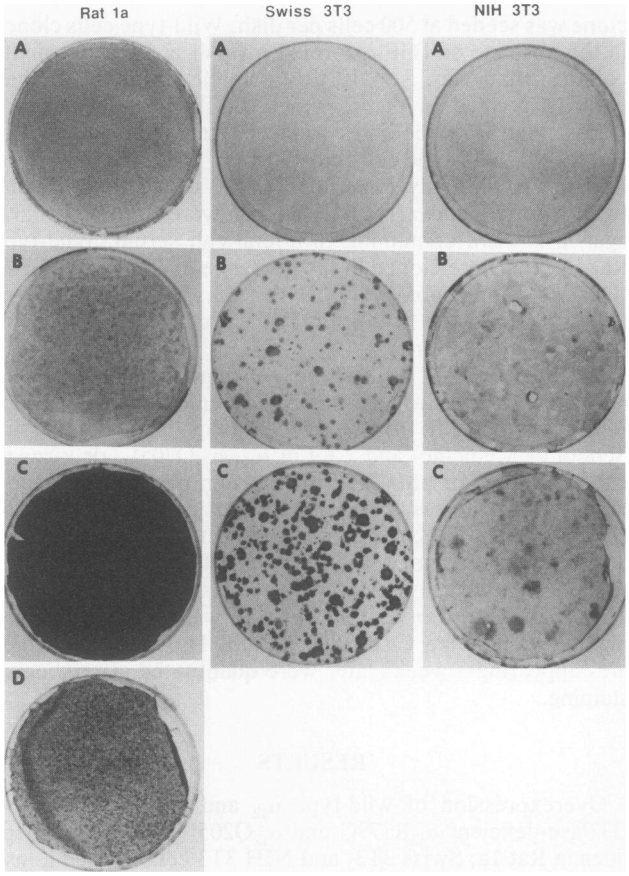

FIG. 1. Altered growth regulation of Rat 1a, Swiss 3T3, and NIH 3T3 cells expressing α_{i2} or GTPase-deficient α_{i2} mutants (α_{i2}Q205L and α_{i2}R179C). The three fibroblast cell lines were infected as described in Materials and Methods with equal titers of virus which encoded G418 resistance only (A), wild-type α_{i2} (B), α_{i2}Q205L (C), or α_{i2}R179C (D). Plates were analyzed 3 weeks after infection by Giemsa staining. Each panel is representative of three different focus-forming assays for each cell type and virus.

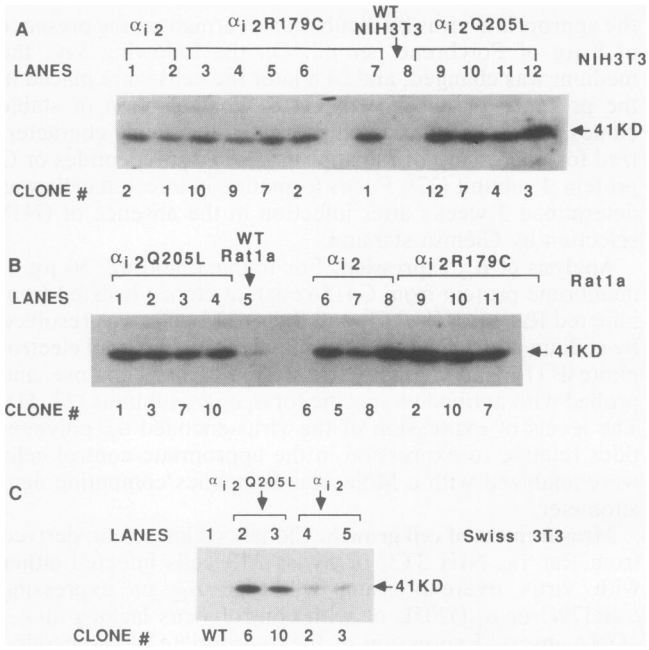

FIG. 2. Analysis of α_{i2}, α_{i2}Q205L, and α_{i2}R179C polypeptide expression in infected NIH 3T3 (A), Rat 1a (B), and Swiss 3T3 (C) cells. Stable clones were generated from virus-infected cells selected in G418 as described in Materials and Methods. The α_{i2}, α_{i2}Q205L, and α_{i2}R179C polypeptides were expressed 3 to 10 times more than the endogenous α_{i2} polypeptide in each cell type. It should be noted that the GTPase-deficient α_{i2}Q205L and α_{i2}R179C polypeptides were consistently expressed at higher levels for all three cell types; this was particularly evident with Swiss 3T3 cells. The basis for the increased expression of the GTPase-deficient α_{i2} polypeptides is presently unclear. WT, wild-type.

fibroblasts infected with viruses encoding wild-type α_{i2}, α_{i2}Q205L, or α_{i2}R179C. Multiple independent clones for each cell type were isolated; these stably expressed the wild-type or mutant α_{i2} at levels 3- to 10-fold greater than levels of the endogenous α_{i2} polypeptide (Fig. 2). None of the clones for the three cell types showed a significant change in G protein β subunit expression (data not shown). In addition, the pertussis toxin-catalyzed ADP-ribosylation of the expressed α_{i2} polypeptides increased proportionately to the fold increase observed by immunoblot analysis. Since association of α_{i2} subunits with βγ complexes is required for pertussis toxin-catalyzed ADP-ribosylation, a significant fraction of the expressed α_{i2} mutant polypeptides must be associated with βγ and capable of coupling to receptors (15, 26).

Growth of Rat 1a, Swiss 3T3, and NIH 3T3 cells overexpressing wild-type α_{i2} or expressing GTPase-deficient α_{i2}. A general property of all three fibroblast cell types was that the expression of the GTPase-deficient α_{i2} mutant polypeptides (α_{i2}Q205L and α_{i2}R179C) caused an increased growth rate, a marked loss of contact inhibition, and increased cell density at both low- and high-level sera (Fig. 3). The phenotypic consequence of wild-type α_{i2} overexpression was more variable in the three fibroblast cell lines. The Rat 1a clone overexpressing wild-type α_{i2} did not dramatically increase cell number relative to a noninfected control clone in low- or high-level serum. This phenotype is probably related in part to the fact that the wild-type Rat 1a cells are small and grow to high monolayer density. In contrast, wild-type Swiss 3T3 and NIH 3T3 cells are larger than Rat 1a cells and inhibit contact at a much lower cell density. Overexpression of the wild-type α_{i2} polypeptide in Swiss 3T3 and NIH 3T3 cells resulted in a fold increase in cell density greater than that observed with Rat 1a cells. The increased cell density of Swiss 3T3 and NIH 3T3 clones was accompanied by a change to a smaller, morphologically altered phenotype characteristic of transformation of these cells by different oncogenes (28, 29). The Swiss 3T3 and NIH 3T3 cells also showed a three- to fivefold increase in cell number and [^{3}H]thymidine incorporation, respectively, in response to high-level serum, indicating that growth factors further stimulated growth of the α_{i2}- and GTPase-deficient α_{i2}-expressing clones. The fold increases of both the growth factor and the serum-induced response were similar for the wild-type cells and α_{i2}-overexpressing and GTPase-deficient α_{i2}-expressing Swiss 3T3 and NIH 3T3 clones, suggesting that the transformedlike phenotype induced by the wild-type and mutant α_{i2} polypeptides was not due simply to an exacerbation of the response to serum growth factors including EGF, platelet-derived growth factor, and insulin. Rather, the GTPase-deficient α_{i2} mutant polypeptides more probably were working independently of the three growth factors, and

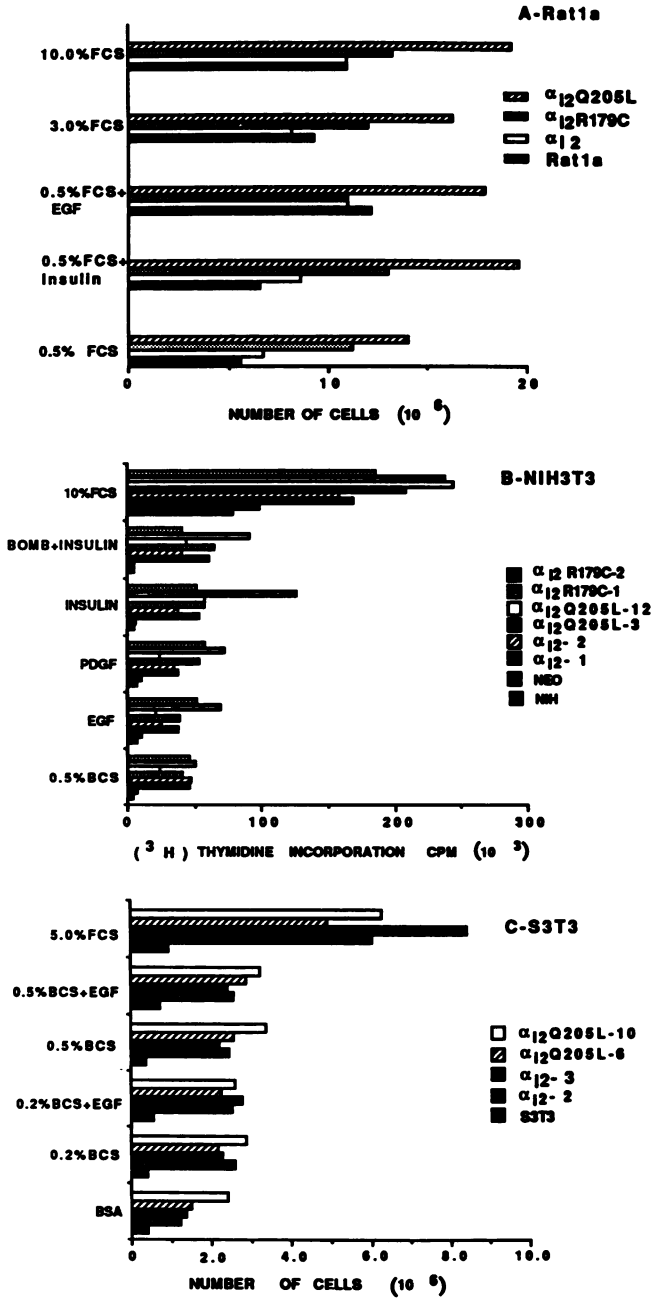

FIG. 3. Growth of Rat 1a (A), NIH 3T3 (B), and Swiss 3T3 (C) cells overexpressing wild-type α_{i2} or expressing the GTPase-deficient α_{i2}Q205L and α_{i2}R179C mutants. Growth of the designated Rat 1a, NIH 3T3, and Swiss 3T3 clones was characterized as described in Materials and Methods. Briefly, 5×10^4 cells (NIH 3T3 and Swiss 3T3) and 7×10^5 cells (Rat 1a) for each clone were seeded in dishes and subsequently growth arrested by incubation for 24 h in DMEM with 0.1% BSA (Rat 1a) or 0.5% calf serum (NIH 3T3 and Swiss 3T3). Cells were then challenged with the indicated growth factor, calf serum (BCS), or fetal calf serum (FCS). Cells were then either harvested and counted 4 days later (Rat 1a and Swiss 3T3) or DNA synthesis assayed (NIH 3T3) 16 h later by [^{3}H]thymidine incorporation. All three cell types expressing the GTPase-deficient α_{i2} mutant polypeptides showed both a loss of contact inhibition and an increased rate of growth resulting from a shortening of the cell cycle in low- and high-level sera relative to the wild-type and neomycin (NEO) (G418)-resistant clones characteristic of the transformed phenotype. BOMB, bombesin.

TABLE 1. Growth in soft agar of Rat 1a clones expressing α_{i2}Q205L and overexpressing wild-type α_{i2}[a]

Rat 1a clone	Clone	No. of colonies (mean ± SEM)	
		Without pertussis toxin	With pertussis toxin
Wild type[b]		61 ± 15	3.5 ± 3.5
Neo[b]		98 ± 6	0
α_{i2}Q205L	1	562 ± 34	534 ± 74
	7	864 ± 4	815 ± 106
α_{i2}R179C	2	680 ± 40	264 ± 24
	8	1,126 ± 14	722 ± 78
α_{i2}	7	497 ± 5	48 ± 4
	8	944 ± 32	77 ± 3

[a] The indicated Rat 1a clones expressing the wild-type α_{i2} or the GTPase-deficient α_{i2} mutants (α_{i2}Q205L and α_{i2}R179C) were cloned in soft agar (5,000 cells per 60-mm dish) in DMEM containing 10% fetal calf serum in the presence or absence of 200 ng of pertussis toxin per ml. Fifteen days after plating, colonies were counted in five dishes for each clone. The numbers represent the average ± standard error of the mean of the number of large colonies per dish for each Rat 1a clone. The results represent three experiments in which different clones and numbers of cells were tested. The cloning efficiencies of α_{i2}-, α_{i2}Q205L-, and α_{i2}R179C-expressing clones were reproducibly 10 to 20%, except in the presence of pertussis toxin, which inhibited the α_{i2} and α_{i2}R179C Rat 1a clones. Pertussis toxin inhibited more than 90% and 40 to 65% of the colony formation with Rat 1a cells overexpressing the wild-type α_{i2} and expressing the α_{i2}R179C polypeptides, respectively. Pertussis toxin had no effect on the cloning efficiencies of the α_{i2}Q205L clones.

[b] The wild-type and neo (no α_{i2} cDNA) clones formed a small number of minute colonies that were 5 to 10 times smaller than the α_{i2}, α_{i2}Q205L, and α_{i2}R179C colonies that were not present with the α_{i2}, α_{i2}Q205L, and α_{i2}R179C clones.

together they gave additive mitogenic responses. In contrast to the growth of NIH 3T3 and Swiss 3T3 cells, the growth of Rat 1a clones overexpressing α_{i2} or expressing the GTPase-deficient α_{i2} mutant polypeptides was not dramatically increased in 10% serum relative to growth in 0.5% serum in the presence or absence of insulin or EGF. The similarity in growth properties of Rat 1a clones in low- and high-level sera is characteristic of the decreased serum requirement of transformed cells. Thus, Rat 1a, Swiss 3T3, and NIH 3T3 cells all show similar but distinguishable losses in growth control (including contact inhibition and serum requirements) when overexpressing the wild-type α_{i2} polypeptide or expressing the GTPase-deficient α_{i2} polypeptides.

Soft-agar colony growth of Rat 1a clones overexpressing α_{i2} or expressing GTPase-deficient α_{i2} mutant polypeptides. Loss of anchorage dependence as measured by growth in soft agar is a characteristic of the transformed phenotype. NIH 3T3 cells overexpressing α_{i2} or expressing the GTPase-deficient α_{i2}Q205L or α_{i2}R179C mutant polypeptide failed to grow in soft agar (data not shown). This was in contrast to the same parental NIH 3T3 clone transfected with the activated c-Ha-*ras* T20 bladder carcinoma gene, which efficiently gave colonies in soft agar. Swiss 3T3 cells expressing α_{i2}Q205L also cloned very poorly in soft agar, indicating that neither the NIH 3T3 nor the Swiss 3T3 cells having an α_{i2}Q205L-induced loss in growth control and contact inhibition had assumed an anchorage-independent phenotype. Unlike the NIH 3T3 and Swiss 3T3 cells, however, Rat 1a cells overexpressing α_{i2} or expressing the α_{i2}Q205L or α_{i2}R179C mutant polypeptide were capable of colony formation in soft

TABLE 2. Effect of anti-TGF-α antibodies on growth of Rat 1a cells in soft agar

Clone	No. of colonies with[a]:		% Inhibition[b]
	No antibody	Anti-TGF-α (6 μg/ml)	
Rat 1a	10[c]	8[c]	
Rat 1a + TGF-α (250 pg/ml)	513	344	33
α_{i2}Q205L-1	549	467	15
α_{i2}Q205L-7	234	162	31
α_{i2}-6	504	431	14
α_{i2}-8	625	429	31
v-*ras*	107	108	0

[a] Numbers are means from duplicate dishes that varied by less than 5%.
[b] Calculated as (control value/anti-TGF-α value) × 100.
[c] Minute colonies 5 to 10 times smaller than wild-type + TGF-α colonies.

agar (Table 1). Neither the parental Rat 1a cells nor the G418-resistant clones infected with virus lacking an α_{i2} cDNA insert were capable of soft-agar colony formation. The relative cloning efficiencies of the α_{i2}, α_{i2}Q205L, and α_{i2}R179C clones were similar but two- to threefold lower than that observed with expression of the c-Ha-*ras* T20 bladder carcinoma gene in Rat 1a cells. Strikingly, treatment of cells with pertussis toxin, which ADP-ribosylates α_{i2} at a cysteine four residues from the C terminus (39), inhibited soft-agar colony growth of the α_{i2}-overexpressing clones and partially inhibited colony growth of the α_{i2}R179C-expressing clones. The pertussis toxin-catalyzed inhibition of soft-agar colony growth demonstrates that the transformed phenotype is directly related to α_{i2} overexpression and not to the mutation of a second gene that is capable of inducing a growth advantage during the clonal-selection procedure. Pertussis toxin had little or no effect on α_{i2}Q205L-expressing clones. The wild-type α_{i2}, α_{i2}Q205L, and α_{i2}R179C polypeptides are all substrates for pertussis toxin (22, 40), in which the consequence of α_{i2} ADP-ribosylation is uncoupling of G_{i2} from receptor activation and a decreased rate of GDP dissociation (2, 11). The almost complete inhibition and partial inhibition by pertussis toxin treatment, respectively, of α_{i2}- and α_{i2}R179C-induced soft-agar colony growth of Rat 1a cells are consistent with the cells' decreased ability to maintain a GTP-ligand conformation relative to the strongly activated α_{i2}Q205L mutant polypeptide (22). The inhibition of soft-agar colony growth of the α_{i2}-overexpressing and α_{i2}R179C-expressing clones suggests that they may have to couple to receptors for activation and that pertussis toxin uncouples receptor interaction. It is presently unclear which receptors, if any, may be involved. In contrast, the α_{i2}Q205L mutant polypeptide is able to maintain the GTP-activated conformation to a significantly greater extent than the α_{i2}R179C mutant polypeptide and is strongly constitutively activated in the absence of receptor coupling.

TABLE 3. Failure of Anti-Ia antibodies to inhibit growth of Rat 1a cells in soft agar

Clone	No. of colonies (mean) with[a]:			% Inhibition by:	
	No antibody	Anti-TGF-α (6 μg/ml)[a]	Anti-Ia (6 μg/ml)[a]	Anti-TGF-α	Anti-Ia
Rat 1a + TGF-α (250 pg/ml)	42, 38 (40)	28, 27 (28)	40, 34 (37)	30	7
α_{i2}-8	63, 58 (61)	41, 39 (40)	55, 58 (56)	34	8
α_{i2}Q205L-7	35, 32 (34)	23, 24 (24)	34, 30 (32)	30	6

[a] Numbers of colonies in duplicate dishes.

TABLE 4. Rat 1a cell DNA synthesis in response to conditioned media[a]

Clone supernatant	[^{3}H]thymidine incorporation (cpm) (wild-type Rat 1a cells)
Rat 1a (clone 1)	3,550
α_{i2}-6	14,180
α_{i2}Q205L-1	6,900
α_{i2}Q205L-10	14,900
α_{i2}R179C-7	4,800
Rat 1a (clone 2)	3,800
10% fetal calf serum	38,000

[a] Stimulation of [^{3}H]thymidine incorporation was measured as described in Materials and Methods in response to the various conditioned media and compared with incorporation in fresh fetal calf serum diluted similarly in DMEM and 0.1% BSA. Fetal calf serum stimulated [^{3}H]thymidine incorporation approximately 10-fold, whereas different clones overexpressing wild-type or expressing mutant α_{i2} polypeptides stimulated DNA synthesis 1.3- to 4-fold relative to wild-type Rat 1a cell-conditioned medium. The results are representative of three independent experiments.

The ability of Rat 1a cells overexpressing α_{i2} or expressing the GTPase-deficient α_{i2}Q205L mutant polypeptide to grow in soft agar, their decreased serum requirement, and their lack of an enhanced growth response in high-level serum contrasted with the properties of NIH 3T3 and Swiss 3T3 cells expressing the same α_{i2} gene products. Rat 1a cells have been previously shown to induce a TGF-α autocrine loop when transfected with an oncogene (20). In fact, transfection of Rat 1a cells with TGF-α cDNA and establishment of a TGF-α autocrine loop are sufficient for soft-agar colony growth (30). Table 2 shows that wild-type Rat 1a cell colony formation in soft agar is, indeed, maintained by added TGF-α. Colony formation induced by TGF-α was also partially inhibited by addition of an anti-TGF-α antibody in the soft agar. The abilities of α_{i2}- and α_{i2}Q205L-expressing Rat 1a cells to form colonies in soft agar were inhibited by anti-TGF-α antibody at levels similar to those observed with wild-type cells grown in the presence of 250 pg of TGF-α per ml (Table 2). Similar concentrations of an anti-Ia antibody of isotype similar to that of the anti-TGF-α antibody added to the soft agar had little effect on colony growth of wild-type Rat 1a cells in the presence of added TGF-α or an α_{i2}Q205L clone, indicating that the inhibition was specific for the anti-TGF-α antibody (Table 3). Supernatants from α_{i2}- and α_{i2}Q205L-expressing clones were also capable of inducing 1.3- to 4-fold-greater DNA synthesis than conditioned medium from wild-type Rat 1a cells (Table 4). Finally, Northern (RNA) blots indicated induction of a low-level expression of TGF-α mRNA in α_{i2}- and α_{i2}Q205L-expressing clones that was not detectable in wild-type Rat 1a cells (data not shown). Cumulatively, the results show that Rat 1a cells overexpressing α_{i2} or expressing the GTPase-deficient mutant polypeptides have induced a growth factor autocrine loop involving TGF-α. This is consistent with the strong mitogenic response normally initiated by the EGF receptor in Rat 1a cells and probably contributes to the decreased serum requirements and soft-agar colony growth of these clones.

Interplay of the α_{i2} and c-*ras* effector pathways in Rat 1a cells. Activation of tyrosine kinase-encoded growth factor receptors such as those for EGF and platelet-derived growth

factor has been shown to transiently activate c-*ras* in several cell types, as measured by an increase in the fraction of immunoprecipitated c-*ras* having GTP bound relative to the fraction having GDP bound (10, 31, 32). We performed similar experiments with Rat 1a cells expressing the GTPase-deficient α_{i2}Q205L mutant polypeptide and found that the maximal EGF receptor activation of c-*ras* was not changed relative to wild-type Rat 1a cells (data not shown). Basal levels of activated c-*ras* were possibly elevated to a small degree in α_{i2}Q205L-expressing clones, which might be related to the TGF-α autocrine loop, but this was a variable result among different clones. The results did suggest that altered growth control induced by α_{i2}Q205L expression did not result from a constitutively enhanced *ras* activation.

To determine, however, whether a functional c-*ras* was required for the α_{i2}- and α_{i2}Q205L-induced changes in Rat 1a cell growth control, cotransfections were performed with the dominant negative Asn-17*ras*^{H} (Ser-17→Asn) mutant polypeptide, which is capable of competitively inhibiting c-*ras*-regulated growth factor responses (4, 8, 36). Figure 4 shows that the dominant negative Asn-17*ras*^{H} mutant polypeptide inhibited serum-stimulated cell growth of c-*ras*-transfected Rat 1a cells. In contrast, the Asn-17*ras*^{H} dominant negative mutant polypeptide did not inhibit serum-stimulated colony formation when cotransfected with either the wild-type α_{i2} or the GTPase-deficient α_{i2}Q205L mutant polypeptide. There is, however, a distinguishable difference between the colony size observed when α_{i2} and α_{i2}Q205L cDNAs are cotransfected with Asn-17*ras*^{H} and the size observed with transfections without the dominant negative *ras* mutant polypeptide. The sizes of the colonies are reproducibly and significantly smaller when Rat 1a cells are cotransfected with the dominant negative *ras* mutant polypeptide. The diminution in colony size was due to a decreased cell number rather than an altered cell shape, indicating that cell growth was slowed but not inhibited by the dominant negative *ras* gene product in α_{i2}- and α_{i2}Q205L-expressing clones. This contrasts with the c-*ras* cotransfections, in which the Asn-17*ras*^{H} dominant negative mutant polypeptide inhibited cell growth. The results demonstrate that overexpression of α_{i2} or expression of α_{i2}Q205L is capable of inducing changes in Rat 1a cell growth regulation that are not dependent upon functional c-*ras* signalling to the same extent as tyrosine kinases such as v-*src*.

Consistent with these findings were a series of control experiments showing that the dominant negative *ras* mutant polypeptide significantly inhibited Rat 1a cell colony formation induced by coexpression with v-*src* (data not shown) as well as c-*ras*. In contrast, the dominant negative *ras* mutant polypeptide did not inhibit the growth-stimulatory action of v-*raf*, indicating that v-*raf* functions independent of c-*ras*. Our findings are similar to those described by Feig and Cooper (8) for v-*src* and v-*raf* in NIH 3T3 cells and indicate that α_{i2} and α_{i2}Q205L are unique membrane-associated gene products capable of altering the regulation of cell growth by a mechanism that is significantly less dependent upon a functional c-*ras* than tyrosine kinases such as v-*src*.

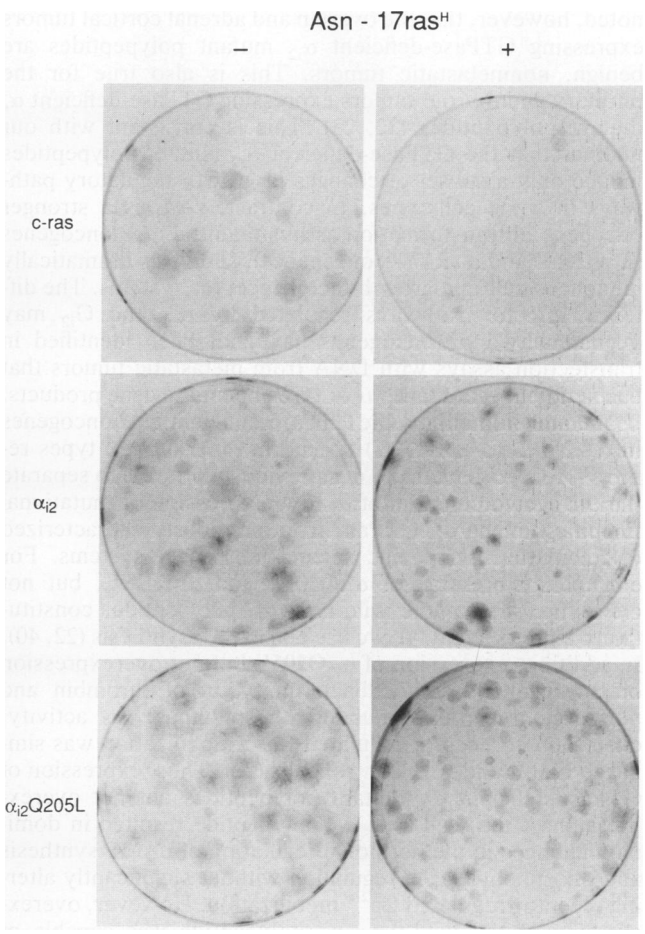

FIG. 4. Influence of the dominant negative Asn-17*ras*^{H} mutant polypeptide on Rat 1a cell growth. Rat 1a cells were cotransfected with Asn-17*ras*^{H} (100 ng) and with c-*ras*, α_{i2}, or α_{i2}Q205L (1 μg) (+). The Asn-17*ras*^{H} and c-*ras* cDNAs were in pZipneo, and the α_{i2} and α_{i2}Q205L cDNAS were in pMV7 having the neomycin resistance gene excised. Control transfections used the neomycin resistance vector in the absence of the Asn-17*ras*^{H} gene (−). G418-resistant colonies were Giemsa stained 3 weeks after transfection and photographed. Numbers of the indicated colonies in the absence and presence, respectively, of the Asn-17*ras*^{H} dominant negative mutant polypeptide were as follows: c-*ras*, 23 and 8; α_{i2}, 55 and 47; and α_{i2}Q205L, 105 and 103. The experiments are representative of five independent transfections. In control experiments with Rat 1a cells, the Asn-17*ras*^{H} mutant inhibited v-*src* transfectants to a significantly greater extent than it did v-*raf* transfectants, as described previously by Feig and Cooper for NIH 3T3 cells (8). However, the dominant negative Asn-17*ras*^{H} mutant polypeptide appeared somewhat less potent in inhibiting serum-stimulated growth of Rat 1a cells than of NIH 3T3 cells. A representative experiment with v-*src* and v-*raf* relative to α_{i2} and α_{i2}Q205L coexpression with Asn-17*ras*^{H} gave numbers of colonies as follows: Asn-17*ras*^{H} + v-*src*, 8; Asn-17*ras*^{H} + v-*raf*, 39; Asn-17*ras*^{H} + α_{i2}, 22; and Asn-17*ras*^{H} + α_{i2}Q205L, 30. Thus, in coexpression experiments with Asn-17*ras*^{H}, the order of potency is v-*raf* > α_{i2}Q205L > α_{i2} > v-*src* in stimulating Rat 1a cell growth.

DISCUSSION

Our results show that expression of GTPase-deficient G_{i2} α subunits and overexpression of the wild-type α_{i2} polypeptide can induce properties characteristic of the transformed phenotype in three different fibroblast cell lines. Predictably, expression of the GTPase-deficient α_{i2} mutant polypeptide was more effective than overexpression of the wild-type α_{i2} subunit. Thus, the GTPase-deficient α_{i2} mutant polypeptides can function as oncogenes.

The presence of GTPase-deficient α_{i2} mutant polypeptides, referred to as *gip2* oncogenes, in ovarian and adrenal cortical tumors (23) would therefore be predicted to contribute to tumorigenic progression in these tissues. It must be

noted, however, that the ovarian and adrenal cortical tumors expressing GTPase-deficient α_{i2} mutant polypeptides are benign, nonmetastatic tumors. This is also true for the pituitary and thyroid tumors expressing GTPase-deficient α_s mutant polypeptides (22, 23). This is consistent with our findings that the GTPase-deficient α_{i2} mutant polypeptides induce only a subset of changes in growth regulatory pathways in some cell types. In contrast, v-*ras* is a stronger oncogene in transformation assays than the *gip2* oncogenes (α_{i2}Q205L and α_{i2}R179C oncogenes), and v-*ras* dramatically influences multiple-growth-factor receptor systems. The different effector pathways regulated by *ras* and G_{i2} may explain why *gip2* oncogenes have not been identified in transfection assays with DNA from metastatic tumors that frequently have mutant *ras* or tyrosine kinase gene products.

The question of how the GTPase-deficient *gip2* oncogenes (α_{i2}Q205L and α_{i2}R179C) transform different cell types remains. It is possible from our previous work (22) to separate the oncogenic potential of α_{i2} overexpression or mutational inhibition of the α_{i2} GTPase from previously characterized α_{i2} regulation of specific second messenger systems. For example, expression of α_{i2}Q205L and α_{i2}R179C, but not overexpression of the wild-type α_{i2} polypeptide, constitutively inhibits adenylyl cyclase and cAMP synthesis (22, 40). In addition, expression of α_{i2}Q205L but not overexpression of α_{i2} results in a feedback inhibition of thrombin and purinergic receptor-stimulated phospholipase A_2 activity, even though receptor-regulated Ca^{2+} mobilization was similar to that found with control cells (22). Thus, expression of GTPase-deficient α_{i2} mutant polypeptides but not overexpression of the wild-type α_{i2} polypeptide resulted in dominant changes in the hormonal regulation of cAMP synthesis and phospholipase A_2 regulation without significantly altering receptor-regulated Ca^{2+} mobilization. However, overexpression of the wild-type α_{i2} polypeptide was capable of inducing characteristics of the transformed phenotype in Rat 1a, NIH 3T3, and Swiss 3T3 cells, indicating its dominant character in growth regulation but not in the control of adenylyl cyclase and phospholipase A_2 activity. We have also observed that expression of the α_{i2}Q205L mutant polypeptide and overexpression of α_{i2} do not dramatically alter hormone and growth factor regulation of phospholipase C activity in several cell types (unpublished observations), indicating that the *gip2* oncogenes do not transform cells by constitutively activating phospholipase C activity. Finally, the finding that expression of GTPase-deficient *gip2* oncogenes and overexpression of α_{i2} can induce a transformed phenotype in Rat 1a cells is substantiated by the findings of Seuwen and coworkers (33) showing that the thrombin mitogenic response requires a G_i protein in CCL39 fibroblasts and that the response is independent of cAMP synthesis and phospholipase C activation. It must be concluded, therefore, that G_{i2} regulates effector enzymes involved in cell growth that have not yet been identified as being coupled to G proteins.

Candidate effector enzymes include tyrosine kinases and phosphotyrosine phosphatases, but no compelling evidence currently exists for a direct coupling of a G protein to either a kinase or a phosphatase. Previous reports of thrombin-stimulated increases in the phosphotyrosine content of proteins (12, 18) have not demonstrated the activation of any specific tyrosine kinase, and it is now clear that tyrosine phosphorylations may be secondary events resulting from intracellular Ca^{2+} transients (13) or protein kinase C activation (6, 17). This is particularly true for the p42 ERK (early response kinase) gene products, also referred to as mitogen-activated protein (MAP) kinases, which have been shown to contain phosphotyrosine after phorbol ester or thrombin treatment of cells (3, 18). The tyrosine phosphorylation of p42 ERK actually may be an autophosphorylation event (9), even though the p42 ERK protein is a serine/threonine kinase for target substrates other than itself. The α_{i2}Q205L-expressing and α_{i2}-overexpressing Rat 1a clones appear to have constitutively elevated the phosphotyrosine content of p42 ERK (unpublished data), suggesting that this pathway may be persistently activated by the *gip2* oncogenes. Additional proteins also appeared to be constitutively tyrosine phosphorylated in α_{i2}- and α_{i2}Q205L-transformed cells, suggesting that tyrosine phosphorylation may be involved in the altered growth regulation of these clones.

Finally, the failure of the dominant negative *ras* mutant polypeptide to significantly inhibit α_{i2}- and α_{i2}Q205L-induced growth of Rat 1a cells suggests that G_{i2} integrates in the network of growth-regulatory metabolic cascades differently from the EGF receptor, *src* tyrosine kinase, and c-*ras*, whose mitogenic responses are blocked by Asn-17*ras*^{H} (4, 8). Candidate effectors might therefore include protein kinases whose mitogenic actions are not inhibited by dominant negative *ras* mutant polypeptides (8). The α_{i2} and α_{i2}Q205L transformation of Rat 1a cells will allow definition of the location of the newly defined *gip2* oncogenes within the network of biochemical events involved in the regulation of cell growth. In this regard, it must be noted that the transformation of Rat 1a cells was selective for α_{i2} polypeptides. We found that neither GTPase-deficient α_s mutants nor GTPase-deficient α_o mutants were capable of transforming Rat 1a cells. It will be of interest, however, to determine whether any of the newly identified G protein α subunits in the α_q or α_{12} families (34, 35) has the ability to alter growth of fibroblasts or whether this is a unique function of α_{i2} polypeptides.

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