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A mutant α subunit of G_{i2} induces neoplastic transformation of Rat-1 cells

(guanine nucleotide-binding protein/oncogene/NIH 3T3 cells)

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ABSTRACT **In a recently discovered class of oncogenes, GTPase-inhibiting mutations constitutively activate α subunits of signal-transducing guanine nucleotide-binding proteins (G proteins). Somatic mutations in a subclass of endocrine tumors are found in the arginine-179 codon of the α subunit of G_{i2} (α_{i2}), creating the putative *gip2* oncogene. We have tested the ability of *gip2* to mediate neoplastic transformation of Rat-1 and NIH 3T3 fibroblasts in tissue culture. Expression of a mutant α_{i2} cDNA encoding cysteine in place of arginine-179 (α_{i2}-R179C) caused Rat-1 cells to grow to a higher density in monolayer culture, to lose anchorage dependence, and to form tumors when injected subcutaneously into nude mice. In contrast, expression of α_{i2}-R179C failed to alter growth or tumorigenicity of NIH 3T3 cells. We conclude that *gip2* is an oncogene, by the criterion that it induces neoplastic transformation of Rat-1 cells. Failure of *gip2* to transform NIH 3T3 cells is in keeping with clinical indications that *gip2* is a tissue-selective oncogene.**

Hormone receptors activate heterotrimeric guanine nucleotide-binding proteins (G proteins) by promoting replacement of GDP by GTP in the guanine nucleotide-binding sites of the G proteins' α subunits. Each GTP-bound α subunit then regulates the activity of its specific downstream effector(s) until an intrinsic GTPase activity converts bound GTP to GDP, turning off the α subunit and returning it to its GDP-bound, inactive state. In the α subunit of G_s (α_s), point mutations (1–4) or cholera toxin-catalyzed covalent modification (5) inhibit this GTPase turnoff mechanism, thereby inducing constitutive activation of adenylyl cyclase, the effector of G_s.

Recently discovered mutations in genes for G protein α subunits have revealed an additional class of human oncogenes. As with many other oncogenes, these α subunit mutations presumably constitutively activate mitogenic signaling pathways that are normally regulated by their corresponding protooncogene products. The *gsp* oncogene results from somatic point mutations that inhibit the GTPase activity of α_s, causing constitutive activation of adenylyl cyclase. By mimicking mitogenic effects of trophic hormones that normally stimulate cAMP synthesis via G_s-coupled receptors, the *gsp* oncogene contributes to the growth of tumors derived from pituitary somatotrophs (2, 6–9) and thyroid cells (7).

A second putative G protein oncogene, *gip2*, results from somatic point mutations in the gene for the α subunit of G_{i2} (α_{i2}) (7). Three inferences suggest that these α_{i2} mutations create an oncogene: (*i*) *gip2* mutations are found in a substantial proportion (≈30%) of a restricted subclass of human tumors—i.e., those derived from the adrenal cortex or from endocrine cells of the ovary (7). (*ii*) *gip2* mutations were found by a method—allele-specific hybridization to tumor DNA—that can only detect clonally expanded alterations in DNA; clonal expansion is a hallmark of neoplasia. (*iii*) *gip2* mutations substitute cysteine or histidine for the arginine residue at position 179 (7); because arginine-179 is homologous to arginine-201 of α_s, the amino acid modified by cholera toxin and the residue altered by most *gsp* mutations, we suspect that its mutational replacement constitutively activates α_{i2} by inhibiting hydrolysis of GTP.

Here we test the hypothesis that *gip2* is an oncogene, by expressing a mutant α_{i2} cDNA in NIH 3T3 and Rat-1 fibroblasts. The mutant α_{i2} contains cysteine at position 179 in place of arginine, hereafter called α_{i2}-R179C; this was the predominant *gip2* mutation in human adrenal and ovarian tumors (7). Rat-1 cells were chosen because of a recent report (10) suggesting that lysophosphatidic acid induces mitogenesis in these cells by stimulating a pertussis toxin-sensitive G protein that also inhibits adenylyl cyclase—i.e., one of the three known G_i proteins, such as G_{i2}. NIH 3T3 cells were chosen because they are commonly used in tissue culture models of neoplastic transformation.

The choice of cell lines has proved crucial. Discovery of *gip2* in a small subset of human tumors suggested that the corresponding normal G protein, G_{i2}, would relay mitogenic signals in a similarly limited subclass of tissue culture cells. In our experiments, α_{i2}-R179C has turned out to be oncogenic in Rat-1 but not in NIH 3T3 cells.

MATERIALS AND METHODS

Plasmids. The *Eco*RI fragment of mouse α_{i2} cDNA (11) was subcloned downstream from a Moloney leukemia virus long terminal repeat in the retroviral expression vector pMV-7 (12) to generate the plasmid α_{i2}-WT/pMV-7, hereafter referred to simply as α_{i2}-WT. pMV-7 also contains the bacterial antibiotic resistance gene for neomycin phosphotransferase under control of the thymidine kinase promoter. The R179C mutation was made by site-directed mutagenesis according to the standard protocol of Bio-Rad's Muta-Gene *in vitro* mutagenesis kit. The *Eco*RI fragment of the mutant α_{i2} was subcloned into pMV-7 to generate the plasmid α_{i2}-R179C/pMV-7, hereafter referred to as α_{i2}-R179C. Frank McCormick (Cetus) provided the pEJ vector containing oncogenic *ras* cDNA (13), hereafter referred to as H-ras^{val12}.

Cell Lines. David Julius (University of California, San Francisco) and Wouter H. Moolenaar (Netherlands Cancer Institute) provided the NIH 3T3 and Rat-1 fibroblasts, respectively. pMV-7, α_{i2}-WT, or α_{i2}-R179C were processed through packaging cell lines and used to infect NIH 3T3 and Rat-1 cells, as described (14). The *ras* plasmid was introduced into Rat-1 cells directly by calcium phosphate precipitation (mammalian cell transfection kit, Specialty Media, Laval-

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Abbreviations: G protein, guanine nucleotide-binding protein; G_i and G_s, G proteins that inhibit and activate adenylyl cyclase, respectively; α_i and α_s, α subunits of G_i and G_s, respectively; CS, calf serum.

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lette, NJ). Infected or transfected cells were propagated in selective medium, made up of Dulbecco's modified Eagle's medium-H21 (DME-H21) with 10% bovine calf serum (CS) and G418 at 0.5 or 0.6 mg/ml (for NIH-3T3 or Rat-1 cells, respectively). All assays were performed on G418-selected pools of cells.

Immunoblots. G418-selected pools of cells were removed from plates with STV (0.05% trypsin and 5 mM EDTA in phosphate-buffered saline) and resuspended in cold lysis buffer (50 mM Tris, pH 7.5/2.5 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride). After a 10-min incubation on ice, cells were lysed by Dounce homogenization. Nuclei were removed by centrifugation at 900 × *g* for 5 min, and a membrane fraction was isolated by centrifugation of the supernatant fraction at 16,000 × *g* for 15 min.

Membrane proteins (100 μg per lane) were resolved on an SDS/12.5% polyacrylamide gel and transferred to nitrocellulose. Blots were blocked for 1 hr in Blotto (50 mM Tris, pH 7.4/150 mM NaCl/5% nonfat dry milk/0.01% sodium azide/0.05% Tween-20/5 mM EDTA) and then incubated overnight in Blotto containing rabbit polyclonal antiserum AS/7 (DuPont/NEN Research Products). Blots were washed twice with Blotto and then incubated for 1 hr in Blotto containing 7.5 μCi (1 Ci = 37 GBq) of ^{125}I-labeled protein A (Amersham). Blots were then washed twice with Blotto and three times with 50 mM Tris, pH 7.5/150 mM NaCl/0.02% Tween-20; antibody–antigen complexes were detected by autoradiography.

Measurement of Cell Growth. Cells were seeded into 24-well plates, and medium (10% CS in DME-H21) was changed every 3 days. Cells were removed from the wells with STV and counted with a Coulter Counter model ZM particle counter.

Growth in Soft Agar. A bottom layer, consisting of 0.7% SeaPlaque low-melting-temperature agarose (FMC) in DME-H21 with 10% CS, was poured into six-well plates (2 ml per well) and allowed to solidify. Cells were suspended in medium (10% CS in DME-H21) with 0.35% SeaPlaque at 5×10^3 cells per 3 ml and poured (3 ml per well) into six-well plates over the bottom layer. The medium was solidified by storage at 4°C for several hours and then incubated at 37°C. After a 16-day incubation, colonies were stained by adding 1 ml of *p*-iodonitrotetrazolium violet at 0.5 mg/ml (Sigma), which is converted to a colored product by live cells only.

Tumors in Nude Mice. Cells were removed from plates with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and suspended in phosphate-buffered saline with Ca^{2+} and Mg^{2+}. Nude mice were subcutaneously injected with the indicated numbers of cells in an injection volume of 25 μl. Four injection sites were tested for each number of cells, except for H-ras^{val12} cells, which were injected at two sites only. Animals were examined daily and sacrificed when tumor size reached ≈7 mm in diameter. The day of tumor appearance and the day of sacrifice were recorded.

RESULTS

Expression, Morphology, and Growth. Expression of endogenous α_i and recombinant α_{i2} was tested by immunoblotting membrane extracts with rabbit antiserum AS/7, which detects α subunits of transducin, G_{i1}, and G_{i2} (15, 16). The total amount of immunoreactive material, detected as a 40-kDa band, was increased ≈2-fold in membranes of NIH 3T3 and Rat-1 cell populations infected with α_{i2}-WT and α_{i2}-R179C, as compared to membranes from cells infected with vector alone (Fig. 1). We have reported elsewhere (17) a similar observation, using a different anti-α_i antiserum, with these NIH 3T3 populations.

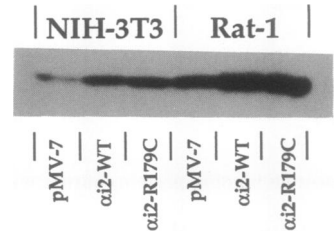

FIG. 1. Expression of recombinant α_{i2}-WT and α_{i2}-R179C in NIH 3T3 and Rat-1 cells. Membrane proteins from G418-selected populations of cells were resolved by SDS/PAGE, transferred to nitrocellulose, and immunoblotted.

Morphologically, Rat-1 cells infected with α_{i2}-R179C appeared slightly more refractile and elongated than control cells and formed more densely packed monolayers (results not shown). Rat-1 cells infected with α_{i2}-WT, however, were indistinguishable from their uninfected counterparts and from cells infected with pMV-7 alone. Neither α_{i2} construct, however, detectably altered the morphology of NIH 3T3 cells. In a focus assay in which infected cells were grown without G418 selection, none of the populations formed morphologically distinguishable foci after 8 weeks in culture.

Growth curves (Fig. 2 *Upper*) showed that Rat-1 cells infected with α_{i2}-R179C reached a saturation density 2- to 3-fold greater than that observed with parental, vector-infected or α_{i2}-WT-infected cells. This increase was similar in extent to that seen with the positive control, cells transformed by H-ras^{val12} (Fig. 1). Both H-ras^{val12} and α_{i2}-R179C cells began to shed from the dish surface after 7 days, while the other Rat-1 lines simply stopped proliferating at about 6 days, at a lower density. Growth curves with NIH 3T3 cells showed no increase in saturation density by either α_{i2}-R179C or α_{i2}-WT, as compared with pMV-7 alone or uninfected cells (Fig. 2 *Lower*). H-ras^{val12} was not tested in NIH 3T3 cells.

Pertussis toxin reduced proliferation of α_{i2}-R179C Rat-1 cells by ≈50% (Fig. 3), to a saturation density at 6 days

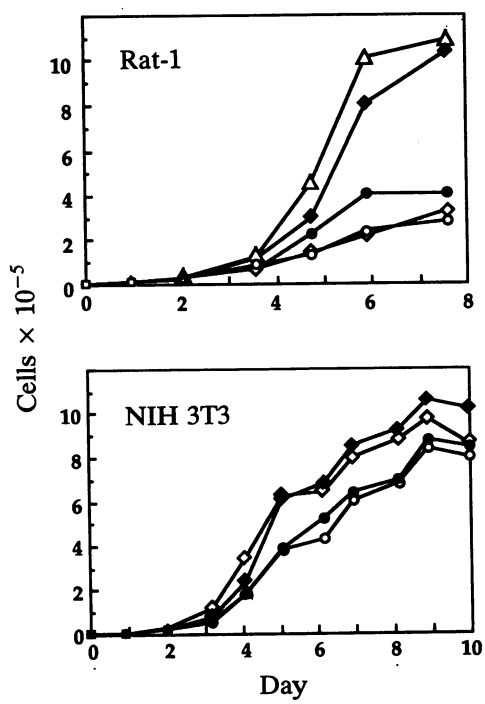

FIG. 2. Growth curves of Rat-1 (*Upper*) and NIH-3T3 (*Lower*) cell lines. Cells were seeded at 8×10^3 per well, and two wells were counted daily for each cell type. ○, Control; ◇, pMV-7; ●, α_{i2}-WT; ◆, α_{i2}-R179C; △, ras.

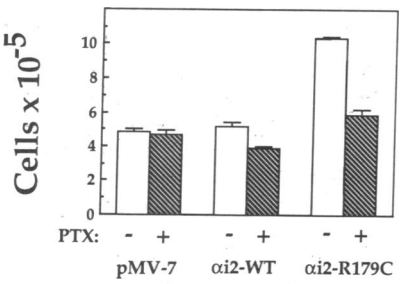

FIG. 3. Effect of pertussis toxin on saturation density. Rat-1 cells infected with pMV-7, α_{i2}-WT, and α_{i2}-R179C were seeded in quadruplicate at 10^4 cells per well and incubated for 6 days in the presence or absence of pertussis toxin (PTX) at 100 ng/ml. Bars indicate the mean ± SD.

similar to that observed with pMV-7. In α_{i2}-WT cells, the toxin caused a smaller decrease (≈20%).

Growth in Soft Agar. Transformed cells in culture often display a loss of anchorage dependence (18), which can be detected by growth of colonies from single cells suspended in soft agar. We plated 5×10^3 cells from different Rat-1 populations in soft agar (Table 1) and counted colonies at 16 days. In all populations tested, some cells (8–16%) were able to form small microcolonies. Only α_{i2}-R179C and H-ras^{val12} cells, however, formed detectable numbers of larger colonies, >175 μm in diameter (Table 1). Colonies formed from H-ras^{val12} cells were more numerous and somewhat larger than those from α_{i2}-R179C cells, however, suggesting that the transforming activity of H-ras^{val12} is greater than that of mutant α_{i2}.

Tumors in Nude Mice. The most stringent criterion for neoplastic transformation is the ability of cells to form tumors *in vivo*. We injected nude mice with G418-selected populations of NIH 3T3 and Rat-1 cells infected with pMV-7, α_{i2}-WT, or α_{i2}-R179C. As a positive control, we also injected Rat-1 cells expressing H-ras^{val12} cDNA. At a dose of 10^6 cells per injection site, none of the NIH 3T3 cell lines tested produced tumors.

As expected, H-ras^{val12} Rat-1 cells induced rapid formation of fast-growing tumors (Fig. 4). After injection of 5×10^5 H-ras^{val12} cells, tumors were detected at 6 days and grew rapidly thereafter; indeed, injected mice were sacrificed at 10 days, when tumor diameters reached the arbitrary cutoff size, a diameter of 7 mm.

α_{i2}-R179C Rat-1 cells also induced formation of tumors in nude mice (Fig. 4). Injection of 10^4 α_{i2}-R179C cells per site produced slow-growing tumors detected at 22 days at two sites and at 43 days at a third site; injection of the same number of α_{i2}-WT or pMV-7 Rat-1 cells produced no detectable tumors.

At a higher cell dose (10^5 per site), both control cells (pMV-7 and α_{i2}-WT) and α_{i2}-R179C-expressing cells produced tumors. However, tumors derived from α_{i2}-R179C cells arose earlier than did the controls and reached a diameter of 7 mm at 38–42 days; tumors derived from control cells, which seemed to grow more slowly, did not attain this size before termination of the experiment (Fig. 4). Tumors formed by injection of 10^5 α_{i2}-R179C-expressing cells arose later and grew more slowly than tumors formed by injection of 5×10^5 H-ras^{val12} cells.

Table 1. Anchorage-independent growth

Cells	No. of colonies >175 μm in diameter
Rat-1	0
pMV-7	0
α_{i2}-WT	0
α_{i2}-R179C	32
H-ras^{val12}	240

Rat-1 cells of the types indicated were seeded in soft agar at 5000 cells per well and incubated for 16 days.

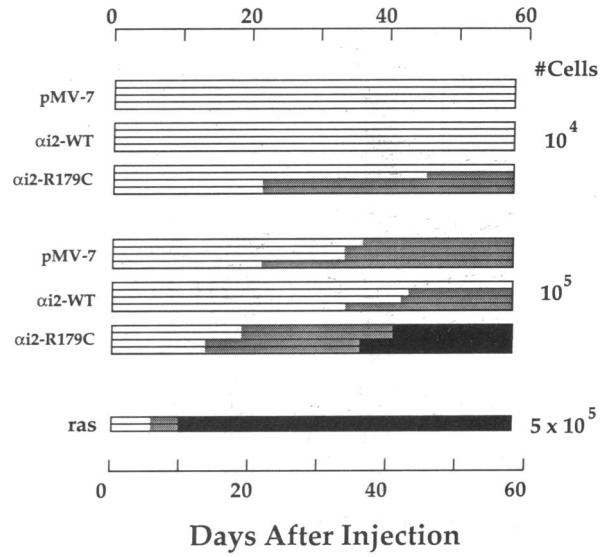

FIG. 4. Tumor formation by infected Rat-1 and *ras*-transformed cells. Nude mice were injected with the indicated Rat-1 cell lines in numbers shown at the right. Subdivisions within each horizontal bar indicate observations at individual injection sites. In each subdivision, the white segment represents tumor latency; the gray segment, which begins on the day the tumor was first detected, represents tumor growth; and the black segment indicates when the tumors reached a diameter of ≈7 mm.

We conclude that α_{i2}-R179C in Rat-1 cells promotes tumorigenesis at a level beyond that seen with appropriate controls.

Effects of cAMP on Growth. Because G_{i2} belongs to a subclass of G proteins that mediate hormonal inhibition of adenylyl cyclase, it seemed reasonable to ask whether cAMP affects growth of Rat-1 and NIH 3T3 cells. Fig. 5 shows that agents that mimic cAMP or elevate cellular cAMP substantially inhibited growth of Rat-1 cells, while the same agents exerted a much smaller effect on growth of NIH 3T3 cells. These agents include cholera toxin, which activates α_s; forskolin, a direct stimulator of adenylyl cyclase; and a cAMP analog, 8-bromo-cAMP.

DISCUSSION

Discovery of the putative oncogene *gip2* in human tumors (7) led to two predictions: (*i*) *gip2* will be found to encode a constitutively active α_{i2} subunit and (*ii*) *gip2* will promote neoplastic transformation in tissue culture cells. As reported elsewhere (17), we tested the first prediction by asking whether mutant α_{i2} can negatively regulate accumulation of cellular cAMP. As expected for a constitutively active α subunit of a G_i protein, stable expression of α_{i2}-R179C in NIH 3T3 cells decreased both forskolin- and hormone-induced cAMP accumulation; transient expression of mutant α_{i2} similarly inhibited cAMP accumulation in 293 cells. α_{i2}-WT had no effect in either expression system. Initial experiments (data not shown) indicate that α_{i2}-R179C inhibits cAMP accumulation in Rat-1 cells as well. These observations show that the R179C mutation in α_{i2} biochemically activates the α subunit's ability to inhibit cAMP accumulation and imply that the mutation will constitutively activate other effector functions of α_{i2}.

The present work tests and verifies the second prediction. Expression of *gip2* in Rat-1 fibroblasts induced neoplastic

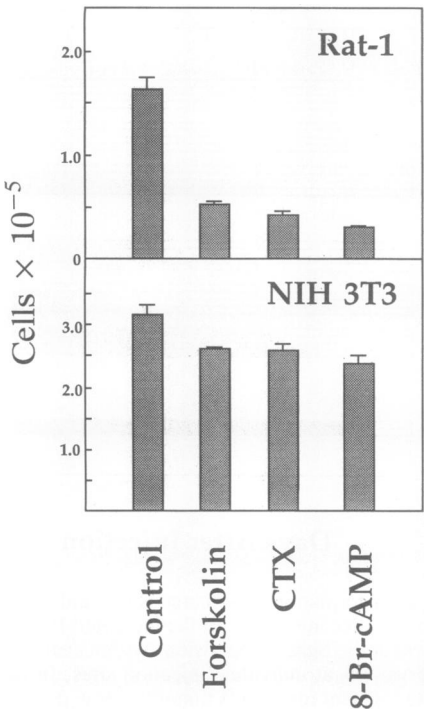

FIG. 5. Effect of cAMP on growth of Rat-1 and NIH 3T3 cell lines. Rat-1 or NIH 3T3 cells were seeded at 2×10^4 cells per well, incubated overnight, and then incubated for 4 days in the presence or absence of the indicated agent at the following concentrations: forskolin at 10 μM; cholera toxin (CTX) at 100 ng/ml; and 8-bromo-cAMP (8-Br-cAMP) at 100 μM.

transformation, as indicated by subtle changes in cell morphology, increased saturation density, and anchorage-independent growth. *gip2*-expressing cells also generated tumors in nude mice at a rate and frequency greater than that observed for control cells, thereby meeting the most stringent criterion of neoplastic transformation.

In contrast to its effect in Rat-1 cells, *gip2* had no obvious effect on the growth of NIH 3T3 cells. Saturation densities of NIH 3T3 cells expressing α_{i2}-R179C were not substantially greater than those of controls (parental, vector-infected, and α_{i2}-WT-infected cells). *gip2* expression did not alter the morphology of NIH 3T3 cells and it did not cause them to form tumors in nude mice. Despite its failure to alter growth of NIH 3T3 cells, α_{i2}-R179C was expressed and biochemically active in these cells, as shown by immunoblots and inhibition of cAMP accumulation (17).

The cell-specific capacity of *gip2* to promote mitogenesis in one cell type, but not in another, is probably responsible for the limited distribution of *gip2* mutations in human tumors: a search for *gip2* mutations in more than 250 human tumors, derived from many different tissues, revealed *gip2* mutations only in tumors derived from adrenal cortex or from ovarian endocrine cells (7). *gip2* mutations will apparently contribute to oncogenesis only in cells that utilize normal α_{i2} to transduce a mitogenic signal, just as *gsp* mutations seem to be oncogenic only in cells that use α_s to transduce a cAMP-mediated mitogenic signal—i.e., in pituitary somatotrophs and in thyroid cells (2, 7).

Pertussis toxin prevented the increase in saturation density attained by Rat-1 cells expressing mutant α_{i2} (Fig. 3). Because the toxin uncouples G_i proteins from activation by hormone receptors (19–21), its effect on proliferation of these cells suggests that the mitogenic effect of the mutant protein depends in part upon its activation by receptors that bind growth factors in the medium; these growth factors, as yet unidentified, could be provided by serum or produced by the cells themselves. This interpretation is based upon the GTPase cycle of G proteins: the R179C mutation inhibits GTP hydrolysis by the mutant α_{i2}, but by itself the mutation does not shift a large enough fraction of α_{i2} into the active GTP-bound state to exert a large mitogenic effect; the slower rate of GTP hydrolysis by the mutant protein does, however, potentiate the effect of growth factors acting on receptors coupled to G_{i2}. In contrast, increased expression of wild-type α_{i2} does not produce a pertussis toxin-sensitive mitogenic effect, presumably because the normal protein hydrolyzes GTP more rapidly and thus lacks the amplifying effect of the mutant protein. The effect of the α_{i2} mutation parallels effects of cholera toxin and GTPase-inhibiting mutations in α_s, both of which potentiate hormonal stimulation of adenylyl cyclase (2, 5).

Agents that elevate or mimic intracellular cAMP markedly inhibited the growth of Rat-1 cells, but only slightly inhibited that of NIH 3T3 cells. This difference raises the possibility that *gip2* stimulates the growth of Rat-1 cells by inhibiting adenylyl cyclase, thereby relieving a tonic inhibitory effect of cellular cAMP on cell proliferation. In agreement with this interpretation, lysophosphatidic acid inhibits adenylyl cyclase and stimulates DNA synthesis in Rat-1 cells, and both effects are blocked by pertussis toxin (10); similar results are reported for several mitogens in CCL39 cells as well (22–24). According to this scenario, *gip2* exerts no mitogenic effect in NIH 3T3 cells—despite its ability to inhibit cAMP accumulation—because cAMP does not negatively regulate proliferation of these cells.

Despite its appealing simplicity, the idea that decreased cellular cAMP universally mediates pertussis toxin-sensitive mitogenic pathways is clearly contradicted by observations in another cell type, Swiss 3T3 cells. Although pertussis toxin blocks effects of several mitogens in Swiss 3T3 cells (25–27), elevated cellular cAMP actually promotes growth of this cell line (ref. 28 and references therein). In this case, mitogenic stimuli relayed through G_i proteins cannot depend on inhibition of adenylyl cyclase and therefore must utilize a different signaling pathway.

Of the other signaling pathways activated by G_i, which ones promote mitogenesis? One obvious alternative pathway is the inositol phospholipid/Ca^{2+} cascade. Results with pharmacological antagonists and/or pertussis toxin have ruled out this alternative in several cell types, including Rat-1 (10) and CCL39 (23, 24) fibroblasts. Analysis of other α_{i2}-mediated signals may help to elucidate the mitogenic pathway triggered by *gip2*.

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