Regulation of the Differentiation of Teratocarcinoma Cells into Primitive Endoderm by $G\alpha_{i2}$

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The amount of the heterotrimeric G protein subunit $G\alpha_{i2}$ decreases after the induction of F9 teratocarcinoma cells to become primitive endoderm in the presence of retinoic acid (RA). The reduction of the $G\alpha_{i2}$ protein in F9 cells by antisense RNA expression was associated with (i) loss of receptor-mediated inhibition of adenylyl cyclase; (ii) decreased cell doubling time; (iii) induction of a primitive, endoderm-like phenotype in the absence of RA; and (iv) production of the differentiation marker tissue-type plasminogen activator. Expression of a constitutively active, mutant $G\alpha_{i2}$ blocked RA-induced differentiation. These data suggest the involvement of $G\alpha_{i2}$ in the control of stem cell differentiation and provide insight into the involvement of G proteins in growth regulation.

The heterotrimeric G proteins regulate a variety of cellular processes (*1–5*). Pertussis toxin–sensitive G proteins $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ mediate transmembrane signaling events, including the inhibition of adenylyl cyclase, the activation of phospholipases A_2 and C, the regulation of ion channels, and mitogenesis (*1–6*). The morphogen RA induces F9 teratocarcinoma embryonic stem cells to differentiate into primitive endoderm-like cells (*7, 8*). Differentiation is accompanied by a decrease in the steady-state amount of the $G\alpha_{i2}$ subunit (*9*). To investigate the function of $G\alpha_{i2}$ in the differentiation of F9 cells into primitive endoderm, we reduced the amount of $G\alpha_{i2}$ in cells by expressing antisense RNA and increased the $G\alpha_{i2}$ activity by the expression of a constitutively active mutant [$Gln^{205} \rightarrow Leu^{205}$ (Q205L)] form of $G\alpha_{i2}$.

Antisense RNA blocks the expression of targeted proteins by hybridizing with the mRNA and preventing translation (*10, 11*). Thirty-nine bases of the 5′ noncoding region immediately upstream of and including the ATG translation initiation codon of $G\alpha_{i2}$ were used as antisense templates (Fig. 1A). This region of $G\alpha_{i2}$ has no similarity with other sequences in GenBank. The retroviral expression vector pLNCX (*12*) with a cytomegalovirus promoter was used to express the antisense RNA for $G\alpha_{i2}$ in the construct pLNCXASGα_{i2} (Fig. 1B). After retroviral infection of F9 stem cells with this construct, a similar construct that contained $G\alpha_{i1}$ sequences (pLNCXASGα_{i1}), or the vector alone (pLNCX), neomycin-resistant colonies were selected and then tested for expression of $G\alpha_{i2}$ by immunoblotting (Fig. 1C). The vector encoding antisense to $G\alpha_{i1}$ (pLNCXASGα_{i1}) was used as a control because F9 stem cells do not express the $G\alpha_{i1}$ protein or mRNA (*13*). The retroviral vector alone, pLNCX, provided another control.

The F9ASGα_{i2} clone that contained the antisense $G\alpha_{i2}$ construct showed a decrease in the expression of $G\alpha_{i2}$ (>85%) when compared to either the F9ASGα_{i1} cells or the wild-type (control) F9 stem cells (Fig. 1C). Staining of immunoblots with antibodies to other G protein subunits [$G\alpha_s$ (Fig. 1C) or Gβ] (*14*)] demonstrated that the decrease in protein expression was specific for $G\alpha_{i2}$ in the F9ASGα_{i2} cells. The inhibitory control of adenylyl cyclase, believed to be mediated by $G\alpha_{i2}$ (*15–17*), was examined in F9ASGα_{i2} cells. The ability of thrombin to inhibit forskolin-stimulated adenylyl cyclase was attenuated in the F9ASGα_{i2} cells (Fig. 2). The suppression of

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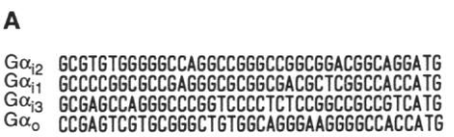

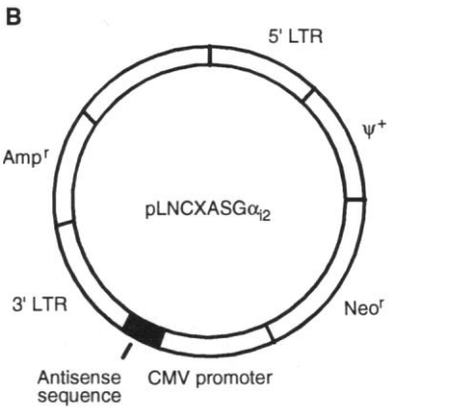

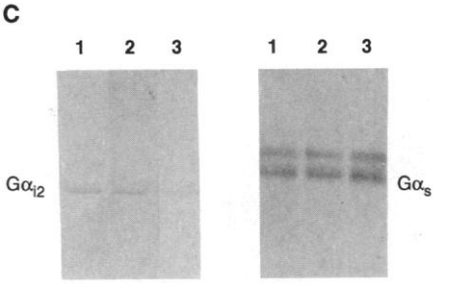

Fig. 1. An antisense RNA construct and its effect on the expression of $G\alpha_{i2}$ in F9 teratocarcinoma cells. (**A**) The 39 bases of the 5′ noncoding region of $G\alpha_{i2}$ were selected for use as an antisense probe to take advantage of the diversity of the nucleotide sequence in this region and to provide specificity. Oligodeoxynucleotides of this sequence and the complementary strand were synthesized commercially (Genosys, Woodlands, Texas). Each oligodeoxynucleotide contained additional bases at both ends to facilitate cloning. (**B**) The pLNCX vector (*12*) contains an ampicillin gene (Amp^r) and neomycin resistance (Neo^r) and retroviral packaging genes (Ψ^+) under the control of the mouse Moloney virus long terminal repeats (5′ and 3′ long terminal repeat). The antisense sequences are transcribed under the control of the cytomegalovirus promoter. The construction of the vectors outlined below was performed with the use of standard techniques (*24*). The oligodeoxynucleotides were hybridized together, and the double-stranded DNA was ligated between the Hind III and Cla I sites of the polylinker region of the retroviral vector pLNCX. Retroviral vectors that contained no insert (pLNCX), antisense sequence to $G\alpha_{i1}$ (pLNCXASGα_{i1}), and antisense sequence to $G\alpha_{i2}$ (pLNCXASGα_{i2}) were transfected into Ψ_{GP+E86} packaging cells (*25*). After selection of neomycin-resistant colonies [G418 (500 μg/ml), Gibco], colonies of packaging cells that had high titer virus production were identified by Northern (RNA) blotting with labeled oligodeoxynucleotides as probes. Virus from these cell lines was then used to infect F9 stem cells that had been pre-incubated in hexadimethrine bromide (Sigma) for 24 hours. Neomycin-resistant F9 colonies were selected and then screened for the expression of $G\alpha_{i2}$ by immunoblotting. (**C**) Immunoblots of cell membranes (100 μg per lane) from a single batch of cells subjected to 10% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antibodies specific to $G\alpha_{i2}$ (antibody CM112, right panel) or $G\alpha_s$ (antibody CM129, left panel). Lanes 1, F9 stem cells; lanes 2, F9 cells infected with the antisense $G\alpha_{i1}$ virus as a control (F9ASGα_{i1}); and lanes 3, F9 cells infected with the antisense $G\alpha_{i2}$ virus (F9ASGα_{i2}). Immune complexes were made visible by use of ^{125}I-labeled iodinated goat anti-rabbit immunoglobulin G as described (*9*). Autoradiograms of the dried immunoblots are shown; we scanned these with an Ultroscan laser densitometer (LKB, Bromma, Sweden) to estimate the relative amount of G protein subunit in each lane. Membranes were collected from the postnuclear supernatant of cell homogenates and were analyzed for G protein content by immunoblotting as described (*9*).

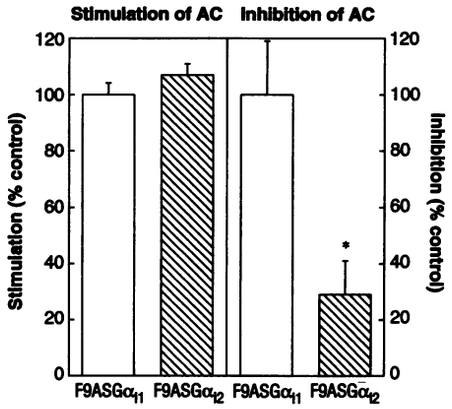

Fig. 2. Attenuation of the inhibition of adenylyl cyclase (AC) by thrombin in F9 cells that expressed antisense $G\alpha_{i2}$ RNA. F9ASGα_{i1} and F9ASGα_{i2} cells (50,000 cells in 100 μl of Krebs' Ringer phosphate buffer) were incubated with various agents for 15 min at 37°C. The reaction was terminated by the addition of HCl (0.1 M final concentration) and heating to 100°C. The amount of cAMP produced was determined by a competitive protein binding assay (*26*). To examine the stimulation of adenylyl cyclase (left panel), we incubated the cells with isoproterenol (100 μM). The amount of stimulation is presented as a multiple of the basal concentration normalized to F9ASGα_{i1} cells; basal levels were 9.1 ± 3.2 and 8.2 ± 2.4 pmol in 10^6 cells in F9ASGα_{i1} and F9ASGα_{i2} cells, respectively. To examine the inhibition of adenylyl cyclase (right panel), we incubated the cells with forskolin (10 μM) in the presence or absence of thrombin (0.1 nM). The amount of forskolin-stimulated cAMP accumulation inhibitable by thrombin is expressed as a percentage of control inhibition (actual inhibition was 46.5 ± 7.6% and 14.3 ± 11.3% in F9ASGα_{i1} and F9ASGα_{i2} cells, respectively). Asterisk, $P < 0.05$ compared to control, Student's t test.

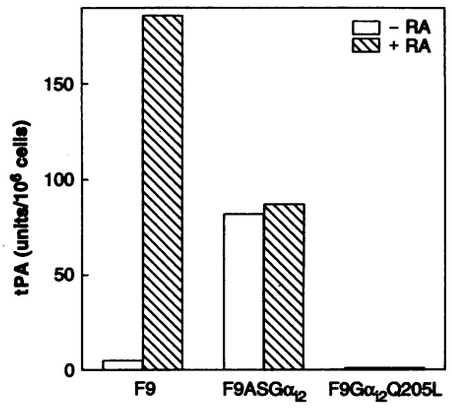

Fig. 4. Analysis of tPA production after inhibition of $G\alpha_{i2}$ expression with antisense RNA or constitutive activation of $G\alpha_{i2}$ with mutant Q205L. F9 stem, F9ASGα_{i2}, and F9Gα_{i2}Q205L cells were plated at a density of 5×10^6 cells in DMEM supplemented with 15% (v/v) fetal calf serum on gelatinized 100-mm dishes in the absence (open bars) or presence (hatched bars) of RA (100 nM). At the end of the fourth day of growth, the culture medium was assayed for tPA activity by the amidolytic assay (*27*). One unit of tPA is arbitrarily defined as that amount of tPA that results in a reaction rate of 10^{-5} ΔA_{405} min^{-2} (change in optical absorbance at 405 nm divided by the square of the time in minutes) (*27*). The data shown are from a single experiment, representative of four separate experiments.

$G\alpha_{i2}$ expression by antisense RNA in a F9ASGα_{i2} clone was sufficient to decrease the inhibitory adenylyl cyclase response by more than 70%. This observation confirms other recent data that implicate $G\alpha_{i2}$ as the mediator of adenylyl cyclase inhibition (*15–17*). Forskolin and isoproterenol stimulated adenylyl cyclase in F9ASGα_{i2} cells to the same extent as in F9ASGα_{i1} and F9 stem cells (Fig. 2).

F9 stem cells in culture exhibit a simple, rounded morphology (Fig. 3A). When induced to differentiate by RA, the cell doubling time is prolonged and the cells assume a morphology characteristic of primitive endoderm—an extended spindle shape with defined foci of cell growth (Fig. 3B). The F9ASGα_{i2} cells displayed a primitive endoderm-like morphology at all stages of growth, forming defined growth foci at confluence (Fig. 3C). Treating F9ASGα_{i2} cells with RA did not result in further changes in morphology (Fig. 3D). In contrast, the F9ASGα_{i1} cells displayed a normal stem cell phenotype in the absence of RA (Fig. 3E) and a primitive endoderm-like phenotype after 4 days of treatment with RA (Fig. 3F).

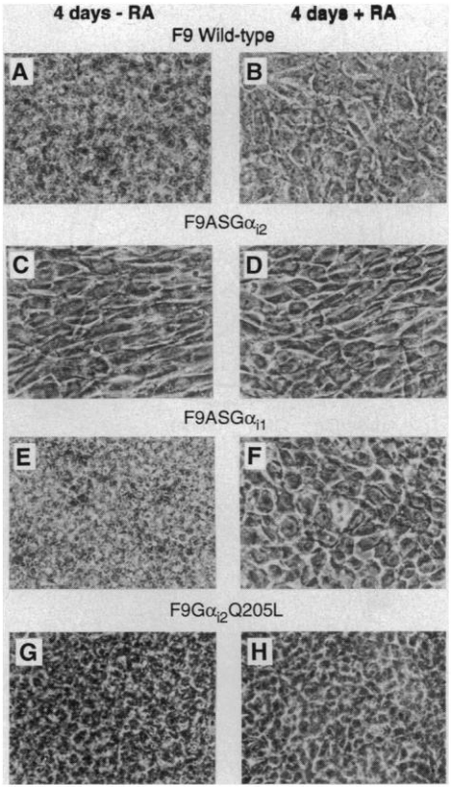

Fig. 3. Regulation of the differentiation of F9 stem cells to primitive endoderm by $G\alpha_{i2}$ activity. (**A** and **B**) F9 stem cells. (**C** and **D**) F9ASGα_{i2} cells. (**E** and **F**) F9ASGα_{i1} cells. (**G** and **H**) F9Gα_{i2}Q205L cells. Cells were plated at a density of 2000 cells per well of eight-well chamber slides (NUNC, Naperville, Illinois) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) fetal calf serum in the absence (left panels) or presence (right panels) of RA (100 nM). The cells were cultured for 4 days and then fixed with 3% (w/v) paraformaldehyde, examined under phase-contrast microscopy, and photographed with a Zeiss Axiophot. The photomicrographs shown are representative of three to seven independent experiments.

When F9 stem cells are treated with RA, the rate of cell growth declines as the number of cells that have terminally differentiated increases (*18*). The doubling time of F9ASGα_{i2} cells was 28 hours in the absence of RA as compared to 16 hours for F9 stem cells. By the criteria of cell morphology, growth rate, and resistance to the effects of RA, the F9ASGα_{i2} cells had differentiated into primitive endoderm.

Tissue-type plasminogen activator (tPA) production is a marker for primitive endoderm. Before differentiation, the F9 stem cells produce no tPA; once differentiated into primitive endoderm, the cells produce and secrete tPA (*8, 18*). The changes in doubling time and morphology of the F9ASGα_{i2} cells in the absence of RA prompted us to assay the ability of these cells to produce tPA. The F9ASGα_{i2} cells produced nearly as much tPA as the RA-induced primitive endoderm cells (Fig. 4). Treatment of F9ASGα_{i2} cells with RA did not induce further production of tPA.

Adenosine 3′,5′-monophosphate (cAMP) is a mitogen for a variety of cell lines in culture (*19*). Basal amounts of cAMP were essentially unaltered; stem, F9ASGα_{i1}, and F9ASGα_{i2} cells contained 7.9 ± 3.2, 9.1 ± 3.2, and 8.2 ± 2.4 pmol of cAMP in 10^6 cells (mean ± SEM, $n = 5$), respectively. Treatment of F9 stem cells for 4 days with dibutyryl cAMP (10 mM) failed to induce either the morphology characteristic of primitive endoderm or the biochemical marker tPA (*8, 14, 18*). These two observations demonstrate that the effects of the decreased expression of $G\alpha_{i2}$ in the differentiation of F9ASGα_{i2} cells are independent of changes in the amount of cAMP.

Because decreased expression of $G\alpha_{i2}$ induced cell differentiation, we investigated if the overexpression of $G\alpha_{i2}$ activity could block RA-induced differentiation. F9 cells were transfected with expression vectors that contained a cDNA encoding a mutant form of $G\alpha_{i2}$, designated $G\alpha_{i2}$Q205L.

$G\alpha_{i2}$Q205L has a point mutation in the guanosine triphosphatase (GTPase) region of the molecule, which renders it constitutively active, independent of hormone-receptor interactions (*15*). This mutation results in the expression of an oncogenic form of $G\alpha_{i2}$, *gip2*, in human tumors (*20*). In addition, the expression of $G\alpha_{i2}$Q205L decreased the doubling time of Rat 1a, Swiss 3T3, and NIH 3T3 cells (*21*). The expression of $G\alpha_{i2}$Q205L (F9$G\alpha_{i2}$Q205L) with the vectors described (*21*) resulted in more than a twofold increase in immunoreactivity in blots of cell membranes stained with antibodies specific to $G\alpha_{i2}$ (*14*). F9 clones that expressed $G\alpha_{i2}$Q205L were refractory to RA-induced differentiation (Fig. 3, G and H). These F9$G\alpha_{i2}$Q205L cells did not produce tPA in response to RA (Fig. 4). Thus, the increased $G\alpha_{i2}$ activity in F9$G\alpha_{i2}$Q205L cells blocked RA-induced differentiation of F9 cells, as judged by both morphological and biochemical criteria.

We have demonstrated the ability of $G\alpha_{i2}$ activity to regulate the differentiation of F9 stem cells into primitive endoderm. Oncogenic transformation leading to proliferation can be thought of as a failure of the cell to differentiate (*6*). The increased activity of $G\alpha_{i2}$ that results from expression GTPase-deficient mutants of $G\alpha_{i2}$ results in the transformation of fibroblasts in cell culture (*21*). Injection of antibodies specific for $G\alpha_{i2}$, in order to block $G\alpha_{i2}$ activity, inhibits serum-stimulated DNA synthesis (*22*). A decrease in $G\alpha_{i2}$ expression in F9ASGα_{i2} cells induced the differentiation of F9 stem cells, whereas an increase in $G\alpha_{i2}$ activity in F9$G\alpha_{i2}$Q205L cells blocked differentiation. These data provide insight into the control of cell growth and differentiation by G proteins and highlight a function other than inhibition of adenylyl cyclase mediated by $G\alpha_{i2}$—namely, control of differentiation.

Note added in proof: Recently (*23*) we demonstrated the critical role of another G protein, $G\alpha_s$, in controlling the rate of differentiation of fibroblasts to adipocytes. In mouse 3T3-L1 fibroblasts, reduction in $G\alpha_s$ expression induced by oligodeoxynucleotides antisense to $G\alpha_s$ accelerated adipogenesis induced by glucocorticoid and methyxanthine.

REFERENCES AND NOTES

1. H. R. Bourne, D. A. Sanders, F. McCormick, *Nature* **348**, 125 (1990).
2. _____, *ibid.* **349**, 117 (1991).
3. L. Birnbaumer, J. Abramowitz, A. M. Brown, *Biochim. Biophys. Acta* **1031**, 163 (1990).
4. A. M. Brown and L. Birnbaumer, *Annu. Rev. Physiol.* **52**, 197 (1990).
5. A. G. Gilman, *Adv. Second Messenger Phosphoprotein Res.* **24**, 51 (1990).
6. J. Pouyssegur and K. Seuwen, *Annu. Rev. Physiol.* **54**, 195 (1992).
7. S. Strickland and V. Mahdavi, *Cell* **15**, 393 (1978).
8. S. Strickland, K. K. Smith, K. R. Marotti, *ibid.* **21**, 347 (1980).
9. P. A. Galvin-Parton, D. C. Watkins, C. C. Malbon, *J. Biol. Chem.* **265**, 17771 (1990).
10. A. R. van der Krol, J. N. M. Mol, A. R. Stuitje, *BioTechniques* **6**, 958 (1988).
11. A. E. Griep and H. Westphal, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6806 (1988).
12. A. D. Miller and G. J. Rosman, *BioTechniques* **7**, 980 (1989).
13. P. Galvin-Parton and C. C. Malbon, unpublished observations.
14. D. C. Watkins and C. C. Malbon, unpublished observations.
15. J. M. Lowndes *et al.*, *J. Biol. Chem.* **266**, 14193 (1991).
16. Y. H. Wong *et al.*, *Nature* **351**, 63 (1991).
17. F. R. McKenzie and G. Milligan, *Biochem. J.* **267**, 391 (1990).
18. A. L. Darrow, R. J. Rickles, S. Strickland, *Methods Enzymol.* **190**, 110 (1990).
19. R. J. Klebe *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9588 (1991).
20. J. Lyons *et al.*, *Science* **249**, 655 (1990).
21. S. K. Gupta *et al.*, *Mol. Cell. Biol.* **12**, 190 (1992).
22. V. J. LaMorte *et al.*, *J. Biol. Chem.* **267**, 691 (1992).
23. H.-Y. Wang, D. C. Watkins, C. C. Malbon, *Nature* **358**, 334 (1992).
24. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989).
25. D. Markowitz, S. Goff, A. Bank, *Virology* **167**, 400 (1988).
26. B. L. Brown *et al.*, *Biochem. J.* **121**, 561 (1971).
27. P. Andrade-Gordon and S. Strickland, *Biochemistry* **25**, 4033 (1986).

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