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Genetics

Embryonic stem cells lacking a functional inhibitory G-protein subunit (α_{i2}) produced by gene targeting of both alleles

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ABSTRACT **The α_{i2} subunit of the inhibitory heterotrimeric guanine nucleotide-binding proteins is highly conserved in mammals and is expressed in all cell types, but its exact function is not yet defined. We have investigated the role of this protein by producing embryonic stem (ES) cells lacking a functional α_{i2} gene. These α_{i2}-null cell lines regulate adenylyl cyclase and grow and differentiate *in vitro* the same as wild-type ES cells. Homologous recombination was used to sequentially inactivate both copies of the α_{i2} gene. The first allele was inactivated by insertion of a neomycin-resistance gene. We modified the hygromycin B-resistance gene for improved expression in ES cells and used this gene to inactivate the remaining normal allele. The techniques used should be generally applicable to other genes whether or not they are expressed in ES cells.**

Heterotrimeric guanine nucleotide-binding proteins (G proteins) (composed of α, β, and γ subunits) comprise a large family of signal transducing proteins. These proteins transmit signals modulating a diverse set of functions, including regulation of cell growth, cAMP levels, phospholipase activity, and ion channel activity. The α subunits bind guanine nucleotides and are thought to determine the functional role of the G protein. The α_i subunits, first defined as inhibitors of adenylyl cyclase, are uncoupled from receptors by pertussis toxin and, therefore, may play a role in any signal transduction pathway inhibited by pertussis toxin [for reviews, see Neer and Clapham (1), Gilman (2), and Raymond *et al.* (3)].

There are three closely related α_i subunits, α_{i1}, α_{i2}, and α_{i3} (2, 4), which have nearly identical activities *in vitro* (5). However, these three subunits have been highly conserved throughout mammalian evolution, suggesting a specific necessary role for each. For example, the human α_{i2} subunit is more similar to the murine or rat α_{i2} subunit (98%) than rat α_{i2} is to either rat α_{i1} (88%) or rat α_{i3} (85%) (4, 6). Whereas α_{i1} is not expressed in some cells, α_{i2} has been found in every cell type, suggesting an important role for this subunit in cell or animal survival (7).

The α_i subunits have been implicated in processes that regulate cell growth and differentiation. Pertussis toxin blocks the mitogenic effects of such agonists as thrombin, bombesin, vasopressin, and bradykinin, which are known to activate the α_i subunit (8–12). Changes in the ratio of the different α_i or α_o subunits alter the cell's growth rate and its responses to mitogens (ref. 13; E.J.N., unpublished results). Further, a mutationally altered α_{i2} subunit has been identified as an oncogene in adrenal and ovarian tumors (14), again suggesting an important role in control of the growth rate.

Because mutationally altered cells lacking a functional α_s subunit (cyc^- cells) were instrumental in defining the function of α_s (2), we predicted that mutationally altered embryonic stem (ES) cells lacking a functional α_{i2} subunit would help define the function of this subunit. We report here the construction of an ES cell line lacking a functional α_{i2} subunit by an extension of the method of gene inactivation by homologous recombination. The availability of an ES cell line lacking α_{i2} should eventually allow the subunit's role in the process of cell growth regulation, differentiation, and signal transduction to be defined. Surprisingly, we found that ES cells lacking a functional α_{i2} subunit (referred to as α_{i2}-null cells) are indistinguishable from wild-type ES cells *in vitro* as determined by several criteria.

With the discovery that gene sequences in mammalian cells could be targeted by homologous recombination, methods for constructing mutationally altered cells that lack particular genes have become available. Over the past several years, a number of groups have demonstrated that heterozygous cells could be constructed in which one of two alleles has been inactivated (15–21). We used this approach to create a cell that is heterozygous for the α_{i2} gene and then extended this method to allow the inactivation of the remaining normal allele to produce a cell line lacking α_{i2}. Unlike the recent report of inactivation of both alleles of *pim-1* (22), our method should be applicable to genes regardless of their expression in ES cells.

MATERIALS AND METHODS

Cloning of the α_{i2} Gene and Construction of Targeting Vectors. The murine α_{i2} gene (λ phage clone α_{i2}-20) was cloned from a bacteriophage λ genomic library (Clontech), and the restriction map was established as described (23). A 6.8-kilobase (kb) *Eco*RI/*Sal* I fragment from λ clone α_{i2}-20 was subcloned, and the location of exon 6 and its associated *Bam*HI site (24) was defined by nucleotide sequencing (data not shown). The position of exon 6 is shown by the vertical bar in Fig. 1*A*. The sixth exon was interrupted in both constructs at a *Bam*HI site by a selectable marker. The *neo* gene was driven by the phosphoglycerate kinase (PGK) promoter region (25), which also contributed the polyadenylylation signal (26). The herpes simplex virus thymidine kinase gene was also driven by the PGK 5′ sequences.

ES Cell Culture. ES cells (line CCE obtained from E. Robertson, Columbia University, New York) were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 0.1 mM 2-mercaptoethanol, and conditioned medium (1:1000) from Chinese hamster ovary cells overexpressing leukemia inhibitory factor (Genetics Institute, Cambridge, MA) to maintain the undifferentiated state (27). Cells were grown to ≈75% confluence and harvested by trypsinization; $\approx 10^7$ cells were suspended in 1 ml of 20 mM Hepes, 145 mM NaCl, and 0.1 mM 2-mercapto-

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Abbreviations: G protein, guanine nucleotide-binding protein; α_i, α subunit of a G protein that inhibits adenylyl cyclase; PGK, phosphoglycerate kinase; BSA, bovine serum albumin; GTP[γ-S], guanosine 5′-[γ-thio]triphosphate; ES, embryonic stem; GANC, gancyclovir.

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ethanol containing 1 pmol of the α_{i2}-neo construct (see Fig. 1*A*). Electroporation was performed by using a Bio-Rad Gene Pulser at 450 V and 250 μF. Cells were then plated at $\approx 5 \times 10^6$ cells per 150-mm gelatinized Petri dish. After 2 days, cells were selected with either G418 at 0.15 mg/ml alone or G418 and gancyclovir (GANC) at 2 μM. Cells were cultured for an additional 7–10 days with daily medium changes, after which surviving colonies were counted, isolated, and expanded. One of the cell lines identified as heterozygous for homologous recombination (17E10) was then transfected with the α_{i2}-hyg construct (see Fig. 1*A*). Transfection and selection were the same as for α_{i2}-neo except that hygromycin B (0.1 mg/ml) was substituted for G418.

Determination of Growth Rates. Cells were grown in 24-well plates. Each cell line was plated at a similar density, and the cell numbers were measured during logarithmic phase growth. Cell number was measured by trypsinizing and counting the cells from each of 9–12 wells at 0, 6, 18, and 24 hr. Three independent lines of α_{i2}-null cells were studied. The best fit line in a semilogarithmic plot was determined for each cell line, and the doubling times and 95% confidence intervals were calculated.

Production of a Modified Hygromycin Marker. PCR amplification (23) of the *hyg* gene with oligomers GCCATGCATCCCGCCGCCACCATGAAAAAGCCTGAACTCACC and CGTCCGAGGGCAAAGGAATAGAGT produced a DNA molecule with *Nsi* I restriction sites at each end, a consensus translational start sequence (28), and the *hyg* gene. After digestion with *Nsi* I, this DNA molecule was inserted between the *Pst* I sites in the PGK-neo vector, replacing the *neo* gene with the modified *hyg* gene to yield the PGK-hyg vector.

Southern and Northern Analyses. DNA was fractionated on 1% agarose gels, transferred to GeneScreen, and hybridized as described (23). RNA from each cell line was fractionated on a formaldehyde/agarose gel (1%), transferred to GeneScreen, hybridized to a 1.4-kb genomic fragment containing exon 4 from the α_{i2} gene (probe A, Fig. 1) as described (23), and washed twice at 70°C in 0.2× standard saline citrate for 20 min.

Western Analysis. Fifty micrograms of total cell lysate or crude particulate fraction was applied to lanes of an SDS/11% PAGE gel. The proteins were transferred to a nitrocellulose filter as described by Towbin *et al.* (29). The filters were blocked with 3% bovine serum albumin (BSA) or with 3% BSA/3% goat serum for 1–2 hr at room temperature or overnight at 4°C. They were incubated with antibody diluted in 3% BSA or 3% BSA/3% goat serum overnight at 4°C. The nitrocellulose filter was washed twice with 10 mM Tris/0.996 NaCl, pH 7.4 (TBS), once with TBS plus 0.05% Nonidet P-40, and again with TBS before incubating with ^{125}I-labeled goat anti-rabbit IgG (ICN). The radioactive IgG was washed from the membrane as described above, and the membrane was used to expose Kodak X-AR film with two screens at −70°C. The antibody used recognizes α_{i1} and α_{i2} approximately equally well [New England Nuclear; NEI-801 (30)].

Adenylyl Cyclase Assays. The adenylyl cyclase assay was performed as described by Salomon (31). The standard assay contained 0.5 mM ATP, 1 μCi (1 Ci = 37 GBq) of [^{32}P]ATP (Amersham), 50 μM cAMP, 5 mM $MgCl_2$, 50 mM Tris (pH 7.6), 1 mM dithiothreitol, BSA (0.1 mg/ml), 10 mM creatine kinase, creatine phosphate (5 units/ml), and 20–30 μg of membrane protein in a total volume of 50 μl. The reaction was carried out at 30°C for 20 min and stopped with a solution containing 8000–15,000 cpm of [^{3}H]cAMP to monitor recovery of [^{32}P]cAMP from the columns. Under these conditions, the reaction was proportional to the amount of protein (data not shown).

Differentiation of ES Cells. ES cells were differentiated first by growth of embryoid bodies for 5–7 days (32) in bacterial plates to avoid attachment. The cells were cultured in medium lacking leukemia inhibitory factor, which is required to maintain ES cells in an undifferentiated state. The embryoid bodies were then transferred to gelatinized glass coverslips and allowed to attach. They were analyzed 5–10 days later for differentiated cells by immunohistochemistry. Primary antibodies were cardiac and skeletal myosin (MF-20; courtesy of D. A. Fischman, Cornell University Medical College, New York), skeletal myosin (MY-32; Sigma), neurofilament 160 (NN18; Sigma), and glial fibrillary acidic protein (GFAP; Sigma). The primary antibody was detected by the Vectastain biotin–avidin–peroxidase procedure according to the manufacturer's instructions (Vector Laboratories).

RESULTS AND DISCUSSION

A targeting vector (α_{i2}-neo; Fig. 1*A*) that contains a segment of the α_{i2} gene interrupted in exon 6 by the selectable marker gene, neomycin resistance (*neo*), was constructed and introduced into ES cells. The *neo* gene used a PGK promoter (a

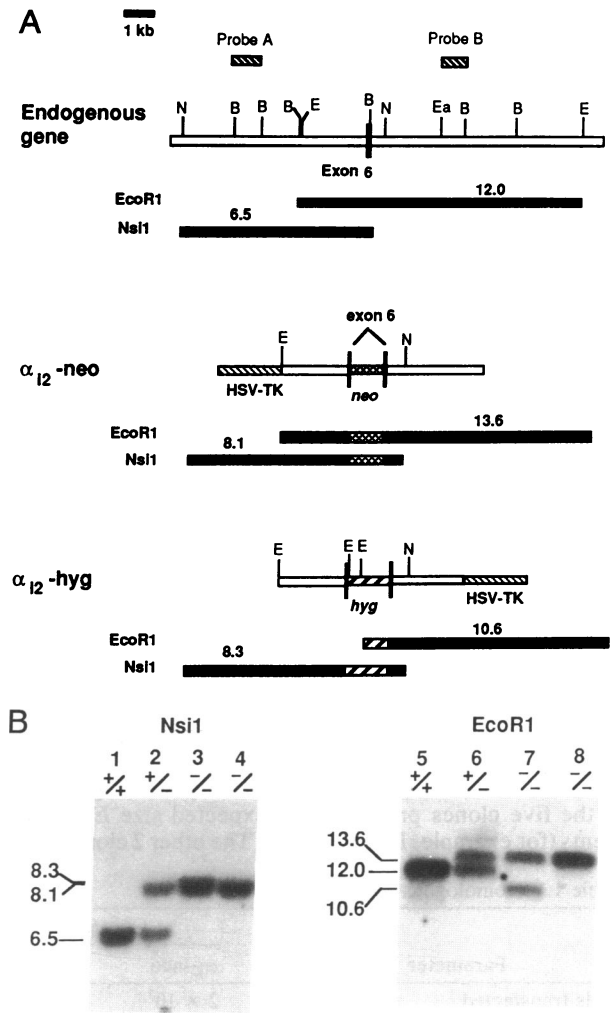

FIG. 1. (*A*) Map of the α_{i2} gene and constructs for homologous recombination. Restriction enzyme sites shown are B, *Bam*HI; N, *Nsi* I; E, *Eco*RI; and Ea, *Eag* I. The solid bars indicate the expected restriction fragments in Southern blots resulting from successful homologous recombination of the targeting constructs. HSV-TK, herpes simplex virus thymidine kinase gene. (*B*) Southern analysis of ES cell DNA obtained from wild-type cells (+/+), cells that were α_{i2} heterozygous (+/−), or two independent α_{i2}-null ES cell lines (−/−). Lanes 1–4, digested with *Nsi* I and hybridized to probe A; lanes 5–8, digested with *Eco*RI and hybridized to probe B. Lanes 1 and 5, wild-type CCE DNA; lanes 2 and 6, DNA from homologous recombinants isolated after transfection with the α_{i2}-neo construct; lanes 3 and 4 and also 7 and 8, DNA from colonies isolated after transfection with the α_{i2}-hyg construct.

gift of M. W. McBurney, University of Ottawa, Ottawa, Canada) because this modified gene produced 20- to 50-fold more neomycin-resistant ES cell colonies per microgram of vector DNA than pMC1-neo or pMC1-polyA (Stratagene) vector DNA. Cells that contained the targeting vector were selected by growth in medium containing the drug G418 and GANC as described (17). Cells that were heterozygous at the α_{i2} locus were detected by Southern blot analysis (Fig. 1*B*) using *Nsi* I- and *Eco*RI-digested ES cell DNA and probes (probes A and B, Fig. 1) derived from the α_{i2} gene outside of the homology region. Of the cell lines that were GANC and G418 resistant, about one in eight was heterozygous at the α_{i2} locus (Table 1).

One of these α_{i2} heterozygous ES cell lines was used as a parent cell for the next step in the production of ES cells lacking a functional α_{i2} gene. To inactivate the remaining allele, we constructed a second targeting vector (α_{i2}-hyg; Fig. 1*A*). Because the α_{i2} heterozygous cells already contained *neo*, a different selectable marker gene, hygromycin B resistance (*hyg*), was used. Available clones of the *hyg* gene had an upstream ATG 10 base pairs 5′ of the translation initiation ATG. To increase expression in mammalian cells, the 5′ end was modified to remove the ATG and to introduce a sequence more closely resembling the mammalian ribosomal binding consensus sequence (28) by using PCR. This modification gave approximately a 10-fold increase in transfection efficiency as judged by the number of hygromycin B-resistant colonies. To obtain the higher levels of *hyg* expression, PGK 5′ and 3′ sequences used in the PGK-neo construct were added to this modified sequence. The final targeting construct was analogous to the neo construct except for the substitution of *hyg* for *neo* and the shortening of the 3′ end of the construct by 700 base pairs to eliminate any homology with probe B from the construct. This construct gave high transfection efficiencies, comparable to PGK-neo, in ES cells.

This targeting vector was introduced into the heterozygous ES cell line, and the cells were selected with hygromycin B and GANC. Five clones out of 78 lacked the 6.5-kb *Nsi* I fragment (for example, Fig. 1*B*, lanes 3 and 4). The 8.3-kb *Nsi* I fragment containing the *neo* gene and the 8.1-kb *Nsi* I fragment containing the *hyg* gene could not be separated by size. To confirm that the cells contained two mutationally altered alleles of the α_{i2} gene, the five clones were examined by Southern blot analysis after *Eco*RI digestion and hybridization with probe B (Fig. 1*B* and data not shown). Because *Eco*RI cleaves the *hyg* gene but not the *neo* gene, an α_{i2} gene interrupted by *hyg* should produce *Eco*RI fragments of different size than an α_{i2} gene interrupted by *neo* (Fig. 1). Three of the five clones produced the expected size *Eco*RI fragments (for example, Fig. 1, lane 7). The other 2 clones clearly lack an unrearranged gene but also appear to lack the expected *Eco*RI fragments (Fig. 1, lane 8). Further characterization of these colonies has shown that they contain a mutation that occurred spontaneously before the α_{i2}-hyg construct transfection. They were selected in hygromycin B because of a random integration of the construct in the genome (data not shown). Because the mutation predated the α_{i2}-hyg transfection, they will not be discussed further here. Three clones did lack a functional α_{i2} gene because their α_{i2} genes had undergone homologous recombination with the targeting vectors. The frequency of these α_{i2}-null ES cells was about 1 in 5×10^5 of the initial cells (Table 1).

Table 1. Homologous recombination of α_{i2} genes

Parameter	Construct	
	α_{i2}-neo	α_{i2}-hyg
Cells transfected	2×10^7	1.2×10^7
$G418^R$ or hygromycin B^R	12,000	2400
$G418^R$ + $GANC^R$ or hygromycin B^R + $GANC^R$	2,700	690
Homologous events/no. analyzed	7/56	3/78*
Homologous events/$G418^R$ or hygromycin B^R	1/35	1/90

The number of colonies reported represents an extrapolation to obtain the number of colonies that would have been detected if all electroporated cells were plated under those conditions. Some colonies (no. analyzed) were isolated, expanded, and analyzed by Southern blotting to identify homologous recombinants. R denotes resistance to the antibiotic.

*Only the colonies that gave predicted sizes for homologous recombination are included.

The targeting vectors were constructed with the assumption that the insertion of a *hyg* or *neo* gene into exon 6 of the α_{i2} gene would prevent the gene from expressing a functional α_{i2} subunit. To test this hypothesis, we assayed for the mRNA and protein products of the inactivated genes. Theoretically, a truncated mRNA could be produced from both of the inactivated alleles. A Northern blot of wild-type, heterozygous, and α_{i2}-null ES cell RNA was hybridized with probe A (Fig. 1), which contains an exon upstream (5′) of the gene interruption site. The autoradiogram revealed α_{i2} mRNA in the wild-type and heterozygous ES cells but not the α_{i2}-null ES cells (Fig. 2*A*). The heterozygote showed a decrease in intensity of the α_{i2} mRNA. Because the α_{i2}-null ES cells did not contain any detectable α_{i2} mRNA, including mRNA forms of unusual size, we concluded that the interrupted α_{i2} gene product was rapidly degraded. Northern analysis of these same cell lines by hybridization to labeled α_{i1} and α_{i3} rat cDNA showed no change in levels, indicating no compensatory changes (data not shown).

To confirm that the α_{i2}-null ES cells lacked normal α_{i2} polypeptide, extracts of these cells were analyzed by Western blotting using an antibody that recognizes both α_{i1} and α_{i2} (Fig. 2*B*). The antibody detected less α_i subunit in the heterozygous cell extracts than in wild-type cell extracts. A small amount of 41-kDa antibody-reactive material was detected in α_{i2}-null ES cell extracts, which probably corresponds to α_{i1}. The Western blots failed to detect any truncated α_{i2} gene product that might be produced by the interrupted gene. We concluded that the α_{i2}-null ES cells are viable despite the fact that they lack normal α_{i2} polypeptide.

We further studied the phenotype of these cells to obtain clues as to the function of the α_{i2} subunit. Because the roles of the G_i proteins and their associated α_i subunits are not completely defined, a number of possible phenotypes might have been altered by the loss of this subunit. We have compared a number of phenotypes of the α_{i2}-null ES cells and the wild-type ES cells and found them to be indistinguishable. For example, the morphology of the wild-type ES cells and the α_{i2}-null ES cells was identical (Fig. 3). We also tested the

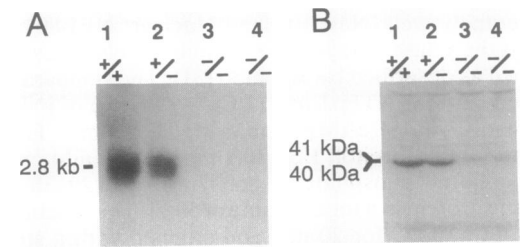

FIG. 2. (*A*) Northern blot of RNA (20 μg) from wild-type (+/+), α_{i2} heterozygous (+/−), or two independent α_{i2}-null ES (−/−) cell lines. The integrity of the RNA was established by the presence of equal amounts of 18S and 28S ribosomal RNA and by the detection of *neo* mRNA in lanes 2, 3, and 4 by rehybridization with a neo probe (not shown). (*B*) Western blot of cell extracts using an antibody (NEI-801) that detects α_{i1} and α_{i2} equally well. The lanes are as indicated in *A*.

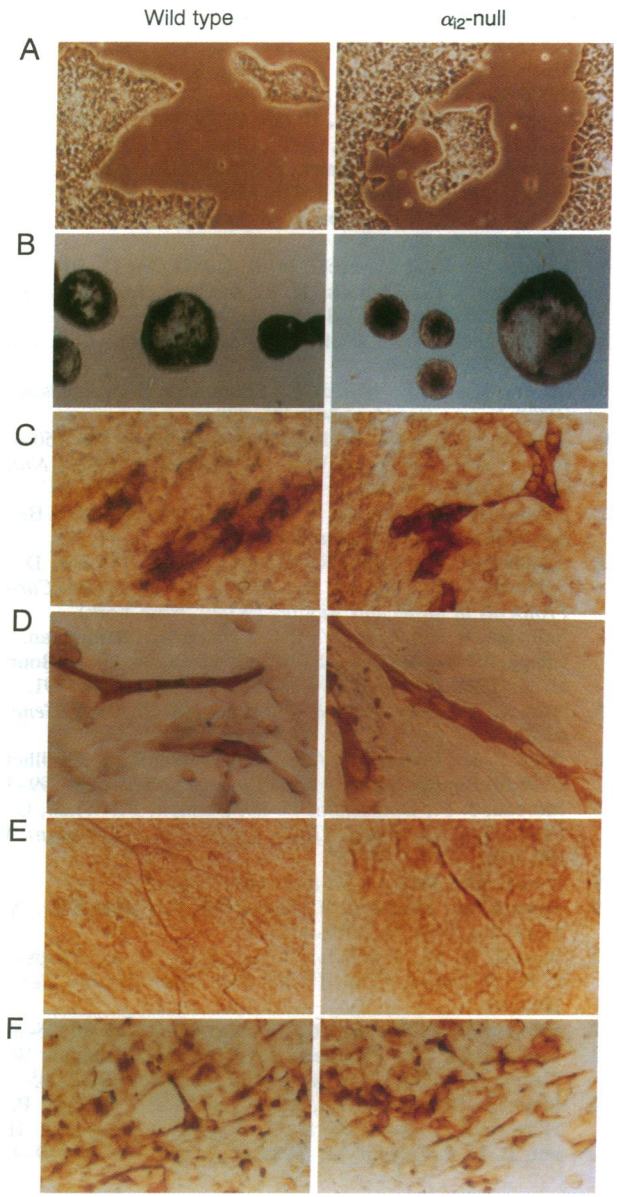

FIG. 3. Photomicrographs of undifferentiated and differentiated wild-type or α_{i2}-null ES cells. Phase-contrast microscopy of undifferentiated cells cultured in leukemia inhibitory factor (*A*; ×140) and embryoid bodies (*B*; ×70). Differentiated cells were incubated with primary antibodies that recognize cardiac myosin in spontaneously beating cells showing the characteristic morphology of cardiac cells (*C*; MF-20; ×280), skeletal myosin (*D*; MY-32; ×280), neurofilament 160 (*E*; NN18; ×280), and glial fibrillary acidic protein (*F*; ×140).

role of the α_{i2} subunit in regulation of growth rates in cultured ES cells. No differences were found in the logarithmic phase growth rates of wild-type, heterozygous, or homozygous α_{i2}-null cell lines (Table 2).

We next investigated the consequences of deleting α_{i2} for regulation of adenylyl cyclase activity. If α_{i2} inhibits adenylyl cyclase, we would predict that its absence would lead to increased activity. Treatment of some cell lines with pertussis toxin to inactivate the α_i family of proteins does cause increased basal and hormone-stimulated adenylyl cyclase activity, suggesting the removal of a constitutive inhibition (33). We found no increase (as compared to wild-type cells) in basal activity or activity stimulated by forskolin, guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) or isoproterenol (Table 3). We therefore conclude that α_{i2} does not produce constitutive inhibition of adenylyl cyclase in ES cells. Further, we did not

Table 2. Doubling times of wild-type and α_{i2}-null cells

Cell line	Doubling time,* hr
Wild type (+/+)	14.1 (12.8–15.8)
Heterozygote (+/−)	12.8 (11.6–14.4)
α_{i2}-null (−/−)	
1	13.6 (11.9–16.1)
2	16.1 (14.1–18.7)
3	14.4 (13.1–16.5)

*Range in parentheses indicates the 95% confidence interval.

see any inhibition of basal or forskolin-stimulated adenylyl cyclase activity (with or without NaCl) by GTP (≤100 mM) in either wild-type or α_{i2}-null cells (Table 3), suggesting that inhibition of adenylyl cyclase activity by α_i subunits is not an important regulatory feature of ES cells.

The complement of hormone receptors expressed in ES cells has not yet been well defined. We therefore screened a number of agonists for their ability to stimulate or inhibit adenylyl cyclase. We found that ES cells have β-adrenergic receptors since adenylyl cyclase was stimulated by isoproterenol (Table 3) and this stimulation was blocked by propranolol (data not shown). No significant inhibition was found with carbachol, serotonin, dopamine, somatostatin, or angiotensin II, indicating either the absence of the receptor or poor coupling in these receptors to inhibition of adenylyl cyclase.

One reason for selecting ES cells for these studies was that they can be differentiated *in vitro* into a variety of different cell types. We therefore differentiated α_{i2}-null ES cells to determine if α_{i2} was required for these processes (Fig. 3). Morphologically distinguishable beating cardiocytes and neurons were visualized. We detected gene products characteristic of these differentiated cells by immunohistochemistry. The presence of other differentiated cell types, skeletal muscle and glial cells, was demonstrated by staining with specific monoclonal antibodies (Fig. 3). In all cases, the differentiated cells from wild-type ES cells were indistinguishable from those from α_{i2}-null ES cells. The present results suggest that many of the signals required to differentiate ES cells do not require the action of the α_{i2} subunit.

We have constructed an α_{i2}-null ES cell using a method of gene inactivation based on targeted homologous recombination using selectable marker genes. A recent report (22) describes a somewhat different approach to the inactivation of genes. In that report, the *pim-1* genes, which are very highly expressed in ES cells (22), were inactivated by *pim-1*-selectable marker fusion genes. Such an approach should only be useful for genes expressed in ES cells. In this report, we describe the production and use of a PGK-hyg construct to express the marker at high levels in ES cells. This vector, along with PGK-neo, should be useful for the targeting of genes regardless of their expression in ES cells.

Table 3. Relative adenylyl cyclase activity

Addition	Wild type	α_{i2}-null
None (basal)	1	1
Isoproterenol (1 μM)	3.1 ± 0.8 (6)	2.5 ± 0.2 (5)
GTP[γ-S] (10 μM)	14 ± 4 (4)	14 ± 5 (8)
Forskolin (10 μM)	15 ± 4 (4)	15 ± 6 (8)
Forskolin (10 μM) + GTP (10 μM)	16 ± 3 (4)	16 ± 5 (8)

The values given are the values relative to basal activity in that experiment ± standard deviation. The number of experiments is shown in parentheses. Basal activities (in pmol·min^{-1}·mg^{-1}) with isoproterenol were 1.4 ± 0.1 (n = 6) for wild-type cells and 1.6 ± 0.4 (n = 5) for α_{i2}-null cells; those with GTP[γ-S] were 3.5 ± 1.2 (n = 4) for wild-type cells and 3.0 ± 0.9 (n = 8) for α_{i2}-null cells; and those with forskolin were 1.7 ± 0.9 (n = 4) for wild-type cells and 1.3 ± 0.4 (n = 8) for α_{i2}-null cells. There was no significant difference when the GTP concentration was increased to 100 μM.

A variety of steps in ES cell differentiation do not require the α_{i2} subunit. Whether other subunits or processes are able to substitute for the α_{i2} subunit or whether this subunit is not involved in any of the complex processes of cellular differentiation is not resolved. We expect that α_{i2}-null ES cells will be an important tool for dissecting the role of the α_{i2} subunit, particularly in differentiated cells. Further analysis of these differentiated cells (such as beating cardiocytes) and chimeric mice produced from these ES cells should help define the role of G_i proteins in the signal transduction process.

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