Induction of Erythroid Differentiation by Altered Gα_{16} Activity as Detected by a Reporter Gene Assay in MB-02 Cells*

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Heterotrimeric G proteins may assume modulatory roles in cellular proliferation and differentiation. The G protein α-subunit Gα_{16}, which is specifically expressed in hematopoietic cells, is highly regulated during differentiation of normal and leukemic cells. In human erythroleukemia cells, suppression of Gα_{16} inhibited cellular growth rates. A reporter gene system was established to assess the role of Gα_{16} on erythroid differentiation of MB-02 erythroleukemia cells. It is based on transient transfection with a plasmid that expresses green fluorescent protein under the control of the β-globin promoter. Expression of Gα_{16} led to a significant increase in green fluorescent protein-positive cells, as did transfection with a Gα_{16} antisense plasmid (154 and 156% of controls, respectively). The GTPase-deficient, constitutively active mutant of Gα_{16}, Gα_{16}R186C, further stimulated differentiation to 195% of control values. Because the effect of Gα_{16} is triggered most efficiently by the GTP-bound protein, an indirect action through interference of overexpressed Gα_{16} with G protein βγ-subunits can be excluded. The corresponding mutant of Gqα (Gqα_R182C), the phylogenetically closest family member of Gα_{16} had no effect. The data define a specific role for Gα_{16}-dependent signal transduction in cellular differentiation: deviations from optimal levels of Gα_{16} functional activity lead to reduced growth rates and promote differentiation in hematopoietic cells.

Receptor-coupled heterotrimeric G proteins are known to mediate signals that modulate growth factor-dependent cellular proliferation (1). G protein-linked second messengers, such as Ca^{2+} or cAMP, as well as G protein βγ-subunits released upon G protein activation, may be involved in this modulation. Ultimately, these signaling elements are thought to act on mitogen-activated protein kinase or on c-Jun amino-terminal kinase modules, which serve as central regulators for cellular growth and differentiation (2, 3). In some endocrine tumors, mutations in clearly defined codons for conserved amino acids result in GTPase-deficient Gα_{16}, which is specifically expressed in hematopoietic cells, is highly regulated during differentiation of normal and leukemic cells. In human erythroleukemia cells, suppression of Gα_{16} inhibited cellular growth rates. A reporter gene system was established to assess the role of Gα_{16} on erythroid differentiation of MB-02 erythroleukemia cells. It is based on transient transfection with a plasmid that expresses green fluorescent protein under the control of the β-globin promoter. Expression of Gα_{16} led to a significant increase in green fluorescent protein-positive cells, as did transfection with a Gα_{16} antisense plasmid (154 and 156% of controls, respectively). The GTPase-deficient, constitutively active mutant of Gα_{16}, Gα_{16}R186C, further stimulated differentiation to 195% of control values. Because the effect of Gα_{16} is triggered most efficiently by the GTP-bound protein, an indirect action through interference of overexpressed Gα_{16} with G protein βγ-subunits can be excluded. The corresponding mutant of Gqα (Gqα_R182C), the phylogenetically closest family member of Gα_{16} had no effect. The data define a specific role for Gα_{16}-dependent signal transduction in cellular differentiation: deviations from optimal levels of Gα_{16} functional activity lead to reduced growth rates and promote differentiation in hematopoietic cells.

Paradoxically, G protein-mediated signaling may also be associated with growth inhibition and cellular differentiation. Overexpression of GTPase-deficient Gαq in rat pheochromocytoma cells induces neurite outgrowth (6). Constitutively active forms of Gα_{13} promote differentiation of P19 mouse embryonal carcinoma cells into an endodermal phenotype (7). Both appear to involve stimulation of the c-Jun amino-terminal kinase pathway. In mouse embryonic stem cells, expression of Gαq or its GTPase-deficient mutant results in adipogenic differentiation (8). Furthermore, expression levels of members of all classes of G protein α-subunits are found to be regulated during cellular differentiation (9–14). These observations indicate a potentially widespread role of G protein α-subunits in differentiation programs. However, conclusive evidence that G protein regulation is the initiating event of the differentiation process is still lacking in most cases.

The molecular cloning of Gα_{16}, a novel member of the Gq family of G proteins, and the analysis of its tissue distribution revealed that it is uniquely expressed in normal and in malignant hematopoietic cells. Its expression is confined to hematopoietic cell lines that were derived from early stages of differentiation and is absent or strongly down-regulated in differentiated normal cells or in leukemia cell lines after induction of differentiation (12, 13, 15–17). In peripheral blood T-lymphocytes, Gα_{16} expression is transiently up-regulated after lymphocyte activation, whereas the expression of Gα_{12} and of Gαq remains unchanged (18). In order to determine whether Gα_{16} modulates T-cell activation, regulative expression of Gα_{16} was disrupted by stably overexpressing Gα_{16} or Gα_{16} antisense RNA in Jurkat T-cells, a human T lymphoma cell line. Activation of Gα_{16}-deregulated Jurkat T-cells was inhibited as demonstrated by a reduced up-regulation of interleukin-2 and of the activation-specific surface antigen CD69 (18). These results suggest a critical role of tightly regulated Gα_{16} expression in lymphocyte activation.

Further information about potential roles of Gα_{16} accumulated mostly from experiments in nonhematopoietic cells by overexpression studies. In Swiss 3T3 fibroblasts, constitutive activation of Gq-dependent pathways by overexpression of GTPase-deficient mutants of Gαq and Gα_{16} results in arrest or in reduced growth in response to platelet-derived growth factor or serum, respectively (19). In small cell lung carcinoma, overexpression of a GTPase-deficient mutant of Gα_{16} inhibits growth (20), and in rat pheochromocytoma cells, it induces differentiation (6). Surprisingly, Gα_{16} has also been reported to be involved in growth stimulatory events. Activation of the receptor for complement fragment C5a in human embryonic kidney cells leads to a pronounced activation of mitogen-activated protein kinase when coexpressed with Gα_{16} (21). Taken together, these findings imply that Gα_{16}-dependent signaling may modulate cellular proliferation or differentiation, depending on the specific cellular environment.

Although Gα_{16} may interact with a broad spectrum of recep-

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tors in some overexpressing systems (22), selective coupling of receptors to G\textsubscript{16}\textsuperscript{a} but not to G\textsubscript{q}, was observed for the C-X-C chemokine interleukin-8, as well as for complement fragment C5a, and for the chemotactic peptide formyl-methionyl-leucylphenylalanine (23–25). In the human erythroleukemia (HEL\textsuperscript{1}) cell line, G\textsubscript{16}\textsuperscript{a} specifically couples to the P2Y\textsubscript{12} (P\textsubscript{2U}) purinoreceptor (26), suggesting that G\textsubscript{16}\textsuperscript{a} might assume specific roles in individual cells or cell lines. However, its role in hematopoietic cells is still poorly defined in view of its lineage-independent but differentiation-stage-dependent expression.

In the present study, we examined the role of G\textsubscript{16}\textsuperscript{a} in growth and differentiation of erythroleukemic cells. A reporter gene assay was established, to detect entry into erythroid differentiation in transiently transfected cells. The results indicate that changes in the expression level and functional activity of G\textsubscript{16}\textsuperscript{a} lead to the induction of differentiation in the factor-dependent erythroleukemia cell line MB-02. The data suggest a new role of G\textsubscript{16}\textsuperscript{a}-dependent signaling in the decision between cellular proliferation and differentiation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cell culture media and medium supplements were purchased from Life Technologies, Inc. Healthy donors who had received no recent medications were the source of human serum, which was heat-inactivated at 56 °C for 30 min and stored at −20 °C for up to 6 months. Granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (Epo), and stem cell factor (SCF) were generous gifts from Werthenstein-Chemie (Schachen, Switzerland), Cilag AG (Schaffhausen, Switzerland), and Dr. E. K. Thomas (Immunex Corp., Seattle, WA), respectively. Unless otherwise mentioned, all chemicals (analytical grade), were from Sigma Chemicals (Buchs, Switzerland) or from Merck AG (Dietikon, Switzerland).

**Cell Culture and Induction of Differentiation**—MB-02 cells (27) were maintained in basal medium (RPMI 1640 medium, 10% human serum, 2 mM Glutamax I (Life Technologies, Inc.), 1 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin), supplemented with 5 ng/ml GM-CSF and kept at 37 °C in a humidified atmosphere of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. Cells were passaged every third day and replated at a density of 4 × 10\textsuperscript{5} cells/ml. The protocol for the induction of differentiation was adapted from Broudy et al. (28). Briefly, cells were washed once with phosphate-buffered saline and plated at a density of 10\textsuperscript{6} cells/ml in fresh basal medium supplemented with 25 μg/ml SCF and 4 units/ml Epo and left undisturbed for a week prior to further routine passaging for another 7–10 days. Hemoglobin-producing cells were detected by benzidine staining of cellular suspensions (29).

The HEL cell lines, 3D4 and 1E3, with suppressed G\textsubscript{16}\textsuperscript{a} expression were generated by stable transfection of parental HEL cells derived from clone 92.1 (American Type Culture Collection, Manassas, VA) with an antisense plasmid to G\textsubscript{16}\textsuperscript{a} and have been described previously (28). G418-resistant cells that expressed wild-type levels of G\textsubscript{16}\textsuperscript{a} were recovered from transfection with pcDNA3 (Inviitrogen, Inc.) by digestion with EcoRI and SalI and then ligated into the unique EcoRI site of pcGFP. As a nonfluorescent control, pcclacZ was constructed replacing GFP in pcGFP with lacZ from the expression vector pCMV-lacZ (a gift from Dr. S. Rusconi, University of Fribourg, Switzerland).

For cotransfection experiments, the different G protein genes were cloned into the expression vector pcDNA3. The plasmids pG16AS and pG16WT, harboring a full-length copy of the human G\textsubscript{16}\textsuperscript{a} cDNA in an antisense or sense orientation, respectively, were described previously (26). In order to generate pG16BC, the GTPase-deficient active mutant of G\textsubscript{16}\textsuperscript{a}, G\textsubscript{16R166C}, was recovered from pVL1939G\textsubscript{a},R166C (a gift from Drs. A. Dietrich and P. Gierschik, University of Ulm, Germany) and cloned into the unique XbaI site of pcDNA3. A plasmid expressing the corresponding GTPase-deficient mutant of G\textsubscript{16}\textsuperscript{a}, pCISG\textsubscript{a},R166C, was a gift of Dr. M. I. Simon (California Institute of Technology, Pasadena, CA). A plasmid encoding the GTPase-deficient mutant of G\textsubscript{16}\textsuperscript{a}, G\textsubscript{16Q205L}, was recovered from the human erythroleukemia cell line MB-02, which was adapted from Broudy et al. (28).

**Cell Transfection**—MB-02 cells were washed with phosphate-buffered saline and resuspended at a density of 2 × 10\textsuperscript{6} cells/ml in phosphate-buffered sucrose (sucrose, 272 mM; MgCl\textsubscript{2}, 1 mM; NaH\textsubscript{2}PO\textsubscript{4}, 7 mM; glucose, 20 mM; KCl, 1 mM; pH 7.4). In transfection experiments, 5 μg of pcGFP in combination with 5 μg of one of the G protein-containing plasmids were used, or 5 μg of pcGFP and 5 μg of pcclacZ (positive control), or 10 μg pcclacZ (nonfluorescent control). 10\textsuperscript{6} cells and the DNA were mixed in the electroporator cuvette (gap width, 4 mm) and equilibrated on ice for 5 min. Cells were then electrically transfected using a Gene Pulser unit (Bio-Rad) that was set to 350 V, 100 μF (exponential decay), and immediately plated into 1 ml of prewarmed (37 °C) complete RPMI medium with human serum and GM-CSF or Epo plus SCF (for noninduced or induced cells, respectively). For the transfection of COS-1 cells, 10\textsuperscript{6} cells in complete Dulbecco's modified Eagle's medium were seeded 48 h before transfection. After 24 h, the culture medium was replaced with Opti-MEM (Life Technologies, Inc.), and cells were transfected with a total of 1 μg of plasmid DNA per dish using the N-11-[2,3-dioleoyl]-propyl-N,N,N-trimethylammoniummethyl sulfate (Boehringer Mannheim) reagent according to the manufacturer's instructions. Cells were harvested after 48 h, and the membrane fraction was analyzed by protein immunoblotting as described previously (28).

**Flow Cytometry**—MB-02 cells were washed three times and resuspended in phosphate-buffered saline (1 ml) supplemented with 22 mM glucose. Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Laser excitation was at 488 nm (argon laser) for GFP, and emission was measured at 515–545 nm. The single color analysis was gated on forward scatter and side scatter. This gate contained all viable cells and excluded cell debris and cellular aggregates. A threshold for intensity of fluorescence was set high enough to exclude autofluorescence and was determined by running samples of untransfected and lacZ-transfected cells prior to the analysis. A total of 10,000 events was counted and analyzed for each sample using the CellQuest software (Becton Dickinson). To correct for variability in transfection efficiencies from experiment to experiment, inducible GFP transfection efficiencies from experiment to experiment, inducible GFP expression was normalized in each experiment to the average of fluorescent cells observed upon expression of GFP under the constitutively active CMV promoter, i.e., after transfection with pcGFP (three to four independent transfections per experiment).

**RESULTS AND DISCUSSION**

**Down-regulation of G\textsubscript{16}\textsuperscript{a} in HEL Cells Impairs Cell Growth**—We recently established several sublines of HEL cells that showed reduced expression of endogenous G\textsubscript{16}\textsuperscript{a} protein after transfection with a plasmid harboring a full-length copy of G\textsubscript{16}\textsuperscript{a} in antisense orientation (26). In these sublines, mobilization of intracellular Ca\textsuperscript{2+} through activation of the P2X\textsubscript{2} (P\textsubscript{2U}) purinoceptor by UTP is impaired, whereas Ca\textsuperscript{2+}-mobilization via other receptors is not or is only partially affected, demonstrating a specific functional defect...
Erythroid Differentiation by Altered Activity of $G_{a16}$

Fig. 1. Expression of antisense RNA to $G_{a16}$ increases population doubling times of HEL cells. Stably transfected HEL cells with reduced levels of $G_{a16}$ expression ($G_{a16}$-suppressed cell lines 3D4 and 1E3) or controls with unaltered $G_{a16}$ expression ($G_{a16}$-normal cell lines 7D6 and 9G10) were seeded at a density of $2 \times 10^5$ cells/ml. Forty-eight hours later, total numbers of viable cells were counted, and population doubling times were calculated. The box plot shows the summary of multiple measurements. The 10th, 25th, 50th (median), 75th, and 90th percentiles of the variables are indicated by vertical bars. One-way analysis of variance indicated significant differences between the means ($p_a < 0.0001$). Post hoc analysis by the method of Bonferroni-Dunn showed that differences between controls and $G_{a16}$-suppressed cell lines are highly significant ($p_a < 0.0014$).

Compared to controls with normal levels of $G_{a16}$, cellular growth was impaired in the $G_{a16}$-deficient sublines (Fig. 1). Population doubling times were significantly higher in the $G_{a16}$-suppressed cell lines 3D4 and 1E3 ($41.4 \pm 1.4$ and $41.2 \pm 1.6$ h, respectively) than in the cell lines 9G10 and 7H6 expressing normal levels of $G_{a16}$ ($34.8 \pm 1.0$, $33.2 \pm 0.6$ h, respectively) (mean $\pm$ S.E.).

The results suggested that $G_{a16}$-mediated cellular signaling may be involved in the regulation of cellular proliferation. However, from these experiments, it could not be established whether $G_{a16}$-mediated inhibition of proliferation was associated with erythroid differentiation in hematopoietic cells, because the factor-independent HEL cells only partially differentiate in response to various inducers (31, 32). To address this question, a factor-dependent cell line, MB-02, was chosen that can be readily differentiated along the erythroid pathway (27, 28).

Differentiation of MB-02 Cells—MB-02 cells maintained in the presence of GM-CSF differentiate along the erythroid pathway upon withdrawal of GM-CSF and subsequent treatment with SCF and Epo (28). Under our experimental conditions, the properties of the cell line corresponded to the ones described in the original literature: treatment for 10 days or longer with SCF and Epo (28). Under our experimental conditions, the presence of GM-CSF differentiate along the erythroid pathway, because the factor-independent HEL cells only partially differentiate in response to various inducers (31, 32).

To address this question, a factor-dependent cell line, MB-02, was chosen that can be readily differentiated along the erythroid pathway (27, 28).

Assembly and Functional Evaluation of the Reporter System—In order to study the effects of G protein overexpression in MB-02 cells, we used a transient transfection system to acutely manipulate G protein levels. As the transfection efficiency is low in hematopoietic cells, potential effects of such treatments could not be studied by biochemical or immunological methods. Thus, a reporter gene assay was established based on a plasmid that was co-transfected with the gene of interest. As a reporter for erythroid differentiation, the nucleotide sequence encoding GFP was placed under the control of the $\beta$-globin promoter in the original plasmid pEV3 (33). In this reporter plasmid (pGFP), the $\beta$-globin promoter is located downstream of locus control region sequences derived from the human $\beta$-globin gene cluster (Fig. 3A). In constructs bearing the locus control region-$\beta$-globin promoter arrangement, strong inducible expression from the $\beta$-globin promoter has been shown upon induction of differentiation in erythroid cells (33). In addition, a plasmid (pGFP) with constitutive expression of GFP under the control of the CMV promoter was constructed by splicing in CMV promoter sequences upstream of the GFP sequence (Fig. 3B).

In order to test whether the reporter plasmid was indeed capable of detecting erythroid differentiation, cells treated with GM-CSF (noninducing conditions) or with SCF/Epo (inducing conditions) were transiently transfected with pGFP or with the control plasmid pCGFP. Expression of GFP was then detected by flow cytometry. As shown in Fig. 4A, the relative number of GFP-expressing cells was 2.7-fold higher in SCF/Epo-treated cells than in GM-CSF-treated controls when normalized to GFP expression under the constitutively active CMV promoter. The relative number of differentiating cells as measured by the reporter assay reached a maximum after 11–14 days of induction (Fig. 4B), consistent with previously published data on SCF/Epo-induced expression of globin proteins in this cell line (28). In order to quantify the extent of $\beta$-globin gene induction in differentiating cells, their fluorescence intensities resulting from the expression of the reporter gene construct were compared with that of noninduced cells. The histogram in Fig. 4C shows an analysis in bins of 0.5 log units over the 4 log units of fluorescence intensities recorded by the FACScan. The first log unit of fluorescence (Fig. 4C, bins 1 and 2) represents autofluorescence as demonstrated by cells that were transfected with lacZ instead of the reporter gene construct. Overall, SCF/Epo-treated cells that were transfected with the reporter plasmid showed a 2.9-fold increase in their number of fluorescent cells contained in bins 3–8 when compared with noninduced cells (averages of 95.2 and 32.5 cells, respectively). The corresponding total fluorescence of induced cells was 4.3-fold higher than in noninduced cells (Fig. 4C).

Thus, SCF/Epo treatment resulted in a substantial increase in the number of fluorescent cells in the gated window (encompassing bins 3–8) but only in a slight increase of average fluorescence per cell (+48%), which can be reflected by higher ratios of fluorescent cells appearing in bins 5–8 than in bins 3 and 4 (3.7, 4.5, 6.5, and 3.3 versus 2.7 and 2.1, respectively). Apparently, cells that undergo spontaneous differentiation under noninducing conditions (see also Fig. 2) show $\beta$-globin promoter activities almost equal to those of cells that were treated with SCF/Epo. Consequently, the number of fluorescent cells rather than gene expression levels on a per cell basis in the gated window was taken as a measure for erythroid induction.

The results shown in Fig. 4A indicate a ratio of differentiated cells of $46 \pm 6$% in SCF/Epo-treated cells. The reporter gene assay closely reflects the relative number of differentiated MB-02 cells, as detected by benzidine staining of cells after treatment with SCF/Epo (Fig. 2B), which resulted in induction rates of $45 \pm 5$% of the population. However, it appears that the reporter assay is more sensitive than benzidine staining at
lower levels of β-globin promoter activity: in noninduced cultures, 6 ± 2% of the cells were benzidine-positive, whereas 17 ± 1% of the cells showed increased fluorescence in the reporter assay (compare Figs. 2B and 4A). In COS-1 cells, transient transfection of pcGFP resulted in strong fluorescence, whereas no fluorescence was observed in cells transfected with piGFP (not shown). These results rule out the possibility that low levels of the reporter gene might have been expressed in the absence of activators of the β-globin promoter. Thus, the reporter system provides a valid and sensitive assay for monitoring erythroid differentiation in MB-02 cells.

**Fig. 2.** Hemoglobin expression and growth characteristics of MB-02 cells upon induction of differentiation by SCF/Epo. A, benzidine staining (dark gray) of hemoglobin in noninduced (maintained in GM-CSF) and induced (SCF/Epo for 14–18 days) MB-02 cells. B, cells treated as in A were counted under the microscope, and the percentage of benzidine positive cells from noninduced and induced cultures was calculated and plotted (mean ± S.E., n = 6). C, MB-02 cells were cultured in the presence of GM-CSF, SCF/Epo, or Epo alone during successive days and counted. The cumulative fold increase in cell numbers is plotted.

**Fig. 3.** Schematic representations of expression plasmids piGFP and pcGFP. A, reporter plasmid piGFP, which expresses GFP upon activation of the β-globin promoter. B, control plasmid pcGFP, which constitutively expresses GFP under the control of the CMV promoter. The construction of the plasmids is described in detail under “Experimental Procedures.”

**Induction of Differentiation in MB-02 Cells upon Changes in G\textsubscript{a16} Expression**—Using the reporter assay, we then examined the effect of G protein expression on β-globin promoter activation in nondifferentiated cells. Cells were co-transfected with the inducible reporter plasmid together with a plasmid encoding either wild-type G\textsubscript{a16} or the GTPase-deficient mutant of G\textsubscript{a16} (G\textsubscript{a16}R186C). Cultures transfected with wild-type G\textsubscript{a16}
showed a significant increase of GFP-expressing cells to 154% of levels observed in control cells transfected with pclacZ (Fig. 5A). Expression of the GTPase-deficient mutant of Gαq, GαqR182C, also was expressed. Gαq represents the phylogenetically closest relative of Gαs, which also belongs to subfamily I of Gα proteins (34). In contrast to GαsL186C, no induction was observed when GαqR182C was expressed (Fig. 5B). Expression of a GTPase-deficient member of the more distant Gαi family, GαiQ205L, resulted in a slight increase to 127 ± 19% (mean ± S.E.) of control values, which, however, was not significantly different from the control (not shown). Although from the same subfamily of G proteins, Gαq apparently is not able to functionally substitute for GαsL in inducing differentiation of MB-02 cells. We previously observed that in HEL cells the P2U purinoceptor specifically couples to GαsL, leading to similarly exclusive GαsL-dependent signaling (26). These experiments demonstrate that the specificity of GαsL-dependent signal transduction is not limited to specificity in the receptor-G protein coupling but may also result from the interaction of GαsL with downstream effector systems. The inducing effect of GαsL appears to depend on functional activity of GαsL, because the GTPase-deficient mutant showed a significantly stronger induction than the wild-type form. The inability of Gαq and of GαiQ to effectively induce β-globin promoter activity indicates that differentiation strictly depends on GαsL rather than being a general effect of G protein β-subunit overexpression.

As HEL cells with suppressed GαsL showed reduced growth rates (Fig. 1) but were not well suited to examine GαsL-dependent effects on cellular differentiation, MB-02 cells were also transfected with a GαsL antisense-plasmid to down-regulate endogenous GαsL. Interestingly, transcription of antisense-RNA also significantly increased the number of GFP-positive cells to 156% of control values (Fig. 5A). Since differentiation is associated with reduced rates of proliferation, this inducing effect of GαsL-down-regulation in MB-02 cells is consistent with its effect on proliferation that was detected in HEL cells (Fig. 1). However, the observation that either reducing or up-regulating the levels of GαsL protein expression triggers differentiation may not easily be reconciled. Although this phenomenon has also been observed for acquiring functional competence of Jurkat T cells (18), the underlying mechanism cannot be deduced from the available data and warrants further investigation. A cell-specific, optimal level of GαsL activity seems to be required for proliferation, and any deviation from it will decrease growth rates and result in increased ratios of differentiating cells.

Expression of Antisense RNA Inhibits GαsL Expression in Transiently Transfected COS-1 Cells—Due to low transfection efficiencies, expression of GαsL protein or antisense-dependent down-regulation of endogenous GαsL could not be verified directly in MB-02 cells. Therefore, expression of GαsL protein and its down-regulation through antisense RNA was verified by protein immunoblotting of solubilized membranes of transiently transfected COS-1 cells. Transfection with GαsLR186C or with wild-type GαsL resulted in a single band with a relative mobility identical to the single immunoreactive band observed in membranes of MB-02 cells (Fig. 6). As expected, in COS-1 cells that were transfected with the control plasmid pclacZ, no immunoreactivity was detected, as these cells do not express endogenous GαsL. Transfection of smaller amounts of the GαsL plasmid resulted in lower expression levels, indicating that under these conditions, plasmid-dependent protein expression was in a dynamic range, thus allowing for the detection of copy number-dependent alterations of expression. As shown in Fig. 6, co-transfection of GαsL with the GαsL antisense construct
interaction of the Gα16 signaling pathway in the induction of differentiation in MB-02 cells. This conclusion is supported by the results from the experimental up- or down-regulation of wild-type Gα16, which is expected to translate into changes in basal levels of GTP-bound Gα16, and consequently into changes of Gα16-dependent signaling activity. It is not known which receptors may engage Gα16 in MB-02 cells. Therefore, an assessment of the role of agonists that activate Gα16-coupled receptors is not yet possible. The observation that the GTPase-deficient Gα16 shows a higher efficacy than the wild-type form also excludes the possibility that Gα16 may act solely as a receptor-independent regulator of G protein signaling, e.g. by capturing βγ-subunits that might have been liberated by activation of other G proteins.

Transfection of MB-02 cells with the Gα16R186C mutant resulted in a 1.9-fold increase of induced cells as compared with the number observed in control-transfected cells (Fig. 5A). This increase is substantial in view of the 2.7-fold increase observed
when cells were induced to differentiate by SCF/Epo (Fig. 4A). Importantly, cells induced with SCF/Epo received the differentiating stimulus for 14 days, whereas transfected cells had to be scored after 3 days due to the transient nature of transfection. It was not possible to subject cells to transfection after 3 days of differentiation by SCF/Epo due to the fragile nature of the cultures at this time point. However, as demonstrated in Fig. 4B, SCF/Epo treatment required several days to fully differentiate MB-02 cells. Thus, G\textsubscript{a16}R186C—and deregulation of G\textsubscript{a16} activity in general—may be considered as potent inducers of differentiation.

In this context, it should be noted that our attempts to generate stable HEL cell lines transfected with G\textsubscript{a16}R186C were not successful. It appeared that expression of constitutively active G\textsubscript{a16} inhibits proliferation in these cells to an extent that precluded the isolation of cell clones. Expression of the functionally similar GTPase-deficient mutant G\textsubscript{a16}Q212L in nonhematopoietic cells apparently led to growth retardation, but stable cell lines could still be established (19). G\textsubscript{a16} may thus act as a much stronger (negative) regulator of proliferation in hematopoietic cells than in other cell lines. Whether this could be caused by a unique coupling of G\textsubscript{a16}-dependent signaling to downstream effector systems or by stronger coupling to effector systems that are used by other members of the G\textsubscript{aQ} family is not known.

In conclusion, in hematopoietic cells an increase of G\textsubscript{a16} function or its down-regulation have profound effects on proliferation and may cause erythroid differentiation in specific cell lines. It remains to be established whether differentiation along the erythroid pathway is specifically determined by G\textsubscript{a16} or whether G\textsubscript{a16} preconditions hematopoietic cells for differentiation independently of lineage determination. Deviations from optimal levels of G\textsubscript{a16} activity seem to be associated with acquiring functional competence by inducing either differentiation or activation of cellular proliferation, as seen in T-lymphocytes (18). One might speculate that agonists employing G\textsubscript{a16}-coupled receptors directly regulate the proportion of functionally competent cells. With the reporter assay based on transient transfection of cells and subsequent analysis by flow cytometry, it should now be possible to identify the G\textsubscript{a16}-dependent signaling pathways involved in these processes. Furthermore, these studies could be extended to primary hematopoietic progenitor cells, in which G protein expression cannot acquire functional competence by inducing either differentiation or activation of cellular proliferation, as seen in T-lymphocytes (18).

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