Expression of the G_s Protein α-Subunit Disrupts the Normal Program of Differentiation in Cultured Murine Myogenic Cells

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Abstract

The manner in which growth factors acting at the cell surface regulate activity of myogenic basic-helix-loop-helix proteins in the nucleus and thus control the fate of committed skeletal myoblasts remains poorly understood. In this study, we report that immunoreactive G_s protein α-subunits ($G_{s\alpha}$) localize to nuclei of proliferating C_2C_{12} myoblasts but not to nuclei of differentiated postmitotic C_2C_{12} myotubes. To explore the biological significance of this observation, we placed a cDNA encoding $G_{s\alpha}$ in an expression vector under the control of a steroid-inducible promoter and isolated colonies of stably transfected C_2C_{12} myoblasts. Dexamethasone-induced expression of activated $G_{s\alpha}$ markedly delayed differentiation in comparison with uninduced stably transfected cells, which differentiated normally in mitogen-depleted media. Northern blot analysis showed that impaired differentiation was associated with delayed up-regulation of MyoD and myogenin and delayed down-regulation of Id, a dominant negative inhibitor of differentiation. Similar impairment of differentiation could not be reproduced in wild-type C_2C_{12} cells by increasing intracellular cAMP either with forskolin or treatment with a cell-permeable cAMP analog. However, treatment of myoblasts with cholera toxin markedly inhibited myogenic differentiation. Taken together, these findings suggest a novel role for $G_{s\alpha}$ in modulating myogenic differentiation. (*J. Clin. Invest.* 1997. 99:67–76.)
Key words: G-proteins • signal transduction • gene expression regulation • developmental cell differentiation • skeletal muscle

Introduction

The interaction of peptide growth factors with cell surface receptors on skeletal myoblasts initiates a complex series of intracellular signaling events that inhibits myogenic differentiation and promotes continued growth and proliferation. This results from repression of expression or inhibition of activity of the myogenic basic-helix-loop-helix (bHLH)[1] proteins (1, 2). These bHLH proteins, also known as the myoD family of myogenic determination factors, are several closely related muscle-specific transcriptional activators that are unique in their ability to transactivate muscle-specific genes, autoregulate their own expression, and orchestrate the entire program of myogenic differentiation in nonmuscle cells. Four myoD family members, MyoD, myogenin, myf-5, and MRF4/myf-6/herculin (3–9), have been identified, along with a related dominant negative HLH protein, known as Id, for "inhibitor of DNA binding" (10). All myoD family members share a common structural motif, the bHLH, which is necessary for formation of heterodimers with ubiquitously expressed factors E12 and E47 (11). These heterodimers exhibit site-specific binding to a *cis*-acting consensus sequence (CANNTG) known as the E-box that is present within many muscle-specific promoters (12). Id is an HLH protein that lacks an adjacent basic region and forms nonfunctional heterodimers with myogenic bHLH proteins and ubiquitous factors, thereby attenuating their DNA binding and transactivation potential (10).

An intriguing property of myogenic bHLH proteins is their sensitivity to growth factor and oncogenic signals that can silence their activities (2). This type of negative regulation is apparent before myogenesis when MyoD and myf5 proteins are present in myoblast nuclei but are unable to activate muscle-specific genes while the cell is exposed to peptide growth factors (3, 5). The effects of a number of growth factors, such as fibroblast growth factor and transforming growth factor type β, on the growth and differentiation of skeletal myoblasts and the activity of myogenic bHLH proteins have been described (13–16). However, the specific signal transduction mechanisms through which growth factors and mitogens acting at the cell surface operate to regulate activity of bHLH proteins in the nucleus remain poorly understood.

A family of heterotrimeric GTP-binding and hydrolyzing proteins (G proteins) is known to play a central role in transducing and modulating extracellular signals. In current paradigms, G proteins act as transducers that couple many cell surface receptors to specific intracellular effector proteins at the plasma membrane (17–19). The classically regulated G_s response involves coupling hormone-stimulated receptors to mediate activation of adenylyl cyclase, leading to an increase in intracellular levels of the second messenger, adenosine 3′,5′-cyclic monophosphate (cAMP). In addition to this effect, the α-subunit of G_s heterotrimers ($G_{s\alpha}$) has also been implicated in modulation of the rate at which murine embryo 3T3-L1 fibroblasts differentiate into adipocytes (20). This new role for $G_{s\alpha}$ operates independently of adenylyl cyclase activation and changes in the concentration of intracellular cAMP (20). The mechanism of this effect on cellular differentiation is presently not known, but it is opposed by G_i, the G protein mediator of inhibitory control of adenylyl cyclase (21).

We have previously shown that a substantial portion of total cellular $G_{s\alpha}$ is not associated with the plasmalemma but in-

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1. *Abbreviations used in this paper:* bHLH, basic-helix-loop-helix; $G_{s\alpha}$, α-subunit of G_s heterotrimers.

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stead localizes to cytoplasmic and nuclear compartments of proliferating S49 lymphoma cells (22). In contrast, we have noted similar cytoplasmic but no specific nuclear $G_{s\alpha}$ immunoreactivity in postmitotic-differentiated cardiac myocytes (23). We have postulated that in addition to its signal transduction role at the plasmalemma, $G_{s\alpha}$ may subserve other important functions during growth and proliferation and that the organization and subcellular compartmentation of G protein subunits within the cell may be an integral component of alternative intracellular and intranuclear signaling functions. The studies described below were undertaken to explore these possibilities in C_2C_{12} cells, a well-established model of myogenic differentiation. In particular, we sought to determine whether $G_{s\alpha}$ might participate in an intracellular signaling cascade that regulates the activity of bHLH proteins in the nucleus and thereby modulates myogenic differentiation.

Methods

Construction of expression vector

The rat $G_{s\alpha}$ cDNA (HA-$G_{s\alpha}$*) that includes a six amino acid hemagglutinin (HA) epitope tag and the R201C point mutation has been described previously (24). This point mutation markedly inhibits the intrinsic GTPase activity of the expressed protein and renders the subunit constitutively active. The cDNA was subcloned into pBluescript SK II as an SpeI-HindIII fragment, excised as a SalI fragment, and ligated into the SalI cloning site of the eukaryotic expression vector pMAMneo (Clontech, Palo Alto, CA).

Cell culture and transfection

The well-characterized murine skeletal myoblast cell line C_2C_{12} (ATCC CRL 1772) was obtained from the American Type Culture Collection (Rockville, MD). C_2C_{12} cells were maintained in a humidified atmosphere of 8% CO_2/92% air and passaged by standard trypsinization in a growth medium consisting of DME supplemented with 20% fetal bovine serum (Gibco BRL, Gaithersburg, MD), penicillin (50 U/ml), and streptomycin (50 mg/ml). Confluent cultures were induced to form myotubes in differentiation medium consisting of DME supplemented with 10% horse serum, penicillin (50 U/ml), and streptomycin (50 mg/ml).

Transfection was performed by the calcium phosphate coprecipitation method described previously (25). After transfection and exposure to glycerol shock, the cells were washed with phosphate-buffered saline, and allowed to recover in DME containing 20% fetal bovine serum for 18–24 h. Cells exhibiting neomycin resistance were selected in medium containing 700 μg/ml G418 (Gibco BRL). After 10–14 d, clonal cell lines were isolated with the use of cloning cylinders. Stably transfected cells were maintained in growth medium containing 700 μg/ml G418 that was replenished every 48 h.

Immunochemical methods

Antiserum. 3A-150 (Gramsch Laboratories, Schwabhausen, Germany) is a rabbit polyclonal antibody directed against a unique COOH-terminal decapeptide epitope of $G_{s\alpha}$ subunits (amino acids 385–394, RMHLRQYELL) (26). It recognizes both 45- and 52-kD splice variants of $G_{s\alpha}$, but does not cross-react with other α or βγ subunits (26). 12CA5 (Boehringer-Mannheim Biochemicals, Indianapolis, IN) is a mouse monoclonal antibody directed against the nonapeptide sequence YPYDVPDYA and derived from the influenza hemagglutinin protein; it has been used extensively to detect this epitope in tagged proteins (27–29).

Immunocytochemistry. C_2C_{12} cells were cultured on collagen-coated glass slides (Nunc, Inc., Naperville, IL), fixed in 4% paraformaldehyde at room temperature, and membrane-permeabilized with methanol at −20°C. The cells were incubated overnight at 4°C in phosphate-buffered saline containing 1% globulin-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 0.15% Triton X-100, 3% goat serum, and either 3A-150 at a dilution of 1:500 or 12CA5 at a dilution of 1:400. The next day, cells were washed extensively and then incubated with the appropriate secondary antibody, either goat anti–rabbit IgG or rabbit anti–mouse IgG conjugated to a CY3 fluorochrome (Jackson ImmunoResearch, West Grove, PA), at a dilution of 1:200 for 3 h at room temperature. Indirect immunofluorescent images were collected and photographed with the use of a Sarastro 2000 laser scanning confocal microscope (Molecular Dynamics, Inc., Sunnyvale, CA) or a conventional epifluorescence microscope.

Immunoprecipitation

Immunoprecipitation of metabolically labeled $G_{s\alpha}$ protein from C_2C_{12} cells was performed according to a method adapted from that of Takizawa et al. (30). C_2C_{12} cells were labeled for 30 minutes in methionine-depleted medium containing [^{35}S]methionine (1,000 Ci/mmol; Amersham, Corp., Arlington Heights, IL). The cells were then rinsed briefly with phosphate-buffered saline and scraped into 1 ml of ice-cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 U/ml aprotinin). Insoluble material was pelleted and removed by centrifugation at 14,000 *g* for 30 minutes at 4°C. The supernatant was incubated with 2 μl of primary antibody and a 20 μl suspension of rProtein A-IPA 300 coated-beads (Repligen, Cambridge, MA) with gentle agitation for 2 h at 4°C. Pellets were collected by centrifugation, washed overnight, and then three additional times for 30 min each in fresh prechilled RIPA buffer. The pellet was then mixed with 25 μl of SDS-PAGE loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 min, and loaded onto a 12.5% SDS-polyacrylamide gel. The gel was fixed in 10% methanol/10% acetic acid, treated with ENH^{3}ANCE (New England Nuclear, Boston, MA), and subjected to fluorography.

Measurement of intracellular cyclic AMP. C_2C_{12} cells were cultured in 60-mm dishes, washed twice with phosphate-buffered saline, and harvested in 140 mM NaCl, 10 mM sodium phosphate, pH 7.2. Cell pellets were resuspended in 0.25 ml of 0.05 M Tris, pH 7.5, 4 mM EDTA, and 50 μM 3-isobutyl-1-methylxanthine to inhibit cyclic nucleotide phosphodiesterase as previously described (31). Samples were briefly sonicated, boiled for 4 min, and centrifuged in a tabletop centrifuge for 5 min to remove cellular debris. 50 μl of the supernatant was assayed for cAMP with a commercial cAMP assay kit (Amersham Corp.) according to the manufacturer's instructions. cAMP values were normalized to the assayed weight of wet tissue or protein.

Analysis of creatine kinase activity. Cells grown in 60-mm dishes were washed twice with phosphate-buffered saline and overlaid with 300 μl of buffer (50 mM Tris-HCl, pH 7.5, 5% glycerol, 5 mM 2-mercaptoethanol). The dishes were frozen at −80°C and thawed, and the supernatant was aspirated and spun at 10,000 rpm at 4°C to remove cellular debris. Total creatine kinase activity was determined from at least two dishes in duplicate for each time point evaluated with the use of a miniature centrifugal analyzer (Gemini, Fairfield, NJ) as described previously (25) and normalized to the protein concentration determined in duplicate by the method of Bradford (Bio-Rad Laboratories, Richmond, CA).

Quantification of myotubes. Dishes of C_2C_{12} cells were washed twice with phosphate-buffered saline and then fixed and stained with Wrights-Giemsa. Myotubes were counted in a minimum of five high-power fields selected at random.

Northern blot analysis. 10–15 μg of total cellular RNA extracted with the use of RNAzol B (Tel-Test, Inc., Friendswood, TX) was fractionated on 1.5% formaldehyde-agarose gels and transferred to maximum strength Nytran membranes (Schleicher & Schuell, Keene, NH). Membranes were prehybridized according to the manufacturer's recommendations and were then hybridized for 16–18 h with cDNA probes labeled by the random primer method (Boehringer-Mannheim). A 1.1-kb myogenin EcoRI cDNA fragment (gift of Eric Olsen, Uni-

versity of Texas, Austin, TX), the 1.8-kb MyoD1 EcoRI cDNA, and 0.9-kb SmaI Id cDNA (gifts of the late Harold Weintraub, University of Washington, Seattle, WA) were used to detect bHLH mRNAs. Hybridization with an 0.6-kb XbaI-HindIII glyceraldehyde 3-phosphate dehydrogenase cDNA fragment (ATCC 57091) was also performed as a control for RNA loading and transfer to membranes.

Statistical methods. Results are expressed as the mean±SD. The significance of differences between group means was determined by a modified Student *t* test (Bonferroni method) after a one-way analysis of variance.

Results

Immunolocalization of $G_{s\alpha}$ protein in myoblasts and myotubes. In previous works, we immunolocalized $G_{s\alpha}$ protein to nuclei of proliferating S49 lymphoma cells but did not detect $G_{s\alpha}$ in nuclei of postmitotic differentiated cardiac myocytes (22, 23). To explore the significance of nuclear $G_{s\alpha}$ in a differentiating cell line, we immunostained proliferating C_2C_{12} myoblasts and differentiated C_2C_{12} myotubes with 3A-150, a specific antiserum raised against an epitope unique to the native $G_{s\alpha}$ subunit. We have previously characterized this antibody and validated its use for immunocytochemical localization of $G_{s\alpha}$ in cells and tissues at the light and electron microscopic levels (22, 23). Proliferating C_2C_{12} myoblasts exhibited a significant fraction of total cellular $G_{s\alpha}$ in cytoplasmic and nuclear compartments (Fig. 1 *A*), similar to our previous findings in S49 lymphoma cells (22). In striking contrast, intranuclear $G_{s\alpha}$ was not detected in C_2C_{12} myotubes (Fig. 1 *B*), similar to our findings in differentiated adult cardiac myocytes (23). This has been a reproducible finding in experiments conducted in our laboratory over a 3-yr period with many independent batches of C_2C_{12} cells. These observations are consistent with the concept that the presence of $G_{s\alpha}$ in the nucleus may be a marker of cell proliferation and its absence a marker of cell differentiation.

Forced expression of $G_{s\alpha}$ in C_2C_{12} myoblasts. To explore the biological significance of these observations, we designed a strategy to permit modulation of the expression of $G_{s\alpha}$. Accordingly, we developed lines of C_2C_{12} myoblasts stably transfected with a plasmid in which expression of a mutationally activated epitope-tagged $G_{s\alpha}$ cDNA was placed under the control of the glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter. We chose to transfect the mutationally activated rather than the native form of $G_{s\alpha}$ because previously published reports have suggested that the active form has reduced membrane avidity (24) and thus may be more likely to localize to intracellular and intranuclear sites. In addition, expression of the active form of the protein circumvented the potential problem of failure of normal cellular mechanisms to activate $G_{s\alpha}$.

Multiple independent G418-resistant clonal lines of C_2C_{12} cells stably transfected with the $G_{s\alpha}$ expression plasmid were established by selection in media containing G418. C_2C_{12} cells stably transfected with the expression vector pMAMneo were

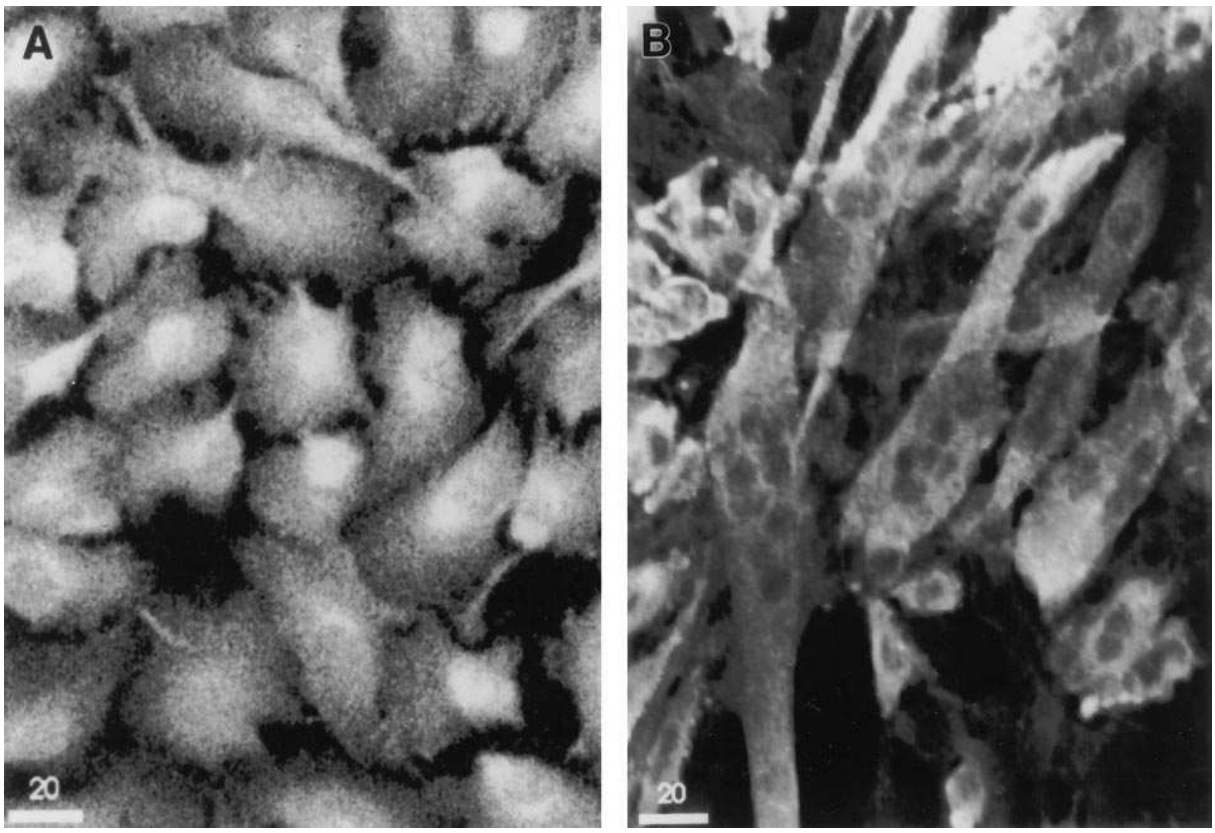

Figure 1. Immunocytochemical localization of $G_{s\alpha}$ in C_2C_{12} cells. C_2C_{12} myoblasts (*A*) and C_2C_{12} myotubes (*B*) were imaged with a confocal laser scanning fluorescent microscope after fixation in 4% paraformaldehyde, membrane permeabilization with cold methanol, and immunostaining with $G_{s\alpha}$-specific primary antiserum (3A-150) and fluorescently labeled secondary antibodies. Each image was obtained by scanning a single 0.6-μm focal plane under identical gain settings. Bar = 20 μm.

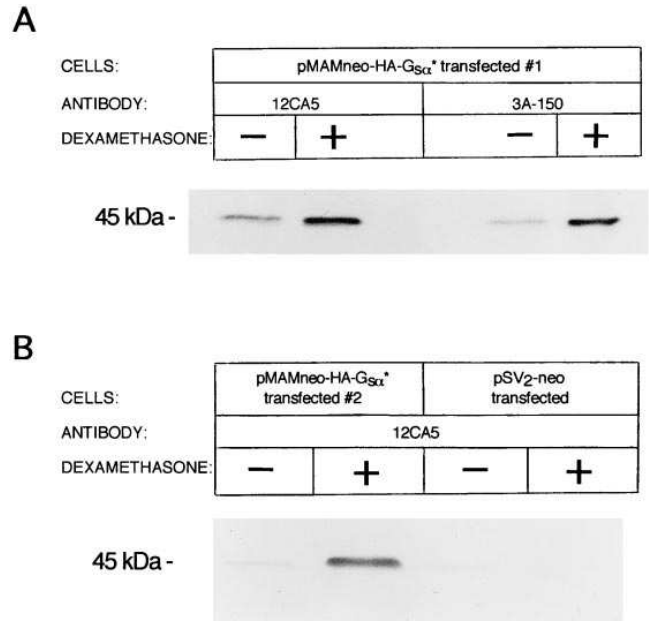

Figure 2. Immunoprecipitation of $G_{s\alpha}$ protein from stably transfected C_2C_{12} cells. (*A*) C_2C_{12} myoblasts stably transfected with the HA-$G_{s\alpha}$* plasmid were grown under control conditions (−) or stimulated (+) with dexamethasone (1 μM) and metabolically labeled with ^{35}S-methionine. A single protein of ~ 45 kD (*arrow*) was immunoprecipitated with either 12CA5, a hemagglutinin epitope-specific mouse monoclonal antibody, or 3A-150, a rabbit polyclonal antiserum directed against a COOH-terminal decapeptide epitope from the $G_{s\alpha}$ subunit. (*B*) C_2C_{12} myoblasts stably transfected with pSV_2neo or the HA-$G_{s\alpha}$* plasmid were grown under control conditions or stimulated with dexamethasone and metabolically labeled as above. Immunoprecipitation was performed with the antibody 12CA5. Results shown in panels *A* and *B* are from lines #1 and #2, two independently isolated lines of HA-$G_{s\alpha}$*-expressing cells (see Table I).

also selected to serve as controls. Myogenic differentiation in these clonal lines was studied both in the presence and absence of dexamethasone. Expression of $G_{s\alpha}$ was demonstrated by immunoprecipitation from a representative cell line that had been metabolically radiolabeled with ^{35}S-methionine. The mouse monoclonal antibody, 12CA5, directed against the hemagglutinin epitope tag, can distinguish expressed $G_{s\alpha}$ from the native protein. As shown in Fig. 2 *A*, a single protein was immunoprecipitated with either 12CA5 or 3A-150, a $G_{s\alpha}$-specific rabbit polyclonal antibody. These proteins comigrated on SDS polyacrylamide gels and showed approximate molecular weights of 45 kD, identical to weights of native $G_{s\alpha}$ immunoprecipitated from nontransfected cells with the antibody 3A-150 (data not shown). Stimulation with dexamethasone (1 μM) up-regulated expression of $G_{s\alpha}$ three- to fourfold, as determined by scanning laser densitometric examination (Ultrascan XL; LKB, Uppsala, Sweden) of the autoradiogram in Fig. 2 *A*. This level of increased expression after dexamethasone stimulation is consistent with previously reported levels of transcriptional up-regulation of cDNAs placed downstream of the inducible promoter in pMAMneo (32, 33). Low-level expression of epitope-tagged $G_{s\alpha}$ detected in the absence of dexamethasone was likely due to leakiness of the MMTV promoter (33) or stimulation by endogenous steroids in serum or the phenol red indicator in DME. As a control for specificity of the 12CA5 antibody, C_2C_{12} cells were selected with G418 after transfection with the irrelevant plasmid pSV_2neo. Immunoprecipitation with the 12CA5 antibody after metabolic labeling in the presence or absence of dexamethasone did not result in any specific radiolabeled band at 45 kD (Fig. 2 *B*).

The subcellular distribution of the expressed epitope-tagged $G_{s\alpha}$ protein in stably transfected C_2C_{12} cells and its response to dexamethasone was demonstrated by indirect immunofluorescence by use of the epitope-specific 12CA5 antibody (Fig. 3). Marked up-regulation of expression of epitope-tagged $G_{s\alpha}$ in response to dexamethasone with prominent intranuclear staining was noted in myoblasts (Fig. 3 *II*). In contrast, stably transfected C_2C_{12} cells that underwent differentiation to myotubes showed marked up-regulation of cytoplasmic staining after stimulation with dexamethasone but still exhibited no significant intranuclear staining (Fig. 3 *II*). These results show that the subcellular distribution of expressed HA-$G_{s\alpha}$* protein closely approximates that of the native $G_{s\alpha}$ subunit (see Fig. 1).

To determine whether expression of $G_{s\alpha}$ in C_2C_{12} cells affected myogenic differentiation, 12 clonal lines of cells stably transfected with the $G_{s\alpha}$-expression plasmid were evaluated. The accumulation of creatine kinase enzyme activity and the extent of formation of myotubes were used as biochemical and morphologic markers of myogenic differentiation. Results from a representative stably transfected clonal line are shown in Fig. 4 *A*. The cells were grown to 80% confluence in growth medium and then switched to differentiation medium either with or without 1 μM dexamethasone. After 4 d in differentiation medium without dexamethasone, the cells fused to form large multinucleated myotubes and accumulated significant creatine kinase enzyme activity. The time course of differentiation was complete after 168 h in differentiation medium. In contrast, when the same clonal line of cells was stimulated with dexamethasone to trigger the expression of $G_{s\alpha}$, myogenic differentiation was markedly delayed, as evidenced by formation of 8.3-fold fewer multinucleated myotubes per high power field (3.0 ± 0.7 vs 24.8 ± 2.6, $n = 5$ random fields, $P < 0.005$) and accumulation of 3.5-fold less creatine kinase activity (98.15 ± 10.12 vs 347.74 ± 41.35 IU/g protein, $n = 3$ plates of cells, $P < 0.005$) at 168 h. Dexamethasone-stimulated cells were observed over 288 h and continued to accumulate creatine kinase enzyme activity and form myotubes at a very low rate. Creatine kinase activity and myotube counts at 288 h were still significantly less than the values determined in the absence of dexamethasone at 168 h, consistent with delay rather than a complete block of differentiation. Qualitatively similar results were noted in 10 of 11 additional independent clonal lines studied by these methods (Table I). The single aberrant clonal line proved to be differentiation defective and completely failed to fuse or up-regulate creatine kinase activity. None of four clonal lines of C_2C_{12} cells stably transfected with the expression vector alone exhibited any significant dexamethasone-induced effect on myogenic differentiation. The results for a representative vector-transfected line are shown in Fig. 4 *B*. These cells differentiated with a time course that closely approximated that of nontransfected C_2C_{12} cells. Because dexamethasone has been shown to have no effect on differentiation of wild-type C_2C_{12} myoblasts (33, 34), additional control experiments with nontransfected cells were not performed. These results show that the expression of activated $G_{s\alpha}$ inhibited myogenic differentiation. The fact that a large number of independent clonal lines demonstrated the same biological effect indicates that inhibi-

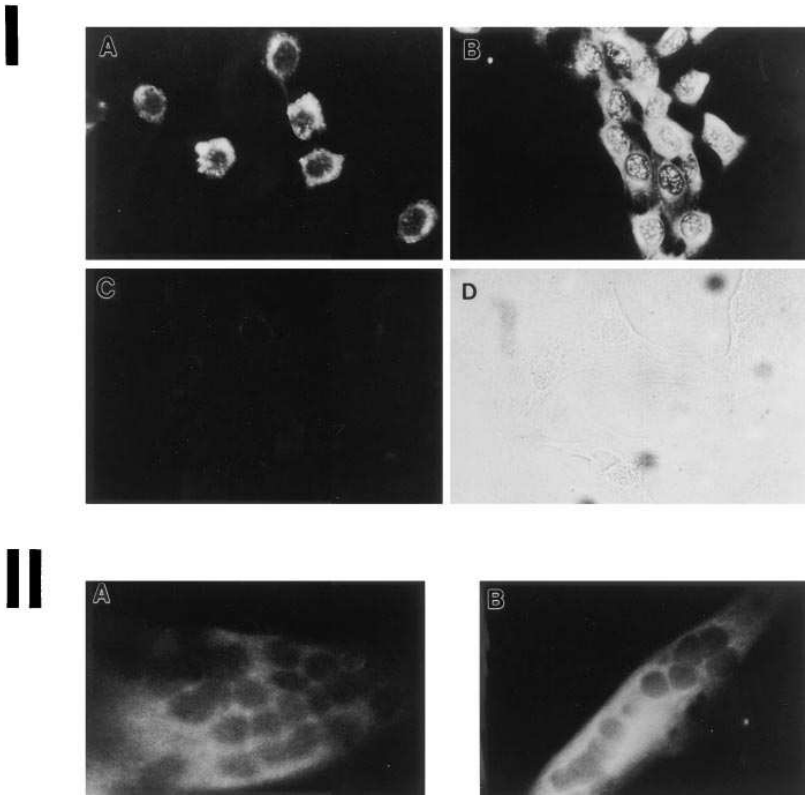

Figure 3. Subcellular localization of expressed HA-$G_{s\alpha}$* in stably transfected C_2C_{12} cells by indirect immunofluorescence. (*I*) Myoblasts. Representative stably transfected C_2C_{12} myoblasts under control (*A*) and 1 μM dexamethasone-stimulated conditions (*B*) were fixed and permeabilized as described in Methods and immunostained with 12CA5 primary antiserum and fluorescently labeled secondary antibody. Cells immunostained with a nonimmune primary antiserum (*C*) were included as a control for background fluorescence. The appearance of the nonimmune control image by phase contrast is included (*D*). (*II*) Myotubes. Stably transfected C_2C_{12} cells were induced to differentiate and then immunostained under control conditions (*A*) or following stimulation with 1 μM dexamethasone (*B*). Cells were processed as above and immunostained with the 12CA5 antibody.

tion of myogenic differentiation was indeed due to the expression and activity of $G_{s\alpha}$ and was not a positional effect mediated by disruption of native genes critical for myogenesis. Furthermore, we were able to subclone a revertant cell line by limiting dilution under partially selective conditions (33). The subclone recovered the ability to differentiate in the presence of dexamethasone with a time course identical to that for wild-type C_2C_{12} cells; it showed creatine kinase activity of 740.6±20 IU/μg protein in the presence of 1 μM dexamethasone after 96 h in differentiation medium. In comparison, a nonrevertant subclone isolated and propagated under identical conditions showed creatine kinase activity of 74.7±1.4 IU/μg protein after 192 h in differentiation medium with dexamethasone. The ability of the cells to revert back to the parental C_2C_{12} phenotype eliminates the possibility that the G418-selected cells were inherently different from wild-type C_2C_{12} myoblasts.

The effect of activated $G_{s\alpha}$ on intracellular cAMP. We assessed the biological activity of $G_{s\alpha}$ expressed in transfected cells in terms of its ability to activate adenylyl cyclase and increase levels of intracellular cAMP. A representative clonal line transfected with the $G_{s\alpha}$ expression plasmid showed a 1.6-fold increase in intracellular cAMP after 18 h of stimulation with dexamethasone (0.02234±0.0041 vs 0.01408±0.0022 pmol/μg wet tissue, $n = 4$, $P < 0.01$). The increase in cAMP over baseline after dexamethasone stimulation is consistent with that previously reported for a related mutationally active form of $G_{s\alpha}$ in a pMAMneo expression plasmid transfected into NIH 3T3 cells (35). In contrast, nontransfected cells and cells transfected with the vector alone did not show statistically significant changes in intracellular cAMP levels after stimulation with dexamethasone (0.0161±0.0004 vs 0.0149±0.0007 pmol/μg wet tissue and 0.0126±0.0005 vs 0.0152±0.0011 pmol/μg wet tissue, respectively, $P > 0.05$, $n = 4$).

Effect of activated $G_{s\alpha}$ expression vs that of intracellular cAMP. To examine the effect of increased intracellular cAMP on differentiation of C_2C_{12} cells we compared cAMP levels under two different conditions. First we measured intracellular cAMP after dexamethasone-induced expression of $G_{s\alpha}$ and

Table I. Inhibition of Myogenic Differentiation in Response to Dexamethasone in Stably Transfected C_2C_{12} Clonal Cell Lines

pMAMneo-HA-$G_{s\alpha}$* clonal cell line	Time course	Myotube counts		% Inhibition
		Unstimulated	Stimulated	
	h			
1	168	24.8±2.6	3.0±0.7	87.9
2	120	25.8±2.3	2.6±1.1	89.9
3	120	28.8±4.2	6.6±2.3	77.1
4	120	31.2±3.6	12.2±3.5	60.9
5	96	32.4±4.6	17.2±2.3	46.9
6	96	30.2±3.5	12.8±1.6	57.6
7	72	31.2±2.8	9.4±1.5	69.9
8	72	32.2±1.9	11.8±5.0	63.4
9	72	32.6±2.7	15.6±1.5	52.1
10	72	32.8±2.6	12.6±3.0	61.6
11	72	29.4±1.7	9.8±2.4	66.7
12‡	168	< 1.0	< 1.0	0

C_2C_{12} cells were transfected with pMAMneo-HA-$G_{s\alpha}$* and selected with G418. Cell lines were isolated with the use of cloning cylinders and expanded in growth medium. Cells from 12 clonal lines were plated, induced to differentiate under control conditions (*unstimulated*) or in the presence of 1 μM dexamethasone (*stimulated*), and stained with Wrights-Giemsa. Myotubes were counted (mean±SD) in at least five high-power fields. ‡Differentiation-defective cell line.

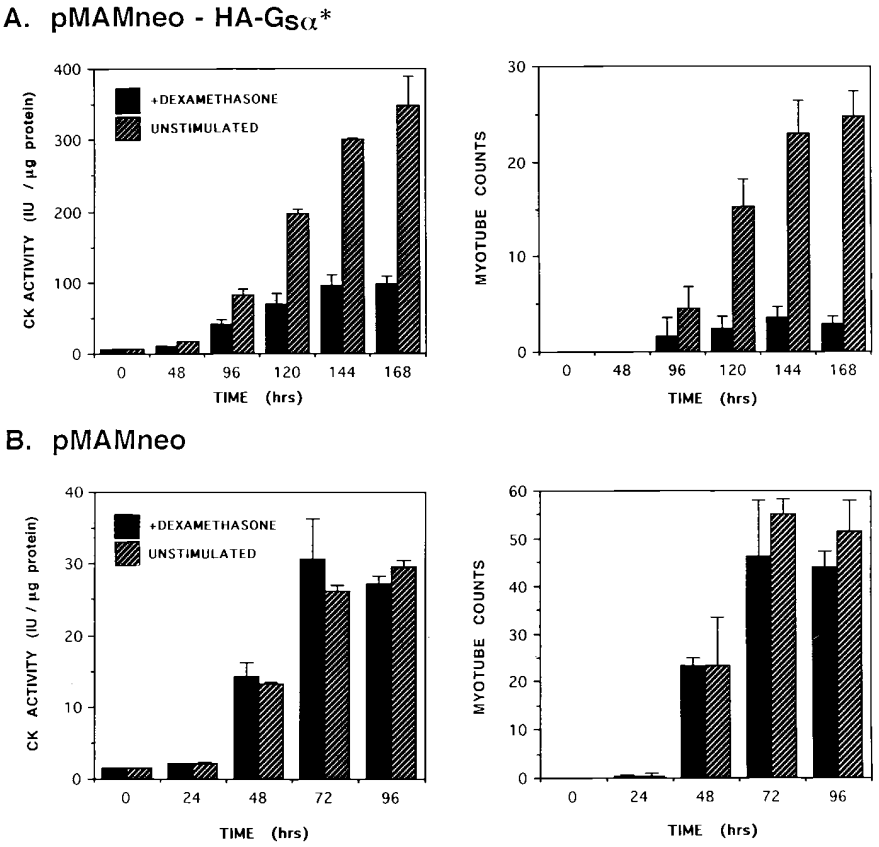

Figure 4. Dexamethasone-induced expression of activated $G_{s\alpha}$ inhibits differentiation of C_2C_{12} cells. The time course of myogenic differentiation as assessed by biochemical and morphologic markers is illustrated for representative clonal lines of C_2C_{12} cells stably transfected with either the $G_{s\alpha}$ expression construct pMAMneo-HA-$G_{s\alpha}$* (*A*) or the expression vector pMAMneo (*B*). Total creatine kinase activity values are the mean±SD determined at the times indicated from three replicate plates of cells from the same clonal line. Myotube values reflect the mean±SD of counts obtained by two independent observers during review of five random high-power fields of a representative plate of cells treated under the conditions indicated.

noted a 1.6-fold increase over control (see above). Second, we induced cells stably transfected with the vector pMAMneo to differentiate in the presence of forskolin, a reagent that stimulates adenylyl cyclase and raises intracellular cAMP independent of $G_{s\alpha}$. Because myogenic cells respond to forskolin rapidly (31, 36), we harvested cells for measurement of cAMP after 1 h. Treatment with forskolin (final concentration 10 μM) resulted in a 1.6-fold increase in intracellular cAMP relative to dimethyl sulphoxide vehicle (0.02425±0.0044 pmol/μg protein vs 0.01543±0.0035 pmol/μg protein, $n = 4$, $P < 0.04$). Although the peak increase in intracellular cAMP in response to forskolin was identical to that seen in cells stably transfected with the $G_{s\alpha}$ expression vector stimulated with dexamethasone, the effects on myogenic differentiation were markedly different. There was no significant difference in creatine kinase activity in forskolin- and vehicle-treated cells after 120 h in differentiation medium (438.42±17.1 vs 373.42±25.3 IU/μg protein, $n = 4$, $P > 0.05$). Because C_2C_{12} cells showed marked toxicity when treated with higher doses of forskolin, as indicated by a large proportion of cells detaching from the plate, we did not attempt to determine whether higher concentrations of forskolin inhibited myogenic differentiation.

To examine the effect of activation of *endogenous* $G_{s\alpha}$ subunits on myogenic differentiation, C_2C_{12} myoblasts were induced that differentiated either under control conditions, in the presence of the cell-permeable cAMP analog 8-Br-cAMP, or in the presence of cholera toxin (Fig. 5). Directly increasing intracellular cAMP levels with 8-Br-cAMP had a small but statistically insignificant effect on the induction of creatine kinase enzyme activity and the formation of myotubes at 48 and 72 h. In marked contrast, treatment with cholera toxin, a reagent that constitutively activates $G_{s\alpha}$ by ADP-ribosylation, had a very significant inhibitory effect on myogenic differentiation. Cholera toxin-treated myoblasts remained viable and retained their ability to differentiate, as assessed by their ability to accumulate creatine kinase activity and form additional myotubes in fresh differentiation medium devoid of cholera toxin. These results demonstrate that both the activation of native $G_{s\alpha}$ subunits and expression of mutationally activated $G_{s\alpha}$ subunits markedly inhibits myogenic differentiation. Although we did not measure adenylyl cyclase activity in any of the cell lines, we infer from these findings that activation of $G_{s\alpha}$ inhibits myogenic differentiation independent of downstream adenylyl cyclase activation and changes in intracellular cAMP.

The effect of forced expression of $G_{s\alpha}$ on myogenic bHLH factors. Myogenic differentiation is regulated by the expression and activity of myogenic determination factors. To gain insight into the molecular mechanism by which expression of $G_{s\alpha}$ affects myogenic differentiation, we performed Northern blot analyses. Plates of a representative clonal line stably transfected with either the expression plasmid or the vector alone were cultured and differentiation was induced in the presence and absence of dexamethasone. RNA was harvested from these cells at selected time points for Northern analysis of bHLH mRNAs. A representative time course of expression of bHLH mRNAs in C_2C_{12} cells stably transfected with the $G_{s\alpha}$-expression plasmid is shown in Fig. 6 *A*. Cells induced to differentiate in the absence of dexamethasone showed an essentially normal program of expression of bHLH factors. C_2C_{12} myoblasts express MyoD both before and after differentiation. Autoactivation, associated with transient up-regulation of MyoD transcripts, contributes to the induction of myogenesis once

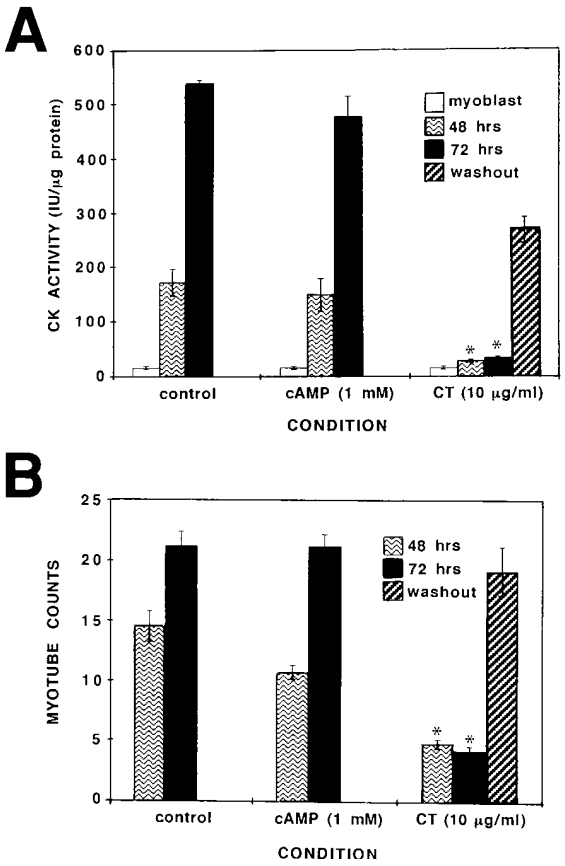

Figure 5. Effect of cAMP and cholera toxin on differentiation of C_2C_{12} cells. C_2C_{12} cells were cultured in differentiation medium (*control*), in differentiation medium supplemented with 8-Br-cAMP (cAMP, final concentration 1 mM), or in cholera toxin (*CT*, final concentration 10 μg/ml) and harvested for measurement of creatine kinase activity and myotube formation after 48 and 72 h. Cells exposed to cholera toxin for 72 h were washed, fed with fresh differentiation medium without cholera toxin, and harvested 96 h later (*washout*). *Statistically significantly different ($P < 0.001$) from control.

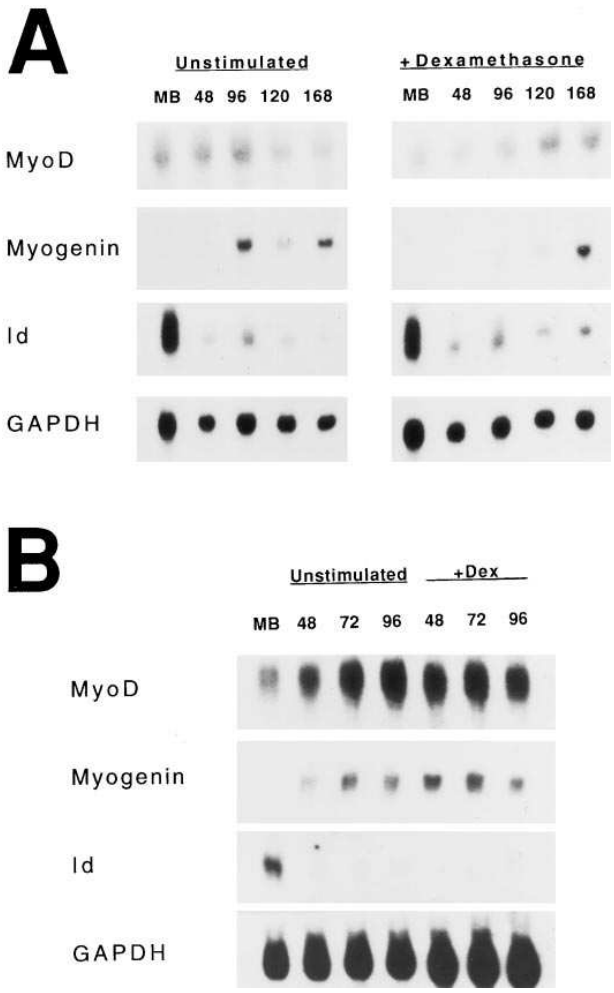

Figure 6. Northern blot analysis of myogenic bHLH regulatory factors. Plates of a representative clonal line of C_2C_{12} cells stably transfected with the $G_{s\alpha}$ expression construct (*A*) or with the vector pMAMneo (*B*) were harvested as myoblasts (*MB*) or induced to differentiate under both control and dexamethasone-stimulated conditions. At the times indicated (h), total cellular RNA was harvested as described under Methods. 15 μg of total cellular RNA was fractionated on 1.5% formaldehyde-agarose gels and subjected to Northern analysis. The membranes were washed and hybridized sequentially with the radiolabeled probes indicated.

differentiation has been triggered (37, 38). Myogenin mRNA is not detectable in undifferentiated myoblasts. During differentiation, transcripts for myogenin appear, peak several hours before other muscle-specific mRNAs, abruptly decline after the stimulus to differentiate, and then rise again so that myogenin mRNA levels remain detectable in differentiated myotubes (4). Transcripts for Id are abundant in undifferentiated myoblasts. After the stimulus to differentiate, Id mRNA is abruptly and continuously down-regulated and becomes virtually undetectable in myotubes (10). As shown in Fig. 6 *A*, dexamethasone-induced expression of $G_{s\alpha}$ in C_2C_{12} myoblasts markedly delayed the normal program of up-regulation of MyoD and myogenin mRNAs and inhibited down-regulation of Id mRNA. In contrast, dexamethasone had no significant effect on the program of expression of myogenic bHLH mRNAs in C_2C_{12} cells stably transfected with the expression vector pMAMneo alone (Fig. 6 *B*).

Discussion

In this report, we describe immunolocalization of $G_{s\alpha}$ protein to cytoplasmic and nuclear compartments of C_2C_{12} myoblasts and the striking absence of intranuclear $G_{s\alpha}$ protein in nuclei of differentiated postmitotic C_2C_{12} myotubes. We have demonstrated in a series of stable transfection experiments an effect of expression of mutationally activated $G_{s\alpha}$ protein on myogenesis relative to uninduced stably transfected control myoblasts. We have also demonstrated that a comparable inhibitory effect can be produced with constitutive activation of endogenous $G_{s\alpha}$ protein in nontransfected C_2C_{12} myoblasts after treatment with cholera toxin. In control experiments, forskolin-induced activation of adenylyl cyclase and treatment with the cell-permeable cAMP analog 8-Br-cAMP alone were insufficient to yield any significant inhibitory effect on myogenesis in this cell line, suggesting an effect of activation of $G_{s\alpha}$ protein on myogenic differentiation independent of cyclase and intracellular cAMP. By Northern analysis, this inhibitory

effect of $G_{s\alpha}$ activation operates through a delay in the normal program of expression of the myogenic bHLH mRNAs.

G protein–mediated signal transduction cascades (36, 39) in addition to cAMP-dependent and independent pathways (35) have all previously been implicated in the regulation of proliferation and differentiation in muscle. Although forskolin and dibutyryl cAMP, acting downstream of $G_{s\alpha}$ in the cAMP signaling pathway, have been shown to inhibit differentiation and the expression of muscle-specific genes in the nonfusing murine muscle cell line BC_3H1 (31), their effect on differentiation in primary and other established skeletal myoblast lines has been far less consistent (31, 36, 39–44). Agents that increase intracellular cAMP have been reported to provoke precocious fusion in some instances (40), and to inhibit myoblast fusion in others (41). In particular, reports of the effects of cAMP on myogenic differentiation in C_2C_{12} cells are highly variable. Whereas a single published report demonstrates morphologic inhibition of differentiation after treatment with dibutyryl cAMP (44), we and others have not noted a comparable effect of cAMP in similar experiments conducted with the same cell line (31, 36). We postulate that this discrepancy may represent a spurious effect of dibutyryl cAMP rather than a true effect of intracellular cAMP because others have demonstrated that butyrate can inhibit myogenesis by interfering with the transcriptional activation function of MyoD and myogenin (45).

In the instances in which cAMP signaling pathways have been shown to regulate myogenic differentiation, the effects are thought to be mediated by cAMP-dependent protein kinase (PKA) via a posttranslational mechanism that selectively represses the transactivating function of the myogenic bHLH factors (43, 44). This posttranslational mechanism presumably involves phosphorylation directed at the basic HLH domain of the myogenic factors and operates through the E-box DNA consensus motif to which these proteins bind (43, 44). In the experiments described in this report we demonstrated that stimulation of $G_{s\alpha}$, either through forced expression of mutationally activated protein or after treatment with cholera toxin, has effects on myogenic differentiation that activation of adenylyl cyclase and increases in intracellular cAMP alone do not. Indeed, the results of this study suggest that $G_{s\alpha}$ activation inhibits myogenic differentiation via a previously undescribed mechanism acting at the level of regulation of mRNAs encoding bHLH proteins and that it occurs upstream of the previously described posttranslational effects on myogenic bHLH proteins mediated by cAMP and PKA.

Apart from cAMP, a role has also been established for products of protooncogenes in the regulatory circuit for transduction of growth factor signals from the cell membrane to the nucleus (46). For example, several members of the *Ras* family of oncogene products, which bind GTP and thereby couple specific growth factor receptors to intracellular effectors, have been shown to inhibit myoblast differentiation when activated by missense mutations that disrupt their endogenous GTPase activity (46–49). By strict analogy with the *Ras* oncogene, it has been proposed that point mutations that disrupt the GTPase activity of $G_{s\alpha}$ should similarly convert it to an oncogene (35, 50–53). Activated mutants of the $G_{s\alpha}$ subunit, however, have never been shown to transform cells in vitro (35, 50, 53). Instead, the opposite appears true. Expression of activated $G_{s\alpha}$ actually suppresses H-Ras transformation in the NIH 3T3 cell line (35). The mutationally activated $G_{s\alpha}$ transfected in our experiments would therefore not be expected to inhibit myogenesis via the previously described protooncogene mechanism.

What, then, might the mechanism of the effect described above, mediated by activation of $G_{s\alpha}$, be? Much attention has been focused recently on the coordination of multiple signal pathways, including the cAMP-PKA pathway and the "Ras pathway," with the series of enzymes known as the mitogen-activated protein (MAP) kinases. Our immunocytochemical observations are consistent with the postulate that inhibition of myogenesis by expression of $G_{s\alpha}$ might be a consequence of the subcellular distribution of this G protein subunit that alters its accessibility to effectors of the MAP kinase pathway in the nucleus. We found that the $G_{s\alpha}$ subunit spatially and temporally localizes to the nucleus, where it can directly modulate transcriptional events that help to establish the differentiated muscle phenotype, in addition to performing its well-characterized signal transduction function at the plasmalemma. These findings would truly represent the type of cAMP-independent and G protein–mediated signal transduction cascade previously postulated to modulate proliferation and differentiation in muscle (42) much like that already shown to modulate adipogenesis in 3T3-L1 fibroblasts (20, 21). These findings are also in keeping with the observation that $G_{s\alpha}$ is 10- to 100-fold more abundant than all the known receptors and effectors that it couples, and that it therefore likely exists in different compartments from them or in otherwise inaccessible states (54). The abundance of endogenous $G_{s\alpha}$ and its potential inaccessibility to cyclase may in part explain the relatively modest effect of forced expression of activated $G_{s\alpha}$ on intracellular cAMP reported in this and previous work (35).

Mechanisms that regulate nuclear protein localization and retention are poorly understood, but are likely important to the processes of development and the regulation of gene expression. Some proteins are known to be targeted to the nucleus by an active nuclear localization sequence (NLS) contained within the protein's primary sequence. These sequences are usually $<$ 8–10 amino acids in length and contain a high proportion of positively charged lysine and arginine amino acid residues (55, 56). We compared nuclear localization motifs previously described (Genetics Computer Group, PROSITE release 7.1) with the amino acid sequence of G_{sa} and were unable to find a canonical NLS.

As a general concept, compartmentation is beginning to emerge as a common theme in signal transduction and is postulated to be a mechanism through which accessibility of signaling molecules to different substrates is regulated and the specificity of diverse physiologic responses is determined (57). Compartmentation of components of tyrosine kinase-mediated signaling pathways (58), several serine and threonine protein phosphatases, and protein kinases (59) have been described. In this report, we present evidence that the α-subunit of G_s-protein heterotrimers exhibits a distinctive developmental pattern of compartmentation in myoblasts distinct from that in myotubes. We offer transfection and expression data as evidence that perturbations that impact on the orderly reorganization of these patterns of compartmentation during development can inhibit the overall process of differentiation.

A plausible explanation for the loss of growth factor responsiveness in terminally differentiated myocytes that have irreversibly exited the cell cycle is the disappearance of one or more components of the signal transduction pathways that link the cell membrane with the nucleus. In light of the data pre-

sented in this report, we propose that the α-subunit of G_s-protein heterotrimers could be such a component.

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