Integrin α Subunit Ratios, Cytoplasmic Domains, and Growth Factor Synergy Regulate Muscle Proliferation and Differentiation

Sarita K. Sastry, Margot Lakonishok,* Dori A. Thomas,* John Muschler, and Alan F. Horwitz*

Departments of Biochemistry and *Cell and Structural Biology, University of Illinois, Urbana, Illinois 61801

Abstract. The role of integrins in muscle differentiation was addressed by ectopic expression of integrin α subunits in primary quail skeletal muscle, a culture system particularly amenable to efficient transfection and expression of exogenous genes. Ectopic expression of either the human α5 subunit or the chicken α6 subunit produced contrasting phenotypes. The α5-transfected myoblasts remain in the proliferative phase and are differentiation inhibited even in confluent cultures. In contrast, myoblasts that overexpress the α6 subunit exhibit inhibited proliferation and substantial differentiation. Antisense suppression of endogenous quail α6 expression inhibits myoblast differentiation resulting in sustained proliferation. These effects of ectopic α subunit expression are mediated, to a large extent, by the cytoplasmic domains. Ectopic expression of chimeric α subunits, $\alpha5^{ex}/6_{cyto}$ and $\alpha6^{ex}/5_{cyto}$, produced phenotypes opposite to those observed with ectopic α5 or α6 expression. Myoblasts that express $\alpha5^{ex}/6_{cyto}$ show decreased proliferation while differentiation is partially restored. In contrast, the $\alpha6^{ex}/5_{cyto}$ transfectants remain in the proliferative phase unless allowed to become confluent for at least 24 h. Furthermore, expression of human α5 subunit cytoplasmic domain truncations, before and after the conserved GFFKR motif, shows that this sequence is important in α5 regulation of differentiation. Ectopic α5 and α6 expression also results in contrasting responses to the mitogenic effects of serum growth factors. Myoblasts expressing the human α5 subunit differentiate only in the absence of serum while differentiation of untransfected and α6-transfected myoblasts is insensitive to serum concentration. Addition of individual, exogenous growth factors to α5-transfected myoblasts results in unique responses that differ from their effects on untransfected cells. Both bFGF or TGFβ inhibit the serum-free differentiation of α5-transfected myoblasts, but differ in that bFGF stimulates proliferation whereas TGF-β inhibits it. Insulin or TGF-α promote proliferation and differentiation of α5-transfected myoblasts; however, insulin alters myotube morphology. TGF-α or PDGF-BB enhance muscle α-actinin organization into myofibrils, which is impaired in differentiated α5 cultures. With the exception of TGF-α, these growth factor effects are not apparent in untransfected myoblasts. Finally, myoblast survival under serum-free conditions is enhanced by ectopic α5 expression only in the presence of bFGF and insulin while TGF-α and TGF-β promote survival of untransfected myoblasts. Our observations demonstrate (*1*) a specificity for integrin α subunits in regulating myoblast proliferation and differentiation; (*2*) that the ratio of integrin expression can affect the decision to proliferate or differentiate; (*3*) a role for the α subunit cytoplasmic domain in mediating proliferative and differentiative signals; and (*4*) the regulation of proliferation, differentiation, cytoskeletal assembly, and cell survival depend critically on the expression levels of different integrins and the growth factor environment in which the cells reside.

A role for the extracellular matrix (ECM)[1] in the regulation of cell differentiation and gene expression has been demonstrated for many different cell types (Adams and Watt, 1993; Damsky and Werb, 1992; Juliano and Haskill, 1993; Lin and Bissell, 1993). For instance, tissue-specific expression of milk proteins in mammary epithelium requires adhesion to basement membrane components (Roskelley et al., 1995) and terminal differentiation of keratinocytes involves changes in adhesion to fibronectin (FN), laminin (LM), and collagen (Adams and Watt, 1990). However, the mechanisms by which the ECM regulates differentiation are currently poorly understood. Skeletal muscle has served as a model system for defining the molecules and mechanisms which govern cell differentiation, including its regulation by the ECM. A requirement for particular ECM components in skeletal

Please address all correspondence to S. Sastry, Department of Cell and Structural Biology, 511 Morrill Hall, 505 S. Goodwin Avenue, Urbana, IL 61801. Tel.: (217) 244-6135; Fax: (217) 244-1648. e-mail: S-SASTRY @UIUC.EDU

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; ECM, extracellular matrix; FACS, fluorescence-activated cell sorting; FN, fibronectin; LM, laminin; UT, untransfected myoblast.

muscle differentiation in vitro is among the early descriptions of the requirement of a matrix molecule for differentiation (Hauschka and Konigsberg, 1966). Adhesion of muscle cells to extracellular matrix components influences such diverse phenomena as cell migration, the proliferative to differentiative transition, muscle morphogenesis, the organization of an elaborate contractile apparatus, and synapse formation (McDonald et al., 1995*a*). It is also apparent that other types of exogenous molecules, e.g., growth factors, regulate muscle differentiation. For example, media rich in growth factors or the presence of bFGF inhibit terminal differentiation and stimulate myoblast proliferation (Clegg et al., 1987). Conversely, the presence of insulin appears to promote differentiation (Florini and Magri, 1989).

Integrins, which are dual receptors for the ECM and the cytoskeleton, are implicated as key players in many aspects of muscle differentiation and development (McDonald et al., 1995*a*); however, the role of specific integrins is only beginning to be addressed. Menko and Boettiger (1987) demonstrated that antibody ligation of the β1 integrin maintains chick myoblasts in the proliferative state. Thus, β1 integrins participate in the transition from proliferation to differentiation. Several recent studies describe the expression and potential functions of a number of different integrins during myogenesis. The α5β1 integrin localizes in adhesion plaques and displays a dynamic and regulated pattern of expression during terminal differentiation suggesting an important role in the early events of myogenesis (Blaschuk and Holland, 1994; Boettiger et al., 1995; Enomoto et al., 1993; Lakonishok et al., 1992). The α4β1 integrin is proposed to function in fusion events during secondary myogenesis (Rosen et al., 1992). The α7β1 integrin localizes in myotendinous junctions (MTJ) (Bao et al., 1993) and several alternately spliced isoforms have been identified which are expressed in skeletal muscle (Collo et al., 1993; Song et al., 1992, 1993; Ziober et al., 1993). The α3 and αv integrins colocalize with nascent myofibrils and with z-bands, respectively, and are thus likely to take part in myofibril organization (McDonald et al., 1995*b*). The α1β1 (Duband et al., 1992), α6β1 (Bronner-Fraser et al., 1992), and α9β1 (Palmer et al., 1993) integrins are also expressed in skeletal muscle; however their functions have not been identified. Although the precise role of these α subunits in muscle differentiation remains unclear, the existing data argue for distinct roles for specific integrins during myogenesis.

We addressed the role of integrins in muscle differentiation by ectopic expression of integrin α subunits in primary quail skeletal muscle cultures. Primary quail myoblasts offer many advantages for the ectopic expression of integrins. They provide a relatively continuous source of replicating myoblasts that are amenable to selection and cloning and can be stably transfected with high efficiency (DiMario et al., 1993). Furthermore, unlike many muscle cell lines which often exhibit abnormal or aborted development (Antin and Ordahl, 1991), myogenic differentiation is more faithfully reproduced in primary muscle cultures. In addition, cloned quail myoblasts, introduced into chick embryos, incorporate into developing embryonic muscle and are thus useful for in vivo studies (Stockdale et al., 1990). Two α subunits were chosen for these studies: the α5 subunit and the α6 subunit. Previous studies suggest that FN and LM play opposing roles in muscle differentiation (von der Mark and Ocalan, 1989; Kosher and Rodgers, 1987; Foster et al., 1987). The α5β1 and α6β1 integrins are specific receptors for FN and LM, respectively, making them attractive candidates to mediate these different signals. Furthermore, the α5 integrin is implicated in transmembrane signaling and gene regulation (Damsky and Werb, 1992; Juliano and Haskill, 1993) and displays a dynamic pattern of expression during muscle differentiation (Blaschuk and Holland, 1994; Enomoto et al., 1993; Lakonishok et al., 1992). The α6 integrin is also implicated in transmembrane signaling (Jewell et al., 1995; Shaw et al., 1993, 1995), and its adhesive function often depends on cell activation by phorbol esters (Delwel et al., 1993; Shaw et al., 1990). In addition, the α6 subunit is expressed throughout myogenesis (Bronner-Fraser et al., 1992; McDonald et al., 1995*a*), suggesting that it plays an important role.

With this system we show that changes in integrin expression have dramatic effects on myoblast proliferation and differentiation. We first demonstrate a specificity for α subunit function during differentiation. Ectopic expression of the α5 subunit inhibits differentiation and maintains myoblasts in the proliferative phase while ectopic α6 expression inhibits proliferation but not differentiation. Antisense suppression of endogenous quail α6 expression also inhibits differentiation resulting in sustained proliferation, suggesting that the ratios of integrin expression are important in the decision to proliferate or differentiate. We also show that the effect of ectopic α5 or α6 expression on proliferation and differentiation is mediated by the α subunit cytoplasmic domain. Finally, we demonstrate that integrin regulation of myoblast proliferation and differentiation depends on the growth factor environment in which the cells reside. The precise phenotype depends on the specific integrin expressed and on the particular growth factor present. Taken together, our results point to an integrated, combinatorial model of signaling events that include growth factors and integrins as regulators of differentiation.

Materials and Methods

Primary Cell Culture

Primary myoblasts were isolated from pectoralis muscle of nine day Japanese quail embryos as described (Konigsberg, 1979). Briefly, myoblasts were dissociated from muscle tissue with 0.1% dispase (Boehringer-Mannheim Corp., Indianapolis, IN) in PBS. The cell suspension was filtered through a Sweeney filter; cells were seeded onto gelatin-coated tissue culture plates (0.1% gelatin in PBS). Myoblast cultures were maintained in complete myoblast medium (DMEM [Sigma Chem. Co., St. Louis, MO] containing 15% horse serum, 5% chick embryo extract, 1% pen/strep, and 1.25 μg/ml fungizone (GIBCO BRL, Gaithersburg, MD). Myoblasts were subcultured in trypsin-EDTA (0.06% trypsin, 0.02% EDTA) and used between passages 1 and 10.

Antibodies and Extracellular Matrix Ligands

The muscle α-actinin specific monoclonal antibody (mAb), 9A2B8, was kindly provided by D. Fishman (Cornell University, New York, NY) as a hybridoma supernatant. The mAb, VIF4, which recognizes the human α5 integrin extracellular domain was a gift of R. Isberg (Tufts University, Boston, MA). The chicken α6 specific polyclonal antibody, α6ex (de Cur-

tis et al., 1991), was provided by L. Reichardt (University of California, San Francisco, CA). The mAb against the extracellular domain of the human α6 integrin, 2B7 (Shaw et al., 1993), was a gift of A. Mercurio (Harvard Medical School, Boston, MA). The mAb against 5-bromo-2'-deoxyuridine (BrdU) was purchased from Sigma Chem. Co. Fibronectin was purified from human plasma by affinity chromatography as described (Ruoslahti et al., 1982). Laminin was isolated from murine Englebreth-Holm-Swarm sarcoma as described (Kleinman et al., 1982).

Vector Construction and Site-directed Mutagenesis

The human α5 cDNA (Argraves et al., 1987) was kindly provided by Dr. L. Reichardt in the eukaryotic expression plasmid, pβApr-1-neo (Gunning et al., 1987) and subcloned into the eukaryotic expression vector, pRSVneo (Reszka et al., 1992), or into the retroviral plasmid 1654 (a gift of J. Majors, Washington University, St. Louis, MO) (Ghattas et al., 1991). All restriction enzymes were purchased from GIBCO-BRL. A 3' 1.2-kb HindIII–DraI fragment of the cDNA was ligated into pTZ18R (USB, Cleveland, OH) at HindIII and SmaI sites in the polylinker. A 1.2-kb HindIII-SalI fragment was then ligated to a 3.0-kb SalI-HindIII piece corresponding to the 5' portion of the α5 cDNA. The resulting 4.2-kb full-length α5 cDNA fragment with SalI at both ends was subcloned into pTZ18R at the SalI site to amplify α5-SalI. The 4.2-kb α5 cDNA was then subcloned into pRSVneo at SalI in the sense orientation and into 1654 at XbaI in both the sense and antisense orientations. Proper insert orientation was determined by restriction endonuclease digestion. The chicken α6A cDNA (de Curtis et al., 1991) was also provided by Dr. L. Reichardt in two fragments, C1 and C5, in λt10 phage. The C1 and C5 fragments were excised with EcoRI and each cloned into pTZ18R at the EcoRI site in the polylinker. The C1 and C5 fragments were partially digested with EcoRI and completely digested with SalI, ligated to each other at EcoRI to produce a 3.4-kb full-length α6-SalI fragment and cloned into pRSVneo at SalI. The chicken α6 antisense (ASα6) plasmid was constructed by excising the full-length α6 cDNA with KpnI, religating to pRSVneo, and screening for inverted orientation by restriction endonuclease digestion. The human α6A cDNA, in the expression plasmid pRc/CMV (Shaw et al., 1993), was a generous gift of Dr. A. Mercurio. The lacZ cDNA was a gift of Dr. R. Blackman (University of Illinois) and was cloned into pRSVneo at KpnI and SalI.

Truncation mutants of the α5 cytoplasmic domain were generated as described (Hayashi et al., 1990) using the Muta-Gene ™ in vitro mutagenesis kit (BioRad Labs., Hercules, CA). Oligonucleotides were synthesized at the University of Illinois Genetic Engineering Facility (Urbana, IL). For the $\alpha5_{GFFKR}$ deletion mutant, a 1.2-kb HindIII–DraI fragment, corresponding to the α5 cytoplasmic domain, was cloned into M13mp18 using HindIII and SmaI. To make the $\alpha5_{cyto}$ deletion, a 2.5-kb XhoI–DraI fragment was cloned into M13mp18 at SalI and SmaI. Oligonucleotides were designed to create a BclI site, which contains an in-frame stop codon, at nucleotide positions 1023 and 1031 (before and after the conserved GFFKR sequence, respectively). Mutants were confirmed by restriction digestion of M13 clones with BclI and by single-stranded DNA sequencing using the dideoxy-chain termination method according to the Sequenase™ protocol (USB). The mutant α5 cytoplasmic domains were subcloned into pUC19 (USB) at HindIII and KpnI, and then into the full-length α5 cDNA in pβApr-neo using complete HindIII-partial NdeI digests. The integrity of subcloning into the β-actin expression vector was verified by DNA sequencing. Full-length mutant α5 cDNAs were subcloned into pRSVneo using KpnI.

Chimeric α Subunit Construction

The $\alpha5^{ex}/\alpha6_{cyto}$ chimeric α subunit was created by cassette mutagenesis using complementary oligonucleotides encoding the entire human α6A cytoplasmic domain (Tamura et al., 1990) with HindIII sites engineered at both ends. This cassette was cloned into the α5 cDNA (in pβApr-neo) at the unique HindIII site which corresponds to the KL sequence of the α5 cytoplasmic domain immediately upstream of the conserved GFFKR region. Correct insert orientation was confirmed by DNA sequencing. To match the α6A cytoplasmic domain sequence (Tamura et al., 1990), an α5 fragment containing the engineered α6A region was cloned into M13mp18 to mutate the KLGFFKR sequence to KCGFFKR using the Muta-Gene kit. To clone the $\alpha5^{ex}/\alpha6_{cyto}$ hybrid cDNA into pRSVneo, a 1.5-kb XhoI–NdeI fragment was inserted into pTZ18R-α5 at unique XhoI and NdeI sites in the α5 cDNA. The full-length $\alpha5^{ex}/\alpha6_{cyto}$ cDNA was cloned into pRSVneo using KpnI.

The $\alpha6^{ex}/\alpha5_{cyto}$ chimeric α subunit was constructed by fusing the extracellular and transmembrane domains of the chicken α6 cDNA with the cytoplasmic domain of the human α5 cDNA. A DraI site was created as a silent mutation at the sequence -FR- of the GFFKR region in both the α5 (α5-DraI) and α6 (α6-DraI) cytoplasmic domains. A 1.2-kb DraI–SalI fragment of the α5-DraI cytoplasmic domain was ligated with a 600-bp band from a BstXI complete-DraI partial digest of α6-DraI, and this 1.8-kb product was cloned into pRSVneo-α6 partially digested with SalI and completely with BstXI (New England Biolabs, Beverly, MA). To match the α5 cytoplasmic domain sequence exactly to the published sequence (Argraves et al., 1987), the KCGFFKR sequence was changed to KLGFFKR by site-directed mutagenesis as described above. The integrity of the gene fusion and mutagenesis were confirmed by DNA sequencing.

Transfection and Flow Cytometry

Replicating myoblasts (4×10^5–5×10^5 cells; passages 1–3) were plated on 60-mm tissue culture plates coated with 0.1% gelatin in complete myoblast medium for 16–20 h. For both transient and stable transfections, 8 μg of plasmid DNA and 50 μg of Lipofectamine™ (GIBCO-BRL) in 0.3 ml of DMEM (Sigma) were added to myoblast cultures, and cells were incubated for 8–16 h in complete myoblast medium. Transfected myoblasts were either washed with DMEM, refed with myoblast medium, and analyzed for transient expression or were trypsinized and plated into selection medium (myoblast medium containing 0.5 mg/ml G418, GIBCO-BRL) on gelatin-coated tissue culture plates. The chicken α6, human α6, and $\alpha6^{ex}/\alpha5_{cyto}$ transfections were selected and maintained on laminin-coated (20 μg/ml) tissue culture plates. Myoblasts were selected in 0.5 mg/ml G418 medium for 7–12 d and then maintained in myoblast medium containing 0.2 mg/ml G418.

Both transient and stable myoblast transfections were analyzed for surface expression by flow cytometry as described (George-Weinstein et al., 1988). Briefly, cells were washed with PBS and detached from plates with 0.02% EDTA in calcium-magnesium free Hepes-Hanks buffer (CMF-HH). The human α5 and mutant α5 transfected myoblasts were stained with a human α5 specific mAb, VIF4, at a 1:5 dilution of hybridoma supernatant in CMF-HH containing 5% goat serum (blocking buffer) followed by an FITC-labeled sheep anti–mouse IgG (Cappell, Durham, NC). Chicken α6A, $\alpha6^{ex}/\alpha5_{cyto}$, and antisense α6-transfected cells were stained with a chick α6 specific polyclonal antibody, α6ex, at 20 μg/ml in blocking buffer and FITC-labeled goat anti–rabbit IgG. Human α6-transfected myoblasts were analyzed with the human α6 specific mAb, 2B7, at 10 μg/ml in blocking buffer. Flow cytometry was performed on a EPICS™ cell sorter (Coulter Electronics, Inc., Miami Lakes, FL) equipped with Cicero™ software for data analysis. The human α5-transfected myoblasts were sorted to enrich for a population that was >90% positive for human α5 expression. To normalize cell surface expression levels, the α5cyto, α5GFFKR, and $\alpha5^{ex}/6_{cyto}$-transfected cells were sorted for surface expression profiles comparable to that of enriched α5 transfectants. The chicken α6-transfected cells were sorted from transient transfections while the $\alpha6^{ex}/\alpha5_{cyto}$ myoblasts were sorted for similar expression as those enriched for chicken α6 overexpression.

Proliferation, Differentiation, and Cell Survival Measurements

Untransfected or transfected myoblasts were trypsinized, washed twice with Puck's Saline G (GIBCO-BRL), and seeded onto fibronectin-(α5-transfected cells; 20 μg/ml) or laminin-(α6-transfected cells; 40 μg/ml) coated 12-mm glass coverslips at a density of 1.6×10^4 cells/cm^2 in complete myoblast medium or 3.2×10^4 cells/cm^2 in serum-free medium (DMEM + 2% bovine serum albumin) and cultured for indicated times. For some experiments, myoblasts were grown in low serum medium (DMEM + 2% horse serum). Growth factors were added to untransfected and α5-transfected myoblasts in serum-free medium at the time of plating and twice daily at concentrations of insulin (Sigma; 10 μg/ml), bFGF (from S. Hauschka and Sigma; 10 ng/ml), TGF-β (Sigma; 1 ng/ml), TGF-α (Calbiochem; 10 ng/ml), PDGF-BB (Sigma; 20 ng/ml).

Myoblasts were immunostained for a muscle specific marker, muscle α-actinin, to determine the extent of biochemical differentiation and myofibrillar organization in the various transfectants. At the time points indicated, coverslips were washed with PBS and fixed with 3% formaldehyde in PBS for 15 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, washed, and blocked in 5% goat serum-PBS for 30 min. Cells were incubated with primary antibody, 9A2B8, (a gift of D. Fishman) an mAb against muscle specific α-actinin (at a 1:5 dilution of hybridoma supernatant in 5% goat serum) for 30 min, and then

with FITC sheep anti-mouse IgG (Cappel, Malvern, PA), rhodamine phalloidin (Molecular Probes), and DAPI (1:2,000 dilution; Sigma) to stain the total nuclei for an additional 30 min. Coverslips were washed and mounted in medium containing elvanol and *p*-phenylenediamine. Fluorescence was observed on an Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). The degree of morphological differentiation is expressed as the fusion index, the percentage of total nuclei in myotubes, and was scored in three independent experiments for five random fields at the indicated time points.

The fraction of proliferating cells was determined by measuring incorporation of BrdU by myoblasts either in complete myoblast medium or in serum-free medium in the presence or absence of individual growth factors. Cells were seeded as described above in the appropriate medium and grown for 12, 24, 48, or 72 h. BrdU (50 μM in DMEM) was added to cultures for 12 h before fixation at indicated time points. Coverslips were fixed for 10 min in 95% ethanol, washed, denatured in 2N HCl for 30 min, and immunostained with an anti-BrdU mAb (Sigma) at a 1:750 dilution in 5% goat serum followed by FITC-sheep anti-mouse IgG and DAPI. The percentage of proliferating cells, the fraction of total nuclei immunopositive for BrdU, was scored for five random fields in three independent experiments. Cell survival for untransfected and α5-transfected myoblasts in serum-free medium or in serum-free medium including growth factors is expressed as a ratio of the average number of nuclei per field in the presence or absence of growth factor to the average number of nuclei per field in serum-free medium alone after 24 h. The data were calculated for five random fields in three independent experiments.

Results

Ectopic Expression of Integrin α Subunits in Primary Muscle

The role of integrins in myogenesis was addressed by ectopic expression of integrin α subunits in primary quail skeletal muscle cultures. These cells transfect stably with high efficiency (DiMario et al., 1993) and most aspects of muscle differentiation are faithfully reproduced in culture. We chose initially to express the α5 and α6 subunits of integrin because they are implicated in myogenesis (Blaschuk and Holland, 1994; Boettiger et al., 1995; Bronner-Fraser et al., 1992; Enomoto et al., 1993; Lakonishok et al., 1992), signal transduction events (Damsky and Werb, 1992; Jewell et al., 1995; Juliano and Haskill, 1993; Shaw et al., 1995) and the control of cell proliferation (Giancotti and Ruoslahti, 1990; Sager et al., 1993; Varner et al., 1995). Replicating, primary quail myoblasts were transfected with the expression plasmids, 1654 (Ghattas et al., 1991) or pRSVneo (Reszka et al., 1992), containing either the human α5 cDNA, the chicken α6 cDNA, or a lacZ control cDNA as well as a neomycin resistance gene as a selectable marker. The lacZ transfection served as a control for both neomycin selection and for ectopic expression of a foreign gene. Transfected cells were analyzed for surface expression by flow cytometry using α subunit specific antibodies as described in Materials and Methods. The α5-transfected myoblasts were analyzed with a human α5 specific mAb, VIF4, while overexpression of the α6 subunit was detected with a polyclonal chicken α6 specific antibody, α6ex, which recognizes both the endogenous quail α6 subunit and the transfected chicken α6 subunit.

As shown in Fig. 1, both integrin α subunits were efficiently expressed on the cell surface at high levels 48–72 h after transfection. The human α5 (hα5) subunit was expressed in 60–80% of the cells (Fig. 1 *A*). Similar expression levels were obtained with both the 1654-α5 and pRSVneo-α5 plasmids. The chicken α6 subunit was initially expressed at levels 2–4-fold greater than the endogenous

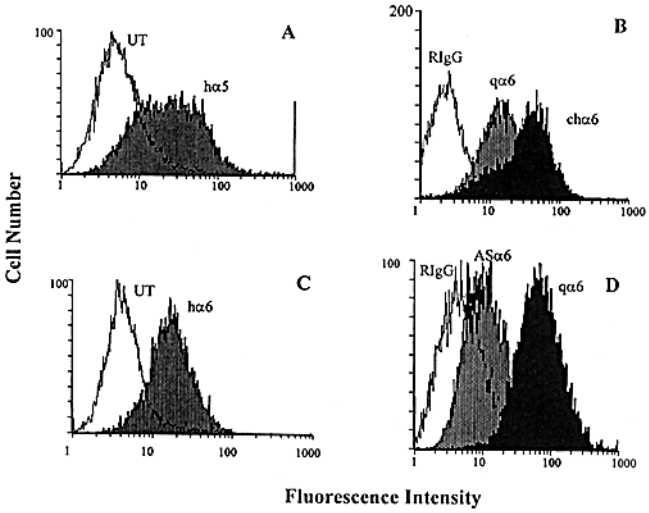

Figure 1. Ectopic expression of integrin α subunits in primary muscle. Primary quail myoblasts were transfected with the human α5 (hα5) subunit (*A*), the chicken α6 (chα6) subunit (*B*), the human α6 (hα6) subunit (*C*), or antisense chicken α6 (ASα6) (*D*) and analyzed for transient cell surface expression by flow cytometry. All subunits are efficiently expressed by 48 h after transfection. Ectopic integrins were detected with α subunit specific antibodies. (*A*) The hα5 specific mAb, VIF4, does not cross react with untransfected myoblasts (UT) and is specific for the transfected subunit. (*B* and *D*) Untransfected, chα6, or ASα6-transfected myoblasts were stained with the chicken α6 specific antibody, α6ex, which recognizes both the endogenous quail α6 (qα6) subunit and the transfected chα6 subunit. (*B*) The shift in the chα6 profile represents the total α6 integrin expressed on the cell surface. (*D*) Expression of the qα6 subunit is efficiently suppressed by ASα6 five days after transfection. A nonspecific rabbit IgG (RIgG) was used as a negative fluorescence control on untransfected myoblasts. (*C*) The 2B7 mAb is specific for the hα6 subunit and does not react with untransfected myoblasts (UT).

quail α6 subunit based on comparison of the mean fluorescence intensities of chicken α6-transfected myoblasts (mean fluorescence intensity = 44) and untransfected controls (mean fluorescence intensity = 15) (Fig. 1 *B*). Since primary quail myoblasts are amenable to selection and cloning, the hα5- and chicken α6-transfected cells were placed in myoblast medium containing G418 to generate stable populations of myoblasts that expressed hα5 or chicken α6. Resistant colonies were pooled and analyzed by flow cytometry for surface expression. The hα5 subunit was stably expressed on the cell surface in 50–80% of neomycin-resistant cells. This level of expression persisted throughout the lifetime of the cells in culture. For the experiments described below, the hα5-transfected cells were enriched by fluorescence-activated cell sorting (FACS) to obtain populations that were 80–90% positive for hα5 expression (See Fig. 6 *A*).

In contrast to the hα5 subunit, we were unable to consistently and reproducibly obtain a myoblast population that stably overexpressed the chicken α6 subunit. FACS analysis showed that chicken α6 expression is lost after 72 h (not shown). This instability of α6 expression is not observed in QT6 fibroblasts or in NIH 3T3 cells where the chicken α6 subunit is expressed after G418 selection and cell sorting

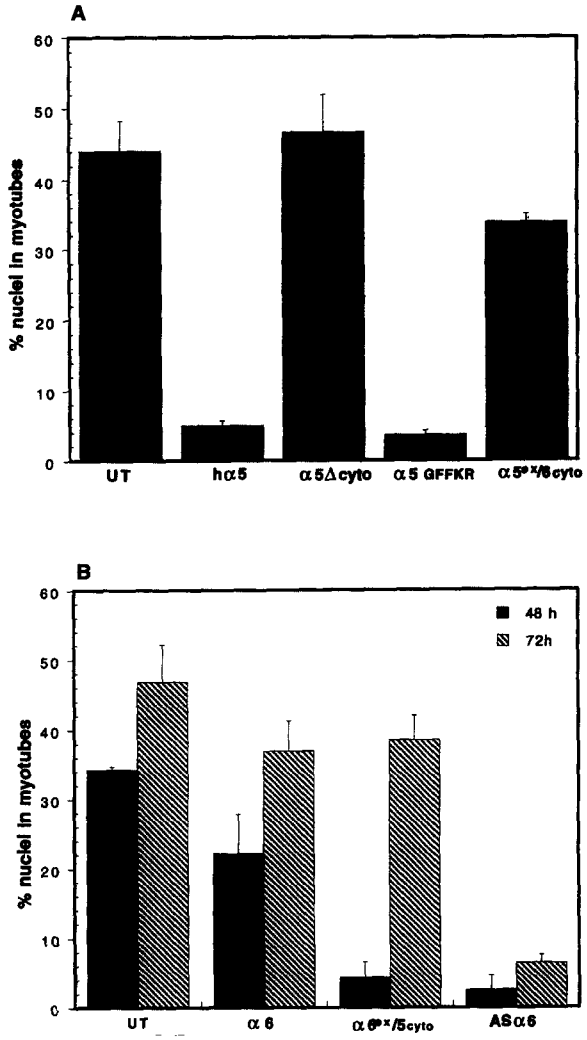

Figure 2. Effect of ectopic α5 (*A*) and α6 expression (*B*), ASα6 suppression (*B*), and cytoplasmic domain alterations (*A* and *B*) on morphological differentiation. Untransfected (UT) and transfected myoblasts were grown in complete myoblast medium for times indicated and immunostained with phalloidin to mark the myotubes and DAPI to visualize the nuclei. The fusion index, the percentage of nuclei residing in multinucleated myotubes, was scored for five random fields and represents an average of three independent experiments. The error bars were calculated as the standard deviations for different fields. (*A*) Untransfected myoblasts (UT) exhibit substantial fusion after 72 h on an FN substrate. Ectopic α5 or α5GFFKR expression inhibits fusion after 72 h whereas myoblasts expressing α5Δcyto or $\alpha5^{ex}/6_{cyto}$ fuse to a similar extent as the untransfected controls. (*B*) On an LM substrate, untransfected cells show significant fusion after 72 h. Cells expressing chicken α6 fuse to a similar extent as the untransfected controls while ASα6 suppression inhibits fusion. The $\alpha6^{ex}/5_{cyto}$ subunit inhibits fusion in 48-h cultures. After 72–96 h, confluent cultures of $\alpha6^{ex}/5_{cyto}$-transfected myoblasts fuse into myotubes.

(not shown). To confirm these results with the chicken α6 subunit, we transfected the human α6 (hα6) cDNA in the expression plasmid, pRc/CMV, which expresses in a number of other cell types (Delwel et al., 1993; Shaw et al., 1993). Expression of the hα6 subunit was also unstable in primary quail myoblasts. The hα6 subunit was initially expressed in 80% of G418-resistant cells (Fig. 1 *C*) at a level comparable to the hα5 subunit. However, this expression was lost after about six to seven days in culture (not shown). Thus, the instability of α6 expression appears to be unique to some cell types or primary cells and does not appear to be a DNA subcloning artifact. Therefore, for most experiments, we relied on cell sorting to enrich transient populations which overexpressed chicken α6 or performed experiments with hα6 expressing myoblasts during a window of 1–5 d after G418 selection when expression was still present on the cell surface.

The α5 and α6 Integrins Act Reciprocally to Regulate Muscle Proliferation and Differentiation

Ectopic expression of the α5 and α6 integrins produced dramatic as well as contrasting effects on myogenesis, the earliest and most striking of which is the effect on the transition between myoblast proliferation and differentiation. In a rich medium containing a high concentration of horse serum and embryo extract, control, untransfected myoblasts (UT) initially replicate, and after 48 or 72 h, a significant fraction of the cells undergo differentiation, as determined by both biochemical and morphological criteria while the remainder of the cells continue to proliferate. Approximately 50% of the total nuclei are found in multinucleated myotubes by 72 h (Fig. 2 *A*). The majority of these cells express muscle specific markers, such as muscle α-actinin, which organize into myofibrils in a striated pattern by 72 or 96 h (Fig. 3 *A*). Myoblasts transfected with a control lacZ plasmid differentiate indistinguishably from UT controls (not shown) demonstrating that expression of a foreign gene per se does not influence proliferation or differentiation.

Ectopic expression of the hα5 integrin inhibits differentiation and maintains myoblasts in the proliferative phase. The hα5-transfected myoblasts proliferate in clusters to form a monolayer with little detectable fusion or biochemical differentiation. The effect of ectopic α5 expression on myoblast proliferation was quantitated by the ability of both untransfected and hα5-transfected myoblasts to incorporate the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU). The percentage of labeled nuclei reflects the fraction of cells that progress through the cell cycle and thus proliferate. As shown in Fig. 4 A, untransfected and hα5-transfected myoblasts initially proliferate to a similar extent after 24 h. Whereas the rate of BrdU incorporation decreases to ~50% in the untransfected controls after 48 h, presumably due to withdrawal from the cell cycle by terminal differentiation, 80–90% of hα5-transfected myoblasts remain in the proliferative phase. After 72 h the percentage of nuclei in hα5-transfected myoblasts that incorporate BrdU decreases, most likely due to density-dependent inhibition of growth. However, as shown in Fig. 2 *A* and Fig. 3 *B*, the hα5-transfected myoblasts do not differentiate. Fewer than 5% of the hα5-transfected myoblasts fuse into myotubes (Fig. 2 *A*) or express muscle α-actinin (Fig. 3 *B*) after 72 h in culture. Although the hα5-transfected myoblasts do not differentiate, they are myogenic since they express the L4 antigen, a cell surface marker for the myogenic lineage (not shown) (George-Weinstein et al.,

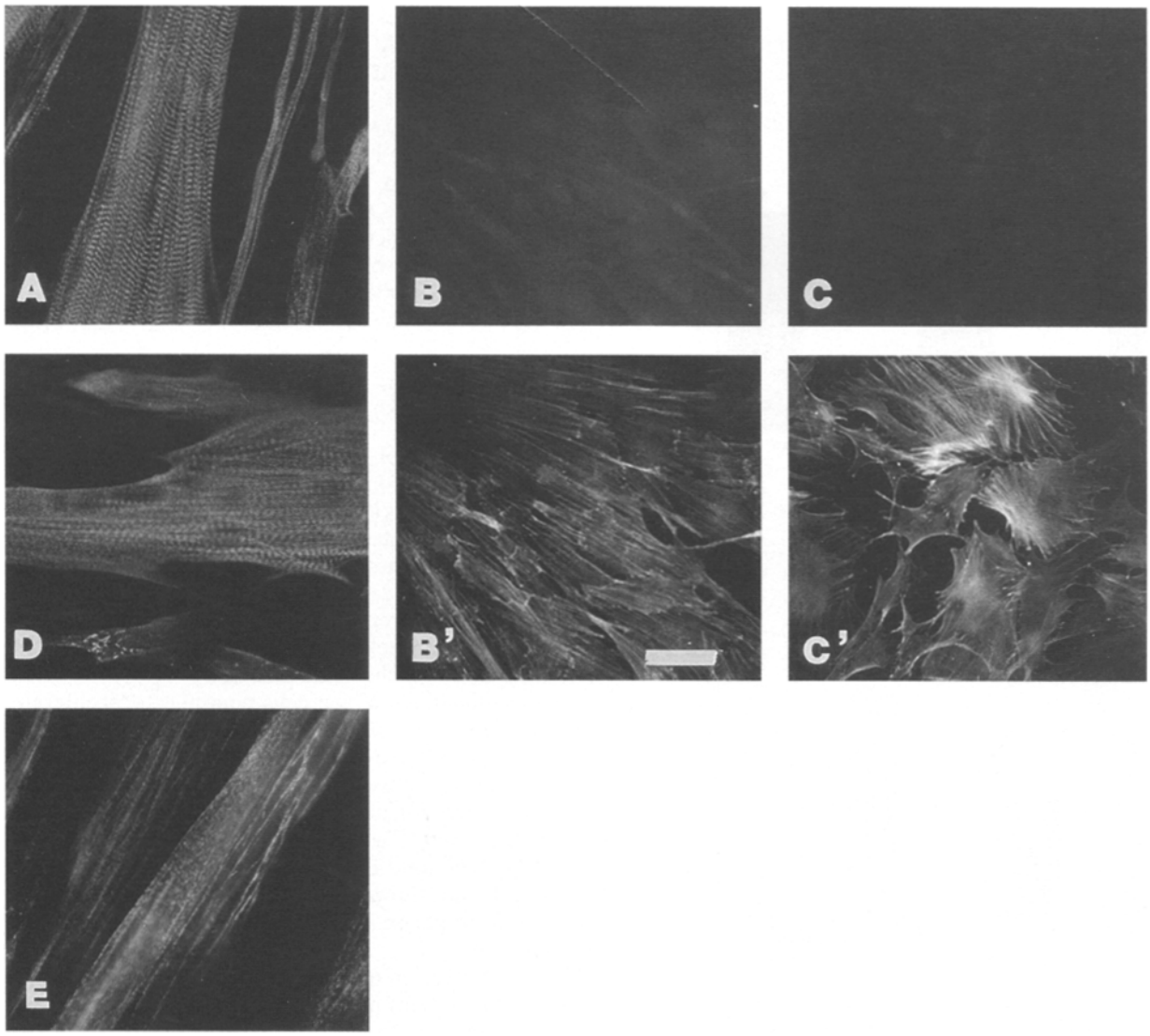

Figure 3. Muscle α-actinin expression and organization in myoblasts transfected with the hα5 subunit or the hα5 subunit with an altered cytoplasmic domain. Untransfected and transfected myoblasts were cultured for 72 h on FN-coated coverslips in complete myoblast medium and immunostained with a muscle α-actinin specific mAb, 9A2B8 (*A–C*, *D*, and *E*). In *B′* and *C′*, cells were costained for actin with rhodamine phalloidin to mark the location of the cells in *B* and *C*. (*A*) Untransfected myoblasts fuse into myotubes, express muscle α-actinin, and organize it into a striated pattern. (*B* and *B′*) Ectopic expression of the hα5 integrin inhibits differentiation. Note that muscle α-actinin is not expressed (*B*) and actin costaining (*B′*) shows that hα5-transfected myoblasts are not fused into myotubes. (*C* and *C′*) Ectopic expression of a truncated hα5 subunit, $\alpha5_{GFFKR}$, which retains the conserved GFFKR region, inhibits differentiation. Muscle α-actinin expression is not detected (*C*) and the cells do not fuse into myotubes (*C′*). (*D*) Myoblasts transfected with the $\alpha5^{ex}/6_{cyto}$ subunit differentiate after 72 h. They fuse into myotubes and express and organize muscle α-actinin in striations. (*E*) Ectopic expression of an hα5 subunit with a deletion of its entire cytoplasmic domain, α5Δcyto, does not inhibit differentiation. Note the presence of myotubes and muscle α-actinin. However, muscle α-actinin is not organized in clear striations. Bars: (*B*, *B′*, *C*, and *C′*) 50 μm; (*A*, *D*, and *E*) 30 μm.

1988). To determine if the effect of ectopic α5 expression on myoblast proliferation and differentiation is specific for the transfected subunit, we expressed a human α5 antisense plasmid containing the full-length human α5 cDNA in an inverted orientation to inhibit the ectopic α5 expression. In FACS enriched cells, human α5 expression was efficiently suppressed (to undetectable levels) by the antisense construct (not shown), and restored a differentiation phenotype to the cells closely resembling that of untransfected myoblasts (not shown).

The inability to obtain a stable population of myoblasts expressing the chicken α6 or human α6 subunits suggested that overexpression of this integrin subunit inhibits cell proliferation. To test this, we determined the ability of myoblasts transiently expressing the hα6 subunit to incorporate BrdU. Under similar growth conditions (density and serum concentration) as the hα5-transfected cells, only 5–10% of myoblasts expressing hα6 incorporate BrdU after 24 h (Fig. 4 *B*). This contrasts both the untransfected controls (Figure 4, *A* and *B*) and the hα5-transfected myoblasts

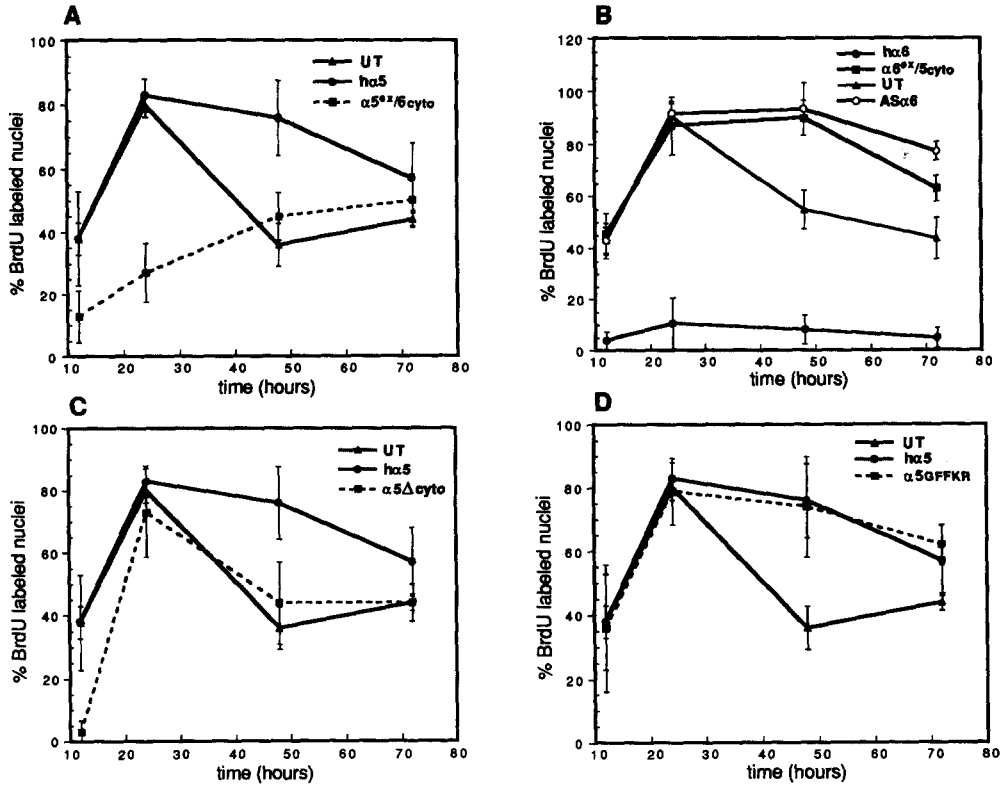

Figure 4. Effect of ectopic α5 (*A*) and α6 expression (*B*), ASα6 suppression (*B*), and cytoplasmic domain alterations (*A*, *C*, and *D*) on myoblast proliferation. Untransfected and transfected myoblasts were grown in complete myoblast medium for 12, 24, 48, and 72 h. Cultures were labeled with 50 μM 5-bromo-2′-deoxyuridine (BrdU) for 12 h before fixation at the indicated time points. The nuclei incorporating BrdU were detected by immunostaining with an anti-BrdU mAb and all nuclei were visualized with DAPI. The percentage of BrdU incorporation was scored from five random fields and represent an average of three independent experiments. The error bars correspond to the standard deviation of percentage of labeled nuclei for different fields. (*A*) Untransfected (UT) and hα5-transfected myoblasts initially proliferate similarly for 24 h. After 48 h, ectopic α5 expression maintains myoblasts in the proliferative phase while proliferation of untransfected myoblasts decreases. Proliferation in myoblasts expressing the $\alpha5^{ex}/6_{cyto}$ subunit is retarded. (*B*) Ectopic hα6 expression inhibits proliferation while ASα6 suppression or expression of the $\alpha6^{ex}/5_{cyto}$ subunit maintain myoblasts in the proliferative phase. (*C*) Myoblasts transfected with the α5Δcyto truncation proliferate much like the untransfected controls. (*D*) Myoblasts expressing α5GFFKR remain in the proliferative phase resembling hα5-transfected myoblasts.

(Fig. 4 *A*) where 80% of the nuclei incorporate BrdU after 24 h. Ectopic α6 expression does not measurably affect the extent of biochemical or morphological differentiation which parallels that observed in untransfected controls cultured on a laminin substrate. At 72 h, 45–50% of untransfected myoblasts fuse into myotubes (Fig. 2 *B*) and muscle α-actinin is expressed and organized in striations (Fig. 5 *A*). Myoblasts that overexpress chicken α6 differentiate within 48–72 h after cell sorting. Nearly 40% of the enriched α6-transfected cells fuse into myotubes after 72 h (Fig. 2 *B*). Furthermore, they express and organize muscle α-actinin into striations (Figure 5 *D*). Thus, in direct contrast to the hα5 subunit, ectopic α6 expression inhibits proliferation but not differentiation.

Since overexpression of the α6 subunit inhibited myoblast proliferation, we next transfected an antisense (AS) chicken α6 cDNA plasmid to inhibit endogenous quail α6 (qα6) expression. The ASα6 construct reduces surface expression of the qα6 subunit to 75% of normal levels after 5 d (Fig. 1 *D*). This reduction of α6 expression results in enhanced proliferation and inhibited differentiation; an effect opposite to that of α6 overexpression. As illustrated in Fig. 4 *B*, 90% of myoblasts with reduced α6 expression incorporate BrdU after 24 h, and this rate of incorporation persists for 48 or 72 h. These cells also exhibit inhibited differentiation. Fewer than 5% of the nuclei reside in myotubes (Fig. 2 *B*) and muscle α-actinin is not expressed in the majority of the cells (Fig. 5 *B*). 8–10 d after transfection, the level of qα6 expression returns to endogenous levels, and the cells differentiate similarly to UT myoblasts (not shown). Therefore, suppression of quail α6 expression, like ectopic expression of the α5 subunit, inhibits myoblast differentiation resulting in sustained proliferation. The overexpression and antisense results together suggest that specific integrins may be critical in the transition between myoblast proliferation and differentiation and that the relative expression levels, i.e., ratios, of different integrins are important in this decision.

The Effects of Ectopic α5 and α6 Expression Are Mediated by the Cytoplasmic Domains

The contrasting effects of ectopic α5 and α6 expression on proliferation and differentiation demonstrate a specificity for integrin α subunits in regulating muscle differentiation. Since integrin α subunits possess distinct cytoplasmic domains (Sastry and Horwitz, 1993), it is likely that the specificity of signals transmitted by different integrin α subunits resides in the cytoplasmic domains. To test this possibility, the cytoplasmic domains of the human α5 subunit and the chicken α6 subunit were exchanged to make chimeric α subunits, $\alpha5^{ex}/6_{cyto}$ or $\alpha6^{ex}/5_{cyto}$. The chimeric α subunits were stably expressed on the cell surface as determined by flow cytometry. The $\alpha5^{ex}/6_{cyto}$ subunit was sorted for comparable surface expression levels as the hα5 subunit (Fig. 6 *D*). The $\alpha6^{ex}/5_{cyto}$ subunit, unlike the chicken

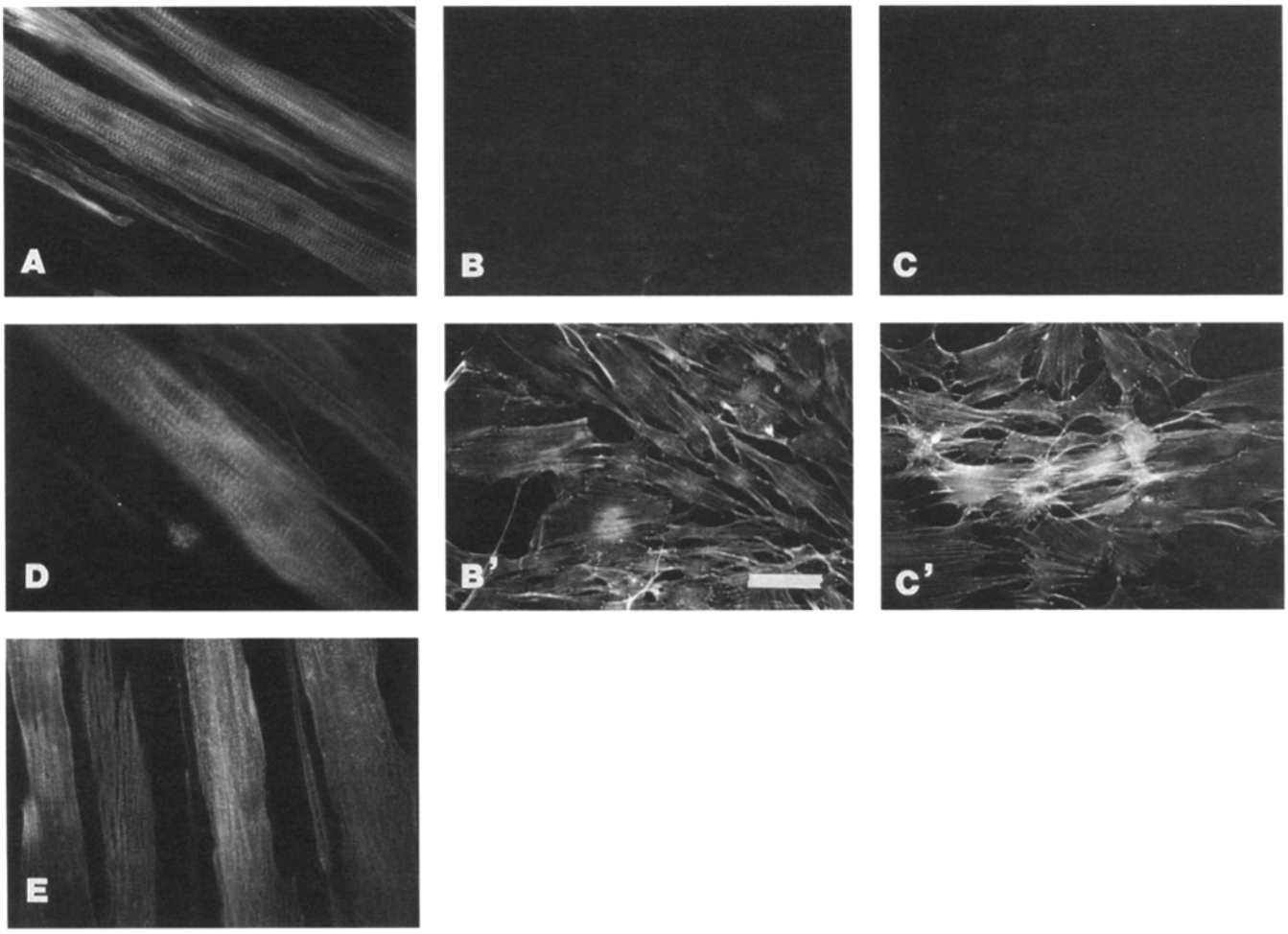

Figure 5. Muscle α-actinin expression and organization in untransfected myoblasts (*A*) or myoblasts transfected with the chicken α6 subunit (*D*), the $\alpha6^{ex}/5_{cyto}$ chimeric subunit (*C* and *E*), or ASα6 (*B*). Untransfected (UT) or transfected myoblasts were cultured on LM-(UT, chicken α6, and $\alpha6^{ex}/5_{cyto}$) or FN (ASα6)-coated coverslips in complete myoblast medium for indicated times and immunostained for muscle α-actinin (*A–C*, *D* and *E*) or phalloidin (*B'* and *C'*). (*A*) Untransfected cells differentiate and fuse into myotubes after 72 h on an LM substrate. Muscle α-actinin is expressed and organized in striations. (*B*) Antisense suppression of endogenous α6 expression inhibits differentiation. Muscle α-actinin expression is not detected in 72-h cultures (*B*) and fusion into myotubes is inhibited (*B'*). (*D*) Ectopic expression of the chicken α6 subunit does not inhibit myotube formation or muscle α-actinin expression and organization in 72-h cultures. (*C*) Expression of a chimeric subunit, $\alpha6^{ex}/5_{cyto}$, inhibits myogenic differentiation in subconfluent, 48-h cultures. Muscle α-actinin expression (*C*) and myotube formation (*C'*) are not observed. (*E*) After 96 h, confluent cultures of α6ex/5cyto-transfected myoblasts differentiate and fuse into myotubes; but muscle α-actinin is not organized in clear striations. Bars: (*B*, *B'*, *C*, and *C'*) 50 μm; (*A*, *D*, and *E*) 30 μm.

α6 subunit, was stably expressed and was sorted for an expression level similar to that in myoblasts transiently overexpressing the chicken α6 subunit (Fig. 6 *E* and *F*).

Ectopic expression of $\alpha5^{ex}/6_{cyto}$ or $\alpha6^{ex}/5_{cyto}$ produces unique effects on myoblast proliferation and differentiation. Under similar growth conditions, the $\alpha5^{ex}/6_{cyto}$-transfected myoblasts exhibit inhibited proliferation when compared to myoblasts transfected with the hα5 subunit. BrdU incorporation shows that cells expressing $\alpha5^{ex}/6_{cyto}$ remain in a prolonged lag phase for 48 h compared to both untransfected and hα5-transfected controls in which 80% of the cells proliferate within 24 h (Fig. 4 *A*). Furthermore, replacement of the α5 cytoplasmic domain with the α6 cytoplasmic domain reverses the inhibition of differentiation observed in hα5-transfected myoblasts. 40% of the $\alpha5^{ex}/6_{cyto}$-transfected cells fuse into multinucleated myotubes after 72 h (Fig. 2 *A*). These cells also express muscle α-actinin which organizes in a striated pattern after 72 h (Fig. 3 *D*). These results demonstrate that the $\alpha5^{ex}/6_{cyto}$ subunit inhibits myoblast proliferation but not differentiation. Thus, the $\alpha5^{ex}/6_{cyto}$-transfected myoblasts tend to behave similarly, but not identically, to myoblasts overexpressing the α6 subunit.

Myoblasts expressing the reverse construct, $\alpha6^{ex}/5_{cyto}$, exhibit density-dependent proliferation and differentiation. As shown in Fig. 4 *B*, 85% of these cells incorporate BrdU after 24 h, and this level is maintained for 48 h. In addition, fewer than 5% of the nuclei reside in myotubes (Fig. 2 *B*) and muscle α-actinin expression is inhibited (Figure 5 *C*). If the $\alpha6^{ex}/5_{cyto}$-transfected myoblasts are

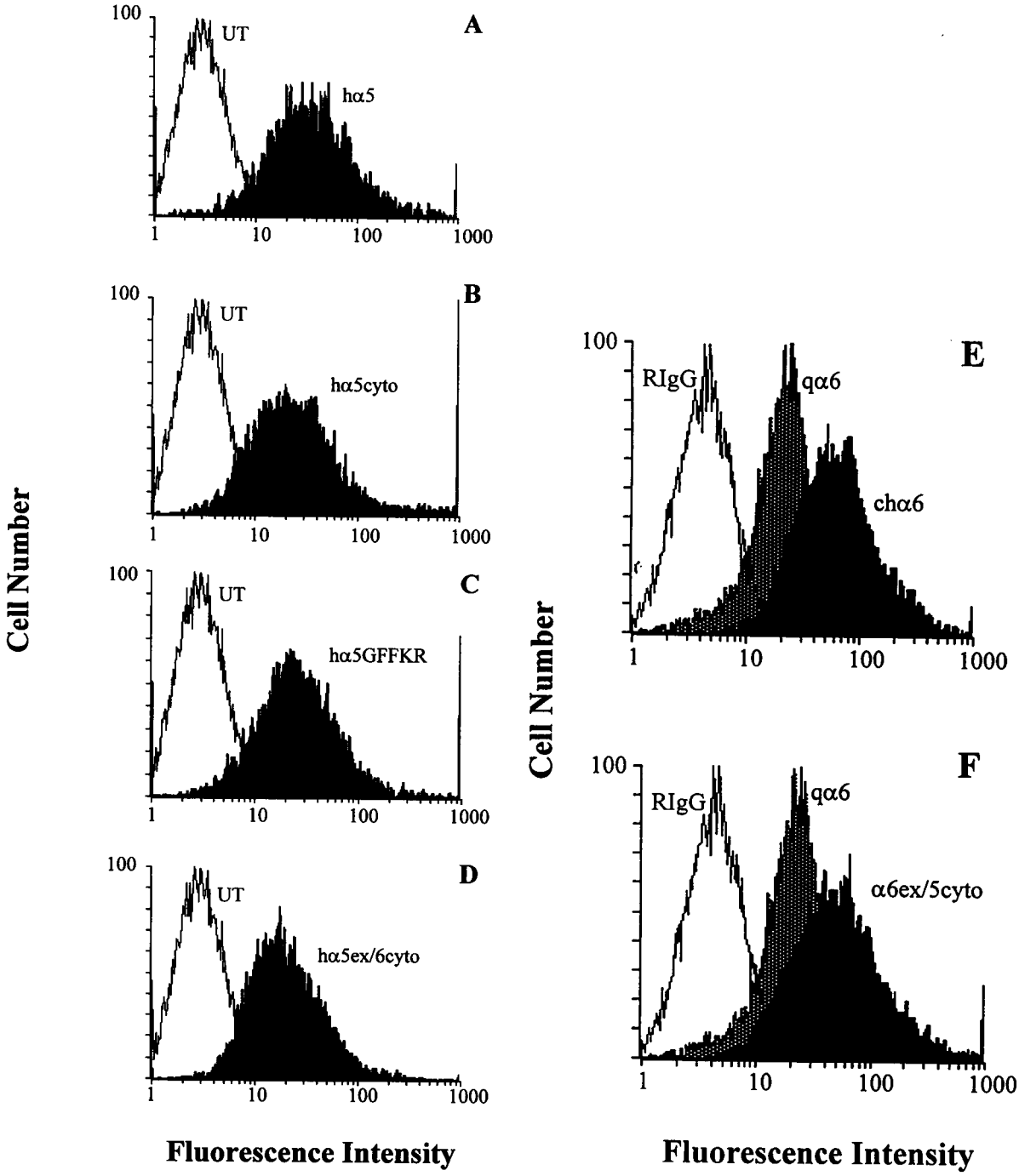

Figure 6. Surface expression levels of the hα5 subunit (*A*), the chicken α6 subunit (*E*), hα5 cytoplasmic domain truncations (*B* and *C*), and chimeric subunits $\alpha5^{ex}/6_{cyto}$ (*D*) and $\alpha6^{ex}/5_{cyto}$ (*F*) in transfected myoblasts used in experiments. Myoblasts were transfected with the pRSVneo plasmid containing the hα5 or chicken α6 integrin cDNAs or cytoplasmic domain mutants and selected for comparable expression levels by fluorescence-activated cell sorting (FACS) to normalize receptor numbers for use in experiments. (*A*) Myoblasts stably expressing the hα5 subunit on the surface were sorted by FACS to enrich for a population that was 80–90% positive for hα5 expression. Myoblasts transfected with the α5Δcyto truncation (*B*), the α5GFFKR truncation (*C*), and the $\alpha5^{ex}/6_{cyto}$ chimeric subunit (*D*) were sorted by FACS for comparable surface expression profiles to that of enriched hα5-transfected myoblasts. (*F*) Myoblasts stably expressing the chimeric subunit, $\alpha6^{ex}/5_{cyto}$, were sorted for similar surface expression as cells transiently overexpressing the chicken α6 subunit (*E*).

subcultured, they remain in the proliferative phase for the life of the cells in culture as discussed above for myoblasts transfected with the hα5 subunit. However, if these cells are allowed to remain confluent for 24–48 h, they undergo synchronous differentiation and 40% of the nuclei reside in myotubes (Fig. 2 *B*). While muscle α-actinin is expressed, it is not organized in striations even after 96 h (Figure 5 *E*). This contrasts the untransfected controls (Fig. 5 *A*) or myoblasts transfected with the chicken α6 subunit (Fig. 5 *D*) in which muscle α-actinin appears stri-

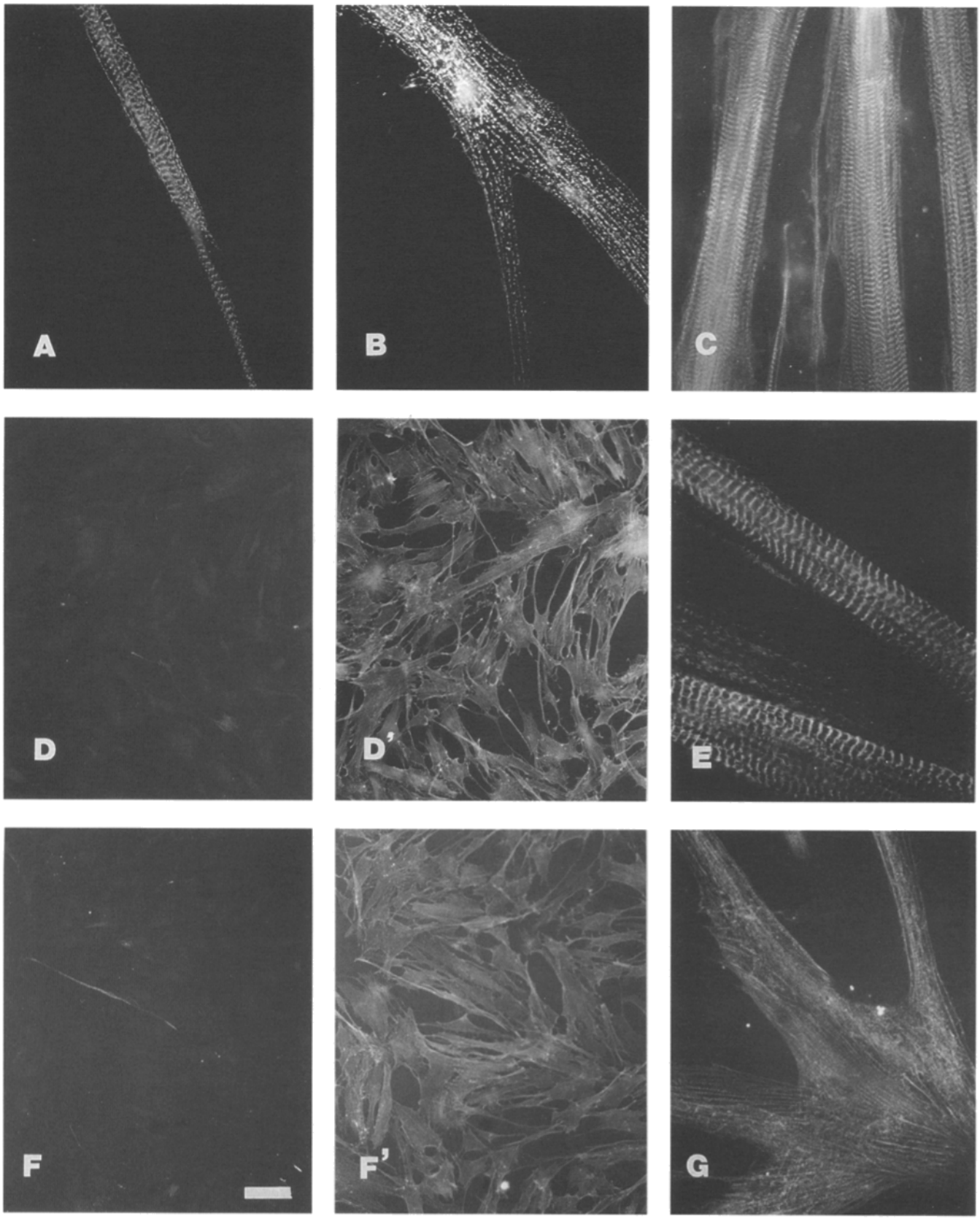

Figure 7. Effect of exogenous growth factors on muscle α-actinin expression and organization in hα5-transfected myoblasts. Untransfected (*A*) or hα5-transfected myoblasts (*B–G*) were cultured on FN-coated coverslips under serum-free conditions in the presence (*C–G*) or absence (*A* and *B*) of individual growth factors. At indicated time points, cells were immunostained with the mAb, 9A2B8, which recognizes muscle α-actinin (*A–C*, *E*, and *G*) or double immunostained for muscle α-actinin (*D* and *F*) and actin (*D'* and *F'*) to mark the location of the cells. (*A*) Untransfected myoblasts fuse into myotubes and express and organize muscle α-actinin into striations after

Table I. Summary of Integrin α Subunit and Cytoplasmic Domain Effects on Muscle Differentiation

Transfected subunit	Proliferation	Differentiation	Myofibril assembly
human α5	+	−	NA
chicken or human α6	−	+	+
AS α6	+	−	NA
α5ex/6cyto	−	+	+
α6ex/5cyto	+	−*	−*
α5Δcyto	+	+	−
$\alpha5_{GFFKR}$	+	−	NA

+, denotes that cells proliferate, differentiate, or have organized myofibrils.
−, denotes that cells exhibit greatly inhibited proliferation, differentiation, or myofibril organization.
NA, not applicable.
*Differentiate if maintained confluent for 24–48 h, however, muscle α-actinin does not striate.

ated within 72 h. Thus, myoblasts expressing the $\alpha6^{ex}/5_{cyto}$ subunit exhibit a phenotype which tends to resemble that of the hα5 subunit. These results further show that the cytoplasmic domain of the α5 subunit positively regulates proliferation whereas the α6 cytoplasmic domain negatively regulates it.

The Conserved GFFKR Region Is Important in α5 Regulation of Differentiation

We next localized active regions of the α5 cytoplasmic domain through ectopic expression of cytoplasmic domain truncation mutants. Since integrin cytoplasmic domains share a highly conserved membrane proximal motif, KXGFFKR, that is implicated in the regulation of α subunit function (O'Toole et al., 1991), we expressed truncation mutants that retained only this sequence ($\alpha5_{GFFKR}$) or that deleted the entire cytoplasmic domain (α5Δcyto). Both of these cytoplasmic domain mutants were efficiently and stably expressed on the cell surface as determined by flow cytometry. To normalize surface receptor numbers, myoblasts expressing α5cyto or $\alpha5_{GFFKR}$ were sorted by FACS to obtain populations with surface expression profiles comparable to that of the hα5 subunit (Fig. 6, *B* and *C*). Ectopic expression of these two deletion mutants resulted in contrasting phenotypes. As shown in Fig. 4 *C*, the α5Δcyto-transfected myoblasts incorporate BrdU to a similar extent as untransfected and hα5-transfected controls after 24 h. Whereas 80% of myoblasts expressing the hα5 subunit remain in the proliferative phase after 48 h, BrdU incorporation decreases to 50% in myoblasts expressing the α5cyto truncation much like in the untransfected control. After 72 h, myoblasts expressing α5Δcyto fuse into myotubes to a similar extent as untransfected myoblasts (Figure 2 *A*). They also express muscle α-actinin, however, muscle α-actinin in the resulting myotubes does not organize in striations (Fig. 3 *E*). Thus, ectopic expression of the α5Δcyto deletion restores differentiation but does not significantly affect the rate of proliferation. On the other hand, myoblasts transfected with the $\alpha5_{GFFKR}$ truncation, behave indistinguishably from hα5-transfected myoblasts. As determined by incorporation of BrdU, 80% of myoblasts expressing $\alpha5_{GFFKR}$ remain in the proliferative phase (Fig. 4 *D*). Furthermore, fewer than 5% of the cells fuse into myotubes (Fig. 2 *A*) or express muscle α-actinin (Fig. 3 *C*). These results show that the ectopic α5 subunit inhibits differentiation and promotes proliferation via its cytoplasmic domain and that the membrane proximal five amino acids are critical in this regulation. The effects of ectopic α5 and α6 expression and cytoplasmic domain alterations on myoblast proliferation, differentiation, and myofibril assembly are summarized in Table I.

Integrin Regulation of Muscle Differentiation Is Modulated by Growth Factors

While ectopic expression of the α5 integrin inhibits differentiation, if hα5-transfected myoblasts are allowed to remain as a confluent monolayer for at least one week to ten days, they synchronously fuse into myotubes and express muscle specific proteins (not shown). The spontaneous differentiation of long term, high density cultures of hα5-transfected myoblasts suggests that sustained proliferation and inhibition of differentiation might arise from an altered sensitivity to serum growth factors. Marked differences in serum requirements for the differentiation of untransfected, hα5-transfected, and chicken α6-transfected myoblasts support this hypothesis. The UT and chicken α6-transfected myoblasts exhibit substantial differentiation in a complete medium, containing a high serum concentration and embryo extract (Fig. 2 *A* and *B*), as well as in low serum (not shown) or serum-free conditions (UT only; Figs. 7 *A* and 8 *A*). In contrast, the hα5-transfected myoblasts terminally differentiate only in a medium containing less than ~2% serum (not shown) including serum-free conditions (Figs. 7 *B* and 8 *A*). Thus, a major effect of ectopic α5 expression is an enhanced susceptibility to the proliferation promoting effects of serum growth factors.

We next compared the effects of individual serum growth factors in the decision of the hα5-transfected myoblasts to proliferate or differentiate under serum-free conditions. Addition of different, exogenous growth factors to hα5-transfected myoblasts in serum-free medium resulted in distinct effects on proliferation and differentiation which were not observed in UT controls. These effects are summarized in Table II. In serum-free medium alone, nearly 60% of the hα5-transfected myoblasts fuse into myotubes after 48 h (Fig. 8 *A*) and express muscle α-actinin. However, in contrast to untransfected cultures where muscle α-actinin organizes in striations (Fig. 7 *A*), muscle α-actinin in differentiated hα5 cultures is not organized in a stri-

48 h in serum-free culture. (*B*) The hα5-transfected myoblasts fuse into myotubes and express muscle α-actinin, however, unlike the untransfected control, muscle α-actinin is not striated in these cultures. bFGF (*D* and *D'*) and TGF-β (*F* and *F'*) inhibit muscle α-actinin expression and fusion into myotubes in hα5-transfected myoblasts. TGF-α (*C*) and PDGF-BB (*E*) enhance myofibril assembly in differentiated hα5 cultures. After 72 h, muscle α-actinin is organized in striations in contrast to serum-free medium alone (*B*). (*G*) Insulin promotes fusion and muscle α-actinin expression in hα5-transfected myoblasts but alters myotube morphology. The myotubes are fat, highly branched and muscle α-actinin organization is impaired. Bars: (*D*, *D'*, *F*, and *F'*) 70 μm; (*A–C*, *E* and *G*) 20 μm.

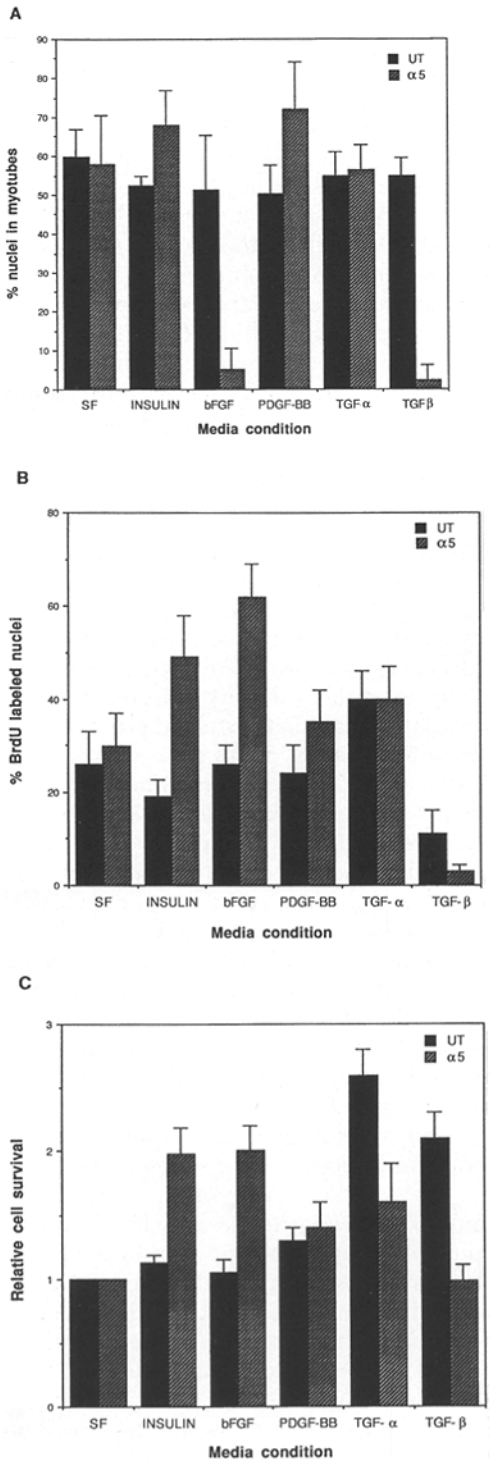

Figure 8. Effect of exogenous growth factors on proliferation, differentiation, and survival of untransfected and hα5-transfected myoblasts. Untransfected and hα5-transfected myoblasts were cultured on FN-coated coverslips under serum-free conditions in the presence or absence of individual growth factors and scored for (*A*) fusion after 48 h, (*B*) BrdU incorporation at 24 h, or (*C*) cell survival at 24 h. Cell survival is expressed as the average number of nuclei per field with or without growth factor relative to serum-free conditions alone. The data were scored from five random fields and are representative of three separate experiments. The error bars correspond to the standard deviations for different fields. (*A*) The fusion index, the percentage of nuclei residing in myotubes, shows that the hα5-transfected myoblasts differentiate substantially after 48 h in the absence of serum. The addition of growth factors to the untransfected myoblasts (UT) does not affect their serum-free differentiation. bFGF and TGF-β inhibit fusion of the hα5-transfected myoblasts while other growth factors have no effect. (*B*) BrdU incorporation in untransfected (UT) and hα5-transfected myoblasts after 24 h shows that bFGF and insulin stimulate proliferation of the hα5-transfected myoblasts but not of untransfected myoblasts. TGF-β inhibits proliferation of both untransfected and hα5-transfected myoblasts while TGF-α promotes proliferation of the untransfected and hα5-transfected myoblasts to the same extent. PDGF-BB has no effect on proliferation. (*C*) After 24 h in serum-free culture, insulin and bFGF promote survival of the hα5-transfected myoblasts while TGF-α and TGF-β promote survival of the untransfected myoblasts.

ated pattern (Fig. 7 *B*). In the presence of bFGF or TGF-β, fewer than 5% of hα5-transfected myoblasts fuse into myotubes (Fig. 8 *A*) or express muscle α-actinin (Fig. 7 *D*, *D'*, *F*, *F'*). Thus, bFGF and TGF-β inhibit the serum-free differentiation of hα5-transfected myoblasts. Furthermore, bFGF and TGF-β produce contrasting effects on proliferation of the hα5-transfected myoblasts. 25–30% of both untransfected and hα5-transfected cells incorporate BrdU in serum-free medium alone (which likely reflects residual growth factor activity) (Fig. 8 *B*). In contrast, 65% of hα5-transfected myoblasts treated with bFGF incorporate BrdU. Hence, the presence of bFGF mimics the mitogenic effects of a rich medium and stimulates proliferation of the hα5-transfected myoblasts. Addition of TGF-β, on the other hand, inhibits proliferation of the hα5-transfected myoblasts (Fig. 8 *B*).

Addition of either insulin, TGF-α, or PDGF-BB produces phenotypes distinct from those just described for bFGF or TGF-β. Insulin promotes both proliferation and differentiation of the hα5-transfected myoblasts, but has no measurable effect on untransfected cells. As shown in Fig. 8 *B*, insulin initially stimulates proliferation in 50–60% of hα5-transfected myoblasts. After 48 h, the hα5-transfected myoblasts differentiate and 70% of the nuclei reside in myotubes (Fig. 8 *A*). The resulting myotubes however are abnormal; they are short, highly branched and muscle α-actinin is not organized in striations (Fig. 7 *G*). TGF-α, like insulin, promotes proliferation, although to a lesser extent (Fig. 8 *B*), as well as differentiation of hα5-transfected myoblasts (Fig. 8 *A*). However, in contrast to insulin or serum-free conditions, TGF-α promotes the organization of muscle α-actinin in striations in the majority of hα5-transfected myotubes (Fig. 7 *C*). Interestingly, among all growth factors tested, only TGF-α stimulates proliferation of the untransfected myoblasts growing under serum-free conditions (Fig. 8 *B*). PDGF-BB has no effect on proliferation or on the serum-free differentiation of hα5-transfected myoblasts (Fig. 8 *A* and *B*); however, like TGF-α, it promotes myofibril organization (Fig. 7 *E*).

While a significant fraction of untransfected and hα5-transfected myoblasts differentiated under serum-free conditions, those cells that did not differentiate survived poorly in the absence of serum or growth factors. After 24 h in serum-free culture, ~35–40% of both the untransfected and hα5-transfected myoblasts detached from the substrate

Table II. Summary of Integrin-Growth Factor Interactions during Muscle Differentiation

	Untransfected myoblasts				hα5-transfected myoblasts			
Media condition	Proliferation	Differentiation	Myofibril assembly	Myoblast survival	Proliferation	Differentiation	Myofibril assembly	Myoblast survival
Complete medium	+	+	+	+	+	−	NA	+
Serum free	−	+	+	−	−	+	−	−
Insulin	−	+	+	−	+	+	−	+
bFGF	−	+	+	−	+	−	NA	+
TGF-β	−	+	+	+	−	−	NA	−
TGF-α	+	+	+	+	+	+	+	−
PDGF-BB	−	+	+	−	−	+	+	−

+, denotes that cells proliferate, differentiate, assemble myofibrils, or survive.
−, denotes that cells display inhibited proliferation, differentiation, myofibril assembly, or survival.
NA, not applicable.

and underwent cell death including nuclear fragmentation and DNA degradation (not shown). The addition of different growth factors to either untransfected myoblasts or hα5-transfected myoblasts had differential effects on myoblast survival. Insulin and bFGF promoted survival of the hα5-transfected myoblasts but not the untransfected myoblasts (Fig. 8 *C*). TGF-α and TGF-β enhanced the survival of the untransfected myoblasts but not the hα5-transfected myoblasts. PDGF-BB protected neither the untransfected nor the hα5-transfected myoblasts. Taken together, these results demonstrate that growth factors elicit different phenotypes for cell differentiation, proliferation, myofibril organization, and cell survival depending on the integrin background of the cells to which they are added.

Discussion

In this study, we addressed the role of integrins in muscle differentiation through ectopic expression of integrin α subunits in primary skeletal muscle. Primary quail muscle is an attractive in vitro system both for the study of differentiation and for the ectopic expression of foreign genes. Since many aspects of muscle differentiation and development are rapidly and faithfully reproduced in these primary cultures, this system enables us to determine the effect of changes in integrin expression at multiple stages of myogenesis. Our results demonstrate the efficient, ectopic expression of integrin α subunits in primary quail myoblasts. The phenotypes we observe support the previous studies of Menko and Boettiger (1987) which suggest that β1 integrins can regulate terminal muscle differentiation. We extend their studies by showing that different integrin α subunits play distinct roles in regulating the transition from proliferation to terminal differentiation and in determining aspects of the differentiated phenotype. These effects appear to result from signaling events mediated by the integrin α subunit cytoplasmic domains. Our data further suggest that the differentiative phenotype is an integrated response to the signals produced by the different integrins expressed on the cell surface and the signals arising from the growth factor environment in which the cells reside. In this context, cell lines, which possess activated signaling pathways, may show responses to different integrins, matrix composition, and growth factors that differ from those in primary cells.

Several previous studies implicate the α5β1 integrin in signaling events that regulate cell proliferation (Giancotti and Ruoslahti, 1990; Varner et al., 1995), differentiation (Adams and Watt, 1990; Boettiger et al., 1995; Hotchin et al., 1993), and apoptosis (Zhang et al., 1995). The α5β1 integrin participates in signal transduction events leading to induction of metalloproteinase expression in rabbit synovial fibroblasts (Werb et al., 1989) and to changes in immediate early gene expression that correlate with proliferation (Varner et al., 1995). Overexpression of the α5 integrin in transformed cell lines suppresses tumorigenicity and anchorage-independent proliferation but promotes proliferation upon substrate attachment (Giancotti and Ruoslahti, 1990; Varner et al., 1995). These findings suggest that the signals mediated by the α5 subunit, which positively signal proliferation in cells adherent to FN and negatively regulate growth in suspension, depend on the state of ligation, i.e., conformation or activation state, of the α5β1 heterodimer (Varner et al., 1995). Our findings support a role for the α5 integrin in promoting anchorage-dependent proliferation. Cell attachment to FN via the α5 integrin also promotes cell survival (Zhang et al., 1995). CHO cells that do not express the α5 subunit undergo apoptosis in the absence of serum. Surprisingly, ectopic expression of the α5 subunit in our system does not spare myoblasts from cell death in serum-free conditions despite the presence of a FN substrate. Thus, cell attachment is not sufficient to protect myoblasts from cell death. Presumably α5 regulation of cell survival is cell-type dependent. However, as discussed below, ectopic α5 expression does affect both myoblast proliferation and survival when myoblasts are grown in the presence of particular growth factors.

The α5β1 integrin also plays an important regulatory role during differentiation. A functional inactivation, resulting in a decreased adhesion to FN and downregulation, of the α5β1 integrin coincides with keratinocyte differentiation (Adams and Watt, 1990; Hotchin, et al., 1993). In muscle, α5β1 is subject to regulation early in myogenesis suggesting an involvement in its terminal differentiation (Enomoto et al., 1993; Lakonishok et al., 1992; Blaschuk and Holland, 1994). The α5β1 integrin is expressed in proliferating myoblasts but is downregulated upon induction of terminal differentiation. This observation suggests that continuous, high-level expression of the α5 integrin coin-

cides with proliferation which is consistent with our observations. There is also evidence that changes in the activation state of the α5 integrin regulate muscle differentiation (Boettiger et al., 1995). Our experiments confirm and extend these previous studies implicating the α5 integrin in myogenesis by demonstrating that the expression level of the α5 subunit can affect the proliferative to differentiative transition and organization of myofibrils.

In contrast to the α5 subunit, considerably less is known regarding the biological function of the α6 integrin. In many systems, the α6 integrin is functionally inactive as a laminin receptor. For example, in macrophages (Shaw et al., 1990) and K562 erythroleukemic cells (Delwel et al., 1993), the adhesive function of α6 requires activation by phorbol esters. In addition, upon attachment of activated macrophages to LM, the α6 integrin participates in signal transduction leading to differential tyrosine phosphorylation of several proteins, which depends on the particular cytoplasmic domain isoform expressed (Shaw et al., 1995). Thus, the α6 integrin is subject to cellular regulation and also acts as a signaling receptor. Our data not only point to a potential role for the α5 integrin in differentiation, they also demonstrate a potential role for the α6 integrin in myogenesis. While ectopic expression of the α5 subunit inhibits differentiation and maintains myoblasts in the proliferative phase, ectopic α6 expression inhibits proliferation but not differentiation. Antisense suppression of endogenous α6 expression inhibits differentiation and promotes proliferation.

Taken together, the effects of ectopic α5 and α6 expression and antisense inhibition of α6 expression on myoblast proliferation and differentiation show that perturbing the existing ratio of α5- and α6-expression affects the decision of myoblasts to proliferate or differentiate. The ratio also appears to contribute to myofibrillar organization since the degree of myofibrillar striation differs in α5- and α6-transfected cells. The opposing effects of α5 and α6 expression on myoblast proliferation and differentiation demonstrate a reciprocity between these two integrin subunits and is consistent with previous studies which show an inverse relationship between α5 and α6 expression during chick embryo development (Bronner-Fraser et al., 1992; Muschler and Horwitz, 1991). However, it is premature to conclude that the ratio of α5/α6 expression, per se, is a physiological regulator of muscle differentiation. We conclude only that changes in integrin ratios can regulate the differentiative state. The relative expression of other integrins also contributes to the proliferative to differentiative transition. Thus, integrin regulation of differentiation is best viewed as an integrated response to the relative expression levels of multiple integrins. An analogous example of complementary signaling is observed with the α5β1 and α4β1 integrins in regulating expression of metalloproteinases in rabbit synovial fibroblasts (Huhtala et al., 1995).

How do these integrins act to regulate differentiation? Several observations suggest that the effects of ectopic α5 and α6 expression are mediated by the α subunit cytoplasmic domain through signal transduction processes. The inhibitory effect of $\alpha5_{ex}/6^{cyto}$ expression on myoblast proliferation and the maintenance of the proliferative phase by expression of $\alpha6_{ex}/5^{cyto}$ show that the α6 cytoplasmic domain is involved in negative regulation of cell growth whereas the α5 cytoplasmic domain transmits proliferative signals. We also show that the conserved, membrane proximal, GFFKR sequence in the α5 cytoplasmic domain is a critical element in the regulation of differentiation via the α5 subunit. The GFFKR sequence regulates the affinity of the αIIbβ3 integrin for fibrinogen (O'Toole et al., 1991). In our system, the role of receptor activation and ligation in mediating the effects of ectopic α5 or α6 is unclear. Both the α5 and $\alpha5_{GFFKR}$-transfected myoblasts, which do not differentiate, and the α5Δcyto and the $\alpha5_{ex}/6^{cyto}$-transfected myoblasts which do differentiate, adhere similarly to a FN substrate and organize cell surface FN similarly (Sastry, S., and M. Lakonishok, unpublished results). However, since these cells secrete FN, it is difficult to test the effects of a single ligand on differentiation. It is also possible that ectopic α5 expression leads to a change in the expression of other integrin subunits either by replacement of endogenous α subunits by the ectopic α5 subunit or by the downregulation of another α subunit. However, surface immunoprecipitations of β1 integrins from α5-transfected myoblasts do not reveal major changes in the profile of α subunits that associate with the β1 subunit when compared to those present in untransfected myoblasts. Among specific α subunits for which suitable antibody probes are available, the level of endogenous quail α5, α6, and α3 expression do not change significantly as a result of ectopic α5 expression (Lakonishok, M., and S. Sastry, unpublished results). We do, however, observe a twofold increase in the total amount of surface β1 and a four to fivefold increase in the total amount of surface α5 expressed in the hα5-transfected myoblasts.

Thus, the most likely explanation for our results is that the integrins activate signaling pathways which interact with growth factor signaling pathways. It is interesting to note that v-*src*–transformed myoblasts possess a phenotype (Alema and Tato, 1987) similar to that of the hα5-transfected myoblasts in our study. Our preliminary studies show significant tyrosine phosphorylation of several proteins with molecular masses in the ranges of 30–34 kD, 60–70 kD, and 120 kD in α5-transfected myoblasts grown in a rich medium but not in untransfected controls (Lakonishok, M., unpublished results). While FAK stands out as an obvious point of convergence between integrin, src-kinases, and growth factor mediated signal transduction pathways (Schlaepfer et al., 1994; Zachary and Rozengurt, 1992), we have not observed changes in its tyrosine phosphorylation that correlate with differentiation. Interestingly, in serum-free conditions, where α5-transfected myoblasts terminally differentiate, the α5 induced tyrosine phosphorylations are suppressed suggesting that α5-mediated signaling depends on the activation of growth factor signaling pathways.

An extensive literature points to growth factors as critical regulators of myogenesis (Florini and Magri, 1989). Our observations demonstrate that growth factor regulation depends on the particular integrins present. This points to an interaction between integrin and growth factor signaling pathways, the consequence of which is that integrin regulation of differentiation depends on the precise growth factor environment and vice versa. For example, the ectopic expression of different integrins shifts the serum response of myoblasts to favor either the prolifera-

tive or differentiative pathway. Ectopic α5 expression enhances myoblast susceptibility to the mitogenic factors in serum whereas ectopic α6 expression abrogates it. Furthermore, ectopic α5 expression alters the response to individual growth factors uniquely to influence differentiation at multiple stages. Similar treatment of untransfected myoblasts does not measurably alter their serum-free differentiation. bFGF, for example, influences the transition from proliferation to differentiation by stimulating proliferation and inhibiting differentiation in the hα5-transfected myoblasts but not in untransfected cells. Insulin, in contrast, promotes both proliferation and differentiation; but the resulting myotubes display an altered morphology and poorly organized myofibrils. PDGF, while having no detectable effect on the decision of α5-transfected myoblasts to proliferate or differentiate, enhances myofibril assembly. TGF-α is unique among the growth factors tested in that it stimulates proliferation, differentiation, and myofibrillar assembly. It produces a phenotype in the α5-transfected myoblasts that most closely resembles untransfected myoblasts grown in a rich medium. The effects of insulin on myotube morphology and TGF-α and PDGF on myofibrillar assembly also demonstrate that these events are regulated, at least in part, by signaling mechanisms and point to a role for integrin-growth factor coupled signaling in muscle morphogenesis. Although the mechanism by which muscle α-actinin becomes striated in the presence of TGF-α or PDGF is unclear, it should be noted that the organization of other cytoskeletal components like titin and myosin heavy chain are not altered by ectopic α5 expression (Lakonishok, M., unpublished results). Finally, it is interesting that the growth factor effects on hα5-transfected, rather than untransfected cells, most closely resemble those described in the literature for muscle cell lines (Florini and Magri, 1989).

The ectopically expressed α5 subunit and growth factors also interact to affect cell survival, a process that is also the result of a signal transduction pathway (Yuan, 1995; Earnshaw, 1995). Ectopic α5 expression does not spare myoblasts from cell death in the absence of serum despite enhanced attachment of the α5-transfected myoblasts to FN compared to untransfected myoblasts. Thus, cell attachment is not sufficient to promote myoblast survival. However, the addition of insulin or bFGF protects hα5-transfected myoblasts from cell death but does not enhance the survival of untransfected myoblasts. In contrast, TGF-α and TGF-β protect the untransfected myoblasts but not the hα5-transfected myoblasts. Therefore, different growth factors differentially influence cell survival of untransfected and hα5 transfected myoblasts, demonstrating further that ectopic α5 expression changes the growth factor response.

Our biological observations complement recent biochemical evidence that demonstrates a coupling between integrin and growth factor signaling pathways (Yamada and Miyamoto, 1995). Integrin engagement is required for PDGF-induced inositol lipid breakdown in fibroblasts (McNamee et al., 1993) and for the association of a novel tyrosine phosphorylated protein with integrin (Bartfeld et al., 1993). In addition, a physical association between IRS-1, a component of the insulin signaling pathway, and the αvβ3 integrin after ligation of the insulin receptor has been demonstrated (Vuori and Ruoslahti, 1994). Another recent report shows that cell attachment to fibronectin causes focal adhesion kinase to associate with proteins in the growth factor–activated Ras/MAP kinase signaling pathway (Schlaepfer et al., 1994).

In summary, the results presented here provide clear biological evidence that integrins and growth factors act together to regulate muscle differentiation. Our data lead to the following conclusions about the role of integrins and growth factors in regulating muscle differentiation. First, different integrins initiate characteristic signals to differentially affect proliferation and differentiation. The ratio of the different integrins expressed and their ligation state are the key parameters. Second, the growth factor environment in which the cell resides influences the integrin mediated signals to uniquely regulate differentiation, proliferation, cytoskeletal assembly, and cell survival. Third, in the absence of a mitogenic stimulus, i.e., serum-free conditions, myoblasts differentiate suggesting that differentiation is the default pathway. Those myoblasts which are unable to differentiate undergo cell death. Fourth, the precise cellular phenotype is a combinatorial response resulting from an integration of signals emanating from specific integrins and particular growth factors. Thus, integrins appear in many respects as functionally equivalent to growth factor receptors.

The authors thank the following for their generous gifts and helpful discussions: Don Fishman (Cornell University, New York, NY); Ralph Isberg (Tufts University, Boston, MA); Steve Hauschka (University of Washington, Seattle, WA); John Majors (Washington University, St. Louis, MO); Lou Reichardt (University of California, San Francisco, CA); Arthur Mercurio (Harvard Medical School, Boston, MA); and Frank Stockdale for many helpful discussions. We thank Gary Durack and Karen Magin of the Flow Cytometry Facility at the University of Illinois for assistance with cell sorting and Matt Plutz for technical assistance. A.F. Horwitz thanks Steve Hughes for particularly helpful discussions about chick vs quail systems and retroviruses.

This research was supported by National Institutes of Health grant GM 23244 to A.F. Horwitz. S.K. Sastry was a trainee on the Cell and Molecular Biology Training Grant.

Received for publication 4 December 1995 and in revised form 6 February 1996.

References

Adams, J., and F. Watt. 1990. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes α5β1 integrin loss from the cell surface. *Cell.* 63:425–435.

Adams, J., and F. Watt. 1993. Regulation of development and differentiation by the extracellular matrix. *Development.* 117:1183–1198.

Alema, S., and F. Tato. 1987. Interaction of retroviral oncogenes with the differentiation program of myogenic cells. *In* Advances in Cancer Research. Vol. 49. Academic Press, Inc., New York. 1–28.

Antin, P., and C. Ordahl. 1991. Isolation and characterization of an avian myogenic cell line. *Dev. Biol.* 143:111–121.

Argraves, W., S. Suzuki, H. Arai, K. Thompson, M. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.

Bao, Z., M. Lakonishok, S. Kaufman, and A. Horwitz. 1993. α7β1 integrin is a component of the myotendinous junction on skeletal muscle. *J. Cell Sci.* 106: 579–590.

Bartfeld, N., E. Pasquale, J. Geltosky, and L. Languino. 1993. The αvβ3 integrin associates with a 190-kDa protein that is phosphorylated on tyrosine in response to platelet-derived growth factor. *J. Biol. Chem.* 268:17270–17276.

Blaschuk, K., and P. Holland. 1994. The regulation of α5β1 integrin expression in human muscle cells. *Dev. Biol.* 164:475–483.

Boettiger, D., M. Enomoto-Iwamoto, H. Yoon, U. Hofer, A. Menko, and R. Chiquet-Ehrismann. 1995. Regulation of integrin α5β1 affinity during myogenic differentiation. *Dev. Biol.* 169:261–272.

Bronner-Fraser, M., M. Artinger, J. Muschler, and A. Horwitz. 1992. Develop-

mentally regulated expression of the α6 integrin in avian embryos. *Development.* 115:197–211.

Clegg, C., T. Linkhart, B. Olwin, and S. Hauschka. 1987. Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J. Cell Biol.* 105:949–956.

Collo, G., L. Starr, and V. Quaranta. 1993. A new isoform of the laminin receptor integrin α7β1 is developmentally regulated during skeletal myogenesis. *J. Biol. Chem.* 268:19019–19024.

Damsky, C., and Z. Werb. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772–781.

de Curtis, I., V. Quaranta, R. Tamura, and L. Reichardt. 1991. Laminin receptors in the retina: sequence analysis of the chick integrin α6 subunit. *J. Cell Biol.* 113:405–416.

Delwel, G., F. Hogervorst, I. Kuikman, M. Paulsson, R. Timpl, and A. Sonnenberg. 1993. Expression and function of the cytoplasmic variants of the integrin α6 subunit in transfected K562 cells: activation dependent adhesion and interaction with isoforms of laminin. *J. Biol. Chem.* 268:25865–25875.

DiMario, J., S. Fernyak, and F. Stockdale. 1993. Myoblasts transferred to the limbs of embryos are committed to specific fibre fates. *Nature (Lond.).* 362: 165–167.

Duband, J., A. Belkin, J. Syfrig, J. Thiery, and V. Koteliansky. 1992. Expression of α1 integrin, a laminin-collagen receptor, during myogenesis and neurogenesis in the avian embryo. *Development.* 116:585–600.

Earnshaw, W. 1995. Nuclear changes in apoptosis. *Curr. Opin. Cell Biol.* 7:337–343.

Enomoto, M., D. Boettiger, and A. Menko. 1993. Alpha 5 integrin is a critical component of adhesion plaques in myogenesis. *Dev. Biol.* 155:180–197.

Foster, R., J. Thompson, and S. Kaufman. 1987. A laminin substrate promotes myogenesis in rat skeletal muscle cultures: analysis of replication and development using anti-desmin and anti-BrdUrd monoclonal antibodies. *Dev. Biol.* 122:11–20.

Florini, J., and K. Magri. 1989. Effects of growth factors on myogenic differentiation. *Am. J. Physiol.* 256:701–711.

George-Weinstein, M., C. Decker, and A. Horwitz. 1988. Combinations of monoclonal antibodies distinguish mesenchymal, myogenic, and chondrogenic precursors of the developing chick embryo. *Dev. Biol.* 125:34–50.

Ghattas, I., J. Sanes, and J. Majors. 1991. The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol. Cell. Biol.* 11: 5848–5859.

Giancotti, F., and E. Ruoslahti. 1990. Elevated levels of the α5β1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.

Gunning, P., J. Leavitt, G. Muscat, S. Ng, and L. Kedes. 1987. A human β-actin expression vector directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA.* 84:4831–4835.

Hauschka, S., and I. Konigsberg. 1966. The influence of collagen on the development of muscle clones. *Proc. Natl. Acad. Sci. USA.* 55:119–126.

Hayashi, Y., B. Haimovich, A. Reszka, D. Boettiger, and A. Horwitz. 1990. Expression and function of chicken integrin β1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. *J. Cell Biol.* 110:175–184.

Hotchin, N., N. Kovach, and F. Watt. 1993. Functional down-regulation of α5β1 integrin in keratinocytes is reversible but commitment to terminal differentiation is not. *J. Cell Sci.* 106:1131–1138.

Huhtala, P., M. Humphries, J. McCarthy, P. Tremble, Z. Werb, and C. Damsky. 1995. Cooperative signaling by α5β1 and α4β1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J. Cell Biol.* 129:867–879.

Jewell, K., C. Kapron-Bras, P. Jeevaratnam, and S. Dedhar. 1995. Stimulation of tyrosine phosphorylation of distinct proteins in response to antibody-mediated ligation and clustering of α3 and α6 integrins. *J. Cell Sci.* 108:1165–1174.

Juliano, R., and S. Haskill. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577–585.

Kleinman, H., M. McGarvey, L. Liotta, P. Robey, K. Tryggvason, and G. Martin. 1982. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry.* 21: 6188–6193.

Konigsberg, I. 1979. Skeletal myoblasts in culture. *In* Methods in Enzymology. Vol. 58. Academic Press, Inc, NY. 511–527.

Kosher, R., and B. Rodgers. 1987. Separation of the myogenic and chondrogenic progenitor cells of undifferentiated limb mesenchyme. *Dev. Biol.* 121: 376–388.

Lakonishok, M., J. Muschler, and A. Horwitz. 1992. The α5β1 integrin associates with a dystrophin-containing lattice during muscle development. *Dev. Biol.* 152:209–220.

Lin, C., and M. Bissell. 1993. Multi-faceted regulation of cell differentiation by the extracellular matrix. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:737–743.

McDonald, K., A. Horwitz, and K. Knudsen. 1995*a*. Adhesion molecules and skeletal myogenesis. *Semin. Dev. Biol.* 6:105–116.

McDonald, K., M. Lakonishok, and A. Horwitz. 1995*b*. αv and α3 integrin subunits are associated with myofibrils during myofibrillogenesis. *J. Cell Sci.* 108:975–983.

McNamee, H., D. Ingber, and M. Schwartz. 1993. Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. *J. Cell Biol.* 121:673–678.

Menko, A., and D. Boettiger. 1987. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. *Cell.* 51:51–57.

Muschler, J., and A. Horwitz. 1991. Down-regulation of the chicken α5β1 integrin fibronectin receptor during development. *Development.* 113:327–337.

O'Toole, T., D. Mandelman, J. Forsyth, S. Shattil, E. Plow, and M. Ginsberg. 1991. Modulation of the affinity of integrin αIIbβ3 (GPIIb-IIIa) by the cytoplasmic domain of αIIb. *Science (Wash. DC).* 254:845–847.

Palmer, E., C. Ruegg, R. Ferrando, R. Pytela, and D. Sheppard. 1993. Sequence and tissue distribution of the integrin α9 subunit, a novel partner of β1 that is widely distributed in epithelia and muscle. *J. Cell Biol.* 123:1289–1297.

Reszka, A., Y. Hayashi, and A. Horwitz. 1992. Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321–1330.

Rosen, G., J. Sanes, R. Lachance, J. Cunningham, J. Roman, and D. Dean. 1992. Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. *Cell.* 69:1107–1119.

Roskelley, C., A. Srebrow, and M. Bissell. 1995. A hierarchy of ECM-mediated signaling regulates tissue-specific gene expression. *Curr. Opin. Cell Biol.* 7: 736–747.

Ruoslahti, E., E. Hayman, M. Pierschbacher, and E. Engvall. 1982. Fibronectin: purification, immunochemical properties, and biological activities. *In* Methods in Enzymology. Vol. 82. W. Cunningham and O. Frederiksen, editors. Academic Press, San Diego, CA. pp. 803–831.

Sager, R., A. Anisowicz, M. Neveu, F. Liang, and G. Sotiropoulou. 1993. Identification by differential display of alpha 6 integrin as a candidate tumor suppressor gene. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:964–970.

Sastry, S., and A. Horwitz. 1993. Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr. Opin. Cell Biol.* 5:819–831.

Schlaepfer, D., S. Hanks, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature (Lond.).* 372:786–791.

Shaw, L., M. Lotz, and A. Mercurio. 1993. Inside-out integrin signaling in macrophages: analysis of the role of the α6Aβ1 and α6Bβ1 integrin variants in laminin adhesion by cDNA expression in an α6 integrin-deficient macrophage cell line. *J. Biol Chem.* 268:11401–11408.

Shaw, L., J. Messier, and A. Mercurio. 1990. The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the α6β1 integrin. *J. Cell Biol.* 110:2167–2174.

Shaw, L., C. Turner, and A. Mercurio. 1995. The α6Aβ1 and α6Bβ1 integrin variants signal differences in the tyrosine phosphorylation of paxillin and other proteins. *J. Biol. Chem.* 270:23648–23652.

Song, W., W. Wang, R. Foster, D. Bielser, and S. Kaufman. 1992. H36-α7 is a novel integrin chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol.* 117:643–657.

Song, W., W. Wang, H. Sato, D. Bielser, and S. Kaufman. 1993. Expression of α7 integrin cytoplasmic domains during skeletal muscle development—alternate forms, conformational change, and homologies with serine/threonine kinases and phosphatases. *J. Cell Sci.* 106:1139–1152.

Stockdale, F., E. Hager, S. Fernyak, and J. DiMario. 1990. Myoblasts, satellite cells, and myoblast transfer. *In* Myoblast Transfer Therapy. R. Griggs and G. Karpati, editors. Plenum Press, New York. 7–11.

Tamura, R., C. Rozzo, L. Starr, J. Chambers, L. Reichardt, H. Cooper, and V. Quaranta. 1990. Epithelial integrin α6β4: complete primary structure of α6 and variant forms of β4. *J. Cell Biol.* 111:1593–1604.

Varner, J., D. Emerson, and R. Juliano. 1995. Integrin α5β1 expression negatively regulates cell growth: reversal by attachment to fibronectin. *Mol. Biol. Cell.* 6:725–740.

von der Mark, K., and M. Ocalan. 1989. Antagonistic effects of laminin and fibronectin on the expression of the myogenic phenotype. *Differentiation.* 40: 150–157.

Vuori, K., and E. Ruoslahti. 1994. Association of insulin receptor substrate-1 with integrins. *Science (Wash. DC).* 266:1576–1578.

Werb, Z., P. Tremble, O. Behrendtsen, E. Crowley, and C. Damsky. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J. Cell Biol.* 109:877–889.

Yamada, K., and S. Miyamoto. 1995. Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.* 7:681–689.

Yuan, J. 1995. Molecular control of life and death. *Curr. Opin. Cell Biol.* 7:211–214.

Zachary, I., and E. Rozengurt. 1992. Focal adhesion kinase (p125FAK): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell.* 71:891–894.

Zhang, Z., K. Vuori, J. Reed, and E. Ruoslahti. 1995. The α5β1 integrin supports survival of cells on fibronectin and upregulates bcl-2 expression. *Proc. Natl. Acad. Sci. USA.* 92:6161–6165.

Ziober, B., M. Vu, N. Waleh, J. Crawford, C. Lin, and R. Kramer. 1993. Alternative extracellular and cytoplasmic domains of the integrin α7 subunit are differentially expressed during development. *J. Biol. Chem.* 268:26773–26783.