

# Quantitative Changes in Integrin and Focal Adhesion Signaling Regulate Myoblast Cell Cycle Withdrawal

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**Abstract.** We previously demonstrated contrasting roles for integrin  $\alpha$  subunits and their cytoplasmic domains in controlling cell cycle withdrawal and the onset of terminal differentiation (Sastry, S., M. Lakonishok, D. Thomas, J. Muschler, and A.F. Horwitz. 1996. *J. Cell Biol.* 133:169–184). Ectopic expression of the integrin  $\alpha 5$  or  $\alpha 6A$  subunit in primary quail myoblasts either decreases or enhances the probability of cell cycle withdrawal, respectively. In this study, we addressed the mechanisms by which changes in integrin  $\alpha$  subunit ratios regulate this decision. Ectopic expression of truncated  $\alpha 5$  or  $\alpha 6A$  indicate that the  $\alpha 5$  cytoplasmic domain is permissive for the proliferative pathway whereas the COOH-terminal 11 amino acids of  $\alpha 6A$  cytoplasmic domain inhibit proliferation and promote differentiation. The  $\alpha 5$  and  $\alpha 6A$  cytoplasmic domains do not appear to initiate these signals directly, but instead regulate  $\beta 1$  signaling. Ectopically expressed IL2R- $\alpha 5$  or IL2R- $\alpha 6A$  have no detectable effect on the myoblast phenotype. However, ectopic expression of the  $\beta 1A$  integrin subunit or IL2R- $\beta 1A$ , autonomously inhibits differentiation and maintains a proliferative state. Perturbing  $\alpha 5$  or  $\alpha 6A$  ratios also significantly affects

activation of  $\beta 1$  integrin signaling pathways. Ectopic  $\alpha 5$  expression enhances expression and activation of paxillin as well as mitogen-activated protein (MAP) kinase with little effect on focal adhesion kinase (FAK). In contrast, ectopic  $\alpha 6A$  expression suppresses FAK and MAP kinase activation with a lesser effect on paxillin. Ectopic expression of wild-type and mutant forms of FAK, paxillin, and MAP/erk kinase (MEK) confirm these correlations. These data demonstrate that (a) proliferative signaling (i.e., inhibition of cell cycle withdrawal and the onset of terminal differentiation) occurs through the  $\beta 1A$  subunit and is modulated by the  $\alpha$  subunit cytoplasmic domains; (b) perturbing  $\alpha$  subunit ratios alters paxillin expression and phosphorylation and FAK and MAP kinase activation; (c) quantitative changes in the level of adhesive signaling through integrins and focal adhesion components regulate the decision of myoblasts to withdraw from the cell cycle, in part via MAP kinase.

**Key words:** MAP kinase • integrins • proliferation • FAK • paxillin

**C**ELL proliferation and differentiation are governed by multiple stimuli including soluble growth factors, the extracellular matrix (Juliano and Haskill, 1993; Adams and Watt, 1993; Roskelley et al., 1995), and direct cell to cell interactions (Gumbiner, 1996). Whereas each of these signals uniquely regulates mitogenic re-

sponses and gene activity, the decision of a cell to proliferate, differentiate, or undergo apoptosis, for example, is an integrated response to its adhesive and growth factor environment (Schwartz and Ingber, 1994; Sastry and Horwitz, 1996). While the mechanisms by which growth factors produce mitogenic responses and regulate gene expression are becoming clearer, the pathways through which adhesive interactions modulate these responses are only beginning to emerge (reviewed in Howe et al., 1998).

Several studies demonstrate that integrins control proliferation and differentiation in numerous cell types (Varnier et al., 1995; Watt et al., 1993). Clustering of integrins on the cell surface with ligand-coated microbeads induces focal adhesion-like structures that recruit numerous mitogenic signaling proteins to integrin receptors which in-

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clude growth factor receptors (Plopper et al., 1995; Miyamoto et al., 1996), mitogen-activated protein (MAP)<sup>1</sup> kinase, lipid second messengers, protein phosphatases, and small GTP-binding proteins (Miyamoto et al., 1995). Thus integrin-associated focal adhesions serve as signaling centers where adhesive and mitogenic pathways can integrate. Numerous physical interactions between integrins or focal adhesion components and mitogenic signaling proteins have been demonstrated. For example, integrins can interact with growth factor receptors through adaptor proteins like IRS-1 (Vuori and Ruoslahti, 1994) and shc (Mainiero et al., 1995; Wary et al., 1996). Focal adhesion kinase (FAK) can interact with PI 3-kinase (Chen and Guan, 1994) and with GRB2 (Schlaepfer et al., 1994). Through its interaction with GRB2, FAK potentially links integrin signaling to the ras/MAP kinase pathway.

Whereas these studies show a biochemical coupling between integrin and growth factor signaling pathways, the functional significance of these interactions in the context of the regulation of proliferation and differentiation is not well understood. MAP kinase stands out as a key point of convergence between integrin and growth factor pathways (Chen et al., 1994; Zhu and Assoian, 1995; Miyamoto et al., 1996; Renshaw et al., 1997) and is required for proliferation of most cells. However, mitogenic responses can be controlled by pathways that do not use MAP kinase (Olson et al., 1995; Klippel et al., 1998). In addition, MAP kinase can modulate other integrin-dependent cell responses including motility (Klemke et al., 1997) and integrin activation (Hughes et al., 1997) suggesting that its activation produces pleiotropic effects. Further, the focal adhesion proteins FAK and paxillin, which are phosphorylated in response to many soluble mitogenic stimuli (reviewed in Sastry and Horwitz, 1996) as well as in response to integrin engagement are likely to play an important role in integrin-growth factor synergy. Although recent studies indicate FAK plays a role in cell survival (Frisch et al., 1996; Hungerford et al., 1996; Ilic et al., 1998) and motility (Ilic et al., 1995; Cary et al., 1996; Gilmore and Romer, 1996), the role of FAK and paxillin, and variations in the level of their activation, in mitogenic signaling is not well understood.

We previously reported contrasting roles for integrin  $\alpha$  subunits in proliferative signaling using myogenic differentiation as a model system (Sastry et al., 1996). Using ectopic expression of integrins in primary quail myoblasts we provided clear biological evidence that integrin  $\alpha$  subunits uniquely alter the response of myoblasts to growth factors. We attributed this effect in part to perturbation of integrin  $\alpha$  subunit ratios (on the order of a three- to fivefold increase in relative expression) which strikingly shifted the probability that a myoblast would either proliferate or withdraw from the cell cycle and initiate terminal differentiation. Ectopic expression of the  $\alpha 5$  integrin enhanced the mitogenic response to favor a much increased probability of proliferation. In contrast, ectopic expression of the  $\alpha 6A$  integrin decreased the probability of continued prolifera-

tion and promoted differentiation. In addition, we also implicated the  $\alpha$  subunit cytoplasmic domains in controlling proliferative versus differentiative signals through integrins.

In this study, we used ectopic expression of these two  $\alpha$  subunits in primary skeletal muscle myoblasts as a convenient tool to drive either the proliferative or differentiative pathway through integrins. We used this approach to (a) assess the relative contribution of the individual  $\alpha$  subunit cytoplasmic domains and (b) identify intracellular targets of integrins that modulate the probability of a myoblast to proliferate or withdraw from the cell cycle and initiate terminal differentiation. First, we demonstrate that the  $\alpha$  subunit cytoplasmic domains indirectly regulate proliferative versus differentiative signals through the  $\beta 1A$  cytoplasmic domain. The  $\alpha 5$  cytoplasmic domain is permissive for proliferative signaling while a discrete region of the  $\alpha 6A$  cytoplasmic domain promotes cell cycle withdrawal. Furthermore, the  $\beta 1A$  cytoplasmic domain is sufficient to initiate proliferative signals and inhibit differentiation. Second, we show that the ectopic  $\alpha$  subunits differentially alter the expression and/or activation of FAK, paxillin, and MAP kinase. Ectopic expression of paxillin or CD2-FAK and their mutants recapitulate the effects of ectopic integrins on myoblast proliferation and differentiation. The effect of ectopic  $\alpha 5$  or  $\alpha 6A$  on proliferation and differentiation can be reversed by altering the relative activity of MAP/erk kinase (MEK), an upstream activator of MAP kinase. These results suggest a model in which proliferative signaling occurs through the integrin  $\beta 1A$  subunit which is modulated by the  $\alpha$  subunit cytoplasmic domains. The level of signaling emanating from the  $\beta 1A$  subunits controls the level of FAK, paxillin, or MAP kinase activation. Thus, in addition to changes in integrin ratios, quantitative changes in the level of focal adhesion signaling or MAP kinase activation shift the probability that a myoblast will proliferate or differentiate.

## Materials and Methods

### Primary Cell Culture

Primary myoblasts were isolated from pectoralis muscle of nine day Japanese quail embryos as previously described (Konigsberg, 1979). In brief, the breast muscle was dissected from the embryo and myoblasts were dissociated from muscle tissue with 0.1% dispase (Sigma Chemical Co.) 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 Chemical Co.] containing 15% horse serum, 5% chick embryo extract, 1% pen/strep, and 1.25 mg/ml fungizone [GIBCO BRL]). 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  $\alpha$ -actinin-specific mAb, 9A2B8, was kindly provided by D. Fishman (Cornell University, New York, NY) as a hybridoma supernatant. mAb VIF4, which recognizes the human  $\alpha 5$  integrin extracellular domain was a gift of R. Isberg (Tufts University, Boston, MA). The chicken  $\alpha 6$ -specific polyclonal antibody,  $\alpha 6ex$  (de Curtis et al., 1991), was provided by L. Reichardt (University of California, San Francisco, CA). mAb 2B7 directed against the extracellular domain of the human  $\alpha 6$  integrin (Shaw et al., 1993), was a gift of A. Mercurio (Harvard Medical School, Boston, MA). The mAb 165 is directed against paxillin (Turner et al., 1990). mAb 2A7 directed against FAK (Kanner et al., 1990) and the polyclonal Ab

1. *Abbreviations used in this paper:* BCA, bichoninic acid; CA-MEK, constitutively active MEK; FAK, focal adhesion kinase; HA, hemagglutinin; MAP, mitogen-activated protein; MEK, MAP/erk kinase; UT, untransfected.

BC3 directed against FAK (Shaller et al., 1992) were gifts of T. Parsons (University of Virginia, Charlottesville, VA). The anti-FAK polyclonal antibody C-20, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-active MAPK polyclonal antibody, which specifically recognizes dually phosphorylated, activated MAPK, was purchased from Promega. The anti-MAPK mAb that recognizes erk-1, as well as the anti-phosphotyrosine antibodies, RC20H and PY20, were purchased from Transduction Laboratories. The anti-hemagglutinin (HA) mAb, 12CA5, was purchased from Boehringer Mannheim. The anti-human CD2 mAb, TS2/18.1.1 was purchased from the Developmental Studies Hybridoma Bank. TS2/16, a mAb against human  $\beta 1$  integrin (Hemler et al., 1984) was from M. Hemler (Dana-Farber Cancer Institute, Boston, MA). Antibody against the human IL2 receptor was purchased from Boehringer Mannheim. Poly-L-lysine was purchased from Sigma Chemical Co. Fibronectin was purified from human plasma by affinity chromatography as previously described (Ruoslahti et al., 1982). Laminin was isolated from murine Engelbreth-Holm-Swarm sarcoma as previously described (Kleinman et al., 1982).

### Expression Vectors

The human  $\alpha 5$  cDNA in pRSVneo and the chicken  $\alpha 6$  cDNA in pRSVneo were described previously (Sastry et al., 1996). The chicken  $\alpha 61044t$  truncation was constructed by first subcloning a 1.6-kb HindIII-SalI fragment of the chicken  $\alpha 6A$  DNA into M13 and then introducing an in-frame BclI site at amino acid position 1044 (de Curtis et al., 1991). 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 (United States Biochemical Corp.). An 800-bp BstXI-SalI fragment containing the mutation was subcloned into pRSVneo $\alpha 6$  partially digested with SalI and completely with BstXI. The human  $\alpha 6A$  and  $\alpha 6B$  cDNAs, in the expression plasmid pRc/CMV (Shaw et al., 1993), were a generous gift of A. Mercurio (Harvard Medical School, Boston, MA). The pRSVneo-CH8 $\beta 1$  plasmid was constructed by subcloning a 1-kb HindIII fragment, containing the CH8 epitope tag, from the CH8 $\beta 1$  pBJ-1 construct received from Y. Takada (Scripps Research Institute, La Jolla, CA) (Takada and Puzon, 1993) into pRSVneo $\beta 1$  expression vector (Reszka et al., 1992). pRSVIL2R- $\alpha 5$  and pRSVIL2R- $\beta 1A$  were constructed by cloning an NheI-XbaI fragment from pCMVIL2R- $\alpha 5$ cyto or pCMVIL2R- $\beta 1A$  plasmids received from Susan LaFlamme (Albany Medical College, Albany, NY; Tahiliani et al., 1997) into the XbaI site of the pRSVneo vector. Clones were screened for orientation by restriction digests. HA-tagged rat MEK1 and HA-tagged rat constitutively active (CA) MEK S218/220D in pCMVneo vector i.e., sodium-deoxycholate, sodium-pyrophosphate, sodium-orthovanadate (Catling et al., 1995) were received from M. Weber (University of Virginia, Charlottesville, VA). The chicken paxillin cDNA, the Y118F, and the S188/190A mutants in the pcDNA3.0neo vector (Brown et al., 1997) were received from C. Turner. CD2FAK, CD2FAK(Y397F), and CD2FAK(K454R) in CDM8 vector (Chan et al., 1994) were received from A. Aruffo.

### Transfection and Flow Cytometry

Cells were transiently or stably transfected using a liposome-DNA solution as previously described previously (Sastry et al., 1996). In brief, replicating myoblasts (passages 1–3) were plated on 60-mm tissue culture plates coated with 0.1% gelatin in complete myoblast medium for 16–20 h. Cells were incubated for 8–16 h in a solution of 8  $\mu$ g of plasmid DNA and 50  $\mu$ g of Lipofectamine (GIBCO BRL) 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 on gelatin-coated tissue culture plates (myoblast medium containing 0.4 mg/ml G418; GIBCO BRL) for 7–12 d and then into myoblast medium containing 0.2 mg/ml G418 (maintenance medium). For transfections with CD2FAK constructs, in order to generate stable populations, myoblasts were cotransfected with a pRSVneo or a pEGFP-C1neo plasmid (Clontech, Palo Alto, CA) at 1:7 ratio (neo resistance gene:CD2FAKcDNA) and selected in G418 as previously described. The chicken  $\alpha 6$ , human  $\alpha 6A$  or  $\alpha 6B$ , and  $\alpha 61044t$  transfections were selected and maintained on laminin-coated (20  $\mu$ g/ml) tissue culture plates. For coexpression of  $\alpha 6A$  integrin and CD2-FAK cells were cotransfected with pRc/CMV $\alpha 6A$  and CDM8CD2FAK vectors at a ratio of 1:7, respectively. Transiently transfected cells were sorted by flow cytometry (see below) for  $\alpha 6A$  expression and the positive cells were grown in G418 containing medium. CDM8CD2FAK vector does not carry a neo resistance gene, therefore, only cells carrying both neo resistance

( $\alpha 6A$  positive) and able to replicate (CD2FAK positive) will survive. Stable populations were analyzed both for  $\alpha 6$  and CD2 expression as described below.

Both transiently and stably transfected (the  $\alpha 5$  phenotype was seen in transient as well as stable transfectants) myoblasts were analyzed for surface expression by flow cytometry as previously described (Sastry et al., 1996). Chicken  $\alpha 6A$  and  $\alpha 61044t$  transfected cells were stained with a chick  $\alpha 6$ -specific polyclonal antibody,  $\alpha 6ex$ , at 20  $\mu$ g/ml in blocking buffer (Hepes-Hanks PBS-CMF with 2% BSA) and FITC-labeled goat anti-rabbit IgG (Cappel). Human  $\alpha 6A$  or B transfected myoblasts were analyzed with the human  $\alpha 6$ -specific mAb, 2B7, at 10  $\mu$ g/ml in blocking buffer. Human  $\alpha 5$  transfected cells were stained with VIF4 mAb. Cells transfected with CD2FAK or its mutants were stained with anti-CD2 mAb TS2/18.1.1. Chicken  $\beta 1$  transfected cells were stained with TS2/16 mAb against the human  $\beta 1$  epitope. IL2R- $\alpha 5$  or IL2R- $\beta 1A$  transfected cells were analyzed with an anti-IL2R antibody. The FACS profiles of the IL2R- $\alpha 5$  and IL2R-cyto-transfected cells were not stable, and we were unable to obtain populations greater than 40% positive, which were used for analysis. Flow cytometry was performed on a EPICS cell sorter (Coulter Electronics, Inc.) equipped with Cicero software for data analysis. As shown in Fig. 1, expression levels were assayed by fluorescence activated cell sorting. These profiles reflect enriched surface expression levels of ectopic integrin subunits in transfected myoblasts used in experiments.

### Cell Extracts, Western Blotting, and Immunoprecipitation

For Western blotting and immunoprecipitation experiments, untransfected and transfected myoblasts were plated on FN (UT and  $\alpha 6$  transfected cells), on LM (UT and chicken  $\alpha 6$  and chicken  $\alpha 61044t$  or human  $\alpha 6A$  transfected cells) or on gelatin (UT, PAX, and CD2FAK transfected cells) for 24 h in complete myoblast medium. Since myoblasts will differentiate in the absence of serum and also secrete their own matrix, we were unable to test the effects of specific matrix ligands on the myoblast response. Therefore, all assays were conducted in the presence of serum and results are presented for steady state conditions. Cells were washed with ice-cold PBS containing 1 mM Na-orthovanadate and lysed in ice-cold modified RIPA extraction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1.0% Triton X-100, 0.25% sodium-deoxycholate, 2 mM EDTA, and 2 mM EGTA) with protease inhibitors (20 mg/ml leupeptin, 0.7 mg/ml pepstatin, 1 mM phenanthroline, 2 mM phenyl-methylsulfonyl-chloride, and 0.05 units aprotinin) and phosphatase inhibitors (30 mM sodium-pyrophosphate, 40 mM NaF, 1 mM sodium-orthovanadate). Protein content of the clarified lysates was determined using the Pierce bicinchoninic acid (BCA) method with bovine serum albumin as the standard.

For phosphotyrosine Western blots, 10–15  $\mu$ g of lysates were separated on 10% SDS-PAGE gels (Laemmli, 1970) under reducing conditions and transferred to nitrocellulose membranes (Towbin et al., 1979). Membranes were blocked in 1% heat denatured BSA in TST buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) overnight at 4°C. Phosphotyrosine containing proteins were detected by incubating the membranes with the anti-phosphotyrosine mAb, PY20, and a secondary horse radish peroxidase (HRP) conjugated anti-mouse antibody (Jackson ImmunoResearch Labs) or with RC20H, a directly conjugated HRP anti-phosphotyrosine Ab. Blots were visualized by chemiluminescence (Pierce Chemical Co.). Membranes were exposed to X-ray film (Kodak, X-OMAT AR) and developed in an automatic film processor. When indicated, membranes were stripped in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM  $\beta$ -mercaptoethanol) for 30 min at 60°C and reprobed with a different antibody.

For anti-MAPK Western blots, cells were trypsinized, washed once with soybean trypsin inhibitor (0.5 mg/ml), washed twice in Puck's Saline G (GIBCO BRL) and resuspended in serum-free medium containing 2% BSA. Cells were held in suspension for 1 h prior to plating on FN or LM in complete myoblast medium for 24 h. Cell extracts were prepared in RIPA buffer as described. 5  $\mu$ g of cell lysates were separated on 12% SDS-PAGE gels under reducing conditions and the proteins transferred to nitrocellulose membranes. The membranes were blocked in 3% nonfat dry milk in TST overnight at 4°C. Active MAPK was detected by an anti-active MAPK pAb (Promega). Membranes were stripped and reprobed for total MAPK with an anti-erk1 mAb (Transduction Labs) or SC-94 anti-erk1 pAb (Santa Cruz Biotechnologies).

For paxillin, FAK, CD2-FAK, and HA immunoblot analysis, 5–20  $\mu$ g cell lysates were resolved on 7.5% SDS-PAGE gels under reducing condi-

tions and proteins transferred to nitrocellulose membranes. Membranes were blocked in TST buffer containing 3% nonfat milk and the proteins were detected with 165 mAb (anti-paxillin), BC3 pAb (anti-FAK), TS2/18.1.1 mAb (anti-CD2), or 12CA5 mAb (anti-HA).

For FAK immunoprecipitations, 100  $\mu$ g of RIPA lysate was mixed with 1  $\mu$ l of anti-FAK mAb, 2A7, 50  $\mu$ l of packed agarose anti-mouse beads (blocked in 5% BSA; Sigma) in a final volume of 500  $\mu$ l. The bead-antibody complex was incubated at 4°C for 2 h with continuous agitation. For paxillin immunoprecipitations, 100  $\mu$ g of cell lysate and 1  $\mu$ l of anti-paxillin mAb, 165 were incubated at 4°C with continuous agitation for 1 h. In a separate tube, 50  $\mu$ l of packed protein A-agarose beads and 30  $\mu$ g/ml rabbit anti-mouse IgG were incubated in lysis buffer for 1 h. The antigen-antibody mixture was then added to rabbit anti-mouse-protein A beads and incubated at 4°C an additional 2 h. The beads were pelleted gently and washed twice with lysis buffer. Bound protein was released from the beads by boiling in 100  $\mu$ l Laemmli sample buffer containing 5%  $\beta$ -mercaptoethanol for 5 min. Equal aliquots of the precipitated protein for each antibody were loaded onto 7% SDS-PAGE gels. The FAK IP was blotted for FAK with C-20 and for phosphoFAK with RC20H. The paxillin IP was blotted for paxillin with the 165 mAb or for phosphopaxillin with RC20H. All immunoprecipitations and Western blots were detected by chemiluminescence.

### Immunofluorescence Staining

Cells were grown on FN- or LM-coated coverslips. Immunostaining was done at room temperature. Cells were rinsed in PBS and fixed with 3% formaldehyde in PBS for 15 min then permeabilized with 0.4% Triton X-100 in PBS for 10 min, washed and blocked in 5% goat serum in PBS (BB) for 30 min. Cells were incubated with primary Ab in BB for 30 min, washed and incubated with FITC- or rhodamine-conjugated secondary Ab (Cappel) and DAPI (Sigma Chemical Co.) for additional 30 min. Coverslips were washed extensively and mounted in medium containing elvanol and *p*-phenylenediamine. Fluorescence was observed on a Zeiss Axioplan microscope.

### Alteration of MAPK activity and differentiation

MAP kinase activity was manipulated in  $\alpha$ 6A transfected myoblasts by coexpression of constitutively activate (CA) MEK1. Myoblasts were cotransfected with pRc/CMV $\alpha$ 6A and the HA-tagged pCMVneoMEK S218/220D vectors at a ratio of 1:7, respectively. Cells were selected in G418 and stable populations were sorted by flow cytometry for human  $\alpha$ 6A expression as described. Cell lysates were analyzed for HA expression by Western blotting as described. Stably cotransfected cells were plated on LM-coated plates and observed for 96 h.

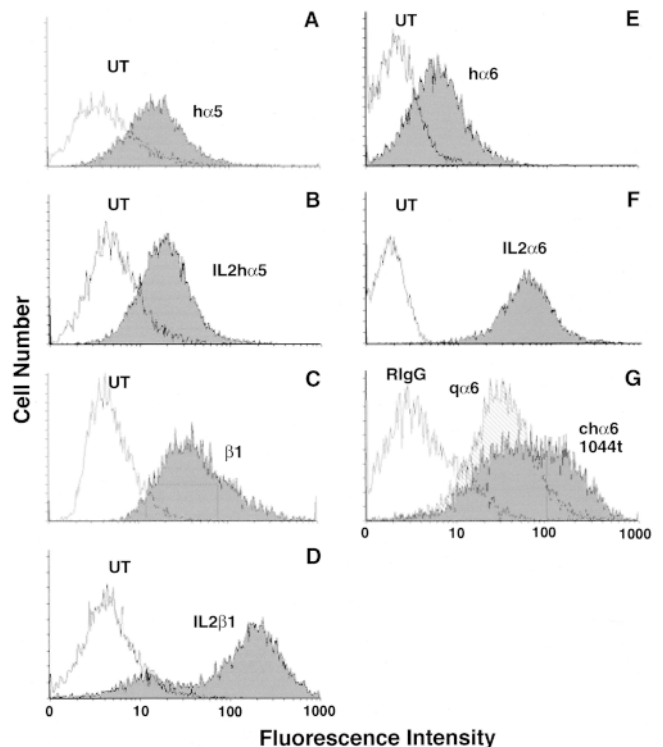
To alter MAP kinase activity in  $\alpha$ 5 transfected myoblasts,  $\alpha$ 5-expressing cells were grown in the presence of the specific MEK inhibitor PD98059 (New England Biolabs) (Alessi et al., 1995). Transfected myoblasts were plated on FN-coated coverslips and on FN-coated TC plates. After 8 h in complete myoblast medium, the first dose of the inhibitor was added to the cells at 1, 10, 25, 50, or 100  $\mu$ M final concentration. Cells were grown for an additional 24 h and a second dose of the inhibitor was added. After 24 and 48 h in presence of the inhibitor, coverslips were fixed and immunostained for DAPI and muscle  $\alpha$ -actinin. At the same time, cells were extracted in RIPA buffer as described and lysates were analyzed by Western blotting for active MAPK and total erk1 expression as described above.

Differentiation was scored using the fusion index, which is the percentage of total nuclei in myotubes as described in Sastry et al. (1996).

## Results

### Integrin $\alpha$ Subunit Cytoplasmic Domains Modulate Proliferative Signals through the $\beta$ 1 Subunit

We recently reported a specificity for integrin  $\alpha$  subunits and their cytoplasmic domains in controlling the proliferative to differentiative transition in primary quail myoblasts (Sastry et al., 1996). Ectopic expression of the human  $\alpha$ 5 integrin subunit ( $\alpha$ 5) enhanced the fraction of myoblasts remaining in the proliferative phase and inhibited the initiation of terminal differentiation. In contrast, ectopic

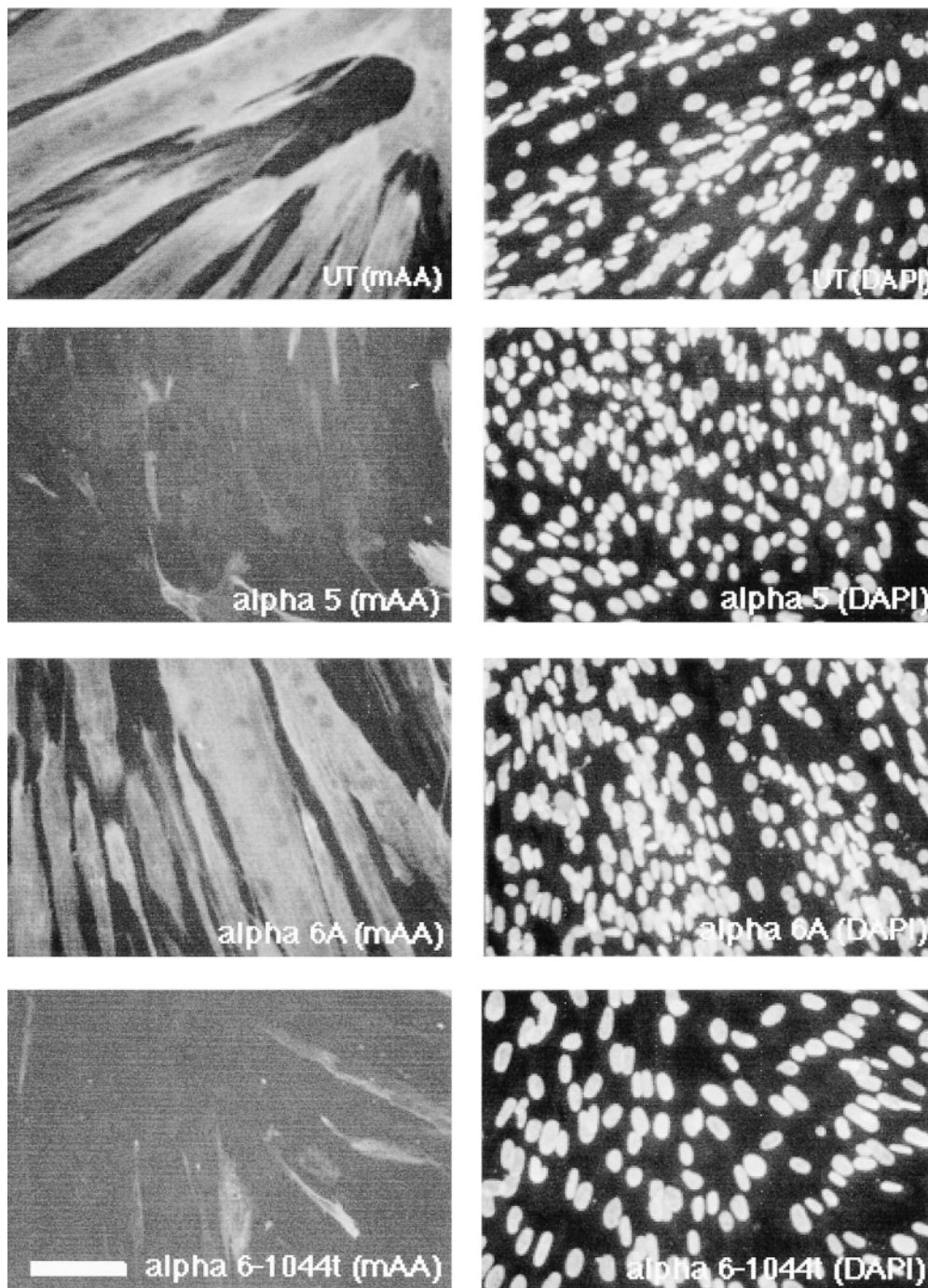


**Figure 1.** Surface expression of ectopic integrins in primary quail myoblasts. Myoblasts were transfected with (A) the human  $\alpha$ 5 ( $\alpha$ 5) subunit, (B) the IL2R $\alpha$ 5 subunit, (C) a chimeric, chicken  $\beta$ 1A subunit, CH8, in which an extracellular region of the chick sequence is replaced by the corresponding human  $\beta$ 1 sequence to create an epitope-tag, (D) the IL2R- $\beta$ 1A subunit, (E) the human  $\alpha$ 6A ( $\alpha$ 6A) subunit, (F) the IL2R- $\alpha$ 6A subunit, or (G) the  $\alpha$ 61044t deletion mutant and analyzed for cell surface expression by flow cytometry. The  $\alpha$ 5 (A) and  $\alpha$ 6A (E) subunits were detected with  $\alpha$ 5 subunit-specific mAb, VIF4, or  $\alpha$ 6A-specific mAb, 2B7, respectively. (C) The CH8- $\beta$ 1A subunit was detected with TS2/16, an anti-human  $\beta$ 1 integrin mAb. An anti-IL2R mAb was used to detect IL2R- $\alpha$ 5 (B), IL2R- $\beta$ 1A (D), and IL2R- $\alpha$ 6A (F). These mAbs do not cross react with UT myoblasts. (G) The  $\alpha$ 61044t deletion was detected with a polyclonal antibody,  $\alpha$ 6ex, which recognizes both the endogenous quail  $\alpha$ 6 subunit as well as the transfected chicken  $\alpha$ 6 subunit. q $\alpha$ 6 is total  $\alpha$ 6 expression in untransfected cells and ch $\alpha$ 6 is total expression in transfected cells. The shift in the expression profile reflects a two- to threefold increase in total  $\alpha$ 6 integrin expression.

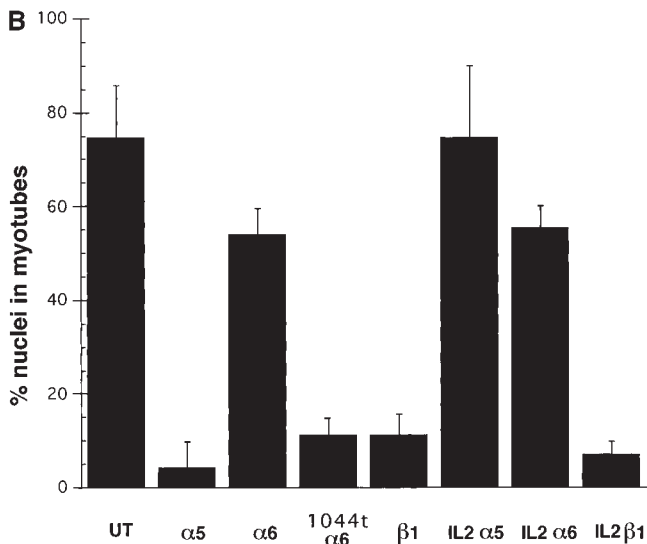
expression of the human  $\alpha$ 6A subunit of integrin ( $\alpha$ 6A) inhibited myoblast proliferation and promoted differentiation. These effects resulted from a three- to fivefold increased surface expression of the  $\alpha$ 5 $\beta$ 1 or the  $\alpha$ 6A $\beta$ 1 integrin (and a two- to threefold increase in total  $\beta$ 1 integrin, see below) with little change in the relative expression of other integrin  $\alpha$  subunits. These findings suggested that the  $\alpha$ 5 cytoplasmic domain promotes proliferative signals whereas the  $\alpha$ 6A cytoplasmic domain inhibits proliferation and enhances the fraction of cells initiating terminal differentiation.

To assess the contribution of these two cytoplasmic domains, we first examined the effect of ectopic  $\alpha$ 5 and  $\alpha$ 6A truncation mutants on myoblast proliferation and differentiation. (Fig. 2 and Table I). As we reported previously,

A



B



**Figure 2.** Effect of ectopic integrin subunits on muscle differentiation. (A) Control untransfected (UT) myoblasts,  $\alpha 5$  transfected myoblasts,  $\alpha 6A$  transfected myoblasts, or  $\alpha 61044t$  transfected myoblasts were plated on coverslips in complete myo medium for 72 h and then double immunostained for a myogenic marker, muscle  $\alpha$ -actinin or DAPI to visualize nuclei. The UT and  $\alpha 6A$  transfected cells show extensive fusion into multinucleated myotubes, alignment of nuclei, and expression of muscle  $\alpha$ -actinin. Myoblasts expressing the  $\alpha 61044t$  subunit do not fuse into myotubes and only a small percentage of cells express muscle  $\alpha$ -actinin. This phenotype resembles that of myoblasts expressing the ectopic  $\alpha 5$  subunit. (B) Fusion index, the percentage of total nuclei in myotubes. Ectopic expression of  $\alpha 61044t$ ,  $\beta 1A$ , or IL2R- $\beta 1A$  inhibit myoblast fusion to a similar extent as the ectopic  $\alpha 5$  subunit after 72 h of culture in serum-containing medium. Fusion of IL2R- $\alpha 5$  or IL2R- $\alpha 6A$  transfected cells resembles that of the UT controls. Bar, 50  $\mu m$ .

ectopic expression of the  $\alpha 5$  truncation,  $\alpha 5$ GFFKR, which retains only the conserved GFFKR sequence, promoted proliferation and inhibited differentiation similar to the wild-type  $\alpha 5$  subunit (Sastry et al., 1996; Table I). These findings suggest that the majority of the  $\alpha 5$  cytoplasmic domain is not required for proliferative signals. On the other hand, ectopic expression of an  $\alpha 6A$  truncation,  $\alpha 61044t$ , which deletes the COOH-terminal 11 amino acid residues, restores proliferative signaling and produces a phenotype similar to that of the ectopic  $\alpha 5$  subunit. Myoblasts expressing  $\alpha 61044t$  remain in the proliferative phase and do not differentiate even in high density cultures (Fig. 2). Like  $\alpha 5$  transfected myoblasts (Fig. 2 A), myoblasts expressing  $\alpha 61044t$  do not express muscle  $\alpha$ -actinin (Fig. 2), a myogenic differentiation marker, and exhibit a fusion index of 5% (Fig. 2 B) after 72 h of culture in a rich medium. This contrasts UT controls and  $\alpha 6A$  transfected cells (Fig. 2) where a significant fraction of cells express muscle  $\alpha$ -actinin and fuse into multinucleated myotubes (Fig. 2 B). Preliminary mapping of the COOH-terminal 11 amino acids points to S1071 (in  $\alpha 6A$ ) as a key residue, since its mutation to alanine produces a phenotype with enhanced proliferation (data not shown). Furthermore, the proliferation inhibiting effect of  $\alpha 6$  integrin is specific for the  $\alpha 6A$  cytoplasmic domain isoform. Ectopic expression of the  $\alpha 6B$  subunit in myoblasts promotes proliferation and inhibits differentiation (data not shown, Table 1). Consistent with these results, the  $\alpha 6A$  isoform is the predominant  $\alpha 6$  integrin expressed in striated muscle (Hogervorst et al., 1993; and our own unpublished observations) and in embryonic cells of determined lineage (Cooper et al., 1991) whereas  $\alpha 6B$  is highly expressed in proliferating, totipotent or undifferentiated ES cells. Taken together, these observations suggest that the  $\alpha 5$  and  $\alpha 6A$  cytoplasmic domains function differently: the  $\alpha 5$  cytoplasmic domains appears permissive whereas a discrete region of the  $\alpha 6A$  cytoplasmic domain is inhibitory with respect to proliferation.

To determine whether the  $\alpha 5$  or  $\alpha 6A$  cytoplasmic domains act directly or indirectly, we assayed the effects of single-subunit cytoplasmic domain chimeras (LaFlamme et al., 1992), IL2R- $\alpha 5$  or IL2R- $\alpha 6A$ , on the ability of myoblasts to proliferate or differentiate. Ectopic expression of either IL2R- $\alpha 5$  or IL2R- $\alpha 6A$  had little detectable effect on myoblast proliferation or differentiation (Fig. 2 B). These cells behaved much like control, untransfected

(UT) myoblasts. Thus the  $\alpha$  subunit cytoplasmic domains do not directly initiate signals for myoblast proliferation or differentiation.

How then do these two integrin  $\alpha$  subunits regulate proliferation and differentiation? Our observation that different integrins, e.g.,  $\alpha 5$ ,  $\alpha 6B$ , and  $\alpha 61044t$ , all produce a similar phenotype suggests an hypothesis in which these  $\alpha$  subunits influence the proliferative signaling through the  $\beta 1$  subunit. In this view the  $\alpha 5$  cytoplasmic domain (as well as that of the  $\alpha 6B$ ,  $\alpha 61044t$ , and perhaps others) would permit signaling through the  $\beta 1$  subunit whereas the  $\alpha 6A$  cytoplasmic domain would inhibit it. Previous studies have shown that the  $\alpha$  subunit cytoplasmic domain can regulate  $\beta 1$  integrin localization to focal adhesions (Briesewitz et al., 1993; Ylanne et al., 1993) and integrin activation (O'Toole et al., 1991); both localization and activation, however, are mediated by the  $\beta$  subunit cytoplasmic domain. Furthermore, the  $\beta 1$  cytoplasmic domain alone, when expressed as a single subunit chimera, IL2R- $\beta 1$ , can activate intracellular signals (Akiyama et al., 1994). To test this hypothesis, we first determined whether overexpression of the chicken  $\beta 1A$  subunit of integrin would increase the fraction of proliferative myoblasts. We chose the  $\beta 1A$  isoform since it is predominant in replicating myoblasts (Belkin et al., 1996). As reported in Sastry et al. (1996) ectopic expression of integrin  $\alpha$  subunits also produces a two- to threefold increase in total  $\beta 1$  expression with little change in relative expression of the other endogenous  $\alpha$  subunit levels. The increase in total  $\beta 1$  expression maintains myoblasts in the proliferative phase and inhibits terminal differentiation. Myoblasts with enhanced  $\beta 1A$  expression grow to confluency but exhibit a fusion index of only  $\sim 10\%$  compared with 60–70% for untransfected cells (Fig. 2 E). Thus, increased  $\beta 1A$  expression produces a phenotype resembling that of increased  $\alpha 5$  subunit expression. This finding agrees with a similar result reported previously for the  $\beta 3$  integrin (Blaschuk et al., 1997).

Next, we addressed whether the  $\beta 1A$  cytoplasmic domain could independently affect myoblast proliferation or differentiation through ectopic expression of the single subunit chimera, IL2R- $\beta 1A$ . Previous studies show that this chimera localizes in focal adhesions (LaFlamme et al., 1992) and mediates enhanced integrin signaling (Akiyama et al., 1994). Myoblasts expressing IL2R- $\beta 1A$  remain replicative and proliferate until confluent with little detectable fusion into myotubes (Fig. 2 B). Like myoblasts expressing ectopic  $\alpha 5$  subunit, they also only exhibit significant cell cycle withdrawal and differentiation if cultured under serum-free conditions (Sastry et al., 1996 and data not shown). These results demonstrate that the  $\beta 1A$  cytoplasmic domain is sufficient to transmit proliferative signals and inhibit differentiation and thus modulate the growth factor response. Further, ectopic expression of IL2R- $\beta 1A$  can rescue the  $\alpha 6A$  phenotype. Myoblasts that coexpress IL2R- $\beta 1A$  and  $\alpha 6A$  integrins proliferate and differentiate like untransfected cells (data not shown; Table I). Taken together, these findings suggest that proliferative signaling through integrins occurs via the  $\beta 1$  subunit and that different  $\alpha$  subunit cytoplasmic domains can modulate these signals. The effects of ectopic integrin subunits on myoblast proliferation and differentiation are summarized in Table I.

Table I. Integrin Subunit Phenotypes

Transfected subunit	Proliferation	Differentiation
Control	+	+
$\alpha 5$	+	–
$\alpha 6A$	–	+
$\alpha 5$ GFFKR	+	–
$\alpha 6$ -1044t	+	–
$\alpha 6B$	+	–
$\beta 1$	+	–
IL2 $\alpha 5$	+	+
IL2 $\beta 1$	+	–
$\alpha 6A$ + IL2 $\beta 1$	+	+

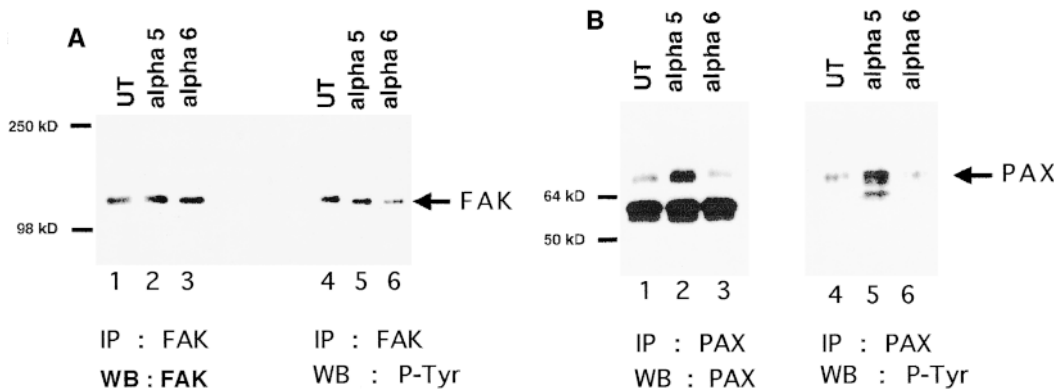
### Ectopic Integrins Regulate FAK and Paxillin

We next sought to determine the effect of ectopic  $\alpha 5$  or  $\alpha 6A$  expression on  $\beta 1A$  integrin signaling pathways. Since integrins stimulate increased tyrosine phosphorylation of several intracellular proteins (Burrige et al., 1992; Kornberg et al., 1992; Bockholdt and Burrige, 1993; Petch et al., 1995; Vuori and Ruoslahti, 1995), we assayed the phosphotyrosine profile of myoblasts expressing different ectopic  $\alpha$  subunits. Immunoblotting with an anti-phosphotyrosine antibody shows that myoblasts ectopically expressing the  $\alpha 5$  integrin contain elevated tyrosine phosphorylation of proteins migrating in the molecular mass ranges of 120–130 and 65–70 kD whereas cells transfected with  $\alpha 6A$  show a marked, general decrease in tyrosine phosphorylation with no additional bands when compared with UT controls (data not shown). These observations are consistent with the phenotypic effects of the ectopic integrins presented above; i.e., the  $\alpha$  subunits do not initiate separate pathways. Thus, ectopic  $\alpha 5$  expression permits enhanced signaling through the  $\beta 1A$  subunit, whereas the  $\alpha 6A$  integrin suppresses these signals.

Next, we pursued the identities of the phosphoproteins migrating at 120–130 and 65–70 kD. Focal adhesion kinase (pp125FAK) and paxillin (pp68) are downstream targets of integrin signaling pathways that migrate in these molecular mass ranges (Burrige et al., 1992). Therefore, we assayed the level of FAK and paxillin tyrosine phosphorylation in UT,  $\alpha 5$ , and  $\alpha 6A$  transfected myoblasts. A Western blot of a FAK immunoprecipitation shows that comparable levels of FAK were precipitated (Fig. 3 A, lanes 1–3). The level of tyrosine phosphorylated FAK decreased in  $\alpha 6A$  (Fig. 3 A, lane 6) versus  $\alpha 5$  transfected (Fig. 3 A, lane 5) or UT myoblasts (Fig. 3 A, lane 4). How-

ever, the level of FAK tyrosine phosphorylation in  $\alpha 5$  and UT myoblasts does not differ (Fig. 3 A, lanes 4 and 5). Immunodepletion of FAK from the lysate, followed by Western blotting the supernatant for phosphotyrosine reveals an additional 120-kD band in the  $\alpha 5$  transfected cells that could account for the observed increase in phosphotyrosine (data not shown). Thus, whereas FAK phosphorylation decreases in myoblasts expressing ectopic  $\alpha 6A$  integrin, it is unaffected by ectopic  $\alpha 5$  expression.

To determine if paxillin phosphorylation is differentially regulated in UT,  $\alpha 5$ , and  $\alpha 6A$  transfected myoblasts, paxillin was immunoprecipitated with an anti-paxillin mAb. In contrast to observations with FAK, we observed a major difference in the level of paxillin expression between UT and  $\alpha 5$  transfected myoblasts. As seen in Fig. 3 B, paxillin is significantly upregulated in  $\alpha 5$  transfected myoblasts (Fig. 3 B, lane 2) when compared to UT (lane 1) or  $\alpha 6A$  transfected (lane 3) myoblasts. The comparable intensity of a 55-kD band in the paxillin immunoprecipitation corresponds to reduced IgG and serves as a loading control. In addition to elevated levels of paxillin, a phosphotyrosine Western blot of the paxillin immunoprecipitation shows a concomitant increase in tyrosine phosphorylation of paxillin in  $\alpha 5$  transfected myoblasts (Fig. 3 B, lane 5) compared to UT myoblasts (lane 4). Tyrosine phosphorylation of paxillin in  $\alpha 6A$  transfected cells (lane 6) is somewhat decreased relative to untransfected cells. The enhanced paxillin expression observed in  $\alpha 5$  transfected myoblasts does not arise as a direct effect of the  $\alpha 5$  integrin. Myoblasts expressing IL2R- $\beta 1A$  also show increased paxillin expression whereas myoblasts expressing IL2R- $\alpha 5$  do not (data not shown). Taken together, these results indicate that enhanced paxillin expression accom-



**Figure 3.** Effect of ectopic  $\alpha 5$  and  $\alpha 6A$  expression on tyrosine phosphorylation of FAK and paxillin. (A) FAK was immunoprecipitated from UT,  $\alpha 5$ , or  $\alpha 6A$  transfected myoblasts with an anti-FAK antibody, mAb 2A7. Cell lysates were normalized for protein content. Immunoprecipitated proteins were detected by Western blot for FAK with an anti-FAK polyclonal antibody, C-20 (left panel, lanes 1–3) and for phosphotyrosine with RC20H (right panel, lanes 4–6). The FAK Western blot shows that equivalent amounts of FAK were isolated from the different cell types. An RC20H, phosphotyrosine, blot of the FAK immunoprecipitation (IP) shows that FAK phosphorylation decreases in  $\alpha 6A$  transfected myoblasts (lane 6) compared with UT (lane 4) or  $\alpha 5$  (lane 5) transfected cells. (B) Paxillin is upregulated in  $\alpha 5$  transfected myoblasts. Paxillin was immunoprecipitated with an anti-paxillin antibody, mAb 165, from equal protein quantities of UT,  $\alpha 5$ , or  $\alpha 6A$  transfected myoblast lysates. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose and Western blotted for paxillin with mAb 165 (lanes 1–3) or phosphopaxillin with RC20H (lanes 4–6).  $\alpha 5$  transfected myoblasts (lane 2) contain significantly elevated levels of paxillin compared to UT myoblasts (lane 1). UT (lane 1) and  $\alpha 6A$  transfected cells (lane 3) contain comparable amounts of paxillin. The paxillin band at 68 kD is indicated by an arrow. The 55-kD IgG band is similar in all three lanes and serves as a loading control. On the right, phosphopaxillin increases in  $\alpha 5$  transfected myoblasts (lane 5) or slightly decreases in  $\alpha 6A$  transfected cells (lane 6) compared to UT (lane 4).

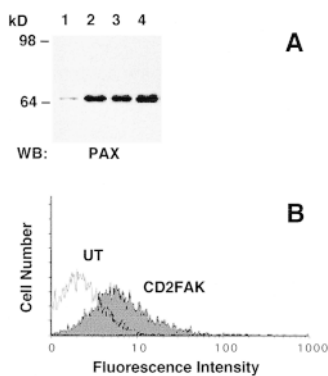
panies increased  $\alpha 5$  (or  $\beta 1A$ ) levels, whereas decreased FAK phosphorylation coincides with increased  $\alpha 6A$  levels. These data also suggest an uncoupling of FAK and paxillin signaling.

### **Paxillin and FAK Regulate the Proliferative to Differentiative Transition**

The altered expression and activation of paxillin and FAK presented above prompted us to examine the effects of ectopic paxillin or FAK expression on myoblast proliferation and differentiation. As shown in Fig. 4 A (lane 2), the level of paxillin expression increases when compared with controls (Fig. 4 A, lane 1) after transfection of a paxillin cDNA. Ectopic expression of wild-type paxillin inhibits differentiation and results in a proliferative phenotype (Fig. 5, Table II). Myoblasts expressing ectopic paxillin proliferate until confluent but neither fuse into multinucleated myotubes (Fig. 5) nor express muscle  $\alpha$ -actinin (Fig. 5). This phenotype is similar to that of  $\alpha 5$  transfected myoblasts (Fig. 2). Thus ectopic paxillin expression alone can recapitulate the effects of the  $\alpha 5$  or IL2R- $\beta 1A$  integrin subunits. Paxillin expression levels in control cells do not differ in replicating myoblasts versus differentiated cultures (data not shown).

Two major sites of phosphorylation in paxillin in response to adhesion to fibronectin are Y118 and S188/190 (Bellis et al., 1997). The tyrosine phosphorylation site, Y118, is also the site phosphorylated by FAK (Bellis et al., 1995). Therefore, we next tested the effect of a Y118F mutation (Fig. 5) or the double mutation S188/190A on the myoblast phenotype. When expressed in myoblasts (Fig. 4 A, lanes 3 and 4), neither of these mutants showed the enhanced proliferation seen when wild-type paxillin was expressed. Instead the paxillin mutants (Fig. 5) exhibited a phenotype characteristic of UT myoblasts (Fig. 5, Table II). This result suggests that these tyrosine and serine phosphorylation sites in paxillin participate in proliferative signaling that regulates myoblast cell cycle withdrawal.

Since FAK phosphorylation decreased in parallel with the inhibition of proliferation in myoblasts expressing  $\alpha 6A$  integrin, we next tested the effect of an ectopic FAK



**Figure 4.** Ectopic expression of adhesive signaling molecules. Myoblasts were transfected with expression plasmids encoding a wild-type chicken paxillin, paxillin mutants Y118F and S188/190A, or a membrane-targeted, chimeric FAK construct, CD2-FAK. (A) Western blot analysis of myoblasts expressing ectopic paxillin (lane 2) or paxillin mutants Y118F (lane 3) or S188/190A (lane 4) shows significant overexpression of these proteins compared with endogenous levels in untransfected myoblasts (lane 1). Paxillin was detected with mAb 165. (B) Expression of CD2-FAK on the cell surface was confirmed by flow cytometry using an anti-CD2 antibody.

mutant, Y397F, which lacks the autophosphorylation site and cannot bind src-family kinases (Schaller and Parsons, 1994), on proliferation and differentiation of myoblasts. Since ectopic expression of soluble FAK often produces short-lived or weak phenotypes (Richardson and Parsons, 1996), we used CD2-FAK, a membrane bound, chimeric FAK construct, which is constitutively active (Chan et al., 1994). Presumably, this results from increased adhesive signaling that arises from its constitutive membrane association and consequent localization in focal adhesions. Ectopic expression of CD2-FAKY397F, inhibits myoblast proliferation while promoting differentiation (Fig. 5). These cells are reminiscent of myoblasts transfected with the  $\alpha 6A$  integrin subunit except that their proliferation is not inhibited as completely. They also show extensive fusion into multinucleated myotubes (Fig. 5). Interestingly, myoblasts transfected with wild-type CD2-FAK (Fig. 4 B), remain proliferative and do not initiate terminal differentiation (Fig. 5) when compared to UT controls. Fewer than 5% of CD2-FAK-expressing myoblasts fuse into multinucleated myotubes (Fig. 5) or express muscle  $\alpha$ -actinin (Fig. 5). Using FACS analysis of propidium iodide labeled cells to measure G1/S progression, CD2-FAK transfected myoblasts show an increased ratio of G2 to G1 cells compared to untransfected cells (data not shown). Similarly, ectopic expression of CD2-FAK454, which is kinase defective, also inhibits differentiation and promotes proliferation (Fig. 5). Ectopic expression of wild-type CD2-FAK in  $\alpha 6A$  transfected myoblasts results in a proliferative phenotype. Myoblasts that coexpress CD2-FAK and  $\alpha 6A$  integrin grow to confluency and do not differentiate (Fig. 5). In sum, the  $\alpha 6A$  phenotype can be recapitulated by expression of a FAK mutant expressed in control myoblasts or rescued by coexpression of an activated form of FAK. These data suggest that one potential mechanism by which  $\alpha 6A$  integrin inhibits myoblast proliferation is through altering FAK phosphorylation. Thus, changes in the level of focal adhesion signaling, through FAK or paxillin, significantly affect the likelihood of myoblasts to proliferate or withdraw from the cell cycle and differentiate. Table II summarizes the effects of ectopic focal adhesion molecules.

**Table II. Focal Adhesion Molecules and Differentiation**

Transfected molecule	Proliferation	Differentiation
Untransfected	+	+
FAK	+	+
FAK(Y397F)	+	+
CD2-FAK	+	-
CD2-FAK(K454R)	+	-
CD2-FAK(Y397F)	+	+
PAX WT	+	-
PAX(S188/190A)	+	+
PAX(Y118F)	+	+

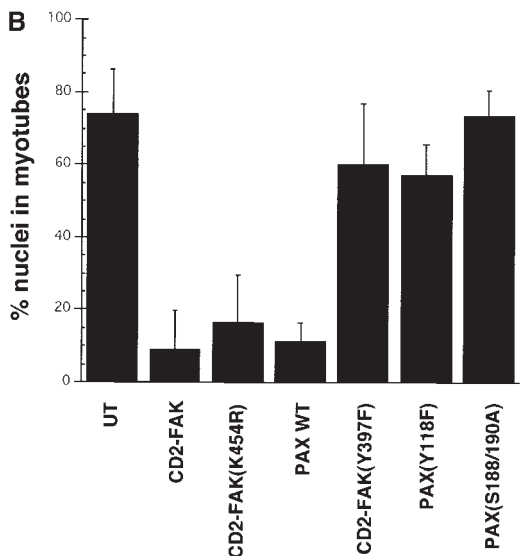
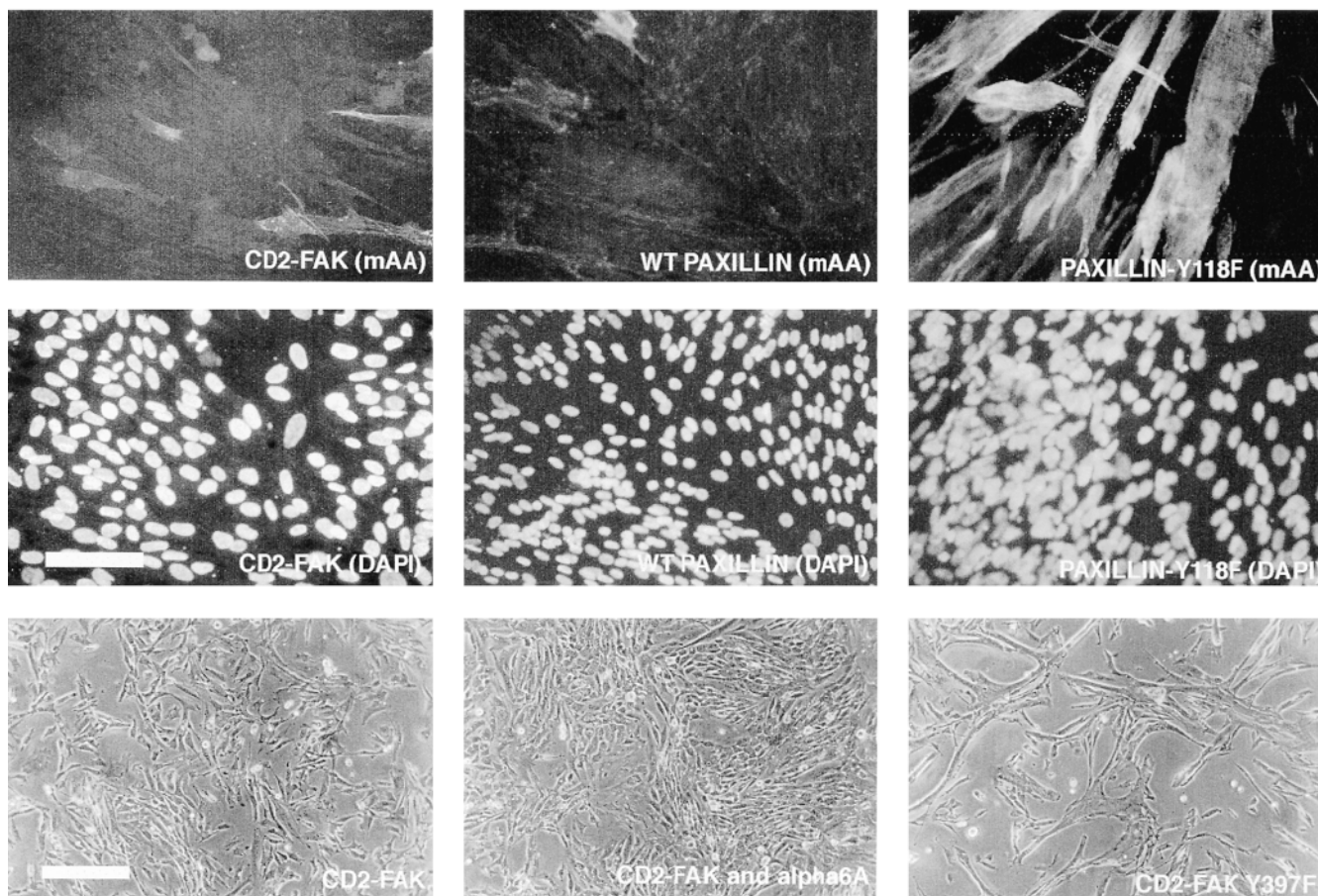
mutant, Y397F, which lacks the autophosphorylation site and cannot bind src-family kinases (Schaller and Parsons, 1994), on proliferation and differentiation of myoblasts. Since ectopic expression of soluble FAK often produces short-lived or weak phenotypes (Richardson and Parsons, 1996), we used CD2-FAK, a membrane bound, chimeric FAK construct, which is constitutively active (Chan et al., 1994). Presumably, this results from increased adhesive signaling that arises from its constitutive membrane association and consequent localization in focal adhesions. Ectopic expression of CD2-FAKY397F, inhibits myoblast proliferation while promoting differentiation (Fig. 5). These cells are reminiscent of myoblasts transfected with the  $\alpha 6A$  integrin subunit except that their proliferation is not inhibited as completely. They also show extensive fusion into multinucleated myotubes (Fig. 5). Interestingly, myoblasts transfected with wild-type CD2-FAK (Fig. 4 B), remain proliferative and do not initiate terminal differentiation (Fig. 5) when compared to UT controls. Fewer than 5% of CD2-FAK-expressing myoblasts fuse into multinucleated myotubes (Fig. 5) or express muscle  $\alpha$ -actinin (Fig. 5). Using FACS analysis of propidium iodide labeled cells to measure G1/S progression, CD2-FAK transfected myoblasts show an increased ratio of G2 to G1 cells compared to untransfected cells (data not shown). Similarly, ectopic expression of CD2-FAK454, which is kinase defective, also inhibits differentiation and promotes proliferation (Fig. 5). Ectopic expression of wild-type CD2-FAK in  $\alpha 6A$  transfected myoblasts results in a proliferative phenotype. Myoblasts that coexpress CD2-FAK and  $\alpha 6A$  integrin grow to confluency and do not differentiate (Fig. 5). In sum, the  $\alpha 6A$  phenotype can be recapitulated by expression of a FAK mutant expressed in control myoblasts or rescued by coexpression of an activated form of FAK. These data suggest that one potential mechanism by which  $\alpha 6A$  integrin inhibits myoblast proliferation is through altering FAK phosphorylation. Thus, changes in the level of focal adhesion signaling, through FAK or paxillin, significantly affect the likelihood of myoblasts to proliferate or withdraw from the cell cycle and differentiate. Table II summarizes the effects of ectopic focal adhesion molecules.

### **MAP Kinase Activity Modulates Integrin-mediated Proliferation and Differentiation**

Several reports implicate MAP kinase in adhesion dependent regulation of proliferation (Chen et al., 1994; Zhu and Assoian, 1995; Wary et al., 1996; Miyamoto et al.,



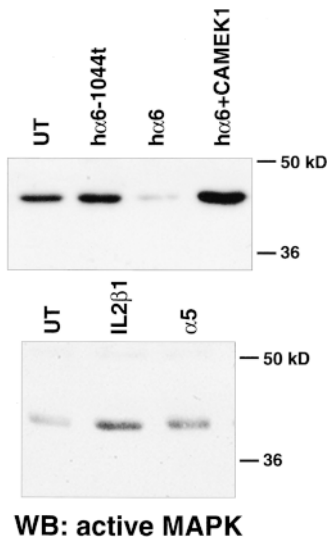
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**Figure 5.** Effect of ectopic adhesive signaling molecules on muscle differentiation. (A) Myoblasts expressing CD2-FAK, wild-type paxillin, or paxillin Y118F were grown in serum-containing medium for 72 h and then double immunostained for muscle  $\alpha$ -actinin (mAA) and DAPI. Both the ectopic CD2-FAK or wild-type paxillin inhibit expression of muscle  $\alpha$ -actinin in the majority of cells. In contrast, paxillin Y118F does not inhibit differentiation. These cells fuse to form multinucleated myotubes and express muscle  $\alpha$ -actinin. Phase contrast pictures show that myoblasts expressing CD2-FAK-Y397 exhibit extensive fusion into myotubes much like control, UT myoblasts, whereas myoblasts that express CD2-FAK or that coexpress  $\alpha$ 6A integrin and CD2-FAK proliferate until confluent with minimal fusion. (B) Fusion index, the percentage of total nuclei in myotubes: CD2-FAK, CD2-FAK K454R, or paxillin expression inhibits myoblast fusion when compared with UT controls. The fusion of paxillin Y118F, paxillin S188/190A, or CD2-FAK-Y397F transfected myoblasts is similar to UT controls. Bars: (fluorescence micrographs) 50  $\mu$ m; (phase micrographs) 35  $\mu$ m.

1996; Lin et al., 1997). In addition, MAP kinase activation plays an important role in muscle differentiation (Bennet and Tonks, 1997). Therefore, we investigated if the ectopic integrins altered MAP kinase activation to control myoblast proliferation and differentiation. MAP kinase activation was assessed by immunoblotting cell lysates with an antibody that specifically recognizes phosphorylated, or

active, forms of the 42- and 44-kD MAP kinases. Quail myoblasts express the 44-kD MAP kinase, erk-1, which was detected using an anti-erk-1 mAb (data not shown). Fig. 6 shows Western blots of active MAP kinase in myoblasts expressing ectopic integrins. In all cases, control or transfected cells were cultured for 24 h in complete serum-containing medium before extraction. The results shown



**Figure 6.** Ectopic integrins regulate MAP kinase activity. Different ectopic integrins alter activation of the level of erk-1 MAP kinase in 24-h cultures grown in serum-containing medium. MAP kinase activity was determined by Western blotting with an antibody that recognizes dually phosphorylated, active MAP kinase. Top panel: ectopic  $\alpha 6A$  expression (lane 3) suppresses MAP kinase activation compared to untransfected controls (lane 1). Ectopic expression of the  $\alpha 6A$  truncation mutant,  $\alpha 61044t$  (lane 2), does not inhibit MAP kinase activation. Coexpression of  $\alpha 6A$  and

constitutive MEK-1 reverses the  $\alpha 6A$  inhibition of MAP kinase activity (lane 4). (Bottom) Ectopic  $\alpha 5$  (lane 3) or IL2R- $\beta 1A$  (lane 2) expression enhance MAP kinase activation compared to untransfected controls (lane 1). The differing intensities for untransfected controls between the top and lower panels reflect different exposure times of the membranes to film.

reflect differences in steady state levels of MAP kinase activity. Compared with UT myoblasts (Fig. 6, bottom, lane 1),  $\alpha 5$  (lane 2), or IL2R- $\beta 1A$  (lane 3) transfected myoblasts contain elevated levels of active MAP kinase. In contrast, the level of active MAP kinase in  $\alpha 6A$  transfected cells is significantly decreased (Fig. 6, top, lane 3) compared to controls (top, lane 1). The different intensities for control levels of active MAP kinase (top versus bottom) is due to different exposure times of the Western blots to film. As presented earlier, the  $\alpha 6A$  cytoplasmic domain truncation,  $\alpha 61044t$ , did not produce the proliferation inhibiting effects of  $\alpha 6A$  integrin, and instead promoted proliferation and inhibited cell cycle withdrawal. This also altered the level of MAP kinase activation, as assayed by Western blotting. Compared with myoblasts expressing  $\alpha 6A$ , those expressing  $\alpha 61044t$  display enhanced levels of active MAP kinase (Fig. 6, top, lane 2). Stripping and reprobing these membranes for total erk1 levels showed similar expression in all cells tested (data not shown). Therefore, the level of MAP kinase activity depends on the expression of specific integrins and their cytoplasmic domains.

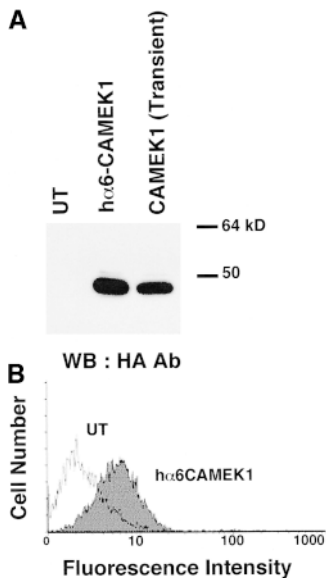
We next investigated whether altering the activation state of MAP kinase could reverse the  $\alpha 5$  or  $\alpha 6A$  induced phenotypes. We manipulated the level of active MAP kinase through overexpression of MEK-1, an upstream activator of MAP kinase, or by addition of PD-98059, a specific MEK inhibitor (Alessi et al., 1995). Cotransfection of myoblasts with constitutively active MEK (CA-MEK; Catling et al., 1995) and  $\alpha 6A$  integrin restores a proliferative phenotype to myoblasts expressing  $\alpha 6A$ . These cells stably express both the  $\alpha 6A$  subunit (Fig. 7 B) and CA-MEK (Fig. 7 A) after drug selection and continue to proliferate for the lifetime of the cells in culture. FACS analysis of propidium iodide labeled cells

shows an increased ratio of G2 to G1 cells in the  $\alpha 6A$ /CA-MEK cotransfectants (data not shown). As reported previously (Sastry et al., 1996), we were unable to isolate cells stably overexpressing only the  $\alpha 6A$  integrin. The  $\alpha 6A$ -CA-MEK transfected cells are similar to the  $\alpha 5$  transfected myoblasts; i.e., they remain proliferative and do not differentiate appreciably (Fig. 7 E) compared with  $\alpha 6A$  transfected (Fig. 7 D) or UT cells (Fig. 7 C). The level of active MAP kinase is enhanced in  $\alpha 6A$ -CA-MEK cells (Fig. 6, top, lane 4) when compared to  $\alpha 6A$  (Fig. 6, top, lane 3) or UT (Fig. 6, top, lane 1) myoblasts. Interestingly, we were unable to obtain stable expression of CA-MEK in untransfected, control myoblasts. Presumably, excessive levels of activated MEK, or MAP kinase, leads to increased cell death or decreased cell growth.

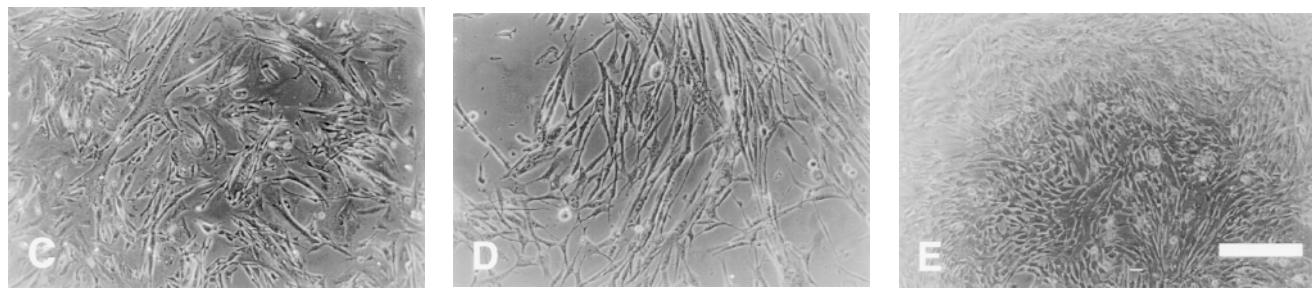
We next determined whether decreasing the level of active MAP kinase, using the specific MEK inhibitor PD-98059, would reverse the  $\alpha 5$  phenotype.  $\alpha 5$  transfected myoblasts were plated onto FN-coated plates in serum-containing medium and allowed to attach for 8–12 h. Increasing concentrations of the MEK inhibitor, PD-98059 were then added for an additional 24–48 h. With increasing inhibitor concentration, the fraction of differentiated cells increased, whereas at high inhibitor concentrations, the total number of cells decreased, presumably due to inhibited proliferation (Fig. 8 and data not shown). After 48 h,  $\alpha 5$  transfected myoblasts treated with a 25  $\mu M$  or greater concentrations (Fig. 8 C) of the MEK inhibitor displayed marked differentiation into myotubes compared with untreated  $\alpha 5$  transfected cells (Fig. 8 B) resembling UT controls (Fig. 8 A). A Western blot for the level of active MAP kinase shows that increasing concentrations of PD-98059 reduces MAP kinase activity in  $\alpha 5$  transfected cells (Fig. 8 E). Taken together, our observations demonstrate that quantitative changes in integrins closely parallels changes in MAP kinase activation. Moreover, the level of active MAP kinase appears to be a critical determinant of myoblast proliferation versus differentiation.

## Discussion

In this study we addressed the mechanisms by which integrin  $\alpha$  subunits modulate intracellular signal transduction events that lead to phenotypic changes in cell proliferation and differentiation. Skeletal myoblasts are well suited for this kind of study. The probability that a myoblast withdraws from the cell cycle and initiates terminal differentiation is highly sensitive to environmental cues including growth factors and ECM components (Sastry et al., 1996). The sharply contrasting effects of ectopic  $\alpha 5$  or  $\alpha 6A$  integrin on myoblast proliferation and differentiation therefore provide a useful system to address  $\alpha$  subunit specific signaling and the mechanisms by which integrins regulate the myoblast decision to withdraw from the cell cycle and initiate terminal differentiation. The data presented here demonstrate that the ectopic  $\alpha$  subunits differentially regulate proliferative signaling through the  $\beta 1$  subunit. They also demonstrate that paxillin, FAK, and MAP kinase serve as important regulators of cell cycle withdrawal and the onset of terminal differentiation. Finally, our results show that the decision to withdraw from the cell cycle and initiate terminal differentiation is highly poised and reg-



**Figure 7.** The effect of ectopic  $\alpha 6$  integrin on proliferation is reversed by MAP kinase activation. (A) Constitutive HA-tagged MEK-1 (CA-MEK1) is efficiently expressed in control (lane 3) and  $h\alpha 6$ A transfected (lane 2) myoblasts in a transient transfection. MEK-1 expression is detected by a Western blot with an antibody specific for the HA epitope. (B) FACS analysis of  $h\alpha 6$ A integrin expression in myoblasts cotransfected with CA-MEK1. The  $h\alpha 6$ A subunit was detected with mAb 2B7, which is specific for  $h\alpha 6$ A. Both the  $h\alpha 6$ A integrin and CA-MEK1 are stably expressed in the double transfected cells after G418 selection. After 72 h of culture in serum-containing medium,  $h\alpha 6$ A/CA-MEK1 transfected myoblasts (E) grow to confluency with little detectable fusion into myotubes while both UT (C) and  $h\alpha 6$ A transfected (D) myoblasts differentiate and form myotubes. Bar, 40  $\mu$ m.

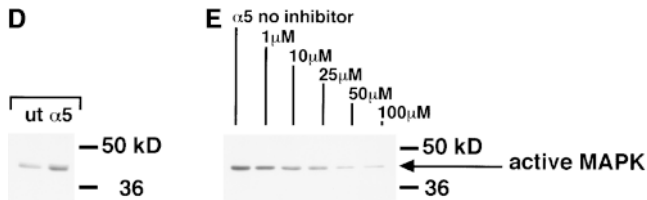
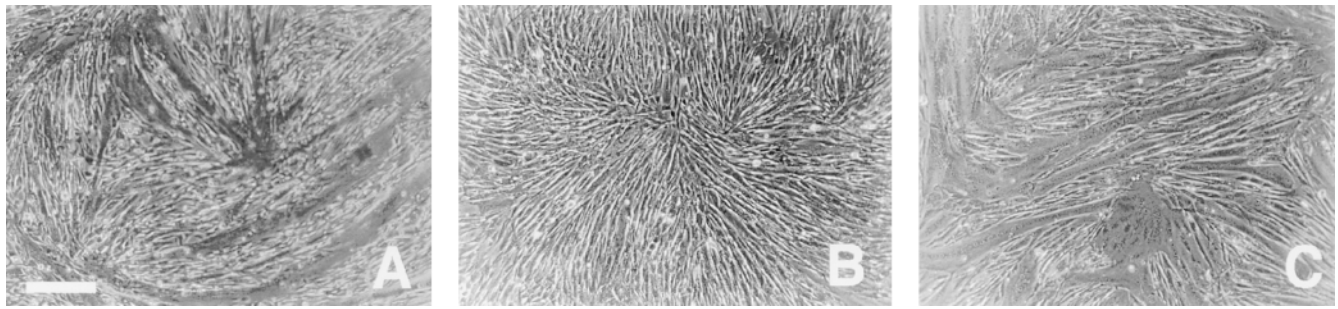


ulated by quantitative changes in the level of MAP kinase activation, which in turn is regulated by quantitative changes in levels of integrin signaling. These observations provide a rationale for: (a) the apparently disparate requirements of different muscle cell lines and primary cultures for proliferation and cell cycle withdrawal, (b) the modulation of integrin subunits during muscle differentiation (Menko and Boettiger, 1987; Muschler and Horwitz, 1991; Bronner-Fraser et al., 1992; Lakonishok et al., 1992; Blaschuk and Holland, 1994; Boettiger et al., 1995) and (c) the hyperproliferative phenotypes observed when integrins are modulated in some other cell types (Carroll et al., 1995).

How does the  $\alpha 5\beta 1$  integrin potentiate signaling while the  $\alpha 6\beta 1$  integrin attenuates it? Our data show that the  $\beta 1$ A subunit (i.e., cytoplasmic domain) is sufficient to transmit proliferative signals. The  $\alpha 5$  subunit is permissive and allows  $\beta 1$  signaling while the  $\alpha 6$ A inhibits it. Previous studies indicate that signaling through integrins requires receptor ligation or clustering into focal adhesions (Kornberg et al., 1991; Miyamoto et al., 1996). In the myoblast system, both the ectopic  $\alpha 5$  and  $\alpha 6$ A subunits form functional receptors with the endogenous  $\beta 1$  subunit for FN or LM, respectively (data not shown, and Sastry et al., 1996). Therefore, the differences in signaling we observe are most likely not due to an inability to bind ligand. Furthermore, since IL2R- $\beta 1$ A alone stimulates proliferation, receptor-ligation does not appear necessary except perhaps to form focal adhesions. Our observation used cells cul-

tured on a complex ECM containing serum and secreted FN, also indicating that receptor ligation may not be a critical factor, for the inhibitory effects of ectopic  $\alpha 6$ . Interestingly, the  $\alpha 5\beta 1$  and the  $\alpha 6\beta 1$  integrins exhibit distinct subcellular distributions in muscle when cultured on the appropriate matrix ligand (Sastry, S., J. Muschler, and M. Lakonishok, unpublished observations). The  $\alpha 5\beta 1$  integrin localizes in focal adhesions on a FN substrate. In contrast, the  $\alpha 6\beta 1$  integrin displays a diffuse cell surface distribution on a laminin substrate. Since the initiating event for signaling through integrins is receptor clustering, the contrasting localizations of  $\alpha 5\beta 1$  and  $\alpha 6\beta 1$  could reflect a difference in their ability to signal. The clustering of integrins recruits signaling proteins into the focal adhesion signaling complex (Miyamoto et al., 1995). In this view, the  $\alpha 5\beta 1$  integrin (or any other  $\beta 1$  integrin with a permissive  $\alpha$  subunit) recruits and activates proteins like FAK, paxillin, and MAP kinase to stimulate signaling. The  $\alpha 6\beta 1$  integrin, though able to bind to LM, is unable to recruit and/or activate the requisite signaling complex.

In the context of a model in which integrin signaling occurs primarily through the  $\beta 1$  subunit, how might the  $\alpha$  subunits regulate signaling? A possible mechanism is that the  $\alpha 5$  and  $\alpha 6$ A cytoplasmic domains differentially regulate the accessibility of binding sites on the  $\beta 1$  cytoplasmic domain. The  $\alpha 5$  subunit (and likely several other  $\alpha$  subunits) would permit exposure of critical binding sites, whereas the  $\alpha 6$ A subunit would mask them. A similar mode of regulation is proposed for the  $\alpha$  subunit cytoplasmic domain



MAP kinase. At greater than 25–50  $\mu\text{M}$  inhibitor, the level of active MAP kinase in  $\alpha 5$  transfected cells is reduced to that observed in UT controls (D). Bar, 30  $\mu\text{m}$ .

**Figure 8.** The effect of ectopic  $\alpha 5$  integrin on proliferation and differentiation requires active MAP kinase. MEK-1 inhibitor, PD98059, was added to  $\alpha 5$  transfected myoblasts. After 72 h in serum-containing medium,  $\alpha 5$  transfected cells treated with PD98059 exhibit extensive fusion into myotubes (C) and resemble control, UT myoblasts (A). (B) Untreated  $\alpha 5$  transfected myoblasts grow to confluency without significant fusion. (E) Increasing concentrations of PD98059 reduce the level of active

in the ligand-dependent localization of integrins in focal adhesions (Briesewitz et al., 1993; Ylanne et al., 1993). Other models of regulation are also possible. They include a steric masking of the  $\beta 1$  cytoplasmic domain by the  $\alpha 6$  cytoplasmic domain through interaction with an inhibitory binding protein, or through an  $\alpha 6$ -mediated signaling event that results in the activation of a phosphatase. The observation that IL2R- $\beta 1A$  expression rescues the  $\alpha 6A$  phenotype, suggests that  $\alpha 6A$  masks some critical site on  $\beta 1A$  cytoplasmic domain. Our observation that ectopic IL2R- $\alpha 6A$  does not inhibit proliferation argues against an  $\alpha 6\beta 1$  binding protein but does not rule it out.

The critical regulatory site(s) within the  $\alpha 6$  cytoplasmic domain appears to reside in the eleven COOH-terminal amino acids of the  $\alpha 6A$  sequence (Table I). This COOH-terminal region of the  $\alpha 6A$  cytoplasmic domain contains two putative serine phosphorylation sites (Shaw et al., 1990; Delwel et al., 1993), which could potentially participate in negative regulation of signaling by the  $\alpha 6A$  integrin. Preliminary mapping of the  $\alpha 6A$  cytoplasmic domain implicates one of these phosphorylation sites in  $\alpha 6A$  (S1071) in inhibition of proliferation (unpublished observations). However, the physiological relevance of  $\alpha 6$  phosphorylation in this system is not yet clear.

Our observations also identify important roles for intracellular components of integrin signaling pathways in regulating cell cycle withdrawal and the onset of terminal differentiation. FAK and paxillin, stand out as potential integrin proximal mediators of adhesive signaling. These proteins are phosphorylated on tyrosine in response to cell adhesion to ECM in many cell types (Guan et al., 1991; Burridge et al., 1992; Hanks 1992; Kornberg et al., 1992; Schaller et al., 1992; Schwartz et al., 1995). In addition, both FAK and paxillin bind to synthetic peptides derived from the  $\beta 1A$  cytoplasmic domain (Schaller et al., 1995). However, their role in cell cycle withdrawal and the onset of terminal differentiation is not well understood. Some evidence implicates FAK in proliferation and cell survival.

Displacement of endogenous FAK from focal adhesions in endothelial cells, through microinjection of the focal adhesion targeting domain, interferes with cell cycle progression (Gilmore and Romer, 1996) resulting in apoptosis. Similarly, microinjection of  $\beta 1A$  peptides corresponding to the FAK binding site, or anti-FAK antibodies induces apoptosis in cultured fibroblasts (Hungerford et al., 1996). Expression of constitutively active FAK, CD2-FAK, in epithelial cells protects them from apoptosis (Frisch et al., 1996).

Our data support a requirement for FAK activation in basal myoblast proliferation; but it does not contribute to the enhanced proliferation, i.e., the inhibited cell cycle withdrawal, observed in cells expressing ectopic  $\alpha 5$ . Phosphorylation of FAK on tyrosine is not significantly altered in myoblasts expressing ectopic  $\alpha 5$  integrin. In most cell types, FAK phosphorylation peaks within 1 h after cell attachment to the ECM (Guan et al., 1991; Burridge et al., 1992; Hanks et al., 1992; Schaller et al., 1992). A transient increase in FAK phosphorylation is observed in  $\alpha 5$  transfected myoblasts after initial attachment on a FN substrate (data not shown). However, we do not observe a sustained increase in FAK activation that coincides with the sustained proliferative phenotype induced by ectopic  $\alpha 5$  integrin. Additionally, overexpression of FAK, as well as several FAK mutants and FRNK (Richardson and Parsons, 1996), had no detectable effect on myoblast proliferation or differentiation (unpublished observations). However, ectopic expression of CD2-FAK, a membrane-bound, activated form of FAK, does promote proliferation and inhibit differentiation, thus resembling the effect of ectopic  $\alpha 5$  or  $\beta 1A$  integrin. Presumably this arises because CD2-FAK is targeted to the membrane and therefore available to recruit additional adhesive signaling complexes. In contrast, the level of FAK phosphorylation is reduced in myoblasts expressing ectopic  $\alpha 6A$  integrin, correlating with the increased cell cycle withdrawal. Ectopic expression of CD2-FAKY397F, which lacks an autophosphorylation

site, acts as a dominant negative and inhibits proliferation and promotes differentiation, thus revealing its role in basal proliferation. This phenotype is similar to but less dramatic than that of ectopic  $\alpha 6A$  integrin expression. It is interesting to note that Y397 is a binding site for src family kinases (Schaller and Parsons, 1994). Finally, coexpression of CD2-FAK and  $\alpha 6A$  results in proliferation. Taken together, these observations are consistent with a role for FAK phosphorylation in the basal level of proliferation seen in control myoblasts and show that it lies on the pathway downstream of the  $\alpha 6A$ -mediated inhibition of the  $\beta 1$  integrin signaling.

In contrast to FAK, both the expression and subsequent tyrosine phosphorylation of paxillin are significantly enhanced in  $\alpha 5$  transfected myoblasts when compared to untransfected or  $\alpha 6A$  transfected cells. A role for paxillin in proliferation or differentiation has not been demonstrated previously. Paxillin is a multidomain adapter protein that mediates numerous interactions with different signaling proteins, including FAK (Schaller and Parsons, 1994). However, paxillin has no known enzymatic activity. Paxillin does contain multiple LIM domains, which are involved in protein-protein interactions and targeting of paxillin to focal adhesions (Turner and Miller, 1994; Brown et al., 1996). Finally, paxillin has been implicated in growth factor dependent differentiation where an increase in its tyrosine phosphorylation correlates with neuronal differentiation (Khan et al., 1995; Leventhal and Feldman, 1996). In myoblasts, the upregulation of paxillin in response to ectopic  $\alpha 5$  integrin coincides with a proliferative phenotype, which can be recapitulated by ectopic expression of paxillin. Our results with paxillin mutants implicate both the Y118F and S188/190A phosphorylation sites as key. Whereas the mechanism by which paxillin inhibits cell cycle withdrawal, i.e., stimulates proliferation, is not known, increased paxillin expression and phosphorylation enhances the formation of adhesive signaling complexes as do CD2-FAK and ectopic  $\alpha 5$  expression (unpublished observation). In contrast to the significant effects of ectopic  $\alpha 5$  expression on paxillin expression and phosphorylation, ectopic  $\alpha 6A$  expression had only a very modest effect on paxillin phosphorylation and none on expression. Coexpression of paxillin and  $\alpha 6A$  did not relieve the  $\alpha 6A$  phenotype (data not shown). Thus the level of paxillin expression and phosphorylation appear to contribute the myoblast decision to proliferate or differentiate. However, it may not play a role in the inhibition of proliferation by ectopic  $\alpha 6A$  expression. It is noteworthy that our results also indicate an uncoupling of FAK and paxillin signaling, since they are not activated in parallel as reported for fibroblasts (BurrIDGE et al., 1992).

Although our results demonstrate an inhibitory role for the  $\alpha 6A$  integrin in signaling, this integrin is capable of activating intracellular signals in other cell systems. In macrophages, for example,  $\alpha 6A\beta 1$ -mediated attachment to laminin leads to increased tyrosine phosphorylation of paxillin (Shaw et al., 1995). In addition, antibody clustering of the  $\alpha 6$  integrin in endothelial cells leads to a distinct profile of tyrosine phosphorylated proteins, which do not include FAK or paxillin (Jewell et al., 1995).

The contrasting effects of ectopic  $\alpha 5$  or  $\alpha 6A$  integrin expression on MAP kinase activation suggests quantitative

changes in MAP kinase activation plays a major role in regulating myoblast cell cycle withdrawal. Perturbing the ratio of different integrins in the cell alters the level of MAP kinase activation. In contrast to our findings, the  $\alpha 6A$  integrin, but not the  $\alpha 6B$  subunit, activates MAP kinase in macrophages (Wei et al., 1998). Cell type differences likely explain these conflicting observations. In the muscle system, the effect of ectopic  $\alpha 5$  or  $\alpha 6A$  integrins and their mutants on MAP kinase activation parallels their phenotypic effects on myoblast proliferation and differentiation. Likewise, perturbing the activation state of MAP kinase, using a MEK inhibitor or CA-MEK, can overcome the integrin induced effect on both MAP kinase activity and the myoblast phenotype. Consistent with these results, the activation of MAP kinase is apparently an important determinant of myogenic differentiation. Inactivation of MAP kinase by overexpression of MAP kinase phosphatase renders C2C12 myoblasts insensitive to mitogenic stimuli, favoring expression of muscle-specific genes like myoD (Bennet and Tonks, 1997). Finally, our data show that MAP kinase activation in myoblasts requires both adhesive and growth factor signals. We do not observe active MAP kinase in the absence of serum or on a non-specific adhesive substrate like poly-L-lysine (unpublished observations). Taken together, these findings reveal a role for integrins in controlling myoblast proliferation and differentiation through a pathway that regulates the level of MAP kinase activation.

The ability of different integrins to activate MAP kinase is proposed to occur via an association of integrins with the adaptor protein, shc. In fibroblasts or endothelial cells,  $\alpha 5\beta 1$  and several other integrins interact with shc and thus can activate MAP kinase whereas the  $\alpha 6$  integrin cannot bind shc or activate MAP kinase (Wary et al., 1996). However, the interaction with shc does not require integrin cytoplasmic domains. Our data do not preclude an involvement of shc; the increase in MAP kinase activation seen in response to the  $\alpha 61044t$  truncation may reflect a difference in the binding of shc, or an unidentified adapter or regulatory protein.

Our observations can be summarized in a working model for adhesive regulation of myoblast withdrawal from the cell cycle and the onset of terminal differentiation. In this model, the myoblast decision to proliferate or withdraw from the cell cycle is regulated, at least in part, by the level of activated MAP kinase. This decision appears highly poised and sensitive to quantitative fluctuations in the level MAP kinase activation: increased MAP kinase activation favoring proliferation and decreased MAP kinase activation favoring cell cycle withdrawal. MAP kinase activation, in turn, is regulated by the signaling emanating from both adhesive and growth factor pathways. Evidently, neither pathway alone is sufficient to sustain proliferation. The absence of either growth factor or adhesive signaling promotes cell cycle withdrawal and the onset of terminal differentiation, which appears to function as a default. At one level, the synergy is compatible with an additive model since increased signaling via either enhanced growth factor or adhesive signaling leads to a decreased probability of cell cycle withdrawal. However, the synergy between the growth factor and adhesive signaling systems likely has interactions that are more consis-

tent with an additive, threshold model, in which signals are required from both the growth factor and adhesive pathways. MAP kinase is not activated and targeted to the nucleus in cells that are either adhering to a nonspecific adhesive substrate like poly-L-lysine or that lack serum growth factors. Recent studies point to RAF as an integration point for adhesive and growth factor signals (Chen et al., 1996; Renshaw et al., 1997; Howe et al., 1998).

This working model provides a rationale for diverse observations on the myoblast decision to proliferate or differentiate. Infection of myoblasts with viruses encoding src family kinases (Alemo and Tato, 1987) or erbB (Olson, 1992), a truncated form of the EGF receptor with intrinsic tyrosine kinase activity, or the ectopic expression of CD2-FAK or paxillin all inhibit cell cycle withdrawal and produce a proliferative phenotype similar to that of myoblasts ectopically expressing the  $\alpha 5$  integrin. It is likely that this arises from increased adhesive and/or growth factor signaling resulting in increased MAP kinase activation. Similarly, the general requirement for high confluency and low serum for differentiation of diverse muscle cell lines likely also appears to reflect enhanced mitogenic signaling, i.e., increased MAP kinase activation, which in this case results from immortalization or adaptation to culture. Previous reports show alterations in the expression of integrin subunits during muscle differentiation, our results raises the possibility that they contribute to cell cycle withdrawal and the onset of terminal differentiation. Finally, our studies appear pertinent to keratinocyte proliferation in vivo as well as to myoblast differentiation in vitro. Transgenic mice expressing two- to threefold increased expression of  $\alpha 5\beta 1$  or  $\alpha 2\beta 1$  integrins exhibit epidermal hyper-proliferation, perturbed keratinocyte differentiation and other features of psoriasis, a skin disease (Carroll et al., 1995). This epidermal hyper-proliferation is similar to our observations on myoblasts expressing ectopic  $\alpha 5$  integrin. Our working model provides a possible mechanism. Therefore, it is likely that quantitative changes in mitogenic signaling through alterations in adhesive signaling produces phenotypic alterations that operate through common mechanisms in diverse systems.

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