Journal of Cellular Biochemistry 101:1394–1408 (2007)

E2F5 and LEK1 Translocation to the Nucleus Is an Early Event Demarcating Myoblast Quiescence

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Abstract Raf/MEK/ERK signaling in skeletal muscle cells affects several aspects of myogenesis that are correlated with the duration and intensity of the input signal. 23A2RafER^{DD} myoblasts directing elevated levels of Raf kinase for 24 h are mitotically inactive. Removal of the stimulus results in cell cycle re-entry and proliferation. Using a proteomic approach, E2F5 and LEK1 were detected in the nuclei of Raf-arrested myoblasts. Disruption of MEK1 activity prevents phosphorylation of ERK1/2 and nuclear translocation of E2F5 and LEK1. The pocket proteins, p107 and p130, remain in the cytoplasm of growth arrested myoblasts irrespective of Raf/ERK activation while pRb translocates to the nucleus. Importantly, both E2F5 and LEK1 are found in the nuclei of non-dividing satellite cells and myonuclei in vivo and in vitro. Our results indicate that Raf-arrested myoblasts may serve as a model system for satellite cell cycle studies and that E2F5 and LEK1 translocation to the nucleus is an important first step during entry into quiescence. J. Cell. Biochem. 101: 1394–1408, 2007.

Key words: E2F5; LEK1; myoblast; quiescent; satellite cell

Satellite cells exist as mononucleated cells between the plasma membrane and basal lamina in skeletal muscle. These muscle stem cells have a low cytoplasm to nucleus ratio, and are fewer in number than normal myonuclei [Mauro, 1961]. In healthy muscle, satellite cells are present in the mitotically inactive state of quiescence [Schultz et al., 1978]. In response to appropriate stimuli, satellite cells activate and commence proliferation [Bischoff, 1990a; Bischoff, 1990b]. Upon cell cycle exit, these myogenic stem cells may either commit to terminal differentiation and fuse into multinucleated myofibers or return to the quiescent state to replenish the pool of available satellite cells.

The transit of a satellite cell back to its normally quiescent state is poorly understood owing in part to the lack of appropriate in vitro models. Primary cultures of rodent satellite cells spontaneously undergo activation leading to proliferation and differentiation [Dhawan and Rando, 2005]. Isolated myofibers with attached satellite cells can be maintained in a quiescent state for upwards of 72 h before entry into the cell cycle [Wozniak and Anderson, 2005]. In both instances, reports of satellite cells traversing into G_0 are absent due to acceptable marker proteins defining entry into the quiescent state. In addition, established methods of cell cycle arrest and maintenance of G_0 such as serum deprivation and cell:cell contact inhibition typically lead to terminal differentiation. Acquisition of fibroblast quiescence by either of these methods leads to clear differences in gene expression profiles indicating that not all states of G_0 are equivalent [Gos et al., 2005]. Confluent myoblasts enter into G_0 prior to terminal differentiation. Growth arrested myoblasts express elevated levels of the cyclin dependent kinase inhibitors (CDKI), p21, p27^{KIP1}, p57^{KIP2}, and p18^{INK4C}, which are necessary for maintenance of G_0 and prevention of myofiber nuclei replication [Walsh, 1997; Myers et al., 2004; Vaccarello et al., 2006]. However, serum starved myoblasts do not faithfully duplicate the quiescent state of

Grant sponsor: USDA-NRI; Grant number: 2003-35206-15297, 2005-35206-1525; Grant sponsor: NIH-NIAMSD; Grant number: R01-AR048830; Grant sponsor: Muscular Dystrophy Association; Grant number: MDA 3685.

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Received 30 October 2006; Accepted 5 December 2006
DOI 10.1002/jcb.21256

satellite cells. Resident satellite cells in rat skeletal muscle contain no detectable p21 protein in contrast to myonuclei, which express both p21 and pRb [Ishido et al., 2004a]. Functional overload models of muscle hypertrophy demonstrate that p21 is expressed in M-cadherin satellite cells beneath the basal lamina 5 days after surgery and expression disappears within 48 h [Ishido et al., 2004b]. These results indicate that CDKI expression is an insufficient monitor of satellite cell quiescence. A recent report elegantly determined that quiescence is a multifaceted process that includes differential gene expression in response to the arrest signal and expression of genes unique to the global G_0 program [Coller et al., 2006]. Importantly, the authors demonstrate that quiescence is not functionally equivalent to cell cycle arrest in myoblasts; quiescent cells are resistant to differentiation whereas growth arrested myoblasts are permissive to terminal differentiation.

Elevated Raf kinase signaling leads to growth arrest in several human and mouse cell lines [Woods et al., 1997; Kerkhoff and Rapp, 1998; Ravi et al., 1998, 1999]. Unlike cultures of primary human fibroblasts [Zhu et al., 1998], initiation of a strong Raf signal in myoblasts does not cause an irreversible senescent phenotype [Dorman and Johnson, 2000; DeChant et al., 2002]. Raf-arrested myoblasts return to the proliferative state upon removal of the input signal. Therefore, myoblasts expressing an inducible Raf kinase may provide a model system to examine the molecular basis for cell cycle entry and exit. The ability of 23A2RafER^{DD} myoblasts to express the satellite cell marker, Pax7, further supports their suitability as a satellite cell line. Using these cells, we report that E2F5 and LEK1 translocation to the nucleus is an early response to Raf-induced growth arrest. Signaling through ERK1/2 is required for translocation. Coincident with nuclear entry of the pocket binding proteins is the traverse of pRb from the cytoplasm to the nucleus. These early events are specific to E2F5, LEK1, and pRb as no changes in the subcellular distribution of the sister proteins, E2F4, p130 or p107, is found. Notably, primary cultures of non-dividing mouse satellite cells contain detectable amounts of E2F5 and LEK1 in the nucleus. As these cells undergo full activation and subsequent proliferation, E2F5 and LEK1 are located principally in the cytoplasm. Thus, 23A2RafER^{DD} myoblasts may serve as a tool for the study of satellite cell entry and exit from G_0.

MATERIALS AND METHODS

Cell Culture

Stock cultures of 23A2RafER^{DD} embryonic mouse myoblasts [Wang et al., 2004] were maintained on 10 cm plastic plates coated with 0.1% gelatin and passaged at approximately 70–75% confluency. Cells were cultivated in basal medium eagle (BME) supplemented with 15% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, 0.1% gentamycin reagent solution, and 10 mM puromycin and incubated in 5% CO_2 at 37°C. All cell culture media, supplements, and sera were purchased from Invitrogen (Carlsbad, CA). Cells (3.5×10^4) for immunofluorescence were cultured on 35 mm glass-bottomed plates (World Precision Inst., Sarasota, FL) coated with 10% BD Matrigel Matrix HC (BD Biosciences, San Jose, CA). Raf activity was induced by the addition of 4-hydroxytamoxifen (4HT) to a final concentration of 1 μM. MEK1/2 activity was inhibited by culture in the presence of 50 μM PD98059 (Invitrogen). When necessary, cells were pulsed with bromodeoxyuridine (BrdU) during the last 30 min of treatment.

To induce growth arrest, subconfluent 23A2 RafER^{DD} myoblasts were washed twice with phosphate buffered saline (PBS), treated with 10 μg/ml protamine sulfate (CalBioChem, San Diego, CA) in serum free BME for 10 min, and washed twice with PBS. Cells were starved in serum-free BME for 1 h prior to induction of Raf activation. Control cells were maintained in the appropriate media supplemented with ethanol.

Nuclear Protein Extracts

Myoblasts were rinsed in cold TBS and scraped from the plates into hypotonic buffer (25 mM Tris, pH 7.5, 1 mM $MgCl_2$, 5 mM KCl, 0.05% NP40, 5 mM orthovanadate, 5 mM sodium fluoride, 5 mM pyrophosphate, 1 mM PMSF, 10 μg/ml aprotinin). Cells were allowed to swell for 15 min on ice in polypropylene Falcon tubes. Lysates were centrifuged at 3,220*g* for 10 min at 4°C in a swinging bucket rotor to recover nuclei. Nuclei were resuspended in high salt buffer (20 mM Tris, pH 8.0, 20% glycerol, 300 mM NaCl, 1.5 mM $MgCl_2$, 200 μM

EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 5 mM orthovanadate, 5 mM pyrophosphate, 5 mM sodium fluoride, 1% NP40, 0.01% SDS) and rocked at 4°C for 1 h. DNA and debris were pelleted for 15 min at 16,100*g* at 4°C. The supernatants containing the nuclear proteins were frozen at −80°C until further use.

Two Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Nuclear proteins were desalted prior to isoelectric focusing (IEF) using D-Salt Excellulose columns (Pierce, Rockford, IL). Proteins were exchanged into IEF sample buffer (4% w/v CHAPS, 7 M urea, 2 M thiourea). Eluates were stored at −80°C or used immediately.

IEF was performed using the Ettan IPGphor II system (Amersham Biosciences, Piscataway, NJ). Precast gel strips with a fixed pI range of 4–7 (Amersham Biosciences) were loaded with 250 μg of protein for 13 cm gels in IEF sample buffer plus 0.4% v/v IPG buffer (Amersham Biosciences) and 0.01% bromophenol blue. IEF strips were placed in the electrophoresis unit and proteins were separated as follows: (1) Rehydration, 16 h, 50 V; (2) 500 V, 500 Vh; (3) 1,000 V, 1,000 Vh; and (4) 8,000 V, 16,000 Vh. Maximum amperage of 50 μA and temperature of 20°C were maintained throughout the course of IEF. Focused gel strips were equilibrated with equilibration buffer (50 mM Tris HCl, pH 8.8, 6 M Urea, 2% SDS, 1% dithiothreitol, 0.01% bromphenol blue) for 15 min and alkylated for 15 min with 2.5% iodoacetamide (Sigma, St. Louis, MO). The gel strips were placed atop 10% SDS polyacrylamide gels and sealed in place with 1% agarose in SDS–PAGE running buffer. Proteins were separated through the polyacrylamide gels at constant amperage (30 mA). Subsequently, gels were fixed in 20% methanol, 10% acetic acid for 1 h at room temperature. Proteins were detected by modified silver staining methodology. Gels were incubated sequentially with gentle shaking as follows: (1) 30% methanol, 2% sodium thiosulfate, and 6.8% (w/v) sodium acetate for 1 h (2) washed five times for 8 min each in ddH_2O (3) 0.25% silver nitrate solution for 1 h (4) washed four times for 1 min in ddH_2O (5) 0.025% (w/v) sodium carbonate, 0.004% formaldehyde (37% stock) until satisfactory color is attained. Development was stopped with ethylenediaminetetraacetic acid (EDTA; 3.65 g EDTA in 250 ml water) for 45 min. Gels were then washed in water.

Mass Spectrophotometry

Proteins of interest were excised and subjected to in-gel tryptic digestion prior to analysis by MALDI-TOF or MALDI MS/MS by the University of Florida Protein Chemistry Core. The type of analysis was determined by the perceived staining intensity of the proteins of interest. For intensely stained proteins, mass spectrometric analysis of the tryptic digests was accomplished by a hybrid quadrupole time-of-flight instrument (QSTAR, Applied Biosystems, Foster City, CA) equipped with the o-MALDI ionization source. A two-point mass calibration was performed in MS/MS mode of operation using the known fragment ion masses of [Glu]-Fibrinopeptide (m/z 175.119 and m/z 1,056.475). Peptide mass fingerprint data generated via the QSTAR were searched against the NCBI nr sequence database using the Mascot (Matrix Science, Boston, MA) database search engine. Probability-based MOWSE scores above the default significant value were considered for protein identification in addition to validation by manual interpretation of the mass spectra.

Proteins stained with moderate intensity were analyzed by capillary rpHPLC separation of protein digests performed on a 10 cm × 75 μm i.d. PepMap C18 column (LC Packings, San Francisco, CA) in combination with a home-built capillary HPLC System operated at a flow rate of 200 nl/min. Inline mass spectrometric analysis of the column eluate was accomplished by a quadrupole ion trap instrument (LCQ, ThermoFinnigan, San Jose, CA) equipped with a nanoelectrospray source. Fragment ion data generated by data dependent acquisition via the LCQ were searched against the NCBI nr sequence database using the SEQUEST (ThermoFinnigan) and Mascot (Matrix Science) database search engines. In general, the score for SEQUEST protein identification was considered significant when dCn was equal to 0.08 or greater and the cross-correlation score (Xcorr) was greater than 2.2. MASCOT probability-based MOWSE scores above the default significant value were considered for protein identification in addition to validation by manual interpretation of the tandem MS data.

Western Blot Analysis

Following Raf activation, plates were washed twice with PBS. Cells were lysed in 4× SDS–PAGE sample buffer (250 mM Tris, pH 6.8, 8% SDS, 40% glycerol, 0.4% β-mercaptoethanol). Lysates were briefly sonicated, heated for 5 min at 95°C, and stored frozen at −20°C until further use. Proteins were separated through 10% polyacrylamide gels and transferred to nitrocellulose. Blots were incubated in 5% nonfat dry milk (NFDM) in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 30 min at room temperature. Primary antibodies were diluted in blocking solution and incubated with the blots overnight at 4°C. Antibodies and dilutions were anti-ERK1/2 and anti-phosphoERK1/2 (1:1,000, Cell Signaling, Danvers, MA), anti-tubulin (1:2,000) and anti-pRb (1:300, Santa Cruz Biotech, Santa Cruz, CA). After washing three times for 5 min each in TBS-T, peroxidase conjugated anti-mouse (or rabbit) antibodies diluted 1:5,000 in blocking solution were added to the blots for 60 min at room temperature. Blots were washed a further three times for 5 min each in TBS-T. Immunoreactive complexes were visualized by enhanced chemiluminescence (Amersham Biosciences) and exposure to X-ray film.

Immunocytochemistry

23A2RafER^{DD} myoblasts were fixed with 4% paraformaldehyde for 20 min at room temperature. For the detection of LEK1, myoblasts were fixed with 70% ethanol for 15 min at room temperature. Nonspecific antigen sites were blocked by incubation in 5% horse serum, 0.1% Triton X-100 in PBS for 60 min at room temperature. Antibody dilutions and sources were anti-p107, anti-p130, anti-pRb, anti-E2F5 (1:50, Santa Cruz Biotech), anti-MyoD (1:30, Vision Biosystems, Norwell, MA), anti-myogenin (1:2,000, F5D, Developmental Studies Hybridoma Bank, University of Iowa, Ames, Iowa), anti-phosphoERK1/2 (1:100, Sigma), and anti-LEK1 (1:300, a kind gift from D. Bader or 1:200, Cocalico Biologicals, Reamstown, PA). After exhaustive washes with PBS, AlexaFluor 488 conjugated anti-mouse and anti-rabbit antibodies diluted 1:500 were added for 60 min at room temperature. Hoechst 33245 diluted to 1 μg/ml in PBS was included as a nuclear stain. Phalloidin-Texas Red (Invitrogen) was used to visualize actin structures. For the detection of LEK, E2F5, and Pax7 in muscle tissue, 8 μm cryosections were collected from the TA and incubated as described. Anti-dystrophin (1:500; AbCam, Cambridge, MA) was used to define the sarcolemma. Fluorescence was detected using a Nikon TE2000 equipped with epifluorescence. Micrographs were imaged using a Nikon Plan Apo 60X oil immersion objective with a numerical aperture of 1.4. Representative images were acquired with NIS-Elements AR 2.3 software using a Nikon DM1200F digital camera. Images were compiled and adjusted for intensity and contrast with Adobe Photoshop CS.

Isolation of Mouse Satellite Cells

Satellite cells were isolated from male retired breeder Balb/c mice (Charles River, Wilmington, MA). Following death by CO_2 inhalation and cervical dislocation, hind limb muscles were removed, rid of connective tissue, and finely minced. Minced muscle was digested with 1.25 mg/ml protease in sterile PBS for 1 h with vortexing every 15 min, as described [Allen and Boxhorn, 1989]. Protease was removed by centrifugation at 1,000*g* and satellite cells were collected from the muscle slurry by differential centrifugation at 400*g*. Satellite cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% horse serum, 1% penicillin/streptomycin, and 0.1% gentamycin reagent solution. Cells were fixed in 4% paraformaldehyde after 24 or 72 h and immunostained, as described above.

RESULTS

Raf-Induced Growth Arrest Is Rapid and Reversible

Previous work from this lab has demonstrated that high levels of Raf activity inhibit mouse myogenesis [Wang et al., 2004]. Elevated Raf function in mesenchymal cells leads to growth arrest [Ravi et al., 1998; Zhu et al., 1998]. To determine the effects of strong Raf activity on myoblast cell cycle kinetics, 23A2RafER^{DD} myoblasts were induced with 1 μM 4HT in low serum media. Cell number was measured after 48 h of treatment. As shown in Figure 1A, control myoblasts doubled during the 2-day period. 23A2RafER^{DD} myoblasts induced to express high amounts of Raf kinase failed to increase in cell number. Raf-induced

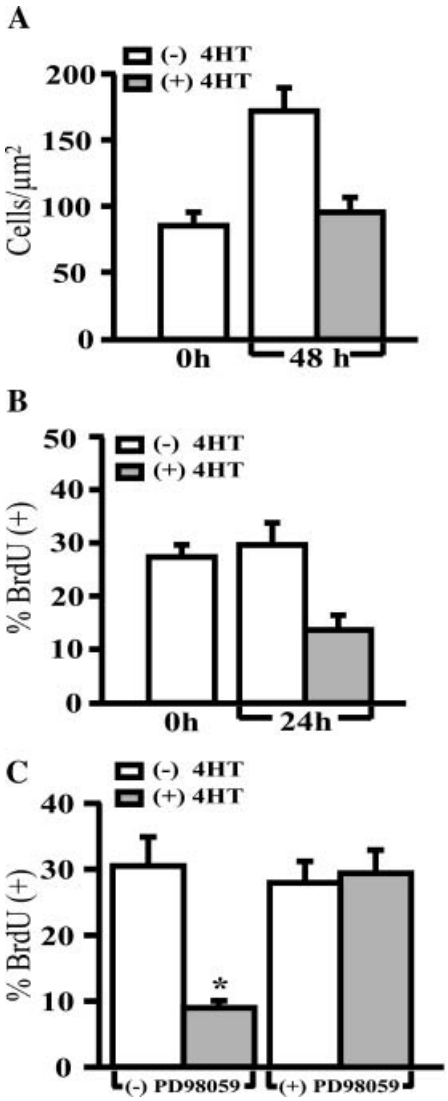

Fig. 1. Elevated Raf signaling leads to cell cycle arrest in myoblasts. 23A2RafER^{DD} myoblasts were cultured for 48 h in the presence or absence of 4HT. Media were replaced with growth medium (BME + 15%FBS) at 24 h. Cells were fixed and counted in six random microscope fields at 200× (**A**). Parallel plates treated as above were treated with 10 μM BrdU for 30 min prior to fixation at 24 h. Cells were immunostained for BrdU incorporation and the numbers of immunopositive cells were counted. Mitotic index was calculated as (BrdU positive nuclei/total nuclei) × 100 (**B**). 23A2RafER^{DD} myoblasts were cultured for 24 h with 1 μM 4HT or vehicle only in the presence or absence of the MEK1 inhibitor PD98059. Cells were fixed and counted in six random microscope fields at 200× (**C**). Means and standard errors for three independent experiments are shown.

myoblast cultures did not exhibit an increase in dead cells or senescent cells, as measured by acidic galactosidase staining (data not shown). To determine if the Raf-imposed growth arrest is reversible, parallel plates of cultures were treated with growth medium and cell number was measured 24 h later. Results demonstrate that removal of 4HT allows the Raf-arrested myoblasts to recommence proliferation (Fig. 1A). Thus, elevated Raf kinase function leads to reversible growth arrest in myoblasts.

To determine how soon the negative effect on mitosis occurs, myoblasts were treated with 1 μM 4HT for 24 h and pulsed with 10 μM BrdU during the final 30 min. Myoblasts were fixed and immunostained for BrdU. 23A2RafER^{DD} myoblasts synthesizing elevated amounts of Raf incorporate significantly less BrdU than control myoblasts, indicating fewer cells in S phase (Fig. 1B). The block to cell cycle progression requires downstream MEK/ERK activity. Treatment of 23A2RafER^{DD} myoblasts with 1 μM 4HT and 50 μM PD98059, a chemical inhibitor of MEK1/2, resulted in no changes in the numbers of BrdU immunopositive nuclei (Fig. 1C). These results demonstrate that Raf/MEK/ERK signaling inhibits myoblast division in a reversible manner.

Raf-Arrested Myoblasts as a Model for Satellite Cell Quiescence

The reversible growth arrest imposed by Raf is a similar feature of myogenic stem cells. Skeletal muscle stem cells or satellite cells exist in mature muscle as quiescent precursors that reactivate the myogenic program during periods of growth and regeneration [Snow, 1977a,b; Schultz et al., 1978; Schultz, 1989]. Thus, 23A2RafER^{DD} myoblasts may be a useful model to examine early events during entry into G_0 or self-renewal. To validate the efficacy of 23A2RafER^{DD} myoblasts as a satellite cell model system, semiconfluent cultures of cells were fixed and immunostained for Pax7. As shown in Figure 2A, 23A2RafER^{DD} myoblasts express Pax7 and the protein is localized predominantly within the nucleus. Approximately 30% of the myoblasts express Pax7. In addition, Pax7 often is localized to one daughter of a mitotic pair. Western analysis indicates that Pax7 protein expression levels do not differ between control and 4HT treated myoblasts (Fig. 2B). Finally, the Raf-induced arrest does not initiate the terminal differentiation program. 23A2RafER^{DD} myoblasts were treated for 1 h with 4HT, fixed and immunostained for myogenin. Control myoblasts receiving vehicle-only exhibit nuclear localized myogenin (Fig. 2C). Cultures of Raf-arrested myoblasts

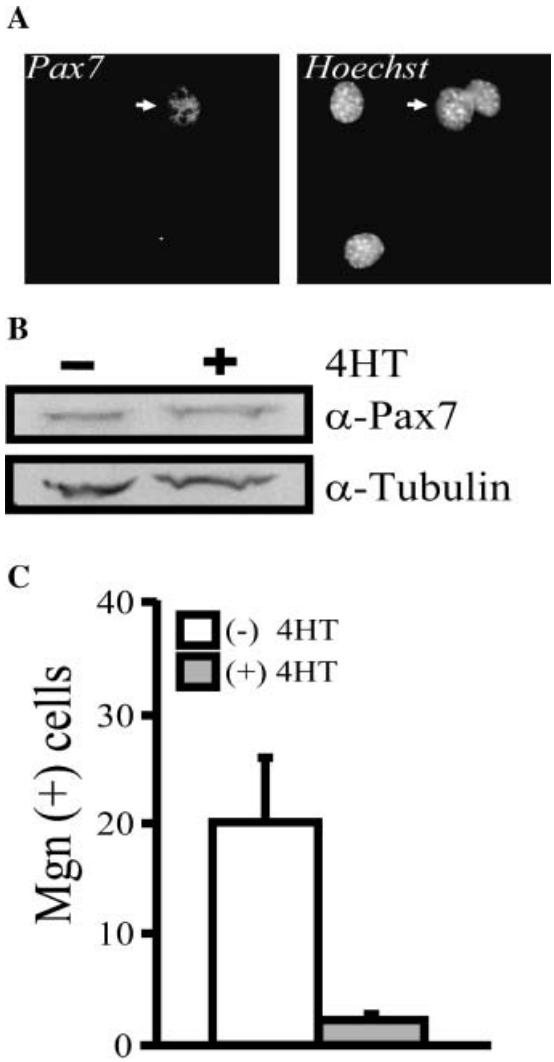

Fig. 2. Mitotically inactive 23A2RafER^{DD} myoblasts express Pax7 but not myogenin. 23A2RafER^{DD} myoblasts were fixed and immunostained for Pax7 expression. Pax7 is expressed in the nucleus of a subset of nuclei. Hoechst stain was used to visualize total nuclei. Arrow indicates Pax7 expressing nuclei (**A**). 23A2RafER^{DD} myoblasts were treated with ethanol or 1 μM 4HT for 24 h. Total cell lysates were analyzed by Western for Pax7 expression. A single immunoreactive band was visualized by chemiluminescence (**B**). 23A2RafER^{DD} myoblasts were treated for 1 h with 1 μM 4HT, fixed and immunostained for myogenin. The numbers of myogenin positive cells were counted in six random microscopic fields at 200-fold magnification (**C**).

contain very few myogenin positive nuclei. In data not shown, no changes in the numbers of MyoD expressing myoblasts were found in response to Raf activation. Thus, subconfluent 23A2RafER^{DD} myoblasts synthesize the requisite protein determinate of the satellite cell lineage and Raf-induced growth arrest does not instigate precocious differentiation.

Elevated Raf Signaling Stimulates Cytoplasmic ERK1/2 Activation

Several proteins demarcate entry of satellite cells into G_1 and the return to a proliferative status. By contrast, few markers exist distinguishing the return to G_0. As a first step toward the definition of G_0 entry, the temporal activation of ERK1/2 was examined. 23A2RafER^{DD} myoblasts were treated with 1 μM 4HT or vehicle-only and replicate plates were fixed at 15 min intervals for 1 h. Total cellular proteins were isolated from plates treated in an analogous manner. Equal amounts of protein were electrophoretically separated and phosphorylated ERK1/2, total ERK1/2 and tubulin expression were measured by Western blot. As shown in Figure 3A, activated ERK2 is apparent within the first 30 min of 4HT treatment and both ERK1 and ERK2 are fully active at 1 h of Raf induction. ERK1/2 activation is detected earlier in 23A2RafER^{DD} myoblasts treated with 1 μM 4HT as measured by immunofluorescent detection of phosphoERK1/2 (Fig. 3B). Control myoblasts treated with ethanol do not display detectable amounts of phosphorylated ERK1/2. Myoblasts expressing inducible Raf contain measurable amounts of activated ERK1/2 as early as 15 min after 4HT treatment. In addition, phosphorylated ERK1/2 is retained in the cytoplasm as predicted for quiescent cells [Ebisuya et al., 2005]. Treatment of 23A2 RafER^{DD} myoblasts with 4HT and PD98059, a chemical inhibitor of MEK function, prevented the activation, and subsequent detection, of cytoplasmic phosphoERK1/2 (data not shown).

Activation of Raf/ERK1/2 Causes Changes in Nuclear Protein Expression Profiles

To identify proteins that are involved in the initial entry into quiescence, nuclear proteins were isolated from 23A2RafER^{DD} myoblasts stimulated for 60 min with 1 μM 4HT or vehicle-only. The 1 h treatment period was selected based upon Western blot demonstration of equal and intense activation of both ERK1 and ERK2 at this time. Control and Raf-induced nuclear protein extracts were separated by 2D PAGE and visualized by silver staining. Gel images were analyzed by Melanie (GeneBio; Geneva, Switzerland), a software program that allows comparisons of 2D PAGE pattern profiles. Protein landmarks were used to align gels and calculate molecular weight and

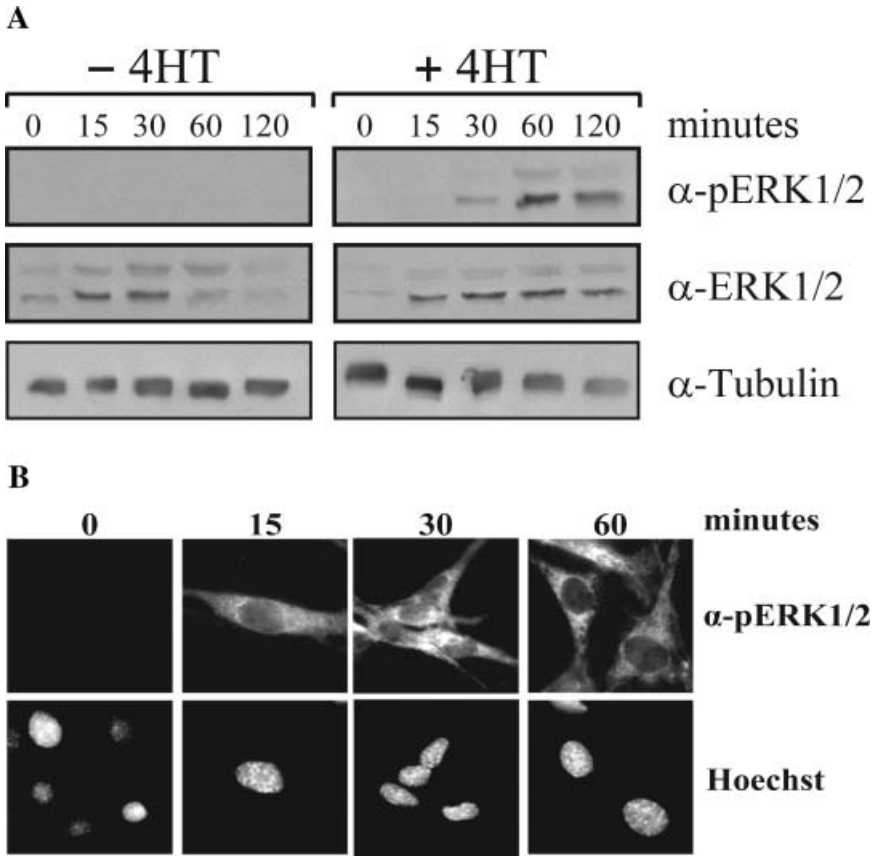

Fig. 3. 23A2RafER^{DD} myoblasts express phosphorERK1/2 in the cytoplasm of Raf-arrested myoblasts. 23A2RafER^{DD} myoblasts were treated with 1 μM 4HT and cultures were lysed with SDS–PAGE sample buffer at 15 min intervals for 60 min. Equal amounts of cellular protein were electrophoretically separated and transferred to nitrocellulose. Blots were probed with anti-phosphoERK1/2, anti-ERK1/2, and anti-tubulin. Immunoreactive complexes were visualized by ECL (**A**). Parallel plates of cells were immunostained with anti-phosphoERK1/2 Nuclei were visualized with Hoechst stain (**B**). Representative photomicrographs at 630× magnification shown.

pI. Proteins unique to 23A2RafER^{DD} control and 4HT treated myoblasts were excised from the gels for subsequent identification (Fig. 4). A total of 57 protein spots were excised from the treatment groups, 24 from the Raf-induced gels and 33 from the control gels. A minimum of four gels was analyzed for each group (control, 4HT). Proteins were digested in-gel with sequencing grade trypsin and peptides were identified by MALDI-TOF and MS/MS (University of Florida Protein Chemistry Core facility). Two proteins corresponding to LEK1 (Fig. 4, arrow a, b) and a single E2F5 protein spot (Fig. 4, arrow c) were identified as exclusive to Raf-expressing myoblasts in all gels analyzed. These proteins were chosen for further study due to their published function as cell cycle modulators [Buck et al., 1995; Sardet et al., 1995; Goodwin et al., 1999; Ashe et al., 2004; Papadimou et al., 2005]. The remaining proteins isolated from growth-arrested and Raf-arrested myoblasts await verification as novel to the respective groups. These results will be published elsewhere.

Raf Signaling Causes Nuclear Translocation of E2F5, LEK1, and PRb

E2F5 and LEK1 are pocket protein binding proteins that are involved in cell cycle control [Buck et al., 1995; Sardet et al., 1995; Goodwin et al., 1999; Ashe et al., 2004; Papadimou et al., 2005]. Both proteins can be found in the cytoplasmic and nuclear compartments. Nuclear translocation of E2F5 is an energy-dependent process that involves the first 56 amino acids [Apostolova et al., 2002]. LEK1 nuclear entry necessitates a functional nuclear localization signal and proteolytic processing of the mature protein form [Goodwin et al., 1999; Ashe et al., 2004]. To validate our MS results and to confirm nuclear retention of proteins,

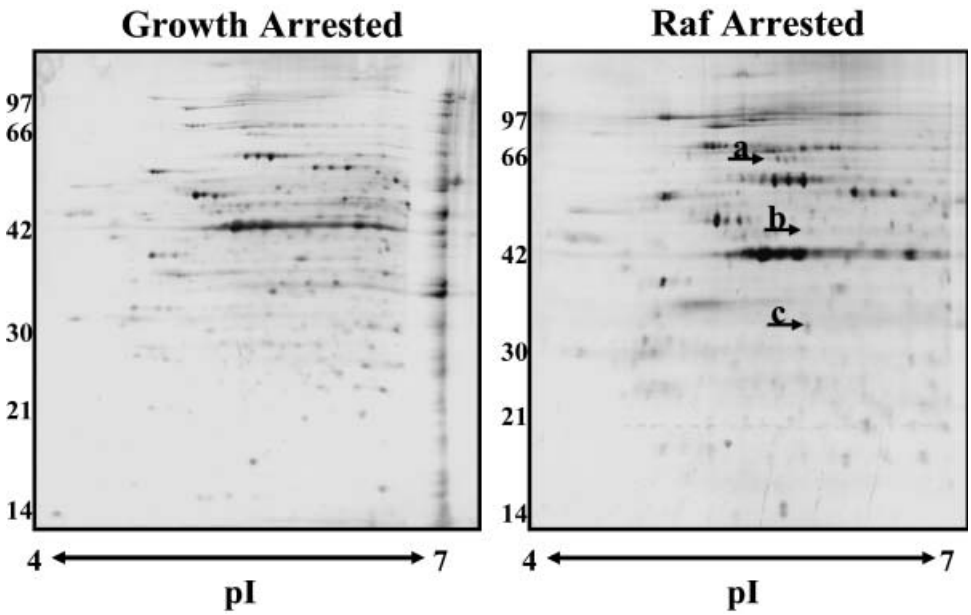

Fig. 4. Representative 2D-PAGE analysis of Raf-induced nuclear proteins. 23A2RafER^{DD} myoblasts were treated for 60 min with 1 μM 4HT or an equal amount of ethanol and nuclear proteins were isolated. Two hundred fifty micrograms of protein from control (Growth Arrested) or treated (Raf Arrested) were separated on pI 4–7 gel strips followed by electrophoresis through 12% polyacrylamide-SDS gels. Proteins were visualized by modified silver stain. Spots were picked, trypsin digested and identified by MALDI-TOF and MS/MS. Proteins corresponding to LEK1 (**a**, **b**) and E2F5 (**c**) are shown.

23A2RafER^{DD} myoblasts were treated for 60 min with 1 μM 4HT. Subsequently, cells were fixed with paraformaldehyde and immunostained for LEK1 and E2F5 expression. Immunofluorescent detection demonstrates that E2F5 is located exclusively in the cytoplasm of growth-arrested myoblasts (Fig. 5). Sustained Raf signaling causes the protein to translocate to the nucleus in approximately 70% of the myoblasts. By contrast, LEK1 was immunolocalized throughout both the cytoplasm and nucleus in control serum starved myoblasts. Exposure to 1 μM 4HT treatment caused a marked redistribution of LEK1 to the nucleus. Residual cytoplasmic LEK1 immunoreactivity was apparent in Raf-induced myoblasts. Redistribution of E2F5 and LEK1 to the nucleus occurs within 15 min of Raf initiated signals.

E2F5 preferentially interacts with the pocket proteins, p130 and p107 [Trimarchi et al., 1998;

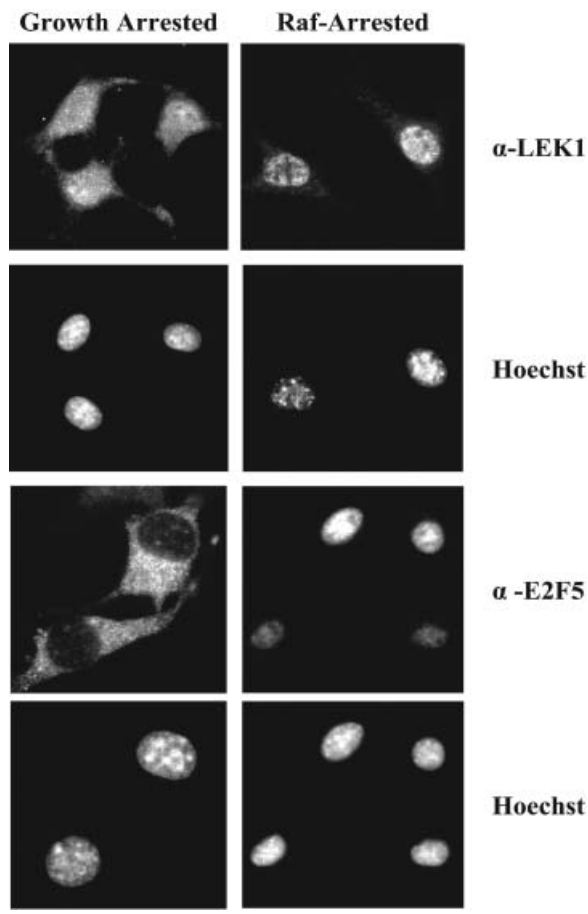

Fig. 5. LEK1 and E2F5 translocate to the nucleus in Raf arrested myoblasts. 23A2RafER^{DD} myoblasts were treated for 60 min with 1 μm 4HT or ethanol (−4HT). Cultures were fixed and immunostained for LEK1 or E2F5. Nuclei were visualized with Hoechst dye. Representative photomicrographs at 630× magnification are shown.

Chen et al., 2002; Gaubatz et al., 2000]. LEK1 binds all three of the pocket proteins through association with an atypical Rb binding site located in the C-terminus [Goodwin et al., 1999; Ashe et al., 2004]. The ability of E2F5 and LEK1 to alter the cell cycle likely requires formation of multiplexes with at least one member of the pocket protein family. Therefore, subcellular changes in the localization of p130, p107 and pRb were monitored in growth-arrested and Raf-arrested myoblasts. In brief, 23A2RafER^{DD} myoblasts were serum starved in the presence or absence of 1 μM 4HT. Myoblasts were fixed and immunostained for the pocket proteins. Immunofluorescent detection revealed no apparent changes in p130 or p107 localization as a function of Raf signaling events (Fig. 6). However, pRb appears to translocate to the nucleus in cells expressing elevated levels of Raf, although some cytoplasmic immunoreactivity exists. Enumeration of the nuclear pRb immunopositive myoblasts indicates that less than 20% of the Raf arrested cells retain pRb exclusively in the cytoplasmic compartment. The nuclear localization of E2F5, LEK1, and pRb is dependent upon an intact MEK/ERK signaling module. Chemical disruption of MEK function completely abolished translocation of the proteins (Fig. 7).

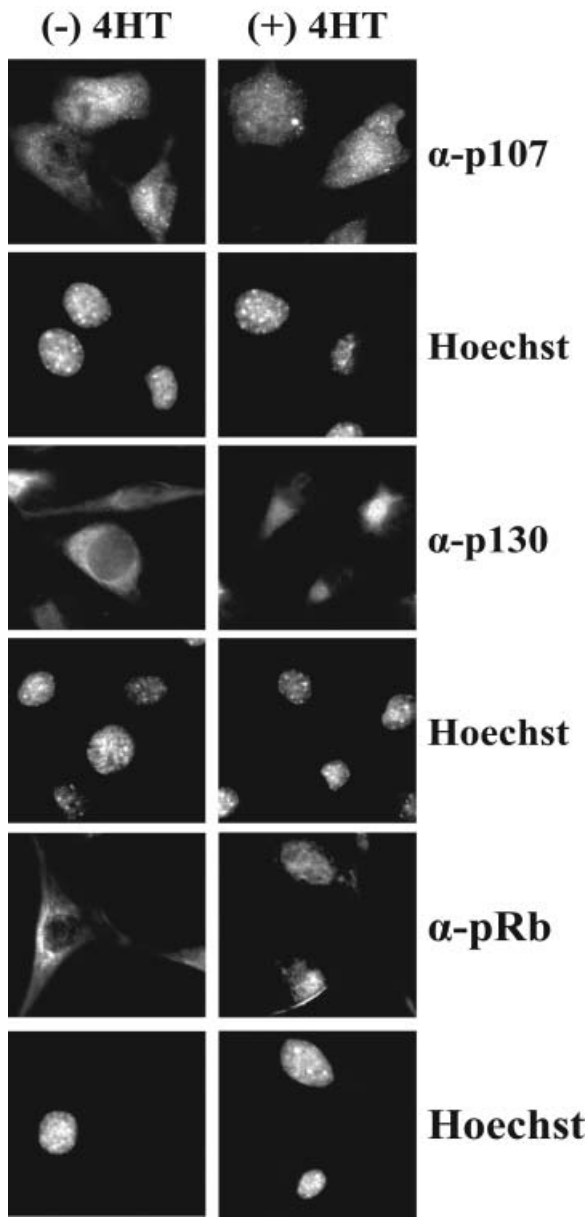

Fig. 6. pRb redistributes to the nucleus in Raf-arrested myoblasts. 23A2RafER^{DD} myoblasts were treated with 1 μM 4HT for 60 min. Parallel cultures were fixed and immunostained for the pocket proteins, p107, p130, and pRb. Representative photomicrographs at 630× magnification are shown.

Quiescent Mouse Satellite Cells Express E2F5 and LEK1 in the Nucleus

The value of a cell line is measured by its ability to reflect the in vivo situation. Raf-arrested myoblasts may represent a suitable system to examine early events leading to quiescence and/or to identify marker proteins associated with G_0 in satellite cells. To this end, cryosections were collected from the tibialis anterior (TA) of adult mice. The tissue sections were incubated with anti-E2F5, anti-LEK1, and anti-Pax7 or anti-dystrophin. Immunofluorescent detection reveals that LEK1 is present in both myonuclei as well as cells contained within the satellite position (data not shown). Colocalization of Pax7 and LEK1 indicates that satellite cells do express the protein although, not in an exclusive manner (Fig. 8A). Pax7$^{(+)}$/LEK$^{(-)}$ muscle cells were apparent. Co-localization of E2F5 and Pax7 clearly demonstrated expression of the cell cycle regulatory protein in satellite cells (Fig. 8B).

To further clarify the presence of E2F5 and LEK1 in putative quiescent satellite cells, primary cultures were established from adult mice. This population of cells remains in a non-dividing state in vitro for upwards of 36 h [Johnson and Allen, 1993, 1995; Smith et al., 1994; Yablonka-Reuveni et al., 1999; Renault et al., 2002]. Primary cultures of satellite cells were cultured in growth medium for 24 h prior to immunostaining for phosphoERK1/2, proliferating cell nuclear antigen (PCNA), Pax7,

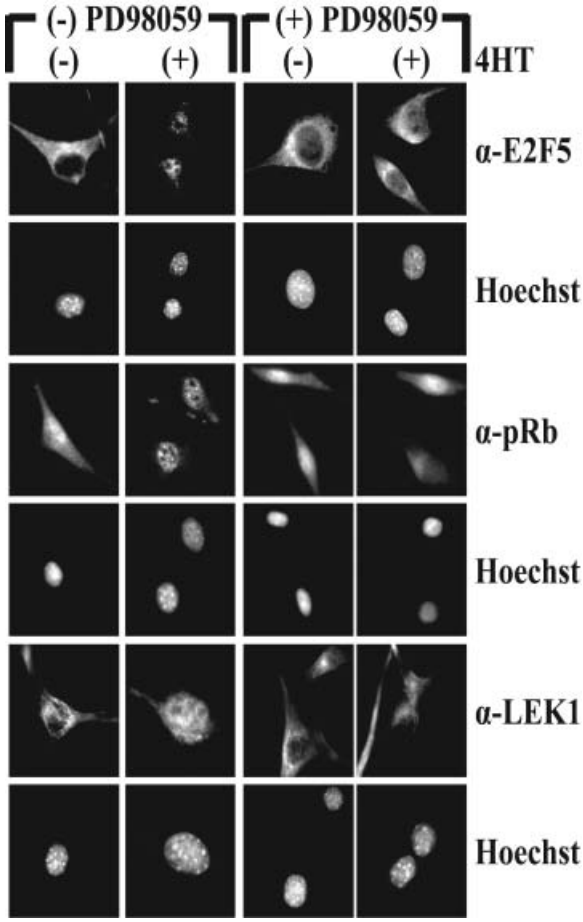

Fig. 7. Translocation of E2F5, LEK1, and pRb requires a functional ERK1/2. 23A2RafER^{DD} myoblasts were treated with 1 μM 4HT or vehicle-only for 60 min in the presence or absence of 50 μM PD98059. Parallel cultures were removed, fixed, and immunostained with anti-E2F5, anti-LEK1, or anti-pRb. Nuclei were visualized with Hoechst dye. Representative photomicrographs at 630× magnification shown. Translocation of E2F5, LEK1, and pRb to the nucleus occurs only in the absence of the MEK inhibitor.

E2F5, and LEK1. Antigens were detected by immunofluorescence and nuclei were visualized by Hoechst stain. Total number of cells and the number of cells expressing the respective proteins were measured. At 24 h, very few PCNA positive cells were present indicating that the satellite cells were not in S phase (Fig. 9A). The heterogeneous cell population contained approximately 80% satellite cells as measured by Pax7 expression. Immunofluorescent staining for E2F5 and LEK1 revealed 82% and 77% of the cells, respectively, expressed the proteins prominently within the nuclear compartment at 24 h post-plating. Based on these values, we deduce that E2F5 and LEK1 are expressed in the nucleus of mitotically inactive satellite cells. This was further clarified by co-localization of the proteins with satellite cell markers. Pax7 expressing satellite cells contain LEK1 in the nuclear compartment (Fig. 9B). Regions of intense anti-LEK1 immunofluorescence do not appear to correlate directly with the corresponding regions of Pax7 expression. Upon full activation of the satellite cells, LEK1 appears to redistribute to the cytoplasmic compartment in the majority of the cells. However, weak nuclear immunostaining with anti-LEK1 is apparent. In a similar manner, abundant amounts of E2F5 were found in the nuclei of M-cadherin immunopositive satellite cells (Fig. 9C). As these cells became mitotically active, the transcriptional repressor was located prominently in the cytoplasmic compartment with weak immunostaining present in the nucleus.

DISCUSSION

Events leading to the terminal differentiation of myoblasts include cell cycle arrest, increased expression of MyoD, p21 and myogenin [Guo et al., 2003]. Critical to formation of mature myocytes is the irreversible entry into G_0 and production of proteins necessary for the prevention of endoreduplication [Gu et al., 1993; Schneider et al., 1994; Huh et al., 2004]. However, many of these same regulatory proteins are believed to participate in the reversible growth arrest displayed by satellite cells. As such, serum-starvation models using myoblast cell lines are often equated with satellite cell self-renewal. Here, we describe an alternate model system to examine myoblast growth kinetics that may more closely reflect G_0 to G_1 transition in satellite cells. 23A2RafER^{DD} myoblasts withdraw from the cell cycle upon induction of elevated levels of Raf and ERK1/2 activity. Release from growth suppression by removal of 4HT allows the cells to progress into the cell cycle, as monitored by thymidine analog incorporation. In addition, ablation of the Raf kinase signal through MEK1 inhibition blocks Raf induced cell cycle arrest and allows continued proliferation. Unlike serum deprivation, Raf-arrested myoblasts fail to express myogenin, a marker of terminal differentiation. Thus, the inability of Raf-induced myoblasts to proliferate does not predispose the cells

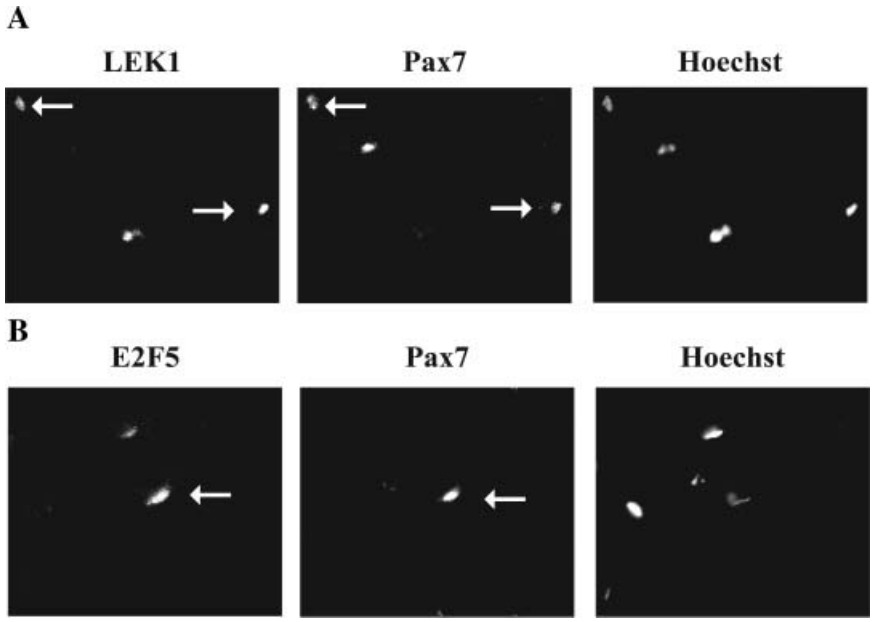

Fig. 8. E2F5 and LEK1 are expressed in satellite cells in vivo. Cryosections (8 μM) were obtained from the mouse TA and co-incubated with rabbit anti-LEK1 and mouse anti-Pax7. Anti-rabbit AlexaFluor 564 and anti-mouse AlexaFluor 488 were used to visualize LEK and Pax7, respectively (**A**). Arrow represents a Pax7 nucleus that does not express LEK1. Rabbit anti-E2F5 and mouse anti-Pax7 were used to co-localize E2F5 to satellite cells. Anti-rabbit AlexaFluor 564 and anti-mouse AlexaFluor 488 were used to visualize E2F5 and Pax7, respectively (**B**).

to fusion and synthesis of muscle specific proteins. The equivalence of satellite cells and $23A2RafER^{DD}$ myoblasts is further supported by their capacity to express Pax7, a hallmark of satellite cells [Seale et al., 2000; Oustanina et al., 2004; Relaix et al., 2005, 2006; Horst et al., 2006; Kuang et al., 2006; Zammit et al., 2006]. While Pax7 is required for maintenance of the

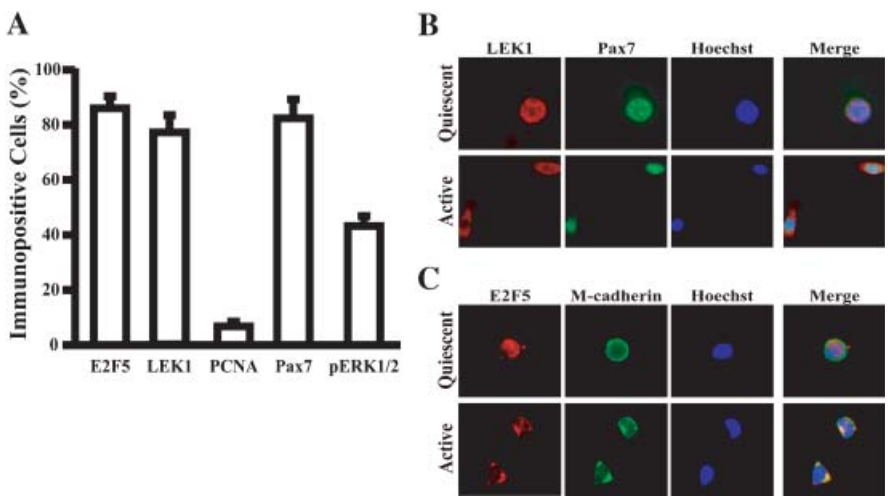

Fig. 9. Quiescent satellite cells express E2F5 and LEK1. Satellite cells were isolated from adult male mice and cultured in growth medium for 24 h. Fixed cells were immunostained for E2F5, LEK1, Pax7, and PCNA. Nuclei were visualized with Hoechst dye. Total number of nuclei and immunopositive nuclei were counted in eight random microscope fields at 200×. Data represent the means and standard errors of three independent isolations and experiments (**A**). Parallel plates of satellite cells were immunostained with rabbit anti-LEK1 and mouse anti-Pax7 (**B**) or mouse anti-E2F5 and rabbit anti-M-cadherin (**C**). Nuclei were visualized with Hoechst dye. LEK1 and E2F5 are predominantly nuclear in non-dividing satellite cells (24 h) and become cytoplasmic in replicating myoblasts (72 h). Representative photographs were captured at 630×.

satellite cell lineage, additional marker proteins include M-cadherin, syndecan 3/4, CD34, and FoxK1 [Garry et al., 1997; Beauchamp et al., 2000; Cornelison et al., 2001, 2004; Sajko et al., 2004]. Initial experiments indicate that M-cadherin, syndecan 3 and 4 are present on 23A2RafER^{DD} myoblasts (Reed and Johnson, unpublished data). Our results present compelling evidence that Raf-arrested myoblasts, which express satellite cell marker proteins, may provide an invaluable model for monitoring self-renewal of muscle stem cells.

E2F5 and LEK1 were identified in a screen for nuclear proteins that may represent markers of entry into quiescence. Both proteins are present in primary cultures of satellite cells isolated from aged mice. Nuclear E2F5 immunofluorescence is present at 24 h post-plating, a time point at which the vast majority of the satellite cells are mitotically inactive. As the satellite cells complete activation (~36 h) and commence proliferation, E2F5 is located predominantly in the cytoplasm. A similar finding was evident for LEK1. Approximately 75% of the satellite cells at 24 h were immunopositive for the centromeric protein with the numbers of cells retaining nuclear LEK1 declining as they became mitotically active. These events duplicate our results using Raf-arrested myoblasts supporting our contention that 23A2RafER^{DD} myoblasts are a model for some aspects of satellite cell biology. However, the Raf-arrested myoblasts are not direct mimics of quiescent satellite cells. The numbers of Raf-arrested nuclei expressing E2F5/LEK1 are greater than the number of Pax7 myoblasts. This would indicate that translocation of the two proteins to the nucleus is not a unique feature to satellite cells but rather a global response of myoblasts to elevated Raf activity. In addition, E2F5 and LEK1 are not expressed exclusively in satellite cells in vivo. Expression of LEK1 and E2F5 in myonuclei suggests that the proteins may be indicators of cell cycle inhibition in all types of myogenic cells.

Withdrawal from the cell cycle in many cell types, including myoblasts, requires the coordinated actions of members of the pocket protein family, pRb, p107, and p130 [Stevens and La Thangue, 2003; Cobrinik, 2005]. These nuclear proteins sequester E2F1, -2, and -3 preventing continued progression through S-phase. One of the immediate responses to Raf-induced growth arrest is the translocation of pRb to the nucleus. This is in stark contrast to serum-starved myoblasts that retain the pocket protein in the cytoplasm. Translocation is dependent upon downstream activation of ERK1/2 as treatment of the cells with the chemical inhibitor, PD98059, prevents detection of nuclear pRb. Western analysis indicates that pRb exists in the hypophosphorylated active form, thus direct phosphorylation of the pRb by ERK1/2 is unlikely to be the cause of translocation (data not shown). The nuclear accumulation of hypophosphorylated pRb in Raf-expressing myoblasts parallels that of murine and human fibroblasts [Sewing et al., 1997; Dasgupta et al., 2004]. However, the cytoplasmic retention of p130 or p107 in Raf-arrested and growth-arrested myoblasts is unique. C2 reserve myoblasts, a satellite cell model, express high levels of p130 following serum deprivation [Carnac et al., 2000]. p130 associates with E2F-containing DNA binding complexes in these myoblasts and p130 is a defining feature of quiescent fibroblasts [Smith et al., 1996, 1998]. The inability of 23A2RafER^{DD} myoblasts to direct p130 to the nucleus under G_0 permissive conditions likely reflects the early time point of analysis. Accumulation of p130 may be required for long-term maintenance of the quiescent state. Indeed, nuclear p130 expression was detected after 4 days of serum starvation in reserve myoblasts, a time very different from that reported here [Carnac et al., 2000]. Further work is required to address the role of the pocket proteins in the early events leading to G_0 versus sustained maintenance of the quiescent state.

The function of LEK1 during the entry into G_0 remains largely undefined. LEK1 is a large protein that contains several protein regulatory motifs including an atypical Rb binding site that can interact with Rb, p107, and p130 [Goodwin et al., 1999; Ashe et al., 2004]. LEK1 is ubiquitously expressed and mRNA levels are downregulated in embryonic mouse tissues as they transit into a non-proliferative state [Papadimou et al., 2005]. The loss of LEK1 expression as cells cease division suggests that LEK1 may be important for continued proliferation and/or maintenance of an undifferentiated state. Indeed, overexpression of a dominant inhibitory LEK1 cDNA in myoblasts leads to premature myofiber formation [Goodwin et al., 1999; Ashe et al., 2004]. Knockdown of LEK1 in mouse embryonic stem cells causes a delay in cardiogenesis typified by reduction in

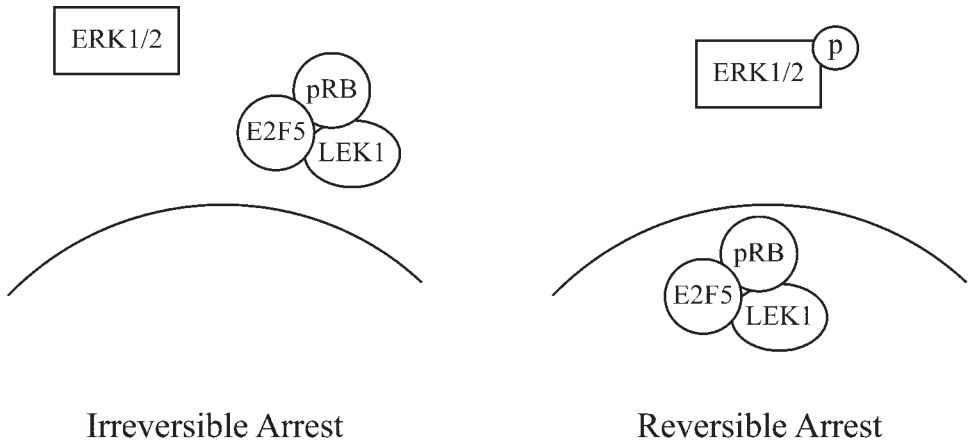

Fig. 10. Nuclear translocation of LEK1, E2F5, and pRb occurs as a result of ERK1/2 activation. Cells entering irreversible growth arrest maintain LEK1, E2F5, and pRb cytoplasmically in the absence of ERK1/2 activation (Irreversible Arrest). Alternatively, cells entering a reversible growth arrest exhibit high levels of ERK1/2 phosphorylation and nuclear translocation of E2F5, LEK1, and pRb (Reversible Arrest).

Nkx2.5 expression and fewer beating embryoid bodies [Papadimou et al., 2005; Puceat, 2005]. A similar finding is apparent in Rb(-/-) ES cells and ES cells whereby the LEK1-Rb interaction is disrupted. LEK1 is abundantly expressed in 23A2 myoblasts with an indiscriminate immunostaining pattern. In response to ERK1/2 activity, LEK1 accumulates in the nucleus although immunodetection reveals this is not exclusive. Nuclear translocation occurs in the same time frame as pRb. Due to the similar translocation kinetics and Rb-binding motif on LEK1, it is possible that LEK1 serves as a shuttle for the efficient localization of pRb to distinct chromosomal locales. The finding of nuclear LEK1 in Raf-arrested cells is intriguing in light of the suggested function of LEK1 as a positive effector of the cell cycle. We propose that LEK1 may function to arrest and maintain the myoblast in a naive state. The undifferentiated immature state may involve LEK1-pRb interaction and/or sequestration of a myogenic bHLH factor. LEK1 contains a protein motif capable of binding E2A proteins [Goodwin et al., 1999]. MyoD homodimers are putative regulatory complexes in myoblasts with MyoD-E47 heterodimers functional in myofibers [Li et al., 1996; Becker et al., 2001; Dhawan and Rando, 2005]. A viable hypothesis includes LEK1 as a sink for E-proteins such that MyoD homodimers predominate and allow for preservation of an undifferentiated state. Alternatively, LEK1 may play a vital anti-apoptotic role in the Raf-arrested cells. Elevated Raf inhibits both differentiation and apoptosis in 23A2 myoblasts [DeChant et al., 2002]. Myoblasts devoid of a functional LEK1 undergo an elevated frequency of apoptosis [Ashe et al., 2004].

In summary, we describe a novel model system for the study of satellite cell activation and self-renewal (Fig. 10). 23A2RafER^{DD} myoblasts induced to activate ERK1/2 enter a reversible state of growth arrest. Unlike serum-starved myoblasts, Raf-arrested myoblasts can re-enter the cell cycle and do not express the early markers of differentiation. It is important to note that this system does not address cell fate determinants or explicitly replicate the life cycle of myoblasts in vivo. However, the evidence provided suggests that this system may be used to examine cell cycle exit without initiating a terminal differentiation program in a method reminiscent of satellite cell self renewal. Using this model system, LEK1 and E2F5 were identified as early determinants of Raf-arrested myoblasts. The proteins translocate to the nucleus as a function of ERK1/2 activity and provoke gene responses necessary to maintain the non-dividing state. This unique model may provide invaluable information regarding control of satellite cell activation and the coordinated reentry into the cell cycle.

ACKNOWLEDGMENTS

The authors are grateful to Scott McClung and Dr. Stanley Stevens at the UF Protein

Chemistry Core Facility for their assistance with mass spectrometry and protein identification. The authors thank Dr. David Bader (Vanderbilt) for supplying anti-LEK1.

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