Rad is temporally regulated within myogenic progenitor cells during skeletal muscle regeneration

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Rad is temporally regulated within myogenic progenitor cells during skeletal muscle regeneration. Am J Physiol Cell Physiol 290: C379–C387, 2006. First published October 12, 2005; doi:10.1152/ajpcell.00270.2005.—The success of using myogenic progenitor cells for therapeutic applications requires an understanding of the intrinsic and extrinsic cues involved in their regulation. Herein we demonstrate the expression pattern and transcriptional regulation of Rad, a prototypical member of a family of novel Ras-related GTPases, during mammalian development and skeletal muscle regeneration. Rad was identified using microarray analysis, which revealed robust upregulation of its expression during skeletal muscle regeneration. Our current findings demonstrate negligible Rad expression with resting adult skeletal muscle; however, after muscle injury, Rad is expressed within the myogenic progenitor cell population. Rad expression is significantly increased and localized to the myogenic progenitor cell population during the early phases of regeneration and within the newly regenerated myofibers during the later phases of regeneration. Immunohistochemical analysis demonstrated that Rad and MyoD are coexpressed with the myogenic progenitor cell population of regenerating skeletal muscle. This expression profile of Rad during skeletal muscle regeneration is consistent with the proposed roles for Rad in the inhibition of L-type Ca\textsuperscript{2+} channel activity and the inhibition of Rho/RhoA kinase activity. We also have demonstrated that known myogenic transcription factors (MEF2, MyoD, and Myf-5) can increase the transcriptional activity of the Rad promoter and that this ability is significantly enhanced by the presence of the Ca\textsuperscript{2+}-dependent phosphatase calcineurin. Furthermore, this enhanced transcriptional activity appears to be dependent on the presence of a conserved NFAT binding motif within the Rad promoter. Taken together, these data define Rad as a novel factor within the myogenic progenitor cells of skeletal muscle and identify key regulators of its transcriptional activity. 

THE CAPACITY FOR SKELETAL MUSCLE GROWTH and regeneration is due to a rare population of undifferentiated progenitor cells resident within adult skeletal muscle. In undisturbed muscle, these cells remain quiescent at the periphery of mature myofibers (10). After muscle damage, these myogenic progenitor cells exit their quiescent state, proliferate, and fuse together or to existing myofibers to regenerate the damaged skeletal muscle (5). From a therapeutic perspective, recent studies have demonstrated that the myogenic progenitor cells from healthy donors may be useful for the treatment of skeletal muscle myopathies such as Duchenne muscular dystrophy (25). Admittedly, this highly promising therapy is still in its infancy, and only through a more thorough understanding of the factors regulating this unique cell population will their therapeutic potential be fully realized.

We previously undertook microarray analysis to define the global molecular events that characterize discrete stages of muscle regeneration (4). On the basis of these studies, we identified a Ras-related protein, termed Rad (Ras associated with diabetes), which was robustly expressed early (6–12 h) after skeletal muscle injury. Rad is the prototypical member of a novel class of 35- to 39-kDa Ras-related GTPases. Rad, as well as other members of this Ras-related GTPase protein subfamily (RGK family members Gem, Kir, Ges, and Rem1/2), possess a highly conserved 88-amino acid NH\textsubscript{2}-terminal extension and a 31-amino acid carboxy terminus that lacks the CAAX-like prenylation motif typical of other Ras proteins (20).

Rad was initially identified because its expression was upregulated in the skeletal muscle of some patients with type 2 diabetes mellitus (20), but it has since been documented to be dysregulated in patients with Duchenne muscular dystrophy (15) and in the amputated limbs of the neut (24). Rad is expressed most highly in the heart, lung, and skeletal muscle of humans and also has been demonstrated to exist in the C2C12 muscle cell line (1, 18, 20).

Although a number of biochemical studies characterizing Rad have been performed (11, 12, 30), the biological function of Rad was not fully understood until recently. Rad, as well as other members of the RGK protein subfamily, has been observed to play key roles in the regulation of intracellular Ca\textsuperscript{2+} dynamics and the remodeling of cell shape. In particular, Rad has been demonstrated to inhibit the activity of L-type Ca\textsuperscript{2+} channels by interacting with the \( \beta \)-subunit (3) and to reduce Rho/RhoA kinase (ROK) signaling by inhibiting ROK-mediated phosphorylation (28). In the present study, we have defined the expression profile of Rad during mammalian development and muscle regeneration, and, for the first time, the key myogenic factors responsible for the transcriptional regulation of Rad within skeletal muscle have been identified.

EXPERIMENTAL PROCEDURES

Animals. Adult male C57BL/6 (wild type) or mdx mice (C57BL/10ScSn) were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were approved and performed according to NIH and University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee guidelines. Like the human form of Duchenne muscular dystrophy, the functional dystrophin protein in

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mdx mice is not expressed, and therefore these animals have routinely been used as an animal model of the disease, although the myopathy observed in the mdx mouse is less severe than that observed in humans.

**Tissue preparations for in situ hybridization.** Acute injury was induced in the gastrocnemius muscles of adult (2–4 mo old) wild-type mice using a 150-μl intramuscular injection of 10 μM cardiototoxic (Naja nigricollis; Calbiochem, La Jolla, CA) (6). The gastrocnemius muscles of these mice were harvested at 6 or 12 h or at 1, 2, 5, 7, or 14 days postinjury. To determine the expression of Rad in tissues undergoing chronic degeneration-regeneration cycles, we harvested the gastrocnemius muscles and diaphragms from adult mdx mice. To assess the spatiotemporal expression of Rad during development, embryos were harvested from timed C57BL/6 pregnant female mice at 8.5, 11.5, and 15.5 days postcoitum.

Embryos and tissues were fixed overnight in 4% paraformaldehyde/diethyl pyrocatechol-PBS after administration of avertin-induced anesthesia and transection perfusion. Paraformaldehyde-fixed tissue was paraffin embedded for rotary microtomy.

Cardiac injury. After they were under continuous anesthesia with 2.0% isoflurane, mice underwent a left thoracotomy/pericardiotomy to induce anesthesia and transcardiac perfusion. Paraformaldehyde-fixed, 4-μm paraffin sections were cut, dehydrated, and dipped in K5 nuclear emulsion gel (Polysciences, Warrington, PA). Autoradiography was undertaken for a 21-day period. The sections were developed, and hybridized at 70°C. Riboprobes were diluted in 50% formamide, 0.1 M NaCl, 20 mM Tris·HCl (pH 8.0), 5 mM EDTA (pH 8.0), 10 μM NaPO₄ (pH 8.0), 10% dextran sulfate, 1 mM MgCl₂, 0.2 mM 2-deoxynucleotide 5'-triphosphate (1,000 Ci/m mole; Amersham, Piscataway, NJ) to produce the respective sense and antisense riboprobes. The Rad riboprobe was ~900 bp in length for sense and antisense probes. The probe was purified using MicroSpin G-50 columns (Amersham), analyzed for integrity, and stored at ~80°C overnight.

In situ hybridization. In situ hybridization technique was performed as previously described (23). Briefly, 5-μm paraffin sections were cut, mounted on Vectabond-coated slides, de waxed, permeabilized, acetylated, and hybridized at 70°C. Riboprobes were diluted in 50% formamide, 0.07 M NaCl, 20 mM Tris·HCl (pH 8.0), 5 mM EDTA (pH 8.0), 10 μM NaPO₄ (pH 8.0), 10% dextran sulfate, 1 mM MgCl₂, and 0.5 mg/ml transfer RNA. After hybridization, the residual pneumothorax was evacuated to restore negative pressure, and buprenorphine (0.05 mg/kg body wt) was administered for postoperative pain control. For each of the protocols (ligation or cryoinjury), mice were euthanized and their hearts excised at day 0 (no injury) or at 1, 3, 7, or 14 days postinjury. Total RNA was harvested, and RT-PCR analysis was performed as described below.

**Riboprobe synthesis.** An I.M.A.G.E. clone (no. 850142; I.M.A.G.E. Consortium) was verified using sequence analysis and prepared for in vitro transcription after restriction enzyme digestion and gel isolation. Linearized template (500 ng) was transcribed using either the T7 or T3 RNA polymerase (Ambion, Austin, TX) with 7.0 μM [α-35S]UTP (1,000 Ci/m mole; Amersham, Piscataway, NJ) to produce the respective sense and antisense riboprobes. The Rad riboprobe was ~900 bp in length for sense and antisense probes. The probe was purified using MicroSpin G-50 columns (Amersham), analyzed for integrity, and stored at ~80°C overnight.

**Transient transfection assays.** For transient DNA transfection, 2 × 10⁵ C2C12 cells per well were added to six-well plates and allowed to adhere overnight. After 24 h, cells were transiently transfected with the Rad promoter-reporter vector and either empty vector (pCI-neo) or overexpression vectors for MyoD (0.5 μg), Myf-5 (0.5 μg), myocyte enhancer factor 2C (0.75 μg), or constitutively active calcineurin A (0.5 μg of pEMSV-MyoD, pEMSV-Myf-5, pCDNA-MEF2C, or pCI-Neo-CaNa) using LipofectAMINE Plus lipid reagent and OptiMEM serum-free medium (Invitrogen) according to the manufacturer’s protocols. Twenty-four hours after transfection, cells were rinsed and harvested using lysis buffer (Promega). Luciferase activity was measured in cell lysates using a luciferase assay system (Promega) according to the supplier’s instructions. Transfection efficiencies were normalized by cotransflecting cells with pCMV-lacZ (0.1 μg) and measuring β-galactosidase expression.

Each experiment was performed in triplicate, and a dose-response curve (using the 2 kb Rad promoter-reporter vector) was performed to determine the optimal DNA concentration for each of the MyoD, Myf-5, and MEF2C overexpression plasmids. The concentration of each overexpression plasmid that provided the greatest luciferase expression from the 2 kb promoter was used in subsequent studies.

**Semiquantitative RT-PCR analyses.** Total RNA was isolated with TriPure isolation reagent (Roche Applied Science, Basel, Switzerland) and was reverse transcribed with SuperScript II (Invitrogen) to generate a promoter fragment 708 bp (0.7 kb) in length. The PCR products were ligated into the PCR2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, CA), digested from the PCR2.1 vector using restriction enzymes BamHI and XhoI, and ligated to the luciferase gene in the linearized pGL3Basic vector (XhoI and BglII; Promega, Madison, WI).

Immunohistochemical analysis of regenerating skeletal muscle. Immunohistochemical detection for Rad and MyoD was undertaken for frozen sections of regenerating skeletal muscle (10 days postinjury). Sections were blocked with appropriate normal sera, and endogenous peroxidase activity was quenched by incubating sections in 0.6% hydrogen peroxide/methanol. Sections were incubated overnight at 4°C with the polyclonal rabbit anti-Rad antiserum (1:50 dilution; generous gift from R. Kahn) and detected using Texas red-conjugated secondary antibody (1:25 dilution). Coexpression of MyoD was performed by incubating the above sections with the MAb MyoD (1:500 dilution; BD Biosciences) for 1 h at room temperature and detected using FITC-conjugated secondary antibody. All negative control sections (PBS substituted for primary antibody) had an absence of signal.

Construction of Rad promoter-reporter plasmid. A 2 kb 5' flanking region of the mouse Rad gene and a 5' deletion promoter fragment were generated by performing PCR from bacterial artificial chromosome clone RPCI-23. The 5' deletion of the 2 kb 5' flanking region generated a promoter fragment 708 bp (0.7 kb) in length. The PCR products were ligated into the PCR2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, CA), digested from the PCR2.1 vector using restriction enzymes BamHI and XhoI, and ligated to the luciferase gene in the linearized pGL3Basic vector (XhoI and BglII; Promega, Madison, WI).

Cell culture. Primary myogenic progenitor cells were harvested from hindlimb skeletal muscle of wild-type neonatal (2 day old) mice (6, 7). For RT-PCR analysis, the mRNA was harvested from these cells after 5 days in culture. C2C12 myogenic cells were grown as monolayers in DMEM supplemented with 20% FBS and antibiotics. This cell line was used for transient transfection of Rad promoter-reporter constructs and various myogenic transcription factors. For RT-PCR analysis, myotube differentiation was promoted by exposing 80% confluent myoblast cultures to differentiation medium (7).
GACCTGAAGCAGAA-3’, Rad reverse primer, 5’-CAAGGGCAGCTTTGAGAAAG-3’; and 18S rRNA forward primer, 5’-GGACCA-GAGCGAAAAGCATTAA-3’, 18S rRNA reverse primer, 5’-TGCCAGAGTCTCGTTCTGTTAT-3’.

**Microscopy and photomicrography.** Rad mRNA expression was visualized using a Leitz Laborlux-S microscope equipped with PlanApochromatic optics, a standard bright-field condenser, and a Mears low-magnification dark-field condenser. Photomicrographs were obtained with an Optronics VI-470 charge-coupled device camera and a Macintosh G3 Power computer using Scion Image 1.62 software.

**Data analysis.** Student’s *t*-tests were performed to identify significant differences (*P* < 0.05) in data obtained from control and experimental samples. Data are presented as means ± SE.

**RESULTS**

**Increased Rad mRNA expression during development.** Northern blot analysis demonstrated an overall increase in Rad mRNA expression during murine development from embryonic day 7 (day E7) to embryonic day 17 (day E17) (Fig. 1A). The 1.6-kb transcript detected using Northern blot analysis corresponded to the size expected for the full Rad mRNA transcript. The increase in Rad expression during development was confirmed and further analyzed using in situ hybridization (Fig. 1, B–D). At embryonic day 8.5 (E8.5), Rad mRNA expression was localized specifically to the developing heart. Persistent expression within the developing cardiac tissue was observed on day E11.5 with expression of Rad also in the developing somites. At day E15.5, Rad expression was observed in all muscle lineages, including the heart, striated muscle tissues (tongue, diaphragm, intercostal, and back musculature), and smooth muscles (esophageal). Note the lack of expression within the developing skeleton, hepatic, visceral, and nervous systems (Fig. 1).

**Regenerating skeletal muscle displays robust Rad expression.** Adult mouse heart demonstrated high Rad mRNA expression levels as detected using Northern blot analysis (Fig. 2A). In contrast to that observed in adult human skeletal muscle (18, 20), resting adult mouse skeletal muscle displayed low Rad mRNA signal (Fig. 2, A and B).

Although Rad expression was low in uninjured skeletal muscle, this expression was greatly elevated in response to

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![Fig. 1](http://ajpcell.physiology.org/)

**Fig. 1.** Rad expression is restricted to the muscle lineages during development. A: Northern blot analysis using total embryo RNA demonstrating an overall increase in Rad expression during embryonic mouse development from embryonic day 7 (E7) to embryonic day 17 (E17). A β-actin probe was used as a loading control. B–D: light (left)- and dark (right)-field images from in situ hybridization analysis of mouse embryonic development showing localization of the early (day E8.5) expression to the developing cardiac muscle (arrowhead) (B), whereas expression of Rad at day E11.5 was also observed within the somites (arrowheads) (C). At day E15.5 (D), Rad expression was observed in all muscle lineages, including the heart, tongue (closed arrowhead), diaphragm (open arrowhead), intercostals and back musculature (arrow), and esophageal smooth muscle. During development, the expression of Rad appears to be localized to the muscle lineages as shown by the lack of expression within the developing skeleton, hepatic, visceral, and nervous systems. Scale bars: A, 200 μm; B: 1 mm; C: 1 mm.
Increased Rad expression in response to damage in adult skeletal muscle. A: Northern blot analysis of adult tissues reveals robust expression within the heart, but negligible amounts are detected in other adult tissues at rest. B: low Rad expression in resting adult skeletal muscle is confirmed using in situ hybridization. Left, light-field image, and right, dark-field image of resting adult skeletal muscle after hybridization with a Rad probe. Scale bar, 100 μm. C: despite the low level expression of Rad at rest, microarray analysis demonstrated that expression was significantly increased as early as 6 h after injury and remained elevated up to 2 days postinjury. Relative change in Rad expression is related to that in resting skeletal muscle, with the actual values plotted on the graph next to the point. D: RT-PCR analysis confirmed the temporal changes in Rad expression observed using microarray analysis. E: RT-PCR performed on proliferating primary myogenic progenitor cells (~50% confluence) demonstrates detectable levels of Rad expression within this cell population. Within the proliferative phase (i.e., myoblast phase, abbreviated as MB in figure), C2C12 cells did not express detectable Rad expression until a high level of confluence (90%) was reached, because no expression was detected at 50% confluence (50%). Upon exposure to differentiation medium, C2C12 cells fuse to form myotubes (MT) and Rad expression is increased at all time points in differentiation medium from 15 h to 4 days. F: in contrast to the temporal regulation of Rad expression in regenerating skeletal muscle, no discernible increase in Rad mRNA expression levels was observed in adult myocardium from 1 to 14 days after ligation (LIG) of the left anterior descending coronary artery or the left ventricular artery cryoinjury (CRYO). Loading control is demonstrated using 18S ribosomal RNA RT-PCR.

Myogenic regulatory factor family of transcription factors bind to a canonical sequence (CANNTG), termed the E-box, on the promoter regions of key muscle-specific genes. The 2-kb promoter region of Rad contains two evolutionarily conserved E-box motifs (total of 4 E-boxes). Although truncation of the 2-kb Rad promoter (to create the 0.7-kb promoter construct) removed the two evolutionarily conserved E-boxes (Fig. 5A), the capacity of MyoD or Myf-5 to transactivate the Rad promoter was not reduced, indicating that the remaining E-box located within the 0.7-kb Rad promoter was capable of activating Rad transcription in response to MyoD and Myf-5 transcription factors (Fig. 5B).
There are two identifiable MEF2 binding motifs within the 2-kb upstream Rad promoter fragment (Fig. 5A). Overexpression of MEF2C in the presence of the Rad 2- and 0.7-kb promoter-reporter constructs resulted in small but significant increases in luciferase expression.

MyoD, Myf-5, or MEF2C overexpression in the COS-7 fibroblast cell line was ineffective in increasing Rad promoter-reporter luciferase expression. This suggests that factors within C2C12 muscle cells, but not in COS-7 cells, were necessary for these transcription factors to function effectively on the Rad promoter (data not shown).

There are two nuclear factor of activated T cell (NFAT) binding motifs located within the 2-kb Rad promoter with the proximal site demonstrating evolutionary conservation (Fig. 5A). Truncation of the 2-kb Rad promoter to generate the 0.7-kb promoter construct removed these NFAT binding motifs. Overexpression of activated calcineurin was capable of transactivating both the 2- and 0.7-kb Rad promoter constructs (Fig. 5C), although the increase observed in association with the 0.7-kb Rad promoter fragment was significantly lower than that observed in association with the 2-kb construct. Because calcineurin has the capacity to act through the transcription factors MyoD and MEF2, the effects of co-overexpressing activated calcineurin and these myogenic transcription factors in the presence of Rad promoter constructs was investigated. As shown in Fig. 5C, overexpression of activated calcineurin with any of the myogenic factors alone or in combination was capable of significantly increasing luciferase expression in the...
2-kb Rad promoter construct above that observed with activated calcineurin alone, with no difference between any of the myogenic factors (MyoD, Myf-5, or MEF2C) regarding their capacity to increase Rad promoter activity in the presence of calcineurin. This increased promoter activity with overexpression of activated calcineurin and the myogenic factors was not observed upon truncation to the 0.7-kb Rad promoter construct (Fig. 5C), suggesting that the NFAT sites may be critical for the transcriptional regulation of Rad in response to the myogenic regulatory factors and MEF2.

**DISCUSSION**

In the present study, we have characterized the expression pattern of Rad during mammalian development and skeletal muscle regeneration and, for the first time, identified key myogenic factors responsible for the transcriptional regulation of Rad within skeletal muscle. Our findings demonstrate minimal Rad expression within resting adult skeletal muscle; however, in response to muscle injury, Rad expression is significantly increased and is localized to the myogenic progenitor cell population during the early phases of regeneration and within the newly regenerated myofibers during the later phases of regeneration. Taken together, our data strongly support the hypothesis that the Rad-positive cells within regenerating skeletal muscle are myogenic progenitor cells. However, future studies are needed to confirm our present findings.

The present study has demonstrated that known myogenic transcription factors can increase the transcriptional activity of the Rad promoter and that this ability is significantly enhanced by activated calcineurin and the presence of a conserved NFAT binding motif within the Rad promoter. On the basis of the current findings, we propose that Rad might play a dual role in the regulation of the myogenic progenitor cell during skeletal muscle regeneration. The early, robust expression of Rad within the myogenic progenitor cell is consistent with the role of Rad in the inhibition of L-type Ca$^{2+}$ channels and thereby the prevention of myoblast differentiation (3, 19), whereas the later expression of Rad within newly regenerated myofibers results in the downregulation of Rho/ROK signaling, which is necessary for myoblast fusion and subsequent terminal differentiation (14, 28).

**Developmental expression of Rad is limited to muscle lineages.** As observed in the day E15.5 embryo, Rad expression is localized to the muscle lineages, with the heart, diaphragm, intercostals, tongue, esophageal smooth muscle, and back musculature all exhibiting high signal intensity (Fig. 1). The lack of expression within the nonmuscle lineages of the developing embryo is consistent with our finding that key muscle regulatory factors are involved in the transcriptional control of Rad expression. In contrast to the developing embryo and adult human skeletal muscle (18, 20), Rad demonstrates low-level expression in resting adult mouse skeletal muscle similar to that observed in the newt homolog of Rad (nRad), which displays ~68% homology to the mouse and human Rad genes (24). Although Rad expression is low in resting skeletal muscle, this level is dramatically increased during skeletal muscle regeneration. We speculate that within skeletal muscle, Rad might play an analogous role during development and regeneration. This assumption is based on the observation that the events associated with development in which muscle precursor cells proliferate and fuse to form muscle fibers recapitulate, in part, events that occur during skeletal muscle regeneration (5).

**Rad expression in regenerating myocardium.** An increase in nRad expression has been observed in the regenerating newt heart after injury (24). Within normal adult mouse myocardium, the high level of Rad expression would suggest an important role for Rad in cardiomyocyte function. This hypothesis is supported by the finding that a nonsynonymous, single-nucleotide polymorphism within the Rad gene has been linked to congestive heart failure (8). Despite an increase in Rad expression after skeletal muscle injury (present study) or myocardial injury in the newt (24), neither ligation nor cryoinjury-induced myocardial damage produced a detectable increase in Rad expression in the present study. Interestingly, like mammalian myocardium, the cardiomyocytes of the adult...
Fig. 5. Transcriptional regulation of Rad by myogenic factors and calcineurin. A: presence of nuclear factor of activated T cell (NFAT), E-box, and myocyte enhancer factor (MEF)2 binding motifs on the Rad 2.0- and 0.7-kb promoter constructs. One NFAT and two E-box binding motifs on the mouse Rad promoter displayed evolutionary conservation as determined using the TRANSFAC database. B: overexpression of MyoD, Myf-5, and MEF2C resulted in a significant increase in luciferase expression in both the 2.0- and 0.7-kb promoter constructs compared with the full-length 2-kb promoter activity. Co-overexpression of MyoD, Myf-5, and MEF2C alone or in combination resulted in a robust increase in 2-kb promoter activity that was significantly greater than that with calcineurin alone (denoted by a). The calcineurin-induced increase in Rad promoter activity was significantly reduced in the 0.7-kb construct compared with the full-length 2-kb construct (denoted by b). n = 3 for each experiment. *P < 0.05, significantly greater than empty vector.
signaling cascade negatively (28), allowing for nuclear translocation of the forkhead in human rhabdomyosarcoma transcription factor and ultimately myoblast fusion (14). Furthermore, Zhu et al. (30) demonstrated an interaction of Rad with the structural protein β-tropomyosin, supporting its role in cell cytoskeletal organization and skeletal muscle differentiation.

Taken together, we propose that Rad might play a dual role within the myogenic progenitor cell population during skeletal muscle regeneration. Our hypothesis is that, initially, Rad prevents myogenic progenitor cell differentiation through inhibition of L-type Ca\(^{2+}\) channel activity. This function would be distinct from its role during the later stages of regeneration, when Rad would inhibit the Rho/ROK signaling cascade, thus allowing for myogenic progenitor cell fusion and ultimately terminal differentiation.

Transcriptional regulation of Rad. On the basis of the localization of Rad expression to the muscle lineage during development and regeneration, we investigated the capacity of myogenic factors known to be important in skeletal muscle regeneration to regulate the transcriptional activity of Rad.

The myogenic regulatory factors MyoD and Myf-5 bind a canonical binding motif known as an E-box and are associated with the regulation of the myogenic progenitor cell population in response to muscle damage (2, 21, 26). Although both MyoD and Myf-5 are important in the activation and proliferation of myogenic progenitor cells, they exhibit distinct expression profiles during skeletal muscle regeneration. On the basis of the microarray analysis results, MyoD displays a temporal expression pattern that parallels that of Rad, whereas Myf-5 is low during the early regenerative phase and is increased during the later stages in which myogenic progenitor cells undergo fusion to regenerate the myofiber population (4). These data suggest that MyoD may be involved in the early regulation of Rad transcription in response to injury, whereas Myf-5 may aid in the transcription of Rad during the later phases of muscle regeneration.

The MADS box (MCM1, agamous deficiens, and serum response factor) transcription factor family member MEF2 has been shown to play a key role in skeletal muscle differentiation (17). Preliminary studies conducted in our laboratory using MEF2 indicator mice (13, 29) confirmed a high level of MEF2 protein expression during the later stages of muscle regeneration in which newly regenerated myofibers were visible (5–10 days postinjury: data not shown). The late expression of MEF2 during skeletal muscle regeneration suggests that this transcription factor is involved in the regulation of Rad expression during myogenic progenitor cell differentiation.

In response to skeletal muscle injury, calcineurin protein expression is upregulated as early as 1 day postinjury and remains elevated for up to 14 days. In addition, the NFATc1 downstream target of calcineurin also displayed an increase in activated (dephosphorylated) protein levels from 1 to 6 days postinjury (22). Whether calcineurin or NFATc1 expression was increased before 1 day was not assessed in the study, however.

The coexpression of activated calcineurin with the other myogenic transcription factors resulted in Rad promoter activity levels that were far greater than expected as an additive effect. This robust increase in Rad promoter activity was dependent on the presence of the 2-kb Rad promoter. We suggest that this increase was dependent on the presence of a conserved NFAT binding motif within the 2-kb Rad promoter, although the reasons for this phenomenon have yet to be elucidated. Although it is conceivable that calcineurin increases the affinity of Myf-5 and MyoD for the evolutionarily conserved E-boxes that are removed in the 0.7-kb promoter construct, this hypothesis would not explain the results observed in association with coexpression of calcineurin and MEF2C.

Conclusion. In summary, we have demonstrated the expression pattern and transcriptional regulation of Rad, a prototypic member of the RGK family of novel Ras-related GTPases, during mammalian development and skeletal muscle regeneration. The expression pattern of Rad during skeletal muscle regeneration is consistent with its proposed role in attenuating myogenic progenitor cell fusion during the initial period after injury and its role in promoting terminal differentiation during the later stages of regeneration. We have also demonstrated that myogenic transcription factors (MEF2, MyoD, and Myf-5) that are expressed during skeletal muscle regeneration can increase the transcriptional activity of the Rad promoter and that this ability is significantly enhanced by the presence of activated calcineurin. Furthermore, this enhanced transcriptional activity appears to be dependent on the presence of a conserved NFAT binding motif within the Rad promoter. Together, these data define Rad as a novel factor within the skeletal muscle regenerative process. Future studies will aid in further elucidation of the role and regulation of this novel protein.

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