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ERK6 is expressed in a developmentally regulated manner in rodent skeletal muscle☆

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Abstract

Rat ERK6, also known as SAPK3 and p38γ, exhibits a distinct pattern of expression during muscle development in vitro and in vivo. Levels of mRNA transcript and protein abundance for ERK6 are increased during the differentiation of 2 rodent myoblast cell lines in culture. This is in contrast to the expression of other MAP kinase family members, namely p42/p44 MAPK and p38 MAPK, whose expression does not change during myogenesis. Similar results are observed in vivo where ERK6 mRNA levels increase with post-natal development in rat hindlimb mixed muscle samples. These results delineate a distinct pattern of ERK6 expression in mature skeletal muscle cells and suggest a specific role for ERK6 in muscle development or muscle function.

Keywords: Kinase; Signaling; Skeletal muscle; ERK; MAPK; Muscle cell line

The mitogen activated protein kinase (MAPK) family of Ser/Thr kinases is comprised of five members that respond to various stimuli and regulate a wide variety of biological responses (reviewed in [1,2]). The five families are the extracellular-regulated protein kinases 1/2 (ERKs), stress-activated protein kinases (SAPKs), c-Jun N-terminal protein kinases (JNKs), ERK3/4, and ERK5. These kinase families differ in their regulation by upstream kinases and in the distinct downstream targets that they phosphorylate. Human ERK6 is a relatively little studied MAPK family member whose expression is predominantly in skeletal muscle [3]. It was also isolated as human p38γ [4] and rat SAPK3 [5]. There is greater than 95% homology at the amino acid level in these isolates, and it is therefore likely that these sequences derive from the same gene. The expression of human ERK6 and human p38γ was reported to be restricted to skeletal muscle when tested in human tissue samples [3,4]. Although rat SAPK3 was predominantly expressed in skeletal muscle, less abundant amounts were also observed in lung, heart, and brain samples from rat tissue [5], a result recently confirmed at the protein level [6].

The genetic program that regulates myogenesis is well understood and members of the muscle regulatory family (MRF) of transcription factors are known to be key modulators of this process [7,8]. These proteins are expressed exclusively in muscle tissue and coordinate the genetic program leading to the expression of enzymes and structural proteins that are important for muscle function. However, the upstream signals that regulate this process are not well defined. Members of the MAPK family have been implicated in controlling various aspects of myoblast growth and differentiation. For example, ERK1/2 (p42/p44 MAPK) has been shown to promote myoblast growth and inhibit muscle cell differentiation [9–11]. In addition, p38α and/or p38β activity is induced during myogenesis [12,13] and may result in phosphorylation of the myocyte enhancer family (MEF2) transcription factor, another protein known to play a role in controlling myogenesis [14]. Although these signal transduction pathways have been implicated in muscle differentiation, the specific

☆ *Abbreviations:* MAPK, mitogen-activated protein kinase; ERK, extracellular-regulated protein kinase; SAPK, stress-activated protein kinase.

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myogenic targets of each signaling pathway are not defined. Because of the high level of expression of ERK6 mRNA in skeletal muscle, we hypothesized that it may function as a key regulator in myogenesis. Here, we describe the cloning of the rat isoform of ERK6 and show that it is expressed predominantly in skeletal muscle. ERK6 is also expressed in various muscle cell lines in a differentiation dependent manner as both ERK6 mRNA and protein are detected in fully differentiated cells, but not in myoblasts. Furthermore, we provide evidence that ERK6 is up-regulated during muscle development in vivo. These data suggest that ERK6 may play a fundamental role in regulating or maintaining the differentiated phenotype.

Materials and methods

Animals. Male and pregnant female Sprague–Dawley rats were purchased from Taconic Breeding Laboratory. The pregnant rats were due within one week of arrival. The animals had free access to water and standard rat chow. Rats were sacrificed by CO_2 asphyxiation and then dissections were performed immediately. On the day of the study, the tissues were frozen in liquid nitrogen and stored at −80 °C until RNA extraction was performed.

Cell lines. The myoblast cell lines, C2C12 [15,16], L6 [17], and Sol8 [18] were maintained at subconfluent conditions in growth media containing DMEM with 4.5 g/L glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% fetal bovine serum (FBS). Near confluent cells (~75–80% confluence) were differentiated by lowering the serum concentration to 2% calf serum (CS) and were maintained for four to five days to obtain myotubes. All cells were grown in a humidified, 37 °C incubator with ambient oxygen and 5% CO_2. The media were changed every 24 h in all experiments.

Isolation of total cellular protein. Cells were washed three times on ice with PBS, pH 7.4, and then lysed on ice for 10 min in 20 mM Tris, pH 7.4, 50 mM NaCl, 2% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, and 1 mM PMSF. Lysates were cleared of insoluble material by centrifugation at 10,000*g* at 4 °C and the resulting supernatant was used for analysis.

Gel electrophoresis and Western blot analysis. Proteins samples were diluted in sample buffer to a final concentration of 50 mM Tris, pH 6.8, and 10% glycerol with or without 100 mM dithiothreitol. Proteins were separated on sodium dodecyl sulfate–polyacrylamide gels [19] and then transferred to 0.2 μm PVDF membrane using a buffer containing 25 mM Tris and 192 mM glycine at 4 °C for 1600 mA h. Membranes were blocked in 10% non-fat milk in PBS-T (PBS plus 0.1% Tween 20), before incubating for 1 h at room temperature with primary antibody. Membranes were then washed in PBS-T and incubated with horseradish-peroxidase conjugated secondary antibodies. Protein bands were detected using enhanced chemiluminescence reagents (NEN). The antibodies used in this study are from the following sources: anti-ERK6 antibody (anti-p38γ) (Dr. Joseph Portanova, Department of Autoinflammatory Disease Research, G.D. Seale, St. Louis, MO), anti-myogenin antibody (F5D monoclonal serum) (Developmental Studies Hybridoma Bank, University of Iowa), anti-pan ERK antibody (Transduction Labs), and anti-pan p38 antibody (Cell Signaling Technology).

RNA isolation. Total RNA was obtained as described by Chomczynski [20]. Cells were washed three times with PBS and then lysed in Solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 0.1 M β-mercaptoethanol). For rat tissue samples, the tissues were pulverized under liquid nitrogen with a mortar and pestle before the addition of Solution D. Sodium acetate, pH 4.2, was added to a final concentration of 200 mM and RNA was extracted with 1 volume of acid phenol (pH 4.2) and 0.1 volume of chloroform:isoamyl alcohol. The aqueous phase was collected and the total RNA was precipitated in isopropanol at −20 °C, followed by centrifugation at 10,000*g* at 4 °C for 20 min.

Poly(A)$^+$ enriched RNA was isolated from 1 mg of total RNA using oligo dT cellulose (Beckton–Dickinson, Franklin Lakes, NJ) according to the manufacturer's instructions. The poly(A)$^+$ RNA was eluted with 3 volumes of 10 mM Tris, pH 7.6, 1 mM EDTA, and 0.05% SDS. The RNA in the eluate was precipitated in 0.3 M sodium acetate, pH 4.2, and 2 volumes of 95% ethanol, followed by centrifugation at 10,000*g* for 20 min at 4 °C.

Labeling of cDNA probes by the random priming method. The cDNA probes were labeled with [^{32}P]dATP by random priming using the method of Feinberg [21]. Approximately 0.1–0.2 μg of denatured cDNA fragment was labeled with 50 μCi [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I (Promega Corp) in a reaction mixture containing random primer, dCTP, dTTP, dGTP, BSA, DTT, and $MgCl_2$. The reaction mixture was incubated at 37 °C for 4–6 h and then labeled probes were purified from unincorporated nucleotides using a NucTrap column as per the manufacturer's instructions (Stratagene, La Jolla, CA). The cDNA fragments used for probes were as follows; rat ERK6 full length cDNA and rat ERK6 *Eco*RI and *Bst*EII digest fragment of 513 bp.

Northern blotting analysis. Equal amounts (20 μg of total RNA or 5 μg of Poly(A)$^+$ RNA) were separated on a 1% agarose/6% formaldehyde gel and transferred to Genescreen nylon membrane (NEN) by capillary action using 10× SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0) as the liquid phase. The RNA was crosslinked to the membrane using the UV Stratalinker (Stratagene). RNA was checked for equal loading and transfer by UV visualization of ethidium bromide rRNA staining (data not shown). Prehybridization of nylon membranes was for 4–6 h at 42 °C in 50% formamide, 4× SSC, 5× Denhardts (0.1% ficoll, 0.1% polyvinyl pyrrolidone, and 0.1% BSA), 0.05 M sodium phosphate, pH 7.0, 0.5 mg/ml sodium pyrophosphate, 1% SDS, and 0.1 mg/ml transfer RNA carrier. Hybridization of the blot was for 16–20 h at 42 °C in 50% formamide, 4× SSC, 1× Denhardts, 0.05 M sodium phosphate, pH 7.0, 0.5 mg/ml sodium pyrophosphate, 1% SDS, and 0.1 mg/ml transfer RNA carrier, and the labeled probe. The specific activity of the probes was at least 10^7 cpm/ml. Blots were washed in a stepwise gradient at moderate stringency (0.2× SSC/0.1% SDS at 42 °C) and exposed to film at −80 °C.

Cloning of rat ERK6. To clone the rat ERK6 cDNA, a fragment of the human ERK6 cDNA was used to screen 5′ Stretch cDNA skeletal muscle library (Clontech, Palo Alto, CA). The probe was prepared by RT/PCR as follows: Human skeletal muscle total RNA (Clonetech) was used as a template in the reverse transcription reaction. One μg of total RNA was annealed to oligo N(6) random hexameric primer (Amersham Pharmacia Biotech) by heating to 65 °C and then cooling on ice. The reverse transcription reaction was carried out as per the manufacturer's instructions. The reaction contained 20 mM DTT, 1 mM dNTP, an RNAse inhibitor; RNAsin, 1000 U of Superscript II reverse transcriptase (Gibco-BRL) in a buffer of 50 mM Tris, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, and 0.1 mg/ml BSA and was incubated at 37 °C for 1 h.

PCR was performed using one quarter of the RT reaction. Primers (Integrated DNA Technology, Coralville, IA) specific for the human ERK6 gene were as follows:

forward primer: 5′ AGTTTCTTTCCCATCCCTACTTCG 3′;

reverse primer: 5′ GTCTATTTCCTTCCAGCCACGC 3′.

In general, PCR mixtures contained the following: 10 μM of each primer, 2.5 mM $MgCl_2$, 1 mM dNTP, 1× PCR buffer plus Q solution, and 2.5 U of Taq polymerase (Qiagen) in a final reaction volume of 30 μl. Reactions were subjected to thermocycling under the following conditions: initial denaturation at 94 °C for 4 min, followed by 35 rounds of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min,

and elongation at 72 °C for 2 min. An extended elongation step at 72 °C for up to 10 min was added at the end of the cycling process. The entire PCR was separated on a 1% agarose gel in TAE (40 mM Tris–acetate and 1 mM EDTA) and the resulting bands were cut from the gel and extracted using a Genelute spin column (Supelco, Bellefonte, PA). The purified PCR products were ligated into the pCR 2.1 vector using the TA Cloning kit as per the manufacturer's instructions (In-Vitrogen, Carlsbad, CA). A 677 bp human ERK6 cDNA fragment was isolated by restriction digestion of the subcloned pCR 2.1 vector with *Eco*RI and subsequently used as a probe to screen a rat skeletal muscle cDNA library (Clontech).

Library plating, filter lifting, and hybridization were performed as described in the manufacturer's instructions (Clonetech). Eight 150 mm agar plates each containing approximately 50,000 plaque forming units (pfu) were transferred to a Colony/Plaque Screen Nylon Hybridization membrane (NEN) and hybridized to the 677 bp human ERK6 probe as described by the manufacturer. Positive plaques were further screened until single positive clones were obtained. Lambda phage cDNA was purified using the NucleoBond Combi kit (Clonetech) and the isolated cDNA's were sequenced using λgt11 primers.

DNA sequencing. Plasmid DNA was sequenced at the DNA CORE at Boston University School of Medicine using a DNA sequencer (model 377) from Applied Biosystems (Foster City, CA).

Results

Cloning of the rat isoform of ERK6

Rat ERK6 cDNA was cloned from a rat skeletal muscle library (Clonetech) based on the assumption that its DNA sequence would be homologous to that of human ERK6 [3]. A cDNA probe encoding nucleotide residues 944–1620 of the human ERK6 coding sequence was made by RT-PCR using a human skeletal muscle cDNA as the template. This probe was used to screen the skeletal muscle cDNA library and eight positive clones were obtained and sequenced. All eight sequences were determined to be from the same gene and encompassed the entire coding region of rat ERK6. Analysis of the obtained sequences by searching GenBank using blastn allowed us to determine that all the cloned cDNA's were 94% homologous to human ERK6 (Accession No. X79483) and 96% homologous to rat (Accession No. X96488) and mouse (Accession No. Y13439) SAPK3 at the DNA level. The slight differences in DNA sequence between rat ERK6 and rat SAPK3 may be due to a polymorphism in the gene.

Tissue distribution of rat ERK6/SAPK3

Human ERK6 and p38γ show an expression pattern that is restricted to skeletal muscle, at least at the level of exposure of the Northern blots shown [3,4], and rat SAPK3 is largely expressed in this tissue [5]. To determine if rat ERK6 also has this specificity, we examined its distribution in various rat tissues by Northern blot analysis. The MAPK family of signaling molecules is highly conserved, particularly in domains I (nucleotide binding domain), VI (Ser/Thr kinase domain), and VIII (Ser/Thr kinase catalytic indicator) and also in the dual phosphorylation site (T–X–Y) in the activation loop. In conserved regions of the rat ERK1, ERK2, and ERK6 sequences, there is as much as 60% identical DNA sequence. Therefore, we used a segment of the cloned ERK6 cDNA as a probe for Northern blot analysis. The full length cDNA was digested simultaneously with the restriction enzymes, *Eco*RI and *Bst*EII, resulting in three fragments. A 513 bp fragment that corresponds to amino acids 16–187 in the ERK6 sequence and encodes the predicted domains I–IV was purified and then used as a probe for Northern blot analysis (Fig. 1). A transcript of approximately 2 kb was the predominant mRNA species identified using this method and it was detected primarily in skeletal muscle (lower arrow). Bands of the same size that were expressed to a much lesser extent were observed in fat, cardiac muscle, and lung tissue. In addition a high molecular weight mRNA species was detected at low levels in both skeletal and cardiac muscle (upper arrow).

ERK6 mRNA expression is induced during muscle cell differentiation

We next determined whether ERK6 was expressed in myoblasts or if its expression was limited to fully differentiated myocytes. Three different skeletal muscle cell lines were examined: mouse C2C12 cells, rat L6 cells, and mouse Sol8 cells. The expression of ERK6 mRNA during the differentiation of each cell line was examined by Northern blot analysis. Myoblasts from each line were switched to 2% calf serum in DMEM (day 0) and total RNA was collected on the indicated days. Northern

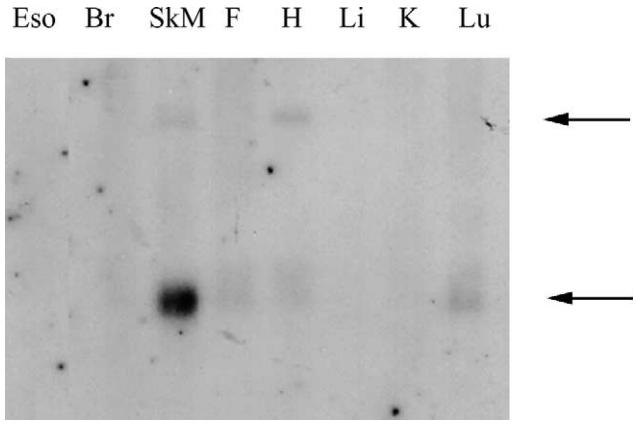

Fig. 1. Determination of ERK6 tissue specificity. Total RNA was isolated from the indicated rat tissues as described in Materials and methods. Equal amounts (20 μg) were separated on an agarose/formaldehyde gel followed by transfer to a nylon membrane. Blots were probed with at least 10^7 cpm/ml of purified ^{32}P-labeled ERK6 cDNA fragment corresponding to a 513 bp *Eco*RI/*Bst*EII fragment. Tissue abbreviations are as follows: Eso, esophagus; Br, brain; SkM, skeletal muscle; F, fat; H, heart (cardiac muscle); Li, liver; K, kidney; and Lu, lung. Arrows indicate the hybridizing bands.

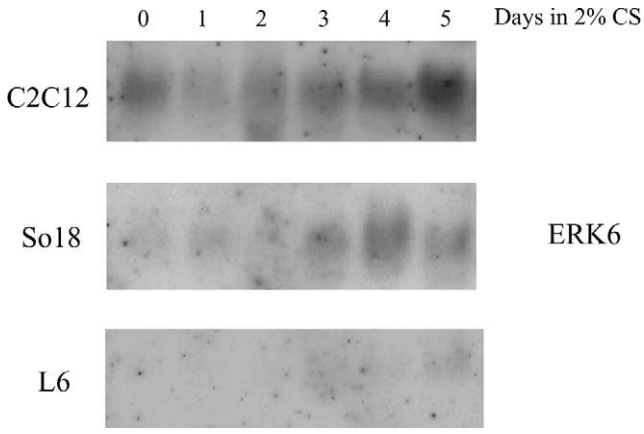

Fig. 2. ERK6 mRNA expression increases during C2C12 and Sol8 differentiation. C2C12, Sol8, and L6 myoblasts were grown to 80% confluency and then switched to 2% CS (day 0). On the indicated day, total RNA was collected as described in Materials and methods. Equal amounts (20 μg) from each sample were separated on a denaturing agarose gel followed by transfer to a nylon membrane. Northern blotting analysis was performed using at least 10^7 cpm/ml of purified ^{32}P-labeled full length ERK6 cDNA probe. These results are representative of two separate experiments.

blotting analysis was performed using full length ERK6 cDNA as a probe (Fig. 2). The expression of ERK6 mRNA was minimal in C2C12 and Sol8 myoblasts, but its expression increased with differentiation of both cell lines. On the other hand, ERK6 mRNA could not be detected in either L6 myoblasts or myocytes.

ERK6 protein is up-regulated during muscle cell differentiation

To determine if the increase in ERK6 mRNA resulted in a concomitant rise in ERK6 protein in the various muscle cells lines Western blot analysis was performed (Fig. 3). Again, myoblasts from each cell line were switched to 2% calf serum (day 0) and total protein was collected on the indicated day. NIH3T3 fibroblast cell lysates were used as a control for non-specific bands. Both C2C12 and Sol8 cells express ERK6 protein starting on day 2 of differentiation and levels increased thereafter (Fig. 3, arrow indicates ERK6). ERK6 protein was not detectable in L6 myoblasts or myotubes and this is consistent with the absence of ERK6 mRNA in these cells as shown in Fig. 2. As a control for differentiation-specific protein expression, the muscle specific transcription factor, myogenin was also examined during the differentiation time course. As expected, myogenin was not appreciably expressed in myoblasts (day 0), but its expression is induced as differentiation progresses. These data also confirm that all of the cell lines tested are differentiation competent and, therefore the reason for the lack of ERK6 in L6 cells is not due to their inability to differentiate. The expression of two other MAPK family members, namely p38 MAPK and p42/p44 MAPK (ERK1/2), was detected in both myoblast and myocyte populations in all three cell lines tested and does not significantly change as differentiation progresses.

ERK6 mRNA expression is up-regulated in rat skeletal muscle during post-natal development

We next determined whether ERK6 expression is induced during rat skeletal muscle development in vivo. Pregnant rats were obtained and following birth of the pups, mixed hindlimb muscle samples were dissected at various time points after birth. Poly(A)$^+$ enriched RNA was isolated and subsequent analysis was by Northern blot using a probe specific for ERK6 (Fig. 4). Little ERK6 mRNA was detected at 2 days after birth, while maximum expression was achieved by 16–19 days postpartum. Thereafter, its level of expression was maintained into adulthood. Taken together, these data sug-

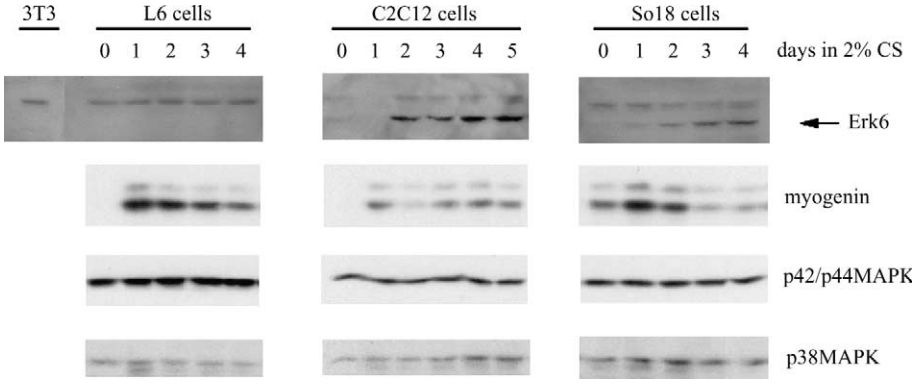

Fig. 3. ERK6 protein expression increases during C2C12 and Sol8 differentiation. C2C12, Sol8, and L6 myoblasts were grown to 80% confluency and then switched to 2% CS (day 0). On the day indicated, total protein was collected from these and NIH-3T3 fibroblasts. Equal amounts (100 μg) were separated by SDS–PAGE and analyzed by Western blot analysis using antibodies specific for ERK6, p38 MAPK, p42/p44 MAPK, and myogenin. Arrow indicates the band corresponding to ERK6. These results are representative of three separate experiments.

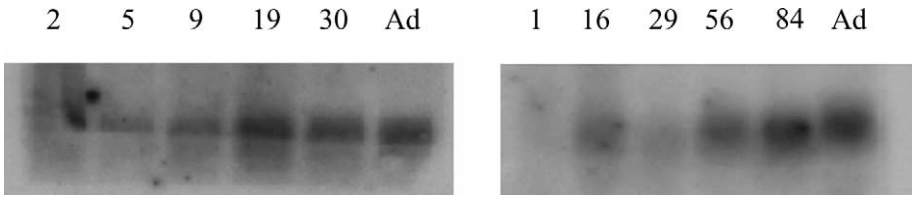

Fig. 4. ERK6 expression is up-regulated during rat development in vivo. Total RNA from rat hindlimb was isolated on the indicated day after birth and then enriched for Poly(A)$^+$ RNA. Equal amounts of RNA (5 μg) from each time point were separated on an agarose/formaldehyde gel, transferred to a nylon membrane, and probed with purified ^{32}P-labeled full length ERK6 cDNA probe. Two individual experiments are shown. Ad, adult.

gest that ERK6 is developmentally regulated and could be involved in regulation of the mature muscle phenotype.

Discussion

To identify signal transduction cascades that regulate skeletal muscle cell differentiation, numerous laboratories have examined known signaling cascades, including those utilizing MAPK/ERK, PI3-K, and SAPK. The expression of these signaling molecules is not tissue restricted and thus the cloning of human ERK6 attracted our interest as a possible mediator of muscle cell differentiation, since it was reported to be expressed highly in human skeletal muscle. Around the same time that human ERK6 was cloned, two related kinases were isolated; p38γ was cloned from a human skeletal muscle cDNA library and SAPK3 from a rat brain cDNA library and both were shown to be highly expressed in skeletal muscle. Upon closer examination of the cDNA sequences, it could be determined that ERK6, p38γ, and SAPK3 were likely to be the same proteins, even though they were classified into three distinct kinase families because of the manner in which they were cloned.

In our effort to identify signaling molecules that might regulate muscle cell differentiation, we cloned the rat isoform of ERK6 based on its homology to the human sequence. Northern blot analysis indicates that rat ERK6 is highly expressed in skeletal muscle, but not elsewhere. Little to no ERK6 is expressed in other muscle types, including cardiac (heart) and smooth (esophagus) muscle, or in other insulin sensitive tissues such as fat and liver. A larger and less abundant transcript is present in skeletal and cardiac muscle, but the nature of this species is not known. A recent manuscript has suggested that SAPK3 (ERK6) is expressed to significant levels in fast, slow, and mixed fiber types of murine skeletal muscle and in cardiac muscle [6].

We also observed that ERK6 expression is induced during post-natal rat development and as differentiation progresses in C2C12 and Sol8 cells. These results are consistent with previous data from C2C12 cells that showed ERK6 transcripts were more abundant in myocytes than myoblasts [3]. Recently, it was reported that ERK6 expression in C2C12 cells is regulated during differentiation by the p38 and mammalian target of rapamycin (mTOR) pathways [12]. This study did not address what extracellular factor(s) may contribute to ERK6 expression, but one could speculate that IGFs are involved since C2C12 cells express these growth factors as they differentiate [22,23], and IGFs are known to promote myogenesis [24,25].

Its distinct tissue distribution, along with its regulated expression during development, suggests that ERK6 has a specialized function in skeletal muscle and perhaps plays a role in achieving or maintaining the mature phenotype. It appears that ERK6 expression alone is not sufficient to promote differentiation, since its forced expression did not result in up-regulation of myogenin in either myoblast or fibroblast cells (data not shown). It is possible that ERK6 is not activated in these transfected cells and its activation could be required to support differentiation. We made numerous attempts to stably transfect C2C12 cells with ERK6 cDNA so that we could determine the possible downstream targets and upstream activators of ERK6. However, in our hands chronic overexpression of ERK6 in C2C12 cells results in their death (data not shown). However, Lechner previously reported that C2C12 myoblasts transduced with wild-type ERK6 displayed an enhanced fusion rate compared to control virus infected cells and a mutant ERK6 (Y185F) could prevent differentiation [3]. Little is known about the factors that activate ERK6 in vivo and the lack of inhibitors that block ERK6 activity [26] has hindered progress. Still, we can speculate that because ERK6 is expressed late during differentiation, it could support late differentiation events such as myoblast fusion or play a role in exercise-regulated metabolism [27].

Acknowledgments

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