Calcineurin Activity Is Required for the Initiation of Skeletal Muscle Differentiation

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Abstract. Differentiation of skeletal muscle myoblasts follows an ordered sequence of events: commitment, cell cycle withdrawal, phenotypic differentiation, and finally cell fusion to form multinucleated myotubes. The molecular signaling pathways that regulate the progression are not well understood. Here we investigate the potential role of calcium and the calcium-dependent phosphatase calcineurin in myogenesis. Commitment, phenotypic differentiation, and cell fusion are identified as distinct calcium-regulated steps, based on the extracellular calcium concentration required for the expression of morphological and biochemical markers specific to each of these stages. Furthermore, differentiation is inhibited at the commitment stage by either treatment with the calcineurin inhibitor cyclosporine A (CSA) or expression of CA IN, a physiological inhibitor of calcineurin. Retroviral-mediated gene transfer of a constitutively active form of calcineurin is able to induce myogenesis only in the presence of extracellular calcium, suggesting that multiple calcium-dependent pathways are required for differentiation. The mechanism by which calcineurin initiates differentiation includes transcriptional activation of myogenin, but does not require the participation of NFAT. We conclude that commitment of skeletal muscle cells to differentiation is calcium and calcineurin-dependent, but NFAT-independent.

Key words: calcium • myogenesis • signal transduction • calcineurin • myogenin

Introduction

Development of skeletal muscle consists of a highly regulated, temporally distinct sequence of events. A model for myogenesis has been proposed by Andres et al. (1996) based on immunofluorescence studies of single cells. In this model, myoblasts first commit to the differentiation pathway, a step marked by the expression of the bHLH transcription factor myogenin, followed by terminal cell cycle withdrawal and the induction of the cell cycle inhibitor p21. In later stages of myogenesis, the cells phenotypically differentiate, marked by contractile gene expression, and then fuse into multinucleated myotubes. That commitment and cell cycle withdrawal is required before phenotypic differentiation and cell fusion is supported by both in vivo and in vitro studies. Mice lacking myogenin have a severe reduction in the appearance of muscle-specific proteins and myotubes (Hasty et al., 1993). Inactivation of two apparently redundant cell cycle inhibitors, p21 and p57, results in a phenotype nearly identical to the inactivation of myogenin (Zhang et al., 1999). Treatment of BC3H-1 cells with a myogenin antisense oligomer blocks myoblast fusion and results in nearly complete inhibition of acetylcholine receptor protein expression (Brunetti and Goldfine, 1990). That phenotypic differentiation precedes cell fusion is supported by numerous studies showing expression of differentiation specific proteins such as acetylcholine receptors (Merlie and Gros, 1976; Bar-Sagi and Prives, 1983), creatine kinase (Merlie and Gros, 1976; Morris and Cole, 1979; Morris and Cole, 1979), myosin heavy chain (Merlie and Gros, 1976; Morris and Cole, 1979), and myogenin (Andres and Walsh, 1996) in mononucleated cells. In addition, fusion-arrested myoblasts have excitation and contraction properties characteristic of myotubes (Constantin et al., 1995).

Calcium regulates several of the later steps required for muscle development. Phenotypic differentiation can be inhibited by lowering extracellular calcium concentration (Shainberg et al., 1969; Morris and Cole, 1979), the addition of EGTA (Morris and Cole, 1979; Salzberg et al., 1995), and the addition of L-type calcium channel inhibitors or the depletion of intracellular calcium stores (Seigneurin-Venin et al., 1996). The molecular mechanisms by which calcium regulates phenotypic differentiation are unknown. Myoblast fusion also demonstrates a requirement
for calcium (Shainberg et al., 1969; Knudsen and Horwitz, 1977), and several molecular targets for calcium have been identified. Calcium is required for glycoprotein interactions at the cell surface (Knudsen, 1985; Knudsen et al., 1990), regulation of cell surface protein phosphorylation (Lognonné and Wahrmann, 1986), and regulation of calcitonin-induced proteolysis of proteins involved in membrane-cytoskeleton stability (Barnoy et al., 1997, 1998; Temm-Grove et al., 1999).

We propose that calcineurin, a calcium-calmodulin-regulated serine/threonine protein phosphatase, is a molecular target of calcium in the regulation of skeletal muscle differentiation at a step before phenotypic differentiation. In previous work, we demonstrated that the calcineurin inhibitor, CSA, blocks differentiation based on the dose-dependent inhibition of creatine kinase and embryonic myosin heavy chain (EMyHC) expression and the formation of multinucleated myotubes (A bbt et al., 1998), suggesting a role for calcineurin in the early stages of myogenesis. However, other cellular targets besides calcineurin may exist for CSA in muscle cells (Lo Russo et al., 1996, 1997). Calcineurin-dependent pathways involving fiber type determination and hypertrophy have been previously described in skeletal muscle. Enhancer elements responding to the calcineurin-regulated transcription factor nuclear factor of activated T cells (NFAT) have been identified in the promoters of slow fiber-specific genes (Chin et al., 1998) and transgenic mice expressing an activated form of calcineurin display an increase in the number of slow fibers (Naya et al., 2000). Hypertrophy of skeletal muscle in response to IGF-1 (M usaro et al., 1999, Semsarian et al., 1999) and to functional overload (Dunn et al., 1999) requires calcineurin activity.

In this study, we test the hypothesis that calcineurin is a key mediator of calcium signals early in the myogenic program using biochemical and genetic modulators of calcineurin activity. We show that in addition to phenotypic differentiation and cell fusion, calcium is required for the commitment to the differentiation pathway. Calcineurin is a necessary molecular target of calcium at the commitment stage and is sufficient, in the presence of adequate calcium, to induce skeletal muscle cells to initiate differentiation. We find that transcriptional activation of myogenin is a component of calcineurin signaling during differentiation, but that NFAT is not a required element of the signaling pathway. We conclude that myogenesis is initiated by a calcineurin-dependent, NFAT-independent pathway.

**Materials and Methods**

**Antisera and Reagents**

Mouse monoclonal antibodies against α-sarcomeric actin (s-actin) and α-tubulin were purchased from Sigma-Aldrich as ascites fluid. Mouse monoclonal antibodies against myogenin (Wright et al., 1991) and EMyHC were purchased from Rando and Blau, 1994). Growth media (GM) consisted of Ham’s F10, 20% FBS, 5 ng/ml bFGF, 200 μM penicillin G, and 200 μg/ml streptomycin. The myoblast cell line L6 was grown in L6 Growth Media (LG M) consisting of DM E M, 10% FBS, 200 U/ml penicillin G, and 200 μg/ml streptomycin. Differentiation was induced by primary cultures grown on E-C-L (U pstate Biotechnology)-coated dishes or L6 cells on uncocated dishes to a low serum, low mitogen differentiation media (DM; DME (1.4 mM Ca²⁺), 2% horse serum, 200 μM penicillin G, 200 μg/ml streptomycin) for 24–48 h. Calcium-free differentiation media (DM-CF) was prepared using CaCl₂-free DME and HS that had been dialyzed twice against PBS using a membrane with a 3,500-kD molecular mass cutoff. DM-CF has a residual calcium concentration of at least 8 μM due to calcium pantetheine in the DM and calcium remaining in dialyzed HS. When necessary, CaCl₂ was added to DM-CF to the desired calcium concentration. Cells were photographed on a Nikon TMS inverted microscope with a Polaroid MircroCam.

**Retroviral Plasmids, Production and Infection, and FACs® Sorting**

The retroviral NFAT-responsive plasmid (pKA7) contains a luciferase coding sequence under the control of a minimal IL-2 promoter with an upstream triplex of the distal IL-2 gene NFAT response element (Abbott et al., 1996). The plasmid pCAGGS ([Amarb et al., 2000]), 2% horse serum, 200 U/ml penicillin G, 200 μg/ml streptomycin) for 24–48 h. Calcium-free differentiation media (DM-CF) was prepared using CaCl₂-free DME and HS that had been dialyzed twice against PBS using a membrane with a 3,500-kD molecular mass cutoff. DM-CF has a residual calcium concentration of at least 8 μM due to calcium pantetheine in the DM and calcium remaining in dialyzed HS. When necessary, CaCl₂ was added to DM-CF to the desired calcium concentration. Cells were photographed on a Nikon TMS inverted microscope with a Polaroid MircroCam.

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Results

Calcium Is Required for Multiple Phases of Myogenesis

To investigate the requirement for calcium at each phase of myogenesis (commitment, phenotypic differentiation, and cell fusion), myoblasts were induced to differentiate in a calcium-free DM containing different concentrations of exogenously added calcium. A shown in Fig. 1a, primary myoblasts show a dose-dependent requirement of calcium for myoblast alignment and fusion. At a 50 μM exogenously added Ca2+, rounded, mononucleated cells are the predominant cell type. A the calcium is increased to 300 μM, the cells are still predominantly mononucleated, but the cells have elongated and aligned next to each other. Significant fusion of myoblasts is not seen until 1,500 μM Ca2+. To determine the requirement for calcium in myogenic phases that can not be distinguished morphologically, we performed immunoblot analyses using antibodies against myogenin, and the sarcomeric proteins EMyHC and actin, markers of commitment and phenotypic differentiation, respectively (Fig. 1b). A III of these markers display a dose-dependent increase in expression in response to increasing concentrations of extracellular Ca2+. Myogenin shows significant expression at 25–50 μM Ca2+, whereas EMyHC and s-actin are not expressed until 150 μM Ca2+. A shown in Fig. 1c, the inhibition of differentiation in calcium-free media is reversible, suggesting that it is not due to cytotoxicity. A after readdition of calcium to the media, the cells express myogenin and EMyHC. Thus, these data suggest that at least three distinct steps in myogenesis are regulated by calcium based on the differing calcium requirements of commitment (25–50 μM), phenotypic differentiation (150 μM), and cell fusion (1,500 μM).

Calcineurin Activity Is Necessary for Myogenic Commitment

Calcineurin is a potential downstream effector of calcium during myogenesis. The importance of calcineurin in mus-
We first expressed CAIN in the presence of an NFAT reporter to test for inhibition of calcineurin activity in skeletal muscle cells (Fig. 3 a). This reporter requires the calcineurin-induced nuclear translocation of NFAT and the induction of AP-1 for transcription of the luciferase cDNA. Stimulation with ionomycin, to activate calcineurin, and PMA, to induce AP-1, in control cells results in an ∼32-fold increase in luciferase levels compared with vehicle-treated control cells. In cells expressing CAIN, ionomycin and PMA treatment yields only an approximately fivefold increase. Thus, CAIN effectively inhibits calcineurin in skeletal muscle cells. In further experiments, L6 myoblasts were infected with a CAIN retrovirus and changed to DM to test whether myogenesis would be inhibited (Fig. 3 b). In L6 cells infected with the CAIN retrovirus, myogenin expression is reduced to ∼7% of control cell levels at 24 h and to ∼39% at 48 h. EMyHC was undetectable in CAIN-expressing cells. We obtained similar results in primary muscle cells (data not shown). As both CSA and CAIN inhibit myogenin and EMyHC expression, we conclude that calcineurin is an essential mediator of the signaling required for myogenesis to occur.

Calcineurin Activity Is Sufficient to Induce Differentiation

To determine if calcineurin activity is sufficient to induce myogenic differentiation, we constructed a retroviral vector that allows for high efficiency gene transfer of a constitutively active form of calcineurin (aCnA). Originally described as a proteolytic fragment of calcineurin, aCnA retains its catalytic activity and sensitivity to CSA, but no longer requires calcium for activity (Manalan and Klee, 1983; O’Keefe et al., 1992). A nNFAT responsive luciferase reporter was used to indirectly test the activity of aCnA in skeletal muscle cells. Cells infected with either control or aCnA retroviruses were induced to differentiate and then treated with vehicle or PMA. Some cultures were pretreated with CSA. As seen in Fig. 4 a, luciferase activity is increased by only ∼1.6-fold in control cells with PMA treatment. By contrast, luciferase activity is increased in aCnA-infected cells by approximately ninefold. The response to PMA is blocked by the addition of CSA. These results confirm the activity of aCnA in skeletal muscle cells.

To examine the effect of aCnA on induction of myogenic differentiation, primary myoblasts were infected based on the reduction in luciferase activity in IP-treated cultures. Data are reported as the fold increase over vehicle-treated control cells. Each bar represents the mean ± SEM of three independent experiments each performed in triplicate. (b) L6 myoblasts were infected with either control or CAIN expression retroviruses and induced to differentiate in DM. Cellular proteins were collected at various time points and analyzed by immunoblotting. CAIN reduces the expression level of both myogenic markers. A portion of a Coomassie-stained membrane demonstrates relative protein loading.
with either control or aCnA retroviruses and maintained in GM that contains 300 μM Ca^{2+} and mitogenic stimuli from both FBS and exogenously added bFGF. Within 48 h after infection, aCnA-infected cells undergo morphologic changes consistent with differentiation, including cell elongation, cell alignment, and occasional cell fusion. The morphologic changes are blocked by the addition of CSA to the media. 

Multiple Calcium-dependent Pathways Initiate Myogenesis

Myogenesis initiated by changing cells from GM to DM requires extracellular calcium (Fig. 1). If calcineurin was the only calcium-dependent pathway required for differentiation, aCnA should allow myogenesis to occur in calcium-free DM. To test this hypothesis, primary myoblasts were infected with either control or aCnA retroviruses and induced to differentiate in calcium-free DM in the presence of different concentrations of exogenously added calcium. Immunoblots were performed using an antibody against myogenin (Fig. 5). At 0 μM Ca^{2+}, neither control nor aCnA-infected cells express myogenin. At 50 μM Ca^{2+} and 1,500 μM Ca^{2+}, myogenin is expressed at equivalent levels in cells infected with either virus. To confirm that the absence of myogenin expression at 0 μM Ca^{2+} was due to the inability of aCnA to substitute for the calcium requirement and not due to inefficient infection, some cultures were maintained in GM. aCnA induces the expression of myogenin in these cells compared with control cells. Myogenin is detected in control cells maintained in GM (compare to Fig. 4 c), most likely due to spontaneous differentiation as a result of cell confluency. Therefore, aCnA is not able to substitute for the calcium requirement during commitment to differentiation, indicating that calcium is necessary for multiple downstream molecular targets. Thus, calcineurin activity is sufficient to induce differentiation only in the presence of adequate levels of calcium.
Transcriptional Activation of Myogenin Is a Component of aCnA-induced Differentiation

Expression of myogenin mRNA and protein mark the transition from a proliferative myoblast to a cell committed to the differentiation pathway. Expression of aCnA can induce differentiation and results in an increase in myogenin protein expression. The increase in the level of myogenin protein could be accounted for by stabilization of either the mRNA or the protein, or by an increase in transcription or translation. To determine the mechanism for the increase in myogenin protein we performed Northern blot analyses on RNA isolated from primary muscle cells infected with either control or aCnA retroviruses (Fig. 6 a). Compared with control cells, the levels of myogenin mRNA increase in aCnA-infected cells at both 24 and 48 h after infection. Therefore, mRNA stabilization or transcriptional activation could account for the increase in myogenin protein levels.

To examine transcriptional activation of the myogenin promoter by aCnA, we constructed a retroviral reporter plasmid that contains 1,565 bp of upstream promoter sequence from the myogenin gene driving luciferase expression. Primary muscle cells containing pMyogLuc were infected with either control or aCnA retroviruses (Fig. 6 b). Luciferase assays were performed on cells after 48 h in GM. Compared with control cells, luciferase levels increase by ~20% in aCnA-infected cells. The increase was not seen with CSA treatment demonstrating that the increase is specific to the expression of aCnA. We conclude that transcriptional activation of the myogenin gene represents a downstream target of calcineurin activity in myogenesis.

NFAT Is Not a Required Downstream Target of Calcineurin During Myogenesis

Transcriptional activation is at least one mechanism whereby calcineurin regulates differentiation. To determine if the transcription factor NFAT is a required downstream target of calcineurin during differentiation, we used a peptide inhibitor of NFAT, GFP-VIVIT (Aramburu et al., 1999). We have shown previously that three NFAT isoforms are expressed in skeletal muscle and are able to translocate to the nucleus at different stages of myogenesis (Abbott et al., 1998). To test whether GFP-VIVIT inhibits NFAT activity in skeletal muscle cells, primary myoblasts were infected with an NFAT reporter and either control or GFP-VIVIT retroviruses and sorted for GFP expression by flow cytometry (Fig. 7 a). Myoblasts were induced to differentiate in DM for 48 h, followed by treatment with ionomycin and PMA for 5 h. Some cultures were also treated with CSA. In control cells, luciferase levels increase ~25-fold, whereas levels in GFP-VIVIT-infected cells increase only ~1.3-fold demonstrating that GFP-VIVIT effectively inhibits NFAT activity in skeletal muscle cells.

To determine if NFAT activity is required during differentiation, L6 muscle cells were infected with either control or GFP-VIVIT retroviruses and induced to differentiate in DM. Immunoblot analysis was performed on cellular proteins using antibodies against myogenin and EMyHC (Fig. 7 b). GFP-VIVIT expression does not inhibit the expression of either myogenin or EMyHC. Paradoxically, we see a slight increase in expression of both markers after 24 h in DM. We obtained similar results in primary muscle cells representitive of two independent experiments.

Figure 5. Multiple calcium-dependent pathways are necessary for initiating myogenesis. Myoblasts were infected with either control (Ctrl) or aCnA retroviruses. Cells were maintained in GM for 6 h and then induced to differentiate in DM-CF containing different concentrations of exogenously added calcium for 36 h. Some cultures were kept in GM for the entire experiment. Cellular proteins were analyzed by immunoblotting using an antibody against myogenin. aCnA-infected cells demonstrate a calcium-dependent expression pattern of myogenin similar to control cells. The absence of the expression of myogenin at 0 μM Ca^{2+} is not due to inefficient infection of the cells, because aCnA-infected cells maintained in GM express myogenin at higher levels than control cells. A portion of a membrane stained for total protein with colloidal gold is shown to indicate relative protein loading. The data are representative of three independent experiments.
channel blockers to inhibit cell fusion and the expression of creatine kinase (Seigneurin-Venin et al., 1996). (c) Extracellular proteins may require calcium for normal activity. The glycoprotein interactions that mediate cell aggregation before fusion require calcium (Knudsen, 1985). A combination of multiple mechanisms at multiple phases is likely to account for the calcium requirement in differentiation.

**Discussion**

The molecular mechanisms that initiate differentiation of myoblasts are not well understood. Changes in muscle regulatory factors such as M y oD, M y f-5, and myogenin do occur early in differentiation (Megeney and Rudnicki, 1995; Rudnicki and Jaenisch, 1995; Buckingham, 1996), but the signaling pathways that regulate these changes have not been delineated. A large body of work has demonstrated that calcium and calcium-dependent pathways are required for myogenesis (Shainberg et al., 1969; Knudsen and Horwitz, 1977; Knudsen, 1985; Lognonne and Wahrmann, 1986; Przybyski et al., 1989; Cottin et al., 1994; Constantin et al., 1995; Barnoy et al., 1997, 1998; Temm-Grove et al., 1999). For the most part, these earlier studies linking calcium to differentiation focused on the clearly identifiable cell fusion event, but since myoblasts pass through multiple, temporally distinct phases before cell fusion, the results of these studies may also reflect calcium-dependent regulation at earlier stages in myogenesis. Thus, it is not exactly clear where calcium acts in the myogenic pathway and whether multiple points of regulation are present. The data presented in this paper suggest that at least three distinct steps require calcium based on their differing requirements for extracellular calcium: commitment to differentiation, phenotypic differentiation, and cell fusion.

Changes in the concentration of extracellular calcium may inhibit differentiation for several reasons. (a) Extremely low levels of extracellular calcium may be toxic to cells. This is unlikely due to the reversibility of the inhibition (Fig. 1 c) that has been noted by us as well as other groups (Shainberg et al., 1969; Przybyski et al., 1989). (b) Calcium available for intracellular signaling processes may be decreased. During differentiation, the total cell calcium increases, and decreases in extracellular calcium produce similar decreases in intracellular calcium (Przybyski et al., 1989). That extracellular calcium is directly needed for intracellular signaling is shown by the ability of calcium
after increasing media calcium concentration (Przybyski et al., 1989). A study by Seigneurin-Venin et al. (1996) is particularly relevant to our work, because it demonstrated the importance of the dihydropyridine receptor in regulating calcium release from intracellular stores for creatine kinase expression. Since the dihydropyridine receptor requirement occurs at a step before phenotypic differentiation, it may therefore represent a pathway for regulating increases in cytosolic calcium necessary for the activation of calcineurin.

The downstream effectors for calcineurin in regulating the initiation of myogenesis are not known. Certainly, transcriptional activation is a component as expression of aCnA-activated transcription from pM yogLUC. However, we detected only a relatively small increase in luciferase levels after expression of aCnA, as compared with the relatively large increase in the level of myogenin mRNA. This suggests that calcineurin-dependent regulatory elements exist in the myogenin promoter outside of the region we used for our reporter. Alternatively, aCnA stabilization may occur as a result of an increase in calcineurin activity. Recently, calcineurin was shown to regulate the stability of the acetylcholinesterase mRNA in C2C12 muscle cells during differentiation (Luo et al., 1999).

The transcription factor NFAT is a potential target of calcineurin during myogenesis. We have previously shown the presence and activity of NFAT in skeletal muscle cells (Abbot et al., 1998). Muscle cells express three NFAT isoforms that differ in their ability to be activated and undergo nuclear translocation at different stages of development. However, our results show that calcineurin substrates other than NFAT are in fact required for differentiation, and novel calcineurin substrates may exist in skeletal muscle cells. The muscle regulatory factor family (M y-f, M yD, myogenin, M RF 4) of transcription factors plays a primary role in regulating myogenesis and are themselves regulated by phosphorylation, and could therefore be potential targets for the phosphatase activity of calcineurin. A III of the muscle regulatory factors are inhibited by cAMP-dependent protein kinase (Li et al., 1992), and M yD activity is inhibited by phosphorylation of a threonine residue at position 115 (Liu et al., 1998). The myocyte enhancer factor (MEF) family of transcription factors also represent potential calcineurin targets. These factors are required for differentiation as a dominant-negative form of MEF2 inhibits differentiation (Ornatsky et al., 1997). The potential for MEFs as calcineurin targets is highlighted by work demonstrating that the myogenin gene is activated by posttranscriptional modifications of pre-existing MEF2 (Buchberger et al., 1994). In addition, a reporter construct containing only a consensus MEF binding site and a TA TA-box was activated after overexpression of a constitutively active form of calcineurin in skeletal muscle cells (Chin et al., 1998).

Our results suggest that calcineurin plays a more fundamental role in myogenesis than has been previously described. Several papers have described the necessity of calcineurin activity during skeletal muscle hypertrophy in vitro and in vivo (Dunn et al., 1999; Usaro et al., 1999; Semsarian et al., 1999). Skeletal muscle hypertrophy using in vivo models requires activation and differentiation of resident satellite cells (Rosenblatt and Parry, 1992; Rosenblatt et al., 1994; Phelan and G onyea, 1997). The results of the in vivo studies on the role of calcineurin in hypertrophy may, therefore, be consistent with our results. If satellite cell differentiation requires calcineurin activity, then inhibition of calcineurin would also inhibit hypertrophy. However, at least one study using a model of functional overload has reported that the prevention of hypertrophy by CSA treatment was not due to an inhibition of satellite cell differentiation (Dunn et al., 1999). Our results and the models of calcineurin-dependent hypertrophy may represent distinct pathways.

In summary, we have defined a calcium-dependent pathway that regulates the commitment of myoblasts to the differentiation pathway. Calcineurin activity is both necessary and sufficient in the presence of adequate extracellular calcium to induce differentiation. This pathway is independent of NFAT activity. Future studies will identify the upstream signals that regulate calcium changes early in myogenesis as well as the downstream targets of calcineurin in skeletal muscle.

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