Stimulation of Slow Skeletal Muscle Fiber Gene Expression by Calcineurin in Vivo*

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Adult skeletal muscle fibers can be categorized into fast and slow twitch subtypes based on specialized contractile and metabolic properties and on distinctive patterns of muscle gene expression. Muscle fiber-type characteristics are dependent on the frequency of motor nerve stimulation and are thought to be controlled by calcium-dependent signaling. The calcium, calmodulin-dependent protein phosphatase, calcineurin, stimulates slow fiber-specific gene promoters in cultured skeletal muscle cells, and the calcineurin inhibitor, cyclosporin A, inhibits slow fiber gene expression in vitro, suggesting a key role of calcineurin in activation of the slow muscle fiber phenotype. Calcineurin has also been shown to induce hypertrophy of cardiac muscle and to mediate the hypertrophic effects of insulin-like growth factor-1 on skeletal myocytes in vitro. To determine whether activated calcineurin was sufficient to induce slow fiber gene expression and hypertrophy in adult skeletal muscle in vivo, we created transgenic mice that expressed activated calcineurin under control of the muscle creatine kinase enhancer. These mice exhibited an increase in slow muscle fibers, but no evidence for skeletal muscle hypertrophy. These results demonstrate that calcineurin activation is sufficient to induce the slow fiber gene regulatory program in vivo and suggest that additional signals are required for skeletal muscle hypertrophy.

Adult skeletal muscle fibers can be generally classified as fast or slow on the basis of their contractile and metabolic properties (1, 2). Certain fast fibers exhibit a glycolytic metabolism, whereas slow twitch fibers are predominantly oxidative. These properties reflect the expression of specific sets of fast and slow contractile protein isoforms of myosin heavy and light chains, tropomyosin, and troponins, as well as myoglobin.

The fiber-type characteristics of adult skeletal muscle are regulated, at least in part, by the frequency of motor nerve stimulation and resulting changes in intracellular calcium levels (3–5). Tonic low frequency stimulation, either from the motor neuron or by direct electrical stimulation of muscle fibers, induces the slow fiber phenotype, whereas desynchronization or lack of electrical stimulation evokes a slow-to-fast fiber conversion (reviewed in Ref. 6). It is well established that slow fibers maintain higher levels of intracellular calcium (7, 8), but the signaling systems responsible for activation of the slow fiber gene program remain poorly understood.

Calcineurin is a calcium-, calmodulin-dependent protein phosphatase activated by changes in intracellular calcium (reviewed in Ref. 9). The properties of calcineurin have been studied most extensively in T cells, whereupon activation in response to T cell receptor signaling, calcineurin dephosphorylates nuclear factor of activated T cells (NFAT)1 proteins, which translocate to the nucleus and act combinatorially with other transcription factors (reviewed in Ref. 10). Calcineurin-dependent activation of NFAT is driven selectively by sustained, low amplitude elevations in intracellular calcium concentrations, while brief calcium transients, even of high amplitude, are insufficient for this purpose (11). Activation of calcineurin-dependent genes in T cells requires costimulation of mitogen-activated and calmodulin-dependent protein kinase signaling pathways (10), which activate the AP-1 and MEF2 transcription factors. Recent studies have also shown that activated calcineurin stimulates transcriptional activity of MEF2 transcription factors (12), expressed in T cells and muscle (reviewed in Ref. 13), through mechanisms that remain to be defined.

Several properties of calcineurin signaling suggest that it may participate in the control of slow skeletal muscle gene expression. First, its selective activation by prolonged elevation of calcium and insensitivity to high amplitude calcium spikes correspond to the type of signaling thought to activate slow fiber-specific genes (9). Second, the control regions of several slow fiber-specific genes contain adjacent binding sites for NFAT and MEF2 factors (14–17). Third, transplant patients maintained on the calcineurin inhibitors, cyclosporin A (CsA) and FK-506, develop skeletal myopathy and a loss of skeletal muscle oxidative capacity, suggesting an important role for calcineurin in regulating muscle function (18).

Recently, we reported that calcineurin up-regulates slow muscle fiber genes in cultured C2C12 muscle cells (15). Transcriptional activation of the myoglobin and slow troponin I (TnI) genes by calcineurin was dependent on adjacent binding sites for NFAT and MEF2. Moreover, treatment of rats with CsA or FK-506 resulted in partial conversion of slow to fast fibers, suggesting that calcineurin activity was required for slow fiber gene expression. However, since systemic administration of CsA can also inhibit calcineurin activity in neurons, potentially altering motor innervation, and also has nonspecific effects, it was unclear whether the decrease in slow fiber gene expression in response to CsA reflected an essential role of calcineurin in skeletal muscle per se.

Calcineurin has also been implicated in hypertrophy of cardiac and skeletal muscle (19–21). In primary cardiomyocytes, calcineurin activation is required for hypertrophic growth in

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1 The abbreviations used are: NFAT, nuclear factor of activated T cells; CsA, cyclosporin A; TnI, troponin I; IGF, insulin-like growth factor; MCK, muscle creatine kinase; hGH, human growth hormone; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s).
response to a variety of agonists (19). Moreover, expression of activated calcineurin in the hearts of transgenic mice results in dramatic myocardial growth that can be partially mimicked by a mutant form of NFAT3 lacking the phosphorylation sites normally dephosphorylated by calcineurin (19). Activated calcineurin has also been shown to induce hypertrophy of skeletal muscle cells in culture, and inhibition of calcineurin activity can block the hypertrophic response to insulin-like growth factor (IGF)-1 (20, 21). CsA has also been reported to inhibit both hypertrophy and fast-to-slow fiber transformations induced in muscles of intact animals by mechanical load (22). While the latter studies suggest an important role for calcineurin in skeletal muscle hypertrophy, they do not indicate whether calcineurin activation alone is sufficient to induce skeletal muscle hypertrophy in vivo or, alternatively, whether other signals are also required to activate calcineurin-dependent genes, as is the case in T cells.

To further investigate the role of calcineurin in the control of skeletal muscle fiber type and hypertrophy, we generated transgenic mice in which activated calcineurin was expressed in skeletal muscle under control of the muscle creatine kinase (MCK) enhancer. These mice show a dramatic increase in slow skeletal muscle fibers, but no evidence for skeletal muscle hypertrophy. We conclude that activated calcineurin is sufficient to activate the slow fiber gene regulatory program in vivo and that additional signals are required for skeletal muscle hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Construction of Skeletal Muscle Calcineurin Transgene**—The 4800-bp base pair upstream region of the mouse MCK gene was released from the pCK4800CAT construct (23) by HindIII digestion and ligated to the HindIII site of plasmid pBlueScript (Stratagene). The activated form of calcineurin (24) was ligated into the EcoRI site followed by the human growth hormone (hGH) polyadenylation (poly(A)) signal. This transgene is referred to as MCK-CN*.

**Creation of Transgenic Mice**—Transgenic mice were created by injection of the MCK-CN* construct into the male pronuclei of fertilized mouse oocytes obtained from the B6C3F1 strain. The MCK-CN* cassette was released from the vector backbone by digestion with either XhoI and SacII or EcoHI, gel-purified, and eluted using Qiagen columns (Qiagen). Stable founder lines were identified initially by Southern analysis and subsequently by PCR using primers specific for the hGH poly(A) signal sequences. Expression of the transgene was assessed by Northern analysis using a calcineurin cDNA as a probe. Histology—Hindlimb muscle (soleus, gastrocnemius, and plantaris), isolated from 2- to 3-month-old nontransgenic and transgenic littermates was flash-frozen in liquid nitrogen. Cryosections were stained histochemically for myosin ATPase activity at pH 4.5–4.6 described previously (25).

**RT-PCR Analysis**—RT-PCR analysis of fiber-specific transcripts was performed under standard techniques. RT-PCR reactions were performed with a PhosphorImager and quantified using ImageQuant Program (Molecular Dynamics).

**RESULTS**

**Characterization of MCK-Calcineurin Transgenic Lines**—To determine whether activated calcineurin was sufficient to modify the properties of skeletal muscle in vivo, we created transgenic mice harboring an expression cassette in which the 4800-bp region upstream of the mouse MCK gene was linked to a cDNA encoding a constitutively active form of the calcineurin A catalytic subunit as well as the hGH poly(A) signal was cloned downstream from the 4800-bp 5′-flanking region of the mouse MCK gene (23). Four independent lines of MCK-calcineurin (MCK-CN*) transgenic mice were obtained. Expression of the transgene was confirmed by Northern analysis using a probe specific for the truncated calcineurin transcript. MCK is normally expressed preferentially in fast skeletal muscle fibers, with detectable but reduced expression in heart and slow skeletal muscle (27). Consistent with the muscle-specific expression of the MCK upstream region, transgene expression was restricted to skeletal and cardiac muscle (Fig. 1). Expression in the heart was only detected upon longer exposure. Calcineurin expression from the transgene was at least 10-fold higher than the level of expression of the endogenous gene in skeletal muscle and about 2-fold higher than the level of expression in brain (Fig. 1B).

Previously, we showed that expression of activated calcineurin in the heart, under control of the α-mycin heavy chain promoter, resulted in severe cardiac hypertrophy, with a 2–3-fold increase in cardiac mass by 4 weeks of age (19). MCK-CN* transgenic mice showed only mild cardiac hypertrophy, reflecting the weak postnatal expression of the MCK upstream region in the heart.

Based on visual inspection and histological sections of skeletal muscle, we found no evidence for skeletal muscle hypertrophy in MCK-CN* transgenic mice. We also determined myofiber cross-sectional areas and weight of soleus and gastrocnemius muscles from wild-type and MCK-CN* transgenic mice, but no statistically significant differences. The only obvious difference in skeletal muscle of transgenic
mice was the deep red appearance of fast fibers, such as the gastrocnemius, which normally appears more pale. This suggested that fast muscles from the transgenics expressed a higher amount of myoglobin, characteristic of slow fibers.

**Increased Number of Slow Skeletal Muscle Fibers in MCK-CN* Transgenic Mice**—To investigate possible changes in fiber type in MCK-CN* transgenic mice, transverse sections of hindlimb muscle were assayed for myosin ATPase activity using a pH-dependent histochemistry that distinguishes slow (dark stained) and fast (unstained) fibers. As shown in Fig. 2A, there was a visible increase in dark stained fibers in MCK-CN* transgenic gastrocnemius, demonstrating a transformation to the slow fiber phenotype.

To further confirm the qualitative changes observed, we quantitated numbers of slow fibers in hindlimbs by counting dark stained cells in cross-sections of the gastrocnemius. Values are the average ± S.E. (n = 5). The difference in group means was significant (p < 0.05).

**Expression of Slow Skeletal Muscle Transcripts in Gastrocnemius of MCK-CN* Transgenic Mice**—We also examined expression of transcripts for several slow and fast fiber-restricted genes in MCK-CN* transgenic mice. Myoglobin, TnI slow, and sarcomeric mitochondrial creatine kinase (sMtCK), which are normally expressed primarily in slow fibers, showed a 2.5 ± 0.3 (n = 3), 3.1 ± 0.7 (n = 3), and 1.8 ± 0.2 (n = 3)-fold increase in expression, respectively, in gastrocnemius of the transgenics (Fig. 3). Conversely, transcripts for parvalbumin and MCK, which are expressed predominantly in fast fibers, were expressed at 0.51 ± 0.06 (n = 3) and 0.65 ± 0.06 (n = 3) of wild-type levels, respectively, in gastrocnemius of transgenics. Together, the above results demonstrated that expression of activated calcineurin in skeletal muscle is sufficient to induce a fast to slow fiber transformation.

**FIG. 2.** Fiber composition of adult hindlimb muscle from nontransgenic and MCK-CN* transgenic mice. A, frozen sections of skeletal muscle from nontransgenic and MCK-CN* transgenic littermates at 2 months of age were assayed for myosin ATPase activity using pH-dependent histochemistry that distinguishes slow (dark stained) and fast (unstained) fibers. B, slow fibers were quantitated by counting dark stained cells in cross-sections of the gastrocnemius. Values are the average ± S.E. (n = 5). The difference in group means was significant (p < 0.05). g, gastrocnemius; p, plantaris; s, soleus.

**FIG. 3.** Analysis of fiber-specific transcripts in nontransgenic and MCK-CN* transgenic mice. Total RNA was isolated from hindlimb muscles following removal of the soleus from 2-month-old mice. Transcripts were amplified by semiquantitative RT-PCR and visualized by a PhosphorImager. L7 transcripts were measured as a control. No transcripts were detected in the absence of reverse transcriptase. Data are expressed as the level of expression relative to the level in wild-type, set at 1 ± S.E. (n = 3). WT, wild-type; Tg, MCK-CN* transgenic.

**DISCUSSION**

The results of this study lead to two important conclusions concerning calcineurin signaling in skeletal muscle. First, they demonstrate that activated calcineurin is sufficient to induce slow fiber gene expression in vivo. Second, they show that calcineurin activation alone is insufficient to induce skeletal muscle hypertrophy in vivo.

The fast and slow muscle fiber phenotypes are thought to be dependent on differences in intracellular calcium resulting from motor innervation (reviewed in Ref. 6). Slow fibers maintain higher intracellular calcium concentrations, evoked by sustained, tonic motor nerve stimulation, whereas fast fibers are characterized by intermittent stimulation and higher amplitude fluctuations in calcium levels (6).

Our results suggest that activated calcineurin partially mimics the effects of tonic motor nerve stimulation on skeletal muscle, resulting in an increase in slow skeletal muscle fibers. However, this fast to slow fiber transformation in response to activated calcineurin is incomplete, in that not all fast fibers undergo this transition. Similarly, systemic administration of CsA to rats causes only a partial loss of slow fibers in vivo (15). These results raise the possibility that there is heterogeneity among fibers with respect to their sensitivity to calcineurin signaling and suggest that other calcium-dependent signaling systems may also be required for determining slow and fast fiber gene expression. In this regard, we have recently found that activated calcium, calmodulin-dependent protein kinase IV (CaMKIV) can up-regulate slow fiber genes and can synergize with calcineurin to stimulate transcriptional activity of the transcription factor MEF2, which binds several slow fiber gene...
regulatory regions. The existence of calcineurin-independent signaling systems for slow fiber gene expression is also suggested by recent studies demonstrating that slow fiber gene expression can be conferred by transcription factors other than those known to be targets for calcineurin (16, 17, 28, 29).

Activated calcineurin is sufficient to induce dramatic hypertrophy of cardiac muscle in vivo (19), as well as skeletal muscle cells in culture (20, 21). Moreover, inhibition of calcineurin blocks IGF-mediated hypertrophy of cultured skeletal muscle cells (20, 21). Why does calcineurin not induce skeletal muscle hypertrophy in vivo? We propose that activated calcineurin may be necessary, but not sufficient, to induce hypertrophic growth of skeletal muscle. Perhaps calcineurin is only one of multiple downstream signaling pathways activated by IGFs and, under the conditions of cell culture systems, activated calcineurin can induce hypertrophy because the other essential pathways are already activated.

Paradoxically, IGF-1 has been shown to induce an increase in glycolytic enzymes, as well as hypertrophy, in cultured skeletal muscle cells (21). Similarly, fast fibers are preferentially hypertrophied in response to IGF-1, additional signaling pathways are also required for slow fiber gene expression. An important goal for the future will be to identify the signaling pathways that cooperate with calcineurin and to determine how these signaling systems are integrated to establish the transcriptional programs that govern hypertrophy and fiber type.

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