A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type

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Slow- and fast-twitch myofibers of adult skeletal muscles express unique sets of muscle-specific genes, and these distinctive programs of gene expression are controlled by variations in motor neuron activity. It is well established that, as a consequence of more frequent neural stimulation, slow fibers maintain higher levels of intracellular free calcium than fast fibers, but the mechanisms by which calcium may function as a messenger linking nerve activity to changes in gene expression in skeletal muscle have been unknown. Here, fiber-type-specific gene expression in skeletal muscles is shown to be controlled by a signaling pathway that involves calcineurin, a cyclosporin-sensitive, calcium-regulated serine/threonine phosphatase. Activation of calcineurin in skeletal myocytes selectively up-regulates slow-fiber-specific gene promoters. Conversely, inhibition of calcineurin activity by administration of cyclosporin A to intact animals promotes slow-to-fast fiber transformation. Transcriptional activation of slow-fiber-specific transcription appears to be mediated by a combinatorial mechanism involving proteins of the NFAT and MEF2 families. These results identify a molecular mechanism by which different patterns of motor nerve activity promote selective changes in gene expression to establish the specialized characteristics of slow and fast myofibers.

[Key Words: Myosin; myoglobin; NFAT; MEF2; cyclosporin A; exercise]

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Calcineurin-dependent signaling mechanisms have previously been characterized extensively in the activation of cyto-kine gene expression in T and B lymphocytes responding to stimuli that elevate intracellular free calcium concentration ([Ca\textsuperscript{2+}]) (Rao et al. 1997). Binding of calcium to a calmodulin–calcineurin complex stimulates serine/threonine phosphatase activity of calcineurin, the major substrates of which are nuclear factor of activated T cells (NFAT) transcription factors. Dephosphorylation of NFAT by calcineurin promotes their translocation from the cytoplasm to the nucleus, where they bind a cognate nucleotide recognition sequence (Rao et al. 1997) and stimulate transcription of target genes that, in lymphocytes, include hematopoietic growth factors (e.g., GM-CSF) and inflammatory cytokines (e.g., IL-2). Recent studies demonstrate that calcineurin activity and the resulting nuclear translocation of NFAT are insensitive to transient, high-amplitude oscillations in [Ca\textsuperscript{2+}] that activate other calcium-dependent events (e.g., NF-κB or c-Jun amino-terminal kinase). Rather, the calcineurin–NFAT pathway responds preferentially to sustained, low-amplitude elevations of [Ca\textsuperscript{2+}] (Timmerman et al. 1996; Dolmetsch et al. 1997). This ability of a calcineurin-dependent signaling pathway to discriminate between different patterns in the amplitude and duration of changes in [Ca\textsuperscript{2+}], in conjunction with previous data characterizing differences in intracellular calcium concentrations among specialized myofiber subtypes, provided the basis for our hypothesis that a calcineurin-dependent pathway influences fiber-type-specific gene expression.

Tonic motor nerve activity at 10–15 Hz is characteristic of slow-twitch fibers (Hennig and Lomo 1985) and results in a sustained elevation of [Ca\textsuperscript{2+}] within a concentration range between 100 and 300 nm (Chin and Allen 1996), a pattern predicted to activate calcineurin. In fast myofibers, resting [Ca\textsuperscript{2+}] is maintained at levels of only 50 nm (Westerblad and Allen 1991), and the high amplitude (∼1 μM) calcium transients evoked by motor nerve activity are predicted to be of insufficient duration to evoke calcineurin-stimulated signaling. Chronic stimulation at 10 Hz of the motor nerve innervating fast myofibers results in sustained elevations of [Ca\textsuperscript{2+}] and promotes fast-to-slow fiber transformation (Williams et al. 1986; Sreter et al. 1987). Calcineurin and several NFAT isoforms are abundant in skeletal muscles (Hoey et al. 1995), though target genes that respond to this pathway in skeletal myocytes have not been identified previously, and a specific role for calcineurin in the control of myofiber specialization has not been proposed previously.

Here we demonstrate that fiber-type-specific gene expression in skeletal muscles is controlled by a signaling mechanism that involves calcineurin, a cyclosporin-sensitive, calcium-regulated serine/threonine phosphatase. This discovery has considerable explanatory power, in that it elucidates plausibly a complete signaling pathway linking motor nerve activity to selective changes in gene expression that establish diversity among myofibers.

Results

Selective activation of slow-fiber-specific promoters by forced expression of a constitutively active form of calcineurin

The myoglobin (Mb) and troponin I slow (TnI) genes are expressed selectively in slow, oxidative skeletal muscle fibers (Levitt et al. 1995; Garry et al. 1996), whereas the muscle creatine kinase (MCK) gene is expressed most abundantly in the fast, glycolytic myofiber subtype (Yamashita and Yoshioka 1991). To test whether these genes might respond differently to a calcineurin-stimulated signaling pathway, we transfected skeletal myogenic cells with reporter genes linked to well-characterized control regions from these genes, along with an expression vector encoding a constitutively active (calcium-insensitive) form of calcineurin that retains sensitivity to inhibition by cyclosporin A (Manalan and Klee 1983; O’Keefe et al. 1992).

Transcriptional activity of the slow-fiber-specific myoglobin and Tnl promoters was stimulated in cultured skeletal myotubes (C2C12) by active calcineurin, as measured by expression of luciferase in cotransfection assays (Fig. 1). In contrast, activity of the fast-fiber-specific MCK promoter, or of other strong (CMV) or weak (minimal TATA element) promoters, was unaffected by activated calcineurin. The induction of the myoglobin promoter in the presence of the calcineurin expression plasmid was inhibited by cyclosporin A. This result indicates the specificity of the response, since the effect of cyclosporin A is to bind cyclophilin and form a protein complex that binds calcineurin and inhibits its protein phosphatase activity (Liu et al. 1991). The same relative potency of calcineurin-dependent transactivation (myoglobin and TnI promoters) was observed in Sol8 myotubes, a different myogenic cell line (data not shown). In contrast, forced expression of activated calcineurin had no effect on promoter activity in undifferentiated myoblasts (not shown) or in 3T3 fibroblasts (Fig. 1), demonstrating a requirement for muscle-specific factors in the calcineurin-stimulated pathway for transcriptional control of the myoglobin and TnI promoters.

Calcineurin-stimulated transactivation of slow-fiber-specific promoters requires nucleotide sequence motifs characteristic of NFAT binding sites

The finding that the myoglobin and TnI promoters can be transcriptionally regulated by a calcineurin-dependent mechanism suggested the participation of NFAT transcription factors in the signaling cascade. Examination...
NFAT binding sites within these transcriptional control regions. Thus, either cryptic binding sites for NFAT proteins that cannot be recognized by inspection of the DNA sequence are present, or calcineurin-dependent signaling to these promoters can be driven without direct DNA binding of NFAT proteins.

Nuclear localization of NFAT proteins in skeletal myocytes is under the control of calcineurin (Fig. 2C), as predicted from previously published results in lymphocytes (Timmerman et al. 1996). A fusion protein linking green fluorescent protein (GFP) to full-length NFATc (NFATc-GFP) is excluded from the nucleus in C2C12 cells under basal conditions, but undergoes nuclear translocation in the presence of activated calcineurin. As controls, an NFATc-GFP fusion protein lacking amino acids 1–318 of NFATc (∆NFATc-GFP) is localized constitutively to the nucleus in the absence of activated calcineurin, whereas native GFP is distributed across both cytoplasmic and nuclear compartments. The amino-terminal segment of NFAT proteins (missing in ∆NFATc-GFP) includes the conserved SPRIEIT motif that constitutes the calcineurin targeting site (Aramburu et al. 1998).

Calcineurin-stimulated transactivation of slow-fiber-specific promoters requires collaboration among multiple transcription factors

Muscle-specific transcription factors are required for calcineurin-dependent activation of the myoglobin and TnIs promoters, since no response was observed in a fibroblast cell background (Fig. 1). Previously, we defined two conserved upstream response elements within the myoglobin promoter, both of which are required for transcriptional activity in skeletal myotubes or cardiac myocytes (Devlin et al. 1989; Bassel-Duby et al. 1993; Grayson et al. 1995, 1998). These CCAC and A/T elements represent binding sites for Sp1 and MEF2 proteins, respectively, and function synergistically in muscle-specific gene regulation (Grayson et al. 1995, 1998). This prior work established a molecular basis for muscle-specific expression of myoglobin, but failed to account for selective expression of myoglobin in slow fiber types, since Sp1 and MEF2 proteins are equally abundant in slow and fast fibers (J. Grayson, R. Bassel-Duby, R.S. Williams, unpubl.).

A myoglobin promoter segment truncated to nucleotides −373 to +7 (Mb380) was sufficient for muscle-specific expression in prior experiments (Devlin et al. 1989; Bassel-Duby et al. 1993; Grayson et al. 1995) and was responsive to calcineurin stimulation in our current studies (Fig. 3A). This region includes the CCAC and A/T motifs required for muscle-specific promoter activity, as well as a putative NFAT response element. Nucleotide substitutions within either the CCAC or A/T elements of Mb380 reduced basal transcription in differentiated myotubes, as observed previously, and abrogated the response to calcineurin (Fig. 3A). Thus, mutations that compromise binding of MEF2, Sp1, or other factors to the CCAC and A/T elements interdict the
calcineurin-stimulated response, even when the NFAT consensus binding motif at −232 remains intact.

Functional interactions between transcription factors binding to motifs within the myoglobin promoter were examined further by linkage of various combinations of multimerized oligonucleotide cassettes representing cognate binding sites for MEF2, Sp1, and NFAT in promoter-reporter constructions. As assessed by cotransfection assays in C2C12 myotubes (Fig. 3B), forced expression of activated calcineurin only marginally enhanced transcription (less than twofold) of a construct bearing multiple copies of the CCAC motif. The response to calcineurin was somewhat more robust (threelfold) if multimers of the A/T element were included within the synthetic promoters, either in the absence of heterologous protein binding sites, or when combined with multimerized CCAC sites. A reporter construction bearing multiple copies of the upstream (−690) NFAT response element (NRE) from the myoglobin promoter was minimally stimulated by activated calcineurin (less than twofold) in this cell background, but a construct combining NRE, A/T, and CCAC motifs was potently transactivated (sixfold). These results demonstrate that collaborative interactions among proteins binding to NRE, A/T, and CCAC elements from the myoglobin promoter are necessary for optimal transduction of the calcineurin-stimulated signal.

Administration of the calcineurin antagonist cyclosporin A to intact animals promotes slow-to-fast fiber transformation

To determine whether calcineurin-dependent activation of slow-fiber-specific promoters observed in cultured myotubes is pertinent to mature myofibers of intact animals, we assessed the proportion of fast versus slow fibers within soleus muscles of rats treated with cyclosporin A, a specific inhibitor of calcineurin (Liu et al. 1991; Clipstone and Crabtree 1992). The intraperitoneal administration of an immunosuppressant dose (5 mg/kg/day) of cyclosporin A for 6 weeks uniformly increased the proportion of fast fibers defined either by histochemical staining of myosin ATPase activity (Brooke and Kaiser 1970), or by specific immunohistochemical staining of fast skeletal myosin (Fig. 4A–D). In soleus muscles of 7 control animals, fast (type II) fibers represented 4%–
24% (mean 14 ± 3%) of the total cell population, whereas 28%–37% (mean 31 ± 1%) of soleus fibers expressed fast myosin in 7 cyclosporin A-treated rats (P < 0.001) (Fig. 4E). This result is consistent with the hypothesis that physiological signals acting to establish and maintain the slow, oxidative myofiber phenotype in intact animals are transduced by a calcineurin-dependent pathway. Interdiction of the calcineurin-signaling pathway with cyclosporin A has reciprocal effects on expression of fast and slow myosin isoforms: Not only is slow myosin expression reduced (Fig. 4A,B), but fast myosin expression is enhanced (Fig. 4C,D). We infer, therefore, that calcineurin-dependent signaling both activates slow-fiber-specific genes and represses the fast fiber-specific program.

Discussion

A molecular basis for control of myofiber specialization by motor nerve activity

The results presented in this paper suggest a molecular model, not previously considered, to explain how motor-nerve activity controls programs of gene expression that define fast and slow subtypes of skeletal myofibers (Fig. 5). The model proposes that tonic motor nerve activity, characteristic of nerves innervating slow muscles, sustains [Ca²⁺], at levels sufficient to activate the calcineurin-NFAT pathway. The protein phosphatase activity of calcineurin leads to dephosphorylation and nuclear localization of NFAT proteins. In the nucleus, NFAT proteins bind DNA in conjunction with other transcriptional regulators, including (but not limited to) MEF2, binding sites for which are clustered in promoter/enhancer regions controlling transcription of genes encoding proteins of the slow-fiber program. In fast fibers, high-amplitude calcium transients stimulated by infrequent, phasic firing of the motor nerve are of insufficient duration to maintain calcineurin in the active state, so NFAT proteins remain phosphorylated and are excluded from the nucleus. When NFAT proteins are unavailable for DNA binding and protein–protein interactions at target promoters, the slow-fiber-specific program is down-regulated, and genes encoding fast-fiber-specific proteins are transcribed.

This model accommodates the well-established associations between motor nerve activity and specialized fiber characteristics described in an extensive literature on muscle plasticity (Michel et al. 1994; Neuf et al. 1996; Williams and Neufier 1996). Fast-to-slow fiber transformation is evoked by increased motor nerve activity, stimulated by cross-innervation, electrical pacing, or exercise training. Slow-to-fast fiber transformation occurs as a consequence of decreased motor nerve activity, resulting from cross-innervation, certain disease states,
hypogravity, or physical inactivity. The new and independent lines of evidence provided here support the contention that calcineurin-dependent signaling is an important mechanism central to these transformations.

Forced expression of constitutively active calcineurin selectively transactivates promoters from two genes that are expressed preferentially in slow versus fast skeletal myofibers. Thus, downstream effectors of a calcineurin-regulated signaling pathway are present and capable of transducing the signal in a muscle-cell background, and transcriptional regulatory elements capable of receiving the signal are present within genes representative of the slow-fiber program. Specific effector molecules appear to include NFAT proteins, as consensus NFAT binding motifs contained within slow-fiber-specific promoters participate in the response to activated calcineurin, and several NFAT isoforms are expressed in skeletal muscle (Hoey et al. 1995). Our data suggest, moreover, that DNA binding of NFAT proteins is not sufficient to transduce the calcineurin-generated signal in skeletal myocytes. Rather, NFAT transcription factors collaborate with MEF2 and other transcriptional regulatory proteins, the correct combination of which is present within differentiated myotubes, but absent from undifferentiated myoblasts or fibroblasts. Previous studies of calcineurin-stimulated transactivation of cytokine gene promoters in T cells, where AP-1 cooperates with NFAT in both DNA binding and transactivation (for review, see Rao et al. 1997), provide a precedent for synergistic combinatorial interactions between NFAT proteins and heterologous transcription factors.

The organization of transcriptional control regions that confer fiber-type-specific expression (Fig. 6) is consistent with this viewpoint. Consensus NFAT binding sequences are conserved in the 5' flanking region of myoglobin and TnIs genes from all vertebrate species in which promoter sequences are available, and in other slow-fiber-specific enhancers (Fig. 6A). Moreover, two studies that have mapped fiber-type-specific enhancers at the highest resolution provide additional support to our hypothesis that a calcineurin–NFAT signaling pathway is important to this mechanism. Buonanno and colleagues (Nakayama et al. 1996) identified a 128-bp element from the rat TnIs gene [slow upstream regulatory element (SURE)] that confers slow-fiber-specific transcription in transgenic mice, and a 144-bp element [fast intronic regulatory element (FIRE)] that directs fast-fiber-specific expression of a different isoform of troponin I (TnIf). These functionally distinctive SURE and FIRE elements contain similar or identical CAGG, CCAC, MEF2, and E box motifs (Fig. 6B), as found in many muscle-specific genes, so the basis for their reciprocal functions in specialized subtypes of myofibers has not been apparent. Examination of the SURE and FIRE elements in light of our new observations reveals a consensual NFAT recognition motif in the TnIs SURE element that is absent from the TnIf FIRE element. In the sarcomeric mitochondrial creatine kinase (sMtCK) gene, a 160-bp upstream element was shown to direct fiber-type-specific gene expression in transgenic mice (Qin et al. 1997). The sMtCK gene is expressed preferentially in slow, oxidative myofibers, in contrast to the MCK isoform (fast-fiber specific) that was studied in our experiments (Fig. 1). Like the myoglobin and TnIs gene enhancers, this sMtCK enhancer includes NFAT recognition motifs (Fig. 6A).

Finally, inhibition of calcineurin phosphatase activity...
in intact animals by administration of cyclosporin A leads to down-regulation of slow and induction of fast-fiber type-specific markers. This observation indicates that the conclusions drawn from experiments in cultured myotubes are likely to be pertinent to mature myofibers of adult animals.

Limitations of this model for fiber type determination

The model illustrated in Figure 5 reconciles our current findings with a substantial body of previous data concerning the regulation of skeletal muscle fiber type by motor nerve activity, the molecular basis for which was not apparent previously. However, for the sake of simplicity and clarity in the conduct and presentation of these initial experiments, we have considered only the distinction between fast and slow skeletal myofibers. Specialization among muscle cells is, of course, much more richly textured. Several additional subtypes of fast-twitch fibers can be defined on the basis of expression of different contractile protein isoforms (type IIa vs. IIb vs. IIx), distinctive metabolic properties [oxidative (IIa) vs. glycolytic (IIb)], or gross appearance [red (I and IIa) vs. white (IIb)]. Additional studies will be required to deter-
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determine the degree to which calcium-regulated, calcineurin-dependent signaling participates in defining the complete spectrum of myofiber subtypes, and the manner in which this pathway interdigitates with other signaling processes.

This working model has other limitations that should be addressed in future studies. Several of the steps in the pathway are inferred by analogy to data acquired in non-muscle-cell types and, therefore, will require direct confirmation in the muscle-cell background. For example, calcium-dependent regulation of calcineurin activity leading to DNA binding of NFAT proteins has not been assessed directly in skeletal muscles, but our model requires only that the fundamental principles established in T lymphocytes—selective activation by sustained but not transient elevations in [Ca\(^{2+}\)](Timmerman et al. 1996; Dolmetsch et al. 1997)—can be extrapolated to predict the control of this pathway within a different cell type. Likewise, the conclusion that NFAT proteins transduce primarily the calcineurin-dependent signal to slow-fiber-specific promoters was suggested by analogy with calcineurin–NFAT signaling in other cell types, and has some experimental support from our current data, but additional studies will be necessary to provide rigorous proof of this concept. Our current data do not exclude the possibility that the calcineurin-stimulated signal is transduced to responsive target genes in skeletal muscle through mechanisms that are independent of NFAT proteins. Although no other transcription factors are known currently to be controlled directly by calcineurin, the activities of several muscle-specific transcription factors are regulated by phosphorylation events (Li et al. 1992) that conceivably could be modulated by calcineurin. In addition, other muscle proteins known to be substrates of calcineurin [ryanodine and inositol 1,4,5-trisphosphate receptors (Cameron et al. 1995; Lam et al. 1995), and dystrophin (Walsh et al. 1995)] could participate plausibly in calcineurin-activated pathways that modulate transcription, without the involvement of NFAT proteins.

Finally, our current data cannot conclusively exclude the possibility that slow-to-fast fiber transformation induced by administration of cyclosporin A to intact animals is attributable to local or systemic drug effects that are unrelated to inhibition of calcineurin, or to inhibition of calcineurin in motor neurons or other cell types that communicate signaling information to skeletal myofibers. The experiments demonstrating calcineurin-dependent transactivation of slow-fiber-specific gene promoters in cultured myotubes support the notion that the effects of cyclosporin on skeletal myofibers of intact animals are cell autonomous, but additional studies will be required to verify this conclusion.

Calcineurin-dependent signaling in cardiac and skeletal muscles

Very recently, a calcineurin-dependent transcriptional pathway was shown to promote hypertrophic growth of the heart (Molkentin et al. 1998). In cardiac myocytes, this pathway was shown to involve collaborative interactions between activated NFAT proteins and GATA4, a cardiac-restricted transcription factor not present in skeletal muscle. Cardiomyocytes express several isoforms of MEF2, and many of the same genes that exhibit slow, oxidative fiber type-specific expression in skeletal muscle are transcriptionally active in cardiac myocytes [e.g., myoglobin or sMtCK (Parsons et al. 1993; Levitt et al. 1995; Qin et al. 1997)]. Thus, the functional interactions between NFAT and MEF2 proteins suggested by our current data in skeletal muscle are likely to be pertinent to the myocardium as well. Future studies will determine whether hypertrophic growth of skeletal muscles also can be stimulated by calcineurin-triggered signaling events, and should identify the molecular mechanisms by which overlapping, but distinctive, sets of genes are regulated by calcineurin in skeletal and cardiac myocytes.

Medical implications of calcineurin-regulated programs of gene expression in skeletal muscles

The discovery of a calcineurin-dependent pathway linked to specialization of myofiber subtypes reveals a potential for immunosuppressant drugs in current clinical use to modify skeletal muscle physiology and exercise performance in patients receiving such therapy. Our model is supported by a previous study in which pharmacologic blockade of calcineurin signaling in rats reduced the capacity for endurance exercise and diminished peak rates of oxidative phosphorylation in mitochondria isolated from skeletal muscles (Mercier et al. 1995). Clinical investigations to determine the degree to which changes in skeletal muscles account for unwanted side effects of immunosuppressant therapy are warranted.

In the long term, the discovery of a calcineurin-regulated pathway controlling fiber specialization in skeletal muscles could lead to novel strategies to enhance human health. For example, slow oxidative fibers are relatively resistant to the progressive myonecrosis that occurs with advancing age in individuals lacking dystrophin (Duchenne's muscular dystrophy) (Webster et al. 1988). A strategy to promote fast-to-slow fiber transformation in these patients may reduce morbidity and prolong life until definitive gene therapy procedures can be developed. Patients with congestive heart failure, regardless of the primary cause, exhibit loss of slow, oxidative myofibers in their skeletal muscles (Mассие et al. 1988; Sabbah et al. 1993), an abnormality that contributes to exercise intolerance in these individuals. A strategy to reverse this process could improve the quality of life of individuals suffering from heart failure, even if cardiac performance remains impaired (Sullivan et al. 1989). A decline in slow fibers is observed as a result of prolonged inactivity or hypogravity (Caiozzo et al. 1994), and the fiber composition of skeletal muscles influences insulin sensitivity (Kong et al. 1994) and lipoprotein metabolism (Tilkkanen et al. 1996). Drugs or gene therapies capable of modifying calcineurin activity selectively in skeletal
muscles plausibly could be used to increase the capacity for endurance exercise and to reduce risk for life-threatening diseases in human subjects.

**Materials and methods**

**Cell culture and transfection conditions**

NIH-3T3 cells or C2C12 myogenic cells were cultured, transfected with plasmid vectors, and assayed for luciferase and β-galactosidase, as described previously (Grayson et al. 1995, 1998). Each 35-mm dish of cells was cotransfected with promoter-reporter plasmid (0.5 µg), an expression plasmid that uses the CMV promoter to force expression of a constitutively active form of calcinurin (Manalan and Klee 1983; O’Keefe et al. 1992), or empty vector (pCI–NEO; 0.5 µg), along with a CMV–lacZ plasmid (0.5 µg) as an internal control for transfection efficiency. For dose-response experiments (Fig. 2), the total input DNA and the amount of promoter-reporter plasmid and CMV–lacZ was held constant, but the ratio of calcinurin expression vector to empty vector varied.

**Plasmid constructions**

The expression plasmid used to stimulate calcinurin-regulated gene transcription was constructed by linking a CMV promoter carried in pCI–NEO (Promega) to a truncated variant of calcinurin A from which the carboxy-terminal region containing the autoinhibitory domain and a portion of the calmodulin-binding domain was deleted (O’Keefe et al. 1992). This form of calcinurin exhibits constitutive phosphatase activity, and is not subject to regulation by calcium-calmodulin in the manner of the native protein (O’Keefe et al. 1992). Promoter-reporter constructs were designed by linking the luciferase gene carried in pGL3 (Promega) to upstream promoter regions from the Mb380, MbaAA/T; MbaCCAC; CCAC-TATA; A/T-TATA; CCAC-A/T-TATA) have been described in previous publications from this laboratory (Bassel-Duby et al. 1993; Grayson et al. 1995, 1998). Reporter constructions bearing five copies of the upstream NFAT response element from the myoglobin promoter (NRE-TATA and NRE-CCAC-A/T-TATA) were based on the oligonucleotide sequence 5′-AACCCGAAATAGTTGCCCCT-3′, and its complementary strand, representing nucleotide positions −694 to −674 in the human myoglobin promoter (underlined bases illustrate the NFAT consensus binding motif).

Putative NFAT binding sites within the myoglobin and troponin I slow promoters were disrupted using a PCR-based mutagenesis procedure, as described (Yang et al. 1997). The specific nucleotide sequence modifications included: myoglobin promoter (−690) AGGAAATA to GTGGACTA and (−232, reverse strand) TGGAAAAG to CTCGGAGA; TnI slow promoter (−738) AGGAAAC to AGCTAGC and (−639) TGGAAACA to TGGAAAC.

**Plasmids used to express NFAT–GFP fusion proteins**

In both NFATc–GFP and ANFATc–GFP, the native leucine residue at position 319 was converted to a methionine initiation codon. In the construct designed to express the truncated NFATc–GFP fusion protein (∆NFATc–GFP), the native leucine residue at position 319 was converted to a methionine initiation codon. In both NFATc–GFP and ∆NFATc–GFP, the native stop codon was replaced with a 7 amino acid insertion preceding the GFP coding sequence.

**Fluorescence microscopy**

An Olympus IMT-2 inverted fluorescence photomicroscope with FITC illumination and detection was used for evaluation and photography of C2C12 cells transfected with GFP expression plasmids. GFP fluorescence (excitation peak = 488 nm, emission peak = 507 nm) was photographed with Kodak Elite II 400 ASA slide film using an Olympus SC 35 SLR camera back.

**Histochemical analysis of fiber type in muscles from intact animals**

Adult rats were treated with cyclosporin A (5 mg/kg) or vehicle administered by intraperitoneal injection daily for 6 weeks. Animal care was in accordance with institutional guidelines. Sections of soleus muscles from 7 animals in each group were stained histochemically for myosin ATPase activity at pH 4.54, as described (Brooke and Kaiser 1970). The proportion of fast and slow fibers was quantified by three observers who were blinded to the treatment status of the animals. Fibers expressing fast myosin were identified in 8 µm cryosections sections of the same muscles, postfixed in 4% paraformaldehyde, by immuno-histochemical analysis using a commercially available mouse monoclonal antibody (MY-32: Sigma, St. Louis, MO; 1:400) and LRSC goat anti-mouse IgG (Jackson Immunocytogenetics, West Grove, PA; 1:50). Nuclei were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR) at 0.6 µg/ml for 10 min.

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