CONTROL OF SKELETAL MUSCLE FIBER TYPES

BY CALCIUM SIGNALING PATHWAYS

APPROVED BY SUPERVISORY COMMITTEE

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TO MY MOM

CONTROL OF SKELETAL MUSCLE FIBER TYPES BY CALCIUM SIGNALING PATHWAYS

by

HAI WU

DISSERTATION

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Different patterns of motor nerve activity drive distinctive programs of gene expression in skeletal muscles, thereby establishing a high degree of metabolic and physiological specialization among myofiber subtypes. Previous studies have demonstrated that calcineurin activity is required to maintain slow myofiber identity. I am interested in

determining the transcription factors downstream of calcineurin and other calcium-regulated signaling pathways in the control of myofiber specialization.

By analyzing two fiber type-specific enhancers, I was able to demonstrate that there are functional NFAT (nuclear factor of activated T cells) and MEF2 (myocyte-specific enhancer factor 2) binding sites within the enhancer of troponin I slow, and both sites are required for slow fiber specific activity of this enhancer.

Next, I identified MEF2 as a target of calcineurin in cultured myogenic cells. Calcineurin physically interacts with MEF2 and dephosphorylates MEF2. C-terminal transactivation domain, but not N-terminal DNA binding domain of MEF2, responds to calcineurin activation. The use of "MEF2 indicator" transgenic mice that harbor a MEF2-dependent lacZ transgene enabled us to monitor the endogenous activities of MEF2 transcription factors. MEF2 is selectively active in slow and oxidative myofibers. Calcineurin is both necessary and sufficient for MEF2 activation in skeletal muscles. I also found a dose-response relationship between calcineurin activity and expression level of slow, oxidative fiber-specific and MEF2 target genes. Furthermore, I observed that functional activity of MEF2 transcription factors was stimulated by sustained periods of endurance exercise or low-frequency motor nerve pacing in a calcineurin-dependent manner.

In addition to calcineurin, CaMKs (calcium, calmodulin-dependent kinases) also transduce their signaling through MEF2. CaMKIV synergistically activates MEF2-dependent gene expression together with calcineurin. Transgenic mice expressing constitutively active CaMKIV in their skeletal muscles showed increased percentage of slow and oxidative

myofibers, which was accompanied by increased mitochondrial biogenesis mediated through the upregulation of PGC-1 (PPARγ co-activator).

Taken together, these findings delineate a molecular pathway in which MEF2 and NFAT integrate signaling inputs from multiple calcium-regulated pathways in the control of skeletal muscle fiber types.

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Chapter I. Introduction

1. Introduction to skeletal muscle fiber types

Classification of vertebrate skeletal muscle fiber types

The adult human musculature is composed of hundreds of muscles, each of which contains different amount of myofibers. These muscles and their comprising myofibers are required to generate a wide range of movement and forces for us to survive. Animals including humans have adapted to these demands by developing specialized fibers that can be categorized under different schemes, such as muscle color, contractile speed, morphologic, metabolic and physiologic characteristics, *etc*.

The redness of a muscle or myofiber correlates with the amount of mitochondria and vasculature within the fiber as well as the concentration of myoglobin. The superficial lateral vastus muscle is almost pure white, while the soleus muscle is deep red. The contractile speed of a myofiber is directly determined by its myosin ATPase activity and also depends on the presence of different calcium handling systems in the sarcoplasmic reticulum as well as specific isoforms of contractile proteins (Reiser *et al*., 1985; Salviati *et al*., 1984). In the same species, the isometric contractile speed of fast fibers is three times as quick as their slow counterparts (Burke *et al*., 1971). Generally speaking, slow myofibers are utilized to maintain posture and antagonize gravity, while fast fibers are engaged in locomotion. Myofibers can also be distinguished by different activities of enzymes involved in energy metabolism. Oxidative fibers rely on oxidative phosphorylation as their energy source, so

they express enzymes involved in TCA cycles and electron transport more than glycolytic fibers, which utilize glycolysis to generate ATP and hence contain more glycolytic enzymes. While slow fibers are red and oxidative, fast fibers can be either red or white, oxidative or glycolytic. For decades, histochemical assays for actomyosin ATPase activity have been used to distinguish fiber types. ATPase activity in type I fibers are acid stable and alkali labile, while the opposite is true for type II fibers (Guth and Samaha, 1969). Type I fibers are slow ones and type II fibers are fast fibers. Type II fibers can be subdivided into IIa, IIb, IIm and IIx/d based upon quantitative differences of ATPase activity after preincubating fiber sections at different pH, as shown in Table 1.

Skeletal myofibers are more heterogeneous when examined at a molecular level than when defined by classification systems mentioned above. Almost every protein involved in contraction (myosin heavy chain, myosin light chain, Troponin I, T, C, actinin, C-protein, α-actin, etc) has at least two isoforms expressed discretely in slow and fast fibers respectively (Schiaffino and Reggiani, 1996). Researchers often use myosin heavy chain (MHC) isoforms to type skeletal myofibers. In adult skeletal muscles, there is good correlation between histochemical fiber types and certain MHC isoforms (Table 1). MHC is encoded by a multi-gene family in all vertebrates, with more than 10 different MHC expressed in embryonic, neonatal and adult mammalian myofibers (Weiss and Leinwand, 1996). Unlike nascent fibers, in which multiple isoforms of MHC coexist, mature adult myofibers normally only express one isoform of MHC. The slow tonic isoform is only found in a narrow range of muscles, including the extraocular and tensor tympani and certain intrafusal fibers. The slow-

Table 1: Adult mammalian skeletal muscle fiber types defined by different schemes

Histochemical Classification			MHC isoform expression	Contraction speed	muscle color	oxidative/ glycolytic
	pH4.4	pH10.4				
Type I	++++	-	slow/cardiac β	slow	red	oxidative
Type IIa	-	++++	IIa	fast	red	oxidative/ glycolytic
Type IIb	+	++++	IIb		white	glycolytic
Type IId/x	++	++++	IId/x		white	glycolytic
Type IIm	+++	++	IIm		n.k.	n.k.

n.k.-not known

twitch isoform is expressed in most slow fibers and is identical to cardiac β isoform. Type IIa, IIb and IIx/d are widely expressed in muscles throughout the body, especially in small mammals that have faster locomotion compared to larger mammals. Type IIeom and IIm are found in extraocular and branchial arch derived muscles, respectively.

Throughout this dissertation paper, I will use slow/fast and type I/II interchangeably and my dissertation research focuses on the dichotomy between adult slow/type I and fast/type II myofibers in rodents. Although skeletal myofibers can be discretely classified based on MHC isoforms and histochemical staining patterns, within each fiber type, there is a wide range of variation in the oxidative /glycolytic enzyme activity (Guth and Yellin, 1971). Type I fibers, together with type IIa fibers, can be loosely labeled as oxidative fibers while other fast fibers are collectively referred to as glycolytic fibers.

Origin of fiber type diversity during embryogenesis in different model organisms

As I will mention later in this chapter, the contraction rate of neonatal and adult muscle fibers can be altered by neuronal activities, which led to the earlier notion that all myoblasts and myotubes are initially identical and subsequent motor neuron innervation determines the myofiber diversity. Since the 1980s, several groups have identified various types of myofibers formed in embryonic chicken and rodent limb muscles that had been denervated, suggesting that at least the initial fiber type diversity was nerve-independent (Butler *et al*., 1982; Condon *et al*., 1990; Crow and Stockdale, 1986). Indeed, from chicken embryos, distinct types of myoblasts were identified that expresse slow or fast MHC isoforms upon differentiation into myotubes (Rutz and Hauschka, 1982). Explant of wing

buds that were not penetrated by nerves into the pelvic area of recipient chicken embryos showed typical fiber diversification of wrist muscles, although they are innervated by lumbar motor neurons (Laing and Lamb, 1983). Collectively, these results suggest that different types of myofibers form from distinct myoblast populations and muscle fibers might determine the properties of motor neurons or be matched with specified motor neurons.

Like chickens, mammalian myogenesis occurs in two waves: primary and secondary (Draeger *et al.*, 1987). In the primordia of most muscles, primary myotubes are linked by gap junctions to form a chemical and electrical syncitium. At this stage, primary myotubes express embryonic and slow isoforms of MHC. Subsequently, primary myotubes move apart and new generations of myoblasts gather around the primary cells and use them as scaffolds to form secondary myotubes. Gap junctions connect the clusters of secondary myotubes with their founding primary ones and they are innervated as a single unit by multiple axon sprouts. Later on, these myotubes separate and become independent contraction units. Most mammalian secondary myotubes express embryonic and perinatal forms of MHC and will develop into fast fibers eventually. Primary myotubes at certain locations later lose slow MHC expression and produce perinatal MHC followed by fast MHC. Some primary myotubes continue slow MHC expression and become adult slow fibers (Rubinstein and Kelly, 1981). Neuromuscular matching ensures that pre-specified slow and fast motor neurons innervate slow and fast fibers respectively.

Unlike vertebrates, in which MHC isoforms are encoded by unique sets of genes, Drosophila only possess one MHC gene, which gives rise to at least 15 MHC isoforms through alternative splicing (Bernstein *et al.*, 1986). Larval and adult musculature have

different MHC composition. Within adult flys, functionally different muscle groups express various MHC splicing isoforms. These alternative exon-encoded regions of Drosophila MHC modulate MHC ATPase activity and actin sliding velocity, mimicking slow/fast property of vertebrate contractile machinery (Collier *et al*., 1990). Muscle-specific splicing factors controlling alternative splicing of MHC have not been identified in Drosophila yet.

More insights on vertebrate fiber type diversification come from studies of zebrafish. In zebrafish, somitic segments are divided into dorsomedial and ventrolateral regions, which form epaxial and hypaxial muscles respectively (Figure 1). The lineage of muscle cells in zebrafish can be tracked and they have three distinctive embryonic fiber types: muscle pioneer slow fibers, non-pioneer slow fibers and fast fibers (Devoto *et al*., 1996). Adaxial cells in the segmental plate adjacent to the notochord elongate and migrate radially to the surface of somite to form a superficial monolayer of embryonic slow myofibers. A small population of these cells express *engrailed* protein and are called pioneer slow fibers, which lie along the dorsal-ventral midline. Fast fibers are formed from cells lateral to adaxial cells in the segmental plate. Sonic hedgehog (Shh) signals emanating form notochord are essential for slow fiber development and ectopic expression of Shh is sufficient to transform the entire myotome into slow fibers (Blagden *et al*., 1997; Currie and Ingham, 1996). Other signaling molecules derived from neural keel (equivalent of neural tube in amniotes), such as TGF-β (transforming growth factor β) family members, can inhibit slow fiber identity (Du *et al*., 1997). The role of Hedgehog signaling in fiber type diversification in mammals has not been established yet. Mammalian secondary fast fibers form on slow primary myofiber scaffolds, reminiscent of the spatial pattern of fast and slow fiber formation in zebrafish.

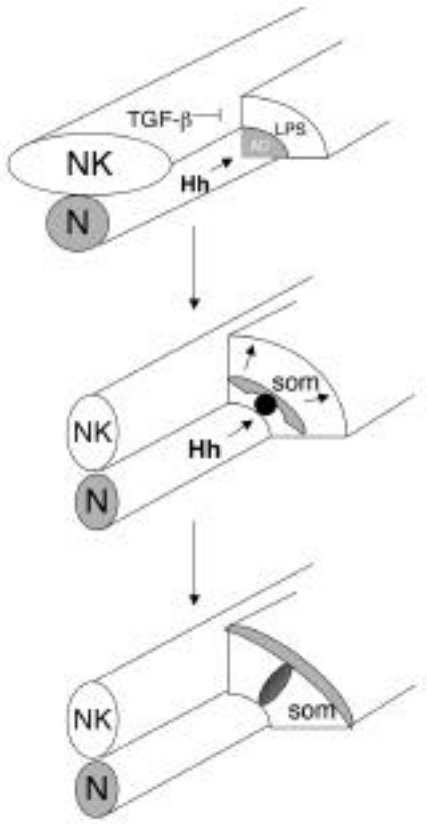


Figure 1. Specification of fiber types in zebrafish embryo. In zebrafish lateral presomitic mesoderm (LPS), sonic hedgehog (Hh) signal from notochord (N) is required for slow fiber (gray) formation in the adaxial (AD) region. TGF-β signal from neural keel (NK) limits the population of slow fibers. A further Hh signal specifies muscle pioneer (black) and non-pioneer (gray) slow fibers in the somites (som). Non-pioneer slow fibers migrate to the somite surface and the lateral somite (white) will differentiate into future fast muscles.

Hedgehog pathway in mammals likely also modulate fiber types of primary and secondary myotube in the absence or presence of motor neuron inputs.

Fiber type switching in adult mammals

As I mentioned previously, neural innervation is not required for initial appearance of distinct slow/fast myoblast populations, but as the animals mature, a number of environmental cues modulate myofiber identities. Among them, neural activity imposed upon myofibers and systemic changes in the hormonal milieu are two major factors (Izumo and Mahdavi, 1986; Pette and Vrbova, 1992). Fiber type diversity is fully established when the animals reach adulthood. At this time, the skeletal muscles still remain plastic, in a sense that they can alter the quantity and/or type of proteins comprising the subcellular compartments in response to stumuli that disrupt their normal homeostasis. By looking into fiber type transformation caused by surgical, electrical or pharmacological interventions, we might gain insights into the mechanisms controlling fiber type determination and maintenance under normal physiological circumstances.

The pioneering work by Buller and his colleagues in 1960s elegantly defined the dominant effect of neural input on muscle phenotypes (Buller *et al*., 1960). As illustrated in figure 2, they switched two nerves innervating fast and slow muscles in adult rabbits. Weeks later, they found that the original fast fibers contracted slower and the previous slow fibers behaved much like fast ones. The transformation of muscles encompasses all functional aspects of myofibers and include the exchange of fast/slow contractile protein isoforms as

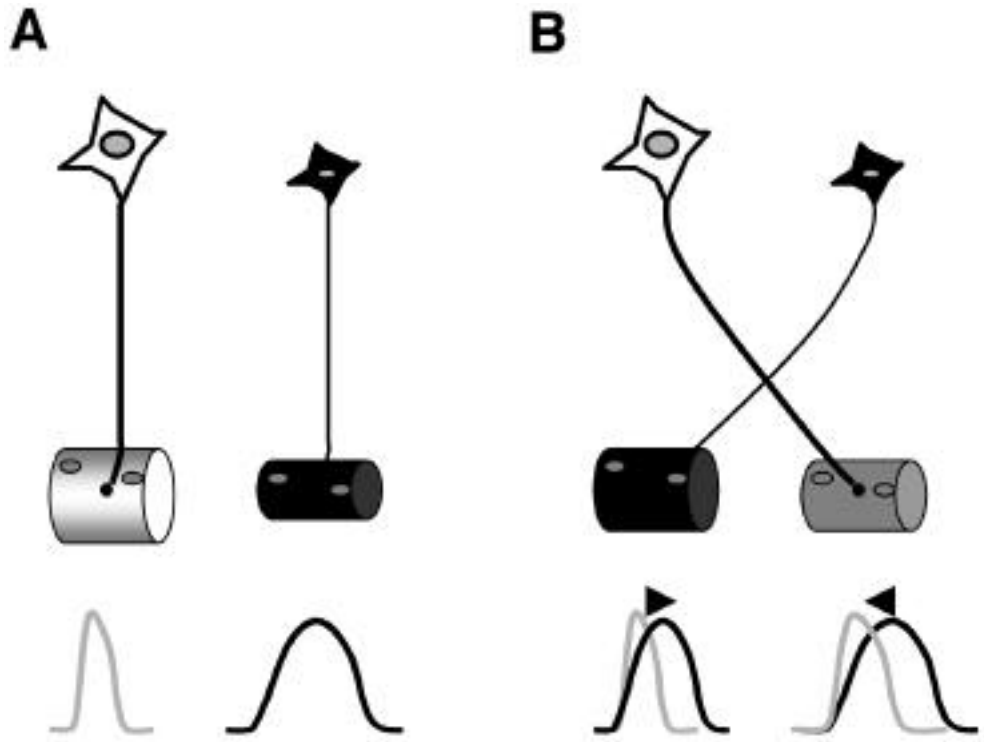


Figure 2. Switch of fiber types by motor nerve cross-innervation. **A.** Before cross-innervation, fast (gray) and slow (black) myofibers are innervated by slow and fast motor neurons, respectively. Contractile speed is illustrated. **B.** After cross-innervation, the original fast fiber behaves like a slow fiber, while the original slow fiber is transformed into a fast one.

well as changes in oxidative/glycolytic enzyme activities (Gunderson *et al*., 1988). This experiment, which highlights the fiber type-determining influence of motor neurons on the myofibers in adult animals, is the starting point for modern studies of fiber type control.

In adult mammals, each myofiber is innervated by only one motor neuron, which also innervates many other fibers. The myofibers innervated by a single motor neuron form a motor unit and the average number of myofibers in a motor unit varies markedly (Kandel *et al*., 1999). All the fibers within a motor unit have very similar physiological properties and express the same MHC isoform such that all fibers innervated by a slow motor neuron express type I MHC and those innervated by fast motor neuron all express type II MHC (Burke *et al*., 1973). The slow motor neuron of slow fibers fires at a frequency of 10-20 Hz and they are active almost continuously because the slow motor units are utilized constantly to maintain posture and antagonize gravity (Hennig and Lomo, 1985). On the other hand, fast motor neurons can have bursts of activity at a frequency of 100 Hz or more during fast locomotion, but this activity is flanked by long periods of neural quiescence (Hennig and Lomo, 1985). Are these different electrical activity inputs from motor neurons accountable for fiber type transformation? Are different trophic factors released by motor neurons into myofibers to effect the switching? Chronic low frequency electrical stimulation of fast muscles, which mimics the tonic firing patterns of slow motor neurons, results in similar transformation of fiber types (Pette and Vrbova, 1992). Conversely, a transformation of slow muscles into fast muscles is achieved by phasic, high-frequency stimulation of muscles (Hamalainen and Pette, 1996). Together, these pacing experiments indicate electrical activities experienced by myofibers cause the shift of fiber types.

Since then, a great number of studies have focused on the effect of neuromuscular activity on muscle phenotype. Functional overload or exercise training results in transformation of pre-existing fast fibers into slow-twitch, oxidative phenotype (Baldwin and Haddad, 2001; Sugiura *et al*., 1993). Conversely, decreased neuromuscular activity induced by spinal cord injury, limb immobilization, hind limb suspension, space flight or blockage of action potential conduction by neurotoxins causes the shift of slow oxidative fibers into fast glycolytic ones, although the extent of transformation varies in different muscles and experimental settings (Baldwin and Haddad, 2001; Talmadge, 2000). The change of muscle phenotype is evident at molecular level, with transition of contractile protein isoforms as well as alteration of glycolytic and oxidative enzymes. MHC isoforms are widely used to measure the direction and extent of fiber type switching, with type I MHC representing the slowest form and type IIb as the fastest one. During transition, the general direction is I ⇔ I&IIa ⇔ IIa ⇔ IIa&IIx ⇔ IIx ⇔ IIx&IIb ⇔ IIb, with the extent of transition depending on the intensity and duration of the stimuli. Fibers within certain muscles have different tendencies to be transformed into another type, perhaps reflecting the embryonic lineage of different fibers (Westgaard and Lomo, 1988).

Neural transmitted impulse pattern is a major influence on fiber type diversity, but additional factors also seem important, among which thyroid hormone (TH) is the most well-documented. TH is obligatory for the attainment of adult MHC phenotype in rats and both hypo and hyper-thyroidism can dramatically affect the fiber type composition in adult muscles (Caiozzo *et al*., 1991; Caiozzo *et al*., 1992). All MHC genes in rats respond to TH in a tissue-specific manner (Izumo and Mahdavi, 1986). Slow type I MHC is always down-

regulated by TH administration, while type IIa MHC shows opposite responses to TH treatment in different muscles. The variation in metabolism and TH receptor isoform availability might contribute to MHC responsiveness to hypo or hyper -thyroidism. The transcriptional control of MHC genes by TH is poorly understood in skeletal muscles since no TH responsive element (TRE) has been found in skeletal MHC genes. It is likely TH exerts its effects on MHC expression through some indirect regulation of other muscle specific factors.

More knowledge of the fundamental mechanisms controlling fiber type diversity will provide a framework for better understanding of the reactions and adaptations of muscles under healthy and diseased states. Fiber type composition of skeletal muscles influences insulin sensitivity and lipoprotein metabolism (Kong *et al*., 1994; Tikkanen *et al*., 1996). Slow oxidative fibers are relatively resistant to progressive myonecrosis that occurs with advancing age in individuals lacking dystrophin (Webster *et al*., 1988). A strategy to promote fast to slow fiber transformation may reduce morbidity and prolong life. Patients with congestive heart failure exhibit loss of slow oxidative fibers, an abnormality that contributes to exercise intolerance (Massie *et al*., 1988). Prolonged inactivity of disabled individuals or exposure to hypogravity during space flight triggers a decline in the number of slow fibers (Caiozzo *et al*., 1994). Measures to modulate fiber type composition could be used to enhance endurance exercise capacity, to reduce risk for life threatening diseases and to maintain fitness of astronauts in outer space adventures. Before these dreams become reality, we need detailed understanding of the mechanisms controlling the complexities of muscle fibers, of which we only have scarce knowledge now. In the next section, I will review some

of the potential signaling pathways and transcription factors that can regulate MHC isoform expression and muscle phenotype.

Transcriptional control of fiber type-specific gene expression

Fiber type determination is a complex process with many facets of gene expression (transcriptional, translational and post-translational) involved. Increasing evidence shows that the major mechanism employed by mammalian myofibers to maintain their diversity is the transcriptional control of differentially expressed isoforms of contractile proteins (Calvo *et al*., 1999). Two approaches are generally used to identify the transcriptional factors dictating fiber type-specific expression. The first is to find regulatory proteins selectively expressed in certain fiber types. The second is to uncover the cis-acting elements that are responsible for fiber type-specific activities of the promoters controlling slow and fast fiber-specific protein in skeletal muscles and then identify the transcriptional factors that bind on these elements. Both strategies have been productive in identifying transcriptional factors involved in fiber type regulation. With the coming of global gene profile assays, such as microarray technology, the first approach will provide us with more potential regulators of fiber type identities.

A lot of signaling molecules, including some transcription factors, have different levels of expression in various myofibers and fiber type transformation alters their expression levels. Neural stimulation provokes changes in the intracellular concentrations of cyclic AMP, nitric oxide, calcium, molecular chaperones and immediate-early gene products, such as c-fos (Michel *et al*., 1994; Neufer *et al*., 1996), but the biggest challenge is to identify

factors that have causal effects on fiber type determination. Recently, an activated Ras-MAPK (mitogen activated protein kinase) pathway has been shown to be able to mimic the effects of slow motor neurons on expression of MHC genes in regenerating fibers (Murgia *et al*., 2000), but the transcription factors mediating this effect have not been identified.

Various transcription factors are known to regulate muscle gene expression, among which, basic helix-loop-helix (bHLH) myogenic regulatory factors, including MyoD, myogenin, myf-5 and MRF-4 are the master regulators for muscle determination and differentiation during embryogenesis (Molkentin and Olson, 1996). They form heterodimers with ubiquitous E proteins and bind to specific DNA elements known as E-boxes. These myogenic factors continue to be expressed in adult skeletal muscles, suggesting more extended roles for these proteins in adult muscles. Indeed, MyoD and myogenin have been implicated as regulators of fiber type phenotype since these two factors display different expression levels in slow versus fast myofibers (Hughes *et al*., 1993). In rat soleus muscles, which contain 90% slow fibers and 10% fast fibers, there is a high level of myogenin mRNA and low level of MyoD mRNA, while in EDL (extensor digitorum longus) muscles that have very few slow fibers, the reverse is true. Furthermore, transformation of slow to fast fibers by several protocols is associated with decreased myogenin and elevated MyoD expression (Voytik *et al*., 1993). Ectopic over-expression of myogenin in fast skeletal muscles induces a shift of enzymatic activities from glycolytic to oxidative metabolism, but this shift toward oxidative direction is not accompanied by changes in MHC isoforms (Hughes *et al*., 1999). Another piece of evidence that argues against the role of MyoD/myogenin in fiber type determination comes from mice lacking MyoD. EDL muscles in these mice have normal

level of fast fibers and the soleus muscles even contain decreased number of slow fibers and elevated proportion of fast fibers. These observations are opposite to what would be expected if the relationship between MyoD/myogenin expression ratio and MHC isoforms is causal (Hughes *et al*., 1997). Since bHLH myogenic factors have multiple antagonizing partners, such as Mist1, MyoR and Id, which can inhibit their function, more genetic analysis are needed to address the potential functions of myogenic regulatory factors in fiber type determination.

Analysis of fiber type-specific promoter/enhancer elements is another powerful approach to identify DNA elements and their binding proteins that can control fiber type specificity. Using this approach, Hardeman's group cloned a novel slow fiber enhancer binding protein, MusTRD1 (muscle TFII-I repeat domain-containing protein 1), which is expressed predominantly in skeletal muscle and exhibits enhancer binding activity preferentially in slow fibers (O'Mahoney *et al*., 1998). *In vivo* reporter plasmid injection showed the enhancer region within troponin I slow (TnIs) gene bound by MusTRD1 is both necessary and sufficient for slow-fiber specific expression of the reporter gene. Transgenic mouse analyses indicated that although this enhancer region is critical for high-level activity of TnI slow promoter, the slow fiber specific expression of a reporter gene is not abolished by mutation of this enhancer. The extended homology between MusTRD1 and TFII-I led to the idea that MusTRD1 might regulate the functions of MEF2, a MADS box family member, and bHLH myogenic regulatory factors since TFII-I is known to interact with several transcription factors, including serum response factor (a MADS family member), USF1 (a

bHLH factor), *etc*. Considering the discrepancies between transgenic mice and plasmid injection experiments, it is not clear what is the exact role of MusTRD1 in fiber type control.

Several other enhancer elements that are necessary and/or sufficient for fiber type-specific expression of reporters have been identified, but the transcription factors bound to these enhancer elements remain elusive (Qin *et al*., 1997; Salminen *et al*., 1994).

2. Calcium ion as a second messenger in skeletal muscles

Calcium handling apparatus in skeletal myofibers

The dominant effects of motor nerve activity on fiber type-specific gene expression in adults led our group to propose several years ago that the relevant signaling pathways controlling fiber type specificity were triggered by changes in intracellular calcium concentration (Chin *et al*., 1998). Before I discuss our model and the experimental evidence that supports it, I would like to briefly introduce the multi-faceted functions of calcium ions in skeletal muscles.

Calcium ions act as second messengers in a variety of cellular contexts, including excitation-contraction coupling (E-C coupling) in muscles, transmitter release from neuronal cells, secretion of hormones, initiation of fertilization, control of cell division, differentiation, survival and apoptosis, *etc* (Berridge *et al*., 2000). In skeletal myofibers, resting intracellular free calcium concentration ($[Ca^{2+}]_i$) is maintained at about 50nM, as in most other types of cells. Depolarization triggered by acetocholine released from nerve endings opens up voltage-sensitive calcium channels (DHPR, dihydropyridine receptor) at the transverse tubules. The physical link between DHPR and the calcium release channel RyR (ryanodine

receptor) localized on the membranes of sarcoplasmic reticulum (SR) results in calcium release from SR into cytosol through RyR and elevates calcium concentration in the cytosol to micromolar range (Figure 3). The binding of free calcium to troponin C on the thin filaments initiates contraction. As cytosolic calcium is taken up into SR and transported back into the extracellular space, muscle relaxes and another E-C coupling is primed to start.

The speed of muscle contraction and relaxation is dependent on the composition of the calcium handling systems in different types of myofibers. The calcium handling apparatus consists of calcium entry channels on cell surface and release channels on SR membranes, calcium pumps, calcium buffering and storage proteins and other calcium-binding molecules not directly involved in calcium cycles (Figure 3). These components display quantitative and/or qualitative differences between slow and fast myofibers (Berchtold *et al*., 2000). Transformation of fiber types as monitored by MHC isoform switch is also reflected by the remodeling of the calcium handling systems (Berchtold *et al*., 2000). The increase of $[Ca^{2+}]_i$ during muscle contraction comes largely from calcium released from SR through RyR1, which is the predominant isoform of RyR in both slow and fast myofibers. Compared to slow fibers, fast fibers have higher density of RyR1 to facilitate fast mobilization of calcium from SR store into cytoplasm (Damiani and Margreth, 1994). Both the rate of calcium release from SR and the open probability of RyR1 are under the control of calcium, nitric oxide and protein partners that interact with RyR1, such as FK506 binding proteins (Berchtold *et al*., 2000). Muscle relaxation is initiated by the fall of intracellular calcium concentration in myofibers after contraction. Fast fibers develop a more efficient system to re-uptake calcium ions back into SR so they relax faster than their slow

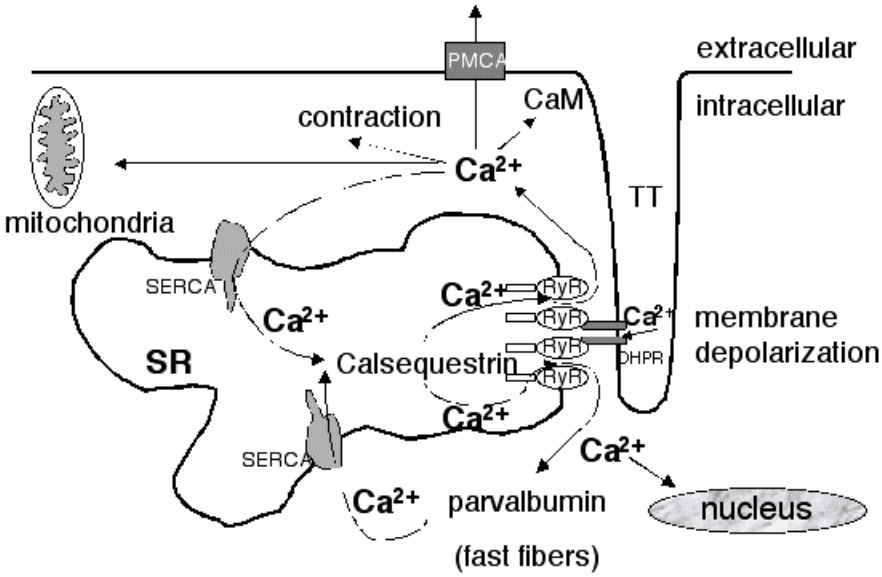


Figure 3. Calcium handling system in skeletal muscles. The propagation of action potential into the transverse tubule system (TT) triggers Ca^{2+} entry through the L-type Ca^{2+} channel (DHPR) and stimulates Ca^{2+} release from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR). Increased cytoplsmic [Ca^{2+}] initiates a variety of signaling pathways, including E-C coupling. $[Ca^{2+}]_i$ is brought down to basal level via several routes. Calcium can be take up into SR by SERCA (sarco-endoplasmic reticulum calcium ATPase) or driven into extracellular space by PMCA (plasma membrane calcium ATPase). Calcium can also be transported into mitochondria and nucleus. Inside SR, calcium is tethered by calsequestrin. In fast skeletal myofibers, parvalbulin in cytoplasm quickly buffers calcium. Once $[Ca^{2+}]_i$ reaches basal level, another cycle of E-C coupling will be initiated.

counterparts. The fastest type IIb fibers show strongest immunoreactivity for parvalbumin, a cytosolic calcium binding protein that helps fast muscles to relax. Ectopic expression of parvalbumin in slow muscles (soleus), which lack the expression of parvalbumin under normal circumstances, shortens relaxation time of soleus, while in a fast muscle (EDL) of the parvalbumin knock out mice, the decrease of calcium concentration after stimulation is 33% slower than that of wild type mice, suggesting parvalbumin is a relaxation factor in fast-twitch fibers (Muntener *et al*., 1995; Schwaller *et al*., 1999).

The active re-uptake of calcium into SR is carried out by sarco(endo)plasmic reticulum calcium ATPase (SERCA), which are encoded by three different genes with alternative splicing variants in rodents (Wuytack *et al*., 1992). SERCA1a is specifically expressed in fast skeletal myofibers and SERCA2a is expressed in slow skeletal fibers as well as in cardiac and smooth muscles. SERCA3 is found in non-muscle cells, such as platelets, lymphoid and endothelial cells. The maximal velocity of calcium transport by SERCA is 2-3 times faster in fast myofibers than in slow ones due to the presence of different SERCA isoforms (Salviati *et al*., 1984). SERCA2a is regulated by phospholamban, which is expressed selectively in slow fibers but not in fast fibers. Phospholamban inhibits calcium ATPase activity by reducing the affinity of SERCA for calcium (Simmerman and Jones, 1998). When phospholamban is phosphorylated by protein kinases as in the case of β-adrenergic stimulation, it dissociates from SERCA2a and the inhibition on SERCA is now relieved. Besides SERCA, plasma membrane calcium ATPase (PMCA) also functions to reduce intracellular calcium concentration. No differences in PMCA expression level or isoforms have been found in slow versus fast fibers (Sacchetto *et al*., 1996).

Calcium ions pumped into SR by SERCAs are transferred to calcium binding proteins localized within SR. Calsequestrin is the major calcium storage protein in muscles SR, with a low affinity but high capacity for calcium binding (Volpe *et al*., 1992). The ubiquitous calreticulin also contributes to calcium storage in SR. Calsequestrin has two isoforms: fast and cardiac. Fast-twitch skeletal fibers express exclusively the fast form of calsequestrin while in slow skeletal myofibers, 75% of calsequestrin is fast form and 25% cardiac form (Damiani and Margreth, 1994). The physiologic significance of calsequestrin composition in the functioning of slow and fast fibers awaits further studies.

Calcium, calmodulin-dependent phosphatase and kinases

In addition to their roles in E-C coupling, changes in $[Ca^{2+}]_i$ can also cause long-term effects in skeletal muscles by mechanisms involving transcriptional regulation of muscle genes. Calmodulin (CaM), a small ubiquitous calcium-binding protein, acts as calcium receptor and transduces the information encoded by calcium changes to a variety of CaM-binding proteins (Zhang and Yuan, 1998), among which calcineurin and CaMKs (calcium, calmodulin-dependent protein kinases) are two families of enzymes known for their effects on transcription factors. Regulation of calcineurin, a Ser/Thr protein phosphatase, and CaMKs, a family of Ser/Thr protein kinases, shares common features including selective recognition of calcium/CaM followed by enzyme activation through relief of intramolecular inhibition (Klee *et al.*, 1988; Lukas *et al*., 1988). Both enzymes are very abundant in brain and immune systems and are expressed at various levels in other tissues.

Calcineurin, also called protein phosphatase 2B (PP2B), is a heterodimeric phosphatase composed of catalytic subunit A (CnA) and regulatory subunit B (CnB). CnB contains four calcium binding E-F hand motifs, but association between CnA and CnB is not affected by calcium binding to CnB (Sikkink *et al*., 1995). The N-terminal myristoylation of CnB might target calcineurin holoenzyme to membranes and increases calcineurin thermal stability (Zhu *et al*., 1995). CnA subunit has a catalytic domain at its N-terminus, followed by a CnB binding helix, a CaM binding domain and an autoinhibitory domain (Figure 4A). In the absence of calcium, CaM cannot bind CnA. Upon calcium binding, CaM gains the ability to associate with CnA. CaM induces comformational changes in the autoinhibitory domain on CnA and displaces this inhibitory domain away from the catalytic domain, resulting in the activation of calcineurin (Kissinger *et al*., 1995). Truncation of the autoinhibitory domain from calcineurin results in a constitutively active form of calcineurin without the requirement for CaM (Klee *et al*., 1988). Calcineurin phosphatase activity is not sensitive to okadaic acid, which can inhibit protein phosphatase 1 and 2A. Immunophilin/immunosupressant complexes, such as cyclophilin/cyclosporin A and FKBP/FK506, are potent inhibitors of calcineurin by non-competitive mechanisms (Liu *et al*., 1991).

The substrate specificity of calcineurin is determined by both the primary sequence and higher order structures of the substrates (Blumenthal *et al*., 1985). The classical substrate for calcineurin is NFAT (nuclear factor of activated T cells) (Crabtree, 2001). Dephosphorylation of NFAT by calcineurin drives NFAT from cytosol into nucleus, where NFAT binds its cognitive DNA elements and turn on the transcription of target genes (Figure 4B). In T lymphocytes, NFAT together with AP-1 family members (c-fos, jun), induces


A
70
333
490
521
CATALYTIC
REGULATORY

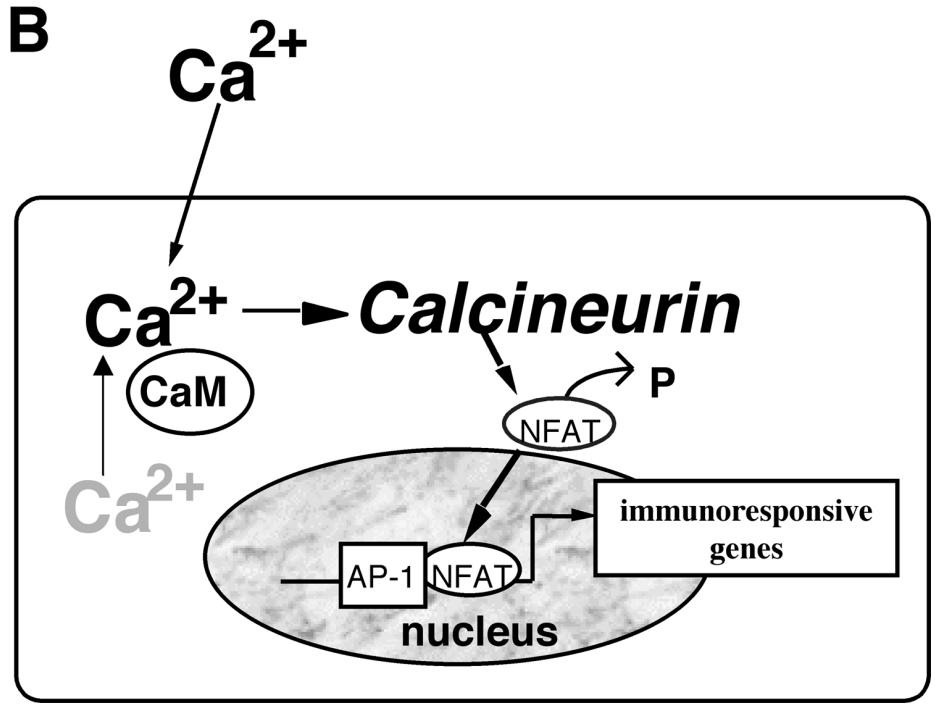

B
Ca2+
Ca2+
Calcineurin
CaM
P
NFAT
Ca2+
immunoresponsive genes
AP-1
NFAT
nucleus

Figure 4. Calcineurin signaling pathway in T lymphocytes. **A.** Schematic representation of murine calcineurin A subunit, with catalytic domain at its N-terminus followed by regulatory domain. The regulatory domain contains the calcineurin B-binding helix, the calmodulin-binding region and the autoinhibitory domain. **B.** Calcineurin-NFAT pathway in T lymphocyte. A rise of the intracellular calcium level through calcium entry (black) and/or calcium release (gray) results in the activation of calcineurin, which dephosphorylates NFAT. Hyper-phosphorylated NFAT proteins reside in the cytoplasm and their dephosphorylation by calcineurin drives the translocation of NFAT into nucleus, where together with AP-1 (Jun and Fos), they bind to the cognate enhancer sequence and turn on the expression of immunoresponsive genes, such as IL-2.

expression of immunoresponsive genes, such as IL-2 (Luo *et al*., 1996). Calcineurin-NFAT pathway regulates neuronal plasticity in response to synaptic activities (Graef *et al*., 1999). In cardiac and skeletal muscle cells, nuclear NFAT triggers hypertrophic responses with the help from other transcription factors, such GATA (Molkentin *et al*., 1998; Musaro *et al*., 1999).

Data from recent studies suggest that calcineurin activity is subject to redox regulation. The catalytic core site of active calcineurin contains an Fe^{3+}-Zn^{2+} dinuclear center. Oxidation of the Fe^{3+}-Zn^{2+} cluster to the diferric state by hydrogen peroxide leads to a loss of calcineurin activity (Yu *et al*., 1997). Superoxide dismutase is able to protect calcineurin from inactivation (Wang *et al*., 1996). Inactivation of calcineurin by hydrogen peroxide is also the result of the oxidative formation of a disulfide bridge involving cystein residues within the catalytic site of calcineurin (Bogumil *et al*., 2000).

CaMK is a family of protein kinases including CaMKI, II, III and IV, each of which has multiple isoforms encoded by different genes or generated through alternative splicing (Lukas *et al*., 1988). Like calcineurin, all CaMK family members are activated, at least partially, by the displacement of an intramolecular antoinhibitory domain from their catalytic domain, but various CaMKs have additional mechanisms to further potentiate their catalytic activities. CaMKII and III can convert themselves to a calcium/CaM-independent state as a result of autophosphorylation (Mukherji and Soderling, 1995; Redpath *et al*., 1993). CaMKII and IV are very abundant in brain and CaMKI and III are ubiquitously expressed (Lukas *et al*., 1998). CaMKI and IV are closely related at primary sequence level with similar modes of activation and substrate preferences. These two are monomeric and trans-phosphorylation of

a conserved threonine residue within the activation loop by upstream calcium/CaM-dependent kinase kinases (CaMKK) increases their catalytic activities (Corcoran and Means, 2001).

CaMKI, II and IV have similar substrate preferences with the minimum consensus sequence as Hyd-X-R-X-X-S/T (Hyr is any hydrophobic amino acid) (Corcoran and Means, 2001). CaMKII homoenzyme is a homo or hetero-multimer of 6-12 subunits, each of which is closely related to others. CaMKII is involved in murine long term potentiation (LTP), a process critical for learning and memory since autophosphorylation of CaMKII allows its kinase activity to persist after the calcium signals subside (Lisman and Zhabotinsky, 2001; Mayford *et al*., 1996). CaMKI and IV can both phosphorylate synapsin I and CREB (cyclic AMP responsive element binding protein). Because of its nuclear localization and its ability to phosphorylate transcription factors, CaMKIV is a very good candidate for the transduction of calcium signaling into gene expression (Soderling, 1999). CaMKIV also mediates depolarization-induced alternative splicing of ion channels in neurons (Xie and Black, 2001). CaMKIII is also called EF-2 kinase because it specifically phosphorylates elongation factor 2 (EF-2) and reduces the affinity of EF-2 for ribosomes (Redpath *et al*., 1993).

Since increases in $[Ca^{2+}]_i$ will activate both calcineurin and CaMK pathways simultaneously, these two enzymes can synergize or antagonize each other under different circumstances. For example, calcineurin and CaMKIV synergistically activate expression of TNF (tumor necrosis factor) family genes, probably through NFAT and ATF (activating transcription factor) (Lobo *et al*., 1999). In T lymphocytes, calcineurin and CaMKIV display synergy in the induction of Nur77, an orphan steroid receptor involved in lymphocyte

apoptosis (Blaeser *et al*., 2000). In hippocampal neurons, CREB phosphorylation and expression of CREB-dependent genes are induced by CaMKIV activation, but activated calcineurin at the same time antagonizes these effects during short period of synaptic activities (Bito *et al*., 1996). Calcineurin indirectly inhibits CREB phosphorylation by dephosphorylating and inactivating phospho-protein inhibitor 1, a PP1 inhibitor. De-inhibited PP1 directly counteracts CaMKIV phosphorylation of CREB.

Signaling specificity conferred by different patterns of calcium transients

Calcium participates in almost every aspect of cellular biological process. How does this simple universal signal have such a profound and diverse effect? The answer lies in the complex spatiotemoral patterns of calcium oscillations, which account for the specificity of calcium signaling (Berridge, 1997). Modulations of calcium localization, duration, amplitude and frequency are exploited to achieve the information-processing diversities of this cation. For example, neuronal differentiation has been shown to be controlled by frequency of spontaneous spikes (Gu and Spitzer, 1995). In lymphocytes, transcription factors are differentially activated by calcium signals in an amplitude-modulated fashion (Dolmetsch *et al*., 1997).

Oscillation of $[Ca^{2+}]_i$ is observed in most cells exposed to external stimuli that trigger calcium mobilization. In non-excitable cells, individual calcium transients last seconds or minutes due to the activation of phosphoinositide cascade and capacitative calcium entry. Frequency of calcium spikes is generally below 1 Hz in these cells. Amplitude and duration of calcium transients in these cells determine the signaling output and oscillation frequency

can further modulate the signaling efficiency especially at low levels of stimulation (Dolmetsch *et al*., 1998). In excitable cells, such as neurons and muscle cells, opening of voltage-sensitive calcium channels and calcium release channels allows the fast flow of large amount of calcium ions into the cytosol and results in a steep rise of calcium concentration that lasts only milliseconds due to the presence of calcium buffering proteins and efficient calcium pumps, as I mentioned previously. The calcium spikes are repeated at frequencies of tens or hundreds of Hz in these cells. During high-frequency stimulation, steady-state intracellular free calcium concentration increases as a result of saturation of the calcium expulsion systems (Carroll *et al*., 1997).

Different patterns of calcium oscillations are sensed by many molecules involved in calcium signal transduction. Calcineurin and CaMKs can decode calcium information in different ways. CaMKII can act as a frequency decoder of calcium spikes (Dekoninck and Schulman, 1998). *In vitro* studies show that at low frequencies (1-10Hz), CaMKII autonomous (calcium/CaM-independent) activity positively correlates with increase of calcium frequency if the duration of each pulse is fixed. Increase of pulse duration shifts the frequency responses of CaMKII such that lower frequency is needed to achieve the same level of CaMKII activation. This unique mode of activation indicates CaMKII can maintain its activity even after the cessation of calcium stimulation and implicates CaMKII in long-term potentiation (LTP) (Lledo *et al*., 1995).

Nuclear translocation of NFAT is a very dynamic process determined by the intimate balance between active nuclear import and export of NFAT (Shibasaki *et al*., 1996; Zhu and McKeon, 1999). Dephosphorylation of NFAT by calcium/CaM-activated calcineurin

unmasks the nuclear localization signal (NLS) on NFAT and drives NFAT into the nucleus, where calcineurin continues to bind to NFAT. The calcineurin-binding site on NFAT overlaps with NFAT nuclear export signal (NES). Binding of calcineurin on NFAT excludes Crm1, the nuclear export signal receptor, from NFAT. When calcium level drops, calcineurin dissociates from NFAT and exposes NES on NFAT to allow the export of NFAT into cytoplasm, where it is re-phosphorylated by a battery of protein kinases, including glycogen synthase kinase 3 (GSK-3), casein kinase I and JNK (c-jun N-terminal kinase) to ensure that NFAT stays in the cytoplasm (Beals *et al*., 1997; Chow *et al*., 1997; Zhu *et al*., 1998). A brief spike of calcium cannot maintain calcineurin/NFAT complex in the nucleus long enough for NFAT to function as a transcription factor. A sustained calcium elevation keeps the association between NFAT and calcineurin and antagonizes the driving force for NFAT nuclear export (Zhu and McKeon, 1999). NFAT is more sensitive to calcium change than other calcium-responsive transcription factors, such as NF-κB and ATF. The enhanced calcium-sensitivity of NFAT enables the selective activation of NFAT by moderate calcium elevation. Moderate increases in $[Ca^{2+}]_i$ (200-400nM) significantly promotes nuclear translocation of NFAT (Dolmetsch *et al*., 1997). The presence of two calcium-binding partners for CnA, CaM and CnB, might contribute to dephosphorylation of NFAT by calcineurin at low calcium levels. CaM and CnB could cooperate in calcium binding and activate CnA at a lower calcium threshold compared to other calcium/CaM-regulated enzymes . Calcium/CaM binding to CnA increases V_{max} of calcineurin catalysis reaction and calcium/CnB binding lowers the K_m and increases the affinity of calcineurin catalytic subunit

for its substrate (Perrino *et al*., 1995; Stemmer and Klee, 1994). In the presence of calcium, CaM and CnB synergistically activate CnA subunit.

Calcium signaling and control of skeletal muscle fiber type

Tonic motor neurons innervating slow-twitch fibers fire at 10-15 Hz almost constantly, resulting in a sustained elevation of $[Ca^{2+}]_i$ within a concentration range between 100nM and 300nM (Chin, 1996)(Figure 5). In fast myofibers, resting $[Ca^{2+}]_i$ is maintained at 50nM, with occasional calcium spikes evoked by fast motor neurons (Schwaller *et al*., 1999) (Figure 5). Chronic treatment of primary cultured rabbit muscle cells with calcium ionophore, which mobilizes calcium ions into the cytosol and increases $[Ca^{2+}]_i$, completely transforms fast MHC-expressing myocytes into cells that express exclusively slow MHC and myosin light chain (MLC) (Kubis *et al*., 1997). Removal of ionophore from culture rapidly reverses the patterns of MHC expression from slow back to fast isoforms. These data indicate that increases of $[Ca^{2+}]_i$ that have been well documented during fast-to-slow fiber transformation is a causal rather than secondary event (Gros, 1997).

As mentioned, sustained moderate elevation of $[Ca^{2+}]_i$ is sufficient to activate calcineurin and maintain its substrate, NFAT, inside the nucleus, while interspersed calcium transients characteristic of fast myofibers are not able to persistently activate calcineuri-NFAT pathway. Several years ago, we proposed that as a consequence of tonic nerve stimulation, slow fibers maintain a sustained elevation of $[Ca^{2+}]_i$ inside the myofibers to activate calcineurin, which in turn controls slow-fiber identity (Chin *et al*., 1998). In fast fibers, this pathway is not activated and slow-fiber specific genes are not induced, leaving

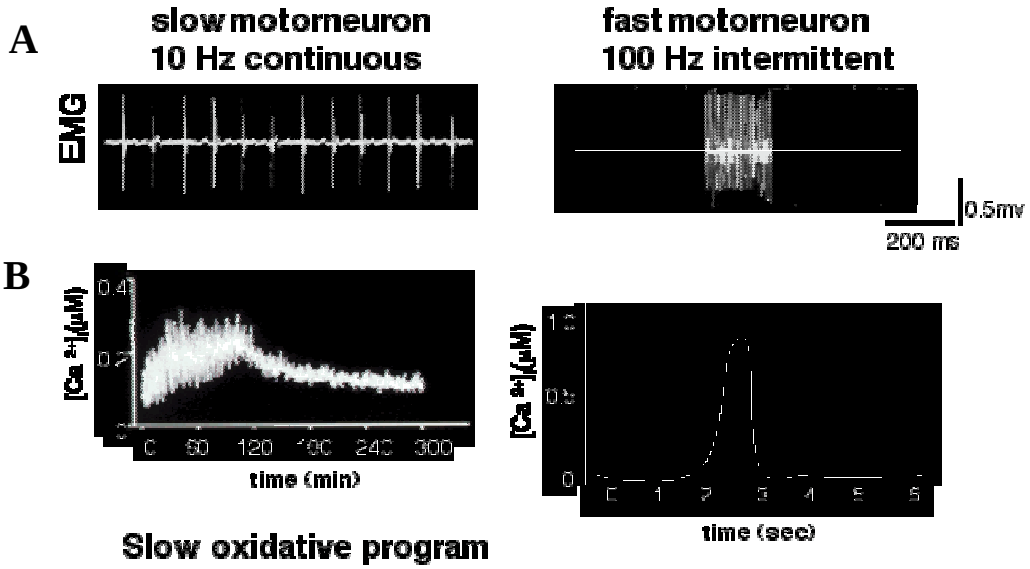


Figure 5. Patterns of motor neuron (MN) activity and calcium transients in slow and fast fibers. **A.** EMG (electromyograph) patterns of slow and fast MN. Each trace represents action potential recorded from a single slow or fast myofiber and reflects the activity of MN. Slow MN fires at low frequency (10Hz) almost constantly, while fast MN has a burst of activity at 100Hz but flanked by long periods of neural quiescence. **B.** As a result of tonic vs. phasic pattern observed in slow and fast MN, the intracellular free calcium concentration ($[Ca^{2+}]_i$)within slow and fast fibers differ. In slow fibers, there is a sustained elevation of $[Ca^{2+}]_i$ at 100-300nM, while in fast myofibers, resting $[Ca^{2+}]_i$ is maintained at 50nM, with occasional calcium spikes evoked by fast motor neurons. Increases of $[Ca^{2+}]_i$ is a causal rather than secondary event for fast-to-slow fiber transformation.

the fibers to adopt a fast phenotype. We demonstrated that activated calcineurin selectively up-regulates slow and oxidative fiber-specific promoters and that pharmacological inhibition of endogenous calcineurin activity in intact rats promotes slow-to-fast fiber transformation. In the soleus muscle of normal rats, 85% of the fibers are slow. Administration of cyclosporin A (CsA), a chemical inhibitor of calcineurin, doubles the amount of fast type II fibers within the rat soleus after six weeks of continuous treatment. Putative NFAT consensus binding sequences are identified from promoter/enhancer regions of TnI slow and myoglobin, two slow & oxidative-specific genes, indicating that NFAT might be the transcription factor mediating calcineurin effect on fiber type-specific gene expression.

The ideas that calcineurin directs myofibers towards the slow fiber phenotype has been supported subsequently by several other groups. Inhibition of endogenous calcineurin results in co-regulation of muscle metabolic components as well as the contractile proteins (Bigard *et al*., 2000). More supporting evidences come from transgenic mice expressing a constitutively active form of calcineurin specifically in their skeletal muscles. In the gastrocnemius muscle of these mice, the number of slow type I fibers is doubled compared to wild type littermates, suggesting that calcineurin is not only required, but also sufficient, at least to certain extent, for the determination of slow fiber type identity (Naya *et al*., 2000). These results further confirm that fiber type switching that results from inhibition of calcineurin by CsA is not due to systemic effect of this drug or effect on motor neurons, but instead is caused by inhibition of calcineurin *per se*.

3. Goals of my dissertation research

The overall goals of my dissertation research are to define the calcium-regulated signaling pathways participating in fiber type control of adult mammals. Specifically, I focused on two calcium, calmodulin-dependent enzymes: calcineurin and CaMKIV, and the downstream transcription factors/co-activators that mediate the effect of these two enzymes over fiber type specialization.

Initially, my attention was directed to the calcineurin-NFAT pathway because biochemical concepts defined in other cell types could be adapted to the skeletal muscle background. As the project evolved, we found that MEF2 transcription factors also were activated by calcineurin. In addition, the synergy between calcineurin and CaMK on MEF2 activation prompted me to investigate the function of CaMK signaling in fiber type control. The following four aims cover the subjects I will discuss in my dissertation:

1. To identify enhancer elements required for fiber-type specific expression of certain genes
2. To define the mechanisms of how calcineurin regulates MEF2 transactivities
3. To determine the physiologic relevance of calcineurin-MEF2 pathway in the process of fiber-type transformation
4. To characterize the function of CaMK signaling in the control of skeletal muscle plasticity

Chapter II: Experimental procedures

1. Tissue culture, cell transfection, virus infection, and reporter gene assays

C2C12 myoblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum and antibiotics (100 U of penicillin and 100 μg of streptomycin per ml). Myotube formation was induced by switching confluent cells to differentiation media (DMEM supplemented with 2% heat-inactivated horse serum, 10 μg/ml insulin (Gibco/BRL) and 10 μg/ml transferrin (Gibco/BRL) for 48 to 72 hours. Differentiation was assessed by the formation of multinucleated myotubes. For transient transfection assays, C2C12 cells were plated 12 hours before transfection in six-well tissue culture dishes at $1.0x10^5$ cells per well, and transfected with 1.6 μg plasmid DNA using Lipofectamine Plus (Gibco/BRL). Cells were harvested 40 hours after transfection with lysis buffer (Promega). Luciferase expression was measured by light emission integrated for the initial 10 seconds at 25°C in the presence of 1mM luciferin (Promega). The efficiency of transfection was determined by cotransfection of pCMV-lacZ. β-galactosidase expression was monitored by adding 3 μl of cell lysate to a final concentration of 0.8 mg of o-nitrophenyl-β-D-galactopyranoside per ml in a buffer of 0.1 M sodium phosphate (PH7.9), 1 mM $MgCl_2$, 45 mM β-mercaptoethanol at a final volume of 300 μl. The samples were incubated at room temperature for 25 minutes before measurement of A_{420}.

Recombinant, replication-defective adenoviruses encoding either Green Fluorescent Protein (Ad-GFP) or a constitutively active form of calcineurin (Ad-CnA*) were generated using standard techniques (Gerard and Meidell, 1995). For gene transfer using adenoviral

vectors, C2C12 cells were first transfected with reporter plasmid as already described, and then exposed to the adenoviral suspension at a multiplicity of infection (MOI) of 10-100. Cells were harvested 24 hours after infection.

2. Plasmid constructs

a.) Reporter constructs

Luciferase reporter plasmids were constructed in pGL3 (Promega) by inserting promoter/enhancer regions from genes encoding human PGC-1, myoglobin (Devlin *et al*., 1989) or the slow and fast isoforms of TnIs (termed SURE and FIRE, respectively (Nakayama *et al*., 1996)). The 3 kb and 2 kb proximal regions from human PGC-1 and myoglobin promoters are cloned into pGL3-basic. pGL3-SURE-TnI95 and pGL3-SUREΔNFAT-TnI95 (reporters for transgenic constructs) plasmids containing wild type SURE enhancer or a mutant SURE that harbors a mutated NFAT binding site, plus a 95 bp minimum promoter from TnI slow gene, were obtained from Dr. A. Buonanno's group (NIH). FIRE enhancer was synthesized using *in vitro* oligo pairs(see below). pGL3-SURE-TATA and FIRE-TATA (used in transfection assays) were constructed by releasing enhancers and subcloning them into SacI/NheI sites of pGL3-TATA, which has a 53 bp TATA sequence from hsp70 promoter inserted into the HindIII site of pGL3 basic vector upstream of luciferase coding region. In addition, a synthetic enhancer constructed by linking 3 copies of a high affinity MEF2 binding sequence from the desmin promoter (termed desMEF2 (Naya *et al*., 1999)) to a minimal TATA sequence to obtain pGL3-desMEF2. β-

galactosidase reporter plasmids pCMV-lacZ (Grayson *et al*., 1995) and desMEF2-lacZ (Naya *et al*., 1999) were described elsewhere.

PCR-based mutagenesis of the SURE and FIRE enhancers was performed by two step PCR and mutations were confirmed by DNA sequencing. The mutant form of desMEF2 enhancer was made by linking three copies of oligo pairs ,which contain the same sequence as desMEF2 but harbor a mutated MEF2 site, and ligating them into NheI site of pGL3-TATA vector. Table 2 lists the primers or oligos used in mutagenesis. For SURE and FIRE enhancer mutations, four primers were used in each case. Two primers (pair1-S and pair2-A in Table 2) sit on the 5' and 3' ends of the target enhancer and the other pair contains sense/anti-sense sequences corresponding to the region to be mutated with the designed mutation at the appropriate position. In the first round of PCR, 5 ng of plasmid DNA was used as template. Two fragments were amplified separately using primer pairs corresponding to the 5' and 3' half of the enhancer region, with mutation introduced at their 3' and 5' ends respectively. Then these two PCR products were mixed and used as template for second round PCR with primers pair1-sense and pair2-antisense. The amplified PCR fragments were gel purified (QIAquick gel extraction kit, Qiagen) and cut with appropriate enzymes and ligated to the original plasmid that has been digested with the same enzymes.

b.) Expression constructs

Expression vectors encoding MEF2A, MEF2C, MEF2D (Black *et al*., 1995), MEF2B (Yu *et al*., 1992), constitutively active forms of calcineurin (O'Keefe *et al*., 1992) and

Table 2. Oligonucleotides used in *de novo* gene synthesis and two-step PCR mutagenesis

Enhancer Name	Primers	Sequence
DesMEF2-mut	sense (S)	CTAGCGGCCTTTCCTTCTCCTCGATCAATGCCAGCTCTGGTATTTCGAATTCG
	anti-sense (A)	CTAGCGAATTCGAAATACCAGAGCTGGCATTGATCGAGGAGAAGGAAAGGCCG
FIRE	pair1:S	ACGGACGGGGTACCTCCCTGAGGAAACCTTATCCTGGAAAATGTGCAGGCACAACACATTGCTGGGAAGAGCAAGGGGGTGGGGGGGGAAA
	pair1:A	ACCCCCTTGCTCTTCCCAGCAATGTTGTGCCTGCACATTTTCCAGGATAAGGTTTCCTCAGGGAGGTACCCCGTCCGT
	pair2:S	GTGCTTCTAAAAATGGCTGGGGAGCTGCACAAGGAGCTGCAGCTGTCTCCTCAGACGCAGCCAGGCTCTAGAGGCAGGCA
	pair2:A	TGCCTGCCTCTAGAGCCTGGCTGCGTCTGAGGAGACAGCTGCAGCTCCTTGTGCAGCTCCCCAGCCATTTTTAGAAGCACTTTCCCCCCCC
FIRE-mutMEF2	pair1:S	CTAGCAAAATAGGCTGTCCC
	pair1:A	CTTGTGCAGCTCCCCAGCTATTTTTAGAAGCACTTTC
	pair2:S	GAAAGTGCTTCTCCCCCTAGCTGGGGAGCTGCACAAG
	pair2:A	GTTCCATCTTCCAGCAGATA
SURE-ΔMEF2	pair1:S	CTAGCAAAATAGGCTGTCCC
	pair1:A	CCACACCTGTTTCCTGGGCATTTTTAGGACTGTTGAT
	pair2:S	ATCAACAGTCCTAAAAATGCCCAGGAAACAGGTGTGG
	pair2:A	GTTCCATCTTCCAGCAGATA

CaMKIV (Ho *et al*., 1996) were described elsewhere. Serine-to-Alanine MEF2A mutants and catalytic inactive calcineurin were generated with the QuikChange™ site-directed mutagenesis kit (Stratagene). Table 3 lists the primers used in single or double amino acid mutagenesis. A c-myc tagged MEF2A cDNA was amplified from pCDNAI/amp-MEF2A (Black *et al*., 1995) and ligated into the EcoRI site of pCI-neo vector (Promega). PCR fragments representing different regions of MEF2A were inserted into EcoRI sites of pM and pVP16 vectors (Clontech) to generate GAL4 and VP16 fusion constructs. The bacterial expression plasmids encoding for GST-MEF2A (amino acid 1-87 or full length MEF2A) were constructed by subcloning EcoRI-cut fragments from pCI-Neo-cMyc-MADS and pCI-Neo-cMyc-MEF2A into the EcoRI site of the pGEX4T-1 vector (Pharmacia). pGEX4T-MEF2A.131-507 was generated by linking a PCR fragment corresponding to amino acids 131-507 of human MEF2A into the EcoRI site of pGEX4T-1. To make transgenic constructs expressing constitutively active calcineurin A under the control of MCK promoter, HA-tagged calcineurin A was released from pCI-Neo-CnA with EcoRI digestion and ligated into pBS-MCK-hGH vector (from Eric Olson's laboratory at UTSW). A PCR fragment representing constitutively active CaMKIV was ligated to the EcoRV site of the same vector to generate another transgenic construct. Table 4 lists the primers used in the construction of these plasmids.

c.) *De novo* synthesis of FIRE enhancer

Table 3. Oligonucleotides used with QuikChange™ site-directed mutagenesis system (Stratagene) to mutate expression plasmids

Mutant constructs✪	Primers (only sequences of sense primers are shown)*
CnA H151-N	GTTTTTACTTCGCGGA**AA<u>C</u>**AATGAATGTAGGCACCTC
MEF2A S255-A	CAAAGTCATGCCTACAAAG**<u>G</u>**<u>CT</u>CCCCCTCCACCAG
MEF2A S304-A	CCCAAAGGATCAGTAGT**<u>G</u>**<u>CT</u>CAAGCCACTCAACCTC
MEF2A S312,319-A	CACTCAACCTCTTGCT**<u>G</u>**<u>CC</u>CCAGTCGTGTCTGTGACA**<u>G</u>**<u>CC</u>CCAAGC TTGCCTC
MEF2A S316-A	CTTGCTACCCCAGTCGTG**<u>G</u>**<u>CT</u>GTGACAACCCCAAGC
MEF2A S321-A	GTCGTGTCTGTGACAACCCCA**<u>GC</u>**<u>C</u>TTGCCTCCGCAAGGACTTGTG
MEF2A S330-A	CGCAAGGACTTGTGTAC**<u>G</u>**<u>CA</u>GCAATGCCGACTGCC
MEF2A S334-A	GTACTCAGCAATGCCG**<u>G</u>**<u>CT</u>GCCTACAACACTG
MEF2A S338-A	CCGACTGCCTACAAC**<u>A</u>**<u>CT</u>GATTATTCACTGAC
MEF2A S344-A	CTGATTATTCACTGACC**<u>GC</u>**<u>C</u>GCTGACCTGTCAGC
MEF2A S348-A	GACCAGCGCTGACCTG**<u>CC</u>**<u>A</u>GCCCTTCAAGGCTTC
MEF2A S355-A	CCTTCAAGGCTTCAAC**<u>GCT</u>**CCAGGAATGCTGTCGC
MEF2A S365-A	GTCGCTGGGACAGGTG**<u>G</u>**<u>C</u>**<u>T</u>**GCCTGGCAGCAGCACC
MEF2A S378-A	CTAGGACAAGCAGCCCTC**<u>GC</u>**<u>C</u>TCTCTTGTTGCTGGAGG
MEF2A S393-A	CAGGGTTCCAATTTA**<u>G</u>**<u>CC</u>ATTAATACCAACC
MEF2A S396-A	CCAATTTATCCATTAAT**<u>G</u>**<u>CC</u>AACCAAAACATCAGC
MEF2A S404-A	CAAAACATCAGCATCAAG**<u>G</u>**<u>CC</u>GAACCGATTTCACCTC
MEF2A S408-A	CAAGTCCGAACCGATT**<u>G</u>**<u>CA</u>CCTCCTCGGGATCG
MEF2A S459-A	CCCCTGTGGACAGTCTG**<u>GC</u>**<u>C</u>AGCTCTAGTAGCTCC
MEF2A S460-A	CTGTGGACAGTCTGAGC**<u>GC</u>**<u>C</u>TCTAGTAGCTCCTATG
MEF2A S464-A	CTGAGCAGCTCTAGTAGC**<u>G</u>**<u>CC</u>TATGATGGCAGTGATC

✪ Numbers within plasmid name indicate the amino acid residues mutated.
* Sequences underlined represent the amino acid residues mutated (from serine/threonine to alanine except CnA, which has a histidine to asparagine mutation) and the highlighted nucleotides are different from wild type constructs.

Table 4. Primers used to generate plasmids expressing different regions of MEF2A

Plasmid✪	Primer pair*
pCI-Neo-cMyc-MEF2A	**S**:CGGAATTCACCATGGAACAAAAACTCATCTCAGAAGAGGAT CTGATGGGGCGGAAGAAAATAC **A**:CGGAATTCTTAGGTCACCCACGCGTCC
pCI-Neo-cMyc-MADS	**S**:CGGAATTCACCATGGAACAAAAACTCATCTCAGAAGAGGAT CTGATGGGGCGGAAGAAAATAC **A**:CGGAATTCCTCAACAATATCCGAGTTGG
pM-MEF2A.1-507	**S**:GCCGAATTCATGGGGCGGAAGAAAATAC **A**:CGGAATTCTTAGGTCACCCACGCGTCC
pM-MEF2A.87-507	**S**:GCCGAATTCACTTTAAGAAAGAAAGGCC **A**:CGGAATTCTTAGGTCACCCACGCGTCC
pM-MEF2A.131-507	**S**:GCCGAATTCCCTGGTCTGCCACCTCAG **A**:CGGAATTCTTAGGTCACCCACGCGTCC
pM-MEF2A.272-507	**S**:GCCGAATTCGATCTTCGAGTTGTCATCC **A**:CGGAATTCTTAGGTCACCCACGCGTCC
pM-MEF2A.299-507	**S**:GCCGAATTCCAAAGGATCAGTAGTTCTC **A**:CGGAATTCTTAGGTCACCCACGCGTCC
pM-MEF2A.131-472	**S**:GCCGAATTCCCTGGTCTGCCACCTCAG **A**:GATGAATTCATCCTCCCGATCACTGCC
pM-MEF2A.272-472	**S**:GCCGAATTCGATCTTCGAGTTGTCATCC **A**:GATGAATTCATCCTCCCGATCACTGCC
pM-MEF2A.299-472	**S**:GCCGAATTCCAAAGGATCAGTAGTTCTC **A**:GATGAATTCATCCTCCCGATCACTGCC
pM-MEF2A.131-373	**S**:GCCGAATTCCCTGGTCTGCCACCTCAG **A**:GATGAATTCTCCTAGGTGGTGCTGCTGC
pM-MEF2A.272-373	**S**:GCCGAATTCGATCTTCGAGTTGTCATCC **A**:GATGAATTCTCCTAGGTGGTGCTGCTGC
pM-MEF2A.299-373	**S**:GCCGAATTCCAAAGGATCAGTAGTTCTC **A**:GATGAATTCTCCTAGGTGGTGCTGCTGC
pM-MEF2A.131-272	**S**:GCCGAATTCCCTGGTCTGCCACCTCAG **A**:GCCGAATTCATCTGGTTTCCTACTGTTC
pVP16-MEF2A.1-115	**S**:GCCGAATTCATGGGGCGGAAGAAAATAC **A**:GCCGAATTCGAATCTGTCCTCCGAGAG
pBS-MCK.hGH.CaMKIV	**S**:CGGCGATATCACCATGGACTACAAGGACGACGATGAC **A**:CGGCGATATCTCAGAGCTTCTTTTGAGCGGTATCC
pGEX4T-MEF2A.131-507	**S**:GCCGAATTCCCTGGTCTGCCACCTCAG **A**:CGGAATTCTTAGGTCACCCACGCGTCC

✪ Numbers within plasmid name indicate the amino acid residues cloned into the vector
* S: sense primer, A: anti-sense primer

FIRE enhancer was synthesized *in vitro* using a two-step annealing protocol with 2 pairs of single-stranded oligos spanning the entire enhancer (Table 2). Each pair corresponds to the 5' and 3' halves of the FIRE and contains an overhang in their ends. After boiling the four oligos for 3 minutes, the solution was cooled slowly to room temperature to allow these oligos to anneal. Double stranded DNA fragments were purified and digested with KpnI/XbaI and ligated into pGL3 vectors with either a minimum TATA sequence from hsp70 (pGL3-TATA-FIRE for transfection assays) or a 95 bp proximal promoter from TnI slow gene (pGL3-TnI95-FIRE for transgenic construct).

3. Preparation of protein from tissues and cultured cells

Muscles and other tissues were minced and then homogenized in lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM DTT and protease inhibitor cocktail(Roche)) using a motor driven micro duncer (Bodine Electric Company). Cell debris were spun down and supernatants were collected and snap frozen in liquid nitrogen. To harvest cytoplasmic and nuclear fractions, C2C12 cells were scraped in lysis buffer (20 mM HEPES, pH 7.6, 10 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 20% glycerol and protease inhibitor cocktail) and spun at 1000g for 1 minute. The supernatant was cytoplasmic fraction. To get nuclear protein, the pellet was resuspended in 4 volumes of lysis buffer supplemented with NaCl to a final concentration of 0.5 M and rocked at 4°C for 1 hour. Supernatant nuclear fractions were harvested by spinning the sample at 12000rpm for 10 minutes.

4. Electrophoretic gel mobility shift assay (EMSA)

A synthetic oligonucleotide representing a high affinity MEF2 binding motif (MCK-MEF2, Table 5) or a NFAT binding motif (IL2-NFAT, Table 5) was annealed to its complementary strand, labeled with polynucleotide kinase and γ-^{32}P-ATP (3000 Ci/mmol, Amersham) and incubated with nuclear protein extracts (15 μg) from C2C12 myotubes in binding buffer (20mM HEPES pH7.6, 50mM KCl, 10% glycerol, 0.2mM EDTA, 1mM DTT and 1μg of polydI-dC per lane) for 20 minutes at room temperature. Competitive binding assays were conducted under the same conditions, with the addition of 0.4 to 1 pmol (20- to 50-fold molar excess) of unlabelled competitor oligonucleotides as listed in Table 5. The identity of MEF2:DNA or NFAT:DNA complexes was verified by pre-incubation of nuclear protein extracts with anti-MEF2 antibody (Santa Cruz), anti-NFAT2 antibody (Affinity Bioreagents) or pre-immune serum for 20 minutes. Complexes were resolved on 4% polyacrylamide gels at 4°C in buffer containing 45 mM Tris, 45 mM borate, 1 mM EDTA, dried and visualized using phosphorimager analysis.

5. Immunoprecipitation and immunoblotting

C2C12 cells transfected with expression plasmids were lysed in modified RIPA buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM sodium vandate, 1% Nonidet P40, 0.5% sodium deoxycholate, 1 mM DTT and proteinase inhibitor cocktail (Roche Molecular Biochemicals)). c-myc or HA tagged proteins were immunoprecipitated with anti-c-myc or anti-HA antibody (Roche Molecular Biochemicals), followed by incubation with protein G

Table 5. Oligonucleotides used in EMSA

Oligo Name	Sequence
MCK-MEF2	5'-GATCCTCTAAAAATAACCCT-3'
SURE-MEF2	5'-GATCTCCTAAAAATACGATC-3'
FIRE-MEF2	5'-GATCTTCTAAAAATGGGATC-3'
IL2-NFAT	5’-AAGGAGGAAAAACTGTTTCAT-3’
IL2-NFAT-mutant	5’-AAGGACCTTAAACTGTTTCAT-3’
Myoglobin-NFAT	5'-AACCAGGAAATAGGATGCCCT-3'
Myoglobin-NFAT-mutant	5'-AACCACCTTATAGGATGCCCT-3'
SURE	5'GCAGGCAGGGTGGGTAAAAATACCCAGGAAACAGGTG-3'
SURE-mutant	5'GCAGGCAGGGTGGGTAAAAATACCCACCTTACAGGTG-3'
FIRE	5'GCAGGCAGGGTGGGTAAAAATGGCTGGGGAGGCTGCACAAGGAGCTCAGCTG-3'

linked to agarose beads (Roche Molecular Biochemicals). After washing the protein complexed beads 5 times in RIPA buffer, the samples were boiled in SDS-PAGE loading buffer, loaded onto a SDS-PAGE and transferred to nitrocellulose paper following standard immunoblotting procedures. Antibodies against calcineurin (Transduction Laboratories), β-galactosidase (Promega), MEF2A (Santa Cruz Biotechnology), MEF2C (gift from John Schwarz, University of Texas Medical School at Houston,), MEF2D (Transduction Laboratories), Phospho-p38 MAP kinase (Cell Signaling Technology), phosphoSerine (Zymed Laboratories Inc.) and α-tubulin (Sigma) were used for immunoblot analyses.

6. *In vitro* protein-protein interaction assay

GST and recombinant GST-MEF2A proteins were purified with glutathione-sepharose beads per the manufacturer's instructions (Pharmacia). ^{35}S-methionine labeled calcineurin A is produced in the TnT *in vitro* coupled transcription/translation system (Promega). For *in vitro* binding assays, equal amount of GST fusion proteins bound to glutathione beads were resuspended in 500 μl of binding buffer (20 mM Tris, pH7.5, 100 mM KCl, 0.1 mM EDTA, 0.05% Nonidet P-40, 10% glycerol and 1 mg/ml BSA). ^{35}S-methionine labeled calcineurin proteins were added to the GST fusion proteins and incubated for 1 hr. The beads were then spun down and washed three times in binding buffer. An equal volume of 2X SDS-PAGE loading buffer was added to the beads and boiled for 3 min. The proteins were resolved on SDS-PAGE and the bound calcineurin was detected by autoradiography.

7. Monitoring phosphorylation status of MEF2

Ionomycin was added to the media of C2C12 myotubes 48 hrs after differentiation at a final concentration of 1 μM, and cells were harvested 4.5 hrs later. Cyclosporin A was included at a final concentration of 250 nM. Nuclear protein extracts were prepared and subjected to immunoblot analysis using a polyclonal antibody recognizing MEF2A (Santa Cruz Biotechnology) and the ECL detection system (Amersham). Aliquots of each nuclear protein extract were incubated with alkaline phosphatase (Roche Molecular Biochemicals) at a concentration of 100 U/ml for 60 minutes at 37° C.

For *in vitro* sample treatment with calcineurin, c-myc-MEF2A was immunoprecipitated from transfected cell lysates using c-myc antibody coupled to protein G agarose beads. The immunoprecipitant was incubated with recombinant human calcineurin (BIOMOL), 0.6 μM calmodulin, 1 mM calcium for 1hr at 30°C or calf intestine alkaline phosphatase (Roche Molecular Biochemicals) for 1hr at 37°C with continuous shaking. The samples were processed for immunoblotting as described above.

8. Assays for constitutively active calcineurin and CaMKIV

The assay for constitutively active calcineurin was performed in a buffer containing 50 mM Tris-HCl, pH 7.5 with 100 mM NaCl, 0.5 mM dithiothreitol, 100 μg/ml bovine serum albumin, 500 nM okadaic acid, 0.1mM RII phosphopeptide (DLDVPIPGRFDRRVpSVAAE, BIOMOL) and 3 mM EGTA. Dialyzed muscle protein extract was added and incubated for 20 min at 30°C, then 500 μl of Biomol Green solution (BIOMOL) was added and further incubated at room temperature for 30 min before OD_{620}

measurement. Standard curves were constructed with free phosphate. Recombinant human calcineurin (BIOMOL) provided a positive control. One unit is defined as the enzyme activity that can release 1 picomole of phosphate from the substrate per minute at 30°C.

To measure constitutively active CaMKIV, total proteins from muscles of transgenic and wild type mice were subjected to immunoprecipitation as described above using an anti-CaMKIV antibody (Transduction Laboratories). After three washes of the protein G agarose immunocomplex with RIPA buffer and two washes with assay buffer for constitutively active CaMKIV (40 mM HEPES, pH 6.8, 1 mM EGTA), CaMKIV substrate peptide (KSDGGVKKRKSSSS, BIOMOL, final concentration 80 μM) and 1 μCi γ-^{32}P-ATP (diluted ten folds in 250 μM cold ATP and 20 mM magnesium chloride) were added. After incubation for 20 minutes at 30 °C with constant shaking, beads were spun down and supernatants were transferred to fresh tubes and TCA (trichloroacetic acid) was added to stop the reaction (final TCA concentration 10%). Each sample was then spotted onto the center of a 2.5 cm circular P81 phosphocellulose paper (Whatman). The papers were washed three times with 0.75% phosphoric acid and once with acetone at room temperature for five minutes each and then transferred to a vial containing 5 ml of scintillation cocktail. CPM was recorded form a scintillation counter. Assays in the absence of the substrate peptide served as controls to subtract background and non-specific kinase activity of the immunocomplex.

9. Northern and Southern blot analysis

Total RNA was prepared from mouse skeletal muscles using RNA STAT-60 (Tel-Test Inc.) following the manufacturer's instructions. Northern blots were performed with 20

μg of total RNA in each lane and probed in Ultrahyb (Ambion) with labeled cDNA probes. For southern blot, total DNA (including genomic and mitochondrial DNA) was isolated from tissues and processed as described (Maniatis *et al*., 1989). To monitor mitochondrial copy numbers, 10 μg total DNA from muscles was digested with NcoI and subjected to southern blot using cytochrome B cDNA as a probe for mitochondrial DNA. The blot was then stripped and hybridized to a cDNA for MCIP1, a nuclear encoded gene.

10. β-Galactosidase Staining and Fiber Typing of Skeletal Myofibers

Dissected muscles were fixed with 2% paraformaldehyde/0.1% glutaraldehyde in PBS on ice for 30-45 minutes followed by washing and X-gal staining (5mM ferrocyanide, 5mM ferricyanide, 2mM MgCl2, 1mg/ml X-gal, 0.01% sodium deoxycholate and 0.02% Nonidet P40) for 1-12 hr at room temperature. Fiber typing was performed with histochemical staining for ATPase activity at pH 4.54 (Brooke and Kaiser, 1970) or a metachromatic dye-ATPase method (Ogilvie and Feeback, 1990).

11. Transgenic mice

After removal of prokaryotic sequences, transgene fragments were introduced by microinjection into fertilized oocytes of C57B6/C3H or C57B6/SJL mice. Transgenic mice containing a lacZ gene under the control of three copies of desmin MEF2 binding elements were described elsewhere (Naya *et al*., 1999). New transgenic lines were generated using the muscle creatine kinase (MCK) gene enhancer/promoter (Sternberg *et al*., 1988) linked to a cDNA encoding an FLAG-tagged form of constitutively active CaMKIV. In addition, five

independent lines of transgenic mice were engineered to express a constitutively active form of calcineurin (CnA*) under the control of the MCK enhancer/promoter. Two of these lines were described elsewhere (Dunn *et al*., 2000; Naya *et al*., 2000). In three new lines, the calcineurin transgene product included an HA-epitope tag for convenient assessment of transgene expression. For SURE-FIRE enhancer analyses, founder mice harboring luciferase cDNAs driven by different enhancers plus a 95 bp proximal promoter from TnIs were generated. The resulting transgenic offspring were identified by Southern blot or PCR analysis of genomic DNA. Tissues were harvested from transgenic animals at 8-10 weeks of age and assayed for expression of transgene (luciferase, calcineurin or CaMKIV), or processed for β-galactosidase assay, or staining for myosin ATPase activity. To inhibit endogenous calcineurin activity, adult mice were injected subcutaneously with cyclosporin A (25 mg/kg) or vehicle daily for 18 days. All experiments involving animals were reviewed and approved by the Institutional Animal Care and Research Advisory Committee.

12. Voluntary wheel running and electrical pacing of the mouse hindlimb muscles

For running experiments, C57B6/C3H mice were individually housed in cages (15 X 32 cm) equipped with running wheels (11 cm in diameter). Animals were maintained on a 12:12 hr cycle of light and dark, and wheel running activity was monitored continuously with a Dataquest Acquisition & Analysis System (Data Sciences International). For long term low-frequency nerve pacing studies, miniature neuromuscular stimulators were purchased from Dr. Jonathan Jarvis at the University of Liverpool, UK (jcj@liverpool.ac.uk). The stimulators were implanted under the dorsal skin of anesthetized mice and leads were

tunneled subcutaneously to the sciatic nerve. After the animals recovered from anesthesia, the circuit was activated by flashes of light transmitted through the skin to deliver supramaximal pulses of 0.2 ms duration at a frequency of 10 Hz. Immediately at the conclusion of the final pacing period, muscles were excised and processed for β-galactosidase staining.

13. Microarray

Cy3-dUTP and Cy5-dUTP labeled fluorescent cDNA probes were prepared by reverse transcription of 10 μg total RNA from plantaris muscles of wild type and transgenic mice, respectively. Detailed procedures could be found at

http://cmgm.stanford.edu/pbrown/protocols/4_human_RNA.html

The probes were incubated overnight at 62°C onto a custom-made microarray chip containing ~8000 clones amplified from skeletal and cardiac muscle libraries. After extensive rinsing, fluorescent images were obtained from a GenePix 4000A microarray scanner (Axon Instruments) and analyzed with GenePix Pro3.0 software (Axon Instruments). The fluorescent intensity ratio of Cy3 versus Cy5 represents differential expression of certain genes in these two samples.

14. Transmission Electron Microscopy

Plantaris muscles were dissected from mice and fixed with 2% glutaraldehyde in PBS and then postfixed in 1% osmium tetroxide in PBS. Samples were subsequently dehydrated

in ethanol and embedded to obtain thin sections that were stained with lead citrate and examined with a transmission electron microscope at a final magnification of 5000X.

Chapter III. Promoter analyses of fiber type-specific genes

1. Introduction

Transcriptional regulation of different isoforms of contractile components is the fundamental mechanism underlying fiber type specificity. The promoter/enhancer regions of these fiber type-specific isoforms provide us with a wealth of information on the transcriptional control of these genes. With more genomes being sequenced, comparing sequences from promoters/enhancers of different gene family members within the same organism and among various species provides clues to the potential regulatory sequences and the bound transcription factors that are responsible for spatiotemporal expression patterns of these genes.

Troponin I (TnI) gene family comprises TnIs, TnIf and TnIc, expressed selectively in slow-twitch skeletal, fast-twitch skeletal and cardiac muscles respectively. The promoter /enhancer sufficient to confer slow or fast fiber-specific expression of TnIs and TnIf genes have been identified using transgenic models (Nakayama *et al*., 1996; Yutzey *et al*., 1989). SURE (slow upstream regulatory element) and FIRE (fast intronic regulatory element) direct reporter expression selectively to slow and fast myofibers in mice. Sequence comparison of these two elements identified several conserved boxes (Figure 6), such as CAGG box, CCAC box, E box and MEF2 binding site, which might be the binding sites for transcription factors common to slow and fast fibers. Interestingly, there is a consensus NFAT binding site unique to TnIs. As I mentioned in Chapter 1, calcineurin is both necessary and partially sufficient for maintenance of slow fiber identity and NFAT is the classical substrate for calcineurin. If

Figure 6. Sequence alignment of SURE and FIRE. Sequences from the sense strand of rat SURE and the anti-sense strand of quail FIRE are shown. Conserved core elements (CAGG box, MEF2 site and E box) are highlighted and marked. The consensus NFAT binding motif (AGGAAA) from SURE is boxed.

slow fiber-specific genes contain functional NFAT binding sites in their promoter, translocation of NFAT and subsequent activation of slow fiber-specific genes in slow-twitch fibers would provide a transcriptional mechanism linking calcineurin activation to fiber type-specific gene expression. Putative NFAT binding sites are also present on the promoters of other slow and oxidative fiber-specific genes, such as myoglobin, sMtCK (sarcomeric mitochondrial creatine kinase), *etc.* (Chin *et al*., 1998)

Initially, I assessed the authenticity of these putative NFAT binding sites. In addition, closer inspection of SURE and FIRE enhancers revealed that the putative MEF2 binding site in FIRE has a G instead of an A in position 9 of the consensus motif (Figure 6). This finding led me to examine the differences between the putative MEF2 binding sites found in these two enhancers both in cultured myogenic cells as well as in intact mice.

2. Results

There are *bona fide* NFAT binding sites within promoter/enhancer regions of slow and oxidative fiber-specific genes

We identified several putative NFAT binding sites, which conform to the consensus NFAT binding motif AGGAAA, from the promoter regions of myoglobin, sMtCK and TnIs (Chin *et al*., 1998). Oligonucleotides spanning these sites were used in EMSA (electrophoretic mobility shift assay) to test their ability to compete with cannonical NFAT responsive elements (NRE) from IL2 promoter for endogenous NFAT protein binding. The binding between radioactive IL2 NRE DNA probe and NFAT protein was competed by molar-excess of non-radioactive IL2 NRE probe as well as NRE derived from myoglobin and TnIs enhancers (Figure 7), but not by an oligonucleotide from FIRE enhancer. Mutation of GGAAA within these NREs into CCTTA abolished their ability to compete with IL2 NRE for endogenous NFAT proteins.

Slow and fast fiber-specific enhancer elements respond differently to MEF2 and calcineurin

The identification of NFAT binding motifs from slow, oxidative fiber-specific promoters/enhancers indicates that NFAT mediates the effect of calcineurin on fiber type-specific gene expression. However, ablation of NFAT binding motifs within slow or oxidative fiber-specific promoter/enhancer regions does not completely abolish transcriptional regulation by calcineurin or fiber type-specific gene expression (Calvo *et al*., 1999; Chin *et al*., 1998). These observations revealed that gene regulatory signals triggered

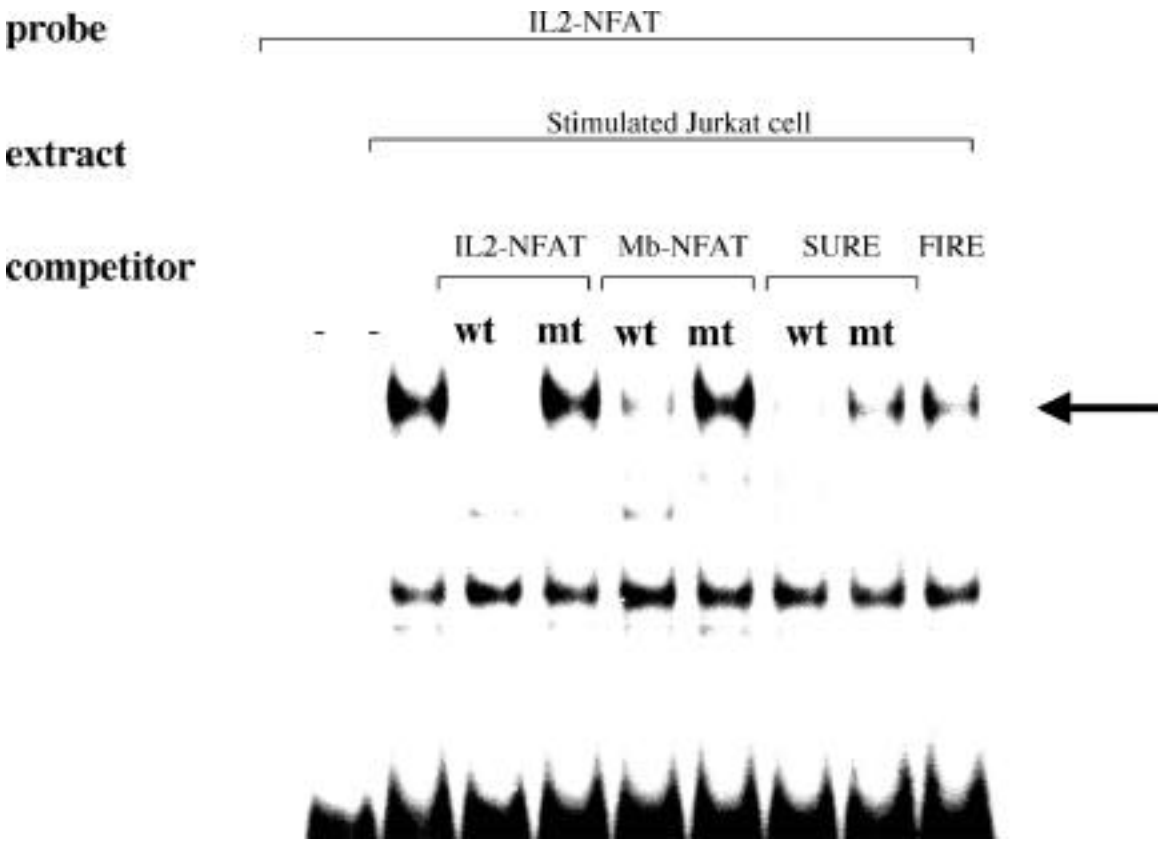


Figure 7. Competitive EMSA of different NFAT binding sites. Nuclear extract from ionomycin-stimulated Jurkat cells was incubated with ^{32}P-labeled oligonucleotide corresponding to NFAT binding site from IL-2 (interleukin-2) promoter and 50 fold molar excess of cold oligonucleotides representing NFAT binding motifs from different promoters (IL-2, Mb: myoglobin, SURE) and a FIRE sequence were used to compete for NFAT protein binding. The DNA-protein complex was indicated by an arrow.

by calcineurin can be transduced to target genes in the absence of DNA binding by NFAT proteins, and suggested that other transcription factors are capable of responding to calcineurin-dependent signals in skeletal muscles.

The fact that NFAT acts with other transcription factors, such as AP-1 in lymphocytes and GATA in cardiac muscles (Macian *et al*., 2001; Molkentin *et al*., 1998), prompted us to look for transcriptional partners for NFAT in skeletal muscles. Myocyte-specific enhancer factor 2 (MEF2) is a family of transcription factors expressed abundantly in muscle cells and is involved in muscle cell differentiation (Black and Olson, 1998). In SURE, a MEF2 binding site locates immediately upstream of the NFAT binding site, suggesting these two transcription factors might cooperate in some fashion to control muscle gene expression. The consensus sequence for MEF2 binding has been defined previously as $CT(A/T)_4ATAA$ (Cserjesi and Olson, 1991). Both SURE and FIRE seem to possess a MEF2 binding motif, but close examination revealed that the putative MEF2 site in FIRE has a G instead of A at the end of the consensus site. Previous studies of DNA binding by MEF2 proteins suggest that this nucleotide substitution in FIRE should reduce the binding affinity for MEF2 by comparison to the corresponding MEF2 binding motif found in SURE (Andres *et al*., 1995; Fickett, 1996; Yu *et al*., 1992). As a direct test of this prediction, oligonucleotides representing MEF2 binding motifs from SURE and FIRE were used in competitive EMSA (Figure 8). MEF2 site from SURE competed well with a high-affinity MEF2 binding motif derived form MCK promoter, but the putative FIRE MEF2 site did not compete at all, indicating that FIRE enhancer does not contain functional MEF2 binding site.

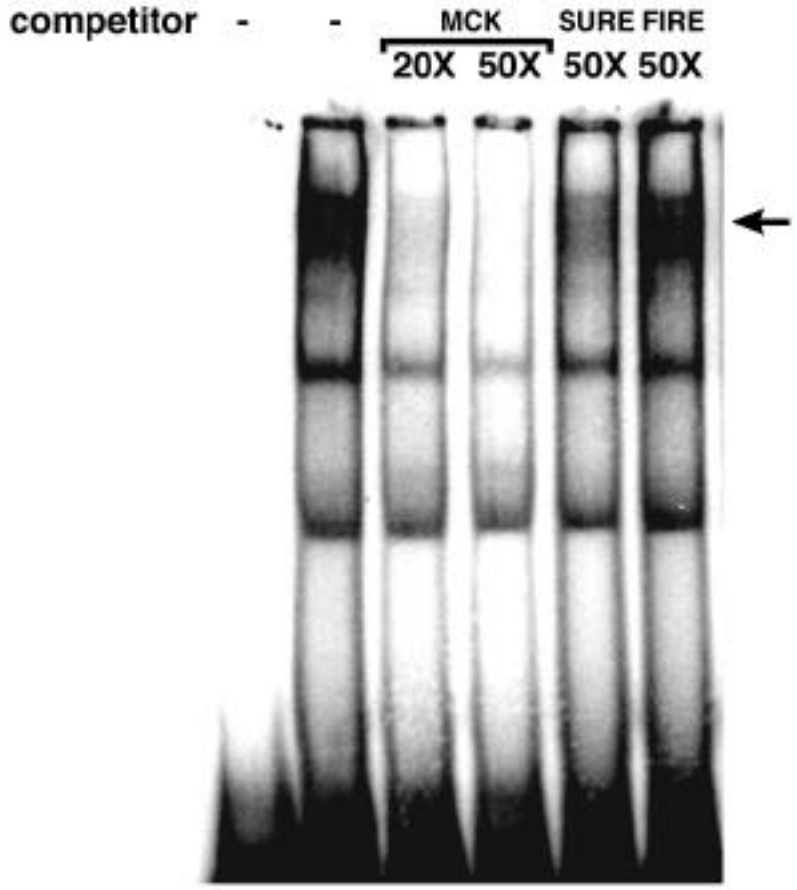


Figure 8. Competitive EMSA of different MEF2 binding sites. Nuclear extract from differentiated C2C12 myotubes was incubated with ^{32}P-labeled MEF2 binding element from MCK (muscle creatine kinase) promoter and 20 or 50 fold molar excess of cold oligonucleotides representing MEF2 binding motifs from different promoters (MCK, SURE and FIRE) were used to compete for MEF2 protein binding. The DNA-protein complex was indicated by an arrow.

The functional difference between MEF2 binding sites from SURE and FIRE are further confirmed by reporter assays in cultured C2C12 myogenic cells. Forced expression of MEF2 proteins in C2C12 myoblasts stimulated the transcriptional activity of the SURE enhancer to a greater degree than the FIRE enhancer, and this difference was accentuated in the presence of constitutively active calcineurin (Figure 9). All of the major isoforms of MEF2 (A, B, C and D) acted preferentially on the slow fiber-specific transcriptional control region. Several luciferase reporters driven by mutant forms of SURE and FIRE were generated by site-directed mutagenesis to test the functional significance of NFAT and MEF2 sites in transducing calcineurin signals onto these fiber type-specific enhancers (Figure 10A). In SUREΔNFAT, NFAT element was replaced by an EcoRI site, and the MEF2 binding site of SURE was converted to the sequence found in FIRE to generate SUREΔMEF2. Also, the non-functional "MEF2 site" from FIRE was changed into the sequence found in SURE to make FIRE-MEF2mut construct. A single nucleotide substitution within the MEF2 binding motif of SURE reduced the response of the SURE enhancer to activated calcineurin in the C2C12 background (Figure 10B). Ablation of the NFAT motif in SURE also reduced the response to activated calcineurin, supporting the notion that both MEF2 and NFAT participate in transducing calcineurin-generated signals to relevant target genes. A single nucleotide substitution that changed the nonfunctional "MEF2 site" from FIRE into a SURE-like MEF2 site increased both the basal activity of FIRE enhancer and its response to calcineurin activation. Thus, MEF2 proteins function more effectively in transcriptional regulation of the SURE enhancer than of the FIRE enhancer, and this differential response is explained, at least in part, by higher affinity binding of MEF2 to SURE than to FIRE.

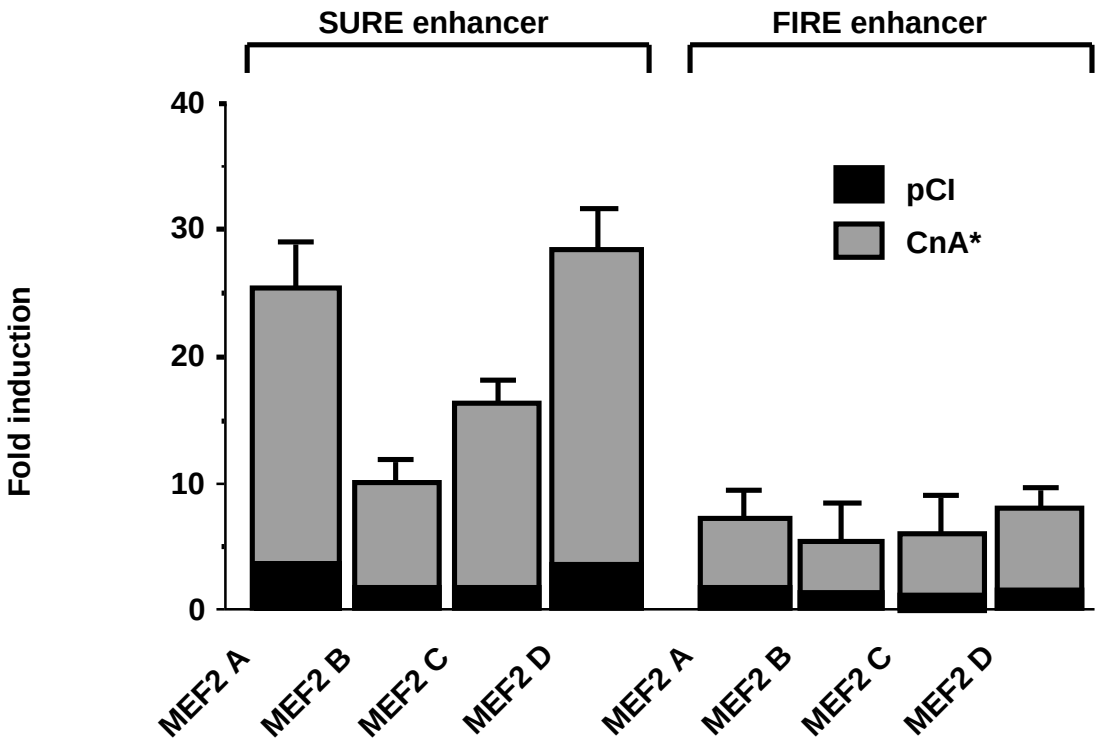
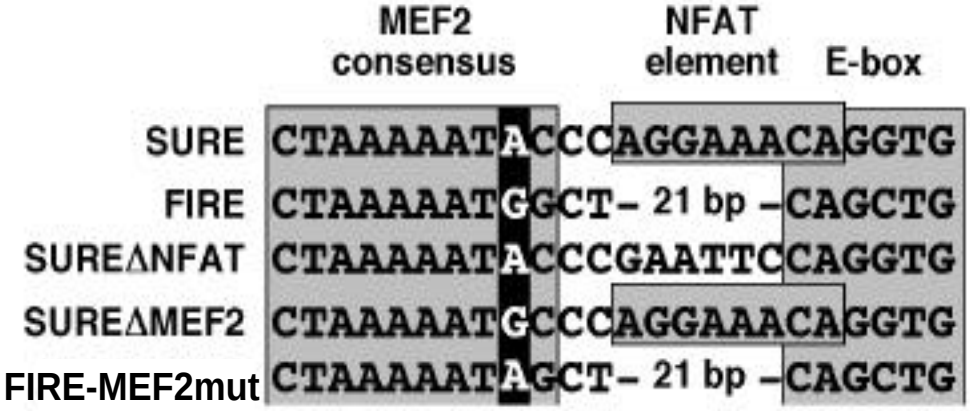


Figure 9. Activation of SURE and FIRE by MEF2 and calcineurin. Expression plasmids containing cDNAs encoding each of the four isoforms of MEF2 were cotransfected with luciferase reporter plasmids controlled by either the SURE or FIRE enhancers. Luciferase activity following forced expression of each MEF2 isoform was measured in the absence (solid bars; pCI) or presence (dashed bars: CnA*) of activated calcineurin. Fold induction is relative to enhancer activity without cotransfected MEF2 or calcineurin.

A


MEF2
consensus
NFAT
element
E-box
SURE CTAAAAATACCCAGGAAACAGGTG
FIRE CTAAAAATGGCT– 21 bp –CAGCTG
SUREΔNFAT CTAAAAATACCCGAATTCCAGGTG
SUREΔMEF2 CTAAAAATGCCCAGGAAACAGGTG
FIRE-MEF2mut CTAAAAATAGCT– 21 bp –CAGCTG

B

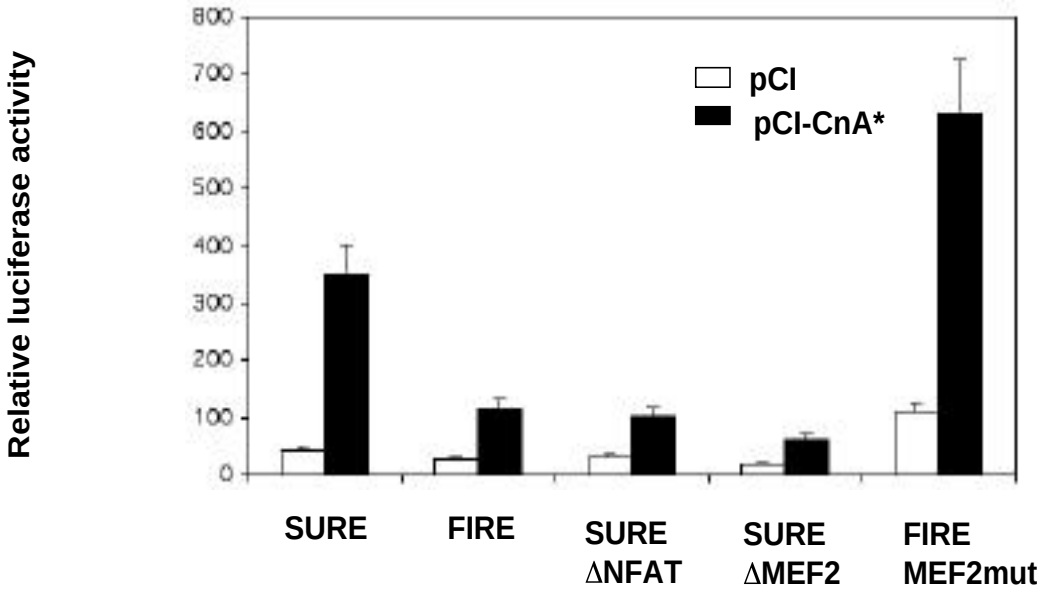

Relative luciferase activity
800
700
600
500
400
300
200
100
0
pCI
pCI-CnA*
SURE
FIRE
SURE ΔNFAT
SURE ΔMEF2
FIRE MEF2mut

Figure 10. Function of MEF2 and NFAT binding sites in the response of SURE and FIRE to calcineurin activation. **A.** Nucleotide sequence alignment of segments of the rat TnI slow (SURE) and quail TnI fast (FIRE) enhancers that include MEF2, NFAT and E box motifs. The putative MEF2 binding site in FIRE has a G nucleotide rather than an A nucleotide in position 9 of the MEF2 consensus binding motif (darkly shaded), and an NFAT consensus binding motif (boxed) found in SURE is not present in FIRE. The sequences of three variant forms of these enhancers generated by site-directed mutagenesis are also illustrated. SUREΔNFAT and SUREΔMEF2 refer to variants in which NFAT and MEF2 binding motifs, respectively, are altered while leaving the remainder of the SURE enhancer sequence intact. The SUREΔMEF2 includes only the indicated single nucleotide substitution within an otherwise intact SURE enhancer. FIRE-MEF2mut has a A instead of G at position 9 of MEF2 consensus. **B.** Luciferase reporter constructs were prepared using the SURE or FIRE enhancers upstream of a minimal TATA sequence from hsp70, or the indicated mutants thereof. Expression of luciferase following transfection of each of these reporter plasmids was assessed in the presence or absence of a constitutively active form of calcineurin (CnA*), and data are expressed as the response to calcineurin. Data mean values of 2 to 4 independent experiments.

MEF2 and NFAT binding sites are required for slow fiber-specific activity of SURE enhancer in transgenic mice

To further address the role of MEF2 and NFAT in fiber type-specific gene expression, promoter/reporter constructs prepared with native or mutated forms of the SURE enhancer (see Figure 10A) were introduced by microinjection into fertilized murine oocytes, and founder transgenic mice were analyzed for reporter gene expression in skeletal muscles and other tissues at 7 to 8 weeks of age.

The native SURE element was expressed preferentially in soleus skeletal muscles as compared either to the plantaris skeletal muscle, or to non-muscle tissues such as kidney (Figure 11A). Since the soleus muscle of the mouse contains approximately 50% slow, oxidative (Type I) fibers, while myofibers of the plantaris muscle are classified primarily as fast, glycolytic (Type IIb), this result confirms the slow fiber specificity of this transcriptional control element described originally by others (Nakayama *et al*., 1996). Mutation of the MEF2 binding motif within SURE (SUREΔMEF2) abolished the selective expression of the SURE enhancer in soleus muscles, although this mutant enhancer retained some degree of muscle-specificity (soleus vs. kidney in Figure 11A). Disruption of the NFAT binding site (SUREΔNFAT) produced a similar effect. The loss of slow fiber-specific expression of the SUREΔMEF2 and SUREΔNFAT constructs, as compared to the native SURE sequence, was attributable to a decline of transgene expression in the soleus, to an increased expression in the plantaris in some animals, and to a greater probability of almost complete silencing of the transgene (Figure 11B). The high variability in absolute levels of

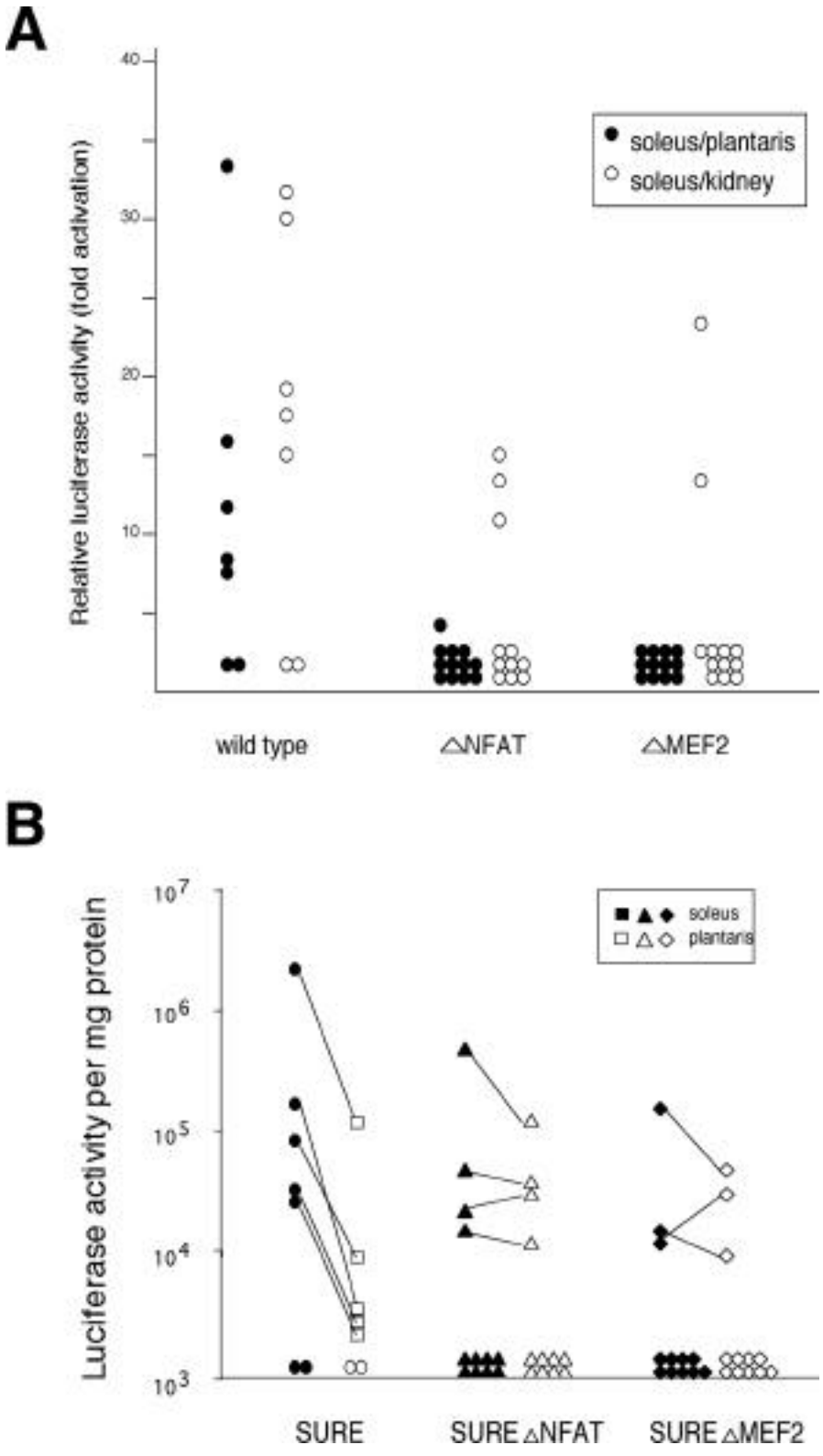
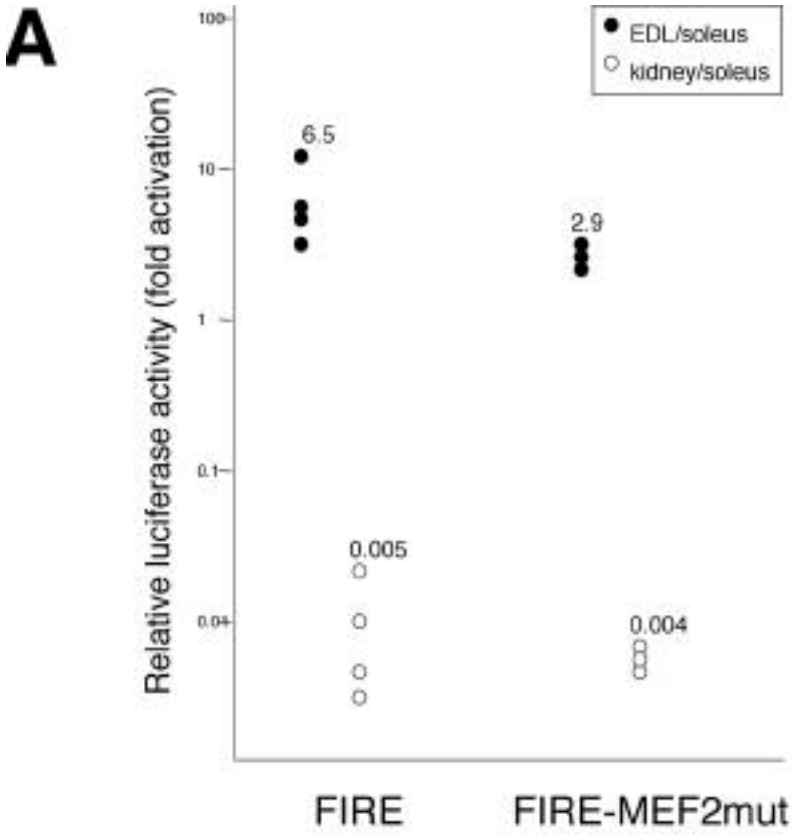

A
Relative luciferase activity (fold activation)
40
30
20
10
soleus/plantaris
soleus/kidney
wild type
△NFAT
△MEF2
B
Luciferase activity per mg protein
10^7
10^6
10^5
10^4
10^3
soleus
plantaris
SURE
SURE △NFAT
SURE △MEF2

Figure 11. Requirement of intact MEF2 and NFAT binding motifs for slow fiber-specific activity of SURE enhancer. Transgenic mice were generated using luciferase reporter constructs controlled by either the native SURE enhancer, or by two variant forms (SUREΔNFAT and SUREΔMEF2; see Figure 10A). Individual transgenic offspring, each representing a different chromosomal insertion event, were examined at 7 weeks of age for luciferase activity in soleus and plantaris muscles, which are enriched in slow, oxidative or fast, glycolytic myofibers respectively, and in kidney. **A.** Data are presented as the ratio of transgene expression in soleus vs. plantaris muscles (filled circles) as a measure of slow fiber-specific transcription, and as the ratio of transgene expression in soleus muscle vs. kidney (open circles) as a measure of muscle-specific transcription. Each point corresponds to an individual transgenic animal. **B.** Data are presented as the log of luciferase activity in the soleus (filled symbols) and plantaris (open symbols) skeletal muscles. Each point corresponds to an individual transgenic animal, and lines connect data from the same animal.

transgene expression among animals with different chromosomal insertion sites (a problem inherent to this experimental design) makes us most confident in conclusions based on ratios (Figure 11A). We interpret these results to indicate that both MEF2 and NFAT participate in regulatory pathways that establish the slow and oxidative myofiber phenotypes.

Introduction of a functional MEF2 site into FIRE diminishes fast fiber specificity of this enhancer

Transgenic mice harboring luciferase reporter under the control of FIRE and FIRE-MEF2mut (Figure 10A) were generated. The native FIRE element was expressed preferentially in fast fiber-dominant skeletal muscles, such as EDL (extensor digitorum longus), as compared either to the soleus skeletal muscle, or to non-muscle tissues such as kidney (Figure 12A). The introduction of a functional MEF2 site into FIRE diminishes fast fiber specificity of this enhancer without changing tissue specificity of FIRE. The reduced ratio of reporter expression in EDL versus soleus is possibly due to the increased expression of transgene in slow fibers (Figure 12B), although more transgenic lines are needed to make definitive conclusions.


A
EDL/soleus
kidney/soleus
Relative luciferase activity (fold activation)
100
10
1
0.1
0.01
6.5
2.9
0.005
0.004
FIRE
FIRE-MEF2mut

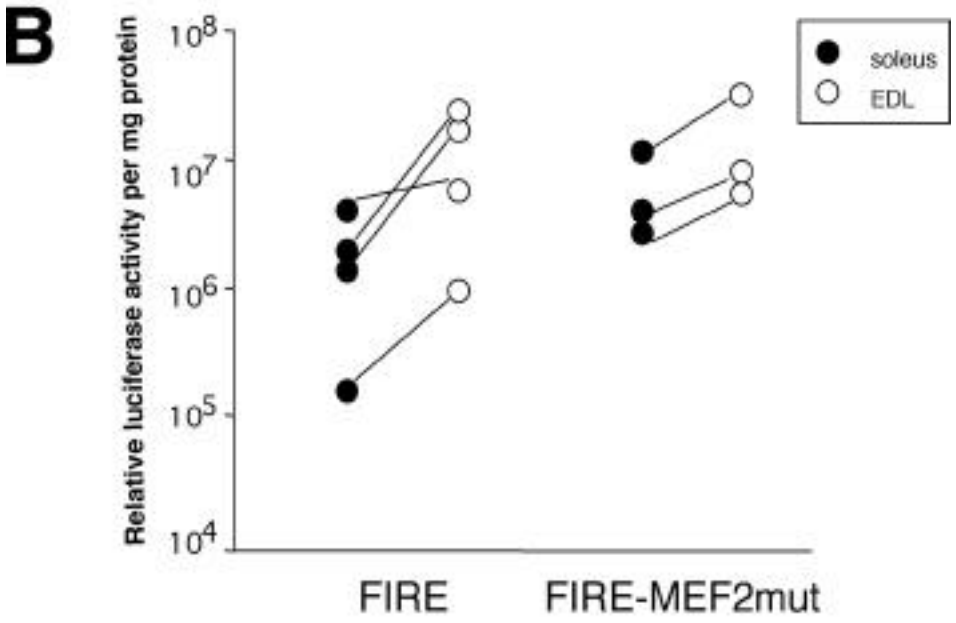

B
soleus
EDL
Relative luciferase activity per mg protein
10^8
10^7
10^6
10^5
10^4
FIRE
FIRE-MEF2mut

Figure 12. Decreased fast fiber specificity of FIRE caused by the introduction of a functional MEF2 binding site into the enhancer. Transgenic mice were generated using luciferase reporter constructs controlled by either the native FIRE enhancer, or by FIRE-MEF2mut (see Figure 10A). Individual transgenic offspring, each representing a different chromosomal insertion event, were examined at 7 weeks of age for luciferase activity in soleus and EDL muscles, which are enriched in slow, oxidative or fast, glycolytic myofibers respectively, and in kidney. **A.** Data are presented as the ratio of transgene expression in EDL vs. soleus muscles (filled circles) as a measure of fast fiber-specific transcription, and as the ratio of transgene expression in kidney vs. soleus muscle (open circles) as a measure of muscle-specific transcription. Each point corresponds to an individual transgenic animal. **B.** Data are presented as the log of luciferase activity in the soleus (filled symbols) and EDL (open symbols) skeletal muscles. Each point corresponds to an individual transgenic animal, and lines connect data from the same animal.

3. Discussions

Our data from cultured myocytes and transgenic mice demonstrate that both NFAT and MEF2 binding sites are necessary for slow fiber-specific activity of SURE and introduction of a functional MEF2 site into FIRE decreases fast fiber specificity of this enhancer. Our findings illustrate some previously unrecognized features of MEF2-dependent gene regulation in skeletal muscle. Direct comparison of well-defined enhancer elements from genes encoding slow and fast muscle isoforms of troponin I demonstrated higher affinity binding of MEF2 to the slow fiber-specific (SURE) enhancer. More avid binding of MEF2 to the SURE sequence, by comparison with binding to the fast fiber-specific (FIRE) sequence, correlated with a greater responsiveness of SURE to transactivation by MEF2. This result appears to be based on a single nucleotide substitution in the MEF2 binding region of SURE.

This notion is supported by examination of transgenic mice developed for the present study. A high affinity binding site for MEF2 appears to be necessary to generate a slow or oxidative fiber-specific pattern of transgene expression. Even a single nucleotide substitution within the MEF2 binding motif of the SURE enhancer, converting it to identity with the lower affinity MEF2 recognition sequence from FIRE, abolished slow fiber-specificity of this transcriptional control region. Ablation of the NFAT binding site within the SURE enhancer produced similar functional consequences. This latter result speaks to the complementary role of NFAT in promoting expression of slow or oxidative fiber-specific genes. The introduction of a functional MEF2 site into FIRE compromises the fast fiber-specific activity

of this enhancer, further supporting our notion that MEF2 binding site is necessary for slow fiber specific gene expression.

This distinction –- lower affinity MEF2 binding in FIRE versus SURE enhancer elements –- while pertinent to expression of troponin I isoforms, is unlikely to represent a universal feature that distinguishes fiber type-selective enhancers. Regulatory regions of certain other fast fiber-selective genes (e.g. muscle creatine kinase) that respond only minimally to calcineurin activation in myotubes, include high affinity binding sites for MEF2 (Gossett *et al*., 1989). Weak binding of MEF2 to certain fast fiber-specific enhancers (e.g. FIRE) represents a mechanism that is relevant only to a subset of fast fiber-specific genes. A second and more generalized mechanism, which I will focus in the following chapters, is based on modulation of the transcriptional activation function of MEF2 by signals that are triggered selectively in slow and oxidative fibers.

Chapter IV. Regulation of MEF2 transactivities by calcineurin

1. Introduction

MEF2 has been implicated as a component of Ca^{2+}-regulated signaling pathways in non-muscle cell types. In T cells, Ca^{2+}-induced release of MEF2 transcription factors from Cabin1 activates MEF2-dependent gene expression and promotes cellular apoptosis through the induction of Nur77 orphan receptors (Youn and Liu, 2000; Youn *et al*., 1999). Cabin1 can bind calcineurin and inhibit the catalytic activity of calcineurin. The association of MEF2 with Cabin1 indicates that MEF2 and calcineurin might co-exist in a protein complex. In B lymphocytes, the Ca^{2+}-responsive and cyclosporin A-sensitive elements within the BZLF1 promoter map to MEF2 binding sites (Liu *et al*., 1997), raising the possibility of calcineurin regulating MEF2 activity in these cells. In cerebellar granule neurons, calcineurin enhances DNA binding ability of MEF2 through dephosphorylation of MEF2 (Mao and Wiedmann, 1999).

The functional interaction between MEF2 and calcineurin has not been firmly established in these studies. Little is known about MEF2 regulation by Ca^{2+}-dependent signaling in skeletal muscles, the tissue with the highest level of MEF2 expression. As mentioned in the previous chapter, mutation of a MEF2 binding site in SURE completely abolished slow fiber-specificity of this enhancer, implying that MEF2 also participates in fiber type-specific gene expression. Since the transactivating function of MEF2 is under the regulation of several signaling pathways, such as MAP kinase (Ornatsky *et al*., 1999; Zhao *et*

al., 1999), CaMK (McKinsey *et al*., 2000a; McKinsey *et al*., 2000b), *etc*, we explored the ability of calcineurin to modulate MEF2 activity.

2. Results

MEF2 transduces calcineurin-dependent activation of fiber type-specific enhancers in cultured myogenic cells

Promoter/reporter constructions were prepared using transcriptional control elements that direct the selective expression of myoglobin in Type I and IIA fibers, or direct slow fiber-specific expression of troponin I (TnIs), as characterized previously by our laboratory and others (Chin *et al*., 1998; Nakayama *et al*., 1996). In addition, we constructed a synthetic enhancer (desMEF2) designed to read out the transactivating function of MEF2, using three copies of a high affinity MEF2 binding site from the desmin gene linked to a minimal promoter. In the absence of exogenous MEF2, forced expression of a constitutively active form of calcineurin (CnA*) up-regulated transcription of these promoter/reporter plasmids by 7 to 10 folds in C2C12 myoblasts (Figure 13A). Forced expression of MEF2A in the absence of calcineurin stimulation produced a detectable but lesser (2- to 6-fold) response. In each case, however, the combined effects of exogenous MEF2A and calcineurin were much greater (20- to 70-fold) than those produced by either stimulus alone. We conclude that MEF2 proteins are capable of transducing calcineurin-generated signals for gene regulation, and that endogenous levels of MEF2 are limiting to this response in this cell background. The effect of calcineurin to up-regulate myoglobin and TnIs enhancer function is not attributable to a generalized stimulation of muscle differentiation (data not shown).

The effect of calcineurin on MEF2-dependent gene expression was specific since a catalytic-inactive form of calcineurin (Youn *et al*., 2000), which had a amino acid

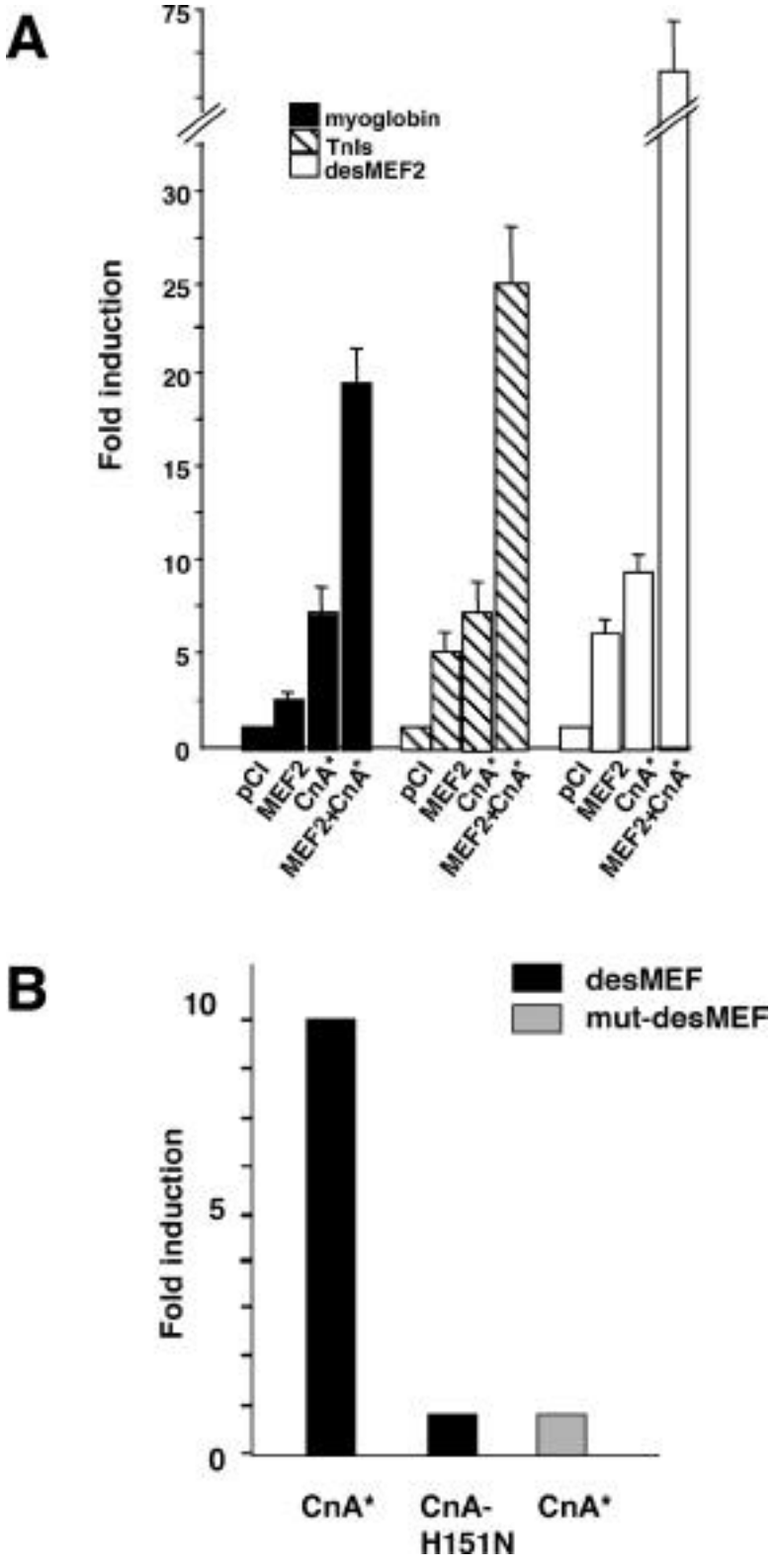

A
75
30
25
20
15
10
5
0
Fold induction
myoglobin
TnIs
desMEF2
pCI
MEF2
CnA*
MEF2+CnA*
pCI
MEF2
CnA*
MEF2+CnA*
pCI
MEF2
CnA*
MEF2+CnA*
B
10
5
0
Fold induction
desMEF
mut-desMEF
CnA*
CnA-H151N
CnA*

Figure 13. Stimulation of MEF2 transactivating function by calcineurin. **A.** Luciferase reporter constructs were prepared using promoter/enhancer elements from the myoglobin or troponin I slow (TnIs) genes, or by three copies of a high affinity MEF2 binding site from the desmin promoter (desMEF2). Expression of luciferase driven by each of these reporter plasmids was determined following co-transfection of expression plasmids containing either no insert (pCI) or cDNAs encoding MEF2A or a constitutively active form of calcineurin (CnA*). Data are expressed relative to the luciferase activity observed in the control state (pCI co-transfection) and represent mean values (+/- SEM) from five or more independent experiments. All results are corrected for variations in transfection efficiency by normalization to expression of a co-transfected pCMV-lacZ plasmid. **B.** mut-desMEF2 luciferase reporter was constructed by mutating the MEF2 binding sites in desMEF2 reporter (see table 2). Histidine 151 was mutated into asparagine in CnA* to obtain the catalytic-inactive CnA-H151N. Two reporters were cotransfected with either CnA* or CnA-H151N and representative fold activation of reporter activity was shown.

substitution at the catalytic core (histidine151→asparagine), was unable to activate MEF2 (Figure 13B). In addition, a luciferase reporter driven by mutant desMEF2 enhancer that has three copies of non-functional MEF2 site was not responsive to calcineurin activation at all.

Lack of change in MEF2 DNA binding affinity in the presence of calcineurin

MEF2 is a bipartite transcription factor with an N-terminal DNA binding domain and a C-terminal transactivation domain (Olson *et al*., 1995; Yu, 1996). To examine whether DNA binding affinity of MEF2 was altered in the presence of activated calcineurin, electrophoretic mobility shift assays (EMSA) were performed using MEF2-binding DNA probe and nuclear extract from C2C12 myotubes infected with recombinant adenovirus expressing either CnA* or GFP (green fluorescent protein) as control. Virally expressed CnA* augmented the expression of a luciferase reporter gene controlled by the desMEF2 synthetic enhancer (Figure 14A), confirming the results produced by plasmid co-transfection (see Figure 13A). EMSA of nuclear proteins extracted from virally infected cells were used to assess the effects of activated calcineurin on DNA binding by MEF2 (Figure 14B). The most slowly migrating band observed by EMSA was identified as a MEF2:DNA complex through competition with unlabelled probe, and by the change in mobility induced by incubation with anti-MEF2 antibodies. These results support the conclusion that calcineurin-dependent stimulation of the transactivating function of MEF2 can occur in the absence of major changes in DNA binding.

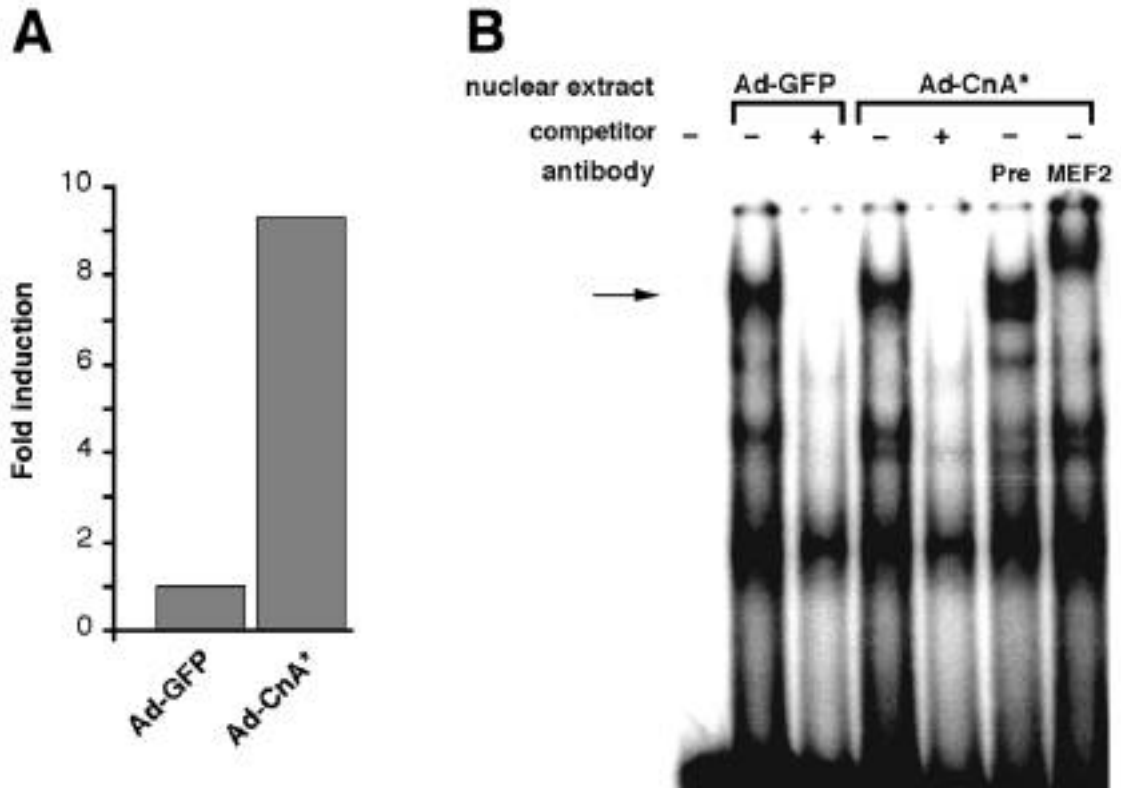


Figure 14. Lack of change in DNA binding affinity of MEF2 in the presence of calcineurin. **A.** C2C12 cells were infected with recombinant adenoviral vectors encoding either Green Fluorescent Protein (Ad-GFP) or a constitutively active form of calcineurin (Ad-CnA*). Luciferase reporter gene expression following transfection of the desMEF2 enhancer construct. Data are presented as in Figure 13 and represent mean values of two independent experiments. **B.** Electrophoretic mobility shift assays (EMSA) of nuclear proteins from adenovirus-infected cells using a high affinity MEF2 binding site as the labeled oligonucleotide probe. The identity of the MEF2:DNA complex (arrow) is confirmed by the change in mobility in the presence of anti-MEF2 antibodies, and by competition with unlabeled MEF2 binding oligonucleotide (competitor). In contrast to the clear functional effect on transcription, no detectable differences in DNA binding by MEF2 are observed in the presence of activated calcineurin. Pre = preimmune serum

C-terminal transactivating domain of MEF2 responds to calcineurin activation

The molecular mechanisms of calcineurin-dependent enhancement of functional properties of MEF2 also were explored in co-transfection experiments using various regions of MEF2 fused either to the transcriptional activation domain (AD) of the herpes simplex viral protein VP16 or to the DNA binding domain (DB) of the yeast transcriptional regulatory protein GAL4. In cultured C2C12 myogenic cells, the ability of a VP16AD:MADS-MEF2 fusion protein (containing only the first 115 amino acids of MEF2 including the DNA binding domain) to activate expression of a luciferase reporter gene controlled by high affinity MEF2 binding sites was enhanced by a constitutively active form of CaMKIV, as previously described (McKinsey *et al*., 2000b), but not by constitutively active calcineurin (Figure 15). This result indicates that calcineurin does not enhance either DNA binding by MEF2 or the recruitment of co-activator or co-repressor proteins to this region of MEF2. In contrast, expression of a luciferase reporter gene controlled by high affinity GAL4 binding sites in the presence of three different GAL4BD:MEF2AD (carboxyl terminal fragments) fusion proteins was stimulated by calcineurin but not by CaMKIV (Figure 15). This latter finding suggests that calcineurin potentiates MEF2 transactivator function in a manner independent of effects on MEF2 DNA binding.

Dephosphorylation of MEF2 by calcineurin *in vivo* and *in vitro*

Hypophosphorylation of MEF2 accompanies its functional activation by calcineurin in neurons (Mao and Wiedmann, 1999). When C2C12 myotubes were exposed to the calcium ionophore ionomycin, a more rapidly migrating form of MEF2A was evident in

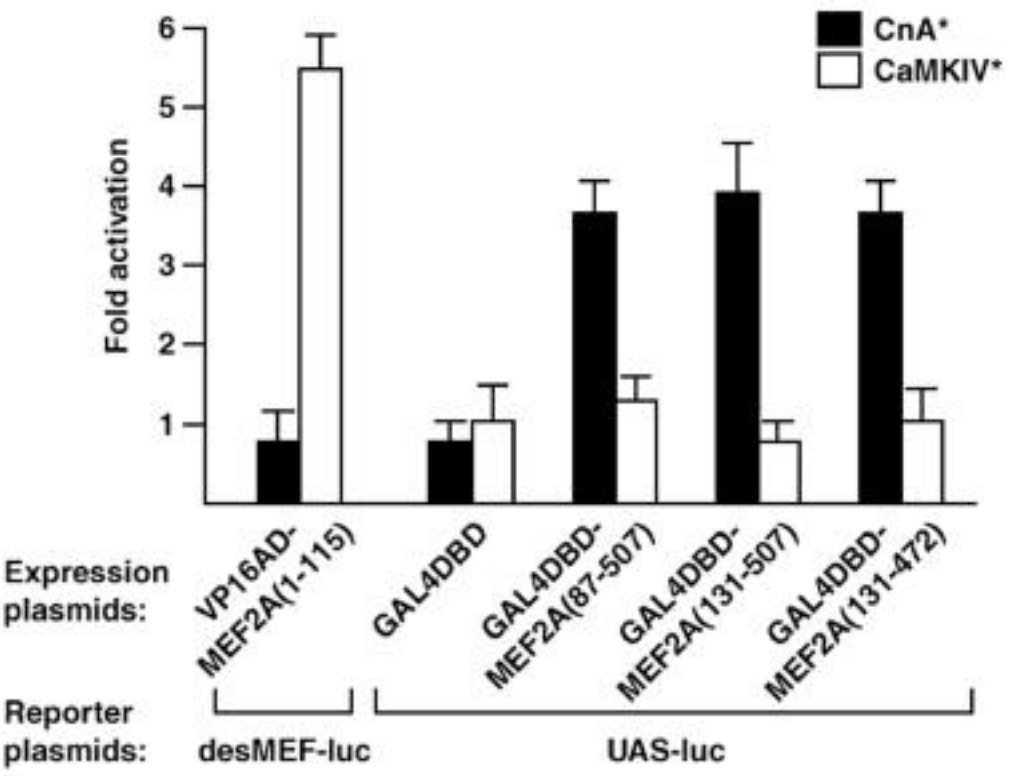


Figure 15. C-terminal transactivating domain of MEF2 responds to calcineurin activation. The indicated segments of MEF2A protein were fused either to a heterologous transactivation domain (VP16 AD) or to a heterologous DNA binding domain (GAL4 DBD) and co-transfected into C2C12 myogenic cells with a luciferase reporter gene controlled by multimerized binding sites for either MEF2 (desMEF2) or GAL4 (UAS). The effects of constitutively active forms of calcineurin CnA* or calmodulin dependent protein kinase IV (CaMKIV*) to alter basal levels of reporter gene expression were calculated as fold activation over basal levels (mean $\pm$ SD in 3-6 independent transfections).

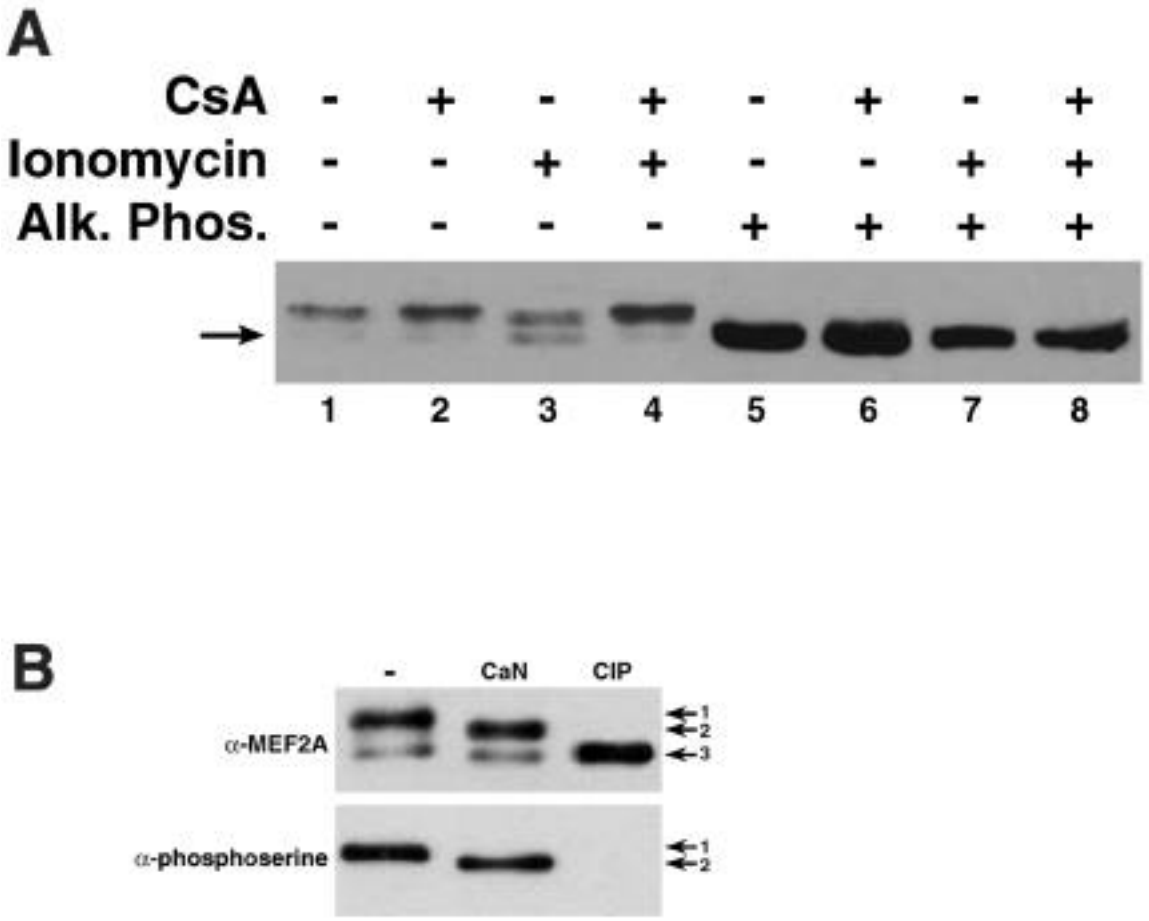


Figure 16. Dephosphorylation of MEF2 by calcineurin *in vivo* and *in vitro.* **A.** Proteins extracted from C2C12 myotubes were separated by SDS-PAGE and immunoblots were probed with anti-MEF2 antibody. A hypophosphorylated form of MEF2A (arrow) is evident in cells exposed to a calcium ionophore (1 μM ionomycin, lane 3). Dephosphorylation of MEF2A in the presence of ionomycin was inhibited by the calcineurin antagonist cyclosporin A (250 nM CsA, lane 4). The identity of the more rapidly migrating band as a hypophosphorylated form of MEF2A was confirmed by incubation of protein extracts with alkaline phosphatase (Alk. Phos., lanes 5-8). Similar findings were observed in each of three independent experiments. **B.** Partially purified MEF2A was incubated *in vitro* with purified calcineurin or calf intestinal phosphatase, and different phosphorylation states of MEF2 (arrows 1, 2, and 3) were identified by mobility during SDS-PAGE as detected by immunoblot (α-MEF2 or α-phophoserine antibodies).

immunoblot assays (Figure 16A, lane 3). The identity of this band as a hypophosphorylated form of MEF2A was confirmed by incubation of protein extracts with alkaline phosphatase (Figure 16A, lanes 5-8). Dephosphorylation of MEF2A in the presence of ionomycin requires calcineurin activity, as determined by the inhibitory effect of the specific calcineurin antagonist cyclosporin A (Figure 16A, lane 4). Incubation of partially purified MEF2A with activated calcineurin *in vitro* converts the major MEF2A band (Figure 16B, arrow 1) to a faster migrating form (Figure 16B, arrow 2), consistent with partial dephosphorylation, based on comparison to the effects of calf intestinal alkaline phosphatase (CIP), which removes all phosphate groups from MEF2 (Figure 16B, arrow 3). The conclusion that calcineurin promotes partial dephosphorylation of MEF2, while certain phosphoserine residues are insensitive to calcineurin, is confirmed by probing the same blot with anti-phosphoserine antibody (Figure 16B).

To define the specific MEF2A serine/threonine (S/T) residues dephosphorylated by calcineurin, twenty conserved S/T within the C-terminal transactivating domain of MEF2A were individually mutated into alanine (A) with the exception of threonines 312 and 319, which were co-mutated. p38 MAPK (mitogen-activated protein kinase) activates MEF2A through phosphorylating these two threonines (Zhao *et al*., 1999). Most of these 19 MEF2A mutants displayed either unaltered or compromised basal activity without change in their responsiveness to calcineurin activition. Only MEF2A-S408A showed increased basal transcriptional activity and decreased responsiveness to calcineurin (Figure 17), in agreement with the notion that calcineurin might dephosphorylate serine 408 and activate MEF2

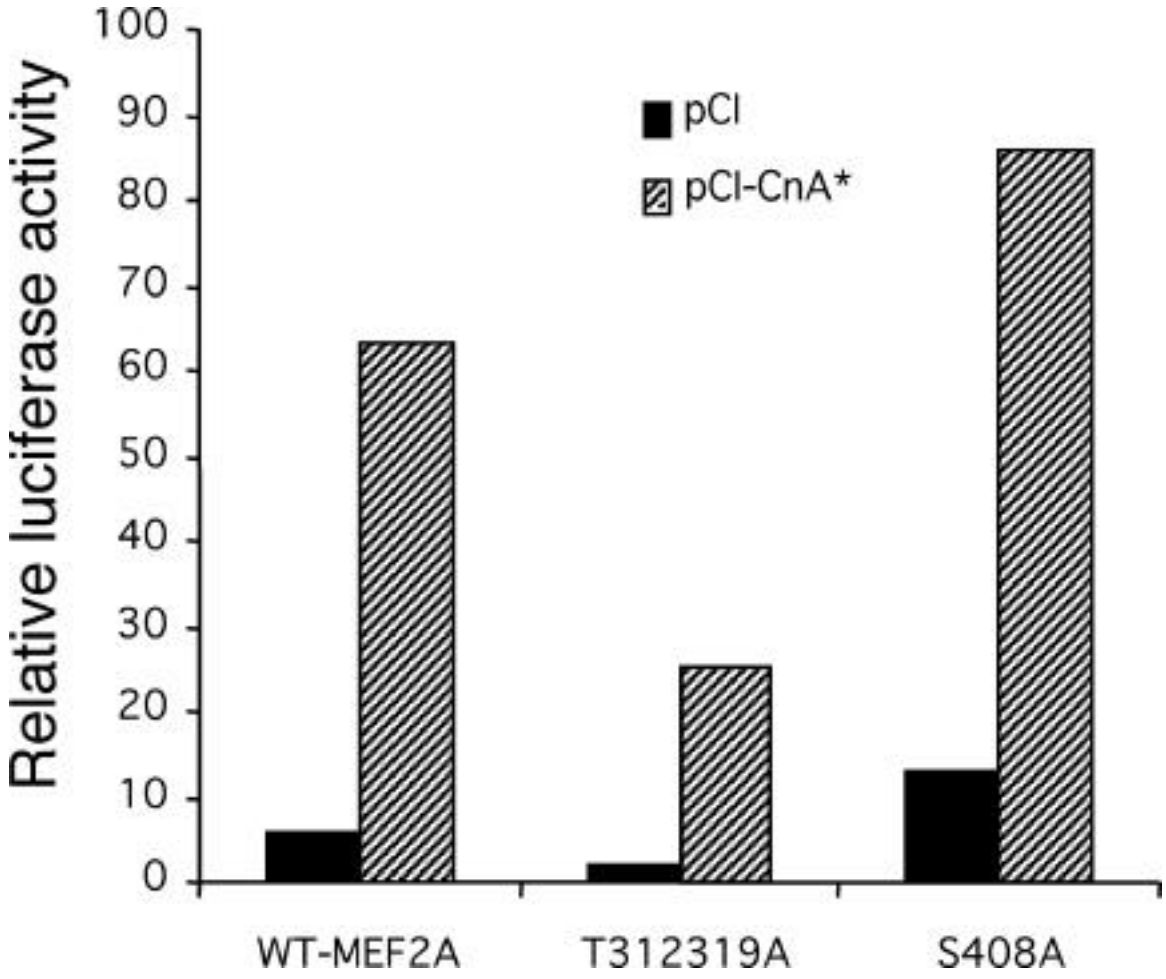


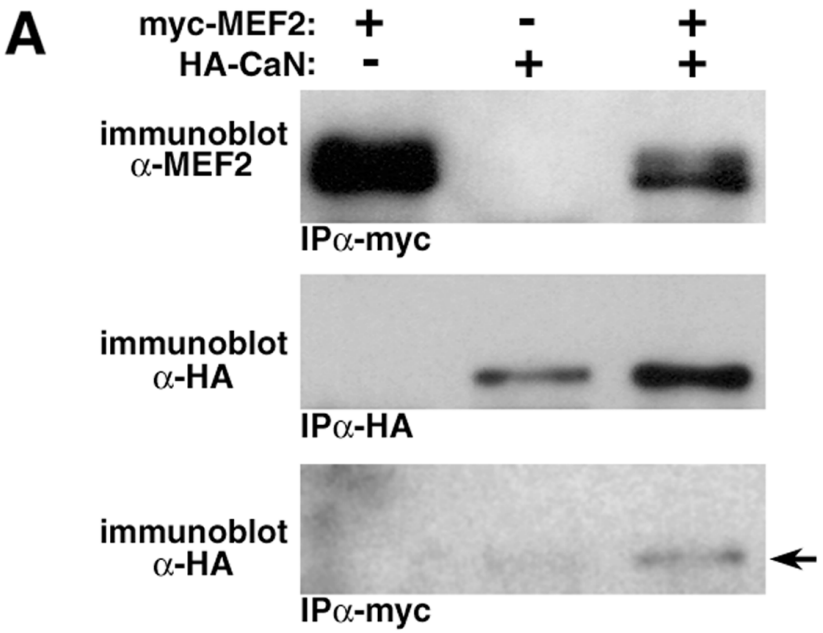
Figure 17. Decreased fold activation of MEF2A-S408A mutant by calcineurin. Wild type (WT) and two mutant MEF2A that have serine/threonine to alanine mutations in their C-terminal domains were tested for their ability to activated MEF2-dependent reporter (desMEF2-luciferase). One mutant MEF2A (T312319A) has lowered basal activity toward desMEF2 reporter but unaltered fold activation in the presence of CnA*. Mutant MEF2-S408A shows increased basal activity but its fold activation by CnA* is moderately decreased.

transcriptional function. Other unknown mechanisms also participate in this calcineurin-MEF2 signaling since MEF2A-S408A is still partially activated by calcineurin. Calcineurin apparently functions independent of p38 MAP kinase pathway since MEF2A- T312319A showed similar response to calcineurin activation compared to wild type MEF2A (Figure 17).

Interaction between MEF2 and calcineurin *in vivo* and *in vitro*

To test whether calcineurin and MEF2 form a physical complex, epitope tagged forms of MEF2A (c-myc tag) and calcineurin A (CnA, HA tag) were expressed in C2C12 myogenic cells, either alone or in combination, and complexes were precipitated with α-HA or α-c-myc antibodies. Immunoprecipitated proteins were identified by immunoblots (α-HA, α-c-myc or α-MEF2A). Calcineurin is present in protein complexes precipitated from muscle cell extracts with antibodies directed against a c-myc epitope tagged form of MEF2 (Figure 18A).

When purified fusion protein containing GST (glutathione S-transferase) and different regions of MEF2A was incubated with ^{35}S-methionine labeled calcineurin, the MEF2A C-terminal (residues 131-507) fusion protein interacted with calcineurin, as well as the full length MEF2A, while the MEF2A N-termianl (residues 1-87) fusion protein did not. These data further support the notion that it is the C-terminal transactivating domain of MEF2A, but not the N-terminal DNA binding domain, that mediates the effect of calcineurin on MEF2A.


A
myc-MEF2: + - +
HA-CaN: - + +
immunoblot
α-MEF2
IPα-myc
immunoblot
α-HA
IPα-HA
immunoblot
α-HA
IPα-myc

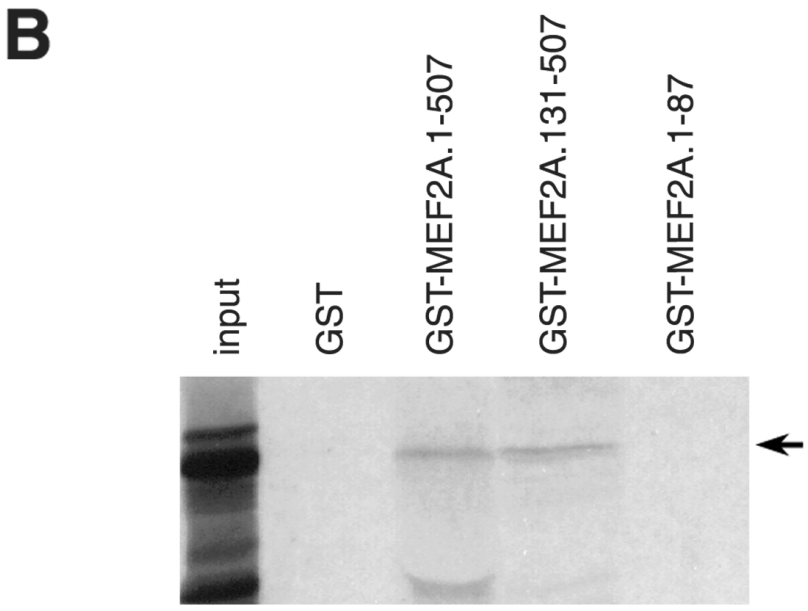

B
input
GST
GST-MEF2A.1-507
GST-MEF2A.131-507
GST-MEF2A.1-87

Figure 18. Physical interaction between MEF2 and calcineurin. **A.** Epitope tagged forms of MEF2A (c-myc tag) and Calcineurin A (CnA, HA tag) were expressed in C2C12 myogenic cells, either alone or in combination, and complexes were precipitated with the indicated antibodies (α-HA or α-c-myc). Immunoprecipitated proteins were identified by immunoblots (α-HA or α-MEF2). **B.** Agarose beads tethering GST alone and GST fusion proteins containing different regions of MEF2A were incubated with ^{35}S-labeled CnA and after extensive washing, the bound CnA was visualized by autoradiography.

The transactivating function of MEF2 within skeletal muscles of adult animals is detectable selectively in the soleus muscle

I have shown that calcineurin activates MEF2 transcriptional functions in cultured myocytes. If this holds true in skeletal muscle tissues, it is likely that MEF2 is more active in slow and oxidative fibers than in fast fibers due to the selective activation of calcineurin in slow and oxidative skeletal myofibers as a result of constant slow motorneuron firing, as we proposed initially in our model. Since MEF2 can act either as an activator or repressor of transcription under different circumstances (McKinsey *et al*., 2000b; Molkentin *et al*., 1995), measurements of MEF2 mRNA and protein expression, or DNA binding activity, provide little information to evaluate the role of MEF2 in gene regulatory responses. The use of "MEF2 indicator mice" (Naya *et al*., 1999), in which the expression of a lacZ transgene is dependent solely on MEF2 transactivator function, provides a readout of MEF2 activity that is suitable for physiological studies of intact animals. In these transgenic mice, a lacZ reporter gene is controlled by a minimal promoter and 3 repeats of a high affinity MEF2 binding site from the desmin gene (desMEF2-lacZ). During embryonic development, expression of lacZ was observed throughout skeletal, cardiac and smooth muscle lineages, mirroring expression of MEF2 proteins. After birth, however, consistently high levels of lacZ expression in these animals are restricted predominantly to the brain (Naya *et al*., 1999). Notably, ß-galactosidase activity is not detectable in cardiac muscle and most skeletal muscles of these animals, despite the clear presence of MEF2 proteins within these tissues. Careful analysis of skeletal muscles of a large number of desMEF2-lacZ transgenic mice

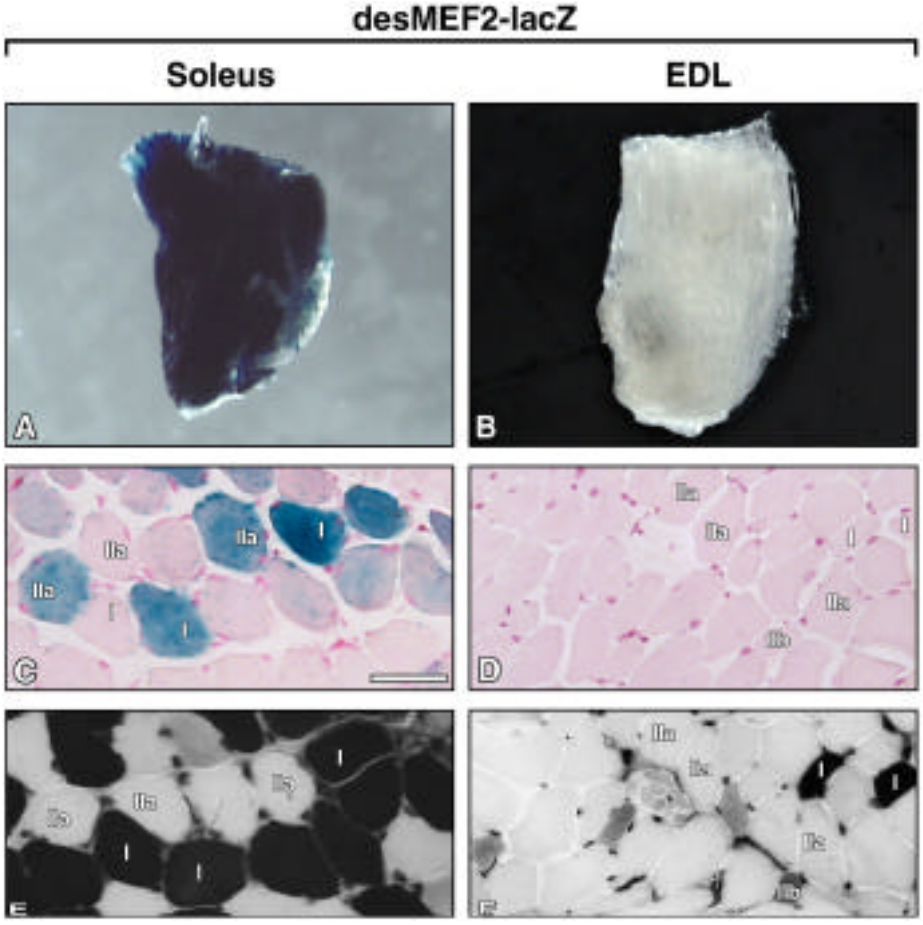

desMEF2-lacZ
Soleus
EDL
A
B
C
D
E
F

Figure 19. Selective activation of MEF2-dependent reporter in soleus. Soleus and extensor digitorum longus (EDL) muscles were dissected from the hindlimbs of desMEF2-lacZ transgenic mice, and stained for β-galactosidase activity. The gross appearance of the muscles (**A,B**), and photomicrographs of serial cross sections stained for β-galactosidase activity (**C,D**) or myosin ATPase activity (**E,F**) are shown. The distribution of ß-galactosidase appeared to be uniform along the length of positively staining fibers (not shown). In E and F, the darkest stained fibers are type I, least stained fibers are type IIa and intermediary stained fibers are type IIb. Bar = 200 microns.

revealed that ß-galactosidase activity is expressed selectively in soleus muscles (Figure 19A and C), while muscles such as the extensor digitorum longus (EDL) that consist predominantly of fast fibers are negative (Figure 19B and D). This phenotype was incompletely penetrant, in that grossly positive ß-galactosidase staining in the soleus was observed in 10 of 95 animals bearing this transgene, as compared to 0 of 95 animals in which ß-galactosidase activity was detectable in the EDL. In cross-sections of these soleus muscles, we observed that the MEF2-dependent reporter gene was activated both in Type I and in Type IIa fibers (Figure 19 E and F).

Calcineurin is necessary and sufficient for MEF2 activation in skeletal muscles

Another transgenic experiment showed that calcineurin can stimulate the transcriptional activation function of MEF2 within intact muscles, as well as in cultured myocytes. The desMEF2-lacZ transgenic line was crossed to a line of transgenic mice that express a constitutively active form of calcineurin under the control of the muscle isoenzyme of creatine kinase (MCK) enhancer. In doubly transgenic animals, desMEF lacZ expression was observed in fibers of the extensor digitorum longus (4 of 10 animals examined) (Figure 20A and B), whereas this muscle was uniformly negative (0 of 95 animals examined) in desMEF2-lacZ mice in the absence of the MCK-calcineurin transgene. In cross-sections of EDL muscles, MEF2-dependent reporter activation occurred in Type IIa fibers (Figure 20C).

Expression of ß-galactosidase is detectable by immunoblot analysis in soleus muscles of sedentary desMEF2-lacZ mice. In five animals treated for 18 days by daily injection of cyclosporin A, a pharmacological inhibitor of calcineurin, expression of the lacZ transgene

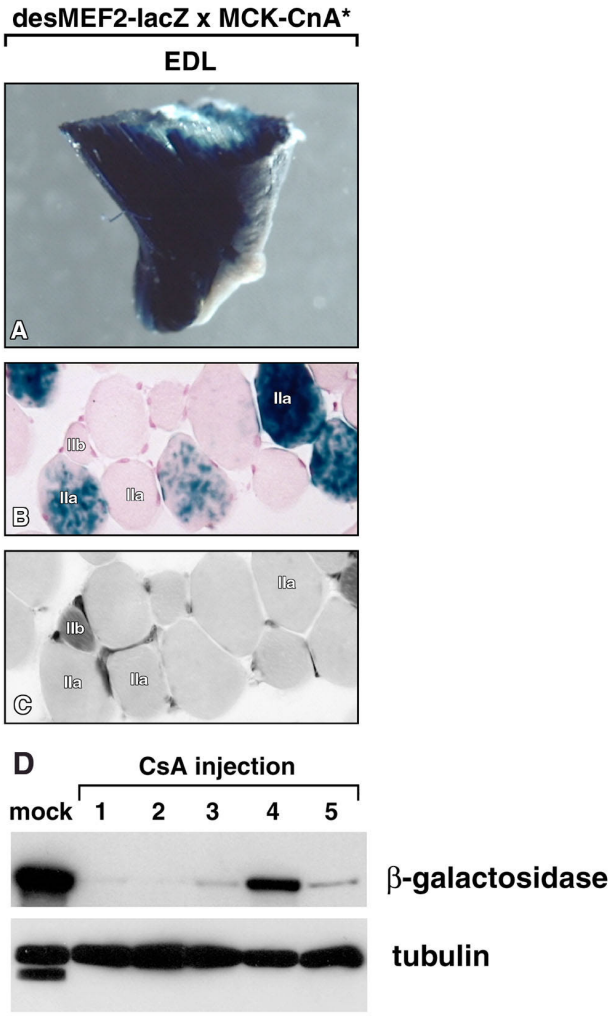

desMEF2-lacZ x MCK-CnA*
EDL
A
IIa
IIb
IIa
IIa
B
IIa
IIb
IIa
IIa
C
D
CsA injection
mock
1
2
3
4
5
β-galactosidase
tubulin

Figure 20. Calcineurin is both necessary and sufficient for activation of MEF2-dependent reporter. Extensor digitorum longus (EDL) muscles were dissected from doubly transgenic mice carrying both the desMEF-lacZ transgene and an MCK-CnA* transgene and stained for β-galactosidase activity. The gross appearance of the muscles (**A**), and photomicrographs of serial cross sections stained for β-galactosidase activity (**B**) or myosin ATPase activity (**C**) are shown. **D.** Protein extracts from soleus muscles of desMEF2-lacZ transgenic mice treated with cyclosporin A or vehicle (PBS buffer) for 18 consecutive days were loaded onto SDS-PAGE gels and expression of β-galactosidase and tubulin (loading control) were detected by western blot.

was uniformly reduced compared to levels found in a pool of soleus muscles from 5 animals treated with vehicle only (mock injection) (Figure 20D). This result indicates that greater transactivating function of MEF2 in soleus muscles of sedentary animals is dependent on calcineurin activity.

A dose-response relationship between calcineurin activity and expression of endogenous MEF2 target genes

Relationships between the enzymatic activity of calcineurin in hindlimb muscles and expression of myoglobin and troponin I slow (TnIs), endogenous genes known to be controlled by MEF2 activity (Grayson *et al*., 1998; Yan *et al*., 2001) and induced by exercise or motor nerve stimulation (Underwood and Williams, 1987), were assessed by comparison of 5 independent lines of transgenic mice engineered to express a constitutively active form of calcineurin under the control of the MCK promoter. Three different transgene constructions were used, as shown in Figure 21A. Other phenotypes associated with two of these lines (Lines 1 and 2 in Figure 21) have been reported elsewhere (Dunn *et al*., 2000; Naya *et al*., 2000), but the remaining 3 lines were generated *de novo* for this purpose, and the data presented here are entirely novel. Although all 5 transgenic lines contain an identical MCK promoter/enhancer fragment (Sternberg *et al*., 1988) and an identical protein coding segment that is translated to produce a truncated, constitutively active, 398 amino acid form of calcineurin A (CnA*), they differ markedly in expression of the CnA* transgene product (Figure 21B). Such highly divergent levels of transgene expression occur as a function of modulatory effects of flanking DNA at different chromosomal insertion sites, and possibly

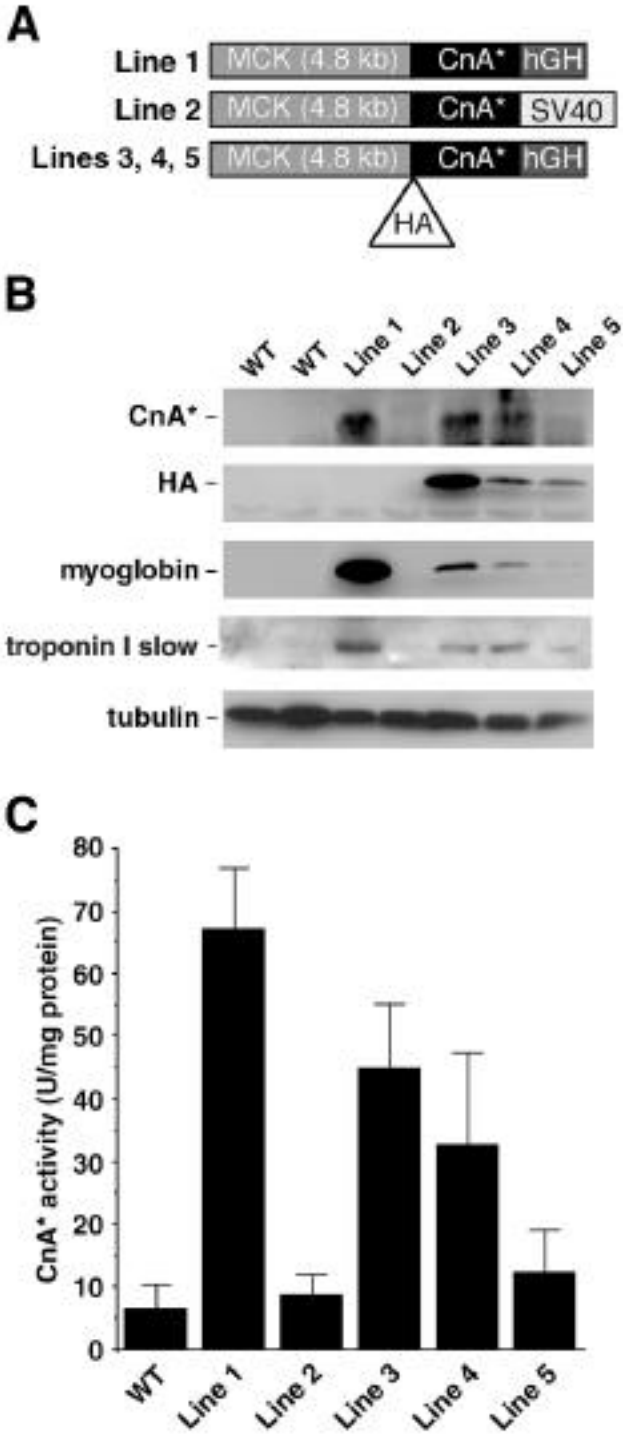

A
Line 1
MCK (4.8 kb)
CnA*
hGH
Line 2
MCK (4.8 kb)
CnA*
SV40
Lines 3, 4, 5
MCK (4.8 kb)
CnA*
hGH
HA
B
WT
WT
Line 1
Line 2
Line 3
Line 4
Line 5
CnA*
HA
myoglobin
troponin I slow
tubulin
C
CnA* activity (U/mg protein)
0
10
20
30
40
50
60
70
80
WT
Line 1
Line 2
Line 3
Line 4
Line 5

Figure 21. A dose response relationship between calcineurin activity and slow, oxidative fiber-specific gene expression in transgenic mice. **A.** Diagram of transgene constructs used to generate 5 independent transgenic lines. **B.** Immunoblots of white vastus lateralis muscle extracts from wild type (WT) and transgenic MCK-CnA* lines using antibodies recognizing calcineurin, HA, myoglobin , troponin I slow and tubulin (loading control). **C.** Calcineurin activity assay of dialyzed against muscle extracts from plantaris muscles. One unit (U) is defined as the amount of enzyme that can release 1 picomole of phosphate from the substrate per minute.

because of differences in 3' UTR and polyadenylation sites. This broad range of CnA* concentrations afforded an opportunity to assess dose-response relationships between the enzymatic activity of calcineurin and expression of MEF2 target genes such as myoglobin and TnIs.

Enzymatic activity of the transgene-derived protein was assessed in the presence of 4 mM EGTA, a condition appropriate to measure activity of the truncated form of calcineurin A that has been engineered to function independently of calcium/calmodulin signaling. Conventional assays of calcineurin are based on the initial rate of dephosphorylation of a synthetic peptide substrate in the presence of saturating concentrations of calcium and calmodulin (Fruman *et al*., 1996). Under these latter conditions, the observed activity bears no predictable relationship to the activation state of endogenous calcineurin *in vivo* or to the level of CnA* protein that is expressed. The conventional assay measures the peak catalytic capacity of calcineurin recovered in the soluble cell fraction, but provides no information about the fraction of that pool that was active in the intact cell or tissue. In contrast, the new data provided here are directly relevant to the *in vivo* activity of the transgene product. As shown in Figure 21C, the line of MCK-CnA* transgenic mice reported by Naya et al. (Naya *et al*., 2000)(Line 1) expressed substantially greater CnA* activity than the mice described by Dunn et al. (Dunn *et al*., 2000) (Line 2), while the newly generated Lines 3-5 expressed intermediate levels. There was some correlation between immunoreactive CnA* protein and catalytic activity of the enzyme (compare Figure 21B and C).

Different levels of CnA* protein expression also correlated directly with the up-regulation of myoglobin and TnIs (Figure 21B), MEF2 target genes and molecular markers

of the Type I and Type IIa (red, oxidative) myofiber subtypes, in skeletal muscles of the distal hind limb. Increased expression of myoglobin and TnIs as a function of CnA* transgene expression in a dose-dependent manner provides *prima facie* evidence for fiber type transformation driven by calcineurin.

3. Discussion

The collective sets of data described in this chapter suggest that MEF2 proteins function in skeletal muscles as downstream effectors of calcineurin signaling pathways that are triggered initially by motor nerve activity, thereby contributing to fiber type-specific gene regulation. Functional activation of MEF2 correlates with its dephosphorylation by calcineurin. The transcriptional response evoked by activated calcineurin is transduced by MEF2 to complex native promoter/enhancer elements from the myoglobin or TnIs genes, and to a synthetic enhancer (desMEF2) constructed solely from MEF2 binding elements. Although MEF2 acts synergistically with NFAT to transactivate enhancers that bind both proteins, our data using the desMEF2 reporter plasmid demonstrate that DNA binding by NFAT is not absolutely required for the ability of MEF2 to transduce calcineurin-dependent signals to target genes.

Our current data should be compared with a recent report that the function of MEF2 as a transcriptional activator is regulated by calcineurin in cultured neurons (Mao and Wiedmann, 1999). Both studies demonstrate functional up-regulation of the transactivating function of MEF2 in the presence of activated calcineurin, corresponding to dephosphorylation of MEF2 through a calcineurin-dependent mechanism. However, calcineurin was observed to stimulate DNA binding of MEF2 in cerebellar granule neurons (Mao and Wiedmann, 1999), while our studies of skeletal myocytes point to a response in which the transactivating function of MEF2 is augmented in the absence of detectable changes in DNA binding. Both phosphorylation and dephosphorylation of S/T residues within the DNA binding domain and transactivating domains of MEF2 have been

demonstrated to activate MEF2 functions. In fibroblasts, DNA binding activity of MEF2 is enhanced by phosphorylation of a serine within MADS box by casine kinase II (Molkentin *et al*., 1996). Phosphorylation of two conserved threonines located at the C-terminus of MEF2A by p38 MAP kinase in 293 cells enhances transactivating function of MEF2A (Zhao *et al*., 1999). Such differences among cell types in the terminal events of otherwise similar MEF2-dependent signaling pathways may contribute to the distinctive programs of gene expression characteristic of each cell background.

Correlation of calcineurin activity and expression of MEF2 target genes underscores an important aspect of studies concerning transgenic mice -- confirming expression of transgene at protein/activity level before attempting to draw any conclusion. Dunn *et al*. (Dunn *et al*., 2000)described that skeletal muscles from a line of transgenic mice (line 2 in Figure 21) expressing CnA* exhibited a phenotype indistinguishable from wild type controls. Upon comparison with four other lines expressing the same transgene under the same promoter, we found the specific mouse line described by Dunn *et al*. had a very low expression of the transgene at protein level. Assay for constitutively active calcineurin confirmed the dramatic differences in transgene activities in various lines of transgenic mice. The line expressing the highest amount of CnA* displayed fiber type transformation and significant induction of slow and oxidative fiber-specific genes, such as myoglobin and TnIs. This observation illustrates the importance of detailed characterization of different lines of transgenic mice prior to the identification of phenotypes.

Chapter V. Involvement of MEF2 and calcineurin in switching of fast glycolytic fibers into slow oxidative ones

1. Introduction

In previous chapters, I showed that MEF2 transcriptional function was selectively active in slow and oxidative fibers and this activation was calcineurin-dependent. Our initial hypothesis that calcineurin serves an important signaling function linking different patterns of motor nerve activity to distinctive programs of gene expression to establish phenotypic diversity among skeletal myofibers has been borne out by these results.

The use of "MEF2 indicator mice" (Naya *et al*., 1999), which harbor a lacZ transgene dependent solely on MEF2 transactivator function, provides a readout of MEF2 activity that is suitable for physiological studies of intact animals. In this chapter, I describe new and rigorous tests of our hypothesis, focusing specifically on the role of MEF2 transcription factors in transducing calcineurin-dependent signals arising as a consequence of muscle work to relevant target genes. The experiments used two different approaches to augment contractile activity in muscles of intact mice: voluntary wheel running and low-frequency electrical stimulation of motor nerves, both of which enhance muscle oxidative capacity and ultimately induce fiber transformation towards slow and oxidative direction (Baldwin and Haddad, 2001; Pette and Vrbova, 1992).

2. Results

Voluntary wheel running in mice promotes transformation of fast glycolytic fibers into slow oxidative ones

When provided access to a running wheel (Figure 22A), mice voluntarily exercised almost continuously for up to 12 hr during the nocturnal phase of their day-night cycle. Activity was monitored and quantified by counting wheel revolutions (Figure 22B). This type of running regimen is proved to be endurance exercise, which promotes oxidative capacity of skeletal muscles and ultimately triggers fiber type switch from fast glycolytic myofibers into slow oxidative ones. mRNA level of myoglobin, an endogenous gene known to be target for MEF2 and responsive to changes in contractile work, became progressively more abundant in skeletal muscles with increased duration of nocturnal wheel running (Figure 22C). The proportion of myofibers expressing Type IIa myosin was increased in running animals, with a reciprocal decline in fibers expressing Type IIb myosin (Figure 22D-F). Thus, sustained periods of repeated muscle contractions associated with wheel running is a good model to study MEF2 functions during fiber type transformation.

MEF2 transcriptional activity is enhanced by spontaneous wheel running and MEF2 activation is dependent upon calcineurin

As little as 3 hr of running was sufficient to generate levels of ß-galactosidase activity detectable by whole mount staining in hind limb muscles in 100% of desMEF2-lacZ animals (Figure 23A). The number of positively stained myofibers and the intensity of staining increased progressively with longer duration of running. Contraction-induced augmentation

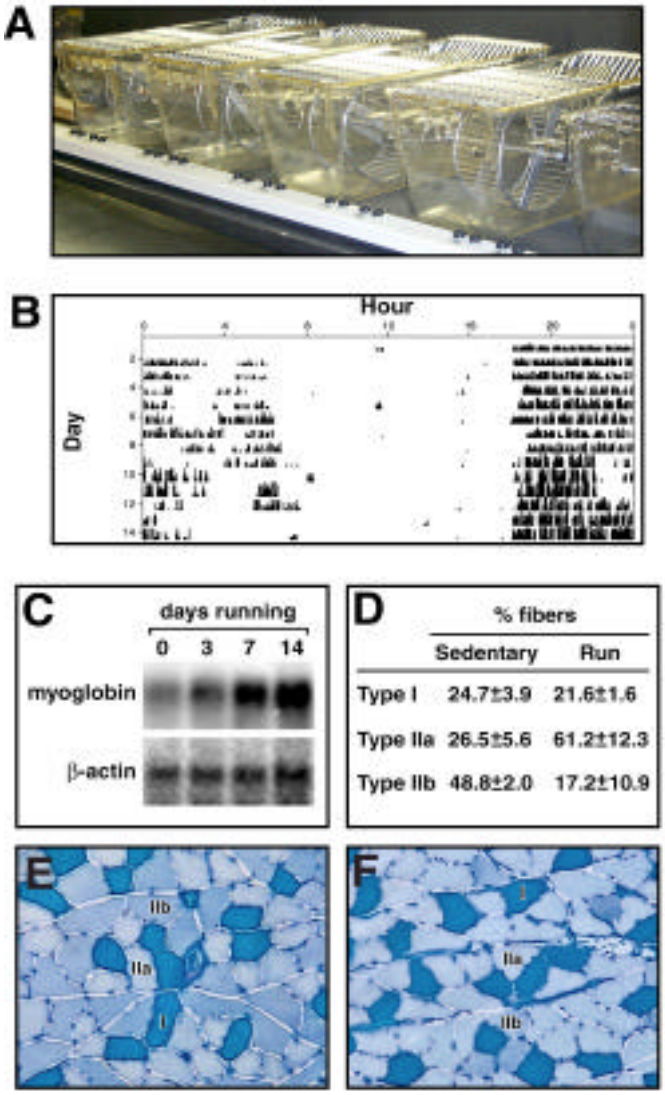

A
B
Hour
Day
C
days running
0 3 7 14
myoglobin
β-actin
D
% fibers
Sedentary Run
Type I 24.7±3.9 21.6±1.6
Type IIa 26.5±5.6 61.2±12.3
Type IIb 48.8±2.0 17.2±10.9
E
IIb
IIa
I
F
IIa
IIb

Figure 22. Voluntary wheel running is an endurance exercise. **A.** Design of cages with running wheels. **B.** Sample recording of 14 days of wheel running activity, recorded as revolutions per minute and depicted by the height of the vertical bars. The recording is continuous and reads from left to right with each line representing a single day. All animals run throughout most of the nocturnal phase of each 12:12 hr light:dark cycle (beginning daily at hr 18 here). **C.** Expression of myoglobin mRNA assessed by Northern blot in plantaris muscles of sedentary (0 days) and exercising mice. ß-actin mRNA provides a loading control. **D.** Specialized myofiber subtypes assessed by myosin ATPase histochemistry reveal fiber type transformation (Type IIb to IIa) in plantaris muscles of running mice. Data represent mean $\pm$ SD in 5 animals within each group. Sample sections of metachromatic staining used for scoring the proportion of myofiber subtypes in sedentary (**E**) and running (**F**) mice are shown. Fibers with dark blue staining are type I and fibers most lightly stained are type IIa. Type IIb fibers show intermediate staining.

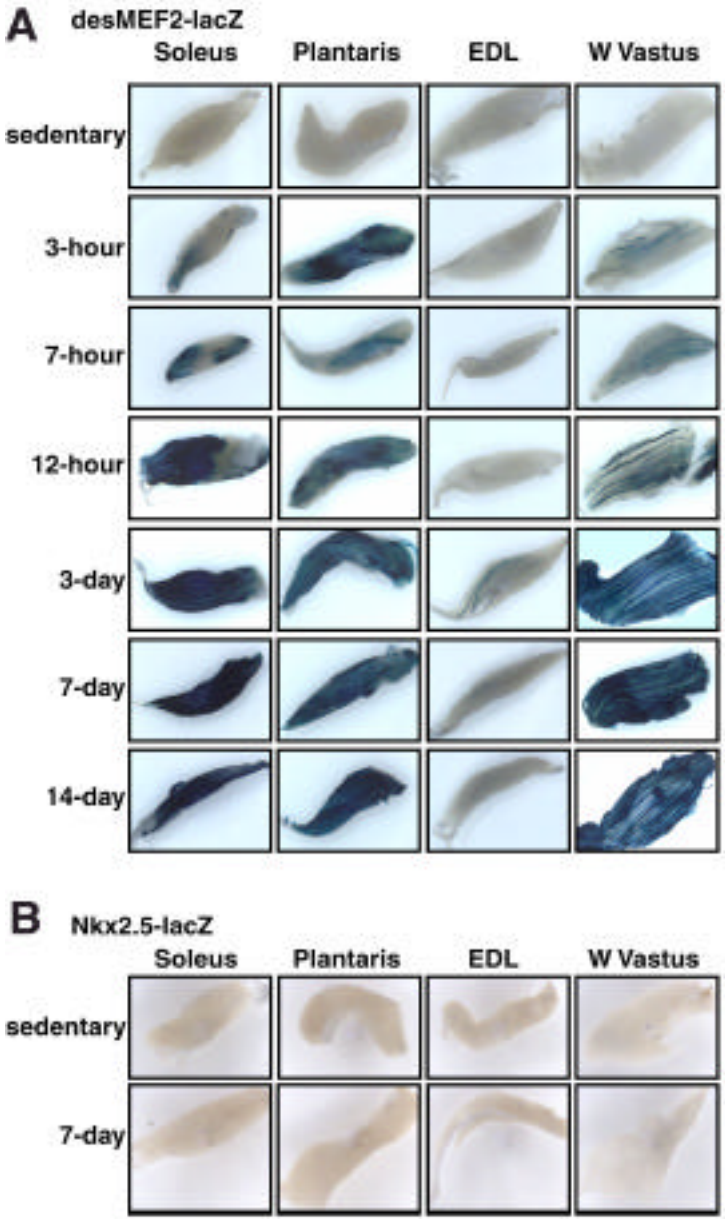

A
desMEF2-lacZ
Soleus
Plantaris
EDL
W Vastus
sedentary
3-hour
7-hour
12-hour
3-day
7-day
14-day
B
Nkx2.5-lacZ
Soleus
Plantaris
EDL
W Vastus
sedentary
7-day

Figure 23. Voluntary wheel running stimulates the transcriptional activation function of MEF2. **A.** Whole mount staining for ß-galactosidase enzymatic activity in individual skeletal muscles (soleus, plantaris, extensor digitorum longus (EDL), and white (W) vastus lateralis) from desMEF2-lacZ transgenic mice. Tissues were acquired from sedentary animals (0 hr) and from mice undergoing voluntary wheel running for the indicated periods of time. Blue staining indicates augmented function of MEF2 as a transcriptional activator in muscles recruited for this locomotor activity (soleus, plantaris, W. vastus but not EDL). Similar results were observed in 3-6 animals assessed at each time point. **B.** Absence of detectable ß-galactosidase activity in muscles from sedentary or exercising mice carrying an Nkx2.5-lacZ transgene.

of the transcriptional activator function of MEF2 proteins, as assessed by this indicator transgene, was evident in soleus, plantaris and white vastus muscles but not in extensor digitorum longus (EDL) of running mice, indicating that the effect was produced only in those muscles recruited to power this particular form of locomotion. The EDL muscle is located in the anterior compartment of the distal hind limb and would be employed to brake the body while descending an incline, but not for continuous assent associated with wheel running. As a negative control, a lacZ transgene controlled by the identical basal promoter (from hsp68) but linked to an enhancer element from the Nkx2.5 gene (Lien *et al*., 1999) showed no induction by wheel running in any muscle (Figure 23B).

Similar results were evident when expression of the lacZ transgene was detected by immunoblot analysis using an antibody directed against ß-galactosidase protein (Figure 24A). As shown in Figure 24B, increased transactivator function of MEF2 induced by running appears to be independent of changes in concentration of major MEF2 isoforms, or of the activation state of p38 MAP kinase, a signaling molecule known to augment MEF2 function (Yang *et al*., 1999; Zhao *et al*., 1999).

Thus, sustained periods of repeated muscle contractions associated with wheel running augment the transcriptional activator function of MEF2 proteins. This effect occurs independently of the preexisting composition of the active muscle with respect to specialized myofiber subtypes, since soleus, plantaris and white vastus show similar responses. Induction of MEF2 function reflects localized neuromuscular activity rather than a systemic response to exercise, since it is limited to muscles recruited for this form of exercise.

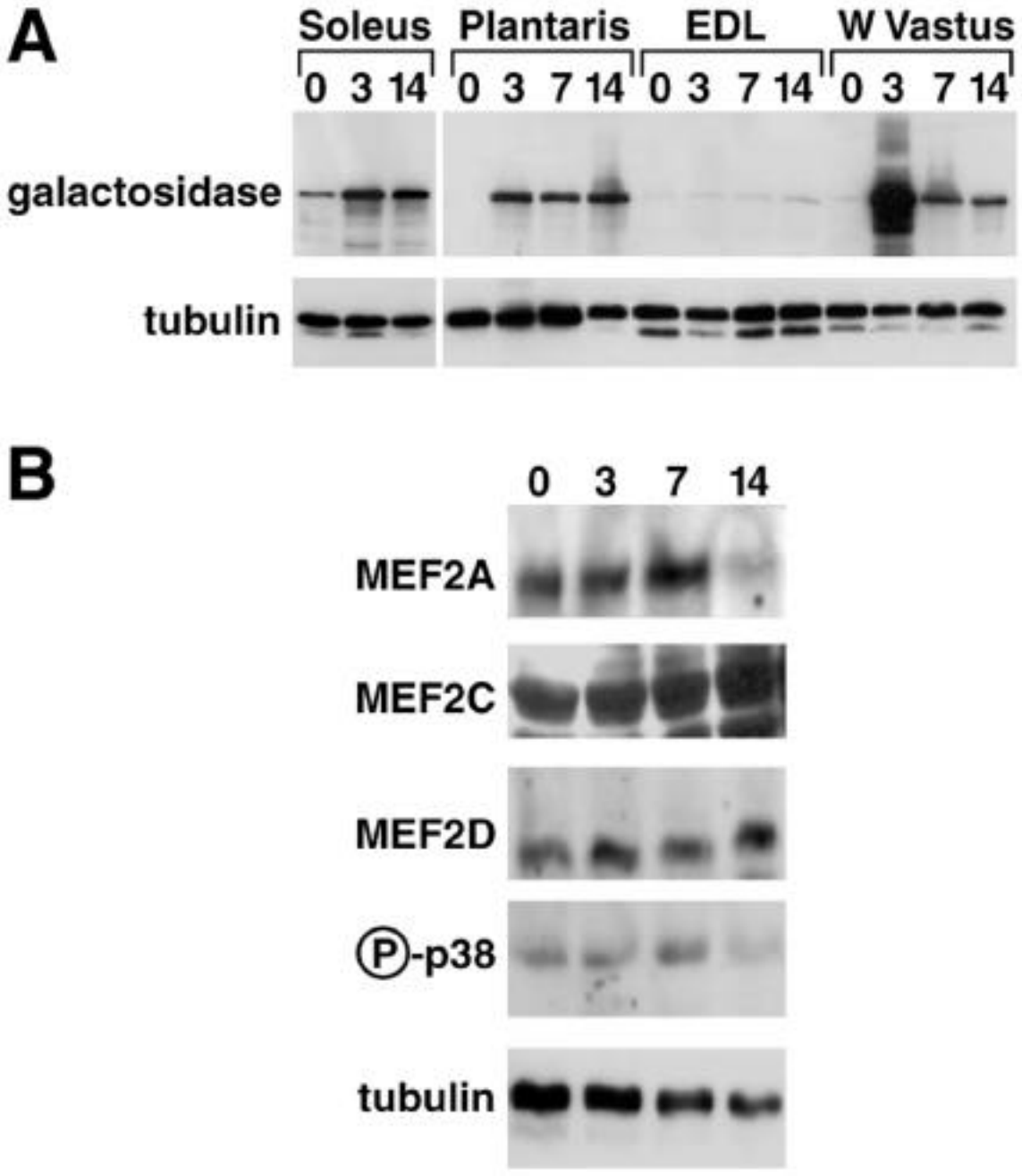

A
Soleus
Plantaris
EDL
W Vastus
0 3 14
0 3 7 14
0 3 7 14
0 3 7 14
galactosidase
tubulin
B
0 3 7 14
MEF2A
MEF2C
MEF2D
Ⓟ-p38
tubulin

Figure 24. Immunoblots of muscle extracts from sedentary and exercising desMEF2-lacZ mice. **A.** Expression of lacZ transgene was detected only in soleus muscles of sedentary animals (0 days), but was up-regulated by running in muscles recruited for this locomotor activity (soleus, plantaris, W. vastus but not EDL). Each lane was loaded with muscle extracts prepared from a pool of 5 animals (10 muscles) and comparable recovery and transfer of each sample was assessed by anti-tubulin antibody. **B.** Up-regulation of desMEF2-lacZ transgene expression in plantaris muscle by running occurs without detectable changes in the abundance of major MEF2 isoforms or of the activated (phosphorylated) form of p38 MAPK.

Because of systemic toxicity associated with the administration of cyclosporin A that could impair running behavior, a different experimental strategy was required to assess the role of calcineurin in mediating contraction-dependent induction of MEF2 function in muscles of animals engaged in spontaneous wheel running. Myocyte-enriched calcineurin interacting protein 1 (MCIP1) functions as an endogenous inhibitor of calcineurin (Rothermel *et al*., 2000; Rothermel *et al*., 2001; Yang *et al*., 2000). Transgenic mice were engineered to express an MCIP1 transgene under the control of the muscle creatine kinase (MCK) gene enhancer, thereby driving expression of MCIP1 selectively in skeletal muscles, particularly fast fibers in which the MCK gene is preferentially active. Doubly transgenic mice that were generated by a genetic cross of desMEF-lacZ animals to an MCK-MCIP1 transgenic line failed to activate the MEF2-dependent indicator gene in the manner seen in the absence of MCIP1 over-expression (Figure 25). These data indicate that calcineurin transduces signals arising as a consequence of muscle contraction to activate MEF2 and stimulate transcription of MEF2 target genes. The use of an MCIP1 transgene to inhibit calcineurin afforded more rigorous conclusions than those based on calcineurin antagonist drugs, since calcineurin is inhibited only within skeletal myofibers, without potentially confounding effects on motor neurons and without systemic toxicity.

Sustained low-frequency electrical stimulation of motor nerve results in activation of MEF2 and this effect also requires calcineurin.

As further proof that calcineurin is required for contraction-dependent activation of MEF2 in skeletal myofibers, desMEF2-lacZ and desMEF2-lacZxMCK-MCIP1 transgenic

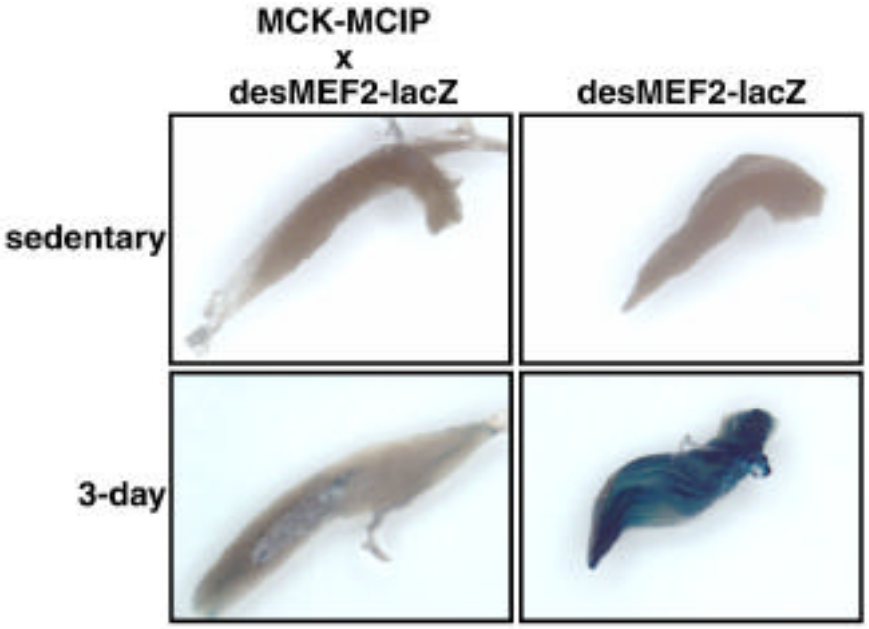


Figure 25. MCIP1 inhibits MEF2 activation by muscle contraction. Histochemical detection of lacZ expression in plantaris muscles of desMEF2-lacZ mice, and in doubly transgenic animals overexpressing MCIP1, a protein inhibitor of calcineurin, in skeletal muscles. Simailar results were observed in 3-6 animals within each group.

mice were subjected to chronic low-frequency electrical stimulation of the motor nerve. This stimulus is known to induce expression of genes characteristic of fatigue-resistant (Type I and IIa) myofibers in a manner similar to the effects of endurance exercise (Mayne *et al*., 1996; Romanul and Van der Meulen, 1966). Sustained periods (28-40 hr) of continuous motor nerve stimulation at 10 Hz produced effects similar to wheel running on lacZ expression, and this response was abrogated either by administration of cyclosporin A (18 days before pacing) or by forced expression of MCIP1 (Figure 26).

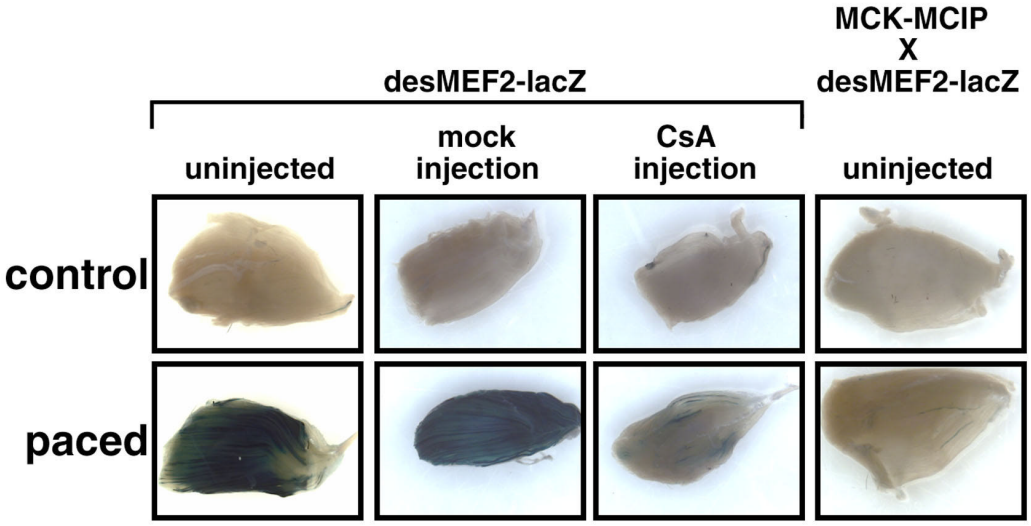


Figure 26. Calcineurin-dependent MEF2 activation by low-frequency motor nerve stimulation. Up-regulation of MEF2 transactivator function by 30 hours of continuous motor nerve pacing (10 Hz) is prevented by administration of cyclosporin A (CsA) or by expression of MCIP1.

3. Discussion

Data gleaned from "MEF2 indicator mice" demonstrate that transcriptional activator function of MEF2 is up-regulated by a calcineurin-dependent pathway in response to contractile activity of skeletal muscles, elicited either through voluntary wheel running or low-frequency nerve pacing. This response requires sustained, repeated patterns of muscle contractions but is independent of the antecedent specialization state (fiber type composition) of the muscle undergoing contractions. We observe that voluntary wheel running consistently activates MEF2 in skeletal muscles, with a time course consistent with a causal role in regulation of endogenous genes that respond to this physiological stimulus.

Kinetic features of contraction-dependent gene regulation in skeletal muscles revealed by our observations merit particular attention . As in classical stimulus-response paradigms, contractile activity of skeletal myofibers promotes transcriptional responses that can be grouped into temporal stages (e.g. immediate early, immediate late, delayed), each characterized by the activation/deactivation of specific sets of genes. A single acute bout of exercise or motor neuron pacing (minutes/hours) induces short-lived changes in expression of certain genes but no stable alterations of muscle phenotype (Michel *et al*., 1994; Neufer *et al*., 1996; Pette and Vrbova, 1999). Remodeling of myofibers from one specialized phenotype to another is a process involving reprogramming of hundreds of genes, and occurs as a cumulative function of repeated, sustained periods of contractile work (days/weeks) (Booth and Baldwin, 1996; Mayne *et al*., 1996; Williams and Neufer, 1996). To review kinetic features of desMEF2-lacZ expression, a few hours are sufficient to evoke a uniformly detectable response to muscle contractions, but maximal activation requires days to weeks.

This time course parallels that of endogenous genes (e.g. myoglobin) that establish specialized myofiber phenotypes. Due to the stable nature of β-galactosidase, it is difficult to examine the deactivation kinetics of MEF2 by monitoring transgene expression in desMEF2-lacZ mice. However, the inducing effects of one week of running are no longer detectable after four weeks of sedentary caging (data not shown), indicating that MEF2 is deactivated after running is terminated. This reversal of contraction-induced transgene activation upon a return to ambient activity resembles that of stable endogenous proteins of the sarcomere or mitochondria (Booth and Baldwin, 1996; Brown *et al*., 1989).

Chapter VI. Control of fiber type specificity and mitochondrial biogenesis by calcium, calmodulin-dependent kinase IV

1. Introduction

Up to now, I have been focusing on calcineurin, which is implicated in the control of skeletal muscle fiber types and can partially transform glycolytic type II fibers into oxidative and mitochondria-enriched type I fibers. The efficiency of fiber type transformation by calcineurin is quite low (Naya *et al*., 2000) compared to the complete fast-to-slow fiber transformation induced by sustained elevation of $[Ca^{2+}]_i$ (Kubis *et al*., 1997), indicating that other calcium-regulated signaling molecules also participate in this process. The premise that changes in regulatory pools of intracellular calcium constitute a proximate stimulus by which the firing pattern of motor neurons controls specific programs of gene expression in skeletal myofibers also suggests that other calcium-regulated signaling pathways, in addition to calcineurin, may be pertinent to fiber type-specific gene transcription. As an initial test of this concept, we examined the potential synergism between calcineurin and calmodulin-dependent protein kinases (CaMK) in the activation of slow/oxidative fiber-specific gene promoters. To further examine the *in vivo* relevance of CaMK signaling pathways in fiber type control, we generated transgenic mouse lines overexpressing a constitutively active form of CaMKIV (CaMKIV*) in their skeletal muscles and found that these mice displayed elevated mitochondrial biogenesis as well as increased slow fiber percentage in their skeletal muscles.

2. Results

Calcineurin-dependent stimulation of the transactivating function of MEF2 is amplified by calmodulin-dependent protein kinase (CaMK) activity

We assessed functional interactions between calcineurin and a calmodulin-dependent protein kinase (CaMKIV) in the control of myoglobin and desMEF2 enhancer function. CaMKIV was shown previously to modify calcineurin-regulated transcriptional responses mediated by the CREB (cAMP responsive element binding protein) transcription factor in hippocampal neurons (Bito *et al.*, 1996), but studies of combinatorial interactions between calcineurin and calmodulin dependent protein kinases in skeletal myocytes have not been reported previously. We observed a pronounced enhancement of calcineurin-induced stimulation of MEF2 transactivator function in the presence of a constitutively active form of CaMKIV (CaMKIV*). This synergistic effect was evident using either the native myoglobin promoter/enhancer or the synthetic desMEF2 enhancer to detect the response (Figure 27). CaMKIV* had only small effects (<5-fold) on transcription of either of these MEF2-dependent enhancers in the absence of concomitant calcineurin activity. In combination, however, CaMKIV and calcineurin evoked a large (35- to 60-fold) response in this assay. These results indicate that MEF2 serves to integrate signaling inputs derived both from calcineurin, and from other calcium-regulated pathways. CaMKI showed similar effects as CaMKIV, but CaMKII and CaMKIII didn't synergize with calcineurin in the activation of MEF2-dependent gene expression (data not shown).

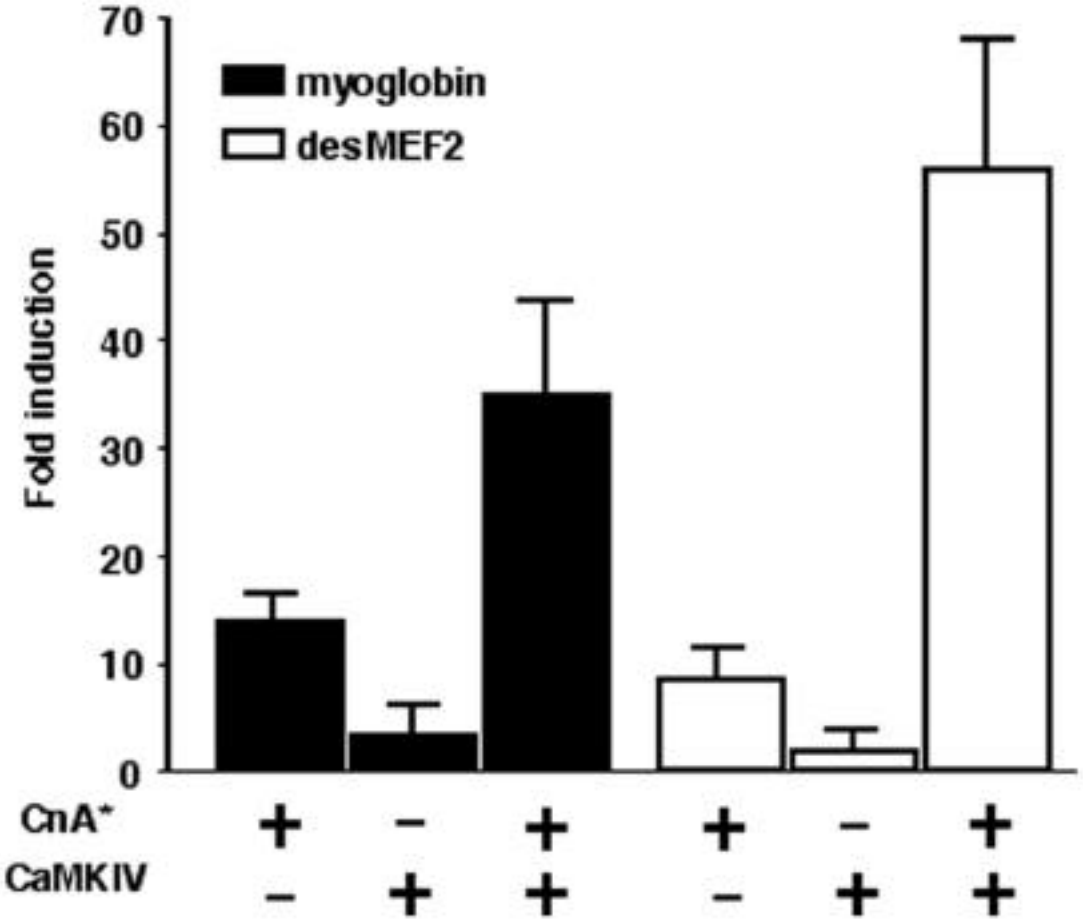
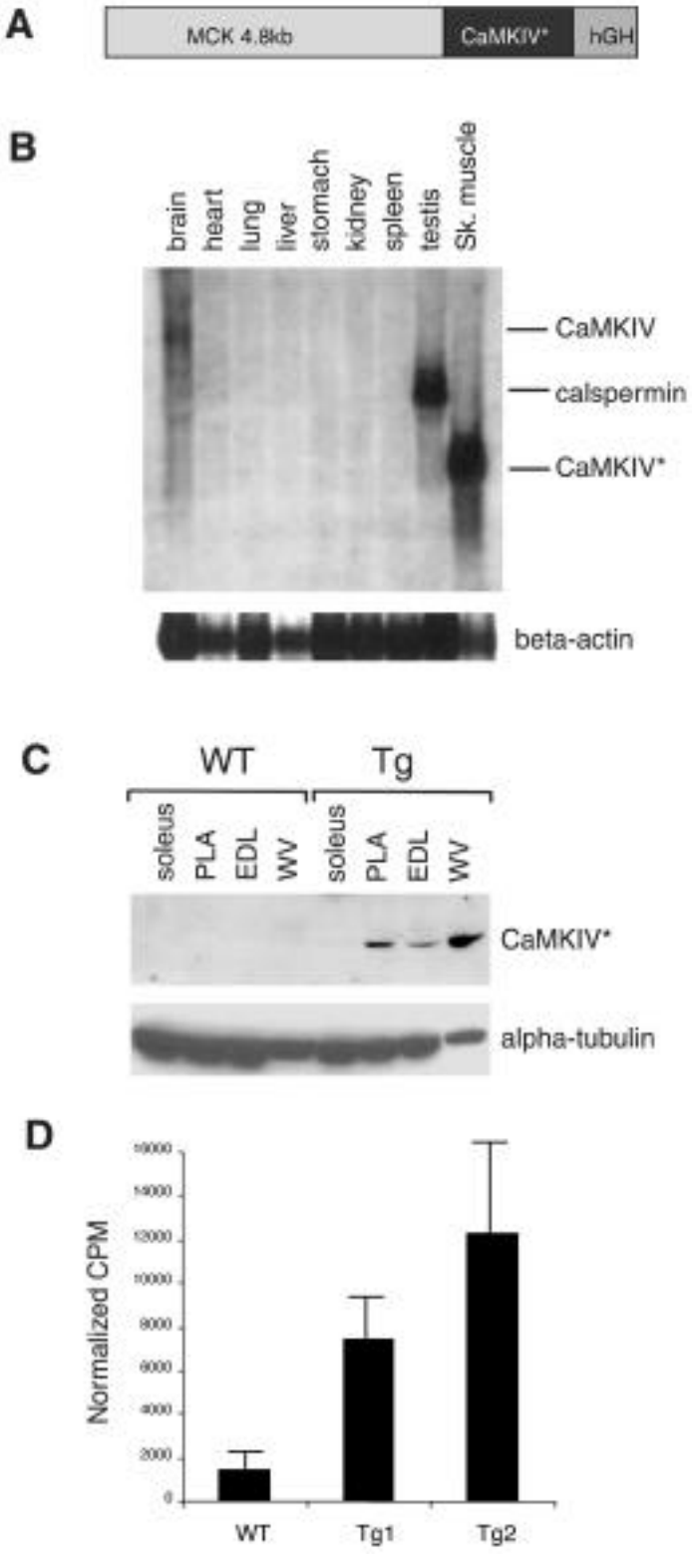


Figure 27. Synergistic activation of MEF2 by calcineurin and CaMKIV. Luciferase reporter plasmids controlled by the myoglobin or desMEF2 enhancers were cotransfected into C2C12 cells with expression plasmids encoding either constitutively active calcineurin A subunit or CaMKIV. Data are presented as in Figure 13, and represent results of 3 or more independent experiments.

Transgenic mouse lines expressing CaMKIV* in their skeletal muscles

To examine the *in vivo* function of CaMK signaling, transgenic mouse lines expressing CaMKIV* were generated. A 4.8 kb proximal region from the promoter of mouse muscle creatine kinase (MCK) gene (Naya *et al*., 2000), which is preferentially active in adult skeletal muscles, was used to drive transgene expression (Figure 28A). CaMKIV* is a truncated form of CaMKIV lacking the C-terminal autoinhibitory domain and the calcium/calmodulin binding domain, and thus does not require calcium or calmodulin for its activation (Passier *et al*., 2000). Northern blot analysis confirmed the expression of transgene in adult skeletal muscles (Figure 28B). Endogenous CaMKIV transcripts, which are longer than the transgene, were detected in brain and testis. Within skeletal muscles, transgene expression was more enriched in muscles composed primarily of type II myofibers, such as plantaris (PLA), extensor digitorum longus (EDL) and white vastus (WV) muscles (Figure 28C). To measure transgene activity directly, CaMKIV was immunoprecipitated from skeletal muscles of two transgenic lines and assayed for its kinase activity toward a peptide substrate in the presence of 1 mM EGTA, which inhibited endogenous CaMK activity in the immunocomplex. Both lines displayed constitutively active CaMKIV activity in their skeletal muscles (Figure 28D).

Enhancement of mitochondrial functions and mitochondrial biogenesis in CaMKIV* transgenic mice


A
MCK 4.8kb
CaMKIV*
hGH
B
brain
heart
lung
liver
stomach
kidney
spleen
testis
Sk. muscle
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calspermin
CaMKIV*
beta-actin
C
WT
Tg
soleus
PLA
EDL
WV
soleus
PLA
EDL
WV
CaMKIV*
alpha-tubulin
D
Normalized CPM
16000
14000
12000
10000
8000
6000
4000
2000
0
WT
Tg1
Tg2

Figure 28. Transgenic mice expressing CaMKIV* in their skeletal muscles. **A.** MCK-CaMKIV* transgene structure. A cDNA encoding a constitutively active form of CaMKIV (CaMKIV*) was inserted between a 4.8 kb MCK gene promoter and human growth hormone (hGH) poly-adenylation signal. **B.** Northern blot analysis of transgene expression in different adult mouse tissues. Endogenous full-length CaMKIV was detected in brain. Calspermin, the testis-specific form of CaMKIV, lacks the C-terminal domain of CaMKIV and the transcript is shorter than the full-length CaMKIV. The constitutively active form of CaMKIV (CaMKIV*) was only expressed in skeletal muscles. β-actin signals were shown to confirm RNA integrity. **C.** Western blot showing expression of transgene at protein level. CaMKIV* was detected in skeletal muscles of transgenic mice (Tg), especially in fast fiber-dominant muscles, such as EDL, PLA and WV. α-tubulin signals were used to show protein loading. **D.** Assay for constitutively active CaMKIV. Protein G-agarose and antibody against CaMKIV were added into dialyzed protein extracts from plantaris muscles of wild type (WT) and two transgenic mouse lines (Tg1, Tg2). The immunoprecipitated CaMKIV complexes were used to measure kinase activity toward a CaMKIV-specific peptide substrate in the presence of 1mM EGTA to inhibit any endogenous CaMK activity. Radioactivity incorporated into the substrate was quantified by scintillation count. Histograms represent mean $\pm$ SEM values of 3 independent assays.

Global gene expression profiles of skeletal muscles from wild type and transgenic mice were compared using DNA microarray technology. Several NADH dehydrogenase subunits showed substantial upregulation in the plantaris muscles from transgenic mice relative to wild type littermates (Figure 29A). These NADH dehydrogenase subunits are encoded by mitochondrial genome and participate in oxidative phosphorylation. Northern blot analyses showed that expression of other mitochondrial genes were also induced in skeletal muscles of transgenic mice (Figure 29B). Expression level of cytochrome B-a mitochondria encoded gene, as well as carnitine palmitoyl transferase 1 (CPT-1)-a nuclear encoded mitochondrial gene, increased in plantaris muscles from two lines of transgenic mice compared to wild type animals. Another nuclear encoded mitochondrial protein, cytochrome C, was also induced by CaMKIV* (Figure 29C). The coordinated upregulation of mitochondrial genes encoded by both nuclear and mitochondrial genomes mimicked the effect caused by chronic muscle electrical stimulation (Williams *et al*., 1987; Williams *et al*., 1986).

Having demonstrated that CaMKIV* enhanced mitochondrial function and oxidative capacity of skeletal muscles, we next tried to identify the factors transducing CaMKIV* signals. PPARγ coactivator 1 (PGC-1) is a newly identified master regulator of mitochondrial functions (Puigserver *et al*., 1998; Wu *et al*., 1999). It activates many aspects of adaptive thermogenesis through upregulation of uncoupling proteins (UCP) in skeletal muscle and brown adipose tissue (BAT). PGC-1 also stimulates the expression of NRF (nuclear respiratory factor) and mtTFA (mitochondrial transcriptional factor A) to trigger

A

Rank	Fold	Gene Name	GenBank ID
1	6.3	NADH dehydrogenase subunit 2	BG792401
2	5.8	NADH dehydrogenase subunit 6	BG791242
3	5.6	NADH dehydrogenase subunit 3	BG795332
4	5.1	NADH dehydrogenase subunit 1	AA499219
5	4.0	Proteasome subunit alpha-5	NM_011967
6	3.9	Transmembrane protein Tm4sf8	NM_019793
7	3.8	Galactokinase (Galk)	NM_016905
8	3.8	Ewing sarcoma homolog (EWS)	BG795113

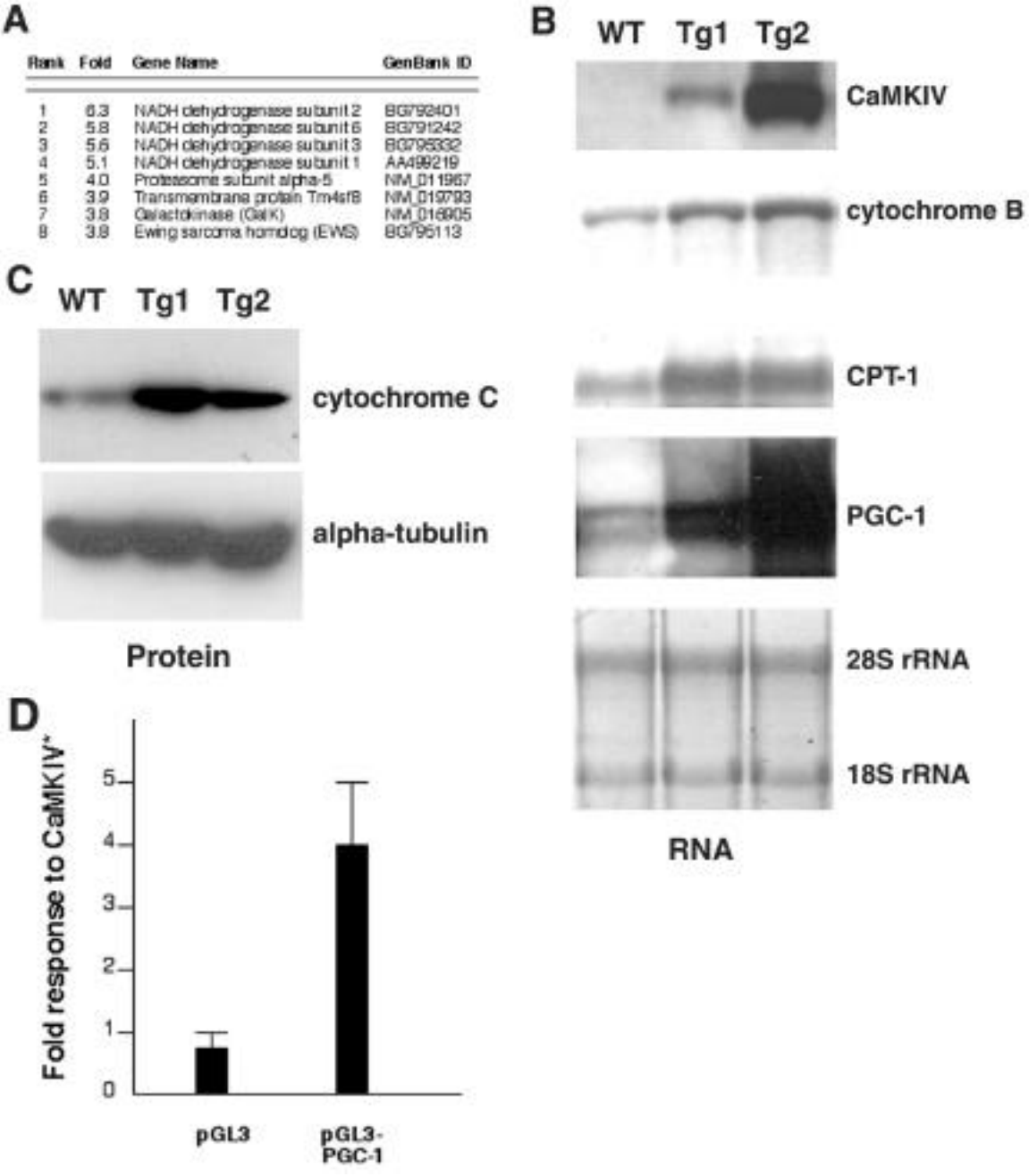

Figure 29. Induction of PGC-1 and enhanced mitochondrial function in skeletal muscles of CaMKIV* transgenic mice. **A.** Gene expression profiling by microarray analysis. Listed here are 8 genes found to be most potently up-regulated in plantaris muscles of CaMKIV* transgenic mice compared to wild type mice. Mitochondria-encoded NADH dehydrogenase subunits showed significant induction by CaMKIV*. **B.** Northern blot analysis using total RNA (20 μg) from plantaris muscles of wild type (WT) and two CaMKIV* transgenic lines (Tg1, Tg2). Cytochrome B-a mitochondrial-encoded gene, and CPT1-a nuclear-encoded mitochondrial gene, showed up-regulation by CaMKIV*. Expression of PGC-1, a master regulator of mitochondrial biogenesis, also increased in the presence of CaMKIV*. An ethidium bromides-stained gel was shown to confirm equal loading of RNA. **C.** Western blot analysis using 50 μg of protein extracts from WV muscles of WT and CaMKIV* mice. Cytochrome C-a nuclear encoded mitochondrial protein, was induced by CaMKIV*. **D.** A 3 kb proximal promoter region of human PGC-1 gene is activated by CaMKIV*. pGL3-PGC-1 construct in which the luciferase is under the control of a 3 kb proximal promoter region of human PGC-1 gene was cotransfected with CaMKIV* into C2C12 myogenic cells. Fold response was determined relative to the basal activity of luciferase reporter in the absence of CaMKIV*. Histograms represent mean $\pm$ SEM values of 3 independent transfections. Transfection efficiency was normalized to expression of cotransfected pCMV-lacZ.

mitochondrial biogenesis. PGC-1 mRNA level is dramatically elevated in both BAT and skeletal muscles upon exposure of the animals to low temperature (Wu *et al*., 1999). The induction of PGC-1 by cold exposure is mediated through β-adrenergic receptors in BAT, but the mechanisms controlling PGC-1 levels in skeletal muscles are not known. In skeletal muscles of CaMKIV* transgenic mice, a significant increase of PGC-1 expression was observed (Figure 29A). A 3 kb proximal promoter region from human PGC-1 gene was activated about 4 folds by CaMKIV* in C2C12 myocytes, suggesting that CaMK signaling induced PGC-1 expression at transcriptional level (Figure 29D). The transcription factors responsible for PGC-1 upregulation are not known yet. CaMKIV has been shown to activate several transcription factors, such as CREB (cAMP responsive element-binding protein) and MEF2 (Bito *et al*., 1996; McKinsey *et al*., 2000b; Passier *et al*., 2000). The involvement of these transcription factors in PGC-1 regulation needs further investigation.

The expression level of mitochondrial genes is proportionate to mitochondrial DNA copy number (Williams, 1986). The relative level of mitochondrial DNA content in skeletal muscles from wild type and CaMKIV* transgenic mice was examined by southern blot analysis. Total genomic DNA (both nuclear and mitochondrial) from skeletal muscles were digested with NcoI to linearize mitochondrial genome. Nuclear and mitochondrial genomic DNA were then quantified with probes hybridized with genes localized on nuclear chromosome and mitochondrial genome. The mitochondrial DNA copy number per nuclear genome increased at least several folds in skeletal muscles from CaMKIV* transgenic mice (Figure 30 A). To investigate whether mitochondrial biogenesis *per se* occurred, transmission

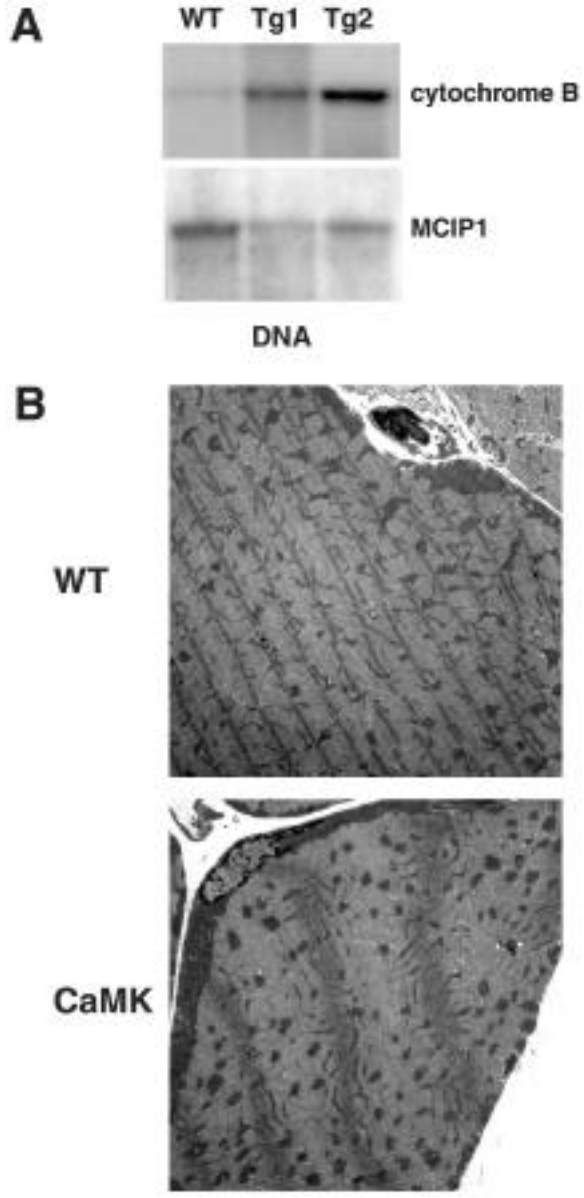
A
WT
Tg1
Tg2
cytochrome B
MCIP1
DNA
B
WT
CaMK

Figure 30. CaMKIV* Stimulates Mitochondrial DNA replication and mitochondrial biogenesis. **A.** Southern blot analysis of mitochondrial and genomic DNA. Total cellular DNA was isolated from gastrocnemius muscles of wild type (WT) and two CaMKIV* transgenic lines (Tg1, Tg2). 20 μg of DNA was digested with NcoI and subjected to Southern blot analysis using cDNA of cytochrome B as a probe for mitochondrial DNA. The membrane was subsequently stripped and reprobed with a cDNA of MCIP1, a nuclear encoded gene. **B.** Transmission electron microscopy of plantaris muscle sections from WT and CaMK transgenic mice. The magnification is 5000X. Mitochondria were seen as black dots in these sections.

electron microscopy was performed on sections prepared from plantaris muscles of wild type and CaMKIV* transgenic mice (Figure 30B). Compared to wild type control, plantaris myofibers from CaMKIV* transgenic mice possessed more total mitochondria and also the subsarcolemmal mitochondria showed a pronounced spreading underneath the muscle fiber surface. These subsarcolemmal mitochondria in close contact with capillary supplies provide the energy for the active transport of metabolites through the sarcolemma in muscle fibers, and they are the limiting factor for endurance performance of the muscles (Muller, 1976).

CaMKIV* transforms fast fibers into slow fibers

Mitochondrial volume density in type I skeletal myofibers is higher than that in type II fibers (Shah and Sahgal, 1991). To evaluate whether mitochondrial biogenesis observed in these CaMKIV* transgenic mice was accompanied by fiber type transformation, sections from plantaris muscles were stained for myosin ATPase activity to distinguish various fiber types (type I, IIa, IIb). In wild type mice (F3 hybrid C57BL/6 X SJL), the average percentage of type I fibers in plantaris muscle is about 2%, while in CaMKIV* transgenic mice, type I fibers accounted for about 10% of the total fibers in plantaris (Figure 31). The introduction of CaMKIV* into skeletal muscles in these transgenic mice mimicked the adaptive responses elicited by chronic muscle electro-stimulation, including both quantitative and qualitative changes in the expression of genes encoding proteins involved directly in muscle contraction (such as myosin) and in the generation of energy from mitochondria (Gauthier *et al.*, 1992; Howald, 1982).

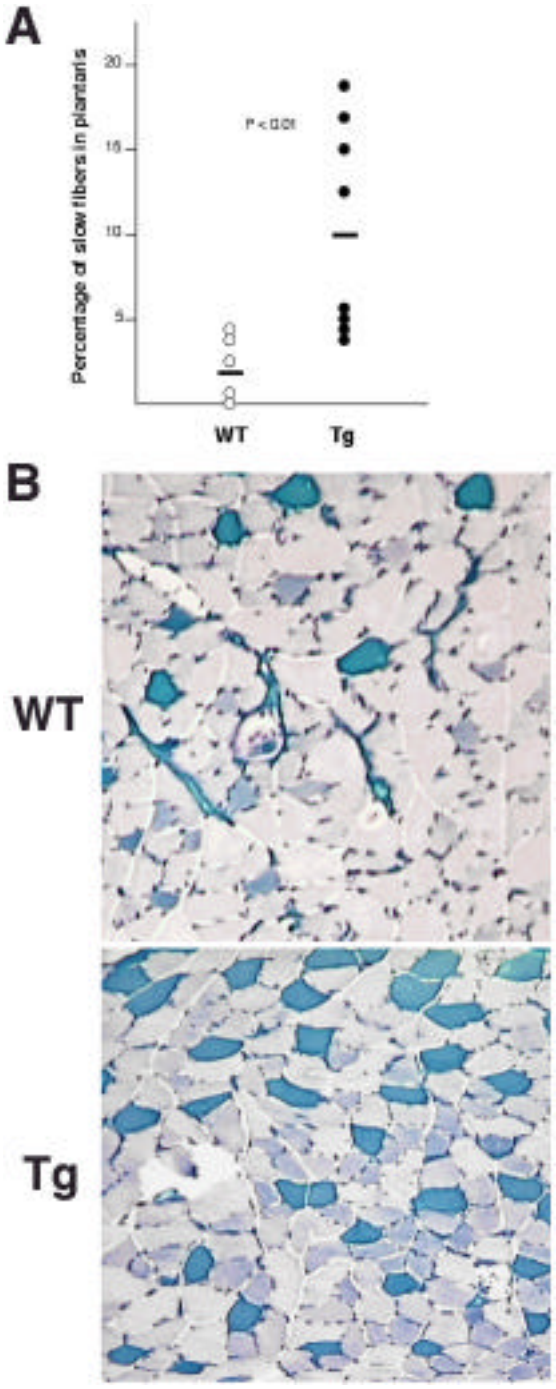
A
Percentage of slow fibers in plantaris
20
15
10
5
P < 0.01
WT
Tg
B
WT
Tg

Figure 31. Fast-to-slow myofiber transformation in CaMKIV* transgenic mice. **A.** Fiber typing of 5 wild type (WT) and 8 transgenic (Tg) mice. The number of slow fibers within plantaris muscles was scored. Circles represent individual animals. The average percentage of slow fibers in PLA muscles is significantly different between WT and Tg mice ($p < 0.01$ by student's test). **B.** Representative fiber typing of plantaris muscles from WT and Tg mice. Myosin-ATPase activity was determined by pH-dependent histochemistry able to distinguish the type I (darkly blue stained), type IIa (unstained) and type IIb (lightly blue stained) myofibers in plantaris muscle sections.

CaMK signaling apparently is sufficient to effect the adaptive changes observed in skeletal muscles experiencing increased contractile activity.

3. Discussion

The oxidative capacity of different myofibers in mammalian skeletal muscles can vary markedly over a range of 10 folds, with type I (slow) fibers the most oxidative and type IIb (fast) fibers the least oxidative (Williams *et al*., 1986). The maximum catalytic capacity of mitochondrial enzymes involved in tricarboxylic acid (TCA) cycle or in the electron transport chain is a determining factor for muscle oxidative capacity (Wang *et al*., 1999). Long term endurance training and chronic low-frequency stimulation of mammalian skeletal muscles trigger mitochondrial biogenesis and therefore, enhance muscle oxidative capacity (Jolesz and Sreter, 1981).

Chronic electrical stimulation of skeletal muscles results in sustained elevation of intracellular free calcium concentration to a range of 100-300 nM, an amplitude sufficient to activate a subset of calcium regulated enzymes, such as calcineurin and CaMK (Alevizopoulos *et al*., 1997; Chin *et al*., 1998). The ability of MEF2 to integrate signals from multiple calcium-regulated signaling proteins, in this case calcineurin and CaMK, is an important feature of our current findings. We showed here that in addition to calcineurin, CaMK signaling pathways also promote transformation of fast myofibers into slow myofibers, accompanied by increased mitochondrial biogenesis.

Mitochondrial defects occur in a variety of degenerative diseases, including several forms of mitochondrial encephlomyopathy (Wallace, 1999). These patients suffer skeletal and cardiac myopathy as a result of mitochondrial DNA mutations maternally inherited or those happen sporadically in muscle cells. Patients with mitochondrial DNA depletion have also been documented (Moraes *et al*., 1991; Spelbrink *et al*., 1998). In these cases, the loss of

mitochondrial DNA in liver and muscle cells is caused by a recessive mutation of a nuclear gene. It remains to be seen whether CaMK/PGC-1 pathway is altered in these patients. Pharmacological modulation of this signaling pathway might prevent or ameliorate the symptoms associated with adult-onset mitochondrial myopathy and potentially improve the health of congestive heart failure patients who exhibit loss of type I skeletal myofibers and intolerance for exercise (Massie *et al*., 1988; Sabbah *et al*., 1993).

Chapter VII. Concluding remarks

The finding by Buller and his colleagues more than four decades ago that motor neurons exerted dominant influence over fiber type-specific gene expression triggered researche aiming at deciphering the molecular mechanisms linking motor neuron activity patterns to skeletal muscle fiber type identities. We reported previously that activated calcineurin selectively up-regulates slow or oxidative fiber-selective gene promoters, and that pharmacologic inhibition of endogenous calcineurin in intact animals promotes slow to fast fiber transformation (Chin *et al*., 1998). The hypothesis that calcineurin activity directs myofibers towards the slow fiber phenotype has been supported subsequently by other investigators (Dunn *et al*., 1999; Naya *et al*., 2000).

My dissertation research focused on the transcription factors mediating the effect of calcineurin on fiber type-specific gene expression. I also explored the function of other calcium, calmodulin-dependent enzymes, such as CaMK, in the control of fiber type plasticity.

First, by analyzing two fiber type-specific enhancers in cultured myogenic cells as well as in transgenic mice, I was able to demonstrate that there are functional NFAT (nuclear factor of activated T cells) and MEF2 (myocyte-specific enhancer factor 2) binding sites within SURE, a slow fiber-specific enhancer, and both sites are required for slow fiber specific activity of this enhancer. Previously unrecognized features of MEF2-dependent gene regulation in skeletal muscle were identified. Direct comparison of well-defined enhancer elements from genes encoding slow and fast muscle isoforms of troponin I demonstrated

higher affinity binding of MEF2 to the slow fiber-specific (SURE) enhancer. More avid binding of MEF2 to the SURE sequence, by comparison with binding to the fast fiber-specific (FIRE) sequence, correlated with a greater responsiveness of SURE to transactivation by MEF2. This result appears to be based on a single nucleotide substitution in the MEF2 binding region of SURE. Weak binding of MEF2 to certain fast fiber-specific enhancers (e.g. FIRE) represents a mechanism that is relevant only to a subset of fast-specific genes.

Activation of MEF2 transcriptional function by calcineurin represents another mechanism by which MEF2 responds to calcium-regulated signals in the control of fiber types. I identified MEF2 as a target of calcineurin in cultured myogenic cells. Calcineurin physically interacts with MEF2 and dephosphorylates MEF2. C-terminal transactivation domain, but not N-terminal DNA binding domain of MEF2, responds to calcineurin activation. The use of “MEF2 indicator mice” (Naya *et al*., 1999), in which the expression of a lacZ transgene is dependent solely on MEF2 transactivator function, enables us to do physiological studies of MEF2 activity in intact animals. By monitoring transgene expression, MEF2 is shown to be selectively active in slow and oxidative myofibers. Through pharmacologic inhibition of calcineurin or genetic crosses of "MEF2 indicator mice" with other transgenic mouse lines expressing activated calcineurin or inhibitor (MCIP1) of calcineurin in their skeletal muscles, it was shown that calcineurin is both necessary and sufficient for MEF2 activation in skeletal muscles. We observed that voluntary wheel running or motor nerve stimulation consistently activates MEF2 in skeletal muscles, with a time course consistent with a causal role of MEF2 in regulation of endogenous genes

that respond to this physiological stimulus. I also found a dose-response relationship between calcineurin activity and expression level of slow, oxidative fiber-specific and MEF2 target genes, such as myoglobin and TnIs.

These new findings, in conjunction with other published work on MEF2 activation by CaMK signaling, are consistent with a three state model for the role of MEF2 in controlling specialized programs of gene expression in skeletal muscles in response to different patterns of contractile work (Figure 32). During prolonged periods of muscle inactivity (State 1), calcineurin and possibly CaMK are inactive, and MEF2 functions primarily as a transcriptional repressor in complex with HDAC proteins. The ability of MEF2 to function in this manner has been established in cultured cells (Lu *et al*., 2000; McKinsey *et al*., 2000a; Miska *et al*., 1999; Zhang *et al*., 2001). Thus, certain MEF2 target genes (e.g. myoglobin) are silenced in fast, glycolytic (Type IIb) fibers of sedentary animals. Other MEF2 target genes, however, could remain transcriptionally active, due to the influence of other transcriptional regulatory events that are sufficient to overcome direct repression by MEF2:HDAC complexes.

State 2 in our model represents the condition found in actively contracting muscles, as evidenced by activation of the desMEF2-lacZ transgene in our current experiments. Contraction is associated with increases in intracellular calcium accessible to the relevant signaling molecules, resulting in activation of calcineurin and CaMK. Activated CaMKI or CaMKIV disrupts the MEF2:HDAC complex, thereby relieving repression, and activated calcineurin stimulates the transactivator function of MEF2. The net effect is up-

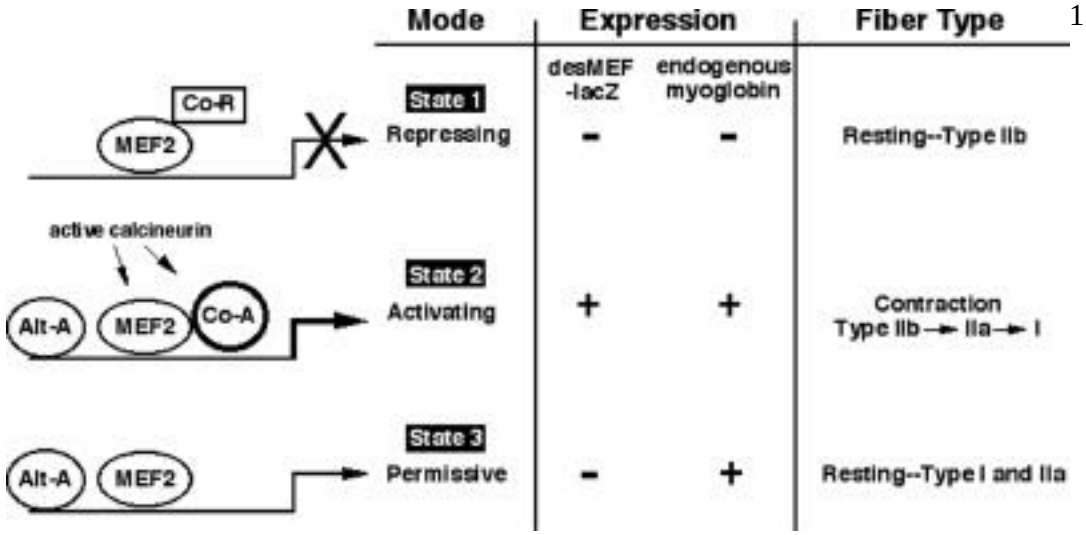

Figure 32. Schematic model of the role of MEF2 in contraction-dependent gene regulation in skeletal muscles. State 1 depicts the condition found in resting Type IIb (fast, glycolytic) myofibers in which neither desMEF2-lacZ nor myoglobin is expressed. State 2 is present during or immediately following sustained periods of contractile activity, as evoked by voluntary wheel running or motor nerve stimulation in our experiments. Both the MEF2-dependent reporter gene and endogenous genes are induced. State 3 describes resting oxidative myofibers (Type I and IIa) that express endogenous myoglobin but do not show evidence for activated MEF2 function as assessed by the desMEF2-lacZ transgene. MEF2 serves repressive, activating or permissive roles in each state, respectively. The maintenance of State 3 requires periodic reinforcement by the mechanisms at work in State 2, or eventually the myofiber reverts to State 1. In addition to MEF2, symbols depict co-repressor (Co-R), co-activator (Co-A), and alternative activator (Alt-A) proteins. Co-A proteins are recruited to DNA by physical interactions with MEF2 while Alt-A proteins bind DNA at sites discrete from MEF2 binding elements. Specific features and implications of this model as discussed in the text.

regulation of previously repressed MEF2 target genes such as myoglobin and troponin I slow. Our present data show that calcineurin is necessary for this state transition, but the model predicts that CaMK, and likely other signaling inputs, are important as well. Individual MEF2 target genes are expected to have different thresholds for the intensity and duration of contractile activity required to drive their promoters from State 1 to State 2, based on differences in binding affinity for MEF2 of nucleotide motifs within promoter/enhancer regions, and on differences in signaling inputs transduced through other transcription factors. State 2 can be established in any myofiber, irrespective of the antecedent state of specialization (fiber type), although stimulus-response thresholds may differ among different fiber types.

State 3 represents the condition defined in our present experiments by Type I and IIa myofibers of sedentary animals that do not express the desMEF2-lacZ gene to measurable levels. MEF2 target genes such as myoglobin are expressed, even though the transactivator function of MEF2 cannot be detected by the indicator transgene. This apparent paradox can be explained in several ways. First, the condition we define as State 3 could occur because the half-life of mRNA and protein products of endogenous genes regulated by MEF2 (e.g. myoglobin) is long relative to the time period in which MEF2 is maintained in its transactivating mode by contractile activity. Thus, the effects of a period of contractile work on expression of target genes could extend for hours or days beyond the time in which MEF2 is functioning as a transcriptional activator. A second explanation is that contractile activity promotes the elaboration and/or activation of other transcription factors (termed "alternative activators" in Figure 32), the half-life of which extends beyond the period in which MEF2 is

active. The effect is to prolong expression of endogenous MEF2 target genes (e.g. myoglobin) beyond the period in which MEF2 itself is active. It is important to note that the desMEF2-lacZ transgene is controlled solely by MEF2 and its associated co-activators and co-repressors, while endogenous genes are subject to control by other classes of transcription factors that employ different nucleotide sequence recognition motifs within promoter/enhancer regions of the relevant genes. Indeed, there is good evidence to support a role of other classes of transcription factors in the maintenance of specialized myofiber phenotypes (Hughes *et al*., 1999; Murgia *et al*., 2000; O'Mahoney *et al*., 1998; Spitz *et al*., 1999; Spitz *et al*., 1998; Yan *et al*., 2001).

According to this conceptual model, State 2 requires the stimulus of contractile activity, the intensity and duration of which (and hence the resulting calcium signals) must be sufficient to convert MEF2 from repressor to transactivator mode. State 3 occurs when such periods of contractile activity are repeated at intervals longer than the half-life of transcriptional complexes formed in State 2, which may include alternative activators distinct from MEF2, of the products of relevant MEF2 target genes. In the absence of sufficiently sustained periods of contractile activity, myofibers revert to State 1. By introducing kinetic considerations, the model provides a plausible explanation for how calcineurin and MEF2 can play central regulatory roles in controlling expression of genes that establish specialized myofiber subtype, even though MEF2 activation by contractions can be detected (transiently) in any myofiber subtype. The model also accounts for the fact that specialized myofiber subtypes we propose to be dependent on a calcineurin/MEF2/NFAT signaling pathway are maintained at times when the transactivator function of MEF2 is undetectable.

I also demonstrated that in addition to calcineurin, other calcium, calmodulin-regulated enzymes, such as CaMK (calcium, calmodulin-dependent kinase), also transduce their signaling through MEF2. CaMKIV synergistically activates MEF2-dependent gene expression together with calcineurin. Transgenic mice expressing constitutively active CaMKIV in their skeletal muscles showed increased percentage of slow myofibers, which was accompanied by increased mitochondrial biogenesis mediated through the upregulation of PGC-1 (PPARγ co-activator).

Taken together, these findings delineate a molecular pathway in which MEF2 integrates signaling inputs from multiple calcium-regulated pathways in the control of skeletal muscle fiber types, as illustrated in figure 33. MEF2, in addition to NFAT, is capable of transducing signals initiated by calcineurin in skeletal myocytes. In addition, MEF2 serves to integrate multiple signaling inputs evoked by motor nerve stimulation that amplify calcineurin-generated responses. The studies in cultured myocytes document the existence of the signaling mechanisms proposed in this model, and data obtained from transgenic mice support its relevance to fiber type determination in intact animals. The experiments reported in this dissertation have focused on skeletal muscle, but it is likely that MEF2 proteins function in cardiac muscle to transduce signals generated by calcineurin-dependent signaling cascades that modulate gene expression and hypertrophic growth (Molkentin *et al*., 1998).

Additional relationships between these pathways and other proteins that govern muscle phenotype undoubtedly await to be revealed. Our model should not be interpreted to suggest that other transcription factors and other signaling pathways are unimportant with

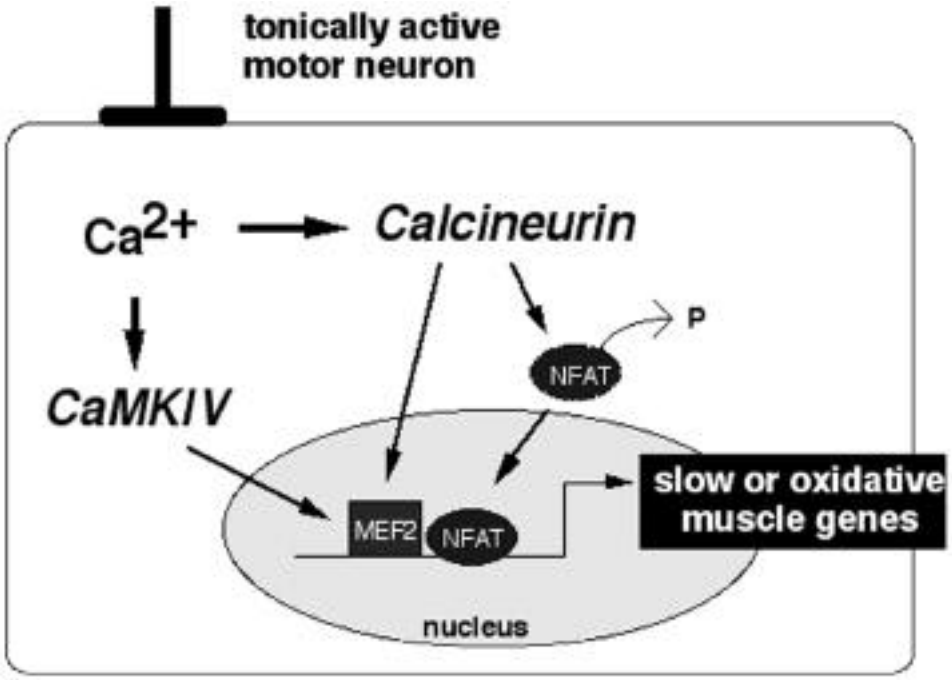

Figure 33. A mechanistic model for control of slow or oxidative fiber-specific gene expression by motor nerve activity. The importance of MEF2 is emphasized as a downstream effector of calcium-dependent changes in gene expression provoked by a tonically active pattern of motor neuron firing. Signals generated by calcineurin augment the transactivating function of MEF2 in a manner that also is increased by concomitant activation of CaMKIV, and by synergistic interactions with NFAT.

respect to fiber type-specific gene expression. To the contrary, a number of interesting observations suggest a role for transcriptional regulatory proteins of the bHLH (Hughes *et al*., 1999), GATA (Musaro *et al*., 1999), SIX (Spitz *et al*., 1998) and nuclear receptor protein families (Spitz *et al*., 1999). In addition, this current model is not yet sufficiently complete to explain the manner in which calcineurin may, under certain conditions, contribute to hypertrophic growth rather than fiber type transformation of skeletal myofibers, as described in recent reports in which muscle growth was stimulated by IGF-1 (Musaro *et al*., 1999; Semsarian *et al*., 1999). MEF2 also interacts with other transcription factors/coactivators/corepressors in the regulation of target genes (Black *et al*., 1998; Morin *et al*., 2000; Quinn *et al*., 2001; Sparrow *et al*., 1999; Youn *et al*., 2000). The involvement of these factors in calcium signaling pathways is clear. Irrespective of these limitations, the model presented here provides a fresh perspective on fiber type-specific gene regulation, and supports a conceptual framework on which a more complete understanding can be constructed as additional experimental results become available.

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