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Molecular Mechanisms Affecting Utrophin Expression in Fast Versus Slow Skeletal Muscles; Possible Therapeutic Targets for Duchenne Muscular Dystrophy

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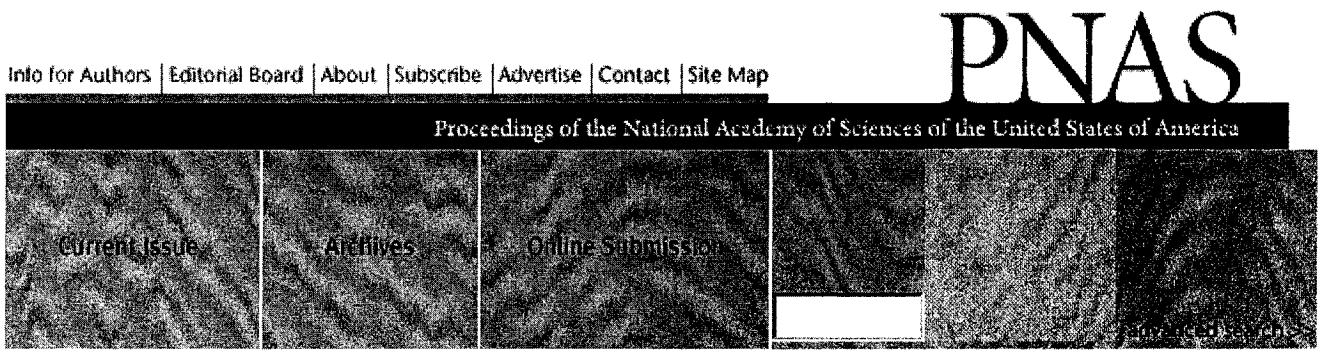

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Abstract

Duchenne muscular dystrophy is a fatal X-linked myopathy characterized by the absence of the cytoskeletal protein dystrophin. The absence of dystrophin leads to impaired sarcolemmal integrity, and inefficient muscle regeneration. Currently, there is no effective therapy to significantly improve the quality of life for DMD patients. One therapeutic approach is to express, at therapeutically relevant levels, a homologous protein that can compensate for dystrophin. Utrophin is one such protein, primarily expressed at the neuromuscular junction, which can compensate for dystrophin function. Therefore, it becomes imperative to identify pathways that regulate the expression of utrophin. Such efforts could identify targets that may be manipulated in attempts to promote utrophin expression along the entire length of dystrophin-deficient myofibers.

In this thesis, we investigate the mechanisms that promote higher levels of utrophin expression in extra-synaptic regions of slow, versus fast muscles. We determined through the use of isoform specific antibodies and primers that the A isoform of utrophin is highly expressed in slow, relative to fast muscles. Direct plasmid injections, and in vitro transfection assays with A-utrophin promoter-reporter constructs determined that peroxisome proliferators activated receptor γ co-factor 1 α (PGC-1α) and calcineurin can affect the transcription of A-utrophin via the transcription factors GABPα and NFATc1, resepectively. Similar techniques using utrophin 3'UTR reporter constructs and in vitro stability assays, also determined that calcineurin activity could affect the stability of A-utrophin transcripts via an AU-rich element (ARE) in the utrophin 3'UTR.

We also interbred transgenic mice with stimulated calcineurin signaling or impaired calmodulin signaling in skeletal muscles, with dystrophin-deficent mdx mice. We found that stimulation of calcineurin activity improved dystrophic pathology, and increased A-utrophin expression. In contrast, inhibition of calmodulin signaling exacerbated dystrophic pathology, impaired pathways that promote the slow myofiber program, decreased GABPα and PGC-1α expression and reduced A-utrophin levels. Collectively, these observations demonstrate that Ca^{+2}/calmodulin regulated effectors such as calcineurin and PGC-1α can regulate the expression of utrophin, and can serve as potential therapeutic targets for DMD.

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Abbreviations

AChR,	acetylcholine receptor
AChE,	acetylcholinesterase
ARE	AU-rich element
β-gal,	β-galactosidase
Ca^{+2}	calcium
CaMK	Ca+2/CaM-dependent kinase
CsA	cyclosporine A
CAT	chloramphenicol transferase
CaM	calmodulin
CaMBP	calmodulin-binding protein
CnA*	constitutively active form of calcineurin
DAB	diaminobenzidene
DAP	dystrophin-associated proteins
DMD	Duchenne muscular dystrophy
EDL	extensor digitorum longus
MEF2	myocyte enhancer factor 2
MyHC	myosin heavy chain
GABP	GA-binding protein
HDACs	histone deacytylases
IGF-1	insulin-like growth factor 1
NFAT	nuclear factor of activated T cells
NMJ	neuromuscular junction

PPAR	peroxisome proliferator activated receptor
PGC-1α	peroxisome proliferator-activated receptor-γ co-factor-1α
PBS	phosphate-buffered saline
SOL	soleus
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
TA	tibialis anterior
TnIs	troponin I slow
UTR	untranslated region
wt	wild type

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Chapter 1

GENERAL INTRODUCTION

I. Duchenne muscular dystrophy and dystrophin

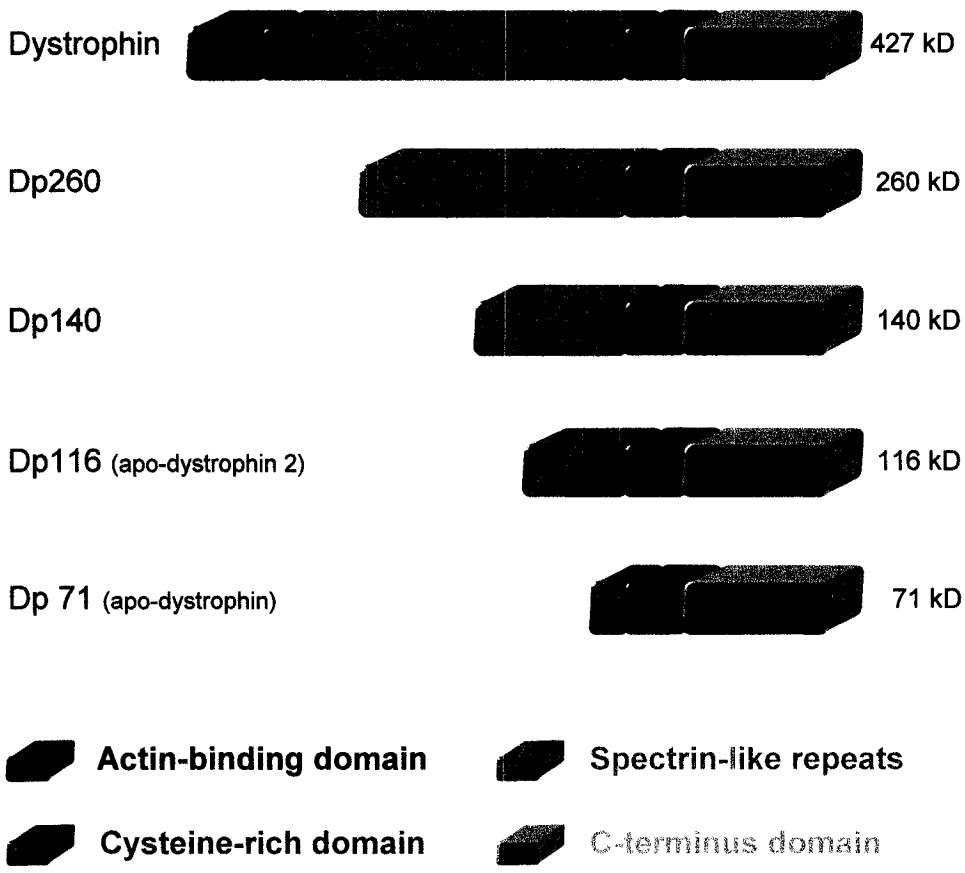
Duchenne muscular dystrophy (DMD) is a severe degenerative disorder of skeletal and cardiac muscles that affects ~1 in 3500 male births (Emery, 1991). Although DMD is present at birth, clinical symptoms are not evident until 3–5 years of age (Jennekins et al., 1991). Initial symptoms include leg weakness, increasing convex curvature of the spine, and a waddle-like gait (Jennekins, 1991). Continuous muscle wasting results in progressively weaker muscles usually leaving DMD patients wheelchair bound by the age of 11 or 12 (Emery, 1993). Eventually individuals afflicted with DMD usually succumb to the disease by the second or third decade of life (Emery, 1993).

Twenty years ago the genetic defect underlying DMD was mapped to chromosome Xp21 in humans (for a review, Monaco et al., 1988 and Worton et al., 1988). This gene is one of the largest identified to date, which could account for its relatively high frequency of mutation (Coffey et al., 1992). The DMD gene product is known as dystrophin (Hoffman et al., 1987; Koenig et al., 1988; Worton et al., 1988). The DMD gene can accommodate production of several dystrophin isoforms through alternative promoter usage, and splicing of pre-mRNA. The predominant dystrophin isoform found in skeletal and cardiac muscles is a ~427 kDa cytoskeletal protein predicted to contain 3685 amino acids. Full-length dystrophin is composed of four distinct structural domains: 1) an N-terminal "actin binding" domain; 2) a middle "rod"

domain consisting of spectrin-like repeats (Davison et al., 1988); 3) a cysteine-rich domain; and 4) a carboxyl-terminal domain (Koenig et al., 1988) (Figure 1.1). Two additional isoforms are considered to be full-length and are expressed in the brain (Boyce et al., 1991; Gorecki et al., 1992). Four other variants contain unique first exons, giving rise to dystrophin proteins of 260 kDa (D'Souza et al., 1995), 140 kDa (Lidov et al., 1995), 116 kDa (Byers et al., 1993), and 71 kDa (Feener et al., 1989; Muntoni et al., 2003) (Figure 1.1). These shorter isoforms lack the actin binding domain, which suggests they may have other functions different from those ascribed to full-length dystrophin.

In skeletal muscle fibers, full-length dystrophin accumulates predominantly at the cytoplasmic face of the sarcolemma (Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988). The distribution of dystrophin along muscle fibers is rather homogeneous, thereby creating a sub membranous mesh of dystrophin molecules. Mutations and/or deletions in the dystrophin gene, as seen in DMD patients, prevent the production of dystrophin, and lead to its complete absence in muscle fibers (Hoffman et al., 1987). A similar, yet milder dystrophy known as Becker muscular dystrophy (BMD) also stems from defects in the dystrophin gene (Monaco et al., 1988). In BMD, however, the nature of the mutations/deletions are such that they allow for synthesis of a truncated yet partly functional dystrophin molecule, likely explaining the milder phenotype observed for BMD patients (Monaco et al., 1988).

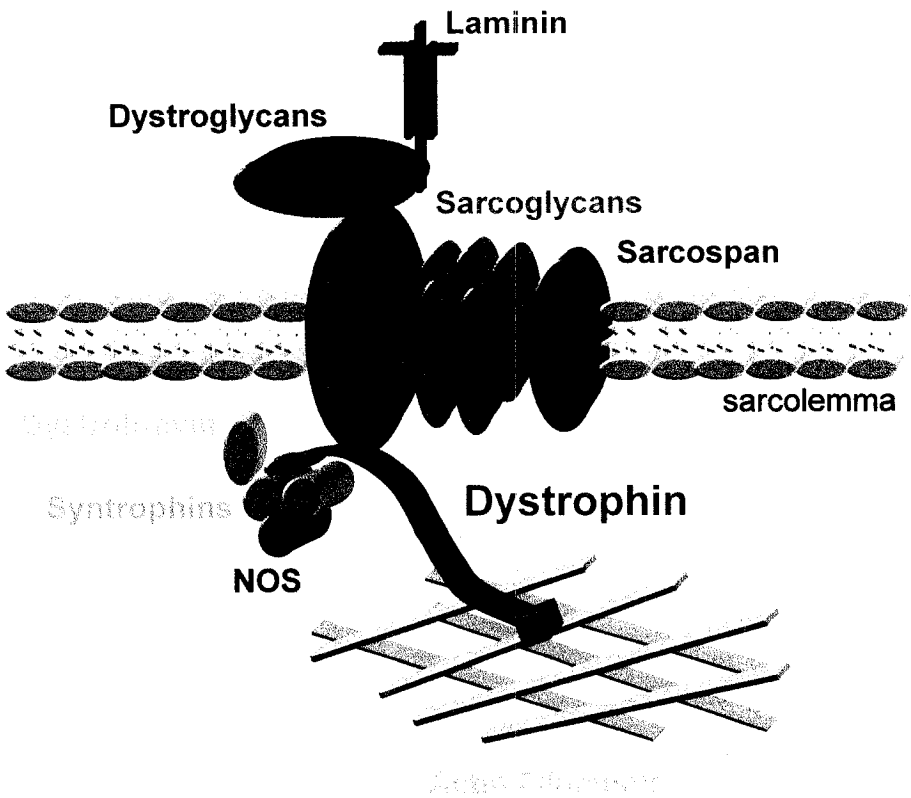
Figure 1.1. Representative diagram of dystrophin isoforms. Shown are the actin-binding domains (blue), spectrin-like repeats (red), cysteine-rich (green) and carboxy-terminal domins (purple). Isoform names are on the left, and molecular masses on the right.


Dystrophin
427 kD
Dp260
260 kD
Dp140
140 kD
Dp116 (apo-dystrophin 2)
116 kD
Dp 71 (apo-dystrophin)
71 kD
Actin-binding domain
Spectrin-like repeats
Cysteine-rich domain
C-terminus domain

I.A. Dystrophin and the Dystrophin Associated Protein Complex

At the sarcolemma, dystrophin is part of a macromolecular group of proteins collectively referred to as the dystrophin-associated protein complex (DAPC) (Ervasti et al., 1991) (Figure 1.2). Several key components of this complex have been studied in recent years, and their interaction patterns and organization support the notion that dystrophin serves to link the intracellular microfilament network of actin to the extracellular matrix (Ervasti et al., 1991; Ervasti et al., 1993). The absence of dystrophin at the sarcolemma results in a dramatic change in the level and localization of the DAPC. For example, studies have reported marked reductions in the overall levels of DAPs in dystrophin-deficient skeletal muscle from DMD patients, and animal models such as the mdx mouse (Bulfield et al., 1984; Ibraghimov-Beskrovnaya et al., 1992; Matsamura et al., 1994). These data indicate that the absence of dystrophin results in the loss of DAPs at the sarcolemma. The absence of this physical link between the interior and exterior of the muscle cell renders the sarcolemma fragile, making muscle fibers susceptible to degeneration during repeated cycles of muscle contraction and relaxation. Support for this model stems primarily from observations showing an increased sensitivity of mdx muscle fibers to mechanical stress (Moens et al., 1993; Petrof et al., 1993). Mutations in the genes that encode some DAPs also cause various forms of muscular dystrophies (for a review, see refs Durbeej et al., 2002; Dalklic et al., 2003). Therefore, dystrophin and its associated proteins assume specific functions at the sarcolemma that are crucial to the integrity of muscle fibers.

Figure 1.2. Schematic diagram of the dystrophin-associated protein complex. Shown is the molecular organization of the dystrophin-glycoprotein complex. Note the role of dystrophin in linking the extracellular matrix to the intracellular actin cytoskeleton, via the DAPC. Symbols; NOS, nitric oxide synthase.


Laminin
Dystroglycans
Sarcoglycans
Sarcospan
sarcolemma
Syntrophins
Dystrophin
NOS

Nonstructural roles have been described for dystrophin, making it a multifunctional protein. The localization of dystrophin, and its associated components, at the sarcolemma places them at an appropriate position to serve as a signaling scaffold that is responsive to extra cellular stressors. For example, binding of the extra cellular matrix ligand laminin to α-dystroglycan results in the recruitment of signaling molecules involved in actin remodeling, such as Rac1 (Oak et al., 2003). Several other signaling molecules interact with components of the dystrophin complex, including nitric oxide synthase (NOS), Grb2, and diacyl-glycerol kinase (Brenman et al., 1995; Young et al., 1995; Abramovici et al., 2003). These observations argue in favor of the notion that the dystrophin associated protein complex is important in stimulating appropriate downstream cascades in response to various extracellular stimuli. Naturally, these signaling events would not function correctly in a dystrophin-deficient muscle, which likely contributes to the disease pathology.

Dystrophin has been proposed to play a role in calcium homeostasis. In mdx mice and DMD patients, intracellular calcium levels are elevated relative to normal controls (Fong et al., 1991; Turner et al., 1991). In cultured DMD and mdx myotubes, increased leak channel activity, affecting the calcium permeability of the sarcolemma, has been reported (Fong et al., 1990; Vandebrouk et al., 2002; Iwata et al., 2003). Young mdx diaphragm muscles isolated before the onset of significant pathology show enhanced calcium influx through calcium/stretch-activated channels (Kumar et al., 2004). The increased influx of calcium leads to the aberrant hyperactivation of signaling cascades involved in the inflammatory response (Kumar et al., 2004). In accordance with a

heightened immune response, elevated expression of inflammatory mediators and chemoattractants has been observed to occur in dystrophin-deficient muscles, prior to the onset of major disease symptoms (Spencer et al., 2001; Porter et al., 2002; Porter et al., 2003). This tendency of dystrophic muscle to trigger inflammation due to aberrant calcium homeostasis may be detrimental to muscle cell survival.

I.B. Up-regulation of a Dystrophin-related Protein

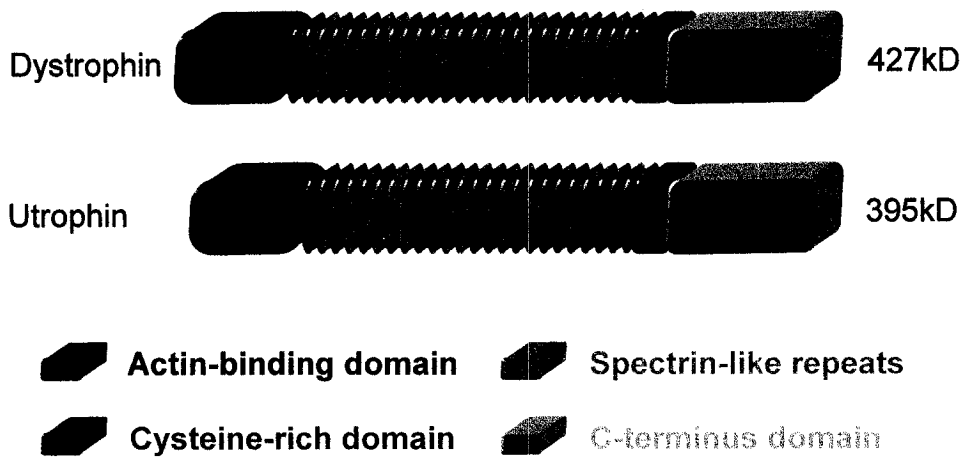
A variety of treatments have been proposed to stem the relentless progression of DMD; those which have been examined suffer from a variety of constraints. For instance, gene based strategies designed to introduce functional dystrophin can trigger immune responses in treated animals to the transgene encoding for dystrophin, or the carrying vector (Muruvue et al., 1999; Nalbantoglu et al., 1999; Gilbert et al., 2003). Pharmacological and cell-based treatments have been shown to suffer from potential adverse side effects, and a lack of a clear understanding of the mechanisms whereby some of these interventions result in functional benefits (Gussoni et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Asakura et al., 2002; Cossu et al., 1997; Bogdanovich et al., 2002). An alternative approach that could evade the negative impact of immunological reactions, and provide a defined target involves up-regulating a protein of therapeutic benefit that is expressed endogenously in dystrophic muscle. The premise of this approach is that the up-regulated protein could compensate for the lack of dystrophin by assuming some, or even all of its functional roles. One such protein is the nonmuscle dystrophin isoform Dp71. However, forced-expression of this dystrophin isoform in mdx

mouse muscle failed to alleviate the dystrophic phenotype (Cox et al., 1994; Greenberg et al., 1994). These results indicate that C-terminal isoforms cannot replace dystrophin or mitigate the dystrophic pathology.

II. The autosomal homologue of dystrophin; utrophin

Utrophin is thus far the only candidate dystrophin-related protein containing all important functional domains found in dystrophin. Utrophin was initially discovered as a transcript of ~ 13 kb in size identified using probes spanning the distinct C-terminal region of dystrophin (Love et al., 1989). At the genomic level the utrophin gene locus is ~ 1Mb in size, and found on chromosome 6 in humans, and 10 in mice (Love et al., 1989; Buckle et al, 1990). DNA and amino acid sequence comparisons revealed that, like dystrophin, utrophin is also a large actin binding protein of 3433 amino acids composed of 4 domains: an N-terminal actin binding domain; rod domain; a cysteine rich domain; and C-terminal domain (Tinsley et al., 1992) (Figure 1.3). The C-terminal, cysteine rich, and N-terminal domains display ~80 % conservation in amino acid composition with dystrophin (Tinsley et al., 1992). Therefore, based on sequence conservation and chromosome localization, utrophin is considered to be the autosomal homologue of dystrophin. The production of specific antibodies to utrophin determined the protein product to have a molecular mass of ~ 395kDa (Khurana et al., 1990). In contrast to full

Figure 1.3. Schematic diagram of full-length utrophin and dystrophin. Shown are the functional domains for dystrophin and utrophin; actin-binding domain (blue); spectrin-like repeats (red); cysteine-rich domain (green); c-terminus (purple). Note that utrophin contains all of the functional domains in dystrophin. The actin-binding, cysteine-rich, and c-terminal domains display ~ 80% conservation in amino acid composition (see text for details). Molecular size of full-length dystrophin and utrophin depicted on the right (adapted from Chakkalakal et al., 2005).

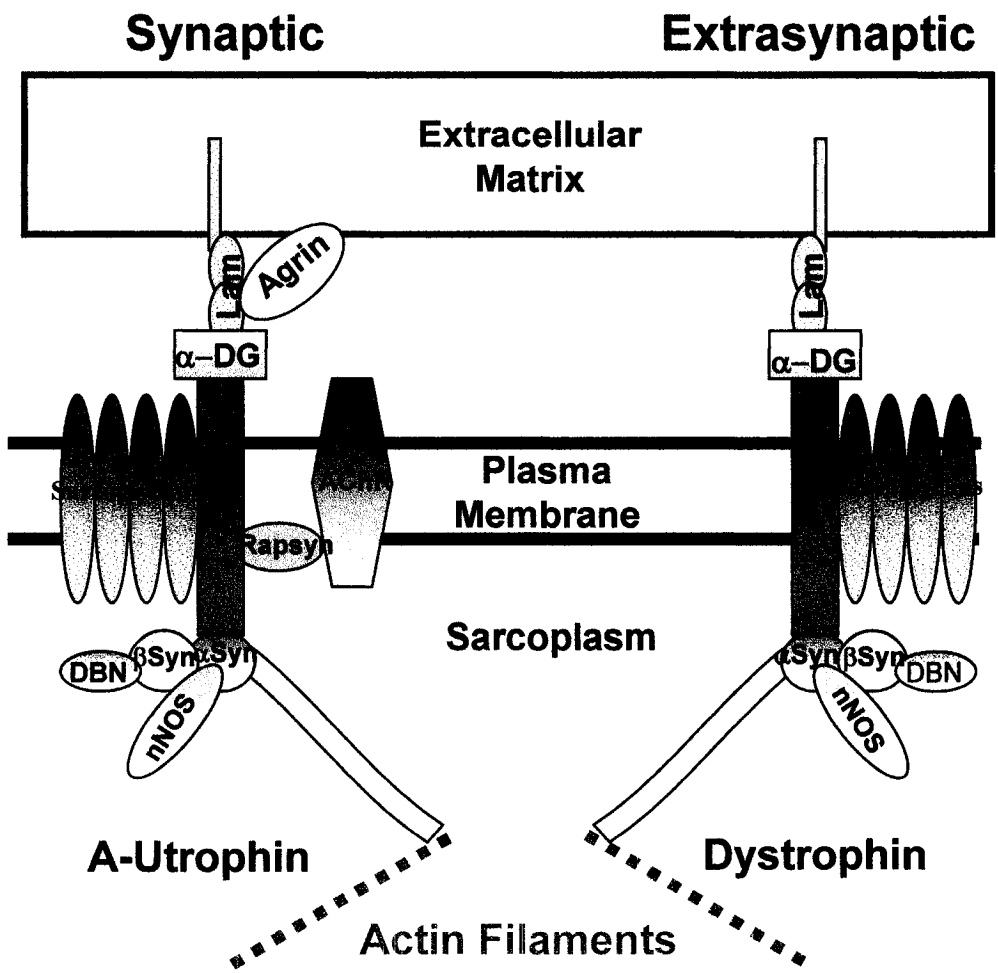

Dystrophin
427kD
Utrophin
395kD
Actin-binding domain
Spectrin-like repeats
Cysteine-rich domain
C-terminus domain

length dystrophin, which is primarily enriched in brain, skeletal and cardiac muscle, utrophin has a more ubiquitous distribution displaying expression in several tissues (Khurana et al., 1990; Ohlendieck et al., 1991; Karpati et al., 1993a; Taylor et al., 1997). Furthermore, in adult skeletal muscle, unlike dystrophin which can be found along the entire length of muscle fibers, utrophin tends to be enriched at the neuromuscular junction, co-localized with acetylcholine receptors (AChRs), and at myotendinous junctions (Khurana et al., 1991; thiMan et al., 1991; Ohlendieck et al., 1991; Takemitsu et al., 1991; Matsumura et al., 1992; Karpati et al., 1993; Taylor et al., 1997; Gramolini et al., 1997; Vater et al., 1998; Stocksley et al., 2005).

II.A. Functions of Utrophin in Skeletal Muscle

In comparing utrophin and dystrophin, a high level of homology is observed in regions known to form interactions with F-actin and DAPs. Therefore, it was hypothesized that, similar to dystrophin, utrophin could also associate with DAPs. Further support for this hypothesis stemmed from the observation of continued localization of DAPs at neuromuscular junctions, concurrent with their loss from extra-synaptic regions in dystrophin-deficient tissues (Matsumura et al., 1992). Through the use of biochemical fractionation it was determined that like dystrophin; utrophin could also associate with DAPs (Matsumura et al., 1992) (Figure 1.4). Based on this observation, it was initially postulated that utrophin together with DAPs may have specific functions in the formation and/or maintenance of the NMJ. To initially test this hypothesis the

Figure 1.4. Schematic diagram of the dystrophin- and utrophin-associated protein complexes. Shown is the molecular organization of both the dystrophin- and utrophin-associated protein complexes within extrasynaptic and synaptic regions, respectively. Note that utrophin, like dystrophin, serves to link the extracellular matrix to the underlying actin cytoskeleton via interactions with the same complex of proteins at synaptic regions. Symbols; DBN, dystrobrevin; Lam, laminin; Syn, syntrophin; nNOS, neuronal nitric oxide synthase; DG, dystroglycan; AChR, acetylcholine receptor.


Synaptic
Extrasynaptic
Extracellular Matrix
Lam
Agrin
α–DG
Lam
α–DG
Plasma Membrane
Rapsyn
Sarcoplasm
DBN
βSyn
αSyn
nNOS
αSyn
βSyn
DBN
nNOS
A-Utrophin
Dystrophin
Actin Filaments

localization of utrophin to spontaneously forming AChR clusters on myotubes was examined (Phillips et al., 1993). This phenomenon mimics some aspects in the early accumulation of AChRs at post-synaptic regions of the developing NMJ (Vogel et al., 1972; Kummer et al., 2005). Examination of these spontaneously forming AChR clusters revealed utrophin to be primarily localized to large clusters rather than small clusters. Due to this observation it was proposed that utrophin could be involved in the maintenance of large AChR rich regions (Phillips et al., 1993).

Support for the involvement of utrophin in the maintenance of large AChR clusters stems from treatment of myotubes with agrin. Agrin is a proteoglycan with important roles in the formation and maintenance of NMJs (Nitkin et al., 1987; Nitkin et al., 1990; Bowe et al., 1995; Sanes and Lichtman, 2001). Agrin, when exogenously applied to cultured myotubes promotes the formation of large, stable AChR clusters (Reist et al., 1992; Gautam, et al., 1996). In this context, utrophin and DAPs become enriched at agrin-induced AChR clusters on myotubes (Campanelli et al., 1994). Consistent with the notion that utrophin participates in the maintenance of large stable AChR clusters; treatment of myotubes with antibodies directed against utrophin significantly impairs agrin-induced AChR cluster formation (Namba and Scheller et al., 1996). Collectively, these studies examining a role for utrophin in agrin-induced AChR cluster formation support a role for utrophin, and DAPs in the maintenance of NMJs.

Studies on transgenic mice further support a role for utrophin in the maintenance of the post-synaptic apparatus. Utrophin-deficient mice have no overt signs of neuromuscular abnormalities. Upon closer inspection, the NMJs of utrophin-deficient mice demonstrate that the density of AChRs in synaptic regions, and the number of junctional folds are significantly reduced in comparison to wild type counter parts (Deconinck et al., 1997a; Grady et al., 1997a). Since utrophin and dystrophin share extensive homology, it was postulated that the subtle effects on NMJ morphology in utrophin-deficient mice are due to compensation by dystrophin. To directly test this hypothesis, utrophin-deficient mice were interbred with mdx animals. Progeny from this interbreeding were obtained that were deficient in both dystrophin and utrophin (mdx/utrophin-/-). Assessment of mdx/utrophin-/- animals revealed an accelerated dystrophic phenotype, relative to mdx, consistent with the notion that utrophin and dystrophin have redundant roles (Deconinck et al., 1997b; Grady et al., 1997b). Although post-synaptic regions were readily apparent, ultra-structural analysis of mdx/utrophin-/- NMJs showed further impairments in junctional folds and the loss of DAPs, in comparison to utophin-/- and wild type NMJs (Deconinck et al., 1997b; Grady et al., 1997b). Collectively these results indicate that NMJs could form, however were not properly maintained in the absence of utrophin and dystrophin. Taken together, these observations reinforce a role for utrophin in the maintenance rather than the formation of NMJs.

II.B. Utrophin: A Plausible Therapeutic Approach for DMD?

Due to sequence conservation and functional redundancy, it was hypothesized that stimulating utrophin expression outside the NMJ fibers could compensate for dystrophin deficiency and alleviate DMD pathology. To initially assess this notion, transgenic mdx mice were generated that over-expressed full length or truncated variants of utrophin along the entire length of skeletal muscle fibers (Tinsley et al., 1996; Deconinck et al., 1997; Tinsley et al., 1998; Rafael et al., 1998). Forced-expression of utrophin in extra-synaptic regions of mdx muscle fibers displayed many improvements in several aspects of the dystrophic pathology. Some of the improvements in dystrophic pathology included restoration of DAPs to the sarcolemma; lower levels of serum creatine kinase; and reduced percentages of centrally nucleated muscle fibers, an indicator of degenerative/regenerative events (Tinsley et al., 1996; Rafael et al., 1998). In addition, forced-expression of utrophin in extra-synaptic regions of mdx skeletal muscles led to improvements in physiological parameters such as the ability to generate contractile force; increased resistance to forced-lengthening contractions; and improved handling of basal cytosolic calcium levels (Deconinck et al., 1997; Tinsley et al., 1998).

Additional mdx transgenic models were generated, in which forced expression of utrophin was either ubiquitous, or could be stimulated at different ages. Ubiquitous forced-expression of utrophin in mdx mice also led to corrections in aspects of dystrophic pathology, with no toxic effects in other tissues (Fisher et al., 2001). To assess the ability of forced utrophin expression to ameliorate the dystrophic pathology at later stages of the disease, mdx mice containing an utrophin transgene whose expression is controlled by a

tetracycline responsive promoter were generated (Squire et al., 2002). In this case, utrophin expression is dependent on the withdrawal of tetracycline from the water of mdx mice containing the tetracycline-sensitive utrophin transgene. Assessment of muscles from these mice revealed that forced expression of utrophin after birth could still correct for various aspects of dystrophic pathology (Squire et al., 2002). Improvements included restoration of DAPs to the sarcolemma; increased force recovery after forced lengthening contractions; and decreased percentages in muscle fibers undergoing degenerative/regenerative events (Squire et al., 2002). Collectively, these studies demonstrate the feasibility of forced utrophin expression as a therapy to treat DMD, since tissue specific induction may not be necessary, and can be initiated after birth.

II.C. Strategies to stimulate utrophin expression

To begin examining treatments designed to induce utrophin expression in extra synaptic regions of dystrophin-deficient muscle fibers, adenoviral vectors containing utrophin mini-genes were generated (Gilbert et al., 1998; Gilbert et al., 1999). Infection of adult mdx mice with adenoviruses containing utrophin transgenes did lead to restoration of DAPs to the sarcolemma, and improvements in muscle pathology, notably decreased collagen content, a marker of muscle necrosis (Gilbert et al., 1998; Gilbert et al., 1999). Consistent with these results, muscles from dystrophin-deficient canines infected with adenoviruses containing utrophin mini-genes also demonstrated restoration of DAPs to the sarcolemma, and improved muscle morphology (Cerletti et al., 2003).

Despite the promising results from these studies, the use of adenoviruses to promote extra-synaptic expression of utrophin in dystrophin-deficient muscle fibers suffers from numerous problems such as relatively inefficient levels of transgene expression, and adverse immune responses (Gilbert et al., 1998; Gilbert et al., 1999; Cerletti et al., 2003).

Various pharmacological agents have been examined to stimulate the endogenous expression of utrophin in dystrophin-deficient muscle. Treatment of dystrophin-deficient human and mouse muscle cells in culture with glucocorticoids can increase endogenous utrophin levels (Passaquin et al., 1993; Pasquini et al., 1995; Courdier-Fruh et al., 2002). Of the many glucocorticoids that have been examined, deflazacort, a derivative of alpha-methyl-prednisolone, has been shown to be very effective as a therapy for DMD. Recently it was demonstrated that deflazacort, in addition to its effects on promoting muscle regeneration, can also increase endogenous levels of utrophin (St-Pierre et al., 2004; Segalat et al., 2005). Substrates for NOS have also been shown to induce endogenous utrophin expression. Treatments of mdx mice with the NOS substrates L-arginine and molsidomine, a pharmacological agent that is converted to a NO donor, can both induce utrophin expression in mdx mice (Chaubourt et al., 1999; Chaubourt et al., 2002; Barton et al., 2005; Voisin et al., 2005). In both cases, stimulation of the NO pathway by L-arginine and molsidomide in mdx mice was also associated with improvements in the dystrophic pathology (Barton et al., 2005; Voisin et al., 2005). Therefore, either glucocorticoids, or pharmacological stimulation of the NO pathway can promote utrophin expression in dystrophin-deficient muscle, and improve the dystrophic pathology.

III. Paradigms and Molecular events Regulating Utrophin Expression

III.A. Utrophin Isoforms

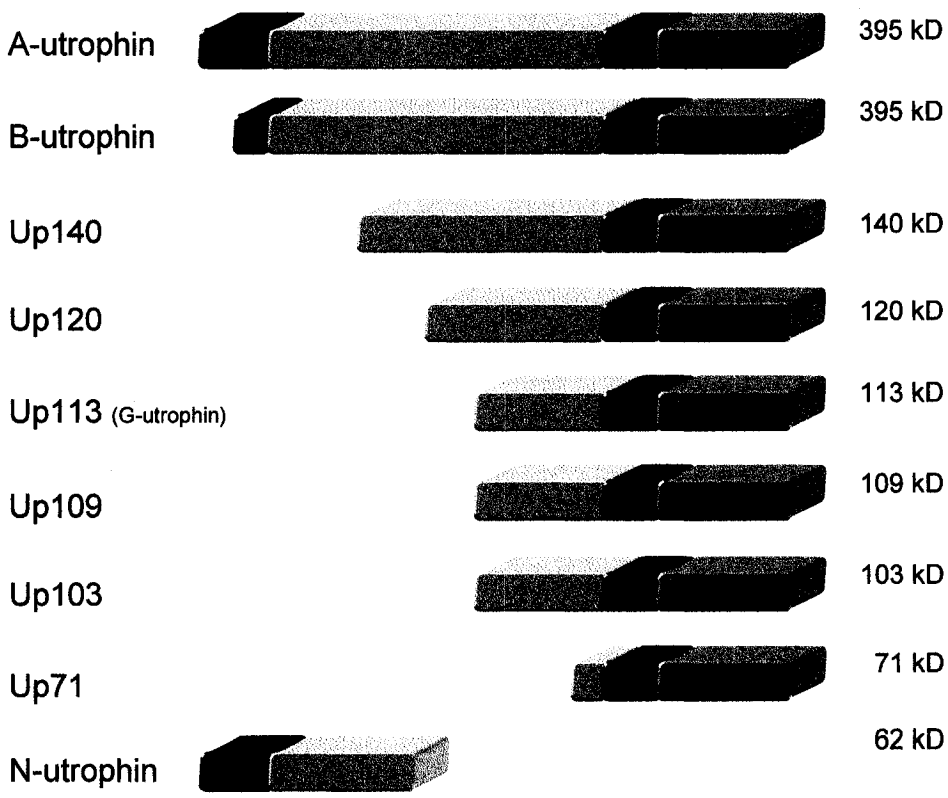
Similar to the dystrophin gene locus, the utrophin gene locus is rather large having the potential to generate different transcript and protein isoforms, through alternative promoter usage and splicing. Indeed, a variety of different utrophin transcripts and proteins have been identified that display tissue differences in expression. To date, two full length utrophin isoforms, A-utrophin and B-utrophin, have been identified. The two isoforms are transcribed from different promoters within the utrophin gene (Dennis et al., 1996; Burton et al., 1999). A-utrophin and B-utrophin mRNAs differ in their 5'untranslated regions (UTRs), and encode for proteins with unique N-terminal regions (Dennis et al., 1996; Burton et al., 1999; Weir et al., 2002). Tissue distribution analysis demonstrates B-utrophin to be primarily expressed in endothelial cells (Weir et al., 2002). In contrast, A-utrophin is ubiquitously expressed, and is the isoform found in skeletal muscle with enrichment at the adult neuromuscular junction (Weir et al., 2002; Stocksley et al., 2005).

In addition to the two full length utrophin transcripts a variety of smaller utrophin variants have been identified. The best characterized of these shorter isoforms is G-utrophin, also known as Up113. G-utrophin is a brain, and peripheral neural tissue specific transcript of 5.5 kb expressed from an internal promoter (Blake et al., 1995). The G-utrophin transcript is predicted to encode for a protein of ~ 113kD (Blake et al., 1995). Similar to the dystrophin transcript isoforms Dp71 and Dp140, PCR-based

screening has identified homologous utrophin transcripts Up71 and Up140 derived from internal promoters (Wilson et al., 1999). Both Up71 and Up140 are ubiquitously expressed, and are predicted to encode for proteins of 71kDa and 150 kDa respectively (Wilson et al., 1999). Examination of transgenic mice deleted for full length utrophin isoforms has uncovered additional short utrophin isoforms, Up120 specifically expressed in kidney glomeruli; Up109 found in fetal dermal tissue; and Up 103 expressed at low levels in testis (Jimenez-Mallebrera et al., 2003). Northern blot analysis of a rat glioma cell line has uncovered the existence of N-utrophin, an isoform that does not contain the C-terminal region (Zuelig et al., 2000). N-utrophin is a truncated utrophin transcript of 3.7kb generated through alternative splicing. The N-utrophin transcript encodes for a 62-kDa protein that possesses only the N-terminal actin binding domain, and part of the rod-domain (Zuelig et al., 2000). An extensive tissue distribution analysis of the N-utrophin isoform is yet to be conducted.

To date 9 different utrophin isoforms have been identified; A-utrophin, B-utrophin, Up140, Up120, Up113 (G-utrophin), Up109, Up103, Up71 and N-utrophin (Figure 1.5). All of the identified utrophin isoforms are expressed from different promoters, with the exception of N-utrophin which is hypothesized to be derived through alternative splicing (Zuelig et al., 2000). A-utrophin is the most extensively studied isoform, as it is the one expressed in skeletal muscle tissue; enriched at the NMJ; and contains all of the functional domains necessary to compensate for dystrophin deficiency.

Figure 1.5. Representative diagram of utrophin isoforms. Shown are the actin-binding domains (blue), spectrin-like repeats (orange), cysteine-rich (green) and carboxy-terminal domains (purple). Isoform names are on the right, and molecular masses on the left.


A-utrophin
395 kD
B-utrophin
395 kD
Up140
140 kD
Up120
120 kD
Up113 (G-utrophin)
113 kD
Up109
109 kD
Up103
103 kD
Up71
71 kD
N-utrophin
62 kD
Actin-binding domain
Cysteine-rich domain
C-terminus domain

III.B. Utrophin expression during skeletal muscle development

In adult skeletal muscle fibers utrophin is preferentially expressed at the NMJ, with very low levels seen in extra-synaptic regions. In contrast, total levels, and extra-synaptic expression of utrophin is higher in embryonic and neonatal muscle in comparison to adult muscle (Schofield et al., 1993; Pons et al., 1994). In neonatal human muscle, utrophin is found along the entire length of the sarcolemma before becoming progressively enriched at the post-synaptic sarcolemma. (Clerk et al., 1993). In murine models, utrophin becomes enriched at post-synaptic regions by embryonic day 13, with residual extra-synaptic expression persisting for approximately 2 weeks post-natal (Khurana et al., 1992; Koga et al., 1993; Stocksley et al., 2005). These observations suggest that under certain conditions, such as early muscle development, utrophin expression can be observed in extra-synaptic regions of muscle fibers.

Myogenic differentiation, the process whereby committed myoblasts fuse to form myotubes, is accompanied by increased expression of many skeletal muscle specific proteins (for review see Charge and Rudnicki, 2004). Increased transcription of skeletal muscle genes during myogenesis involves the contribution of a variety of different cellular and molecular events. Of these events, the binding of the basic helix-loop-helix (bHLH) transcription factors of the MyoD family to E-box motifs in target promoters is the best characterized (for review see Charge and Rudnicki, 2004). Studies have shown utrophin transcription increases during myogenic differentiation. Specifically, RT-PCR and nuclear run-on analysis indicate that both utrophin mRNA levels and transcriptional activity increase by ~ 2 fold upon differentiation of myoblasts to myotubes (Gramolini et

al., 1999; Perkins et al., 2002). Consistent with the observation that utrophin expression increases during myotube formation, the A-utrophin promoter contains a functional E-box motif capable of binding the myogenic factors (Perkins et al., 2002). Accordingly, forced expression of the bHLH myogenic transcription factors MyoD, MRF4, Myf5 and myogenin, in cultured muscle cells, is capable of stimulating A-utrophin promoter reporter activity (Perkins et al., 2002).

The regulation of utrophin expression during myogenesis occurs at multiple levels. In addition to alterations in transcription, post-transcriptional mechanisms can also affect utrophin expression during myotube formation. In particular, the sub cellular distribution of utrophin transcripts has been shown to change upon myotube formation (Gramolini et al., 2001a). This altered distribution is reflected by the enrichment of utrophin mRNA to cytoskeletal bound polysomes, sites of highly active translation (Gramolini et al., 2001a). 3' untranslated regions (UTRs) of host mRNAs have important roles in a variety of post-transcriptional processes that can impact on gene expression (for review see Ross et al., 1995; Bevilaqua et al., 2003). For instance, the 3'UTR can serve as a control point for the regulated turnover, translation and targeting of mRNAs (Ross et al., 1995; Bevilaqua et al., 2003). In accordance with these roles, the utrophin 3'UTR is able to target mRNAs to cytoskeletal bound polysomes upon differentiation of myoblasts to myotubes (Gramolini, 2001a). Therefore, during myogenesis both transcriptional and post-transcriptional mechanisms, through distinct regulatory regions, can alter utrophin expression.

The regeneration of skeletal muscle after damage, or due to disease is a condition that replicates many events seen in early skeletal muscle development. Upon injury,

quiescent primordial muscle cells called satellite cells are activated, and fuse with damaged myofibers. The fusion competent myofibers transition to centrally nucleated myotubes. These myotubes eventually reform healthy myofibers, with peripherally located nuclei (Charge and Rudnicki, 2004). Examination of skeletal muscles during early stages of toxin induced regeneration demonstrates robust increases in A-utrophin mRNA levels (Galvagni et al., 2002). These early inductions in A-utrophin mRNA levels have been attributed to transcriptional mechanisms involving the A-utrophin promoter, and an intronic region called the downstream utrophin enhancer (Galvagni et al., 2000; Galvagni et al., 2002). This enhancer region is hypothesized to interact with the A-utrophin promoter in a synergistic manner to induce utrophin transcriptional activity during early stages of skeletal muscle regeneration (Galvagni et al., 2002).

In addition to transcriptional mechanisms, post-transcriptional mechanisms also exert an important influence on A-utrophin expression during skeletal muscle regeneration. Despite the involvement of transcriptional mechanisms during early stages of toxin induced skeletal muscle regeneration, small inductions in A-utrophin mRNA are observed at later stages (Galvagni et al., 2002; Stocksley et al., 2005). Paradoxically, these small elevations in A-utrophin mRNA levels are accompanied by large increases in A-utrophin protein levels (Gramolini et al., 1999; Weir et al., 2004; Miura et al., 2005). Similar observations of discordant A-utrophin mRNA and protein levels have been seen in regenerating muscle from mdx mice, and patients afflicted with DMD and inflammatory myopathies (Karpati et al., 1993; Gramolini et al., 1999, Roma et al., 2004). In this context, the A-utrophin 5'UTR has been shown to promote A-utrophin translation during toxin induced skeletal muscle regeneration through an internal

ribosomal entry site (Miura et al., 2005). Hence, similar to the observations made in differentiating muscle cells, both transcriptional and post-transcriptional mechanisms can affect A-utrophin expression during skeletal muscle regeneration.

III.C. Utrophin expression at the neuromuscular junction

The NMJ employs a variety of mechanisms to ensure the proper localization of synaptic proteins in a spatially restricted manner to allow for proper neurotransmission (see for review Sanes and Lichtman, 1999; Sanes and Lichtman, 2001). A variety of pathways have been implicated in the enrichment of AChRs in close apposition to the nerve terminal. One well characterized mechanism involves the receptor tyrosine kinase muscle specific kinase (MuSK), and the cystoskeletal protein rapsyn. Both MuSK and rapsyn have important roles in the initial accumulation, and expression of AChRs in central regions of muscle fibers (Gautam et al., 1995; DeChiara et al., 1996; Sanes et al., 2001). Subsequently, nerve derived factors such as agrin and acetylcholine work in a concerted manner to promote the consolidation of AChRs specifically beneath the nerve terminal (Lin et al., 2005; Misgeld et al., 2005). Specialization of the NMJ is also achieved by the enrichment of several mRNAs encoding key synaptic proteins including AChR subunits, acetylcholinesterase (AChE) and MuSK at the post-synaptic regions of skeletal muscle fibers (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman et al., 1989; Jasmin et al., 1993; Michel et al., 1994; Fromm et al., 1998; Meier et al., 1998; Schaeffer et al., 1998; Moore et al., 2001; Kishi et al., 2005). In agreement with the localization of synaptic mRNAs at the NMJ, in-situ hybridization demonstrates that A-

utrophin transcripts are also enriched at post-synaptic regions (Gramolini et al., 1997; Vater et al., 1998; Stocksley et al., 2005).

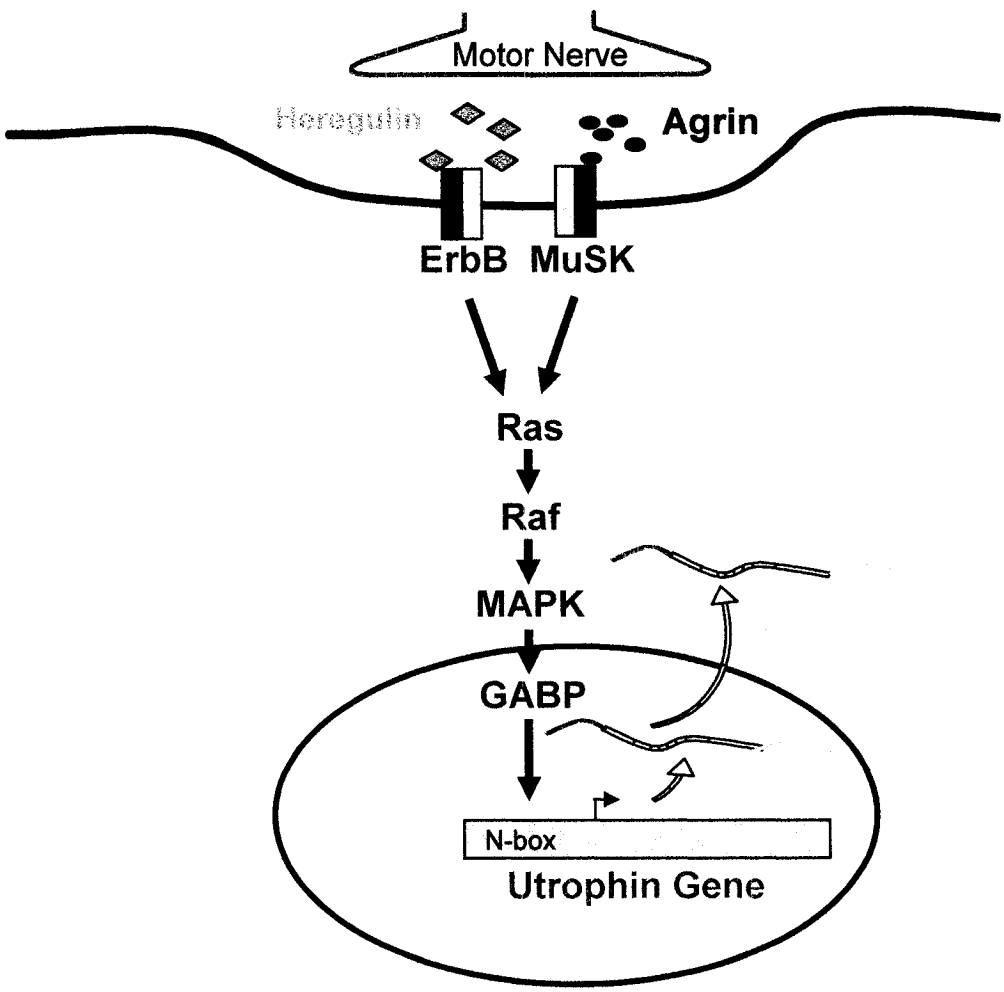
The accumulation of synaptic mRNAs at post-synaptic regions involves transcriptional activation from specialized nuclei at the sub synaptic sarcolemma, together with repression of expression from extra-synaptic nuclei mediated by electrical activity (see for review Schaeffer et al., 2001). In accordance with the enrichment of A-utrophin mRNAs at the NMJ, characterization of adult transgenic mice containing A-utrophin promoter-reporter NLS transgenes display high levels of reporter activity from synaptic nuclei, relative to extra-synaptic nuclei (Stocksley et al., 2005). Furthermore, direct injection of plasmids containing A-utrophin promoter-reporter NLS transgenes also show reporter activity primarily at synaptic nuclei (Gramolini et al., 1997). Taken together, transgenic and direct plasmid injection studies demonstrate that A-utrophin transcription is preferentially active at the NMJ.

The nerve can affect the expression of synaptic genes through a variety of different mechanisms. Sciatic nerve trans-section, a paradigm of muscle denervation, leads to large inductions in the expression of synaptic mRNAs, such as AChR subunits (see for review Duclert and Changeaux, 1995). Under similar circumstances, utrophin expression has been observed to increase, however only moderately (Jasmin et al., 1995). Furthermore, studies whereby foreign nerves are used to induce ectopic synapse formation strengthen the notion that the neural innervation can influence utrophin expression (Gramolini, 1997). In addition to activity, the nerve has been shown to influence the expression of synaptic genes through the release of secreted factors (see for review Schaeffer et al., 2001). Although a variety of neurally secreted factors have been

examined for their ability to stimulate the expression of synaptic genes, two neurally secreted factors, neuregulin1/ARIA and agrin, are the best characterized (Fromm et al., 1998; Meier et al., 1998; Schaeffer et al., 1998; Schaeffer et al., 2001; Lacazette et al., 2003). The cascade of events whereby agrin and neuregulin1/ARIA regulate the transcription of synaptic genes begins with their binding to, and subsequent activation of the putative receptors MuSK and ErbB respectively (Schaeffer et al., 2001; Lacazette et al., 2003). Once MuSK and ErbB have been activated, they initiate downstream events involving the Ras/MAPK pathway. This cascade of events promotes the binding of the ets-related GABPα/β transcription factor complex to N-box motifs, found on the promoters of some synaptic genes including MuSK, AChR subunits and A-utrophin (Fromm et al., 1998; Gramolini et al., 1998; Gramolini et al., 1999; Meier et al., 1998; Schaeffer et al., 1998; Schaeffer et al., 2001; Lacazette et al., 2003) (Figure 1.6).

Treatment of C2C12 myotubes with neuregulin1 or agrin has been shown to increase utrophin expression, transcriptional activity and A-utrophin promoter-reporter activity (Gramolini et al., 1998; Gramolini et al., 1999; Khurana et al., 1999). The responsiveness of A-utrophin promoter-reporter constructs to agrin and neuregulin treatments is lost upon mutation of the conserved N-box motif (Gramolini et al., 1998; Gramolini et al., 1999; Khurana et al., 1999). In accordance with a role for the N-box motif in regulating utrophin transcriptional activity, forced expression of GABPα/β also leads to inductions in A-utrophin promoter-reporter activity (Gramolini et al., 1999; Khurana et al., 1999). The A-utrophin promoter also contains binding sites for the transcription factors Sp1 and Sp3. Both Sp1 and Sp3 have been observed to cooperate

Figure 1.6. Schematic diagram of known regulatory mechanisms controlling utrophin expression at the neuromuscular junction. The release of agrin and heregulin (ARIA, neuregulin) from nerve terminals initiates a cascade of intracellular events. These events promote the binding and transcriptional activity of the transcription factor complex GABPα/β to the utrophin promoter (see text for details). These pathways assist in the higher levels of utrophin expression in synaptic relative to extra-synaptic regions.


Motor Nerve
Heregulin
Agrin
ErbB
MuSK
Ras
Raf
MAPK
GABP
N-box
Utrophin Gene

with GABPα/β in a synergistic manner to super-induce A-utrophin transcriptional activity (Galvagni et al., 2001; Gyrd-Hansen et al., 2002). Although the signaling events that regulate the ability of Sp1, Sp3 and GABPα/β to cooperate to stimulate A-utrophin transcriptional activity remain to be characterized, the involvement of phosphatases has been suggested (Rodova et al., 2004). Collectively, through a myriad of approaches, it has been shown that A-utrophin transcriptional activity is enriched at NMJs, and this enrichment occurs via signaling pathways and effectors regulated by neurally secreted factors. In this context, treatment of mdx mice with heregulin, a related molecule to neuregulin1/ARIA, has been shown to stimulate utrophin expression and correct for aspects of dystrophic pathology (Krag et al., 2004).

III.D. Utrophin expression and skeletal muscle fiber type

Skeletal muscle is composed of individual fibers that can differ in their metabolic capabilities, and speed of contraction (Schiaffino et al., 1996). Phenotypic differences between individual muscle fibers stem from a variety of factors including intrinsic developmental programs, hormonal influences and the patterns of activity provided by innervating motor neurons (Hennig et al., 1985; Gunning et al., 1991). Collectively, both intrinsic and extrinsic factors work in a coordinated fashion to specify distinct gene expression profiles between fast contracting glycolytic muscles, and their slower contracting more oxidative counterparts (see below). For instance, in comparison to fast glycolytic muscles slow oxidative muscles display higher levels of residual expression in extra-synaptic regions for a variety of different synaptic proteins including

acetylcholinesterase (AChE), collagen Q1 (ColQ1), and the AChRα subunit (Michel et al., 1994; Kues et al., 1995; Krejci et al., 1999). The observed differences in the spatial distribution of synaptic mRNAs between fast and slow muscles suggest that either pathways and/or proteins that help to spatially restrict mRNAs to the synapse may be found outside the NMJ in slow muscle fibers; or additional factors found in slow muscle fibers can promote the expression of synaptic genes.

Similar to other synaptic proteins, it has been shown that utrophin expression is also found at higher levels in extra-synaptic regions of slow muscle fibers. For instance, levels of utrophin and its mRNA have been shown to be 3-4-fold higher in slow contracting muscles such as the soleus (Gramolini et al., 2001b). Examination of transgenic mice containing an A-utrophin promoter-reporter transgene demonstrates only a modest, ~1.3 fold, higher reporter activity in slow muscles relative, to fast muscles (Stocksley et al., 2005). In agreement with the modest differences in A-utrophin promoter activity between fast and slow muscles, it has also been shown that the utrophin 3'UTR is capable of increasing the expression of reporter transcripts in slow soleus (SOL) muscles relative to fast extensor digitorum longus (EDL) muscles (Gramolini et al, 2001b). Interestingly, the utrophin 3'UTR has been shown to be involved in post-transcriptional processes such as mRNA stability and targeting (Gramolini 2001a). Taken together, these results indicate that the greater levels of utrophin in slow muscle results from the involvement of both transcriptional mechanisms acting on the promoter, and post-transcriptional events likely involving mRNA stability and the 3'UTR.

IV. Skeletal Muscle Diversity

Skeletal muscle fiber type diversity is associated with differences in metabolic and contractile capabilities (Brook and Kaiser, 1970; Peter et al., 1972). On this basis, skeletal muscles are classified into different categories based on their speeds of contraction, and propensity for oxidative versus glycolytic metabolism (Brook and Kaiser, 1970; Peter et al., 1972). Type I slow muscle fibers contract at slower rates and, are considered to be fatigue resistant due to their reliance on oxidative metabolism. In contrast, type II fast fatigable muscle fibers, contract at faster rates, and rely more on glycolytic metabolism. The maintenance of distinct muscle fiber phenotypes involves the regulated expression of different isoforms of proteins involved in contraction and metabolism (Schiaffino and Reggiani, 1996). Among the genes that display fiber type specific expression of different isoforms, is the family of myosin heavy chain (MyHC) contractile proteins (I, IIa, IIx, and IIb) (Reiser et al., 1985; Staron and Pette, 1986; Schiaffino et al., 1989; Schiaffino and Reggiani, 1996). Velocity of muscle fiber shortening during contraction is related to MyHC isoform expression based on the following continuum, from slowest to fastest, I<IIA<IIX<IIB (Reiser et al., 1985). Accordingly, type I slow muscle fibers primarily express MyHC I, whereas type II fast muscle fibers can be sub-divided into three categories based on the expression of MyHC IIA, IIX, or IIB (Schiaffino et al., 1989). Taking into account both contractile and metabolic capabilities, type I and IIA muscle fibers are considered to be oxidative; type IIX muscle fibers are of intermediate metabolism; and type IIB muscle fibers rely primarily on glycolytic metabolism (Rivero et al., 1999).

Despite the importance of developmental and hormonal factors, neural innervation patterns can profoundly influence the specification, and maintenance of skeletal muscle fiber diversity (Vrbova, 1963; Pette and Vrbova, 1985; Williams et al., 1986; Pette and Staron, 1997; Gunning and Hardeman, 1993). Fast muscle fibers typically receive phasic, high frequency stimulation from their respective innervating motor neurons (Hennig and Lomo, 1985). In contrast, slow muscle fibers receive tonic low frequency stimulations from innervating slow motor neurons (Hennig and Lomo, 1985). Cross-innervation studies, where nervous input or activity patterns for one type of muscle fiber are placed on another type, demonstrate that the intensity and pattern of nerve mediated activity can impact on the expression of contractile and metabolic proteins in skeletal muscle (Williams et al., 1986; Ausoni et al., 1990; Pette et al., 1992). Since nerve elicited activity can impact on the phenotype of individual skeletal muscle fibers, there has been a considerable effort in trying to identify factors with the ability to modulate the expression of fiber type specific genes in response to neural activity.

One candidate is the second messenger Ca^{+2}, whose intracellular levels in skeletal muscle are influenced by motor nerve elicited activity patterns (Sreter et al., 1987). Consistent with differences in the patterns of neural stimulation received by fast and slow muscles, the levels and mechanics of intracellular Ca^{+2} also differ between the two types of muscles. Tonic patterns of activity received by slow muscle fibers lead to intracellular Ca^{+2} levels that oscillate between 100-300nM (Chin et al., 1996). In contrast, fast muscle fibers at rest maintain low levels of intracellular Ca^{+2} with high transient amplitudes occurring during contraction (Westerblad et al., 1991). Studies in cultured muscle cells provided initial observations for the involvement of intracellular

Ca^{+2} levels as a factor that could regulate the expression of fiber type genes. Pharmacological elevation of intracellular Ca^{+2} levels with Ca^{+2} ionophores in primary muscle cultures was shown to induce the expression of MyHC I, and other slow contractile and oxidative genes (Kubis et al., 1997). These observations suggested that changes in intracellular Ca^{+2} levels could serve as a second messenger to link nerve evoked activity patterns to distinct profiles of gene expression. For this reason, it was postulated that Ca^{+2} regulated effectors, such as enzymes or transcription factors, serve important roles in the specification of contractile, and metabolic capabilities of muscle fibers.

V. Calcineurin

In different cellular systems, including skeletal muscle, the enzyme calcineurin has been implicated in numerous paradigms of gene expression that are derived from changes in intracellular Ca^{+2} levels (Ho et al., 1998; Crabtree, 2000; Tomita et al., 2002; Hogan et al., 2003; Schulz and Yutzey, 2004). Calcineurin, also referred to as protein phosphatase 2B (PP2B), is a ubiquitously expressed Ca^{+2}/calmodulin (CaM)-dependent serine/threonine phosphatase found at relatively high levels in muscle and neurons (Klee et al., 1979; Klee et al., 1998; Olson and Williams, 2000). Calcineurin is a heterodimer consisting of a catalytic subunit (CnA) (Figure 1.7) and a regulatory subunit (CnB) (Kincaid et al., 1990; Watanabe et al., 1995; Rusnak and Mertz, 2000). In mammals, there are 3 CnA isoforms γ, α and β. The γ isoform is specifically expressed in testes,

Figure 1.7. Schematic diagram depicting the functional domains for the calcineurin, catalytic, subunit A. Shown is the organization of the N-Term (N-terminal), catalytic, calcineurin B (CnB) subunit binding, calmodulin (CaM), and autoinhibitory (AI) domains. Note deletion of the autoinhibitory domain renders the calcineurin A subunit constitutively active (see text for details) (adapted from Rusnak and Mertz 2000).

Calcineurin A Domain Structure

whereas the α and β isoforms display a ubiquitous pattern of expression (Muramatsu et al., 1992; Klee et al., 1998; Crabtree, 1999; Rusnak and Mertz, 2000). The conserved domains of CnA consist of a catalytic domain, and three regulatory domains clustered at the C-terminal end of the protein. The regulatory domains consist of the CaM-binding domain (Kincaid et al., 1988); CnB-binding domain (Sikkink et al., 1995); and the auto-inhibitory domain (Hashimoto et al., 1990) (Figure 1.7). The auto-inhibitory domain inhibits the activity of calcineurin in the absence of CaM and Ca^{+2} (Perrino et al., 1995). Deletions of portions of the C-terminal domain containing the auto-inhibitory domain lead to constitutive activity of calcineurin, independent of Ca^{+2} and CaM (O'Keefe et al., 1992; Perrino et al., 1999). The CnB subunit contains 4 Ca^{+2}-binding EF-hand domains, and forms tight associations with the CnA subunit (Aitken et al., 1984; Kakalis et al., 1995; Sikkink et al., 1995). In agreement with CnB possessing multiple sites for Ca^{+2} binding, the function of the regulatory subunit is to recruit Ca^{+2} to the CnA catalytic subunit to stimulate phophatase activity (Perrino et al., 1995).

V.A. Calcineurin inhibitors

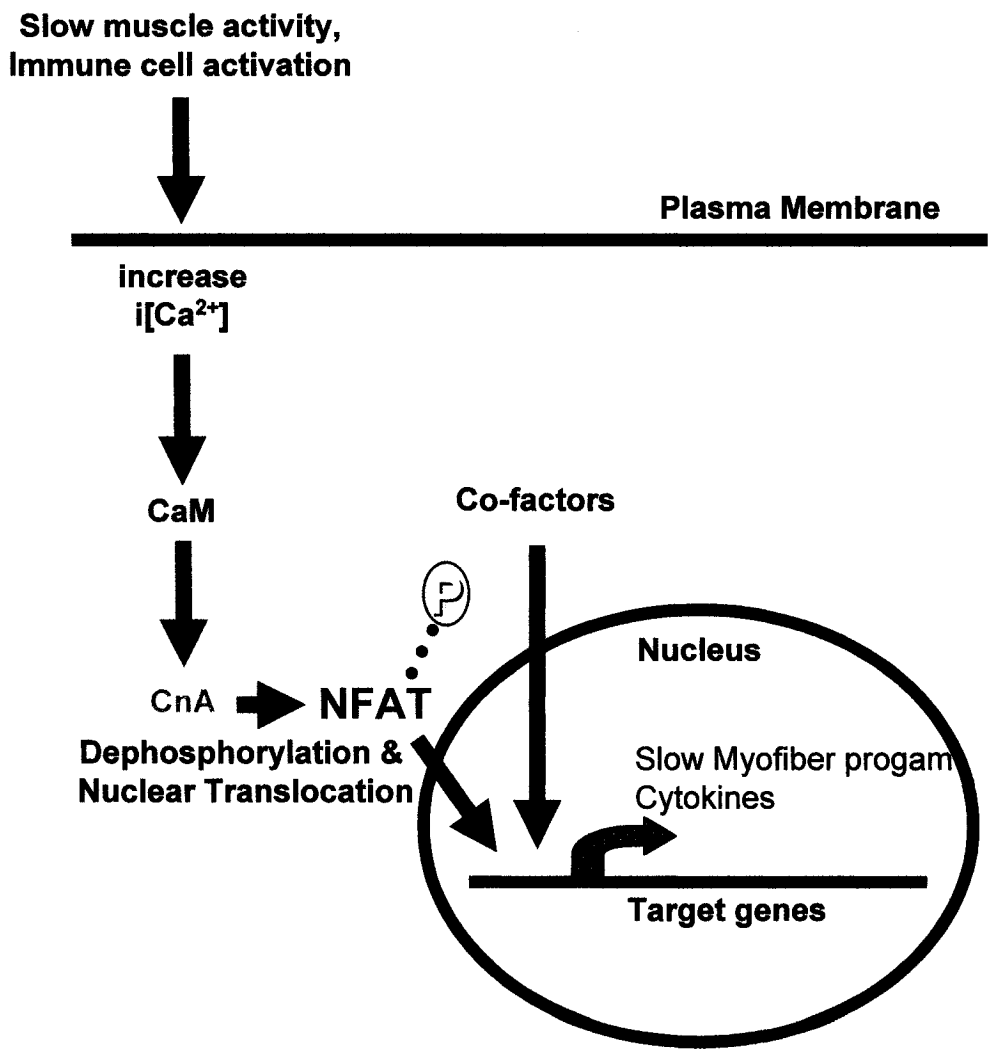
Calcineurin signaling can be inhibited through different mechanisms, including the development of specific drugs and peptides, and/or by endogenously expressed inhibitory proteins (Lin et al., 1991; Crabtree, 2000; Rothermal et al., 2003; Schulz and Yutzey, 2004). Although a variety of calcineurin inhibitors have been identified, the two most often used in studies are the immunosuppressive drugs, FK506 and cyclosporine A (CsA). FK506 and CsA form complexes with the endogenously expressed proteins

cyclophilin and FK506 binding protein 12 (FKBP12), respectively (Lin et al., 1991; Flanagan et al., 1991). After formation, these complexes bind to the CnA subunit, thus inhibiting the activity of calcineurin. Among endogenously expressed inhibitors, modulatory calcineurin-interacting protein 1(MCIP1) also known as Down syndrome critical region 1 (DSCR1) and cain/cabin1, are the best characterized for roles in skeletal muscle physiology (see for review Rothermal et al., 2003; Schiaffino et al., 2002). MCIP1 is relatively abundant in skeletal muscle (Rothermal et al., 2000). In contrast, cain/cabin is abundantly expressed in the brain, with only low levels in skeletal muscle (Lai et al., 1998). Both MCIP1 and cain/cabin1 bind to the CnA and inhibit calcineurin activity (Crabtree, 2000). Other proteins that have been shown to interact with, and inhibit calcineurin have been identified; however their roles in skeletal muscle physiology and development remain to be characterized (Crabtree, 2000).

V.B. Calcineurin/NFAT signaling

Calcineurin-dependent signaling mechanisms have well characterized roles in the activation of cytokine gene expression in both T and B lymphocytes (see for review Hogan et al., 2003). Upon activation, lymphocytes experience elevated levels of intracellular Ca^{+2} that lead to the association of CaM with calcineurin, thus stimulating phosphatase activity (Timmerman et al., 1996; Dolmetsch at al., 1997; Hogan at al., 2003). Subsequently, calcineurin dephosphorylates members of the nuclear factor of activated T cells (NFAT) transcription factor family (c1, c2, c3, c4) (see for review, Hogan et al., 2003) (Figure 1.8). The dephosphorylation of NFATs leads to their nuclear

Figure 1.8. Diagram of calcineurin/NFAT signaling. Shown are the events that lead to the activation of the calcineurin/NFAT pathway. Stimuli that promote immune cell activation, or slow muscle activity patterns lead to characteristic increases in intracellular Ca^{+2}. These events lead to the activation of calcineurin's phosphatase activity by CaM and Ca^{+2}. Upon activation, calcineurin dephosphorylates the downstream effector NFAT. Once in the nucleus, NFAT binds to target promoters and stimulates the transcription of target genes, including those that specify the slow myofiber program in skeletal muscle, and cytokines in immune cells.


Slow muscle activity,
Immune cell activation
Plasma Membrane
increase
i[Ca2+]
CaM
Co-factors
P
Nucleus
CnA
NFAT
Dephosphorylation &
Nuclear Translocation
Slow Myofiber progam
Cytokines
Target genes

translocation, where they bind to NFAT-binding sites found in the promoter regions of target genes, including cytokines, stimulating their expression (Figure 1.8) (O'Keefe et al., 1992; Crabtree, 2000; Olson, 2000 and Williams; Hogan et al., 2003). Examination of calcineurin/NFAT signaling during lymphocyte activation indicate that this pathway is activated in response to sustained low intracellular Ca^{+2} levels, in the range of (200nM-400nM) (Timmerman et al., 1996; Dolmetsch et al., 1997). In contrast, using the same lymphocyte cell system higher levels of intracellular Ca^{+2} do not promote the calcineurin/NFAT pathway.

VI. Calcineurin/NFAT signaling and muscle fiber type

Considering the similarities in intracellular Ca^{+2} levels that activate calcineurin/NFAT signaling in lymphocytes and those normally found in slow muscle fibers, Chin et al., postulated that this pathway may specify aspects of the slow muscle fiber nuclear program (Chin et al., 1998). In their study, they demonstrated that forced expression of constitutively active CnA (CnA*) deleted for the auto-inhibitory domain in C2C12 muscle cells promoted the nuclear translocation of NFATc1, and increased reporter activity driven by slow muscle fiber specific promoters. They, and others, also observed that this stimulation of slow muscle fiber specific promoter-reporter activity by CnA* is abrogated upon mutation of NFAT binding sites (Chin et al., 1998; Wu et al., 2000). In contrast, Chin et al., also observed that CnA* was unable to stimulate reporter activity driven by promoters that are primarily active in fast glycolytic muscle fibers.

Other studies have supported the notion, that calcineurin has an important role in promoting the slow muscle fiber program. Infection of fibroblasts induced to differentiate into the muscle lineage with adenoviruses containing transgenes encoding for CnA* increases expression of slow contractile genes, such as MyHC I (Delling et al., 2000). In-vivo, administration of the calcineurin inhibitors CsA and FK506, or injection of plasmids containing transgenes encoding for cain/cabin result in a lower percentage of type I oxidative muscle fibers in the slow soleus muscle (Chin et al., 1998; Serrano et al., 2001; Pallafacchina et al., 2002). Furthermore, transgenic inhibition of calcineurin in-vivo, either through knockout of CnA subunits, or forced expression of an endogenous inhibitor of calcineurin MCIP leads to a decrease in the percentage of type I slow muscle fibers (Parsons et al., 2003; Oh et al., 2005). In accordance with these observations, lines of transgenic mice containing a CnA* transgene expressed in all skeletal muscle types leads to the conversion of myofibers to a slow phenotype (Naya et al., 2000; Wu et al., 2001; Talmadge et al., 2004).

VI.A. Mechanisms of Calcineurin/NFAT activation in muscle

Numerous studies have been conducted to examine the mechanisms whereby calcineurin/NFAT signaling contributes to the specification of the slow myofiber program. Cultured C2C12 muscle cells treated with dosages of Ca^{+2} ionophore that raise intracellular Ca^{+2} levels to those found in slow muscle fibers lead to elevated expression of MyHC I, which is inhibited by CsA treatment (Kubis et al., 1997; Meissner et al., 2001; Kubis et al., 2003). Furthermore, stimulation of cultured muscle fibers with slow

muscle patterns of activity promotes the expression of MyHC I in a calcineurin sensitive manner (Kubis et al., 2002; Kubis et al., 2003). The stimulated expression of MyHC I in response to ionophore treatments, and slow patterned activity corresponds to the ability of these paradigms to promote the nuclear translocation of NFATc1, in a calcineurin-dependent manner (Liu et al., 2001; Kubis et al., 2002; Kubis et al., 2003). For instance, in muscle fibers expressing NFATc1-green fluorescent protein (GFP) fusion proteins, fluorescence is normally observed outside of nuclei. However, these muscle fibers when stimulated with slow muscle activity patterns, or treated with appropriate dosages of Ca^{+2} ionophore, promote in a CsA-sensitive manner the translocation of NFATc1-GFP to nuclei (Liu et al., 2001). In contrast, fast stimulation patterns or vehicle treatments do not promote nuclear translocation of NFATc1-GFP (Liu et al., 2001). Collectively, these in vitro studies implicate neural activity patterns, and intracellular Ca^{+2} levels as upstream effectors that regulate the ability of calcineurin/NFAT signaling to promote the expression of genes that characterize the slow myofiber program (Figure 1.8).

VI.B. Influence of nerve derived activity on calcineurin signaling

Increased load and activity are stimuli that can alter the phenotypic characteristics of skeletal muscle. Functional overload is a method whereby the ablation of postural muscles, the gastrocnemius and soleus, places an increased load on the plantaris, a muscle predominately comprised of fast muscle fibers (Olha et al., 1988). The increased load on the plantaris leads to hypertrophy of this muscle, and shifts towards a slower metabolic phenotype (Kandarian et al., 1994; Dunn et al., 1997; Dunn et al., 1999).

These adaptations are prevented upon prior treatment of functionally overloaded mice with the calcineurin inhibitors CsA and FK506 (Dunn et al., 1999). Functional overload also fails to induce a shift in fiber type, towards a slow phenotype, in plantaris muscles from CnA null mice, or mice expressing a dominant negative protein for CaM (Dunn et al., 2000; Parsons et al., 2004). Innervation by slow motor neurons exerts a strong influence, in vivo, on the ability of calcineurin/NFAT signaling to promote the expression of slow contractile proteins. In regenerating slow, soleus, muscles expression of MyHC I and MyHC I promoter-reporter activity is increased in the presence of slow motor neuron innervation (Serrano et al., 2001). Within this paradigm, the effects of slow motor neurons on the transcription of MyHCI are prevented by treatment of mice with CsA, FK506, or the forced expression of cain/cabin1 (Serrano et al., 2001; Pallafachina et al., 2002). Furthermore, the ability of slow patterned activity from slow motor neurons to promote the transcription of MyHC I is dependent on NFAT activity. This was exemplified by the forced expression of VIVIT, a small peptide inhibitor for the NFAT family of transcription factors (Crabtree, 2000; McCullagh et al., 2004). Similar to calcineurin inhibition, forced expression of VIVIT prevents slow motor neuron activity induced expression of MyHC 1 in regenerating slow soleus muscles (McCullagh et al., 2004). Conversely, forced expression of constitutively active NFATc1 in soleus muscles increased MyHC I promoter-reporter activity, however could not elevate reporter expression in fast muscles (McCullagh et al., 2004).

The affects of calcineurin signaling on the specification of muscle fiber types at different developmental stages was examined with muscles from mice containing a transgene that encodes for the calcineurin inhibitor MCIP1. These mice were designed to

over-express MCIP1 at very early stages of skeletal muscle development. Muscles from MCIP1 over-expressing transgenic mice displayed decreases in the percentages of slow muscle fibers, in mature appropriately innervated muscle fibers beginning at approximately 7 days post-natal (Oh et al., 2005). This decrease in the percentage of slow muscle fibers corresponded to reduced expression of slow contractile proteins, such as MyHC I and troponin I slow (Oh et al., 2005). In contrast, analysis of muscles from MCIP1 over-expressing transgenic mice at age E14.5, did not display any defects in the expression of slow contractile proteins (Oh et al., 2005). These data support a role for calcineurin signaling in the maintenance of the slow myofiber program, which is dependent upon the innervation patterns and intracellular environment observed in mature slow muscles.

Other studies have suggested that additional pathways to calcineurin/NFAT signaling function to maintain the slow myofiber program. Some studies have demonstrated that transfection of plasmids containing a transgene that encodes for CnA* into C2C12 muscle cells, in addition to activating slow muscle promoters could also increase reporter activity from fast muscle promoters, such as MyHC IIB (Swoap et al., 2000; Allen et al., 2001). Another group also administered CsA to rodents and found no change in fiber type proportions in fast or slow muscle (Biring et al., 1998). This study differed from other studies which demonstrated changes in fiber type with CsA administration in experimental set up including the use of different CsA dosages, and modes of delivery (Biring et al., 1998; Chin et al., 1998). These differences in methodology could explain why in some instances there may be no effects of CsA administration on muscle fiber type. Mutation of the NFAT site in the slow upstream

regulatory element (SURE), a cis-element found in promoters capable of conferring high levels of reporter activity to slow muscle fibers, was reported not to be sufficient to attenuate this fiber type specific effect (Calvo et al., 1999; Calvo et al., 2001). Muscles from knock out mice for the CnA α and β subunits display reduced proportions of slow myofibers; however this effect could not be entirely explained by reduced NFAT activity (Parsons et al., 2003; Parsons et al., 2004). Collectively these studies indicate that calcineurin may function via as yet to be identified mechanisms and effectors to promote the slow myofiber program.

VI.B. Ca^{+2} regulated factors and the slow myofiber program

In addition to calcineurin/NFAT signaling other Ca^{+2} regulated signals and effectors function in the specification, and maintenance of the slow myofiber program. The myocyte enhancer factor 2 (MEF2) family of transcription factors are calcium regulated transcription factors that participate in the survival, and development of a variety of tissues, including skeletal muscles (see for review Black and Olson, 1998). Examination of promoters for slow muscle fiber specific genes initiated interest in MEF2, as a transcription factor that could promote the expression of slow contractile proteins. Mutation of MEF2 binding sites in SUREs was observed to be sufficient to abrogate the ability of this regulatory element to drive high levels of reporter activity in slow muscle fibers (Calvo et al., 1999; Wu et al., 2000; Calvo et al., 2001). Additional evidence for the involvement of MEF2 in the specification, and maintenance of the slow myofiber program is provided by examination of MEF2-dependent promoter-reporter transgenic

mice. These mice contain a reporter transgene whose expression is controlled by a regulatory element with 3 copies of the consensus MEF2 binding site (Naya et al., 1999; Wu et al., 2000; Wu et al., 2001). Initial examination of these mice indicated that MEF2-dependent reporter activity is high early in skeletal muscle development (Naya et al., 1999). Although MEF-2 dependent reporter activity is severely attenuated in adult myofibers, it remains prominent in adult slow muscles relative to fast muscles (Naya et al., 1999; Wu et al., 2000; Wu et al., 2001). In accordance with the potential for MEF2-dependent transcription to promote the expression of slow myofiber genes, stimulation of fast muscles with activity patterns normally found in slow muscles leads to a substantial increase in MEF2-dependent reporter activity (Wu et al., 2000; Wu et al., 2001). Calcineurin has been shown to regulate the ability of MEF2 to promote the transcription of slow contractile proteins. For instance, forced expression of CnA* stimulates reporter activity driven by a MEF2-dependent regulatory element (Wu et al., 2000; Handschin et al., 2003). In addition, increases in MEF2-dependent reporter activity in fast muscles stimulated with slow patterns of activity are attenuated when calcineurin signaling is inhibited (Wu et al., 2001). Stimulated calcineurin activity can also directly affect MEF-2 post-translationally by modulating its phosphorylation status, an event hypothesized to promote MEF2-dependent transcriptional activity (Wu et al., 2001).

Other calcium regulated enzymes can function with calcineurin to promote the slow myofiber program. For instance, stimulation of the Ca^{+2}/CaM-dependent protein kinases (CaMKs) have been shown to augment the ability of calcineurin to promote the transcription of slow myofiber genes, and MEF2-dependent transcriptional activity (Wu et al., 2001; Handschin et al., 2003). Furthermore, stimulation of CaMK activity can

directly regulate MEF2 transcriptional activity during myogenic differentiation, and upon stimulation of muscle fibers with slow patterns of activity (McKinsey et al., 2000; Wu et al., 2000; Liu et al., 2005). In these contexts, CaMKs when activated by rises in intracellular Ca+2 and CaM, phosphorylate the chromatin remodeling proteins, histone deacytelases (HDACs) 4 and 5 (McKinsey et al., 2000; Liu et al., 2005). Phosphorylation of HDACs by CaMK results in the disassociation of these proteins from MEF2. The disassociation of HDACs 4 and 5 prevents their ability to inhibit of MEF-2 dependent transcription, a phenomenon important for early stages of myogenesis, and the expression of slow oxidative myofiber genes (McKinsey et al., 2000; Liu et al., 2005).

Skeletal muscles from transgenic mice bearing transgenes encoding constitutively active CaMK variants display conversion of myofibers to a slow contractile oxidative phenotype (Wu et al., 2002). This conversion of myofibers to a slow phenotype by CaMK corresponds with increased expression of the transcriptional co-activator peroxisome proliferator activated receptor-γ co-activator 1α (PGC-1 α). PGC-1α is a co-factor that does not contain inherent transcriptional activity; however it is able to associate with a variety of different transcriptional regulators (Lin et al., 2005). Expression of PGC-1α, and its association with transcriptional regulators are well characterized events that promote nuclear paradigms involved in oxidative metabolism and mitochondrial biogenesis (see for review Lin et al., 2005). Notably, the expression of PGC-1α in adipose and muscular tissues promotes the activity of the transcription factors nuclear respiratory factor (NRF) 1 and NRF 2α (human homologue of GABPα), an event inherent in the promotion of oxidative metabolism and mitochondrial biogenesis in these cellular systems (Lin et al, 2002; Handschin et al, 2003; see for review Lin et al., 2005).

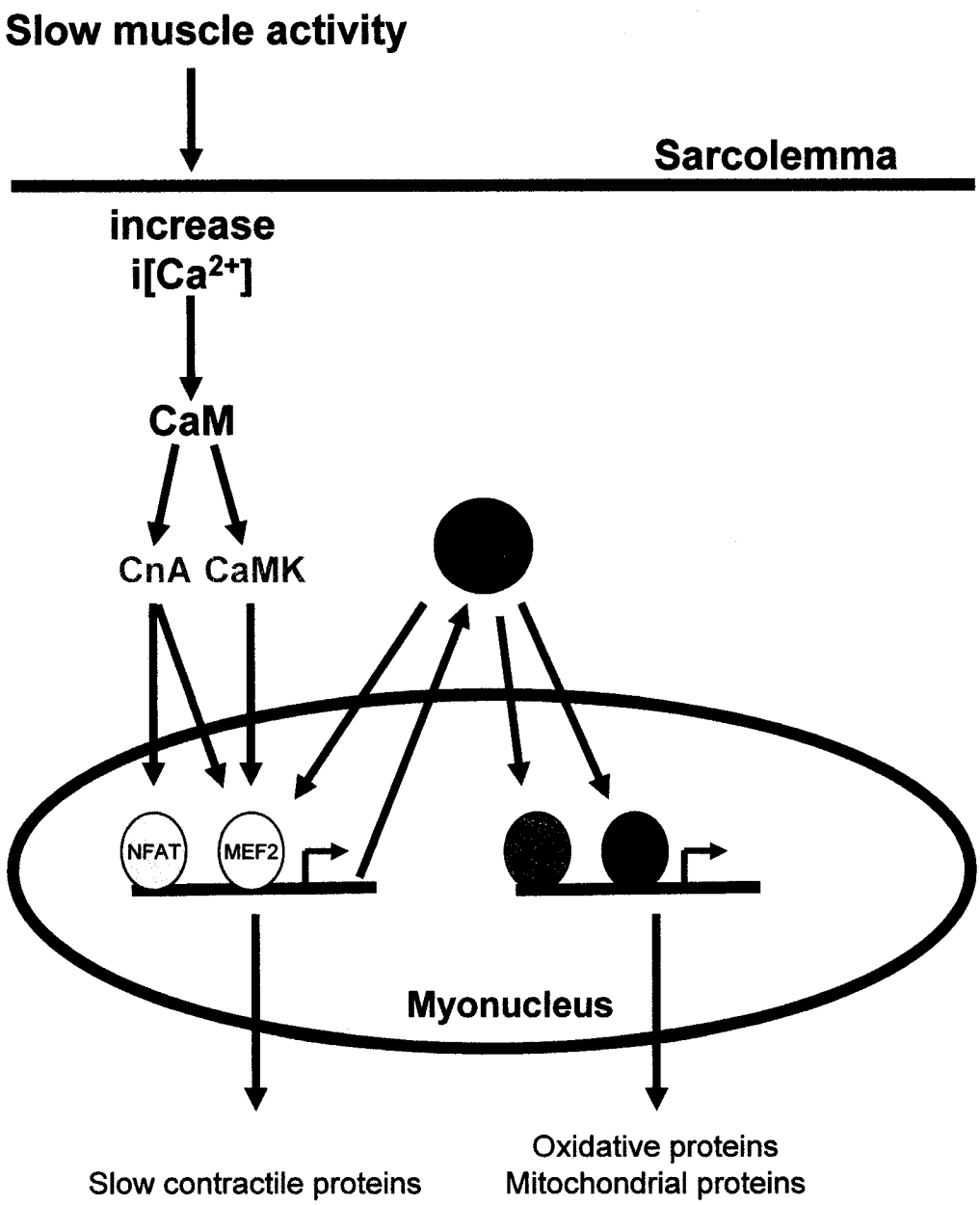
Consistent with roles in the regulation of metabolism, the expression of PGC-1 α is high in tissues that rely primarily on oxidative metabolism, and are high in mitochondrial content (Lin et al., 2002; Lin et al., 2005). In the case of skeletal muscle this relationship holds true, as PGC-1 α is found to be highly expressed in slow oxidative muscle fibers, relative to faster glyocolytic counterparts (Lin et al., 2002). To examine the effects of PGC-1α on muscle fiber diversity, transgenic mice were generated with forced expression of PGC-1 α specifically in skeletal muscle. Examination of these mice demonstrated that forced expression of PGC-1α, in all muscle types, promotes the conversion of myofibers to a slow contractile, more oxidative, phenotype (Lin et al., 2002).

VI.D. Regulation of PGC-1α and the slow myofiber program

The ability of PGC-1α to promote the transcription of slow contractile proteins has been shown to involve both calcineurin and CaMK signaling. Experiments in C2C12 muscle cells indicate that PGC-1α, calcineurin, CaMK, MEF2c and NFATc1 all function in a concerted manner to increase the transcription of genes that specify the slow contractile, oxidative, phenotype (Lin et al., 2002; Handschin et al., 2003; Lin et al., 2005). For example, transfection of plasmids containing transgenes encoding for MEF2c and PGC-1α function to stimulate the transcription and expression of PGC-1α itself (Handschin et al., 2003). Furthermore, transfection studies in C2C12 muscle cells also demonstrate that activated forms of calcineurin, CaMK, NFATc1 and MEF2c can also work together to stimulate PGC-1α transcriptional activity and expression (Handschin et

al., 2003). Taken together these observations indicate that PGC-1α cooperates with calcineurin/NFAT, CaMK, and MEF2C in an autoregulatory loop that maintains high levels of its own expression in skeletal muscle. Based on these observations, it is hypothesized that this autoregulatory loop serves to maintain the expression of genes that characterize the slow contractile oxidative myofiber phenotype (Handschin et al., 2003). In this context, forced expression of PGC-1α, in C2C12 muscle cells, also promotes the expression and transcriptional activity of NRF1 and GABPα. In addition to its roles in regulating the expression of synaptic genes, such as A-utrophin, GABPα together with NRF1 has been shown to promote the expression of genes involved in oxidative metabolism and mitochondrial biogenesis (Gramolini et al., 1999; Schaeffer et al., 2001; Mootha et al., 2004). Therefore, PGC-1α functions with the calcium regulated effectors calcineurin, CaMK, MEF2C, and NFATc1, and via the transcription factors GABPα and NRF1 to promote and maintain the slow contractile oxidative myofiber program (Lin et al., 2002; Handschin et al., 2003; Mootha et al., 2004; Lin et al., 2005) (Figure 1.9).

Figure 1.9. Model for the specification of the slow contractile, oxidative, muscle fiber program. Slow muscle activity leads to increased intracellular Ca^{+2} (i[Ca^{+2}]) levels. Increased i[Ca^{+2}] together with CaM, in turn, activates calcineurin and CaMK. Activated calcineurin and CaMK promote MEF2 and NFAT-dependent transcription of slow contractile proteins and PGC-1α. Subsequently, PGC-1α acts in an autoregulatory manner to maintain high levels of its own, and slow contractile protein expression. PGC-1α also promotes the activity and expression of the transcription factors nuclear respiratory factor (NRF) 1 and GABPα. Both NRF1 and GABPα promote the expression of genes involved in oxidative metabolism and mitochondrial biogenesis.


Slow muscle activity
Sarcolemma
increase
i[Ca2+]
CaM
CnA CaMK
NFAT
MEF2
Myonucleus
Slow contractile proteins
Oxidative proteins
Mitochondrial proteins

VII. Statement of problems and objectives

In the past, there have been observations that slow muscle fibers are more resistant than fast muscle fibers, to the pathological consequences associated with the absence of functional dystrophin (Webster et al., 1988; Moens et al., 1993). Type II fast muscles in DMD patients have been observed to undergo rounds of degeneration and regeneration prior to type I slow muscle fibers (Webster et al., 1988). Furthermore, adult mdx fast muscles are more sensitive to damage inducing forced lengthening contractions, compared to their slower counterparts (Moens et al., 1993). This sensitivity of mdx fast muscles to stretch mediated damage is rescued upon over-expression of utrophin (Tinsley et al., 1996). Differences in the resistance to dystrophin-deficiency between fast and slow muscle fibers may stem from; the capacity of slow muscle to express higher levels of extra-synaptic utrophin (Gramolini et al., 2001, Stocksley et al., 2005). Therefore, the identification of factors or pathways that promote the slow oxidative muscle fiber program may provide therapeutic targets that can be manipulated to promote utrophin expression in DMD muscle.

In this thesis we were interested in: *1) examining the influence of signaling pathways that promote the slow myofiber program on utrophin expression; and 2) determining the consequences of altering the activity of signaling pathways that promote the slow myofiber program, in dystrophin-deficient muscles, on utrophin expression and the dystrophic pathology.* Hypothesis: Pathways with roles in the specification of the slow contractile, oxidative, phenotype regulate fiber type differences in utrophin

expression, and can function as therapeutic targets for DMD. In this thesis, we tested our hypothesis through the following objectives:

1. To determine if signaling pathways that promote the slow contractile, oxidative, myofiber program can regulate utrophin expression (Chapter 2 and 3).

Utrophin mRNA and protein levels are known to be higher in slow contractile, oxidative, muscles relative to fast contractile, glycolytic, muscles (Gramolini et al., 2001). Since stimulation of both calcineurin/NFAT and PGC-1α/GABPα signaling can promote the expression of genes indicative of the slow contractile, oxidative, program it is possible that both pathways can similarly influence utrophin expression. Therefore, we will begin to investigate the ability of calcineurin and PGC-1α signaling to regulate utrophin expression by examining: 1) which full-length utrophin isoform (A or B) is found at higher levels in slow muscle relative to fast muscles; 2) the affects of calcineurin/NFAT signaling on A-utrophin transcription, and endogenous expression using both in vitro and in vivo models; 3) the affects of altered PGC-1α levels, on utrophin expression using both in vitro and in vivo models. Hypothesis: Signaling pathways that are involved in the promotion of the slow myofiber program can affect utrophin expression.

2. To determine if signaling pathways that promote the slow contractile, oxidative, myofiber program are involved in post-transcriptional mechanisms that regulate utrophin expression (Chapter 4).

Higher levels of utrophin expression in slow muscles relative to fast muscles have been shown to involve both transcriptional and post-transcriptional mechanisms (Gramolini et al., 2001; Stocksley et al., 2005). Calcineurin has been shown to promote the slow myofiber program in part through transcriptional mechanisms involving the transcription factor NFAT; however additional, as yet to be characterized pathways are also likely to be involved (Parsons et al., 2003; Parsons et al., 2004). Since the utrophin 3'UTR can promote higher levels of reporter mRNA in slow muscles relative to fast muscles, we wanted to determine the mechanisms whereby this region can mediate this effect (Gramolini et al., 2001). Furthermore, we wanted to determine if calcineurin signaling could also affect the regulation of mRNA levels via events targeting the utrophin 3'UTR. Therefore, we began to examine the ability of calcineurin signaling, and the mechanisms employed by the utrophin 3'UTR to influence utrophin mRNA expression post-transcriptionally in fast and slow skeletal muscles by examining: 1) the stability of A-utrophin and utrophin 3'UTR reporter mRNAs in the presence of fast and slow skeletal muscle protein extract, with an in vitro stability assay; 2) cis-elements in the utrophin 3'UTR responsible for mediating differences in mRNA expression between fast and slow muscles; 3) the influence of altered calcineurin signaling on A-utrophin mRNA stability, and utrophin 3'UTR reporter mRNA levels and stability. Hypothesis: Cis-elements in the utrophin 3'UTR an affect utrophin expression post-transcriptionally in a calcineurin-sensitive manner.

3. To determine the affects of stimulating, or inhibiting signaling pathways that promote the slow contractile, oxidative, myofiber program in dystrophin-deficient muscles, on utrophin expression and the dystrophic pathology (Chapter 5 and 6).

There has been a considerable effort to identify mechanisms that can promote utrophin expression in dystrophin-deficient muscle, so as to correct or attenuate the dystrophic pathology. A variety of approaches have been used to promote utrophin expression along the length of myofibers, including adenoviruses, glucocorticoid treatments, heregulin treatments and stimulation of nitric oxide synthase activity with NO-donors (Gilbert et al., 1998; Chaubourt et al., 1999; Gilbert et al., 1999; Chaubourt et al., 2002; Cerletti et al., 2003; Krag et al., 2004; St-Pierre et al., 2004; Barton et al., 2005; Segalat et al., 2005; Voisin et al., 2005). All of these methods have resulted in varying degrees of utrophin upregulation, and correction of dystrophic pathology. Therefore, since utrophin expression is seen at a higher level in slow muscles relative to fast muscles, we will determine the effects of stimulating or inhibiting pathways involved in the specification of the slow myofiber program in dystrophin-deficient muscles on utrophin expression, and the dystrophic pathology. We will analyze muscles from mdx mice containing a transgene encoding either a constitutively active variant of calcineurin, or a small peptide inhibitor for calmodulin for: 1) affects on factors that influence the expression of slow oxidative myofiber genes; 2) muscle fiber type shifts; 3) affects on utrophin expression; and 4) different aspects of the dystrophic pathology, such as membrane integrity and muscle morphology. Hypothesis: Stimulation of signaling pathways that promote the slow myofiber program can increase endogenous utrophin expression in dystrophin-deficient muscles, and attenuate dystrophic pathology.

Conversely, inhibition of these pathways will decrease utrophin expression in dystrophin-deficient muscles, and exacerbate the dystrophic pathology.

Chapter 2

Expression of utrophin A mRNA correlates with the oxidative capacity of skeletal muscle fiber types and is regulated by calcineurin/NFAT Signaling

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Contribution from authors

Joe V. Chakkalakal and Bernard Jasmin wrote manuscript. Joe V. Chakkalakal performed all of the RT-PCR analysis, direct plasmid injections, cell culture transfections, some of the immunofluorescence analysis, and assisted in characterization of utrophin A antibody. Mark Stocksley assisted in the generation of utrophin A antibody, and performed some of the immunofluorscence analyses. Lindsay Angus performed gel shift assay for NFATc1. Julie Deschenes-Furry assisted in isolation of mRNA from some muscles. Mary-Ann Harrison, Eva Chin and Robin Michel provided muscles from functionally overloaded, CsA treated and CnA* transgenic mice, as part of an on-going collaboration. Eva Chin provided pCnA* plasmids. Simon St-Pierre and Lynn Megeney provided plasmids containing transgenes encoding for constitutively nuclear NFATc1.

Abstract

Utrophin levels have recently been shown to be more abundant in slow versus fast muscles but the nature of the molecular events underlying this difference remains to be fully elucidated. Here, we determined whether this difference is due to the expression of utrophin A or B, and examined whether transcriptional regulatory mechanisms are also involved. Immunofluorescence experiments revealed that slower fibers contain significantly more utrophin A in extrasynaptic regions as compared to fast fibers. Single fiber RT-PCR analysis demonstrated that expression of utrophin A transcripts correlates with the oxidative capacity of muscle fibers, with cells expressing myosin heavy chain I and IIa demonstrating the highest levels. Functional muscle overload, which stimulates expression of a slower, more oxidative phenotype, induced a significant increase in utrophin A mRNA levels. Since calcineurin has been implicated in controlling this slower, high oxidative myofiber program, we examined expression of utrophin A transcripts in muscles having altered calcineurin activity. Calcineurin inhibition resulted in an 80% decrease in utrophin A mRNA levels. Conversely, muscles from transgenic mice expressing an active form of calcineurin, displayed higher levels of utrophin A transcripts. Electrophoretic mobility shift and supershift assays revealed the presence of an NFAT binding site in the utrophin A promoter. Transfection and direct gene transfer studies showed that active forms of calcineurin or nuclear NFATc1, transactivate the utrophin A promoter. Together, these results indicate that expression of utrophin A is related to the oxidative capacity of muscle fibers, and implicate calcineurin and its effector NFAT in this mechanism.

Introduction

Ever since its initial characterization in the early 90's, there has been considerable interest in understanding the mechanisms regulating the expression of utrophin in skeletal muscle. This can be partially attributed to the fact that utrophin accumulates at the level of the neuromuscular junction where it appears to participate in the full differentiation of the postsynaptic membrane domain (1-3). In addition, because of its high degree of sequence identity with dystrophin, utrophin is also a solid candidate in a therapeutic strategy aimed at increasing the expression of a functional substitute for dystrophin in muscle of patients afflicted with Duchenne muscular dystrophy (DMD).

Several studies have shown that utrophin levels can be modulated according to the state of innervation and differentiation of muscle cells. For example, myogenic differentiation leads to a 2-fold increase in the expression of utrophin transcripts (4). Additionally, muscle denervation (5) as well as regeneration has been shown to also affect expression of utrophin (6, 7). In this context, several laboratories have now identified specific transcription factors and promoter elements that are important for regulating the abundance and localization of utrophin transcripts within muscle fibers (8-15). Of particular relevance, it was shown recently that utrophin can be transcribed from two different promoters resulting in the expression of utrophin A and B transcripts that differ in their 5' end (16).

In a recent study, we examined the expression and localization of utrophin in slow versus fast muscles. Using a combination of approaches, we found that in comparison to the fast extensor digitorum longus (EDL) muscle, the slow soleus contains 3- to 4-fold more utrophin mRNA (17). Accordingly, these findings may have important functional

implications in designing a therapeutic strategy based on utrophin upregulation since it is known that fast fibers are preferentially affected in DMD (18). In the present study, we have capitalized on a combination of approaches to determine whether this difference between fast and slow muscles is due to the expression of utrophin A or B. Additionally, we have also examined whether transcriptional regulatory mechanisms are also involved in the differential pattern of expression of this protein (see 17).

Materials and Methods

Animal Care and Protocols. EDL and soleus muscles from control C57BL/6 mice were dissected and subsequently used for immunofluorescence experiments or to isolate single muscle fibers (see below). Soleus muscles were also used in direct gene transfer studies (see below). Bilateral functional overload of the plantaris muscle was performed by surgically removing the soleus and gastrocnemius muscles from both hindlimbs (19, 20). Injection of mice with cyclosporine A (CsA) (25 mg/kg; 2X per day) or vehicle was performed as previously described in detail (20). Two to 4 weeks later plantaris muscles were removed, rapidly frozen in liquid nitrogen and stored at -80oC until further analysis. Transgenic mice expressing a constitutively active form of calcineurin (CnA* Tg) have been previously characterized (21).

Production of Utrophin A antibody. A polyclonal antibody for utrophin A was raised in New Zealand white rabbits against the synthetic peptide NH2 CMAKYGDLEARPDDGQNEFSD-COOH (Dalton Chemical Laboratories Inc., Toronto, ON) coupled to keyhole lymphocyte hemocyanin to increase immunogenicity (Covance Research Products Inc., Denver, PA). The peptide was injected intradermally in the back or subcutaneously in the neck, at a concentration of 250 mg/ml at alternating 3 week intervals. Production bleeds of antisera were collected 1 week after each respective peptide boost.

Immunoblotting. Total protein was extracted from muscle cells with RIPA buffer containing anti-proteases. Forty to 50 μg of protein was electrophoresed on 5.5 % SDS-polyacrylamide gels and transferred onto PVDF membranes (Immobilon P; Millipore, Bedford, MA). After pre-incubation, the membrane was incubated with the rabbit anti-utrophin A antibody (see above). The blot was then washed, incubated with a horse radish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory; West Grove, PA) and revealed using an enhanced chemiluminescence kit (Pierce Laboratories).

Immunofluorescence. The presence of utrophin was detected on serial cross-sections of mouse soleus, EDL, plantaris and TA muscles with either a mouse monoclonal antibody (2002 catalog, number: NCL-DRP2, Nova Castra, Newcastle upon Tyne, UK) using a mouse on mouse kit (M.O.M; Vector Laboratories), or with a polyclonal anti-utrophin antibody (2002 catalog, number C-19: SC-7459; Santa-Cruz Biotechnology Inc; Santa Cruz, CA.). These two antibodies recognize separate regions of utrophin, i.e., C-19 recognizes the C-terminus whereas DRP2 is against the N-terminus. In separate experiments, sections were incubated with a polyclonal utrophin A antibody (see above). Specificity of the labeling was determined by preabsorption of utrophin A antibodies with the peptide used to raise this antibody. Furthermore, the specificity of the 3 antibodies was ascertained by using muscles from utrophin-deficient and mdx mice. To label acetylcholine receptors, Alexa 488-conjugated a-bungarotoxin was used (Molecular Probes; Eugene, OR). MyHC IIb was detected using the BF-F3 antibody (German collection of microorganisms and cell culture)(22, 23). Sections were viewed with a

Zeiss Axioskop-2 microscope and quantitative analyses were performed using Scion Image software.

Single Fiber Isolation. Single fibers from EDL and soleus muscles were isolated as previously described (24).

Expression of Utrophin A Promoter-Reporter Gene Constructs. We used the same utrophin A promoter-LacZ reporter gene construct as previously described (8, 10). In addition, we used plasmids that contained a constitutively active form of calcineurin (CnA*) or a constitutively nuclear form of NFATc1 (nNFATc1) (25, 26). The nNFATc1 plasmid was prepared by RT-PCR using mouse skeletal muscle total RNA and primers that selectively amplify an NFATc1 fragment that lacks the coding region for the first 250 amino acids (27).

Mouse myogenic C2C12 cells were cultured (see 8, 10, 28) and transfected with the utrophin A promoter-reporter construct alone, or together with pCnA* or pnNFATc1 plasmids, using the lipofectamine reagent (Gibco/BRL, Burlington. ON). In all these studies, a constitutively expressed chloramphenicol acetyltransferase (CAT) plasmid (Promega) was also included to control for transfection efficiency.

Direct gene transfer was performed on mouse soleus muscles as described in detail elsewhere (8, 9, 17). The soleus muscles were isolated and injected with 10 ml of a solution containing the appropriate plasmids (utrophin A promoter, pCnA* and pCAT) diluted at a concentration of 2-4 mg/ml. Seven days later, injected muscles were excised, immediately frozen in liquid nitrogen, and total RNA was extracted.

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted using TriPure (Boehringer Mannheim) as recommended by the manufacturer. Quantitative RT-PCR was carried out to determine the relative abundance of total utrophin (both A and B together), utrophin A and B separately, and myosin heavy chain (MyHC) transcripts in EDL and soleus single muscle fibers, in whole muscles from mice subjected to different experimental treatments, and to determine the abundance of both lacZ and CAT transcripts in transfected C2C12 muscle cells and in transduced soleus muscles. These assays were performed as previously described (10, 17, 28). Primers that selectively amplified total utrophin, utrophin A, utrophin B, S12 rRNA, MyHC I, IIa, IIx , IIb, LacZ and CAT were designed on the basis of available sequences (16, 17, 29). Cycle numbers varied depending on the primers used and were within the linear range (17, 30). For amplification of LacZ and CAT mRNAs, samples were first digested using DNase 1 to eliminate plasmid contamination (17). In all these assays, negative controls consisted of RT-mixtures in which total RNA was replaced with RNase-free water. PCR products were first visualized on 1% agarose gels containing ethidium bromide. For quantitative measurements, PCR products were separated and visualized on agarose gels containing the fluorescent dye Vistra Green (Amersham; Arlington Heights, IL). Values obtained for utrophin, utrophin A and utrophin B were standardized relative to the amount of S12 rRNA present in the same sample. Values obtained for LacZ were standardized relative to the amount of CAT expressed in the same sample.

Electrophoretic Mobility Shift Assay (EMSA). Total muscle protein extraction and EMSAs were performed as previously described (10, 28). The sequences of the synthetic 32P-labeled oligonucleotides encompassing the NFAT binding site in the utrophin A promoter were (in 5' - 3' orientation) gtg cat att gga aaa cag aaa aat (sense) and att ttt ctg ttt tcc aat atg cac (antisense). For competition and supershift experiments, the samples were incubated with a 200X molar excess of unlabeled oligonucleotides or with 2.5 μl of a commercially available NFATc1 antibody (Santa Cruz Biotechnology), respectively.

Statistics. Two tailed Student's t-tests, analysis of variance and regression analysis were used to analyze the data. Means + SEM are presented throughout.

Results

Serial cross-sections were used in immunofluorescence experiments to detect both utrophin and MyHC IIb. Exposure times were increased when images were taken to examine extrasynaptic utrophin between fibers. As shown in Figure 2.1A and B, muscle fibers expressing MyHC IIb contain little or no extrasynaptic utrophin. By contrast, MyHC IIb-negative fibers displayed a clear signal for utrophin at their sarcolemma. It is important to note that identical results were obtained using another commercially available antibody (that recognizes a different region of utrophin; data not shown) and on muscle sections obtained from mdx mice (Figure 2.1 C and D) thereby ruling out the possibility of cross-reactivity with dystrophin.

We next examined whether this fiber type-specific pattern of expression could be attributed to the presence of utrophin A (see 31). We therefore generated a rabbit polyclonal antibody against a specific peptide sequence in the distinct N-terminal region of utrophin A (16). Western blot analysis demonstrated that this antibody recognizes a single high-molecular mass protein (Figure 2.2A). Fluorescence studies using this antibody clearly showed labeling of neuromuscular junctions (Figure 2.2B and C). Importantly, this utrophin A immunoreactivity was effectively competed by first incubating the antibody serum with the original peptide (Figure 2.2D and E). Furthermore, no immunostaining was detected in junctional regions of utrophin-deficient mice (Figure 2.2 F and G). In both the peptide block experiment and on muscle sections from utrophin-deficient mice, no immunoreactivity was observed in extrasynaptic regions (Figure 2.2 H and I). Together, these experiments highlight the specificity of the

Figure 2.1. Localization of utrophin in extrasynaptic compartments of type IIb-negative fibers. Shown are representative examples of photomicrographs of serial sections processed to detect utrophin using the DRP2 antibody (A, C) and MyHC IIb (B, D) by immunofluorescence. Note the lack (or low level) of utrophin staining in extrasynaptic regions of IIb fibers from both normal (A,B) and mdx (C, D) mice. * denote the same fibers identified on these serial sections. Images for utrophin were taken with increased exposure times to show the difference in the staining pattern between fiber types.

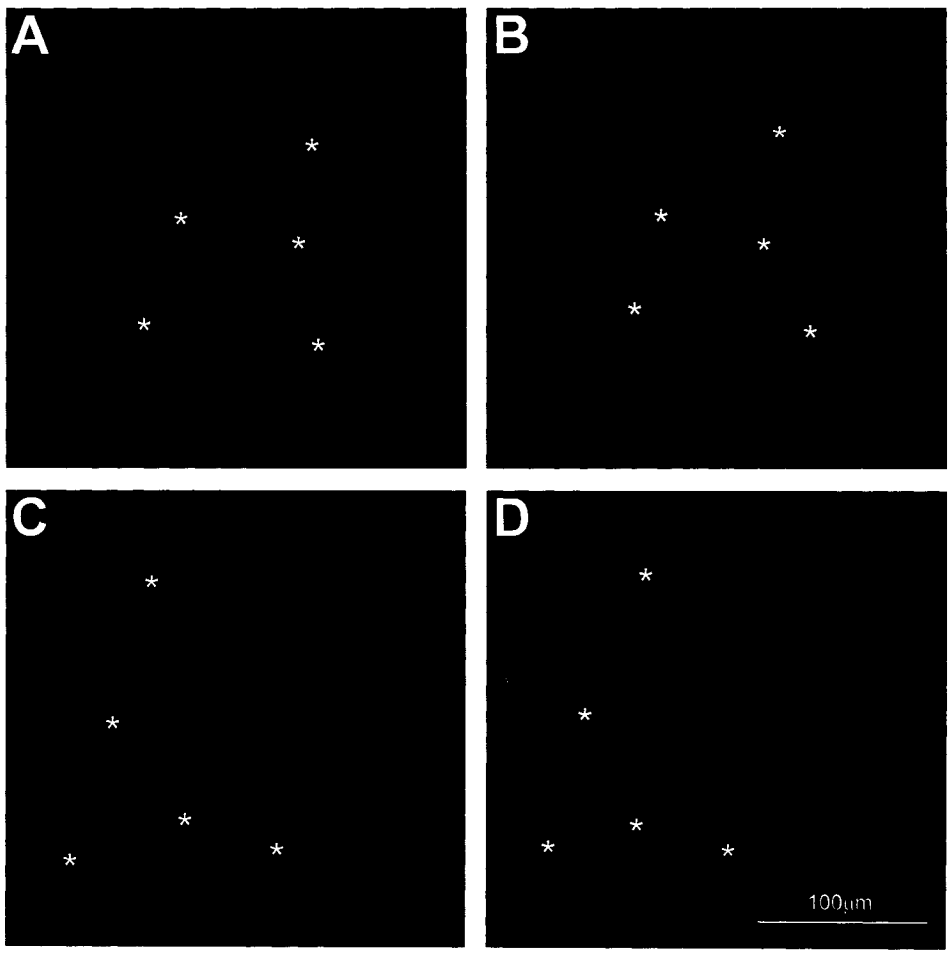
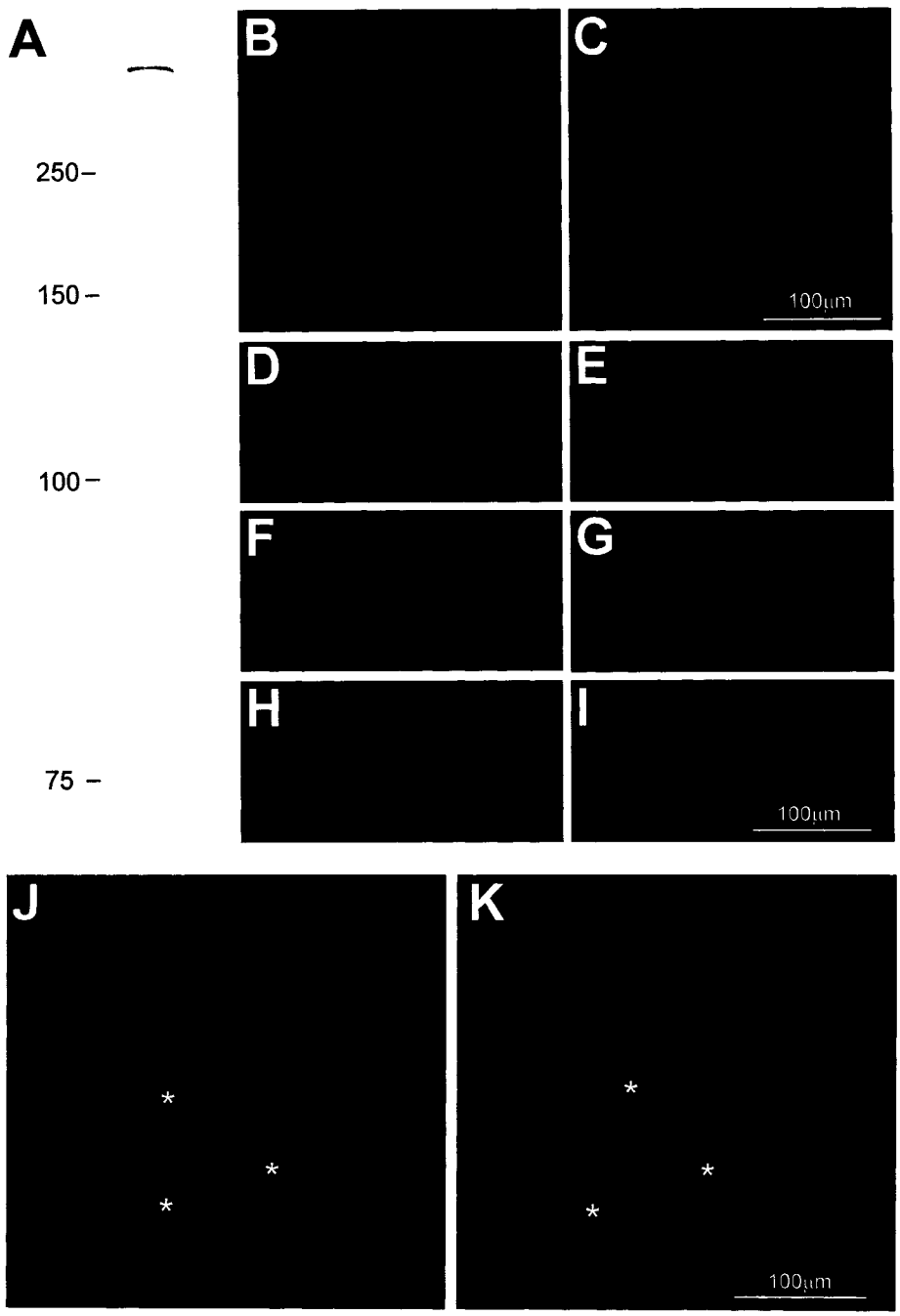

A
B
C
D
100μm

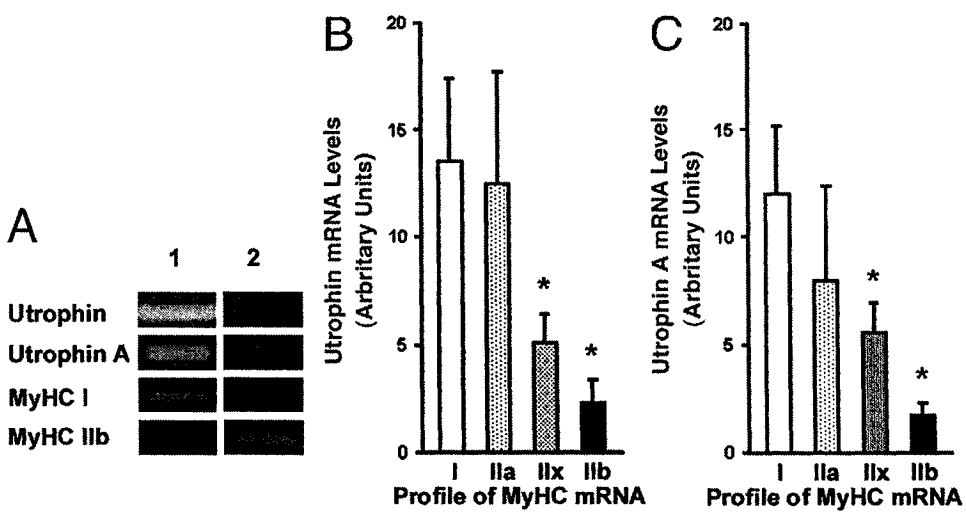
Figure 2.2. Extrasynaptic utrophin is the A isoform. A) shows that a polyclonal antibody raised against utrophin A recognizes a single high molecular mass band in Western blots using muscle proteins. B) and C) show the presence of AChR and utrophin A in cryostat sections of muscle fibers, respectively. Note the accumulation of utrophin A at the neuromuscular junctions. Preincubation of the rabbit serum with the utrophin A peptide, used to raise the antibody, completely abolished the utrophin A labeling (E) at AChR-rich regions (D). This labeling was also absent at AChR rich-regions from utrophin-deficient mice (F and G). In both the peptide block experiments (H) and in sections from utrophin-deficient mice (I), no labeling could be observed using this utrophin A antibody in extrasynaptic compartment of muscle fibers. Double immunofluorescence experiments showed that utrophin A (J) is expressed at the sarcolemma of MyHC IIb-negative fibers (K) and is thus not confined to junctional regions. * denote the same fibers identified in these serial sections.


A
250–
150–
100–
75 –
B
C
100μm
D
E
F
G
H
I
100μm
J
K
100μm

utrophin A antibody and its lack of cross-reactivity with dystrophin. Using this antibody, we observed that MyHC IIb-negative fibers showed higher extrasynaptic expression of utrophin A at the sarcolemma (Figure 2.2J and K). These data indicate therefore that the fiber type differences seen in utrophin expression (see Figure 2.1) are caused by differences in the expression pattern of utrophin A.

We attempted to establish a correlation between the level of utrophin expression and the MyHC profile of muscle fibers which is known to reflect not only the speed characteristics of individual fibers, but also their reliance on oxidative capacity. To this end, we performed RT-PCR analysis on single fibers isolated from soleus and EDL muscles. Our initial findings demonstrated that fibers expressing MyHC I contained more utrophin transcripts (A and B together) and that this difference was caused by a greater abundance of utrophin A mRNAs (Figure 2.3A). Fibers expressing MyHC IIb mRNA contained much less utrophin transcripts. Since these single fiber experiments displayed such a striking pattern, we counted the number of utrophin-positive fibers expressing a particular isoform of MyHC transcript. We found that 94% of single fibers positive for MyHC I mRNA were also positive for utrophin transcripts. By contrast, less than 50% of IIb fibers expressed detectable levels of utrophin mRNA. The relative abundance of utrophin mRNA was also dramatically different amongst the various fiber types. As illustrated in Figure 2.3B, utrophin mRNA levels were significantly ($P < 0.05$) higher in fibers expressing MyHC I and IIa in comparison to those fibers expressing MyHC IIx and IIb. RT-PCR experiments using primers specific for utrophin A and B transcripts further revealed that this difference was in fact due to the presence of utrophin

Figure 2.3. Expression of utrophin and utrophin A mRNAs in single fibers. (A) Examples of ethidium bromide-stained agarose gels showing PCR products obtained from two single fibers (1 and 2) for total utrophin, utrophin A, MyHC I and MyHC IIb. Note the presence of strong utrophin signals in the fiber expressing MyHC I. Quantitation of the RT-PCR data show that MyHC I and IIa fibers contained considerably more total utrophin (B) and utrophin A (C) transcripts. * denotes significant differences from type I ($P < 0.05$). A total of 60 fibers were analyzed from three different mice. Mean + SEM are shown.


A
1
2
Utrophin
Utrophin A
MyHC I
MyHC IIb
B
Utrophin mRNA Levels (Arbritary Units)
20
15
10
5
0
*
*
I
IIa
IIx
IIb
Profile of MyHC mRNA
C
Utrophin A mRNA Levels (Arbritary Units)
20
15
10
5
0
*
*
I
IIa
IIx
IIb
Profile of MyHC mRNA

A mRNAs (Figure 2.3C). Transcripts encoding utrophin B were low and did not vary according to the MyHC profile (data not shown). As displayed in Figure 2.3B and C, there also appeared to be a progressive decline in the pattern of utrophin expression based on the continuum of fiber types. In fact, a significant correlation ($P < 0.05$) was seen between the mRNA levels of utrophin A and combined MyHC I and IIa ($r = 0.76$), but not between utrophin A and MyHC IIb ($r = 0.20$).

To determine if adaptive changes resulting in a shift of muscle fiber type towards a slower phenotype could increase utrophin mRNA expression in whole muscle, we subjected plantaris muscle to functional overload since this experimental paradigm is well known to increase the proportion of slower, more oxidative muscle fibers (19, 23). In comparison to sham-operated animals, plantaris muscles that were overloaded demonstrated the typical hypertrophic response as determined by wet muscle mass (increased by ~ 2-fold) and a decrease in the level of MyHC IIb transcripts ($P < 0.05$) (Figure 2.4)(19). Analysis of utrophin mRNA levels standardized to S 12 rRNA, indicated that overload induced a 2-fold increase ($P < 0.05$) in the abundance of utrophin transcripts (Figure 2.4). As observed with the single muscle fibers, this increased expression of utrophin mRNA was largely caused by an induction in the levels of utrophin A transcripts (Figure 2.4) and protein (not shown).

Given these findings and the fact that calcineurin has been recently implicated in regulating the expression of a slower, more oxidative phenotype in muscle fibers (26, 32-35), we also examined the levels of utrophin A mRNA expression in muscle presenting altered levels of calcineurin activity. In comparison to mice treated with a vehicle

Figure 2.4. Expression of utrophin transcripts is increased in overloaded plantaris muscle. (A) Examples of ethidium bromide-stained agarose gels showing PCR products obtained from control (CTL) and overloaded (OV) muscles for total utrophin, utrophin A and MyHC IIb transcripts. Note the increased utrophin and utrophin A mRNAs concurrent with a decrease in MyHC IIb transcripts in OV muscles (B). * denotes significant differences from control ($P < 0.05$). N = 3 for CTL and N = 6 for OV. Mean + SEM are shown.

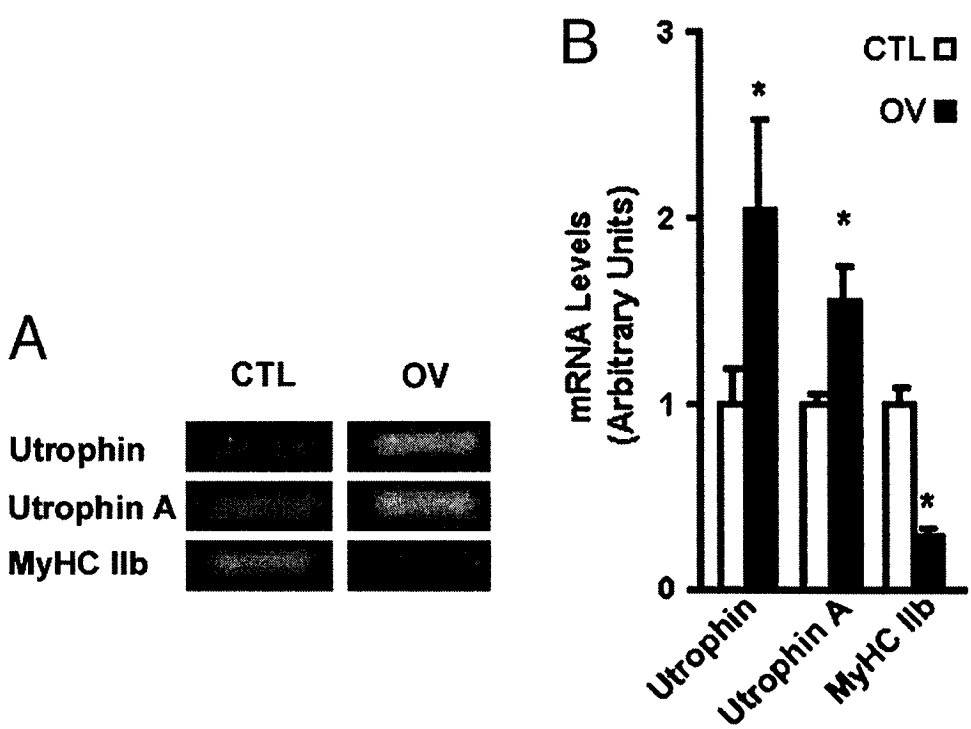
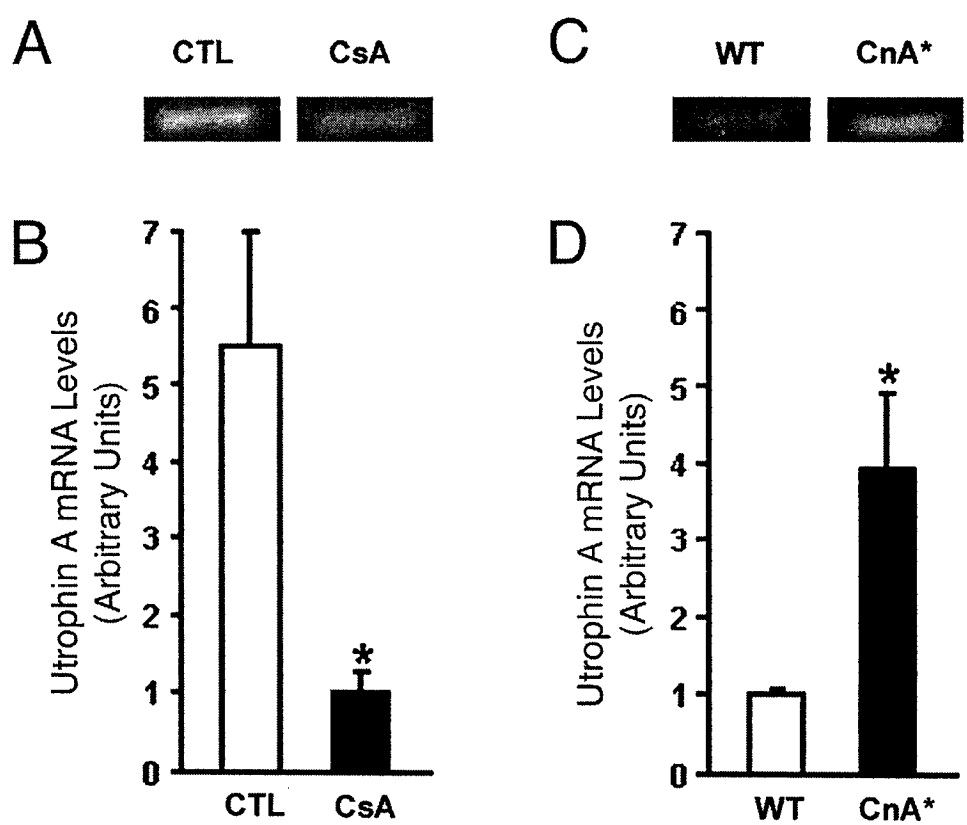

A
CTL
OV
Utrophin
Utrophin A
MyHC IIb
B
CTL
OV
mRNA Levels (Arbitrary Units)
0
1
2
3
*
*
*
Utrophin
Utrophin A
MyHC IIb

Figure 2.5. Expression of utrophin A mRNA is regulated by calcineurin. A) and C) are examples of ethidium bromide-stained agarose gels showing PCR products for utrophin A mRNA in control (CTL) and cyclosporine (CsA)-treated muscles, and in wildtype (WT) and transgenic (CnA*) muscles, respectively. Quantitation showed that in comparison to vehicle-treated CTL muscles, muscles from CsA-treated mice contained significantly less utrophin A transcripts (B). By contrast, muscles from CnA* transgenic mice contained significantly more utrophin A transcripts than WT mice (D). * denotes significant differences ($P < 0.05$). Muscles from 3 to 6 mice were analyzed for each group. Mean + SEM are shown.


A
CTL
CsA
C
WT
CnA*
B
Utrophin A mRNA Levels
(Arbitrary Units)
0
1
2
3
4
5
6
7
*
CTL
CsA
D
Utrophin A mRNA Levels
(Arbitrary Units)
0
1
2
3
4
5
6
7
*
WT
CnA*

solution, analysis of muscle from mice treated with CsA showed that calcineurin inhibition led to an ~ 80% decrease in utrophin A mRNA levels (Figure 2.5A and B). To further characterize the apparent relationship between utrophin mRNA expression and calcineurin, we determined the levels of utrophin A and B mRNAs in transgenic mice (CnA* mice) engineered to express a constitutively active form of calcineurin (21) which can stimulate the slower, high oxidative fiber myogenic program (34). Examination of these mice revealed that soleus muscles from CnA* mice contained significantly more utrophin A transcripts ($P < 0.05$) in comparison to wild-type animals (Figure 2.5C and D). Immunofluorescence experiments showed that individual soleus muscle fibers from CnA* mice expressed considerably more utrophin ($P < 0.05$; ~ 70 to 120% more) at the extrasynaptic sarcolemma in comparison to the levels seen in muscle from age-matched controls (Figure 2.6).

Since the effects of calcineurin on expression of the slower, high oxidative myofiber program involve transcriptional mechanisms (26, 33, 36, 37) we examined whether the mouse and human utrophin A promoters contain a consensus sequence for NFAT, a transcription factor whose nuclear translocation is regulated by calcineurin. Sequence analysis revealed the presence of a putative NFAT binding site in both the human and mouse promoters upstream of previously characterized N– and E-box motifs (8, 9, 15, 38)(Figure 2.7A). EMSAs performed using an oligonucleotide that encompasses the NFAT site in the utrophin A promoter showed that muscle proteins could specifically interact with this sequence because this binding was effectively competed upon incubation with a 200X molar excess of unlabeled probe (Figure 2.7B). Supershift assays confirmed that NFATc1 could directly interact with this region in the

utrophin A promoter. Co-transfection studies using C2C12 muscle cells demonstrated that a constitutively active CnA* or nuclear NFATc1, could induce a significant increase ($P < 0.05$) in the activity of the utrophin A promoter (Figure 2.7C). Direct plasmid injection performed in soleus muscles further confirmed that calcineurin affects the activity of the utrophin A promoter in vivo (Figure 2.7D).

Figure 2.6. Increase in utrophin A expression in muscle fibers from CnA* mice. Immunofluorescene experiments using the utrophin A antibody on soleus muscle sections from control (panels A and C) and CnA* (panels B and D). Note that muscles from CnA* mice express high levels of utrophin A at the sarcolemma of each individual fibers.

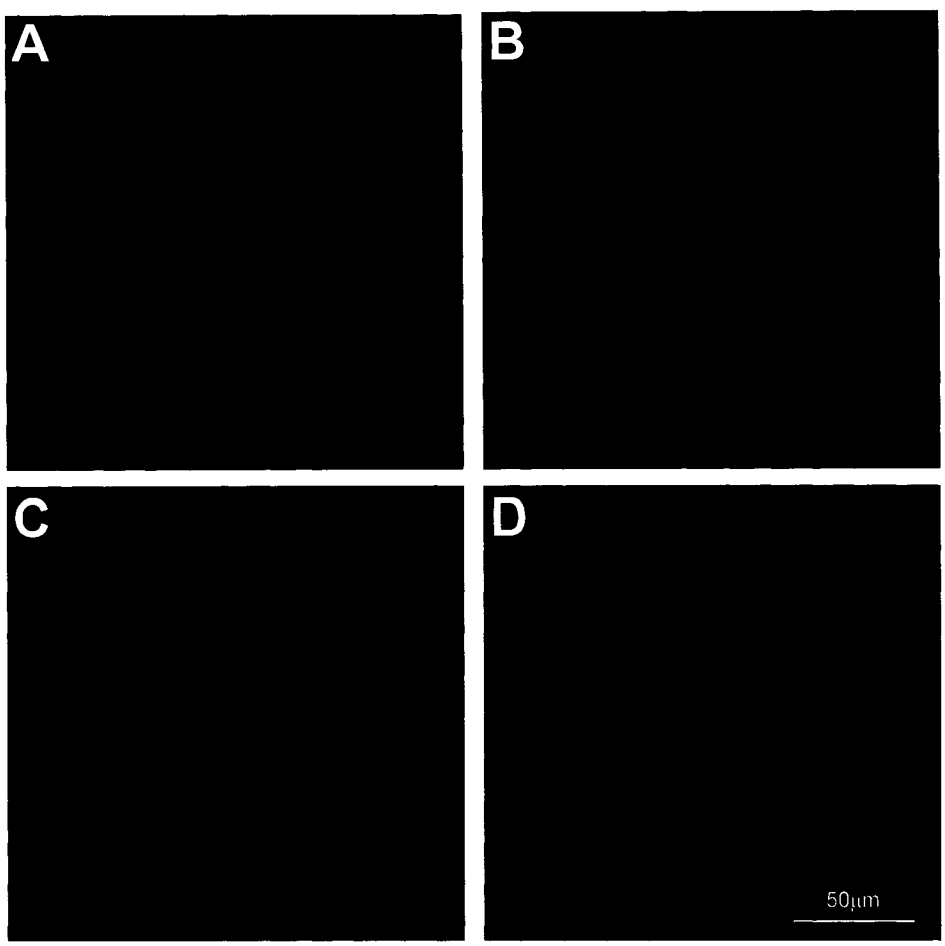
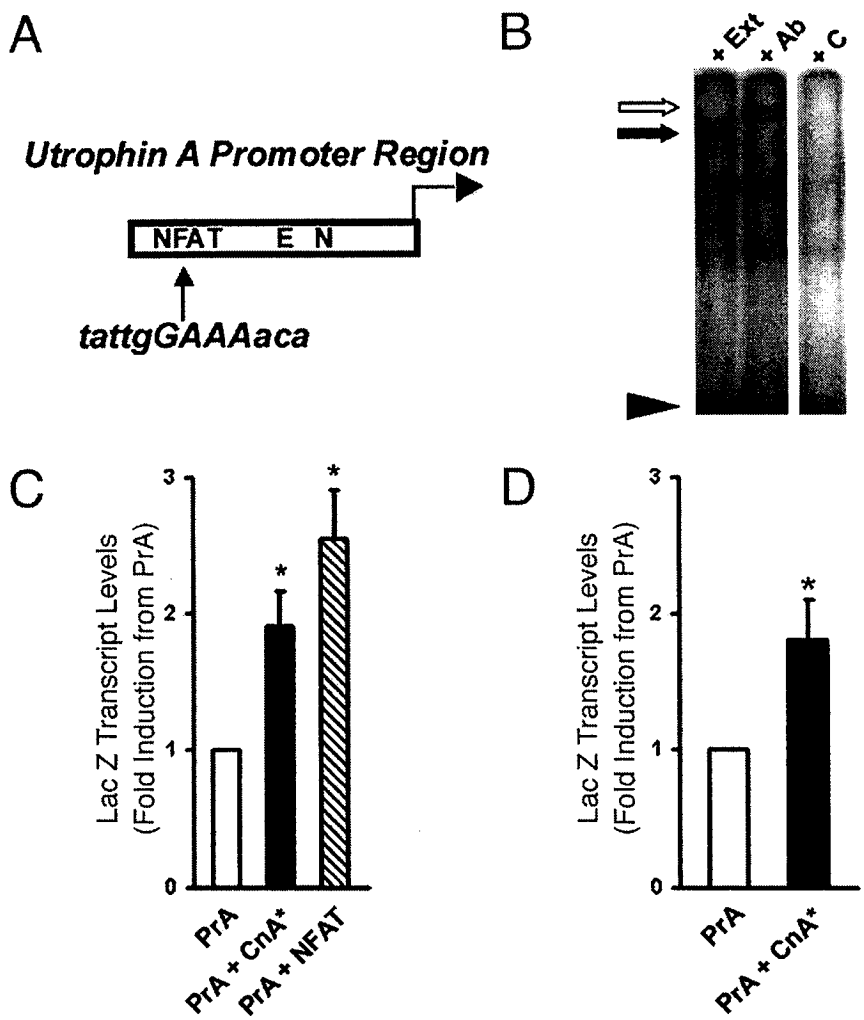

A
B
C
D
50μm

Figure 2.7. Activity of the utrophin A promoter is modulated by calcineurin and NFAT. A) schematic representation of a putative NFAT site in the utrophin A promoter. B) EMSAs using protein extracts (lane: + Ext) incubated with 32P-labeled double-stranded oligonucleotides corresponding to the NFAT motif (black arrow). Note the specific binding activity that is competed by a 200X molar excess of unlabeled probe (lane: + C). This binding can be supershifted by adding NFATc1 antibodies in the reaction mixture (white arrow; lane: + Ab). C) C2C12 muscle cells transfected with plasmids containing the utrophin A promoter-LacZ reporter gene alone or with expression vectors containing CnA* or nNFATc1. D) Mouse soleus muscles were transduced with plasmids containing the utrophin A promoter-reporter gene alone or with an expression vector containing CnA*. Note that in these two sets of experiments, expression of active CnA* or nuclear NFATc1 increased ($P < 0.05$) the activity of the utrophin A promoter as measured by the relative abundance of LacZ transcripts. For the transfection experiments, N = 2 to 4 in duplicate. For the injection, N = 4 mice. Mean + SEM are shown.


A
Utrophin A Promoter Region
NFAT E N
tattgGAAAaca
B
+ Ext
+ Ab
+ C
C
Lac Z Transcript Levels
(Fold Induction from PrA)
0
1
2
3
*
*
PrA
PrA + CnA*
PrA + NFAT
D
Lac Z Transcript Levels
(Fold Induction from PrA)
0
1
2
3
*
PrA
PrA + CnA*

Discussion

We have used single fiber RT-PCR and immunofluorescence to determine the expression level of utrophin and the MyHC profile of individual muscle fibers. Our results demonstrate that fibers expressing MyHC I and IIa contain significantly more utrophin than type IIx and IIb fibers and that its expression is not confined to junctional regions. Using isoform specific PCR primers and antibodies, we were also able to show that the increased utrophin expression in type I and IIA fibers is due to a greater expression of utrophin A. This particular isoform of utrophin is preferentially expressed at the level of the postsynaptic membrane of the neuromuscular junction (31). It is therefore interesting to note that under specific conditions, expression of this utrophin isoform extends well into extrasynaptic compartments of muscle fibers.

Our findings showed that the relative abundance of utrophin A mRNAs correlates well with fiber type characteristics. In fact, it appears that the expression level of utrophin correlates more with the oxidative capacity of the fibers rather than with their contractile, speed-related properties per se. It is well established that type I and IIa fibers display slower MyHC and higher oxidative capacity (19, 39, 40). Fast type IIb fibers, on the other hand, display high energy utilization characteristics and rely more on glycolytic metabolism to derive ATP while type IIx fibers lay somewhere in between these two classes in terms of the source of their energy supply. Accordingly, it seems reasonable to argue that utrophin A expression is directly related to the oxidative capacity and mitochondrial content of the fibers as opposed to their speed

of contraction. Interestingly, this relationship may even extend to the subcellular level since, similar to utrophin A, mitochondria and enzymes of oxidative metabolism accumulate within the postsynaptic sarcoplasm (41).

Given that type I and IIa fibers have smaller diameters, an alternative explanation is that utrophin expression may be related to the size of individual muscle fibers. However, our experiments employing functional overload allowed us to rule out this possibility since it is well established that functional overload, which caused a significant increase in utrophin A expression, results in a dramatic doubling in the size of all MyHC fiber types (19, 20, 23). In fact, the shift towards slower MyHC profiles and improved oxidative metabolism (21, 32) that also occurs within overloaded muscles fits nicely with our view that utrophin A expression correlates with the oxidative capacity of muscle fibers.

One of the key questions that arise based on these findings concerns the nature of the signaling pathways involved in controlling utrophin A expression in oxidative fibers as well as in muscle subjected to functional overload. One common feature, due to their recruitment properties, is that oxidative fibers and overloaded muscle fibers likely present sustained levels of intracellular calcium (35, 42). In this context, it is well established that calcium activates calcineurin via calmodulin which in turn affects muscle characteristics by stimulating the expression of a slower and more oxidative phenotype (20, 32, 43, 44). In our experiments, inhibition of calcineurin with CsA caused a large decrease in utrophin A expression. This result is in fact in excellent agreement with previous findings showing a reduction in oxidative capacity (37, 45) and an increase in the percentage of fast fibers in CsA-treated rodents (26, 37,). Furthermore, in our study,

we also showed that muscles from transgenic mice expressing a constitutively active form of calcineurin contained ~ 4-fold more utrophin A mRNA. Since it has been shown that mice which express an activated form of calcineurin harbor a greater proportion of slower and oxidative fibers (34, 46), these results further support the link between utrophin A expression and the profile of muscle fibers, and clearly implicates calcium and calcineurin as key mediators.

It is now well documented that the slower, oxidative myofiber program is under the influence of sustained calcium influx (caused by tonic electrical activity) which in turn, can activate calcium-dependent transcription pathways such as the calcineurin/NFAT signaling cascade (26, 37). Calcineurin dephosphorylates NFAT factors allowing them to travel to the nucleus where they can exert their transcriptional effects by binding to the promoter region of target genes (26, 36, 44, 46, 47). We have shown in the present study, that the utrophin A promoter contains an NFAT binding site, and that calcineurin and NFATc1 can increase the transcriptional activity of this promoter in cultured myogenic cells as well as in muscle in vivo. In this context, we have previously shown that post-transcriptional events acting via the 3' untranslated region (UTR) of utrophin transcripts can, at least partially, account for the increased expression of utrophin transcripts in slow versus fast muscles (17). Together with the findings of the present study, these results indicate that the regulation of utrophin A in extrasynaptic compartments of slow, oxidative muscle fibers involves the complex interplay between transcriptional and post-transcriptional mechanisms. In one possible scenario, calcineurin could increase the transcriptional activity of the utrophin A promoter while also affecting through an unknown signaling cascade, the stability of existing transcripts.

One of the questions raised by our findings concerns the functional significance of having more utrophin A in the extrasynaptic compartment of slow, oxidative fibers. Although there are no clear data suggesting a preferential role in slow fibers for utrophin A, several possibilities may be envisaged. For example, it is possible that slow fibers may contain more dystroglycan thereby allowing for the association of extrasynaptic utrophin with available binding sites at the sarcolemma. In addition, it appears plausible that utrophin-containing dystrophin complex may associate with different signaling molecules in contrast to complexes containing dystrophin. A third possibility is that utrophin containing complexes may be more or less stable than those containing dystrophin. Finally, the differential binding characteristics of utrophin versus dystrophin for actin may result in differences in the remodelling of actin filaments in response to mechanical stimulation (48, 49).

The results of the present study may have some important clinical implications. For example, fast IIb fibers from DMD muscles are known to be more prone to degeneration (18) while the abundance of MyHC IIx transcripts is reduced in DMD patients (50). It appears likely therefore that based on our findings, fibers expressing MyHC I and IIa can somewhat compensate for the lack of dystrophin by allowing utrophin A to interact with the dystrophin complex at the sarcolemma. This could indeed be the case since as part of the current study; we have observed that expression of utrophin A in mdx mouse muscle is fiber type-specific. In support of this, it is important to note also that a slow muscle from the mdx mouse contains more utrophin than fast muscles (for example 51). As expected on the basis of the foregoing discussion, mdx EDL muscles are known to be more sensitive to the effects of eccentric contractions than

soleus muscles (52). Importantly, this greater sensitivity of mdx EDL fibers to mechanical stress is rescued upon transgenic expression of utrophin in fast muscles (53). Together, these observations suggest that utrophin A may therefore serve a protective role in slower, oxidative fibers from DMD patients.

The reduction in utrophin A expression following calcineurin inhibition with cyclosporine is troubling particularly since previous studies have used calcineurin-inhibiting immunosuppressants in various therapeutic strategies for DMD. Based on our findings, it appears that the use of these calcineurin inhibitors could somewhat mask any beneficial effects linked to particular DMD therapies. Finally, the identification of calcineurin and NFAT as signaling molecules involved in controlling utrophin A expression in muscle provides a unique opportunity to design pharmacological interventions aimed at increasing the endogenous levels of utrophin in muscle fibers from DMD patients.

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Chapter 3

Calcineurin/NFAT Signaling, Together with GABP and PGC-1α, Drives Utrophin Gene Expression at the Neuromuscular Junction

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Contribution from authors

Lindsay Angus and Bernard Jasmin wrote majority of manuscript. Lindsay Angus performed promoter A utrophin NFAT mutation analysis, Chromatin immunoprecipitation assays, immunoprecipitation assays, and cell culture transfections. Joe V. Chakkalakal wrote portions of the manuscript, assisted in immunofluorescence analysis for CnA, NFATc1, PGC-1α, cell culture transfections, performed immunofluorescence analysis for utrophin A, performed western blot for HA-tagged PGC-1α, and performed RT-PCR analysis on PGC-1α stable cells. Alexander Mejat and Laurent Schaeffer perfomed electroporations, and RT-PCR analysis of synaptic regions. Joe Eibl, Eva Chin and Robin Michel provided muscles from CsA treated and CnA* transgenic mice, as part of an on-going collaboration. Eva Chin provided pCnA* plasmids. Lynn Megeney provided plasmids containing transgenes encoding for constitutively nuclear NFATc1.

ABSTRACT

We examined whether calcineurin/NFAT signaling plays a role in specifically directing the expression of utrophin in the synaptic compartment of muscle fibers. Immunofluorescence experiments revealed the accumulation of components of the calcineurin/NFAT signaling cascade within the postsynaptic membrane domain of the neuromuscular junction. RT-PCR analysis using synaptic versus extrasynaptic regions of muscle fibers confirmed these findings by showing an accumulation of calcineurin transcripts within the synaptic compartment. We also examined the effect of calcineurin on utrophin gene expression. Pharmacological inhibition of calcineurin in mice with either cyclosporine A or FK506 resulted in a marked decrease in utrophin A expression at synaptic sites whereas constitutive activation of calcineurin had the opposite effect. Mutation of the previously identified NFAT binding site in the utrophin A promoter region followed by direct gene transfer studies in mouse muscle, led to an inhibition in the synaptic expression of a LacZ reporter construct. Transfection assays performed with cultured myogenic cells indicated that calcineurin acts additively with GABP to transactivate utrophin A gene expression. Since both GABP and calcineurin-mediated pathways are targeted by PGC-1α, we examined whether this co-factor contributes to utrophin gene expression. In vitro and in vivo transfection experiments showed that PGC-1α alone induces transcription from the utrophin A promoter. Interestingly, this induction is largely potentiated by co-expression of PGC-1α with GABP. Together, these studies indicate that the synaptic expression of utrophin is also driven by calcineurin/NFAT signaling and occurs in conjunction with signaling events that involve GABP and PGC-1α.

INTRODUCTION

The neuromuscular junction (NMJ) serves as an excellent model system for examining the events regulating the formation, maintenance and plasticity of synapses primarily due to its simplicity and accessibility in the peripheral nervous system. In particular, considerable emphasis has been placed on the elucidation of the mechanisms responsible for the accumulation of synaptic proteins at the postsynaptic membrane. Transcripts encoding numerous synaptic proteins, including the acetylcholine receptor (AChR) subunits, acetylcholinesterase (AChE) and utrophin, have been shown to accumulate at the NMJ as a result of the local transcriptional induction of their genes within subsynaptic nuclei (for review see Sanes and Lichtman, 2001; Burden, 2002; Mejat et al., 2003; Sunesen and Changeux, 2003). A current model to explain this local transcriptional control posits that nerve-derived factors such as neuregulin, bind to receptors located on the postsynaptic membrane and initiate a series of signaling events that ultimately result in the local activation of synaptic genes. In this context, several studies have highlighted the importance of the ets-related transcription factor GABP and of its DNA binding site, termed the N-box, in regulating synaptic gene expression in muscle fibers (for review see Burden, 2002; Mejat et al., 2003). However, not all synaptic genes contain an N-box motif in their 5' regulatory regions, nor are all synaptically expressed genes dependent upon GABP for their expression (de Kerchove et al., 2002), indicating that additional pathways contribute to synapse-specific gene expression in muscle. Furthermore, expression of several synaptic proteins in muscle is not restricted to the NMJ but clearly extends into extra-synaptic compartments of these cells (see for example Michel et al., 1994; Kues et al., 1995; Krejci et al., 1999).

Recently, calcineurin/NFAT signaling has emerged as a key pathway controlling expression of contractile proteins and oxidative enzymes along muscle fibers leading to the notion that calcineurin/NFAT controls the slower, more highly oxidative and metabolically efficient myogenic program (see for review Olson and Williams, 2000; Schiaffino and Serrano, 2002; Bassel-Duby and Olson, 2003; Rothermel et al., 2003; Schulz and Yutzey, 2004; Michel et al., 2004). In this context, utrophin is a large cytoskeletal protein that displays a high degree of sequence identity with dystrophin (see for review (Krag et al., 2001; Blake et al., 2002; Jasmin et al., 2002). In contrast to dystrophin, which is present at the sarcolemma along muscle fibers, utrophin accumulates preferentially at the NMJ where it appears to participate in the maturation of the postsynaptic apparatus. In a series of recent studies, we documented the expression of utrophin in extra-synaptic regions of muscle fibers, and further demonstrated that such expression occurred specifically in slower (type I and IIa), more highly oxidative fibers. Additionally, we showed that expression of utrophin outside junctional compartments is under the direct influence of calcineurin/NFAT signaling (Chakkalakal et al., 2003); see also (Cohen and Randall, 2004; Lee et al., 2004).

Based on these observations, we decided to examine whether the calcineurin/NFAT pathway plays a role in specifically regulating utrophin gene expression at the NMJ. In addition, we investigated whether calcineurin cooperates with the well characterized GABP/N-box pathway to further stimulate utrophin gene expression in skeletal muscle.

MATERIALS AND METHODS

Animal Care and Protocols. All care and experimental procedures were performed in accordance with the Canadian Council of Animal Care guidelines and approved by the Institutional Animal Care and Research Advisory Committee (Laurentian University) or by the University of Ottawa Animal Care and Use Committee or in accordance with recommendations made by the Comite Rhone Alpes d'Ethique pour l'Experimentation Animale.

Here, CD-1 mice were treated twice daily for 14 days with either cyclosporine A (CsA; 25 mg/kg, s.c.), FK506 (5 mg/kg, s.c.) or vehicle (as controls) as previously described (Dunn et al., 1999). To verify that drug treatments were effective, plasma levels of CsA or FK506 were measured (Toronto Medical Laboratories) following two weeks of administration. In CsA-treated animals, plasma levels were 808 ± 70 ng/ml as compared to vehicle-treated animals (<30 ng/ml). In FK506-treated animals, plasma levels were 26.9 ± 1.7 ng/ml as compared to vehicle-treated animals (<2 ng/ml). Open field testing was also performed and all animals, regardless of treatment, showed comparable levels of locomotor activity. Two weeks later, muscles were excised, frozen and subsequently processed for immunofluorescence (see below). Muscles from transgenic mice overexpressing a constitutively active form of calcineurin (CnA*) were also harvested (Dunn et al., 2000) and their muscles similarly processed for immunofluorescence. In these experiments, all surgical procedures were performed under pentobarbital anaesthesia.

In separate experiments, diaphragm muscles from OF1 mice were isolated and synaptic versus extrasynaptic regions were visualized by AChE staining (Karnovsky and

Roots, 1964). Synaptic and extrasynaptic regions from stained tissues were micro-dissected under binocular magnification, and total RNA was extracted as described below. For these experimental procedures, mice were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally. In these experiments, similar observations were obtained using mice of different strains and following procedures performed in the different contributing laboratories.

In Vivo Electroporation. Five μg of total plasmid DNA (pcDNA3 used as a control vector or the same vector containing a cDNA for the peroxisome proliferator-activated receptor-γ co-factor-1α (PGC-1α) carrying also a HA tag, and termed pcDNA3-HA-hPGC1α, (Knutti et al., 2000) and 2 μg of pECFP-Nuc (Clontech, Palo Alto, CA) resuspended in 30 μl of 0.9 % NaCl, were injected in tibialis anterior (TA) muscles of 6 week-old male OF1 mice anesthetized with intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). One cm2 plaque electrodes were then placed on each side of the leg and eight 200 V/cm pulses of 20 ms were applied to the injected muscles at 2 Hz. Seven days after injection, the injected muscles were micro-dissected under a fluorescence binocular microscope (SZX12, Olympus) to isolate CFP-positive fibers, i.e, those successfully transduced and expressing the plasmids, and to determine the levels of several mRNAs encoding synaptic proteins in these single muscle fibers (see below). Specifically, electroporated TA muscles were quickly dissected in 1X PBS. Groups of CFP-positive fibers containing 10 to 20 fibers, were isolated using ultrafine forceps (Moria, N°5). Non-positive extremities of the fibers were eliminated and CFP-positive

portions of the muscle were placed in RLT buffer (RNeasy mini Kit, Qiagen, Chatsworth, CA) to prevent RNA degradation.

RNA Extraction and Real Time RT-PCR. Real time RT-PCR was performed using total RNA isolated from synaptic and extrasynaptic regions of diaphragm muscles, and from micro-dissected CFP-positive fibers, with the RNeasy mini (Qiagen, Chatsworth, CA) RNA extraction kit according to the manufacturer's recommendations with the addition of proteinase K and DNase treatments. Reverse transcription was performed using the superscript II kit (Invitrogen Life Technologies, Carlsbad, CA) with random hexamer primers. A 1 μl sample of 1:10 diluted cDNA was analyzed by real-time quantitative PCR (Light Cycler, Roche) using QuantiTect SYBR Green PCR kit (Qiagen, Chatsworth, CA) as described by the manufacturer in the presence of 1 μM of the respective forward and reverse primers. The sequences of the primers were as follows: β-actin forward 5'-ccctgtatgcctctggtcgt-3', reverse 5'-atggcgtgagggagagcat-3'; ErbB3 forward 5'-cttacgggacacaatgctga-3', reverse 5'-gcatggctggagttggtatt-3'; AChR-α subunit forward 5'-acctggacctatgacggctct-3', reverse 5'-agttactcaggtcgggctggt-3'; AChR-ε subunit forward 5'-cttggtgctgctcgcttactt-3', reverse 5'-cgttgatagagaccgtgcatttc-3'; calcineurin A forward 5'-cgattctccgacaggaaaaa-3', reverse 5'-aaggcccacaaatacagcac-3'; GABP-α forward 5'-ggggaacagaacaggaaaca-3', reverse 5'-ccgtaatgcacggctaagtt-3'; GABP-β forward 5'-ctccgagccggtgtaagtag-3', reverse 5'-cctccagcttctgcctgtag-3'; UCP3 forward 5'-atgagttttgcctccattcg-3', reverse 5'-ccagttcccaagcgtatcat-3'; cytochrome c forward 5'-ccaaatctccacggtctgtt-3', reverse 5'-gtctgccctttctccctttct-3'; cytochrome oxidase IV forward 5'-actacccctttgcctgatgtg-3', reverse 5'-gcccacaactgtcttccatt-3';

utrophin A forward 5'-ggcaggaagattgcacaagt-3', reverse 5'-ctgctagccaagtcccagag-3'; and hsp48 forward 5'-cgggaaagagctgaaaattg-3', reverse 5'-agaatccgacaccaaactgc-3'.
In the case of micro-dissected CFP-positive fibers obtained following in vivo electroporation, relative cDNA measurements of synapse-specific genes, i.e., utrophin, AChR α and ε, were normalized to presynaptic ErbB3 cDNAs. The expression of all other transcripts was normalized to β-actin cDNAs. In the case of synaptic and extrasynaptic whole diaphragm samples, relative cDNA measurements were also normalized to β–actin cDNA levels.

Immunofluorescence Staining. Cryostat sections of TA or plantaris muscles from CD-1 or C57BL/6 mice were labeled to detect by immunofluorescence the presence of calcineurin A (AB1698, Chemicon International, Temecula, CA), NFATc1 (sc-7294, Santa Cruz Biotechnology, Santa Cruz, CA), utrophin A (Chakkalakal et al., 2003) or PGC-1α (sc-13067, Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (Chakkalakal et al., 2004;Chakkalakal et al., 2003). To localize NMJs, AChR were labeled with Alexa 488-conjugated α–bungarotoxin (B-13422, Molecular Probes, Eugene, Oregon). Quantitation of protein expression at the NMJ was performed using muscle cryostat sections from 3 different animals as described in detail elsewhere (Arikawa-Hirasawa et al., 2002). Briefly, longitudinal sections of plantaris muscles taken from wildtype, CnA*, vehicle-, CsA- and FK506-treated animals were mounted on the same slide to ensure that the fluorescent intensities were taken at comparable exposures with similar background. The intensity of utrophin A staining was quantified using the Northern Eclipse Imaging system and values were standardized to the area occupied by

the NMJ as determined by α-bungarotoxin staining. These procedures were repeated on 3 separate occasions using muscles taken from 3 different animals per experimental group to account for differences in variability.

Cell Culture. Mouse C2C12 muscle cells (ATCC, Manassas, VA) were cultured on Matrigel (Collaborative Biomedical Products, Bedford, MA)-coated plates and maintained in Dulbecco's modified Eagle's medium (Life Sciences/Life Technologies, Inc., Burlington, Ontario) supplemented with 10% fetal bovine serum, 20% horse serum and 100units/ml penicillin-streptomycin at 37OC and 5% CO2. Culture media were changed every 48 hours.

Stable Expression of PGC-1α in Myogenic Cells. C2C12 cells were transfected with 1 υg of pcDNA3-HA-hPGC1α using LipofectAMINE according to the manufacturer's instruction (Invitrogen Life Technologies, Carlsbad, CA). Pooled stable transfectants were selected in D-MEM containing 1 mg/ml G-418 (Gibco BRL, Grand Island, NY). Cells designated as 'control' were stably transfected with an empty vector. Western blot analysis was performed using anti-HA antibody (H 9658, Sigma, St Louis, MO) to confirm that the cells were overexpressing PGC-1α.

InVitro Transfections. C2C12 myoblasts were transfected using LipofectAMINE (Invitrogen Life Technologies, Carlsbad, CA). The utrophin plasmids used for transfections included a 1.3 kb utrophin A-specific promoter region linked to a lacZ reporter gene, designated UA (see (Gramolini et al., 1997) and a mutated version of UA,

designated mutUA, in which the previously identified upstream NFAT binding site (Chakkalakal et al., 2003) was mutated by site-directed mutagenesis such that tattgGAAaaca was altered to tattgCTTaaca (mutation is underlined). Mutagenesis was performed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen Life Technologies, Carlsbad, CA). Expression vectors for GABPα, GABPβ, PGC-1α, a constitutively active form of calcineurin (CnA*), and a nuclear-localized form of NFATc1 (nNFATc1) were co-transfected in various combinations with UA or mutUA. These various plasmids have been described elsewhere (Chakkalakal et al., 2003;Schaeffer et al., 1998;Dunn et al., 2000;Knutti et al., 2000). To control for the efficiency of transfection, a plasmid encoding the chloramphenicol transferase (CAT) gene driven by the SV40 promoter (Promega, Madison, WI) was always co-transfected. Following transfection, the myoblasts were maintained for 24 hrs in growth medium and then harvested for analysis of reporter gene expression by quantitative RT-PCR analysis. In additional experiments, cells were transfected with the CnA* expression vector and treated with 5 μM CsA for 48 hrs prior to harvesting in order to demonstrate that activity of CnA is necessary for the observed effects.

RNA Extraction and RT-PCR. RNA was extracted from transfected cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells were lysed with 1 ml of Trizol per 35mm dish and mixed with chloroform prior to centrifugation at 12,000 X g. Isopropanol was then used to precipitate the RNA from the aqueous layer and subsequent washes of the RNA pellets with ethanol were performed. To eliminate contaminating traces of plasmid DNA

(Gramolini et al., 2001), the RNA samples were treated with DNase I (Fermentas Life Sciences, Burlington, ON) as recommended by the manufacturer. Briefly, aliquots of RNA were treated with DNase I for 2.5 hrs at 37^{O}C and then re-isolated by phenol-chloroform extraction. Total RNA was quantified using the Amersham Pharmacia Biotech Gene Quant II RNA/DNA Spectrophotometer and subjected to quantitative RT-PCR as described elsewhere (Gramolini et al., 2001;Chakkalakal et al., 2003). Briefly, RT was performed for 45 min at 42OC with 100 ng of total RNA using random hexamer primers. The resulting LacZ and CAT cDNAs were then amplified by PCR as described previously (Gramolini et al., 2001;Chakkalakal et al., 2003). The relative abundance of the PCR products was assessed by agarose gel electrophoresis, using ethidium bromide-staining for visualization and the fluorescent dye VistraGreen (Amersham Pharmacia Biotech, Arlington Heights, IL) for quantification. The efficiency of all transfections was assessed by analyzing the expression of a co-transfected plasmid encoding the CAT gene and normalizing LacZ reporter-gene expression to CAT mRNA expression. In all experiments, negative controls, consisting of RNase-free water instead of RNA and samples in which the reverse transcriptase was omitted, were always performed and never revealed the presence of contaminating transcripts or cDNAs.

Direct Gene Transfer. Direct gene transfer experiments were performed as previously described (Gramolini et al., 1997;Gramolini et al., 1998). Briefly, 50 μg of plasmid DNA containing either the UA or mutUA promoter-reporter gene constructs were directly injected into the TA muscle of 4 week-old C57BL/6 mice. During this experimental procedure, mice were anesthetized by exposure to halothane gas and were continuously

monitored for a response to tail and toe pinch in order to ensure that an acceptable level of anesthesia was maintained. Seven days later, the mice were euthanized with a lethal dose of pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario) and the muscles were excised and frozen in melting isopentane. Cryostat sections were later processed for β-galactosidase (β-gal) and AChE activity to identify expression of the reporter gene and to localize NMJs, respectively. The percentage of synaptic events was quantified as previously described elsewhere in detail (Duclert et al., 1996;Gramolini et al., 1997).

Statistical Analysis. Statistical analysis was performed using analysis of variance or Student's t-tests, and the statistical significance was set at $P < 0.05$. A minimum of three independent experiments were performed and the data are presented as means ± SEM.

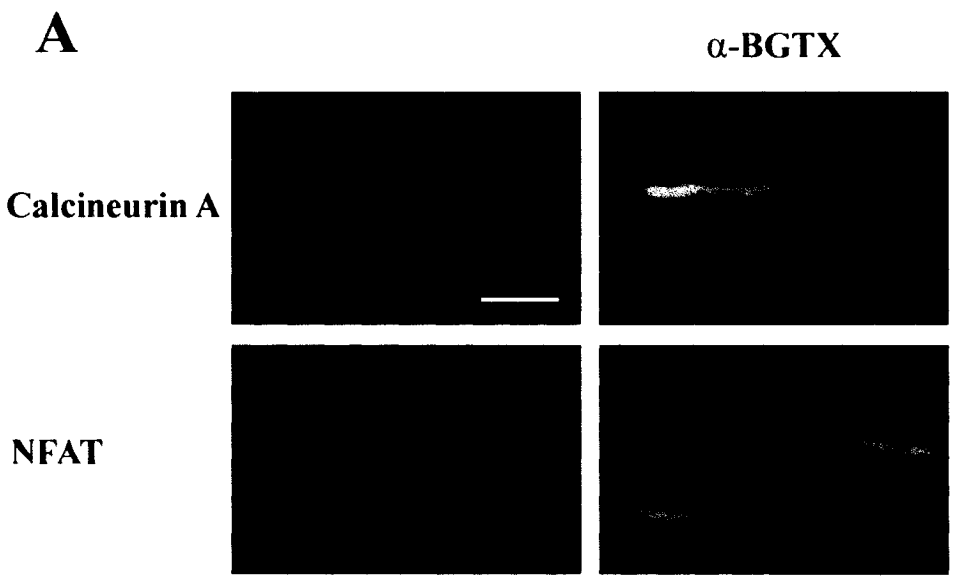
RESULTS

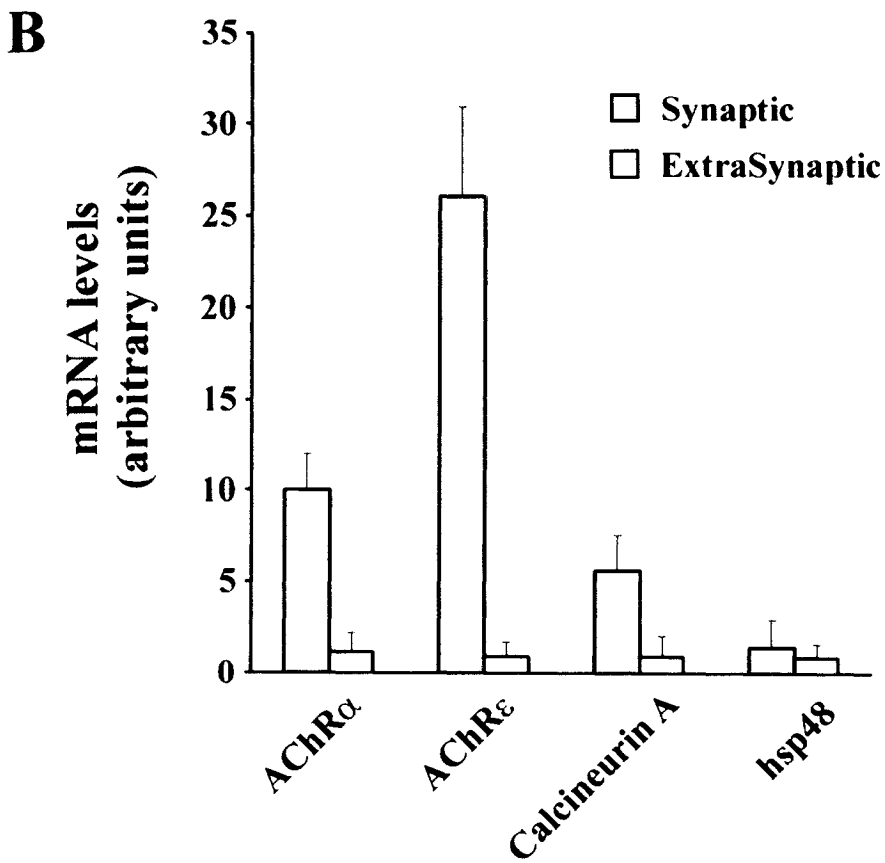
Calcineurin/NFAT Regulates Utrophin Gene Expression at the Neuromuscular Junction

In the present study, we sought to determine if the calcineurin/NFAT pathway is active within the postsynaptic membrane domain of the NMJ and whether it specifically regulates utrophin gene expression. To address this issue, we initially tested for localization of components of the calcineurin pathway at the NMJ and examined whether perturbations of this pathway alter the synaptic expression of utrophin. Immunofluorescence experiments performed on cryostat sections showed, as expected, diffuse expression of both calcineurin and its downstream effector NFATc1 throughout extrasynaptic regions of muscle fibers (not shown). However, we observed a more extensive labelling of calcineurin and NFATc1 in junctional regions identified by α-bungarotoxin labelling of AChR (Figure 3.1A). A similar staining pattern for calcineurin was also observed in short-term (24-48 hours) denervated muscle indicating that the observed calcineurin staining is not associated with presynaptic structures (results not shown).

To confirm these findings, we performed a series of RT-PCR analyses using total RNA isolated from micro-dissected synaptic and extrasynaptic regions of whole hemi-diaphragm muscles. Using this sensitive technique, we focused on the relative abundance of calcineurin A mRNA rather than protein levels due to the limited size of the micro-dissected samples. As shown in Figure 3.1B, isolation of synaptic regions was confirmed by the presence of a considerable amount of transcripts encoding the AChRα and ε

Figure 3.1. Localization of Calcineurin and NFAT at the Neuromuscular Junction. Double fluorescence experiments were performed using Alexa 488-conjugated α-bungarotoxin to localize NMJs via AChR labelling and antibodies to either calcineurin or NFATc1. (A) shows the accumulation of both calcineurin and its downstream effector NFATc1 in synaptic regions of muscle fibers. Bar in (A) represents 25 μm. (B) corresponds to the results of RT-PCR analysis performed on synaptic versus extrasynaptic regions of diaphragm muscles. All data were standardized according to the amount of β-actin mRNA present in the same samples. Note that in agreement with the immunofluorescence data, there is an enrichment of calcineurin A transcripts in synaptic compartments of muscle fibers.


A
α-BGTX
Calcineurin A
NFAT

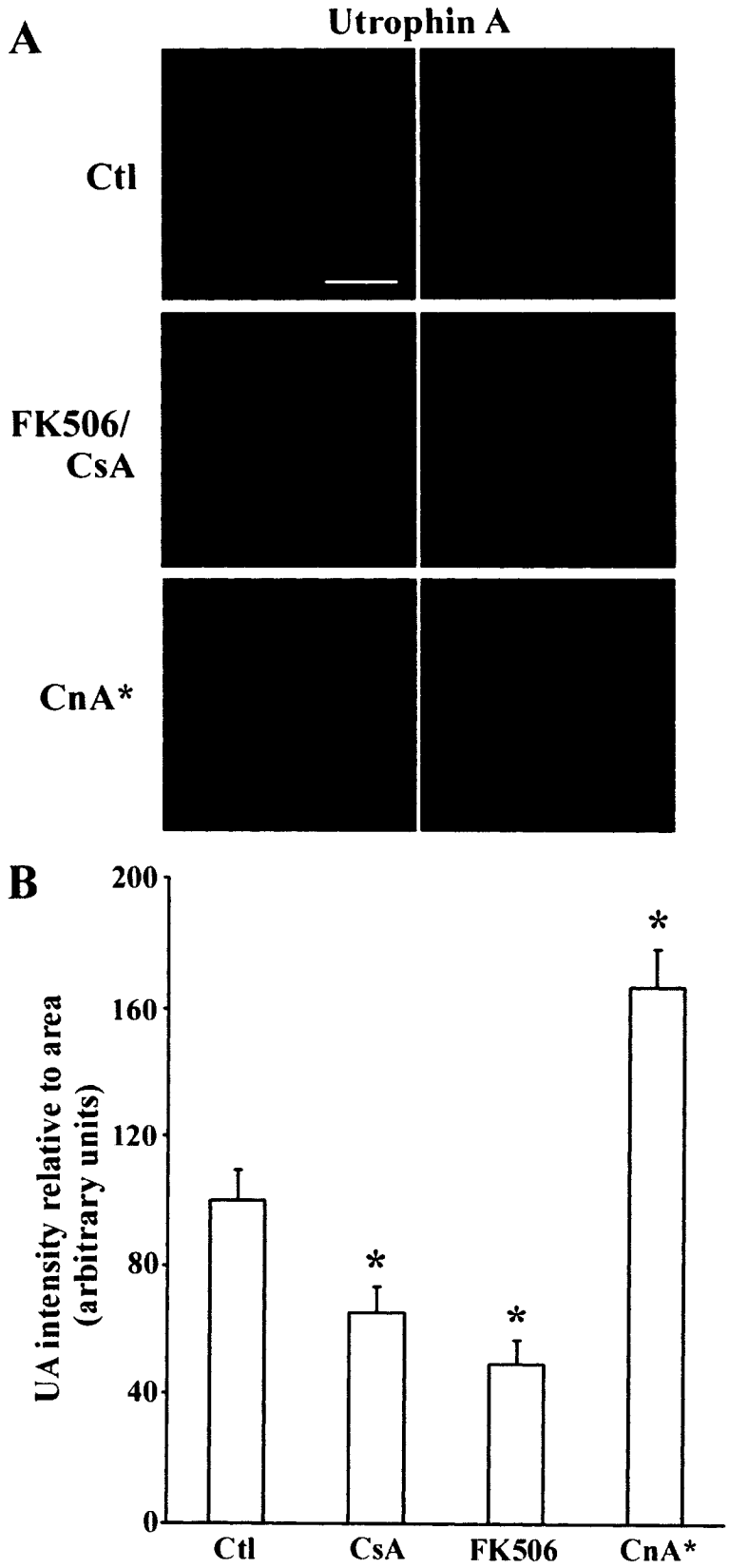

B
mRNA levels
(arbitrary units)
35
30
25
20
15
10
5
0
Synaptic
ExtraSynaptic
AChRα
AChRε
Calcineurin A
hsp48

subunits in these samples. These experiments also revealed an enrichment of mRNAs encoding calcineurin A in synaptic versus extrasynaptic regions of muscle fibers. By contrast, hsp48 mRNAs were relatively equal between synaptic and extrasynaptic compartments. Together, these experiments show that protein components of the calcineurin pathway along with their transcripts accumulate at the NMJ.

Next, we sought to determine whether the calcineurin pathway actively regulates utrophin expression at the NMJ. To this end, we pharmacologically inhibited the calcineurin pathway and subsequently examined the pattern of utrophin expression within the postsynaptic sarcoplasm by immunofluorescence. Mice were thus treated with the calcineurin inhibitors CsA or FK506 and two weeks later, plantaris muscles were excised and the synaptic expression of utrophin A assessed by immunofluorescence. Immunofluorescence experiments are necessary over Western blots in this case since: i) we were interested in specifically examining the sub-cellular localization of utrophin following these drug treatments; and ii) postsynaptic membrane domains occupy less than 0.1% of the total surface of muscle fibers.

As expected, we observed a strong accumulation of utrophin A at the neuromuscular junctions in plantaris muscles obtained from control, vehicle-treated animals (Figure 3.2A). In sharp contrast however, the synaptic expression of utrophin A was markedly reduced in muscles from either CsA- or FK506-treated mice (Figure 3.2A). Quantitative analyses revealed that the intensity of utrophin A staining at the NMJ was decreased by 35-50% ($P < 0.05$) under these two conditions (Figure 3.2B). Of note, a similar reduction in the expression of AChRα protein was also observed at the NMJ under these conditions (25-30%; $P < 0.05$). Additional experiments showed that the

Figure 3.2. Calcineurin Regulates Expression of Utrophin A at Synaptic Sites. Double fluorescence experiments were performed on cryostat sections of muscles from control (Ctl), FK506- or CsA-treated mice, and MCK-CnA* (CnA*) over-expressing transgenic mice to detect expression of utrophin A specifically at NMJs. Panels in (A) represent utrophin A staining at NMJs as determined by co-incident AChR staining using α-bungarotoxin (not shown). Note the pronounced reductions in utrophin A expression in synaptic regions of muscles following inhibition of calcineurin with FK506 or CsA, and the increased synaptic expression of utrophin A (UA) in CnA* transgenic mice (B). Bar in (A) represents 50 μm. Astericks represent a significant difference ($P < 0.05$) versus control.


A
Utrophin A
Ctl
FK506/
CsA
CnA*
B
UA intensity relative to area
(arbitrary units)
200
160
120
80
40
0
*
*
*
Ctl
CsA
FK506
CnA*

reduction in utrophin A staining following these drug treatments, was paralleled by a 30-35% reduction ($P < 0.05$) in the relative abundance of utrophin A transcripts in the synaptic compartment of treated versus control (vehicle) diaphragm samples as determined by quantitatitve RT-PCR assays. Finally, immunofluorescence experiments conducted on muscles from transgenic mice overexpressing a constitutively active form of calcineurin (CnA*) showed in this case, in agreement with the calcineurin-inhibition experiments, an increase in utrophin A staining at the NMJ (65%; $P < 0.05$, Figure 3.2B). Thus, perturbations of the calcineurin pathway clearly affect expression of utrophin A at the mammalian NMJ.

To determine whether these effects of calcineurin were a direct consequence of transcriptional changes in the activity of the utrophin gene specifically within synaptic myonuclei, we mutated the consensus NFAT binding site that we previously identified in the utrophin A promoter region (Chakkalakal et al., 2003). In vitro studies confirmed that the mutated utrophin A promoter abrogated expression of the reporter LacZ gene following NFAT overexpression in cultured myogenic cells (see below). To assess whether this NFAT site is involved in directing synapse-specific expression of the utrophin gene in vivo, we directly injected mouse TA muscles with either wild-type (designated UA) or mutated (designated mutUA) promoter-reporter gene constructs. For these experiments, we chose to inject a fast muscle in order to avoid the contaminating effects of extra-synaptic utrophin A expression, which is characteristic of slower (type I and IIa), more oxidative fibers (see (Chakkalakal et al., 2003). Seven days later, we determined the pattern of expression of the LacZ gene along muscle fibers using a quantitative approach previously described (Duclert et al., 1996; Gramolini et al., 1997).

Expression of the reporter gene as well as the presence of NMJs in these cryostat sections can be easily monitored by histochemical staining of β-gal (which accumulates in myonuclei due to the presence of an nls) and AChE activity. Every observed cluster of myonuclei staining positively for β-gal was considered an event, and co-incident β-gal staining of myonuclei with AChE staining of NMJ indicated a synaptic event (see (Duclert et al., 1996). In these experiments, we noted in agreement with previous findings (see Gramolini et al., 1997), that approximately 75% of all the events associated with expression of the reporter gene driven by the wild type utrophin A promoter occurred in synaptic regions. Mutation of the consensus NFAT binding site considerably reduced ($P < 0.05$) the percentage of synaptic events by 40% thereby indicating that the NFAT site located within the utrophin A promoter region specifically contributes to the synaptic expression of this gene (data not shown). Taken together, these results demonstrate the calcineurin/NFAT pathway actively regulates the expression of utrophin at the NMJ by stimulating the synapse-specific transcription of the utrophin gene.

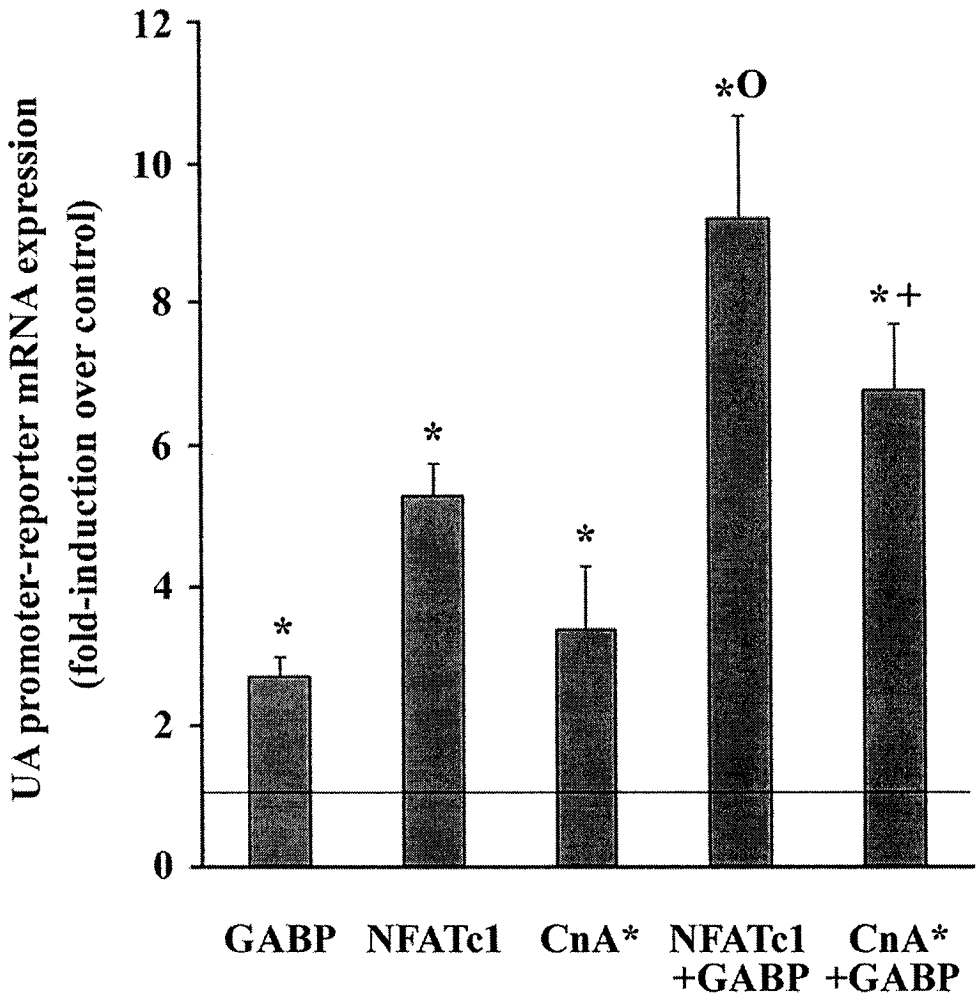
Additive Effects of Calcineurin and GABP on Utrophin Gene Expression

Both the calcineurin/NFAT and GABP/N-box pathways are known to transcriptionally regulate the expression of the utrophin gene (see Introduction). Since both pathways are active at the level of the post-synaptic membrane domain (this study; and (Gramolini et al., 1999; Khurana et al., 1999), we sought to determine whether these pathways cooperate in the induction of utrophin gene expression. To this end, we capitalized on the use of cultured C2C12 myogenic cells which have been extensively used in the past to study synaptic gene regulation and post-synaptic membrane

differentiation. To address this issue, cultured myoblasts were first co-transfected with the UA promoter-reporter gene construct and expression vectors encoding a constitutively active form of calcineurin (CnA*) or a nuclear-localized form of NFATc1 (nNFATc1). As observed previously (see Chakkalakal et al., 2003), CnA* or nNFATc1 expressed alone resulted in an ~ 3.0-5.5-fold ($P < 0.05$) increase in UA promoter-reporter gene expression (Figure 3.3). We then performed several additional experiments to further highlight the importance of the calcineurin signaling cascade in the regulation of utrophin gene expression. As described above, we first mutated the NFAT site previously identified within the promoter region of the utrophin A gene and confirmed that incorporation of the mutation inhibited the expression of the LacZ reporter gene following NFAT overexpression in C2C12 myoblasts. Moreover, we observed that the enzymatic activity of calcineurin was necessary for these effects since cyclosporine treatment of cells co-transfected with the CnA* expression vector and the utrophin A promoter-reporter construct, prevented this transcriptional induction. Taken together, these results further demonstrate the involvement of the calcineurin/NFAT signaling cascade in the regulation of utrophin A gene expression and show the direct involvement of these cis- and trans-acting elements.

We next determined whether the calcineurin/NFAT pathway co-operates with GABP to induce utrophin gene expression. To address this issue, cultured myoblasts were co-transfected with various combinations of UA promoter-reporter gene constructs and expression vectors encoding CnA*, nuclear NFATc1 (nNFATc1) and GABPα/β. As observed previously (Gramolini et al., 1998;Gramolini et al., 1999;Khurana et al., 1999), expression of GABPα/β alone resulted in an ~ 2.5-fold ($P < 0.05$) increase in UA

Figure 3.3. Calcineurin/NFAT Acts Additively with GABP to Induce Utrophin Gene Expression. C2C12 muscle cells were co-transfected with the wild type utrophin A (UA) promoter-Lac Z reporter gene construct, alone or in various combinations with expression vectors containing GABPα/β, nNFATc1 or CnA*. Following transfection, the levels of reporter gene expression were assessed by RT-PCR. The line across represents the control level of UA promoter-reporter gene expression seen in these cells. To control the efficiency of transfection, a plasmid encoding the CAT gene driven by the SV40 promoter (Promega) was always co-transfected and the LacZ data were standardized accordingly. Astericks represent a difference ($P < 0.05$) versus UA alone; an open circle represents a difference ($P < 0.05$) versus nNFATc1 or GABP alone; and a cross represents a difference ($P < 0.05$) versus CnA* or GABP alone. Mean ± SEM are shown. A minimum of three independent experiments in triplicate were performed.


UA promoter-reporter mRNA expression
(fold-induction over control)
12
10
8
6
4
2
0
*
*
*
*O
*+
GABP
NFATc1
CnA*
NFATc1 +GABP
CnA* +GABP

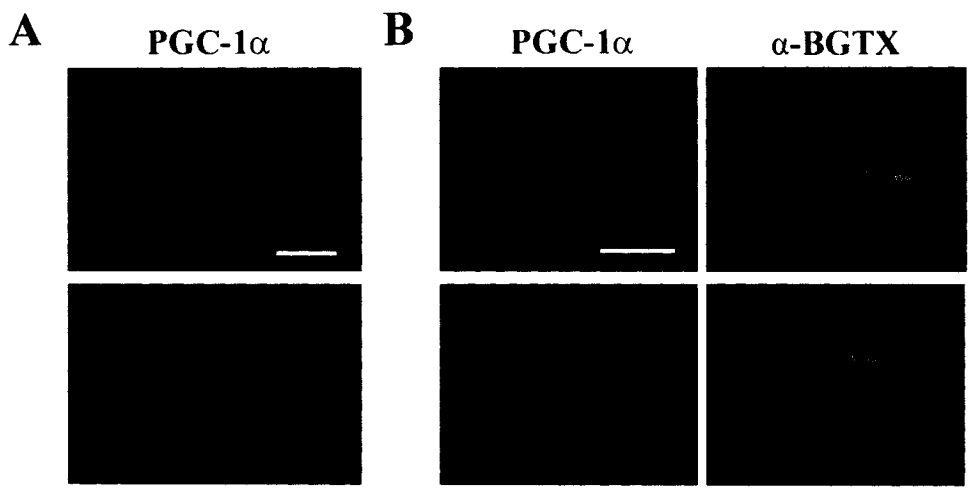
promoter-reporter gene expression (Figure 3.3). Moreover, expression of either CnA* or nNFATc1, with GABPα/β potentiated UA promoter-reporter gene expression to ~7-10-fold of control ($P < 0.05$). These results suggest that the calcineurin/NFAT and GABP/N-box pathways act independently to additively, but not synergistically, stimulate transcription of the utrophin gene at the NMJ.

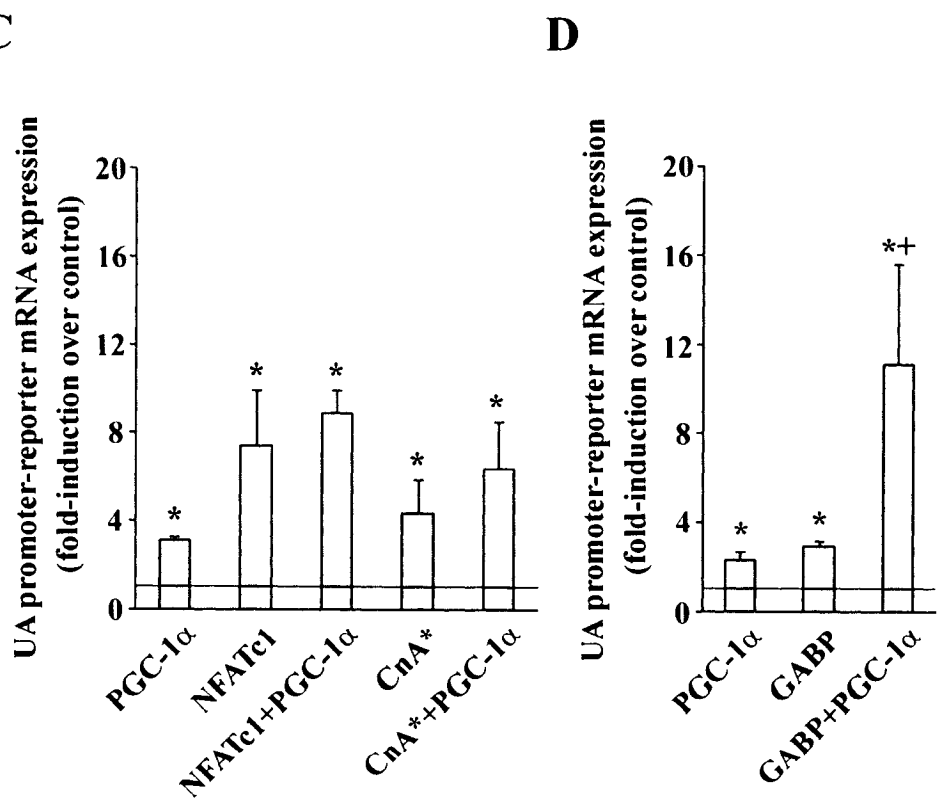
PGC-1α is Synaptically Expressed and Stimulates Expression of Utrophin

The mechanisms by which the co-factor PCG-1α mediates gene expression have been shown to involve both GABP and the calcineurin signaling cascade (Wu et al., 1999;Lin et al., 2002). Given that utrophin expression is under the control of both calcineurin- and GABP-mediated pathways (this study and (Gramolini et al., 1999;Khurana et al., 1999;Chakkalakal et al., 2003), we tested whether PGC-1α is also involved in the regulation of gene expression at the NMJ. To address this issue, we first examined the expression pattern of PGC-1α along muscle fibers by immunofluorescence. As expected (see (Lin et al., 2002)), PGC-1α is expressed throughout muscle fibers (Figure 3.4A). However, we detected a clear accumulation of PGC-1α at the NMJ (Figure 3.4B), suggesting an enhancement of PGC-1α function within the postsynaptic sarcoplasm.

We next determined whether PGC-1α co-activates the effects of known inducers of utrophin gene expression. We thus performed a series of transfection assays in cultured myogenic cells using utrophin A promoter-reporter constructs with PGC-1α alone or with CnA*, nNFATc1 or GABPα/β. PGC-1α alone increased transcription of the UA

Figure 3.4. PCG-1α is Expressed at the NMJ and Acts Synergistically with GABP. Representative examples showing the localization of PGC-1α throughout muscle fibers (both panels in (A)) and its synaptic co-localization (with AChR in (B) panels) in mouse muscles. (C) and (D) show the results of transfection experiments using C2C12 muscle cells and the UA promoter-LacZ reporter gene construct alone or co-transfected with various combinations of expression vectors containing PGC-1α, nNFATc1, CnA* or GABPα/β. Following transfection, the levels of reporter gene expression were determined by RT-PCR. The lines across in (C) and (D) represent the control level of UA promoter-reporter gene expression seen in these cells. To control for the efficiency of transfection, a plasmid encoding the CAT gene driven by the SV40 promoter was always co-transfected and the LacZ data were standardized accordingly. Astericks represent a difference ($P < 0.05$) versus UA alone; and a cross represents a difference ($P < 0.05$) versus GABP or PGC-1α alone. Mean ± SEM are shown. A minimum of three independent experiments in triplicate were performed. Bar in (A) represents 50 μm, bar in (B) represents 25 μm.


A
PGC-1α
B
PGC-1α
α-BGTX

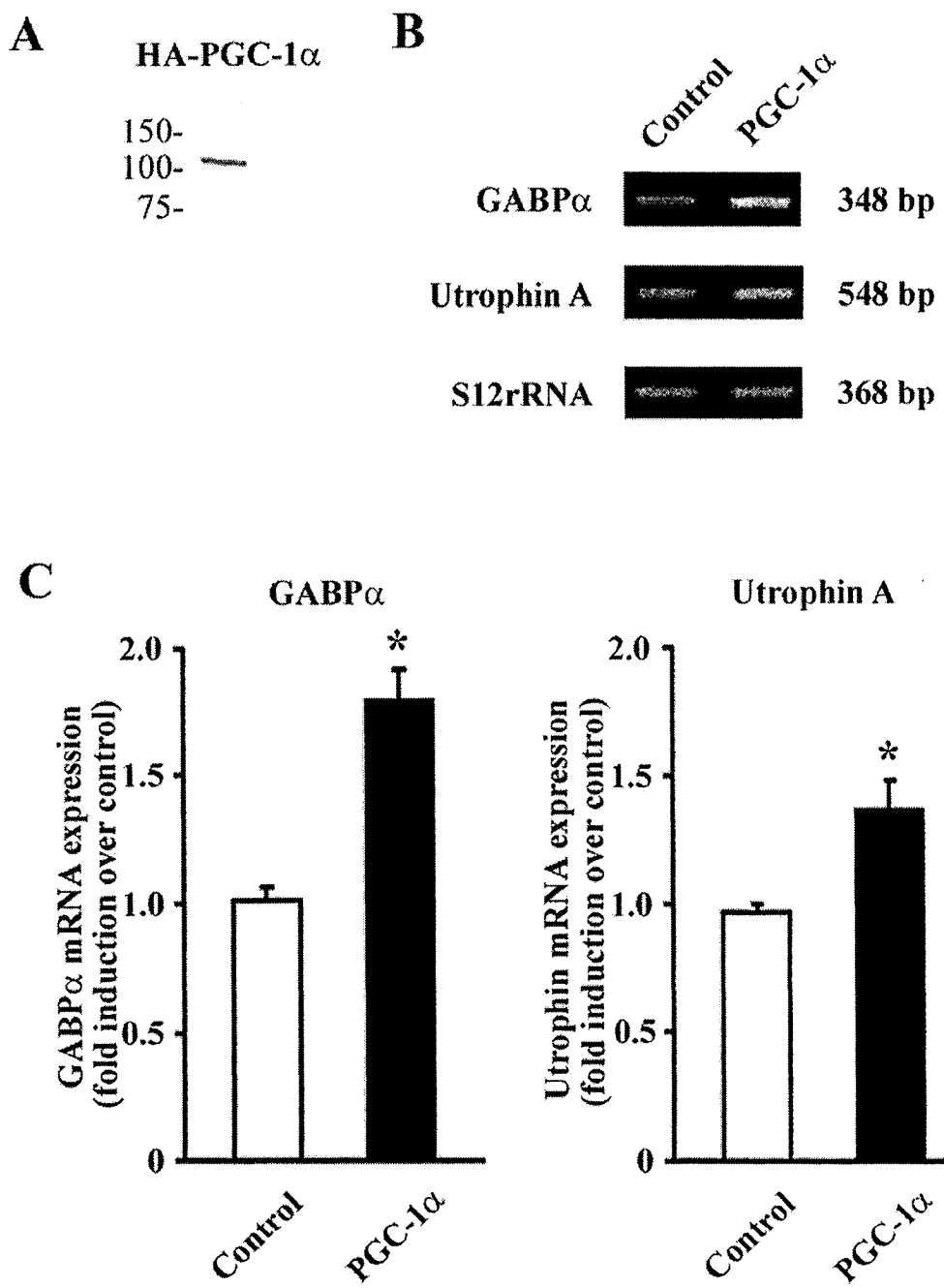

C
D
UA promoter-reporter mRNA expression
(fold-induction over control)
20
16
12
8
4
0
*
*
*
*
*
PGC-1α
NFATc1
NFATc1+PGC-1α
CnA*
CnA*+PGC-1α
UA promoter-reporter mRNA expression
(fold-induction over control)
20
16
12
8
4
0
*
*
*+
PGC-1α
GABP
GABP+PGC-1α

promoter-reporter construct by ~3-fold (Figure 3.4C and D). Co-transfection with an expression vectors for PGC-1α and CnA* or nNFATc1 modestly increased transactivation of the utrophin A promoter (Figure 3.4C). However, co-transfection of PGC-1α and GABPα/β had a dramatic impact on expression of the UA promoter-reporter gene which clearly and significantly ($P < 0.05$) exceeded the effects seen with GABPα/β alone (Figure 3.4D). These results thus indicate that PGC-1α functions predominantly via GABP to enhance utrophin gene expression at the NMJ.

Several mechanisms may be envisaged to explain the effect of PGC-1α on GABP-mediated gene expression, including a direct effect of PGC-1α on GABP gene expression and/or interaction of PGC-1α with GABP. To address these possibilities, we performed two sets of experiments. First, we generated stable myogenic cell lines expressing PGC-1α and examined whether the endogenous mRNA levels of GABPα were increased. Expression of PGC-1α in stably transfected cells was confirmed by Western blot analysis using an antibody recognizing the HA tag (Figure 3.5A). Quantitative RT-PCR assays indicated that stable expression of PGC-1α in myogenic cells increased the expression of GABPα transcripts by approximately 2-fold ($P < 0.05$) as compared to control cells stably transfected with the empty vector (Figure 3.5B and C). As expected based on the promoter-reporter data shown in Figure 3.4, stable expression of PGC-1α also increased the endogenous expression of utrophin A transcripts (Figure 3.5B and C).

Second, PGC-1α may also mediate its effects on utrophin gene expression by interacting with, and potentiating the transactivation potential of GABP. However, consistent with recent reports (Scarpulla, 2002a; Baar, 2004), we were unable in the

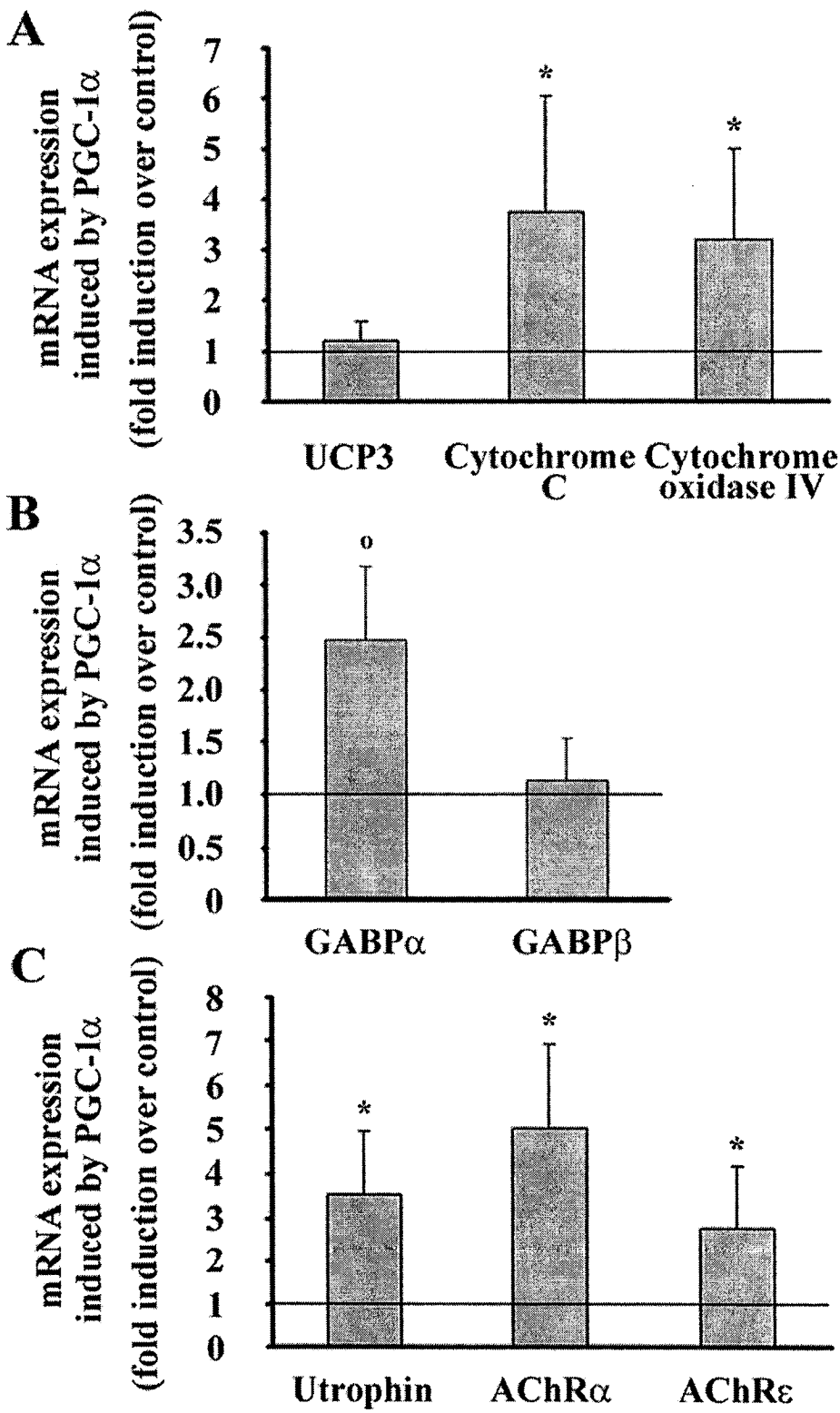
Figure 3.5. PGC-1α Induces Expression of GABPα in Myogenic Cells. C2C12 cells were stably transfected with an expression vector for PGC-1α and endogenous expression of GABP and utrophin transcripts was determined. In (A), PGC-1α expression in stable cell lines was confirmed by Western blot analysis using antibodies against the HA tag present in the construct. In (B) and (C), expression of GABPα and utrophin A transcript levels in cells stably transfected with PGC-1α or an empty vector (used for control cells) was assessed by RT-PCR. In (B), representative ethidium bromide-stained agarose gels showing RT-PCR products are shown. (C) represents the quantitation of the RT-PCR experiments. In these experiments, levels of GABPα and utrophin A mRNAs were normalized using S12 rRNA as a control. Asterick represent significant differences ($P < 0.05$). Mean ± SEM are shown.


A
HA-PGC-1α
150-
100-
75-
B
Control
PGC-1α
GABPα
348 bp
Utrophin A
548 bp
S12rRNA
368 bp
C
GABPα
Utrophin A
GABPα mRNA expression (fold induction over control)
Utrophin mRNA expression (fold induction over control)
2.0
1.5
1.0
0.5
0
*
Control
PGC-1α

present study, to obtain evidence for an interaction between GABP and PGC-1α using co-immunoprecipitation experiments as well as ChIP assays (data not shown) suggesting that the primary mechanism of action of PGC-1α is through stimulation of GABP expression.

Finally, we confirmed the above findings in vivo and extended the influence of PGC-1α to other synaptically-expressed genes. For this analysis, the expression vector for PGC-1α was transduced into mouse muscle fibers by electroporation and individual muscle fibers were micro-dissected and subsequently analyzed by real time RT-PCR for changes in the levels of endogenous transcripts encoding GABPα and β, utrophin A and the model synaptic genes AChRα and β. Control experiments consisted of analyzing transcript levels in single fibers transduced with the empty pcDNA3 plasmid. Figure 3.6A confirmed that expression of PGC-1α induced ($P < 0.05$) the expression of genes involved in oxidative metabolism such as cytochrome c and cytochrome oxidase IV, both well-known targets of PGC-1α (Wu et al., 1999;Lin et al., 2002). As expected, transcript levels of UCP3 remained unchanged ($P > 0.05$) further confirming the specificity of our approach. Examination of the levels of GABPα and β mRNA in these transduced muscle fibers showed a specific induction of GABPα following expression of PGC-1α (Figure 3.6B). Furthermore, PGC-1α stimulated expression of utrophin A mRNAs as well as transcripts encoding other synaptically-expressed genes, i.e., AChRα and ε (see Figure 3.6C). Collectively, these results show that PGC-1α mediates the expression of utrophin at the NMJ via GABP and that this effect can be extended to the regulation of other synaptically-expressed genes.

Figure 3.6. PGC-1α Increases Expression of Utrophin and GABP In Vivo. Plasmids containing an expression vector for PGC-1α were electroporated into mouse skeletal muscle. Seven days following transduction, the levels of endogenous transcripts were assessed by real time RT-PCR. Panel (A) shows transcript levels for cytochrome c and cytochrome oxidase IV which are known to be regulated by PGC-1α, and UCP3 used a control to show the specificity. Panel (B) shows that PGC-1α increases selectively the expression of GABPα. Panel (C) demonstrates in vivo that PGC-1α regulates utrophin A mRNA levels as well as other transcripts encoding synaptic proteins. Control muscles were electroporated with an empty vector. Astericks represent significant differences ($P < 0.05$), an open circle represents $P=0.052$.


A
mRNA expression induced by PGC-1α
(fold induction over control)
0
1
2
3
4
5
6
7
*
*
UCP3
Cytochrome C
Cytochrome oxidase IV
B
mRNA expression induced by PGC-1α
(fold induction over control)
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
o
GABPα
GABPβ
C
mRNA expression induced by PGC-1α
(fold induction over control)
0
1
2
3
4
5
6
7
8
*
*
*
Utrophin
AChRα
AChRε

DISCUSSION

We previously showed that utrophin expression extends beyond the junctional compartment of muscle fibers and that this expression correlates with the metabolic efficiency of muscle fibers (Gramolini et al., 2001;Chakkalakal et al., 2003). Moreover, we found in these initial experiments that extrasynaptic expression of utrophin is partially mediated by calcineurin which is a known regulator of the slower, high oxidative, myogenic program. Here, several approaches allow us to extend these findings considerably by showing that calcineurin specifically participates in the synaptic regulation of utrophin in muscle. These studies not only demonstrate the importance of an active calcineurin/NFAT cascade in regulating utrophin expression within postsynaptic myonuclei but, importantly, they are the first to identify calcineurin as a positive regulator of gene expression at the NMJ. Together with the demonstrations of the role of Kaiso-δ catenin (Rodova et al., 2004), Wnt signaling (Kim et al., 2003) and purinergic receptor-mediated signaling (Choi et al., 2003;Tsim et al., 2003;Tung et al., 2004), our findings highlight the complexity and plethora of signaling pathways that contribute to the regulation of synaptically-expressed genes at the NMJ.

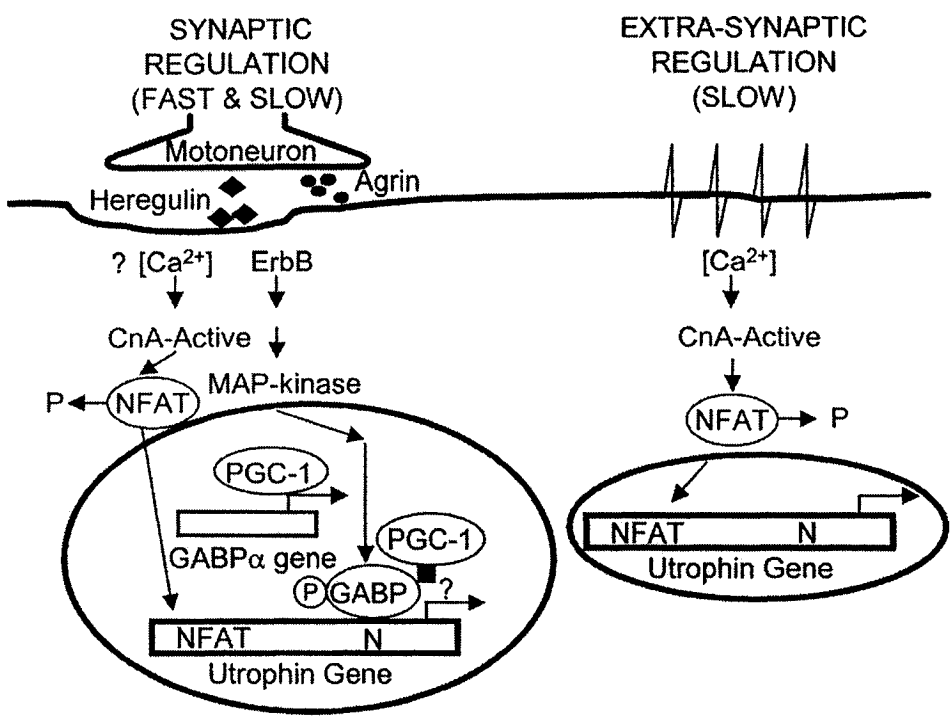
A key question arising from these observations deals with the mechanisms leading to activation of calcineurin within the postsynaptic sarcoplasm. Calcineurin is a calcium-dependent phosphatase and its activation relies on calcium fluxes typically seen in slower, highly oxidative, myofibers (see for review Schiaffino and Serrano, 2002; Bassel-Duby and Olson, 2003; Rothermel et al., 2003; Schulz and Yutzey, 2004; Michel et al., 2004). Calcium transients have been described at the NMJs of mouse muscle and are known to occur in response to in-coming electrical activity. Studies describing these

slow calcium transients suggest they arise from AChR channels or from IP3 receptors localized in the sarcoplasmic reticulum of NMJs and are distinct from muscle contractile events (Dezaki et al., 1997;Powell et al., 2003). While the role of calcium transients within the postsynaptic sarcoplasm has yet to be resolved, it has been suggested that they are involved in the stabilization of the postsynaptic apparatus and in the mechanisms regulating local synaptic gene expression (Megeath and Fallon, 1998; Carrasco et al., 2003; Powell et al., 2003). Together, these observations suggest that calcium waves likely occur at the NMJ where they activate calcineurin which in turn regulates the expression of synaptic genes including utrophin.

We recently proposed a model in which utrophin gene expression in muscle is regulated by different mechanisms along muscle fibers (Chakkalakal et al., 2003). In this model, extrasynaptic expression of utrophin is regulated by calcineurin/NFAT in slower (type I and IIa myosin heavy chains), high oxidative, fibers, whereas at the NMJ of both fast and slow fibers, utrophin gene expression occurs via the activation of GABP and the N-box motif. In support of this model, we previously demonstrated that: i) utrophin A transcript and protein levels are higher in extrasynaptic regions of slow versus fast muscle fibers (Gramolini et al., 2001); and ii) this difference in utrophin A expression correlates with the contractile speed and oxidative status of individual muscle fibers (Chakkalakal et al., 2003).

Here, we refine this model to include the novel and additional contribution of calcineurin/NFAT as a positive regulator of synaptic gene expression (Figure 3.7). Furthermore, our data lead us to propose that the calcineurin/NFAT and GABP/N-box pathways cooperate to induce the transcriptional activity of utrophin at the NMJ. In this

Figure 3.7. Model Depicting the Regulatory Events Controlling Utrophin Gene Expression Along Skeletal Muscle Fibers. In synaptic regions of fast and slow muscle fibers, nerve-derived factors stimulate the ras-MAP kinase pathway leading to phosphorylation of GABP and its binding to the N-box motif of the utrophin A promoter. In extrasynaptic compartments of slower muscle fibers, tonic electrical activity triggers specific patterns of calcium fluxes, which in turn activate calcineurin leading to the dephosphorylation of NFAT. Once dephosphorylated, NFAT is free to travel to myonuclei to transactivate the utrophin A promoter. This pathway is not active in fast muscle fibers thereby explaining the absence of utrophin in their extrasynaptic regions (not shown). Based on our current findings, we can now extend this model to include the important contribution of calcineurin/NFAT and PGC-1α in the synaptic regions of muscle fibers. See Discussion for more detail.


SYNAPTIC
REGULATION
(FAST & SLOW)
EXTRA-SYNAPTIC
REGULATION
(SLOW)
Motoneuron
Agrin
Heregulin
? [Ca2+]
ErbB
[Ca2+]
CnA-Active
CnA-Active
MAP-kinase
P
NFAT
NFAT
P
PGC-1
PGC-1
GABPα gene
P
GABP
?
NFAT
N
Utrophin Gene
NFAT
N
Utrophin Gene

context, a recent study showed that calcineurin plays a role in the redistribution of pre-synthesized AChR molecules following agrin treatment of cultured myotubes (Madhavan et al., 2003). Collectively, findings from these studies clearly indicate that calcineurin can assume multiple functions according to the state of differentiation, maturation and innervation of the postsynaptic apparatus.

Co-activators contribute to gene expression by conferring transactivating potential to transcription factors rather than binding DNA directly. One of these co-activators, PGC-1α has been described as a master regulator of mitochondrial biogenesis and mitochondrial metabolism (Puigserver et al., 1998; Lehman et al., 2000; Lin et al., 2002; Scarpulla, 2002b), reviewed in (Puigserver and Spiegelman, 2003). Accordingly, PGC-1α is expressed predominantly in tissues with high mitochondrial content, including muscle (Puigserver et al., 1998; Knutti et al., 2000; Lin et al., 2002). Recently, PGC-1α was shown to direct slow fiber-type transitions in transgenic animals and to co-activate calcineurin signaling in muscles (Lin et al., 2002). Expression of PGC-1α is also known to stimulate expression of enzymes involved in oxidative metabolism and to induce expression of the transcription factor NRF2 in skeletal muscle cells (Wu et al., 1999).

NRF2 was initially identified as a transcriptional regulator of cytochrome oxidase genes and has since been shown to regulate expression of numerous genes involved in oxidative metabolism (Virbasius and Scarpulla, 1991; Virbasius and Scarpulla, 1994; Au and Scheffler, 1998; Wu et al., 1999; Kelly and Scarpulla, 2004). Further investigations indicate that NRF2 can bind to known ets sites on promoter regions of various genes and that it is a human homologue of the ets-related transcription factor GABP (Virbasius and Scarpulla, 1991; Virbasius et al., 1993; Scarpulla, 2002b). This is particularly interesting

and important since, as described above, GABP is also a key transacting factor at the NMJ known to direct the synaptic expression of several genes (see also Schaeffer et al., 1998). Within the postsynaptic compartment, GABP binds to a sequence present in the regulatory regions of several synaptic genes, termed the N-box element, which contains the core ets binding sequence GGA also recognized by NRF2. Thus, regulation of oxidative and synaptic gene expression in skeletal muscle appears to be mediated by the same transacting factor, namely GABP/NRF2.

Given that PGC-1α is known to exert its effects through GABP/NRF2 as well as the calcineurin pathway which, as described previously, are both important regulators of utrophin gene expression (Figure 3.7), we examined whether this co-factor also participates in the regulation of utrophin. To this end, we showed that PGC-1α expression is enhanced in synaptic regions of muscle fibers and that PGC-1α increases utrophin A gene expression both in cultured myogenic cells and in muscle in vivo. These results show that utrophin is a downstream target gene of PGC-1α signaling. These observations can be extended to include the regulation of other synaptically-expressed genes since transcripts encoding AChRα and AChRε subunits are also induced following ectopic expression of PGC-1α in muscle fibers.

Based on our findings, PGC-1α appears to mediate its most dramatic effect on utrophin expression by synergizing with GABP/NRF2 rather than by co-activating the calcineurin/NFAT pathway. Specifically, we showed that PGC-1α preferentially stimulates expression of GABPα, and not β, in cultured myogenic cells and muscle fibers. These results are in excellent agreement with previous studies showing that NRF2a (known also as GABPα) is induced by PGC-1α (Wu et al., 1999; Mootha et al.,

2004). Since GABPα transcripts accumulate at the NMJ (Schaeffer et al., 1998), this preferential induction of GABPα by PGC-1α is not surprising. Given the ability of GABP to induce utrophin gene expression, these findings collectively indicate that induction of GABPα by PGC-1α represents a key mechanism by which PGC-1α contributes to utrophin expression at the NMJ. This scenario further supports previous observations showing that GABP is an important mediator and co-activator of PGC-1α-induced gene expression (Mootha et al., 2004).

While PGC-1α and GABP are expressed throughout muscle fibers (see Results and Schaeffer et al., 1998; Lin et al., 2002), we propose that their important synergistic effect on utrophin gene expression likely occurs only at the NMJ. PGC-1α and GABPα both show enhanced expression within synaptic regions of muscle fibers (see Results and Schaeffer et al., 1998). Furthermore, the phosphorylation events that confer transactivating potential to GABP are likely confined to the NMJ since they are initiated by the release of nerve-derived trophic agents such as heregulin and their binding to appropriate receptors (Schaeffer et al., 1998; Fromm and Burden, 2001; Sunesen et al., 2003). Finally, PGC-1α activity is regulated by p38 MAPK and the activation of p38 MAPK in response to neuregulin has recently been described in myogenic cells (Canto et al., 2004), which further supports a physiological role for PGC-1α at the NMJ.

The regulation of utrophin gene expression at the NMJ can now be envisioned to include induction or co-activation through additional factors of GABP by PGC-1α. A key denominator arising from these findings is that synaptic gene expression appears to occur via molecules localized to the NMJ that also control expression of genes involved in oxidative metabolism (PGC-1α, GABP, calcineurin and NFAT, see Figure 3.7).

Consistent with this notion, the postsynaptic region of muscle fibers is known to be rich in mitochondria and oxidative enzymes (see Jasmin et al., 1995 and references therein). An attractive possibility is that the mechanisms presiding over utrophin expression at the NMJ involve multiple signaling pathways that cross-talk to co-ordinately regulate synaptic gene expression and oxidative metabolism in order to ensure an adequate supply of key proteins within the postsynaptic membrane domain allowing for efficient and sustained neurotransmission. A recent study (Lin et al., 2004) described the reduced expression of brain-specific and mitochondrial genes in PGC-1α-deficient mice and associated abnormalities in neuronal function, suggesting that PGC-1α also mediates the co-ordinated expression of genes in the CNS.

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Chapter 4

Calcineurin signaling differentially regulates A-utrophin mRNA stability in fast versus slow muscle via an AU-rich element.

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Joe V. Chakkalakal and Bernard Jasmin wrote manuscript. Joe V. Chakkalakal performed all experiments with some technical assistance. Robin Michel provided muscles from CsA and FK506 treated mice, as part of an on-going collaboration. Pedro Miura assisted in subcloning ΔARE utrophin 3'UTR

ABSTRACT

Although higher levels of A-utrophin mRNA seen in slow muscles can be attributed to calcineurin signaling and transcriptional mechanisms, post transcriptional mechanisms have also been implicated in this paradigm. In this study, we examined the post transcriptional mechanisms involved in promoting higher levels of A-utrophin expression in slow versus fast muscles. We observed that the stability of A-utrophin mRNA is lowered in the presence of fast skeletal muscle extract. Direct plasmid injections of reporter constructs, containing full length or truncated variants of the utrophin 3'untranslated region (UTR), revealed that an upstream region, 264 nucleotides in length, is sufficient to confer higher levels of reporter mRNA expression in slow muscles, in comparison to fast muscles. Inspection of this region uncovered the presence of a conserved AU-rich element (ARE) that suppressed the expression of reporter mRNAs in culture. In addition, the stability of reporter mRNAs fused to the utrophin 3'UTR was lower in the presence of fast muscle extract. This destabilization effect was not apparent upon deletion of the conserved ARE. We also observed that calcineurin signaling can affect A-utrophin mRNA stability, utrophin 3'UTR reporter activity and affect the turnover of reporter mRNAs fused to the utrophin 3'UTR, through an ARE dependent mechanism. Taken together, these results indicate that ARE-mediated mRNA decay is an important factor that regulates the expression of A-utrophin mRNA. Furthermore, these findings provide novel targets to design therapies aimed at elevating endogenous levels of A-utrophin in muscle fibers, from patients afflicted with Duchenne muscular dystrophy (DMD).

INTRODUCTION

Ever since its discovery there has been a considerable effort to decipher the molecular mechanisms that regulate the expression of the cytoskeletal protein utrophin (7, 48). These efforts are due, in part, to the accumulation of utrophin at the neuromuscular junction (NMJ), where it participates in the differentiation of post-synaptic regions (46, 63, 66, and 71). In addition, increased expression of utrophin in extra-synaptic regions of muscle fibers is considered to be a potential therapeutic strategy for the treatment of DMD (7, 20, 48, and 58). The therapeutic potential of utrophin upregulation stems from its high degree of sequence and functional homology with dystrophin, the protein whose absence from the sarcolemma is the molecular signature for DMD (1, 7, 42, 52 and 55). This notion is reinforced by observations that forced expression of utrophin in extra-synaptic regions of dystrophin-deficient animal models can correct dystrophic pathology (16, 76 and 79). Therefore, the elucidation of mechanisms that regulate the expression of utrophin, in muscle fibers, can provide insights into synaptic gene expression and provide possible therapeutic targets for DMD.

Two full length utrophin isoforms, A-utrophin and B-utrophin, have been characterized that are transcribed from two different promoters (15, 27). The mRNAs for these two isoforms differ in their 5'UTRs, and translate into proteins that differ in their N-terminal regions (15, 82). B-utrophin has been observed to be preferentially expressed in endothelial cells (82). In contrast, A-utrophin is the isoform found in skeletal muscle with preferential accumulation in post synaptic regions (77, 82). Studies have shown that the state of differentiation and innervation of muscle fibers can influence the expression patterns of A-utrophin (32, 34, 36, 43, 69 and 77). Within these

physiological contexts, alterations in A-utrophin mRNA levels are controlled primarily by transcriptional mechanisms (44). During differentiation of muscle cells, in culture, elevations in both A-utrophin expression and transcriptional activity have been observed (36, 69). In mature muscle fibers, A-utrophin mRNAs accumulate in synaptic regions, due to transcriptional mechanisms (34, 77 and 81). Specifically, the nerve derived factors, agrin and neuregulin, have been shown to activate downstream signaling cascades, that promote the binding of the transcription factor complex GABP α/β to N-box motifs in the promoters of some synaptic genes including A-utrophin (35, 37 and 47).

Despite the preferential accumulation of A-utrophin mRNAs in synaptic regions, mature slow contracting, oxidative, muscle fibers express higher levels of A-utrophin in extra-synaptic regions, in comparison to faster contracting, glycolytic, counterparts (17, 39 and 77). In a series of studies, we demonstrated the involvement of signaling pathways that promote the expression of slow muscle fiber genes in regulating the expression of A-utrophin (2, 17, 19 and 21). One pathway involves calcineurin, a Ca^{+2}/calmodulin regulated phosphatase activated by distinct rises in intracellular Ca^{+2} similar to those observed in innervated slow muscle fibers (26, 49, 51, 56, 61, 65, 67, 68 and 73). Upon activation, calcineurin dephosphorylates target transcription factors, such as MEF2 and NFAT, allowing them to promote the expression of target genes including those that specify the slow oxidative myofiber program, and A-utrophin (2, 17, 26, 51, 73 and 86). Of particular relevance, we have shown that dystrophin-deficient muscle fibers with activated calcineurin display increased sarcolemmal levels of A-utrophin, together with fiber type shifts towards a slower phenotype and attenuations in aspects of muscle

pathology (19). Conversely, inhibition of calcineurin activity, in mdx muscles, exacerbates the dystrophic phenotype, and decreases A-utrophin expression (21).

In recent years it has become apparent that in addition to transcription, post-transcriptional mechanisms can greatly influence the expression of genes in skeletal muscle (18). For instance, mRNAs encoding for myogenic regulatory factors, such as MyoD and myogenin, and synaptic proteins, including acetylcholinesterase and α-dystrobrevin1, can be regulated at multiple levels with effects on processes involving mRNA stability, targeting and translation (8, 14, 28, 29, and 62). Recently, the contribution of post-transcriptional mechanisms has been shown to play an important role in the regulation of A-utrophin expression in skeletal muscle (39, 40, 44, and 57). Specifically, the A-utrophin mRNA contains exceptionally long 5' and 3' UTRs, that can regulate the translation, stability and targeting of mRNAs (44). For instance, diseased and toxin induced regenerating skeletal muscle display increased levels of A-utophin protein that are not accompanied by similar increases in transcriptional activity (38, 57). Internal ribosomal entry site (IRES) mediated translation, acting on the A-utrophin 5'UTR, is one mechanism that contributes to the induction of A-utrophin protein during skeletal muscle regeneration (57). During myogenesis utrophin transcripts preferentially localize to cytoskeletal bound polysomes (40). Examination of the mechanisms involved in this targeting demonstrate the importance of specific regions in the utrophin 3'UTR (40).

Transcriptional mechanisms can only partially explain the higher levels of A-utrophin mRNA observed in slow muscles relative to fast muscles (17, 18, 39, and 77). In particular, the utrophin 3'UTR has been implicated in post-transcriptional

mechanisms, likely involving mRNA stability, that function to promote higher levels of A-utrophin expression in slow oxidative muscle fibers, in comparison to fast glycolytic muscle fibers (17, 39, 77). Despite the implication that the utrophin 3'UTR may exert a stabilizing effect on A-utrophin mRNA in slow muscles, relative to fast muscles, the molecular determinants of this effect remain to be characterized. Therefore, in the present study we examined the turnover of A-utrophin mRNA using an in vitro stability assay with protein extracts from fast and slow skeletal muscle. In addition we also conducted a series of experiments to begin to decipher the signaling pathways, and cis-elements in the utrophin 3'UTR that are involved in post transcriptional regulation of A-utrophin expression in fast and slow muscles.

METHODS

Animal care and protocols. All animal care and experimental procedures were performed in accordance with the Canadian Council of Animal Care guidelines, and were approved by the Institutional Animal Care and Research Advisory Committee of Laurentian University, or by the University of Ottawa Animal Care and Use Committee. In the present study, C57BL6 mice were treated twice daily for 14 days with either cyclosporine A (CsA; 25 mg/kg administered subcutaneously), FK506 (5 mg/kg administered subcutaneously), or vehicle (control) as previously described (2). Soleus (SOL) muscles and extensor digitorum longus (EDL) muscles from control or treated mice were then excised, and subsequently processed for in vitro stability assays (see below). In separate experiments, EDL and SOL muscles that were injected with utrophin 3'UTR reporter constructs (see below) were excised from C57BL6 mice, and subsequently processed for RT-PCR analysis (see below).

Cell Culture. Mouse C2C12 cells (ATCC, Manassas, VA) were cultured on 6-well culture dishes coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum, 292 ng/ml L-glutamine, and 100 units/ml penicillin-streptomycin in a humidified chamber at 37 °C with 5% CO2. Confluent myoblasts were then induced to differentiate into myotubes by replacing the growth medium with differentiation medium containing low serum (2% horse serum) for 3 days.

In Vitro Stability Assays. Proteins were extracted from EDL, SOL and treated SOL muscles using homogenization buffer (0.01M Tris pH 8.0, 0.01M KCl, 0.0015M $MgCl^2$, 2.5% IGEPAL (Sigma-Aldrich, Oakville, ON), Protease Inhibitor complete mini tablet as per the manufacturer's recommendations (Roche Applied Science, Laval, QC)). After homogenization, the extract was centrifuged at 3500g. The pellet was subsequently vortexed and incubated at 4C in extraction buffer (0.02M Tris pH 8.0, 0.45M NaCl, 0.01 EDTA, Protease Inhibitor complete mini tablet as per the manufacturer's recommendations (Roche Applied Science)). After incubation, the pelleted fraction was centrifuged at 14000g and the supernatant was collected for subsequent use in stability assays. RNA used in stability assays was isolated from C2C12 myotubes using TriPure reagent (Boehringer Mannheim, Laval, QC). For determination of LacZ reporter mRNA half-life, RNA was isolated from C2C12 myotubes that had been transfected with the appropriate reporter constructs (see below).

Degradation assays were preformed as described elsewhere, with some modifications (12, 13). Briefly, RNA and protein extracts were incubated together in degradation buffer (0.01M Tris pH 7.4, 0.1M KOAc, 0.002M MgOAc, 0.002M DTT, 0.0001M Spermine, 0.001M ATP, 0.0004 M GTP, 0.01M Phosphcreatine, 1ug Creatine Phosphokinase, 20U SUPERNasin (Ambion, Austin TX), 0.25 ug/ul extract, 0.2 ug/ul RNA) for 30 and 60 mins at 37C. Time 0 was taken as RNA incubated in buffer without protein extract for 1 min at 37C. The reactions were stopped by addition of 200 ul of phenol/chloroform. RNA was then precipitated with isopropanol in the presence of yeast tRNA (10 ug) (Sigma-Aldrich) as a carrier. The values of A-utrophin, LacZ and S12rRNA transcript remaining at each time point were determined through RT-PCR

analysis (see below). Values were then plotted on a logarithmic scale as a function of time, and the half-life was extrapolated using line equations (72). Four separate experiments were conducted, using 4 different extracts. Half-life values were then determined relative to appropriate controls in each individual experiment. Relative half-life values were then averaged and compared between samples.

Plasmid Construction. The 3'UTR of the mouse full-length ~2 kb utrophin mRNA was isolated and subcloned into a LacZ reporter construct driven by the cytomegalovirus (CMV) promoter (pCMVsport from Invitrogen) as described previously (40). Truncated variants of the utrophin 3'UTR were also generated encompassing the first 596 and 332 nucleotides in a similar manner, as described previously (40). To generate the utrophin 3'-UTR fragment without the conserved 20 nt AU-rich element (ARE), we employed a PCR-based protocol described elsewhere (28). Briefly, the utrophin full-length 3'-UTR cDNA was used as a template, and two rounds of PCR amplification were first performed with the following primers: 5'-CATAATGGTAAAACAGTCAAATAA-3' and the flanking primer 5'-TGGTCTCCTCGAGGCATCTATCCAGCCAG-3' (reaction 1); and the flanking primer 5'-TGGTCTCAAGCTTGGTATGTAACAGATTAG-3' and primer 5'-ATCAGCCATACCAAACGAATAGAT-3' (reaction 2). To remove the ARE region, we performed a third round of PCR, with the reaction 1 and reaction 2 PCR products, using the two flanking primers listed above to yield a product containing the utrophin 3'-UTR, without the conserved 20 nt ARE region (3'UTR ΔARE) as confirmed by sequencing.

Transfection and Direct Plasmid Injection. Plasmid DNA was prepared using the Mega-Prep kit (Qiagen, Mississauga, ON). DNA pellets were re-suspended in 10 mM Tris-HCl, pH 8.5. Transfections were performed with the Lipofectamine reagent kit (Invitrogen) according to the manufacturer's instructions. Briefly, C2C12 myoblasts at 80% confluency were transfected with 1ug of utrophin 3'UTR-reporter constructs together with, 1ug of pCIneo (Promega) or a construct containing a constitutively active variant of calcineurin (pCnA*) (26, 64), by using lipofectamine reagent (Invitrogen). Total RNA was extracted, and the levels of reporter LacZ mRNA and neomycin mRNA (to control for transfection efficiency) were determined by RT-PCR analysis (see below). Transfections examining the effects of deleting the 20 nt ARE from the utrophin 3'UTR were done in a similar fashion. Briefly, 2ug of pCMVsport containing a LacZ gene fused to the SV40 late polyadenylation signal (SV40 3'UTR), utrophin 3'UTR full-length and 3'UTR ΔARE reporter constructs were transfected, however the levels of reporter LacZ mRNA were standardized to ampicillin mRNA transcribed on the same construct by a separate promoter.

Direct gene transfer was performed on mouse EDL and SOL muscles as described in detail elsewhere (39). The EDL and SOL muscles were isolated, and injected with 10 ul of a solution containing either the utrophin 3'UTR full-length, 3'UTR ΔARE, 3'UTR 596, and 3'UTR 332, together with pCAT control plasmids, used to control for injection efficiency, diluted at a concentration of 2–4 ug/ul. Seven days later, injected muscles were excised and immediately frozen in liquid nitrogen, and total RNA was extracted, for subsequent analysis by RT-PCR (see below).

RNA Extraction and RT-PCR. Total RNA was extracted by using TriPure (Boehringer Mannheim) as recommended by the manufacturer. Quantitative RT-PCR was carried out to determine the relative abundance of A-utrophin and S12rRNA in EDL and SOL muscles; A-utrophin, S12rRNA and LacZ transcripts remaining at each time point during the in-vitro stability assays; LacZ and CAT mRNA levels after direct plasmid injections; LacZ and neomycin mRNA levels in co-transfection experiments of the utrophin 3'UTR constructs with pCIneo or pCnA* in C2C12 muscle cells; and Lac Z and ampicillin transcripts in C2C12 muscle cells transfected with utrophin 3'UTR full-length, parental or 3'UTR ΔARE constructs. These assays were performed using previously described protocols and primers (17, 22, 28 and 39). Cycle numbers varied depending on the primers used and were within the linear range (22, 28 and 39). For amplification of LacZ, CAT, neomycin and ampicillin mRNAs, samples were first digested, by using DNase 1 to eliminate plasmid contamination (17, 39). In all these assays, negative controls consisted of reverse transcription-mixtures in which total RNA was replaced with RNase-free water. PCR products were first visualized on 1% agarose gels containing ethidium bromide.

The labeling intensity of the PCR product which is linearly related to the abundance of cDNAs was quantified using Kodak digital science 1D Image analysis software. For direct plasmid injections, values obtained for LacZ were standardized relative to the amount of CAT. For transfection studies, in C2C12 muscle cells, involving the utrophin 3'UTR reporter and pCnA* constructs, values obtained for LacZ were standardized relative to the amount of neomycin. For transfection studies in C2C12 muscle cells, involving the utrophin 3'UTR full-length, SV40 3'UTR and 3'UTR ΔARE

reporter-constructs, values obtained for LacZ were standardized to the amount of ampicillin (28).

Statistical Analysis. Analysis of variance (ANOVA) and F-tests, or two-tailed Student's t-tests were used to analyze the data. Means +/- SEM are presented throughout. All statistics were done using Microsoft Excel Analysis ToolPak.

RESULTS

A-utrophin mRNA expression and stability is greater in slow muscles in comparison to fast muscles. In an initial series of experiments, we examined the levels of A-utrophin mRNA in fast and slow muscles by RT-PCR. As shown in Figure 4.1A, the levels of A-utrophin mRNA in slow SOL muscles were much higher in comparison to fast EDL muscles. Quantitative analysis revealed that A-utrophin mRNA levels were ~ 300% higher ($P<0.05$) in SOL muscles in comparison to EDL muscles (Figure 4.1B). The higher levels of A-utrophin mRNA in slow muscles, relative to fast muscles, are in agreement with previous observations (17, 39).

To determine if the stability of A-utrophin transcripts is different in fast versus slow muscles we employed a previously described in vitro stability assay (12, 13). With this assay, we examined the degradation of A-utrophin mRNA in the presence of either fast (i.e. EDL) or slow (i.e. SOL) skeletal muscle protein extract. Total RNA from 3 day-old C2C12 myotubes was incubated with either fast or slow skeletal muscle protein extracts for different time durations in a degradation buffer (12, 13). Subsequently, the amount of A-utrophin mRNA remaining at each time point was determined, and used to calculate mRNA decay kinetics (see materials and methods). In the presence of slow muscle extracts, A-utrophin mRNA appeared to degrade at a slower rate in comparison to incubation with fast muscle extracts (Figure 4.2A). Evaluation of mRNA half-life values revealed that A-utrophin degraded at twice the rate ($P<0.05$) in the presence of fast skeletal muscle extracts, relative to incubation with slow skeletal muscle extracts (Figure 4.2B). As a control, we observed no differences in the rate of degradation of S12rRNA upon incubation with fast or slow muscle extracts (Figure 4.2A). These data indicate that

Figure 4.1. Levels of A-utrophin mRNA are higher in slow skeletal muscles relative to fast skeletal muscles. A) Examples of ethidium bromide-stained A-utrophin and S12rRNA PCR products from EDL and SOL muscles. B) Quantification of A-utrophin mRNA levels in EDL muscles expressed as a percentage of those found in SOL muscles ($p<0.05$, $n = 3$ experiments) standardized to S12rRNA. Note the greater amount of A-utophin mRNA in SOL muscles relative to EDL muscles. * indicates a significant difference from EDL ($p < 0.05$; $n = 3$).

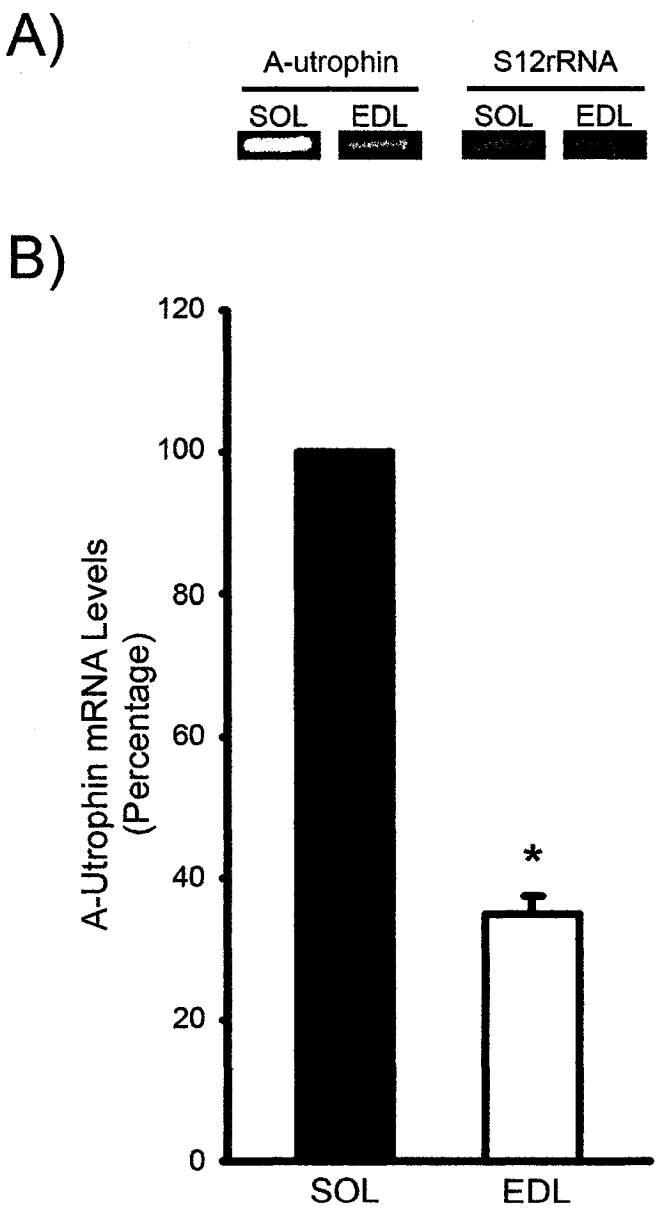
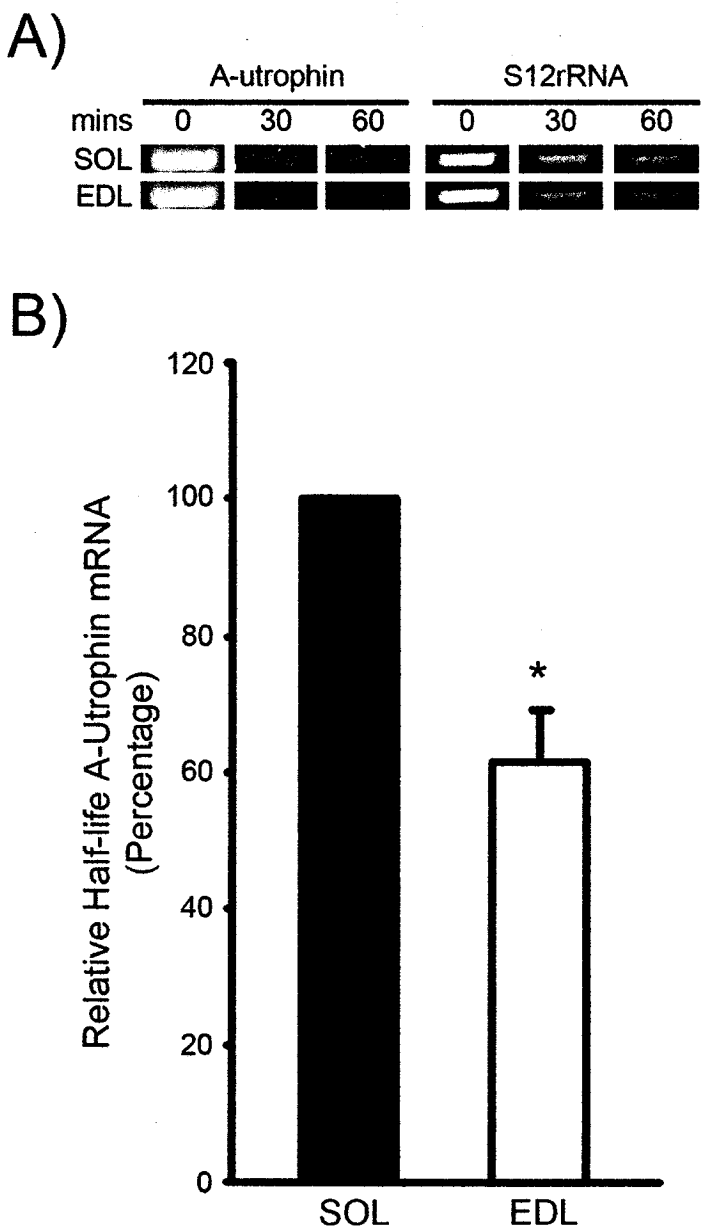

A)
A-utrophin
S12rRNA
SOL
EDL
SOL
EDL
B)
A-Utrophin mRNA Levels
(Percentage)
120
100
80
60
40
20
0
*
SOL
EDL

Figure 4.2. A-utrophin mRNA decays at a faster rate in fast muscle. In vitro stability assays were performed with protein extracts from EDL and SOL skeletal muscle and RNA from 3 day old C2C12 myotubes. A) Example of ethidium bromide stained agarose gels displaying A-utrophin and S12rRNA PCR products following 0, 30, and 60 min of incubation with protein extracts. Note the greater amount of A-utrophin PCR product remaining after 30 and 60 min incubations with the SOL (slow) muscle protein extracts versus incubation with the EDL (fast) muscle protein extracts. B) Quantification of the relative half-life values expressed as a percentage of half life values obtained for A-utrophin mRNA incubated with SOL protein extract. Note the significant decrease in A-utrophin mRNA half life upon incubation with EDL skeletal muscle protein extract relative to SOL skeletal muscle protein extract. * indicates a significant difference from EDL ($p < 0.05$; $n = 4$ independent experiments).

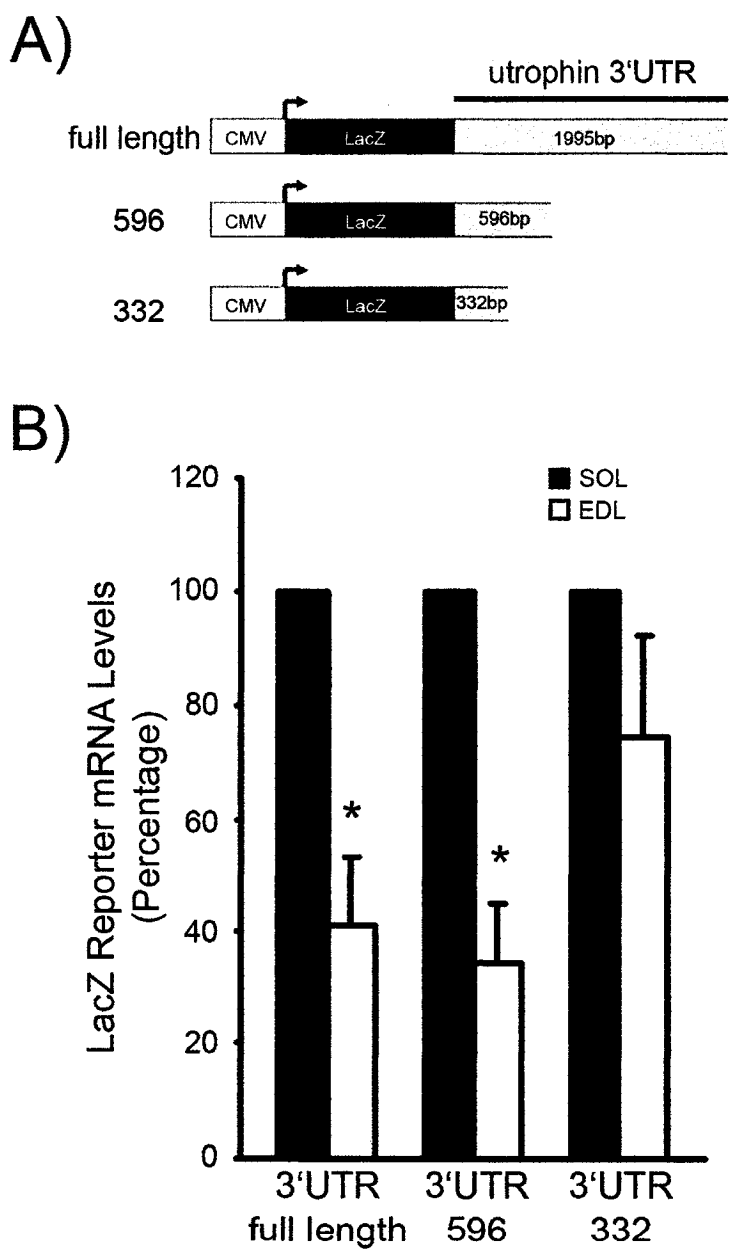

A)
A-utrophin
S12rRNA
mins
0
30
60
0
30
60
SOL
EDL
B)
Relative Half-life A-Utrophin mRNA (Percentage)
120
100
80
60
40
20
0
*
SOL
EDL

the higher levels of A-utrophin in slow muscles relative to fast muscles, occurs in part at the level of mRNA stability.

A cis-element in the utrophin 3'UTR differentially regulates reporter expression in slow versus fast muscles. The 3'UTRs of host mRNAs can function to regulate the levels of mRNAs present in a cell (3, 6, 41, and 72). This ability to control mRNA levels, for the most part, stems from the presence of cis-elements within the 3'UTR (3, 6, 24, 72, 41, 84, and 85). Using direct plasmid injection of deletion reporter constructs in EDL and SOL muscles of mice, (see Figure 4.3A) we next sought to determine cis-elements in the mouse utrophin 3'UTR capable of conferring differences in mRNA expression between fast and slow muscles. Consistent with previous observations (39), direct plasmid injection of an utrophin 3'UTR full-length reporter construct led to ~ 2.5 fold ($P<0.05$) higher levels of reporter mRNA in SOL muscles, relative to EDL muscles (Figure 4.3B).

Similar to the results obtained with the utrophin 3'UTR full-length reporter construct, direct plasmid injection of a reporter construct containing the first 596 nucleotides of the utrophin 3'UTR also led to ~ 2.5 fold ($P<0.05$) higher levels of reporter mRNAs in SOL muscles relative to EDL muscles (Figure 4.3B). In contrast, the levels of reporter mRNAs fused to the first 332 nucleotides of the utrophin 3'UTR were not significantly different ($P>0.05$) between EDL and SOL muscles (Figure 4.3B). Furthermore, the levels of reporter mRNAs fused to the first 332 nucleotides of the utrophin 3'UTR in EDL muscles increased to values comparable to those seen in SOL muscles. Collectively, these data suggest that nucleotides 332-596 of the utrophin 3'UTR

Figure 4.3. Nucleotides 332-596 in the utrophin 3'-UTR are sufficient to suppress reporter expression in fast muscles. A) Schematic diagram displaying reporter contructs with the full length ~2kb utrophin 3'UTR, the first 596 bps of the utrophin 3'UTR, and the first 332 bps downstream of the LacZ reporter construct used in our studies. B) Mouse EDL and SOL muscles were transduced with plasmids containing the utrophin 3'UTR full length, 3'UTR 596, and the 3'UTR 332 reporter contruscts. Note that, LacZ reporter mRNA levels are ~ 60% lower in EDL muscles when fused to either the utrophin 3'UTR full length or 3'UTR 596 relative to SOL muscles, however when LacZ mRNAs are fused to the 3'UTR 332, reporter mRNA levels in EDL muscles are comparable to those seen in SOL muscles. * indicates a significant difference from EDL ($p < 0.05$; n of 4-5 animals were used for each construct in independent experiments).

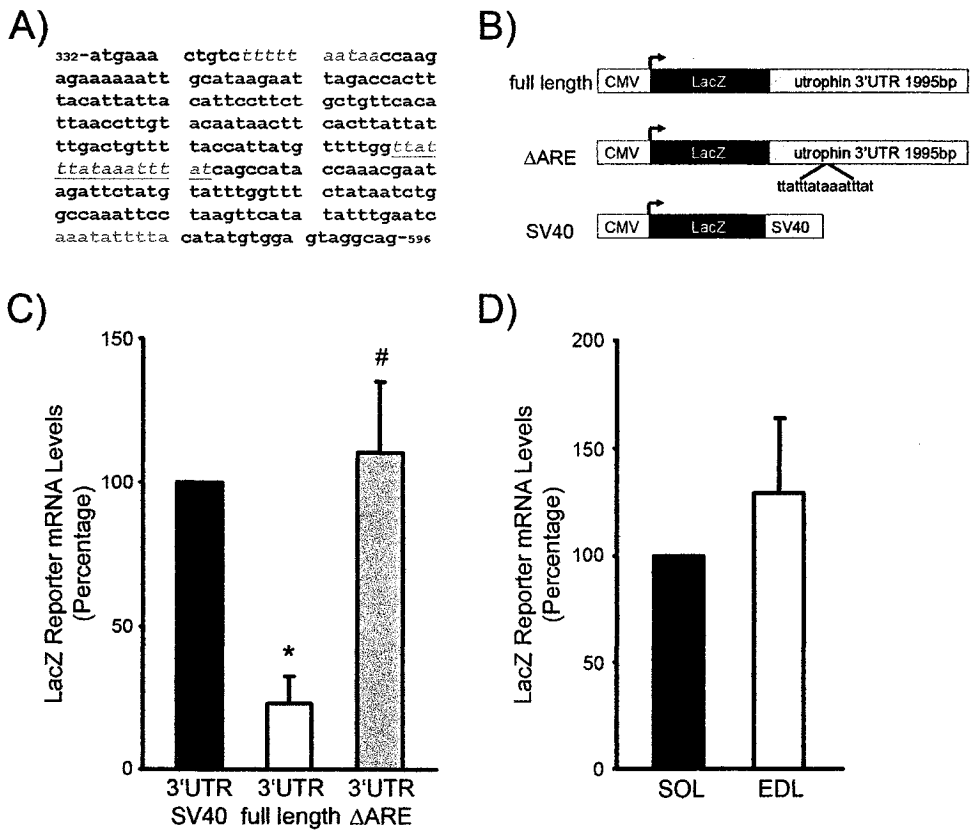

A)
utrophin 3'UTR
full length
CMV
LacZ
1995bp
596
CMV
LacZ
596bp
332
CMV
LacZ
332bp
B)
SOL
EDL
LacZ Reporter mRNA Levels
(Percentage)
120
100
80
60
40
20
0
*
*
3'UTR
full length
3'UTR
596
3'UTR
332

contain elements capable of suppressing reporter mRNA levels in EDL muscles relative to SOL muscles.

The utrophin 3'UTR 332-596 region contains multiple AU-rich elements (AREs). Sequence analysis of region 332-596 in the utrophin 3'UTR revealed that 72 % of the nucleotides were either adenosine or uridine. Within these 264 nucleotides, we found 3 potential AREs, elements that have important roles in paradigms involving the regulated turnover of mRNAs (Figure 4.4A, grey characters) (24, 25, 41, 50, 74, 78 and 88). Species comparison revealed one of the three AREs to be conserved between mouse and human utrophin 3'UTR sequences (Figure 4.4A, underlined grey characters).

To determine if the conserved ARE in the mouse utrophin 3'UTR may contribute to the regulation of A-utrophin mRNAs, we generated full-length utrophin 3'UTR reporter constructs deleted for this minimal region (Figure 4.4B). To initially examine the effects of deleting the ARE, we transfected C2C12 muscle cells with reporter constructs containing either the utrophin 3'UTR full-length, 3'UTR ΔARE, or the LacZ gene fused to SV40 3'UTR (see materials and methods). After 3 days of differentiation, transfected cells were harvested, and reporter mRNA levels examined. RT-PCR analysis of total mRNA isolated from transfected muscle cells, revealed that the presence of the utrophin 3'UTR full-length decreased reporter mRNA levels by ~ 80% ($P<0.05$), relative to the presence of the SV40 3'UTR (Figure 4.4C). In contrast, the levels of reporter mRNA fused to utrophin 3'UTR ΔARE were ~ 4 fold ($P<0.05$) higher relative to the

Figure 4.4. The utrophin 3'-UTR contains a conserved ARE. A) Sequence of region between nucleotides 332 and 596 of the utrophin 3'UTR. Grey characters denote the 3 potential ARE sequences, underlined is the ARE that is conserved between mouse and human that was subsequently deleted for analysis. B) Schematics displaying the utrophin 3'UTR full length, 3'UTR deleted for the conserved ARE (3'UTR ΔARE) and the SV40 late polyadenylation signal (3'UTR SV40) downstream of the LacZ reporter construct used in our studies. C) Analysis of LacZ mRNA levels, from C2C12 3 day old myotubes that were transfected with SV40 3'UTR, utrophin 3'UTR full length, and 3'UTR ΔARE reporter contructs. Note the loss in LacZ reporter mRNA expression when fused to the utrophin 3'UTR full length relative to SV40 3'UTR. Also note the increase in LacZ mRNA levels upon deletion of the conserved ARE in the utrophin 3'UTR, relative to the utrophin 3'UTR full length (compare 3'UTR full length and 3'UTR ΔARE) * indicates a significant difference relative to SV40 3'UTR, # indicates a significant difference relative to 3'UTR full length (* ($p < 0.05$); # ($p < 0.01$) n = 3 independent experiments done in triplicate). D) Mouse EDL and SOL muscles were injected with plasmids containing the utrophin 3'UTR ΔARE reporter constructs. Note that, LacZ reporter mRNA levels are comparable between EDL and SOL muscles when LacZ mRNAs are fused to the 3'UTR ΔARE (n of 5 animals were used independent experiments).


A)
332-atgaaa ctgtctttttt aataaccaag
agaaaaaatt gcataagaat tagaccactt
tacattatta cattccttct gctgttcaca
ttaaccttgt acaataactt cacttattat
ttgactgttt taccattatg ttttggttat
ttataaattt atcagccata ccaaacgaat
agattctatg tatttggttt ctataatctg
gccaaattcc taagttcata tatttgaatc
aaatatttta catatgtgga gtaggcag-596
B)
full length
CMV
LacZ
utrophin 3'UTR 1995bp
ΔARE
CMV
LacZ
utrophin 3'UTR 1995bp
ttatttataaatttat
SV40
CMV
LacZ
SV40
C)
LacZ Reporter mRNA Levels (Percentage)
150
100
50
0
#
*
3'UTR SV40
3'UTR full length
3'UTR ΔARE
D)
LacZ Reporter mRNA Levels (Percentage)
200
150
100
50
0
SOL
EDL

presence of the utrophin 3'UTR full-length (Figure 4.4C). Furthermore, utrophin 3'UTR ΔARE, relative to SV40 3'UTR, reporter mRNA expression was not significantly different ($P>0.05$) (Figure 4.4C).

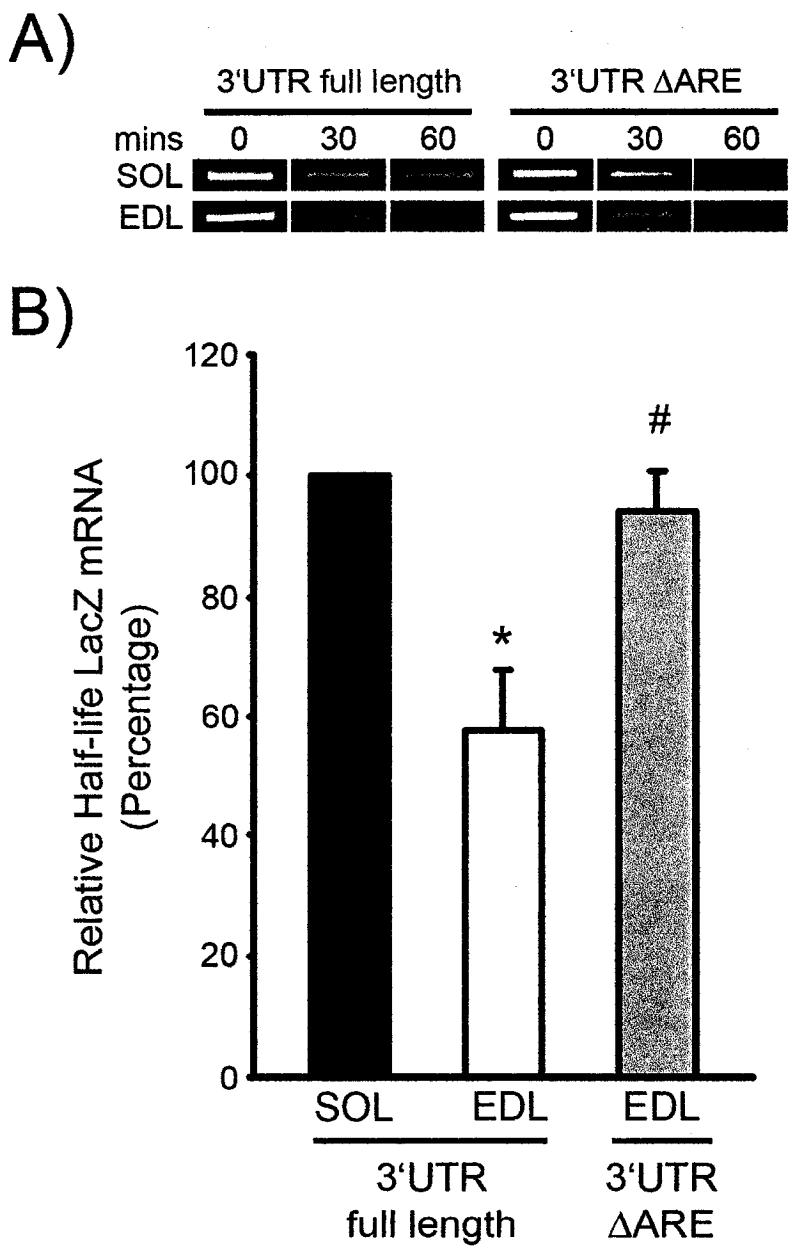
Next, we determined if the conserved ARE found in the utrophin 3'UTR could function in vivo. To examine the function of the utrophin 3'UTR ARE in a physiological context, we performed direct plasmid injection of 3'UTR full-length and 3'UTR ΔARE reporter constructs into EDL and SOL muscles. As seen in our previous work (and in Figure 4.3) direct plasmid injection of a 3'UTR full-length reporter construct resulted in ~ 2-3 fold ($P<0.05$) higher reporter mRNA levels in SOL muscles relative to EDL muscles (39). In contrast, direct plasmid injection of a 3'UTR ΔARE reporter construct did not reveal any significant differences in reporter mRNA levels between EDL and SOL muscles (Figure 4.4D). These observations are consistent with the notion that the ARE in the utrophin 3'UTR is a suppressive element for mRNA stability in vivo.

The conserved ARE in the utrophin 3'UTR functions as a stability element in the presence of fast and slow muscle protein extracts. Since A-utrophin displays differential mRNA stability in the presence of either fast or slow skeletal muscle extract, we next examined if the utrophin 3'UTR could confer differences in mRNA stability under similar conditions. Therefore, we performed in vitro stability assays, whereby mRNA collected from C2C12 muscle cells transfected with either utrophin 3'UTR full-length or 3'UTR ΔARE reporter constructs were incubated with either fast or slow skeletal muscle extract (as described above). Consistent with the ability of the utrophin 3'UTR to promote higher levels of reporter mRNA expression in slow muscles relative to

fast muscles (see Figure 4.3), in vitro stability assays also demonstrated stabilization of reporter mRNAs fused to the utrophin 3'UTR in the presence of slow skeletal muscle extract relative to fast skeletal muscle extract (Figure 4.5A). Quantitative comparisons of utrophin 3'UTR full-length reporter mRNA half-life values demonstrated a ~ 50% ($P<0.05$) decrease in this parameter upon incubation with fast muscle extracts versus slow muscle extracts (Figure 4.5B).

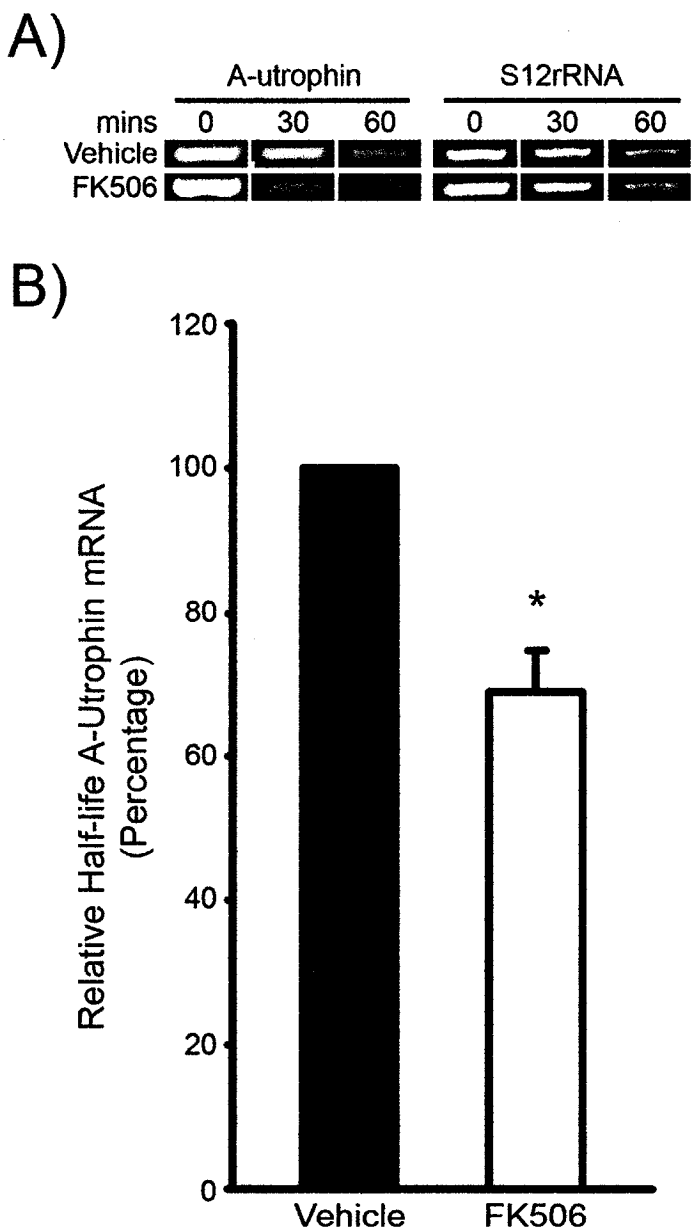
Deletion of the ARE from the utrophin 3'UTR, although not affecting the stability of reporter mRNAs incubated with slow skeletal muscle extract (Figure 4.5A), did have a stabilizing influence on reporter mRNAs incubated with fast skeletal muscle extract (Figure 4.5A). Indeed, quantitative analysis revealed that deletion of the ARE from the utrophin 3'UTR led to a more than 1.5 fold ($P<0.05$) increase in reporter mRNA half-life relative to mRNAs fused to the utrophin 3'UTR full-length upon incubation with fast EDL skeletal muscle extract (Figure 4.5B). We also did not observe any significant difference ($P>0.05$) in the half-life for utrophin 3'UTR ΔARE reporter mRNAs incubated with fast muscle extract relative to utrophin 3'UTR full-length reporter mRNAs incubated with slow muscle extract (Figure 4.5B). As a control, we observed no difference in the degradation of reporter mRNAs fused to the SV40 3'UTR upon incubation with either fast or slow muscle protein extract (data not shown). Therefore, the conserved ARE in the utrophin 3'UTR serves as an instability element in the presence of fast muscle protein extract.

Figure 4.5. The conserved ARE in the utrophin 3'UTR confers faster rates of decay of reporter mRNAs in fast skeletal muscle. A) Example of ethidium bromide stained agarose gels displaying LacZ PCR products, from in vitro stability assays, following 0, 30, and 60 min of incubation with EDL or SOL protein extracts. Note the greater amount of LacZ mRNA fused to the utrophin 3'UTR Full remaining after 30 and 60 min incubations with the SOL (slow) muscle protein extracts versus incubation with the EDL (fast) muscle protein extracts. Also note the higher levels of LacZ mRNA remaining after 30 and 60 minute incubations with EDL protein extract upon deletion of the conserved ARE in the utrophin 3'UTR (compare EDL 3'UTR full length to EDL 3'UTR ΔARE). B) Quantification of the relative half-life values expressed as a percentage of half life values obtained for LacZ mRNA fused to the utrophin 3'UTR full length incubated with SOL protein extract. Note the significant decrease in half-life for LacZ mRNA fused to the utrophin 3'UTR full length upon incubation with EDL skeletal muscle protein extract, relative to SOL skeletal muscle protein extract. Also note the significant increase in half-life for LacZ mRNA upon deletion of the conserved ARE in the utrophin 3'UTR, relative to utrophin 3'UTR full length (compare EDL 3'UTR full length to EDL 3'UTR ΔARE). * indicates a significant difference from EDL ($p < 0.05$; n = 4 independent experiments).


A)
3'UTR full length
3'UTR ΔARE
mins
0
30
60
0
30
60
SOL
EDL
B)
Relative Half-life LacZ mRNA (Percentage)
120
100
80
60
40
20
0
*
#
SOL
EDL
EDL
3'UTR full length
3'UTR ΔARE

Promotion of A-utrophin mRNA decay in the presence of protein extract from muscles treated with calcineurin inhibitors. Calcineurin has been shown to have important roles in maintaining the expression of genes indicative of the slow myofiber program (26, 65 and 73). Recently we have shown that stimulation of calcineurin signaling promotes the expression of A-utrophin mRNA through modest increases in transcriptional activity from the A-utrophin promoter (2, 17, and 77). We sought to determine if altered calcineurin signaling can also affect A-utrophin mRNA turnover. To this end, we first performed in vitro stability assays. In these assays, mRNA from C2C12 myotubes was incubated with skeletal muscle protein extract taken from animals treated with the specific calcineurin inhibitor FK506 or a vehicle control. Determination of half-life values demonstrated that A-utrophin decayed at an accelerated rate in the presence of SOL muscle extracts obtained from FK506-treated mice, relative to vehicle controls (Figure 4.6A). Quantitative assessment of half-life values revealed a ~ 30% ($P<0.05$) decrease in the amount of time required for A-utrophin mRNA to decay to half-maximal values when incubated with SOL muscle extracts from FK506 mice, in comparison to vehicle controls (Figure 4.6B). Similar results were obtained when in vitro stability assays were conducted with SOL protein extracts from mice treated with another calcineurin inhibitor, cyclosporine A (CsA) (data not shown). Analysis of S12 rRNA decay did not reveal any differences upon incubation with SOL protein extracts taken from vehicle or FK506 treated mice (Figure 4.6A).

Figure 4.6. Calcineurin signaling regulates A-utrophin mRNA stability. In vitro stability assays were performed with SOL protein extracts from mice treated with vehicle or the calcineurin inhibitor FK506, and RNA from 3 day old C2C12 myotubes. A) Example of ethidium bromide stained agarose gels displaying A-utrophin and S12rRNA cDNA products following 0, 30, and 60 min of incubation with protein extracts. Note the greater amount of A-utrophin mRNA remaining after 30 and 60 min incubations with SOL extracts from vehicle treated mice versus incubation with SOL extracts from mice treated with FK506. B) Quantification of the relative half-life values expressed as a percentage of half-life values obtained for A-utrophin incubated with SOL extracts from vehicle treated mice. Note the significant decrease in half-life for A-utrophin upon incubation with SOL extracts from FK506 treated mice, relative to SOL extracts from vehicle treated mice. * indicates a significant difference from vehicle ($p < 0.05$; $n = 3$ independent experiments).


A)
A-utrophin
S12rRNA
mins
0
30
60
0
30
60
Vehicle
FK506
B)
Relative Half-life A-Utrophin mRNA (Percentage)
120
100
80
60
40
20
0
*
Vehicle
FK506

Due to the negative effects of calcineurin inhibition on the stability of A-utrophin mRNA, we next examined how the altered activity of this enzyme could impact the ability of the utrophin 3'UTR to regulate mRNA expression. Initially, we studied the effects of stimulated calcineurin activity on utrophin 3'UTR reporter mRNA levels in cultured C2C12 muscle cells. For these experiments, we co-transfected constructs containing a transgene that encodes for a constitutively active variant of calcineurin (pCnA*) together with utrophin 3'UTR full-length reporter constructs (17, 26, and 64). In accordance with the ability of calcineurin activity to regulate the stability of A-utrophin transcripts, we observed that co-transfection of a utrophin 3'UTR full-length reporter construct with pCnA* led to ~ 2 fold ($P<0.05$) increase in reporter mRNA expression in comparison to empty vector controls (pCIneo) (Figure 4.7). Similarly, co-transfection of pCnA* with a deletion reporter construct containing the first 596 nucleotides of the utrophin 3'UTR also led to a ~ 2 fold ($P<0.05$) induction in reporter mRNA levels in comparison to controls (Figure 4.7). In contrast, this difference was abrogated upon further truncation of the 3'UTR to the first 332 nucleotides (Figure 4.7). Therefore, nucleotides 332-596 in the utrophin 3'UTR, in addition to functioning as a minimal region required to maintain differences in mRNA expression between fast and slow muscles, also is a calcineurin responsive element.

Next, we wanted to determine the effects of altered calcineurin signaling on mRNA stability mediated by the utrophin 3'UTR. Therefore, we performed in vitro stability assays with SOL muscle protein extracts from mice treated with either FK506 or vehicle, in the presence of mRNA from C2C12 muscle cells that were transfected with either utrophin 3'UTR full-length or 3'UTR ΔARE reporter constructs. The decay of

reporter mRNAs fused to the utrophin 3'UTR upon incubation with SOL muscle extracts from FK506 treated mice increased relative to vehicle controls (Figure 4.8A). This observation is consistent with the effect of calcineurin inhibitors on the stability of A-utrophin mRNA, and the ability of active calcineurin to promote utrophin 3'UTR reporter activity. Quantitative assessment of the half-life of reporter mRNAs fused to the utrophin 3'UTR revealed that upon incubation with FK506, this parameter decreased ~ 25% ($P<0.05$) relative to controls (Figure 4.8B). In contrast, utrophin 3'UTR ΔARE reporter mRNA half-life values, in the presence of SOL extracts from FK506 treated mice, were similar to those obtained for reporter mRNAs fused to the utrophin 3'UTR full-length that had been incubated with SOL extracts from vehicle treated mice (Figure 4.8B). These results suggest that inhibition of calcineurin signaling can promote the degradation of mRNAs through the conserved ARE found in the utrophin 3'UTR.

Figure 4.7. Stimulation of calcineurin activity promotes utrophin 3'UTR reporter activity. C2C12 myotubes were transfected with utrophin 3'UTR full length, 3'UTR 596 and 3'UTR 332 reporter constructs, together with either empty vector (pCIneo) or constructs containing a constitutively active variant of calcineurin (pCnA*). Note that, LacZ reporter mRNA levels, fused to either the utrophin 3'UTR full length or 3'UTR 596, are ~ 50% lower in myotubes transfected with pCIneo relative to myotubes transfected with pCnA*. This difference is lost when LacZ mRNAs are fused to the 3'UTR 332. * indicates a significant difference from pCIneo ($p < 0.05$; n of 4 experiments done in triplicate).

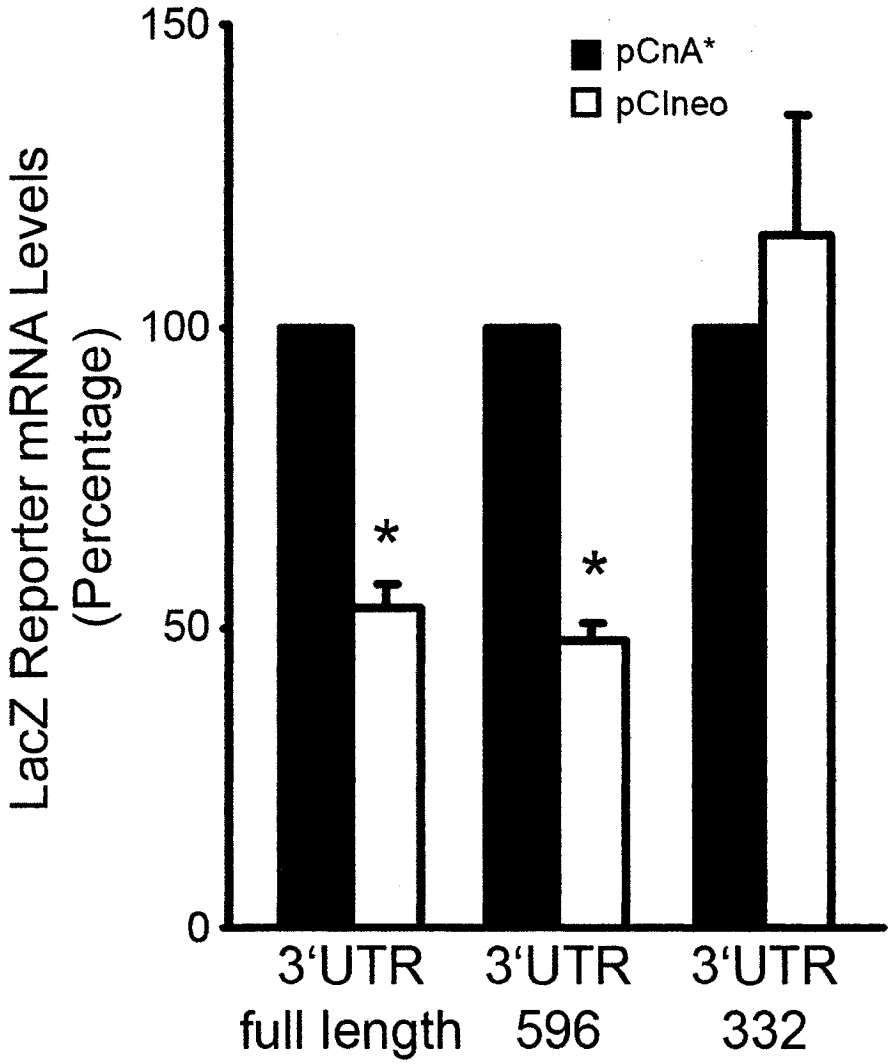
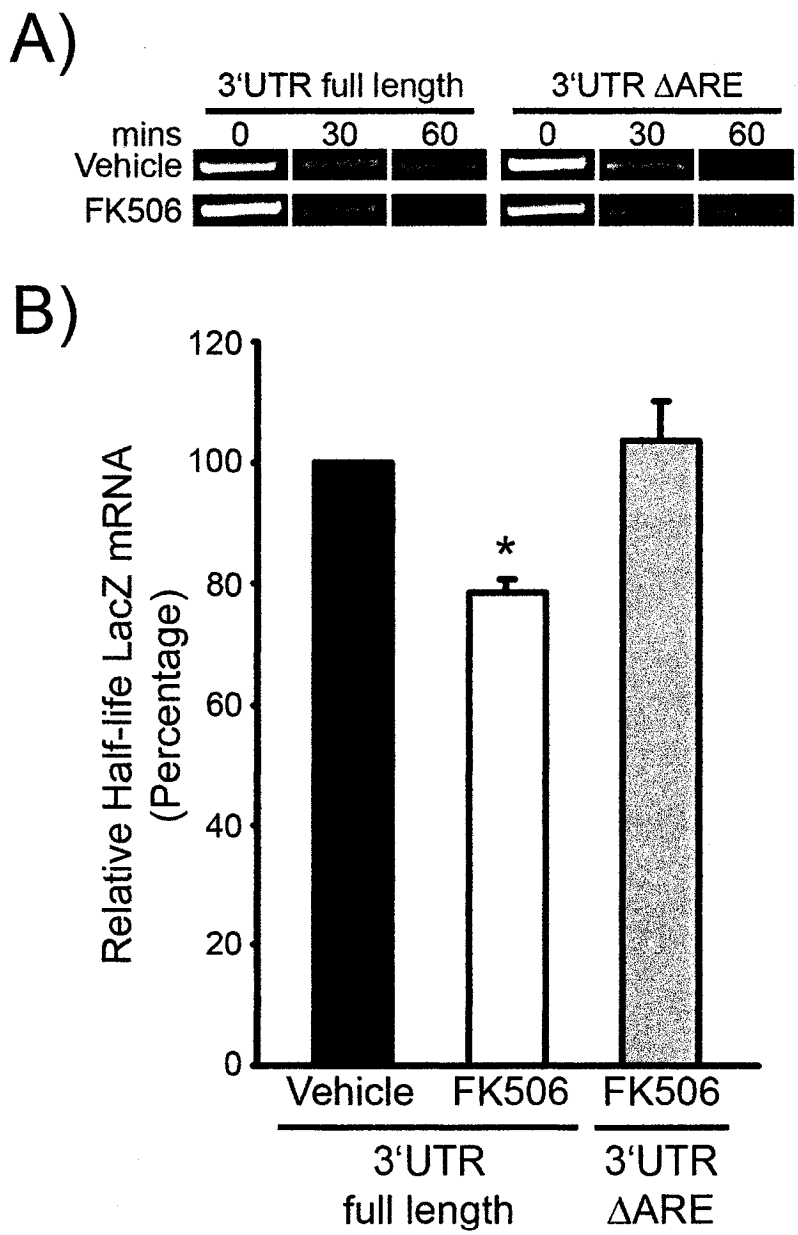

150
100
50
0
pCnA*
pCIneo
LacZ Reporter mRNA Levels
(Percentage)
*
*
3'UTR
full length
3'UTR
596
3'UTR
332

Figure 4.8. Calcineurin signaling regulates utrophin 3'UTR reporter mRNA stability through the conserved ARE. In vitro stability assays were performed with SOL extracts from mice treated with vehicle or FK506, and RNA from 3 day old C2C12 myotubes that had been transfected with utrophin 3'UTR full length or 3'UTR ΔARE reporter constructs. A) Example of ethidium bromide stained agarose gels displaying LacZ cDNA products following 0, 30, and 60 min of incubation with protein extracts. Note the greater amount of LacZ mRNA fused to the utrophin 3'UTR remaining after 30 and 60 min incubations with SOL extracts from vehicle treated mice, versus incubation with SOL extracts from FK506 treated mice. Also note the loss of responsiveness to FK506 upon deletion of the ARE (compare vehicle utrophin 3'UTR full length to FK506 utrophin 3'UTR ΔARE). B) Quantification of the relative half-life values expressed as a percentage of half-life values obtained for LacZ mRNA fused to the utrophin 3'UTR incubated with SOL extract from vehicle treated mice. Note the significant decrease in half-life for LacZ mRNA upon incubation with SOL extract from FK506 treated mice, relative to SOL extract from vehicle treated mice. Also, note the loss of responsiveness to FK506 upon deletion of the ARE. * indicates a significant difference from utrophin 3'UTR full length ($p < 0.05$; $n = 3$ independent experiments).


A)
3'UTR full length
3'UTR ΔARE
mins
0
30
60
0
30
60
Vehicle
FK506
B)
Relative Half-life LacZ mRNA (Percentage)
120
100
80
60
40
20
0
*
Vehicle
FK506
FK506
3'UTR full length
3'UTR ΔARE

DISCUSSION

In this study, through the use of complimentary approaches, we were able to determine some of the molecular events involved in the post-transcriptional regulation of A-utrophin in fast and slow muscles. The application of the in vitro stability assay allowed us to measure A-utrophin mRNA degradation in the presence of distinct skeletal muscle extracts from fast and slow muscles (12, 13). This analysis enabled us to determine that A-utrophin mRNA degrades at a faster rate in the presence of fast skeletal muscle protein extract. In accordance with this observation, we also observed that reporter mRNAs fused to the utrophin 3'UTR decayed at a faster rate upon incubation with fast skeletal muscle extract. Within the utrophin 3'UTR, we uncovered a conserved ARE with a prominent role in conferring higher levels of A-utrophin expression in slow muscles, in comparison to fast muscles. We also demonstrated that the activity of calcineurin, an enzyme with roles in the maintenance of the slow myofiber program, can affect the stability of A-utrophin mRNAs, and utrophin 3'UTR reporter activity and stability via the conserved ARE. The results from this study, together with previous observations, establish that both transcriptional and post-transcriptional mechanisms promote higher levels of A-utrophin expression in slow oxidative muscle fibers, in comparison to fast glycolytic muscle fibers (17, 39 and 77).

A-utrophin mRNA stability in skeletal muscle

Post-transcriptional mechanisms are becoming recognized as important regulatory processes that can regulate the expression of genes in skeletal muscle (18). Fast and slow skeletal muscles, in addition to differing in the profiles of genes expressed, also differ in

the patterns of activity they receive from innervating motor neurons (70). In the present study, we show that stability of A-utrophin mRNA is differentially affected in fast versus slow skeletal muscle. Similar to A-utrophin, the expression of other transcripts in skeletal muscle are known to be regulated post-transcriptionally in response to altered activity. For example, denervation of muscle fibers leads to an almost complete disappearance for transcripts encoding AChE (8). The large reductions in the levels of AChE transcripts, in response to denervation, result from a reduced mRNA half-life for this transcript with only negligible changes in transcription from this gene (8). Post-transcriptional mechanisms also have important roles in promoting the expression of the mitochondrial protein cytochrome c in response to increased skeletal muscle activity (30, 87). In this case, transcriptional mechanisms could account for later increases in the expression of cytochrome c in response to elevated skeletal muscle activity; however the use of in-vitro stability assays determined that changes in mRNA stability were involved in early inductions (30). These observations, together with the present study, implicate mRNA turnover as an important mechanism that regulates the expression of genes in skeletal muscle in response to altered activity (18).

Implication of a conserved ARE in the utrophin 3'UTR

It has been well established that ARE mediated mRNA turnover is an important regulatory process controlling the expression of mRNAs in many cellular systems (see for review 3, 6, 24, and 41). The importance of ARE elements in regulating mRNA turnover was exemplified by studies conducted on the ARE containing granulocyte-macrophage colony stimulating factor (GM-CSF) 3'UTR (74). Fusion of the ARE

containing GM-CSF 3'UTR to globin mRNA promoted the degradation of this normally stable transcript, thereby providing the first functional demonstration of the importance of ARE motifs in mRNA turnover (74). Now a larger percentage of genes have been identified that contain AREs in their 3'UTRs, many of which are involved in a variety of important physiological processes in different tissues (3, 41).

Inspection of the utrophin 3'UTR ARE reveals the presence of two AUUUA motifs within a conserved region, 20 nucleotides in length, overlapping with a UUAUUUA (U/A) (U/A) nonamer. The presence of the two AUUUA motifs and the nonamer suggested that this particular ARE shared characteristics consistent with motifs involved in mRNA degradation (3, 50 and 88). Based on these observations we deleted the conserved ARE found in the utrophin 3'UTR. Deletion of this element resulted in the loss of suppressive activity conferred by the utrophin 3'UTR on reporter mRNAs in muscle culture. In vitro stability assays demonstrated that deletion of the ARE could rescue the reduced half life of reporter mRNAs fused to the utrophin 3'UTR that had been incubated with EDL skeletal muscle extract. Therefore, the utrophin 3'UTR ARE functions as a destabilizing element that promotes the degradation of mRNAs in the presence of fast muscle extract.

Mechanisms of ARE-regulated A-utrophin mRNA stability

Thus far, using other cellular systems, it has been shown that ARE containing transcripts are rapidly deadenylated and degraded by one of two processes. One involves the association of the exosome, a large multiprotein enzymatic complex, to ARE containing transcripts (25, 59). The association of the exosome subsequently targets

ARE containing mRNAs for degradation (25, 59). The second pathway involves a dynamic equilibrium whereby ARE containing mRNAs are targeted to cellular entities, processing (P) bodies or stress granules (45, 83). P-bodies are regions that contain factors that promote the degradation of mRNAs. In contrast, stress granules, in response to physiological stressors, sequester mRNAs in a stable state prior to their targeting either for extra rounds of translation at polysomes, or to P-bodies for degradation (45, 83). In this context, the 264 nucleotide 332-596 region of the utrophin 3'UTR has previously been shown to have important roles in the targeting of mRNAs to cytoskeletal bound polysomes during myogenesis (40). Since the same minimal region in the utrophin 3'UTR can regulate the stability and localization of mRNAs, it is possible that in the contexts of fast and slow skeletal muscle the processes of stability, and targeting of A-utrophin transcripts are linked. Therefore, deciphering the intracellular sites of enrichment for A-utrophin transcripts in fast and slow skeletal muscle may provide important insights into the mechanisms that regulate its turnover.

The ability of AREs to affect the turnover rates of host mRNAs involves the recruitment of ARE-binding proteins (ARE-bps) (see for review 3, 6, 41 and 85). The binding of ARE-bps to AREs can lead to either the stabilization or accelerated degradation of mRNAs. ARE-bps such as AUF1 and KRSP, are examples of trans-factors that promote the degradation of ARE containing mRNAs (3, 10, 11, 33, and 84). In contrast, the Hu family of proteins (HuR, B, C and D), are the most well characterized ARE-bps known to promote the stabilization of mRNAs (3, 6, 9 and 85). In skeletal muscle, HuR has been shown to associate with the AREs for MyoD, myogenin, p21 and AChE (28, 29 and 80). In this context, HuR levels and localization have been shown to

have important roles in the stabilization of these transcripts during myogeneis (28, 29 and 80). Conversely, activation of p38 MAPK signaling promotes the stabilization of MyoD, myogenin and p21 mRNAs, by preventing the binding of KRSP to AREs found in their 3'UTRs (14).

Chronic stimulation of skeletal muscle leads to increased expression of cytochrome c with a loss in the binding of a potentially destabilizing 37 kDa protein to the cytochrome c 3'UTR (87). Considering that chronic stimulation promotes the conversion of skeletal muscles to a slower oxidative phenotype (70), the above observations, concerning the association of trans-factors to the cytochrome c 3'UTR, are consistent with the notion that fast muscle extracts contain a factor that promotes the destabilization of target mRNAs. Previously, through UV crosslinking assays, we had shown greater binding activity for a 37 kDa factor to the utrophin 3'UTR in the presence of fast muscle protein extract relative to slow muscle extracts (39). Collectively, these observations suggest that conversion to a slow myofiber phenotype can lead to a loss in the association of a potentially destabilizing factor to the 3'UTRs for cytochrome c and possibly utrophin. The size range of this probable destabilizing protein is consistent with the ARE-bp AUF1 (10, 11). Based on the forgoing discussion it would be of interest to determine the activity and expression of AUF1 in fast and slow skeletal muscle, and whether it binds to the utrophin 3'UTR.

Calcineurin and post-transcriptional regulation

Different rates of muscle activity generated by stimulation patterns provided by innervating motor neurons can influence the expression of mRNAs, through changes in

intracellular Ca^{+2} release (5). In skeletal muscle, changes in intracellular calcium have been linked to alterations in the turnover of mRNAs for AChE and the inwardly rectifying potassium channel 1 (53, 75). It is now well established that the activity of calcineurin, a Ca^{+2}/calmodulin regulated phosphatase, takes part in the maintenance of the slow fiber phenotype by regulating the expression of slow myofiber genes (26, 61, 65, 67 and 68). Recent examination of calcineurin null mice, although confirming roles for calcineurin in the promotion of the slow fiber phenotype, suggested that this phosphatase could regulate the maintenance of the slow fiber type phenotype through additional factors other than NFAT and MEF2 (68). In this context, alterations of calcineurin activity in skeletal muscle cell culture have been shown to influence the stabilization of AChE mRNAs during myogeneic differentiation (54). Interestingly, calcineurin has also been shown to regulate ARE-mediated mRNA turnover. For example, calcineurin inhibition leads to the destabilization of IL-3 mRNAs (60). This destabilizing effect of calcineurin inhibition is lost upon deletion of the ARE from the IL-3 3'UTR (60). In addition, inhibition of calcineurin activity has been shown to alter parathyroid mRNA levels, and promote the binding of AUF1 to the parathyroid 3'UTR (4). In this study, we demonstrate that calcineurin activity can influence the turnover of A-utrophin mRNA, utrophin 3'UTR reporter activity and stability through an ARE dependent mechanism. Therefore, these results suggest that post-transcriptional mechanisms, at the level of ARE regulated mRNA stability, provide an additional mechanism whereby calcineurin can promote the expression of genes that characterize the slow myofiber phenotype.

Conclusions

Collectively the results from this study uncover a novel mode of regulation for A-utrophin expression in skeletal muscle. Importantly, the results from this study have implications for the design of pharmacological strategies aimed at elevating utrophin expression, to functionally compensate for dystrophin-deficiency in DMD muscles. For example, a potential pharmacological strategy designed on the basis for its ability to stimulate A-utrophin promoter activity could be considered a failure due to the lack of increased A-utophin mRNA levels. The lack of increased A-utrophin mRNA, despite stimulation of transcriptional activity, may reflect the rapid destabilization of newly transcribed A-utrophin mRNAs. Previously we observed that calcineurin could effect the expression of A-utrophin mRNA, in part through transcriptional mechanisms (17). Recently, we have shown that promotion of calcineurin signaling in dystrophin-deficient muscle increases A-utrophin expression and alleviates dystrophic pathology (19, 21). In the present study we observe that calcineurin activity can also effect the stability of A-utrophin mRNA by acting on a conserved ARE found in the utrophin 3'UTR. Taken together these observations suggest that future work aimed at identifying strategies to promote the expression of endogenous A-utrophin should focus on targets, such as calcineurin, that have the ability to promote A-utrophin transcription, and subsequently maintain A-utrophin mRNA levels through post-transcriptional mechanisms

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Chapter 5

Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice

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Contribution from authors

Joe V. Chakkalakal and Bernard Jasmin wrote manuscript. Joe V. Chakkalakal performed all RT-PCR analysis, immunofluorescence analysis, western blot analysis and histological analysis with technical assistance from John Lunde. Mary-Ann Harrison, Eva Chin and Robin Michel provided muscles from CnA* and mdx/CnA* transgenic mice, as part of an on-going collaboration. Salvatore Carbonetto provided affinity purified β-dystroglycan antibody.

ABSTRACT

Utrophin has been studied extensively in recent years in an effort to find a cure for Duchenne muscular dystrophy. In this context, we previously showed that mice expressing enhanced muscle calcineurin activity (CnA*) displayed elevated levels of utrophin around their sarcolemma. In the present study, we therefore crossed CnA* mice with mdx mice to determine the suitability of elevating calcineurin activity in preventing the dystrophic pathology. Muscles from mdx/CnA* displayed increased nuclear localization of NFATc1 and a fiber type shift towards a slower phenotype. Measurements of utrophin levels in mdx/CnA* muscles revealed an ~ 2 fold induction in utrophin expression. Consistent with this induction, we also observed that members of the dystrophin-associated protein (DAP) complex were present at the sarcolemma of mdx/CnA* mouse muscle. This restoration of the utrophin-DAP complex was accompanied by significant reductions in the extent of central nucleation and fiber size variability. Importantly, assessment of myofiber sarcolemmal damage, as monitored by the intracellular presence of IgM and albumin as well as by Evans blue uptake in vivo, revealed a net amelioration of membrane integrity. Finally, immunofluorescence experiments using Mac-1 antibodies showed a reduction in the number of infiltrating immune cells in muscles from mdx/CnA* mice. These results show that elevated calcineurin activity attenuates the dystrophic pathology and thus provides an effective target for pharmacological intervention.

INTRODUCTION

Over the last 15 years, a large number of laboratories have focused their efforts on trying to develop an effective therapy for Duchenne Muscular Dystrophy (DMD). Although several therapeutic strategies have been explored including gene transfer and cell therapy (1-4), there is currently no cure for this disease. One proposed strategy aimed at alleviating the debilitating symptoms of DMD involves the up-regulation of the autosomal homologue of dystrophin referred to as utrophin (5-7). In skeletal muscle, utrophin is known to accumulate preferentially at the post-synaptic membrane of the neuromuscular junction though a low level of expression in extra-synaptic compartments can also be detected in slow myofibers (8,9). Studies using either transgenic mouse models or adenoviral-based gene transfer have shown that increasing utrophin levels can rescue muscle fibers by decreasing the extent of the dystrophic pathology in both canine and mouse models of DMD (10-12). The demonstration in these studies that utrophin can functionally compensate for the lack of dystrophin shows the importance of defining the mechanisms regulating utrophin in skeletal muscle. Elucidation of these mechanisms will identify specific molecular targets for which pharmacological interventions, aimed at increasing the abundance of utrophin throughout muscle fibers, may be designed.

Within the last few years, several groups have shown that expression of utrophin can vary according to the state of differentiation and innervation of muscle cells (6). In this context, it has been shown that expression of utrophin is regulated at different levels since it appears to involve the contribution of transcriptional events (see for review 6,7)) as well as post-transcriptional mechanisms (8,13-15). Recently, we reported that slower, high oxidative, muscle fibers contained significantly more utrophin in comparison to

faster, more glycolytic counterparts (8,16 see also 9). Using a series of complementary approaches, we were also able to demonstrate that the increased expression of utrophin in slower, high oxidative, fibers was due to greater levels of the utrophin A isoform and involved the calcineurin/NFAT signaling cascade acting on the utrophin A promoter (16). Specifically, we showed in these recent studies that activated calcineurin and constitutively nuclear NFATc1 could directly transactivate the utrophin A promoter in myogenic cells in culture and in vivo (16).

Given these findings, we decided to examine whether stimulation of the calcineurin pathway could attenuate the dystrophic pathology in mdx mice. To this end, we crossed mice that expressed an activated form of calcineurin (CnA*) in their skeletal muscle with mdx mice. We then determined the levels of utrophin expression in these transgenic mdx mice and examined several indices that reflect the extent of the muscle pathology. Our results demonstrate that utrophin A expression is indeed under the control of calcineurin signaling and that increased utrophin A expression achieved under these conditions, can have significant beneficial effects on dystrophic muscle fibers in vivo. These findings have significant implications in assessing the suitability of calcineurin activation as a therapeutic avenue for DMD.

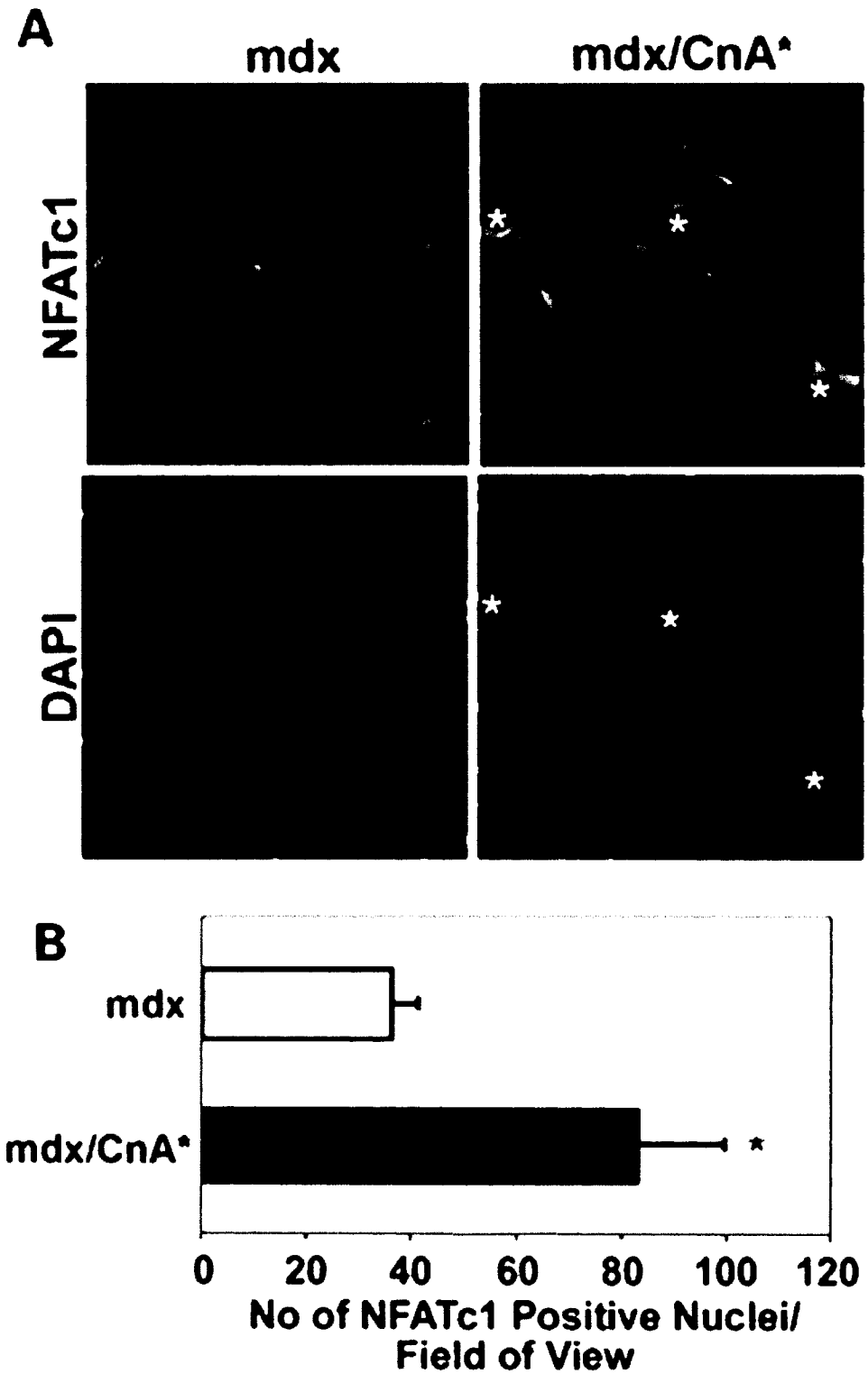
RESULTS

Stimulation of the Calcineurin Pathway in mdx Mouse Muscle. Stimulation of calcineurin activity is known to result in the dephosphorylation of NFAT transcription factors leading to their translocation to nuclei where they can increase transcription of target genes (17,18). The CnA* mice used to generate mdx/CnA* mice have been shown previously to display increased levels of activated calcineurin mRNA and an increase in calcineurin activity (19). We thus initially performed a series of immunofluorescence experiments to ascertain that muscles from mdx/CnA* mice contain a higher number of NFAT-positive nuclei compared to muscles obtained from mdx mice. As shown in Figure 5.1A, staining for NFATc1 in mdx tissue appeared diffuse and cytoplasmic. In contrast, staining of NFATc1 in mdx/CnA* muscles displayed a preferential accumulation in myonuclei with a reduction in the diffuse cytoplasmic staining pattern seen in mdx tissue. This is expected given that expression of the transgene is under the control of the muscle-specific creatine kinase promoter. In these experiments, controls performed by omitting the primary antibody resulted in an absence of labeling (data not shown). Comparison of the average number of NFATc1-positive nuclei revealed a > 2-fold increase in the number of myonuclei stained for NFATc1 in mdx/CnA* mice (Figure 5.1B). These findings not only confirm the genotype of mdx/CnA* mice, but further our previous contentions (19, 20) that expression of this transgene results in a functional stimulation of the calcineurin signaling pathway.

Since calcineurin signaling has been shown to be involved in controlling the slow oxidative myofiber program and, ultimately, the transcriptional activity of slower isoforms of myosin heavy chain (MyHC) II genes (17, 18, 20-23), we next sought to

Figure 5.1. Enhanced localization of NFATc1 in myonuclei of mdx/CnA* mice.

Shown are representative photomicrographs of cross sections from mdx and mdx/CnA* muscles double labeled to detect NFATc1 and myonuclei with DAPI. * denotes NFATc1 labeling and corresponding nuclei (A). Quantification of the number of nuclei stained for NFATc1 show that mdx/CnA* muscles contain considerably more positive nuclei in 20x cross-sectional views (B). * denotes significant difference from mdx ($P < 0.05$), n = 4 animals per group, 3-4 20x sections from each animal (B) . Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests.


A
mdx
mdx/CnA*
NFATc1
DAPI
B
mdx
mdx/CnA*
0
20
40
60
80
100
120
No of NFATc1 Positive Nuclei/
Field of View

determine whether a fiber type shift occurred in mdx/CnA* muscles. Using isoform-specific antibodies in immunocytochemical experiments, we observed an increase in the percentage of MyHC IIa fibers which was accompanied by a parallel reduction in the percentage of MyHC IIb fibers in mdx/CnA* muscles (Figure 5.2). These results demonstrate that muscle-specific expression of an activated calcineurin transgene in an mdx background is capable of inducing a fiber type shift towards a slower phenotype.

Changes in Utrophin Expression in mdx/CnA* Mouse Muscle. Recently, we showed that levels of utrophin A in myofibers are positively correlated with the expression of slower myosin heavy chain isoforms (16). In the present study, immunoblotting experiments using muscle proteins revealed an ~ 2-fold induction in utrophin levels in muscles from mdx/CnA* versus mdx mice (Figure 5.3A). In agreement with the immunoblot data, immunofluorescence experiments using three different antibodies showed an increased expression of utrophin (Figure 5.3B, and 5.5A,B). These experiments further revealed that the increase in extra synaptic utrophin A occurred at the level of the sarcolemma and led to a more homogeneous pattern of utrophin expression amongst myofibers. RT-PCR analysis using RNA extracted from muscles isolated from mdx/CnA* mice revealed an ~ 2 fold induction ($P < 0.05$) in utrophin transcripts (Figure 5.4A). Using isoform-specific primers, we also demonstrated that this increase was accompanied by a corresponding induction in utrophin A mRNA with utrophin B transcripts remaining largely unchanged (Figure 5.4).

The induction in utrophin expression in mdx/CnA* mouse muscle prompted us to examine the localization of several components of the dystrophin-associated protein

Figure 5.2. mdx/CnA* mice display shifts in fiber type towards a slower phenotype. Shown are representative photomicrographs of cross sections from mdx and mdx/CnA* muscles processed to detect MyHC IIb or MyHC IIa by immunocytochemsitry (A). Quantification revealed a significant induction in MyHC IIa expressing fibers and a reduction in MyHC IIb (B). * denotes significant difference from mdx ($P< 0.05$), n = 4 animals per group. Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests.

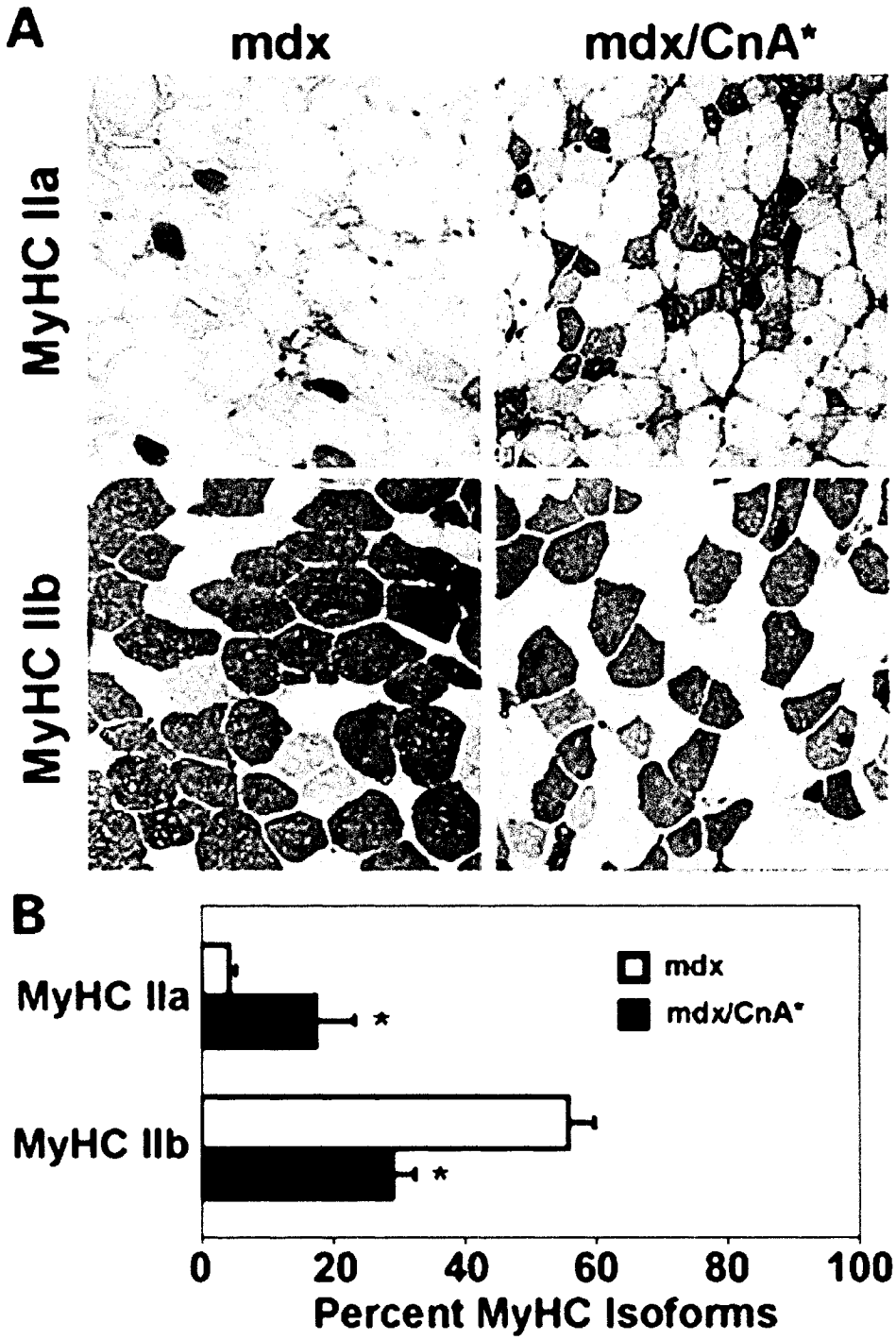

A
mdx
mdx/CnA*
MyHC IIa
MyHC IIb
B
MyHC IIa
MyHC IIb
mdx
mdx/CnA*
*
*
0
20
40
60
80
100
Percent MyHC Isoforms

Figure 5.3. Increase in utrophin and utrophin A protein levels in mdx/CnA* muscle fibers. (A) Representative immunoblot for utrophin. Note the increased levels of utrophin in mdx/CnA* muscles. Immunofluorescence analyses demonstrated that muscles from mdx/CnA* mice express high levels of both utrophin and utrophin A at the sarcolemma of each individual fiber (B). * denotes significant difference from mdx ($P < 0.05$), n = 3 animals per group. Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests.

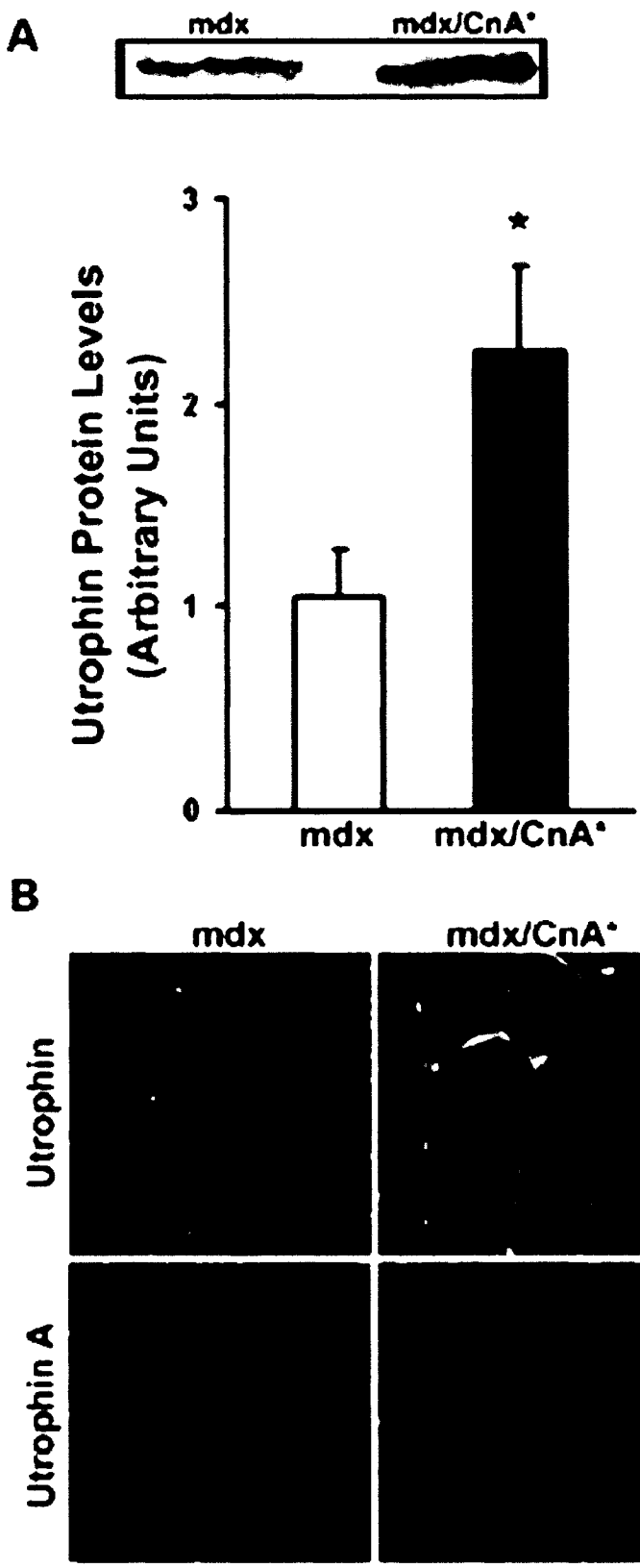
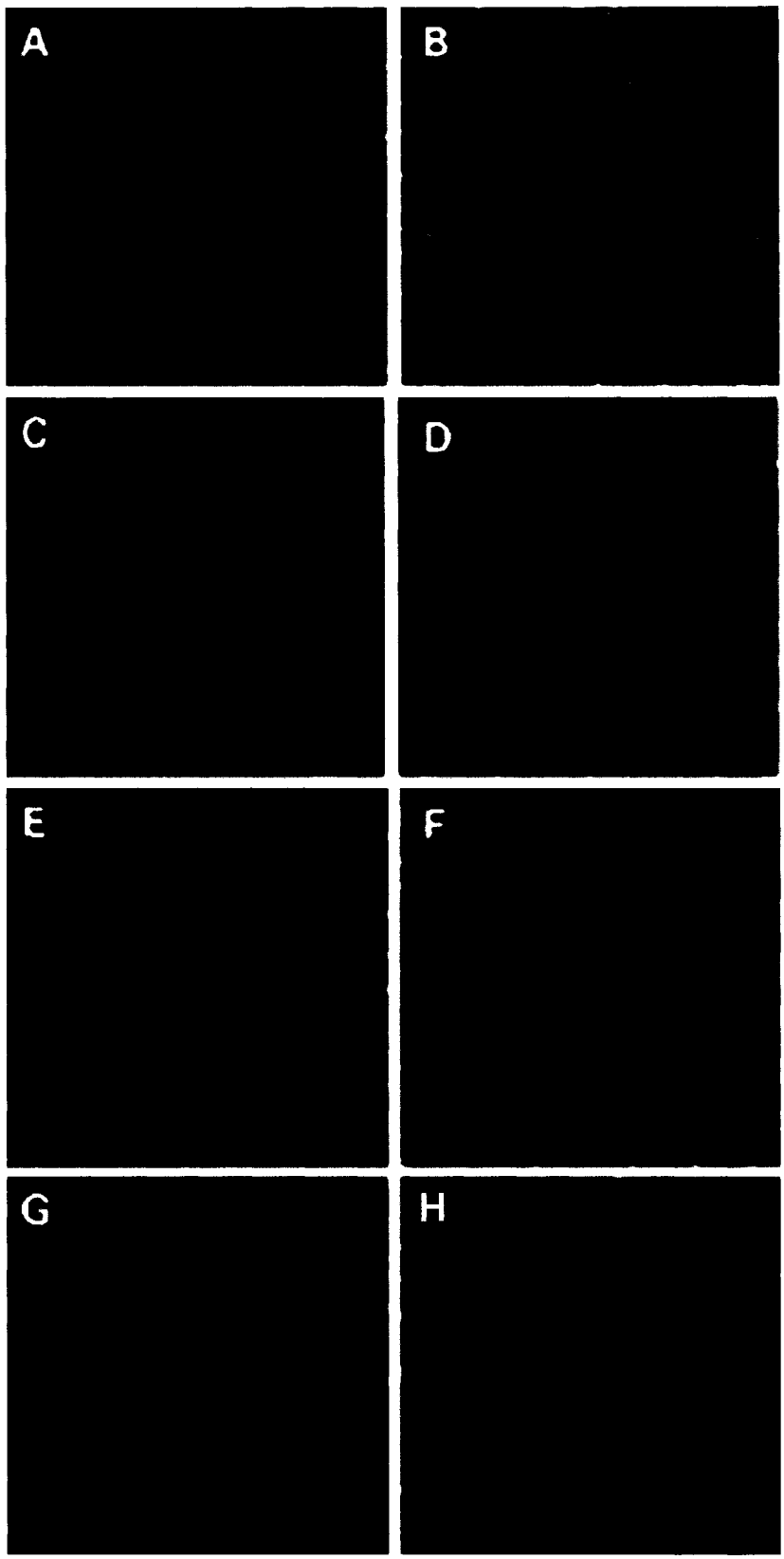

A
mdx
mdx/CnA*
Utrophin Protein Levels
(Arbitrary Units)
3
2
1
0
*
mdx
mdx/CnA*
B
mdx
mdx/CnA*
Utrophin
Utrophin A

Figure 5.4. Increase in utrophin and utrophin A mRNA expression in mdx/CnA* muscle fibers. (A) Examples of ethidium bromide stained gels showing PCR products from muscles from mdx and mdx/CnA* for total utrophin, utrophin A and utrophin B. (B) Note the increased levels of utrophin and utrophin A mdx/CnA* without any change in utrophin B levels. * denotes significant difference ($P < 0.05$), n = 6-8 animals per group. Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests.


A
B
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H

complex to determine whether they also accumulated at the sarcolemma of individual myofibers. As shown in Figure 5.5, immunofluorescence experiments demonstrated a greater presence of β–dystroglycan, syntrophin and nNOS at the sarcolemma of mdx/CnA* muscles.

Improvement in Morphological Features of mdx/CnA* Mouse Muscle. The induction of utrophin and the restoration of dystrophin-associated proteins has been shown by others to have significant beneficial effects in mdx mouse muscles (see Introduction). To determine whether stimulation of the calcineurin signaling pathway could result in an attenuation of the dystrophic pathology, we examined several morphological parameters in muscles from mdx/CnA* mice. Hematoxylin and eosin staining of muscle sections revealed a healthier appearance of mdx/CnA* muscle fibers in comparison to mdx counterparts (Figure 5.6A). An assessment of the percentage of central nucleation seen in mdx/CnA* muscles showed an ~ 20% reduction of this parameter (Figure 5.6B), in agreement with other studies that used different paradigms to improve the dystrophic phenotype (11, 24, 25). Importantly, the reduction in the percentage of central-nucleated fibers illustrates the enhanced survival capacity of original myofibers in mdx/CnA* mouse muscle (26).

Among the several abnormal morphological indices, dystrophic muscle fibers are known to display greater variations in their cross-sectional area (for example 27). Assessment of fiber size variability as determined by the averaged standard deviation of the cross sectional areas of myofibers, showed greater homogeneity in mdx/CnA* muscles (Figure 5.6A and C). This correction in the morphological features of muscles

Figure 5.5. Increase in sarcolemmal expression of dystrophin associated proteins. Shown are representative photomicrographs of cross sections from muscles from mdx and mdx/CnA* animals processed to detect utrophin (A,B), β–dystroglycan (C,D), syntrophin (E,F) and nNOS (G,H). Immunofluorescence analyses demonstrated that muscles from mdx/CnA* mice express high sarcolemmal levels of utrophin, β–dystroglycan, syntrophin and neuronal nitric oxide synthase.

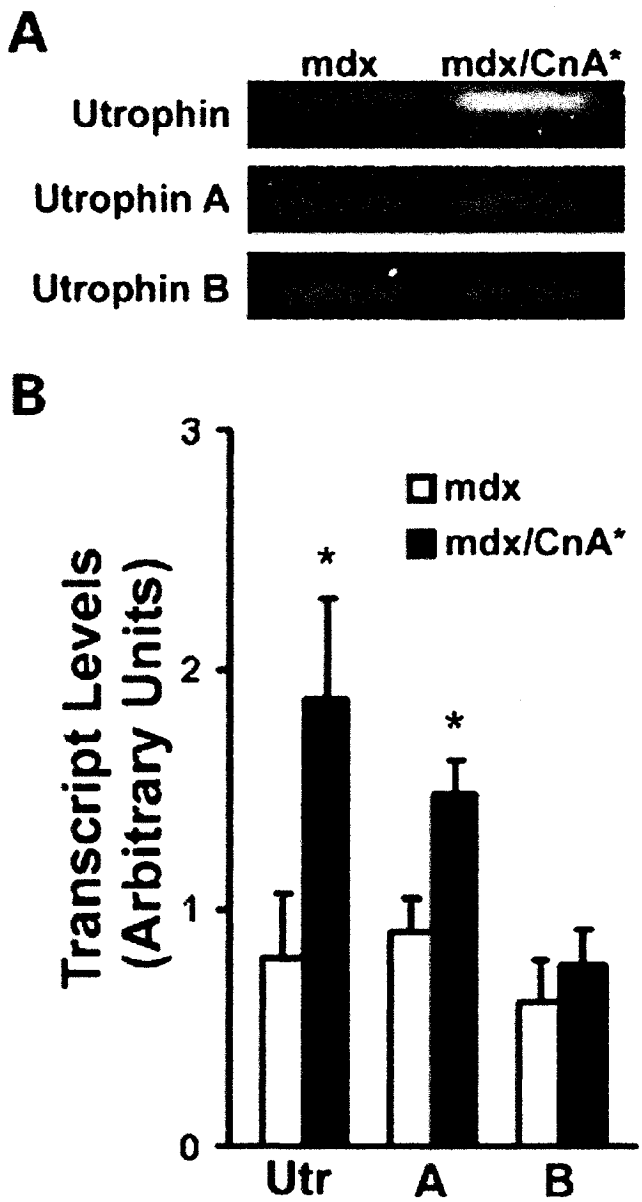
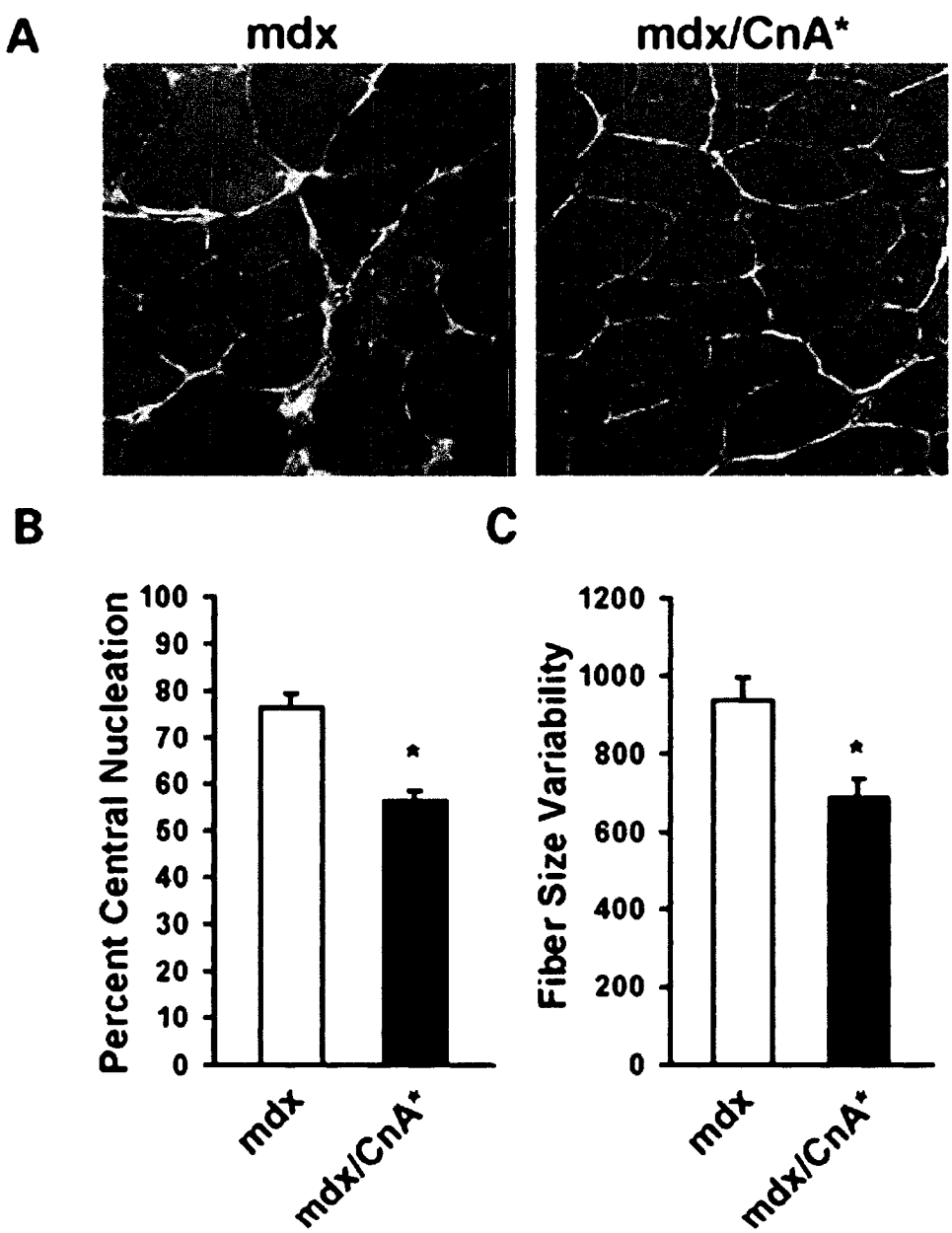

A
mdx
mdx/CnA*
Utrophin
Utrophin A
Utrophin B
B
Transcript Levels
(Arbitrary Units)
3
2
1
0
mdx
mdx/CnA*
*
*
Utr
A
B

Figure 5.6. Reduction in central nucleation and fiber size variability in mdx/CnA* muscle fibers. Shown are representative photomicrographs of cross sections from mdx and mdx/CnA* muscles processed for hematoxylin and eosin staining (A). Myofibers from mdx/CnA* animals display a reduction in central nucleation (B). Similarly, mdx/CnA* myofibers displayed a reduction in fiber size variability (C). * denotes significant difference from mdx ($P < 0.05$), n = 4 animals per group. Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests.


A
mdx
mdx/CnA*
B
Percent Central Nucleation
100
90
80
70
60
50
40
30
20
10
0
*
mdx
mdx/CnA*
C
Fiber Size Variability
1200
1000
800
600
400
200
0
*
mdx
mdx/CnA*

from mdx/CnA* mice are similar to those reported by others using different paradigms and correlates favorably with the functional improvements seen in dystrophic muscle (24).

Improved Sarcolemmal Integrity of mdx/CnA* Myofibers. We also determined the percentage of fibers staining positively for IgM (mass of 900 kD) and albumin (mass of 65 kD) taken as indices of membrane stability. Normally, these serum components are extracellular but sarcolemmal disruption, caused by the dystrophic pathology, confers membrane leakiness thereby allowing serum proteins to penetrate myofibers (5,28). As shown in Figure 5.7, immunofluorescence experiments showed that approximately 10% of the fibers in muscles from four to seven week-old mdx mice stained for IgM. Other studies using different paradigms have found a similar percentage of damaged fibers in mdx muscles (10, 24, 29). Transgenic expression of CnA* led to a significant reduction ($P < 0.05$) in the percentage of IgM-positive fibers. Additional studies performed on older mice revealed that this beneficial effect was still present in 14 week-old mice. Similar results were also obtained in experiments using cytoplasmic labeling of albumin as a marker of membrane disruption (data not shown).

To further examine the beneficial effects of utrophin up-regulation on the integrity of the sarcolemma in mdx/CnA* muscles, we injected mice with the small (1 kD) dye Evans blue and monitored the uptake of the dye in myofibers using fluorescence microscopy as described previously (28). As shown in Figure 5.8, there was a significant reduction in the levels of Evans blue uptake in myofibers of mdx/CnA* mice compared to mdx counterparts. In agreement with our data showing an improvement in membrane

Figure 5.7. Reduction in membrane lesions in mdx/CnA* mice. Cross sections from 4-7 and 14 week old muscles demonstrate a reduction in the occurrence of cytoplasmic staining for IgM (A). Assesment of the percentage of fibers stained for IgM revealed an approximately 2 fold reduction in mdx/CnA* in comparison to age-matched mdx mice (B). * denotes significant difference from mdx ($P< 0.05$), n = 3-4 animals per group. Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests.

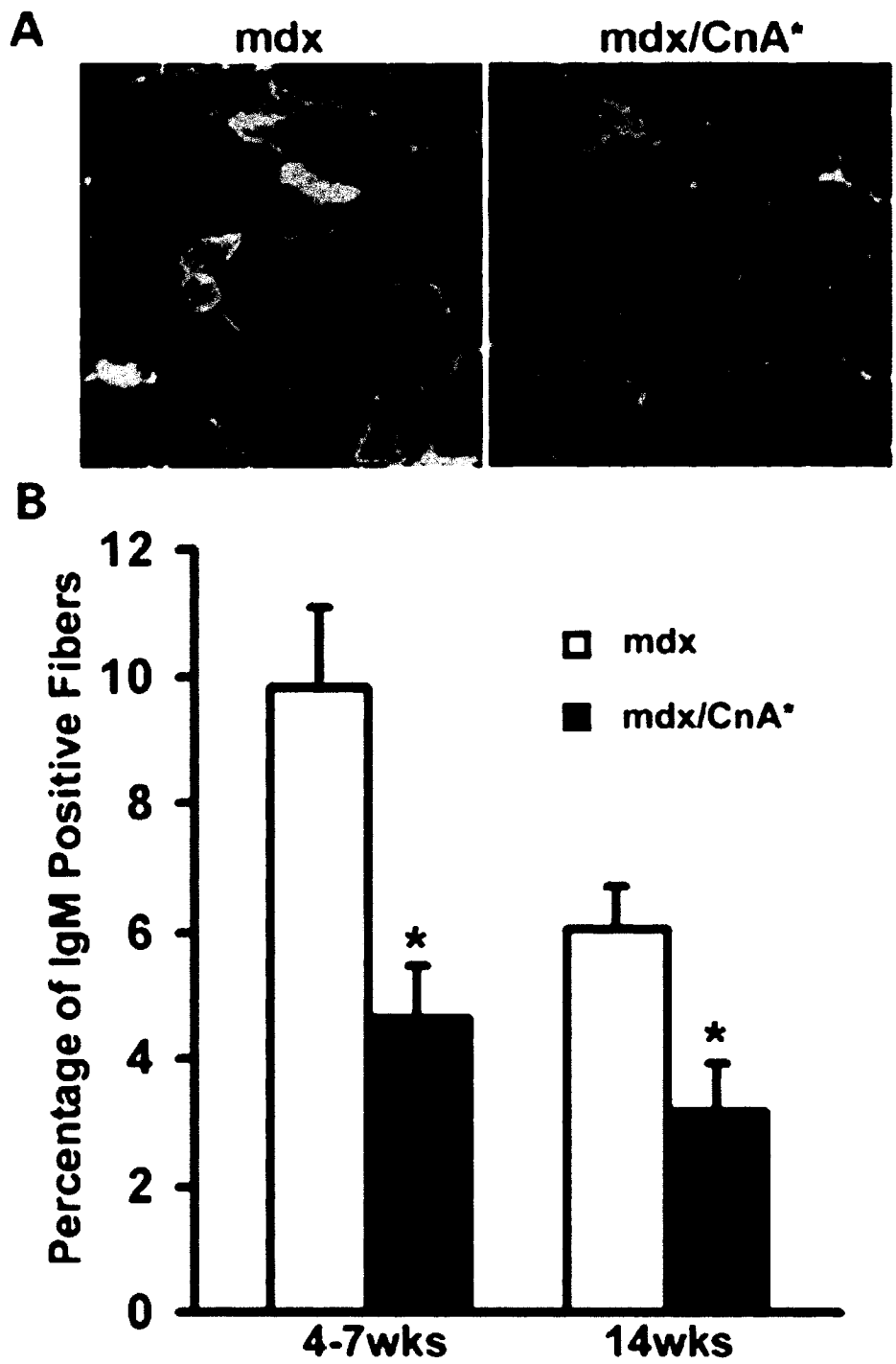
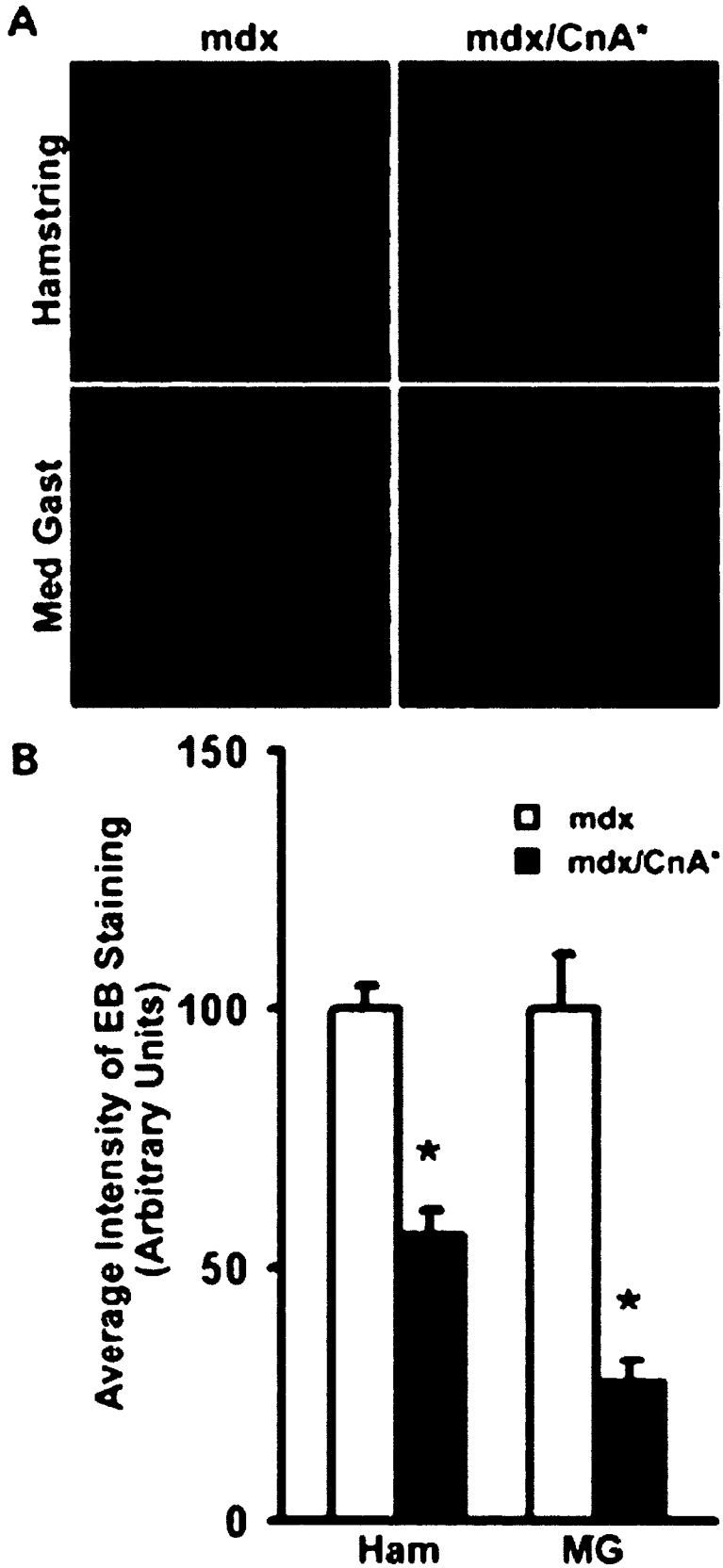

A
mdx
mdx/CnA*
B
Percentage of IgM Positive Fibers
12
10
8
6
4
2
0
mdx
mdx/CnA*
*
*
4-7wks
14wks

Figure 5.8. Reduction in the levels of Evans blue staining in skeletal muscles from mdx/CnA* mice. Cross sectional views of Evan blue dye-positive regions of both medial gastrocnemius (MG) and hamstring (Ham) muscles from mice intravenously injected demonstrate levels of dye infiltration. Assessment of Evans blue dye staining in damaged regions of mdx/CnA* hamstring and medial gastrocnemius muscles revealed an approximately 2 and 3 fold reductions, respectively, in comparison to age matched mdx mice. * denotes significant difference from mdx ($P< 0.05$), n = 4 animals per group. Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests. integrity in muscles from mdx/CnA* mice, serum creatine kinase activity was also reduced by ~50% in these animals (data not shown).

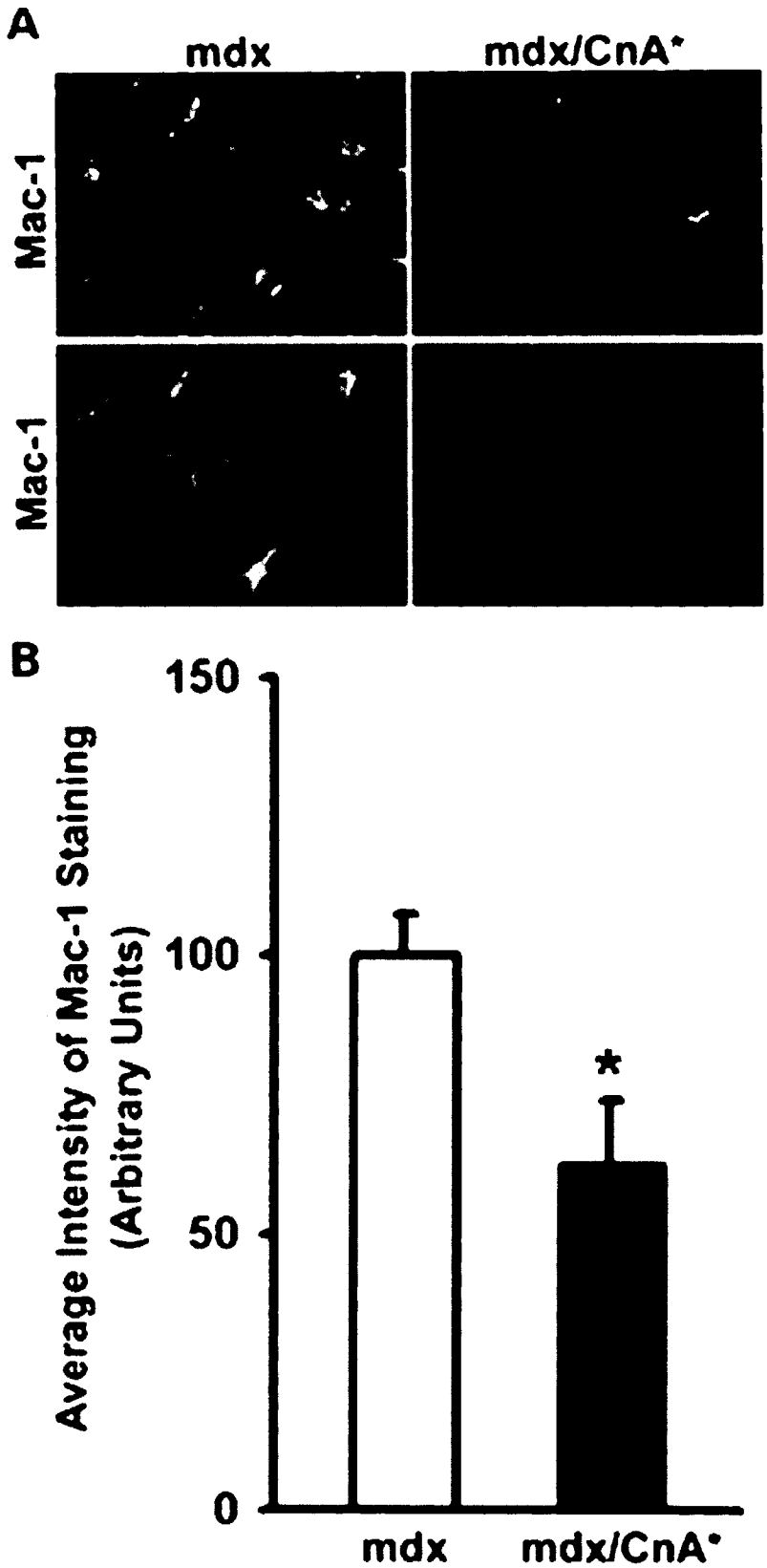

A
mdx
mdx/CnA*
Hamstring
Med Gast
B
150
100
50
0
Average Intensity of EB Staining (Arbitrary Units)
mdx
mdx/CnA*
*
*
Ham
MG

integrity in muscles from mdx/CnA* mice, serum creatine kinase activity was also reduced by ~50% in these animals (data not shown).

Attenuation of the Inflammatory Response in mdx/CnA* Mouse Muscle. Several recent studies demonstrate that the dystrophic process in mdx mouse muscle is accompanied by a marked inflammatory response (30, 31). Therefore, in a last set of experiments, we determined whether muscles from mdx/CnA* mice contained fewer immune cells as identified by immunofluorescence experiments using Mac-1 antibodies which recognize several cells involved in the immune response. As shown in Figure 5.9, we noted a significant reduction in the levels of Mac-1 immunolabeling in muscles from mdx/CnA* mice. In these experiments, we observed similar findings using different muscles including the hamstring, gastrocnemius and diaphragm (data not shown).

Figure 5.9. Reduction in the levels of infiltrating immune cells in mdx/CnA* mice.

Muscle cross sections demonstrate a reduction in the occurrence of Mac-1 staining, a marker for invading cells involved in the inflammatory response (A). Assessment of the levels of Mac-1 positive regions revealed an approximately 2 fold reduction in mdx/CnA* in comparison to age-matched mdx mice (B). * denotes significant difference from mdx ($P < 0.05$), n = 3-4 animals per group. Mean +/- SEM are shown. Statistical analysis was conducted using student's t-tests.


A
mdx
mdx/CnA*
Mac-1
Mac-1
B
Average Intensity of Mac-1 Staining
(Arbitrary Units)
150
100
50
0
*
mdx
mdx/CnA*

DISCUSSION

We have previously shown utrophin levels to be positively correlated with the expression of slower MyHC isoforms and to be regulated, at least partially, by calcineurin/NFAT signaling (16). In the present study, we significantly extend these initial observations by showing using immunofluorescence that transgenic expression of an activated form of calcineurin in an mdx background results in an increase in the number of NFATc1-positive myonuclei. In turn, enhanced calcineurin signaling in an mdx background increases the expression of MyHC IIa and utrophin A (16, 22, 23). Within the last several years, numerous studies have shown the remarkable beneficial effects of increased levels of utrophin at the sarcolemma of mdx mice (5, 10, 11, 32, 33). Coherent with these earlier reports, we used a battery of different markers to show significant improvements in several morphological features normally associated with the dystrophic process. Taken together, the results of the present study clearly highlight in an in-vivo dystrophic model, the benefits of calcineurin activation in attenuating the disease phenotype.

Normally, activation of calcineurin/NFAT gene expression involves sustained increases in the intracellular levels of calcium in a pattern similar to that seen in slower contracting muscles (34-36). Therefore, treatments designed to enhance calcineurin activity could potentially involve either the direct stimulation of calcineurin activity or sustained elevations in intracellular calcium levels. Although still controversial, abnormal calcium handling has been postulated to be an important factor resulting in the progression of the dystrophic pathology (37). Such disease progression via impaired calcium kinetics may occur as a result of alterations in the normal activation of the

calcineurin/NFATc1 signaling pathway. In this context, modulations in calcium levels can also result in the activation of other transcriptional networks including those involving the stress-activated MAPK JNK1 pathway (38). Interestingly, the activation of this pathway has been shown to be associated with the progression of the dystrophic pathology and the higher JNK1 activity seen in dystrophic muscles can result in increased interactions with NFATc1 thereby displacing it from the nucleus (39). Therefore, imbalances in calcium homeostasis in dystrophic muscles may result in the activation of pathways that antagonize calcineurin/NFAT signaling resulting in impaired gene expression eventually triggering muscle necrosis/apoptosis.

Although it appears likely, based on our previous (16) and current findings that NFATc1 is directly involved in the transcriptional regulation of utrophin A, it is not possible to completely rule out the contribution of other transcription factors. For instance, MEF2 and SRF are factors known to be capable of responding to various nerve-dependent calcium-regulated signals and have been shown to be implicated in the expression of fiber type specific genes and cytokine production in lymphocytes together with NFAT (19, 40, 41). Given that mdx myofibers have elevated levels of intracellular calcium (37), it is plausible that MEF 2 and SRF or other, as yet to be identified, calcium-regulated transcription factors, act alone or in concert with NFATc1 to stimulate the expression of MyHC IIa and utrophin A in mdx/CnA* muscles.

Pharmacological strategies enhancing calcineurin activity in combination with other proposed therapies could thus provide an effective treatment for counteracting the devastating symptoms caused by dystrophin deficiency. In our system, the ability of enhanced calcineurin activity to potentiate endogenous utrophin levels coupled with the

potential to attenuate the inflammatory response may promote survival of dystrophin-deficient fibers. In this context, calcineurin/NFAT signaling has been shown to regulate the expression of IL-4 in myogenic cells which promotes the fusion of myoblasts with myotubes (42). This apparent paradox, whereby enhanced calcineurin activity results in a reduction in the number of infiltrating immune cells in muscle (this study) while stimulating cytokine production (42), may be reconciled if we consider the selectivity of NFAT factors in preferentially regulating the expression of specific cytokines (43). Alternatively, it appears likely that the role of IL-4 in promoting myoblast fusion may be most relevant during the early stages of myogenesis in our mdx/CnA* mice whereas in mature muscles from these mice, the induction of utrophin A and of the dystrophin-associated proteins preserves the integrity of existing fibers and hence results in an attenuation of the inflammatory response. It will be interesting in future studies to examine whether myoblast fusion proceeds normally in these mice and whether cytokine production is affected by enhanced calcineurin activation. In any case, the ability of calcineurin to both elevate utrophin expression and attenuate the immune response in mdx muscles is relevant particularly towards the design of future therapies involving the use of the immunosuppressants and calcineurin inhibitors, cyclosporine A and FK506, that could have deleterious effects on DMD patients by decreasing expression of utrophin in their muscles (see also 16).

Through these studies (8,16), we have effectively shown an association between utrophin A expression with both calcineurin signaling and the slower oxidative myogenic program that can serve as the basis for designing a therapeutic strategy for the treatment of DMD. This is particularly relevant considering that fast MyHC IIb fibers are

preferentially affected in DMD patients (44). In the present study, we show beneficial effects stemming from a fiber type shift towards a slower more oxidative phenotype in dystrophin-deficient muscles. Interestingly, such a shift in fiber type towards a slower more oxidative phenotype has previously been shown to result in corrections of the dystrophic pathology. Specifically, mdx mice with ADR (arrested development of righting response) mutations resulting in myotonia and enhanced muscle activity show reductions in various markers of the dystrophic pathology with a corresponding shift in muscle fiber type to a more oxidative phenotype (45, 46). Based on the foregoing discussion, it is tempting to speculate that the improved phenotype of ADR-mdx mice is a result of increased utrophin expression.

MATERIALS/METHODS

Generation of CnA* Tg/mdx Mice. Transgenic mice expressing a constitutively active form of calcineurin have been characterized (19). Female mdx mice were paired with male CnA* transgenic mice resulting in male dystrophic pups. Male pups that expressed the transgene were identified through PCR screening of genomic DNA extracted from tail tissue. The absence of dystrophin from these mice was confirmed by immunolabelling of muscle sections using dystrophin antibodies (Santa-Cruz Biotech). Male pups that did not incorporate the CnA* transgene were used as dystrophic controls.

Immunofluorescence. Detection of sarcolemmal utrophin and utrophin A were conducted as described (16). Briefly, cross sections were incubated with H-300 polyclonal antibodies recognizing the C-terminus of utrophin (Santa-Cruz Biotech), NCL-DRP2 (Novocastra) recognizing the N-terminus of utrophin or with previously characterized antibodies generated in our laboratory specific for residues in the N-termini of utrophin A (16). Detection of sarcolemmal levels of dystrophin-associated proteins was done using the following antibodies: 1351 recognizing syntrophin, anti-nNOS (Zymed) and β–dystroglycan.

The β–dystroglycan antibody was made in rabbits to a synthetic peptide encompassing the last 15 amino acids. Antiserum was subsequently purified on a peptide affinity column. Western blotting using microsomes from rat muscle or brain homogenates, or following immunoprecipitation from Triton X-100 extracts and fractionation by SDS-PAGE, reveals a specific band of 43 kDa. Affinity chromatography

with this antibody followed by electrophoresis and mass spectrometry yields an unambiguous match with the sequence of rat β–dystroglycan.

Assessment of NFATc1 nuclear localization was performed in a similar manner using polyclonal NFATc1 antibodies (Santa-Cruz Biotech). The number of myonuclei positively staining for NFATc1 was counted in 3- 4 20x cross sectional views of myofibers from the midbelly of muscles from 3-4 animals per group. These values were then averaged and compared between samples. Sections were viewed with a Zeiss Axioskop-2 microscope with 10X and 20X plan-neofluor objectives and imaged using a digital camera (DVK). The images were captured and analyzed with Northern Eclipse software and Adobe Photoshop.

Western blot Analysis. Protein extraction and quantitation was conducted as described in detail elsewhere (47). Briefly, skeletal muscles were harvested and crushed in extraction buffer (pH 6.8) consisting of 5mM Tris, 10%SDS, 0.2M DTT, 1mM EDTA, and protease inhibitor (Roche) subsequently boiled and centrifuged for 10 min at 10 000 x g. Supernatant was separated with one part used for determination of protein concentration using a BCA protein assay (Pierce) and the other for electrophoresis analysis. Total protein for each sample (100 μg) was diluted in a loading dye consisting of Tris, 10%SDS, 2-mercaptoethanol and bromophenol blue and then separated on a 6% SDS-PAGE gel with 5% stacking at 75V for 6 hrs. The gels were then transferred onto nitrocellulose membranes (BioRad) overnight at 4C. Membranes were then stained with ponceau red to ensure equal loading of protein sample. Blots were incubated with the NCL-DRP2 primary antibody, washed thoroughly and incubated with peroxidase-labeled,

anti-mouse secondary antibodies (Jackson Labs). The presence of utrophin was detected using ECL reagents (Perkin-Elmer). The utrophin bands were visualized and their intensity determined using image analysis software (Kodak Image Analysis Software).

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted using TriPure (Boehringer Mannheim) as recommended by the manufacturer. Quantitative RT-PCR was carried out to determine the relative abundance of total utrophin (both A and B together), utrophin A and utrophin B using primers that selectively amplify total utrophin, utrophin A, utrophin B, and S12 rRNA (8, 16). Cycling conditions were optimized for all targets. In all these assays, negative controls consisted of RT-mixtures in which total RNA was replaced with RNase-free water. PCR products were first visualized on 1% agarose gels containing ethidium bromide (Sigma-Aldrich). For quantitative measurements, PCR products were separated and visualized on agarose gels containing the fluorescent dye Vistra Green (Amersham). The labeling intensity of the PCR product which is linearly related to the abundance of cDNAs, was quantified using a Storm PhosphorImager (Molecular Dynamics). Values obtained for utrophin, utrophin A and utrophin B were standardized relative to the amount of S12 rRNA present in the same sample. For all quantitative measurements, PCR experiments were performed during the linear range of amplification as described and shown in detail in our previous work (48, 49).

Muscle Fiber Typing. Muscle cross-sections were incubated for 1 hr with primary antibodies recognizing either MyHC IIa or IIb (see 16)), washed with PBS and incubated with affinity purified goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (Jackson Labs Inc.). Following several washes with PBS, the sections were incubated for 10 min with DAB media made up of 3'3-diaminobenzidine, deionised water, 2X buffer (0.2M Tris-HCl pH 7.6) and 1% hydrogen peroxide (Sigma-Aldrich). The sections were subsequently washed with running tap water, dehydrated using alcohol solutions, cleared with Xylene and mounted with permount. Using light microscopy, the percentage of fibers staining for MyHC IIa or IIb were determined from 3-4 20x cross sectional views of myofibers from the midbelly of muscles from 3-4 animals per group. The percentage for both MyHC isoforms were then averaged and compared between samples.

Assessment of Muscle Fiber Size and Central Nucleation. Cross-sections of muscles were stained with hematoxylin and eosin, dehydrated through a series of alcohol solutions, cleared with xylene and mounted using permount (Fisher Scientific). The extent of regeneration occurring in muscles was determined by comparing the averaged percentage of central nuclei between samples. Variability of muscle fiber size was determined because an increase in this value has been shown to be a pathological feature of mdx muscles (24, 27). Cross sectional areas for each individual fiber were measured using Northern Eclipse Software. Variability in fiber size was determined by averaging the standard deviations from 3-4 20x cross sectional views of myofibers from the

midbelly of muscles from 3-4 animals per group and comparing this value between samples.

Assessment of Muscle Membrane Damage. Sarcolemmal integrity was assessed by evaluating the levels of cytoplasmic staining within muscle fibers for the normally membrane impermeable markers IgM and albumin (28). Muscle cross-sections were incubated for 1 hr with fluorescein-conjugated IgM anti-mouse secondary antibodies (Sigma-Aldrich), or with and anti-albumin antibody subsequently revealed in this case with an AlexaFluor 594 secondary antibody (Molecular Probes). Sections were viewed using fluorescent microscopy to identify injured fibers. The percentage of injured fibers was then determined from 3-4 10x cross sectional views of myofibers from the midbelly of muscles from 3-4 animals per group. The percentage of damaged fibers was then averaged and compared between samples.

Evans Blue Uptake and Staining. Evans blue dye injections were performed as described elsewhere (28). Briefly, 50 μl/10 mg of b.w. of Evan blue dye was injected intravenously. Six to 12 hr later, muscles were isolated and frozen in melting isopentane. Prior to observing the sections under the microscope, muscle sections were incubated in ice-cold acetone for 10 min, washed 3x 10 min with PBS and mounted with Vectashield mounting medium (Vector laboratories). The presence of Evan blue dye in myofibers was observed under fluorescence microscopy and the intensity level was determined using northern Eclipse software by converting images to 8 bit gray scale and determining the total and average gray intensity taken as a measure of Evans blue dye fluorescence in

the entire area of each 10x cross-section. The average gray intensity was then compared between groups n = 4 animals per group. Three 10x cross-sections per animal was used to obtain average intensities.

Assessment of Inflammation. The extent of inflammation was determined by monitoring the presence of Mac-1 immunoreactivity using a commercially available antibody (BD Biosciences Pharmigen). Levels of Mac-1 staining was determined using northern eclipse software by converting images to 8 bit gray scale and determining the total and average gray intensity taken as a measure of Mac-1 labeling in the entire area of each 10x cross-section. The average gray intensity was then compared between groups (N = 3-4 animals per group) using three 10x cross sections per animal.

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Chapter 6

Targeted inhibition of Ca^{+2}/calmodulin signaling exacerbates the dystrophic phenotype in mdx mouse muscle

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Contribution from authors

Joe V. Chakkalakal and Bernard Jasmin wrote manuscript. Joe V. Chakkalakal performed all RT-PCR analysis, immunofluorescence analysis, and histological analysis with technical assistance from John Lunde, Amanda Bradford and Kim Wong. Stephanie Michel, Eva Chin and Robin Michel provided muscles from CaMBP* and mdx/CaMBP* transgenic mice, as part of an on-going collaboration. Stephanie Michel performed NFATc1 counts.

Abstract

In the present study, we crossbred mdx mice with transgenic mice expressing a small peptide inhibitor for calmodulin (CaM), known as the CaM-binding protein (CaMBP), driven by the slow fiber-specific Troponin I slow (TnIs) promoter. This strategy allowed us to determine the impact of interfering with Ca^{+2}/CaM-based signaling in dystrophin-deficient slow myofibers. Consistent with impairments in the Ca^{+2}/CaM-regulated enzymes calcineurin and Ca^{+2}/CaM-dependent kinase (CaMK), the nuclear accumulation of NFATc1 and MEF2C was reduced in slow fibers from mdx/CaMBP mice. We also detected significant reductions in the levels of PGC-1α and GABPα mRNAs in slow fiber-rich soleus muscles of mdx/CaMBP mice. In parallel, we observed significantly lower expression of myosin heavy chain I (MyHC) mRNA in mdx/CaMBP soleus muscles. This correlated with fiber type shifts towards a faster phenotype. Examination of mdx/CaMBP slow muscle fibers revealed significant reductions in A-utrophin, a therapeutically relevant protein that can compensate for the lack of dystrophin in skeletal muscle. In accordance with lower levels of A-utrophin, we noted a clear exacerbation of the dystrophic phenotype in mdx/CaMBP slow fibers as exemplified by several pathological indices. These results firmly establish Ca^{+2}/CaM-based signaling as key to regulating expression of A-utrophin in muscle. Furthermore, this study illustrates the therapeutic potential of using targets of Ca^{+2}/CaM-based signaling as a strategy for treating Duchenne muscular dystrophy (DMD). Finally, our results further support the concept that strategies aimed at promoting the slow oxidative myofiber program in muscle may be effective in altering the relentless progression of DMD.

Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked myopathy characterized by exhaustive cycles of muscle degeneration and regeneration (1). The molecular defect defining DMD is the loss of sarcolemmal expression of the cytoskeletal protein dystrophin (2-5). Dystrophin is part of a trans-membranous structure referred to as the dystrophin-associated protein complex (DAPC). The DAPC provides a link between the extracellular matrix and the underlying filamentous actin network (6-8). Mutations in the dystrophin gene prevent synthesis of the full-length protein and leads to loss of the DAPC. The loss of this structural linkage causes instability of the sarcolemma (7-9). Although a variety of approaches are currently envisaged to treat DMD, their development and eventual implementation are complicated due to the multi-faceted nature of the disease (5, 10-12). Amongst the various therapeutic strategies, one is aimed at stimulating expression of booster genes whose proteins can effectively compensate for the lack of dystrophin (4, 13, 14).

Utrophin was initially identified in a screen to find genes that could compensate functionally for dytrophin-deficiency (15). Further characterization of utrophin has shown that this protein shares high sequence similarity with dystrophin and that it can in fact associate with members of the DAPC (15-17). Coherent with these characteristics, over-expression of utrophin in skeletal muscle of mdx mice, an animal model of DMD, results in a near complete correction of the dystrophic phenotype (18-20).

The relevance of utrophin as a therapeutic target for DMD has prompted considerable efforts, over the last decade, in deciphering the regulatory networks that control its expression in muscle. Although utrophin is expressed in most tissues, it

accumulates preferentially, in mature skeletal muscle fibers, at the level of the post synaptic membrane of the neuromuscular junction (NMJ) (21-24). To date, two full-length utrophin isoforms have been characterized. A-utrophin is expressed in skeletal muscle, and is the form enriched within the post synaptic sarcoplasm, whereas B-utrophin appears primarily confined to endothelial cells (25-27). Although a variety of regulatory pathways are envisaged to explain the restricted expression of A-utrophin in synaptic regions of muscle, transcriptional events remain the most well characterized (28). Within the post synaptic regions of muscle fibers, A-utrophin expression appears to be under the influence of nerve-derived factors such as agrin and heregulin; both promote transcriptional cascades that stimulate the activity of the transcription factor complex GABPα/β (24, 29-31). GABPα/β, when activated, is able to bind to the N-box motif found in the A-utrophin promoter (30, 31). Subsequently, the binding of GABPα/β to the N-box stimulates expression of A-utrophin in post synaptic myonuclei (30, 31).

In addition to its synaptic accumulation, recent studies from our laboratory have shown that slow muscle fibers express A-utrophin in their extra synaptic compartments (27, 32). In this context, we have also shown that expression of A-utrophin in slow muscle fibers is regulated, at least in part, via signaling pathways important for sustaining the slower, high oxidative, myofiber phenotype (26, 33, 34). Specifically, we demonstrated the ability of calcineurin/NFAT signaling and that of the transcriptional co-activator PGC-1α, in the regulation of A-utrophin expression in muscle (26, 33, 34). We also showed in these studies that over-expression of PGC-1α stimulates A-utrophin transcription, through a mechanism involving increased levels of GABPα (34). These observations suggest that stimulation of calcineurin/NFAT and PGC-1α, pathways

known to promote the slower, more oxidative, myofiber program, may help stem the progression of DMD pathology by stimulating expression of A-utrophin (see 33).

Calcium, together with CaM, serves as an upstream regulator for calcineurin and CaM kinases (CaMK) in specifying the slower, high oxidative, myofiber program (35-40). In this context, activation of calcineurin's phosphatase activity by Ca^{+2}/CaM leads to the dephosphorylation, nuclear translocation, and binding of NFAT to target promoters (41-44). These events enable NFAT to stimulate expression of genes associated with the slow, high oxidative, program in muscle (41-44). Chronically increased activity of skeletal muscles, that promotes the slower contractile phenotype, leads to characteristic sustained low amplitude oscillatory profiles of intracellular Ca^{+2} which, together with CaM, activate calcineurin and CaMK (36, 42, 45). In turn, stimulated CaMK liberates MEF2 from its association with histone deacytylases (HDACs) (42, 45, 46). This event allows MEF2 to translocate to the nucleus, where it can be dephosphorylated by calcineurin and stimulate transcription of target genes including PGC-1α (36, 37).

Ca^{+2}/CaM-regulated factors such as calcineurin/NFAT and PGC-1α are now known to be able to regulate expression of A-utrophin in skeletal muscle (26, 33, 34). Therefore, we decided to examine the impact of attenuating CaM-based signaling in mdx slow myofibers on expression of A-utrophin and the dystrophic pathology. To this end, we crossbred mice expressing a transgene encoding a small peptide inhibitor for CaM, called CaM-binding protein (CaMBP), fused to the troponin I slow (TnIs) promoter (47, 48), with mdx mice. In contrast to using immunosuppressants to inhibit calcineurin signaling systemically, this targeted genetic approach aimed at a specific muscle fiber type, allowed us to avoid affecting other physiological processes such as immune cell

activation which is known to promote the dystrophic pathology and is also regulated by CaM signaling (49, 50). Knockdown of Ca^{+2}/CaM signaling specifically within mdx slow muscle fibers led to a reduction in the levels of PGC-1α, GABPα, MyHC I and A-utrophin. Consequently, impaired Ca^{+2}/CaM-based signaling in slow muscle fibers within mixed muscles of mdx mice led to a clear segregated exacerbation of the dystrophic phenotype in these fibers. Collectively, results of the present study are consistent with the notion that promotion of the slow myofiber program through CaM-regulated pathways may be an effective therapeutic strategy to counteract the relentless progression of DMD.

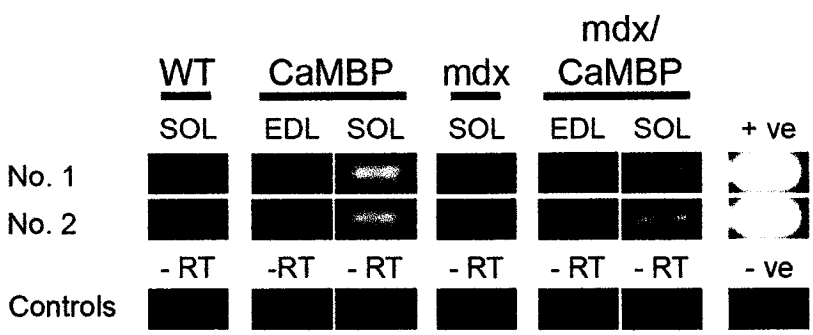
Results

Generation of mdx/CaMBP Mice and Impairment of Downstream Ca^{+2}/CaM-based Signaling. To assess the contribution of CaM-based signaling in a dystrophin-deficient background, we generated mdx mice expressing a CaMBP transgene specifically in muscle fibers expressing the slow phenotype. The CaMBP transgene encodes a protein that serves as a specific inhibitor for CaM and, consequently, downstream pathways involving Ca^{+2}/CaM-regulated enzymes such as calcineurin and CaMK, are attenuated (47, 48). Expression of the CaMBP transgene is controlled by the TnIs promoter known to restrict expression of transgenes to mature slow muscle fibers (48, 51, 52, 53). Previous characterization of TnIs -CaMBP mice used in the present study determined that under resting conditions, muscles from these animals displayed a normal phenotype (48). Furthermore, similar to the ability of calcineurin inhibition to prevent skeletal muscle adaptations in response to functional overload, muscle fibers from TnIs-CaMBP mice also display impaired responsiveness to this paradigm (48, 54). Therefore, the CaMBP mice used to generate the mdx/CaMBP animals do have normal skeletal muscle development, and only display phenotypes consistent with impaired Ca^{+2}/CaM signaling under specific conditions.

PCR-based screening was initially used to correctly identify animals that had incorporated the CaMBP transgene (data not shown). We next examined total RNA from EDL and soleus muscles from wildtype (wt), CaMBP, mdx and mdx/CaMBP mice for CaMBP expression. RT-PCR analysis clearly demonstrates expression of CaMBP solely in soleus muscles from transgene positive animals without detectable levels in EDL

Figure 6.1. Expression of CaMBP in mdx/CaMBP fast and slow muscles.

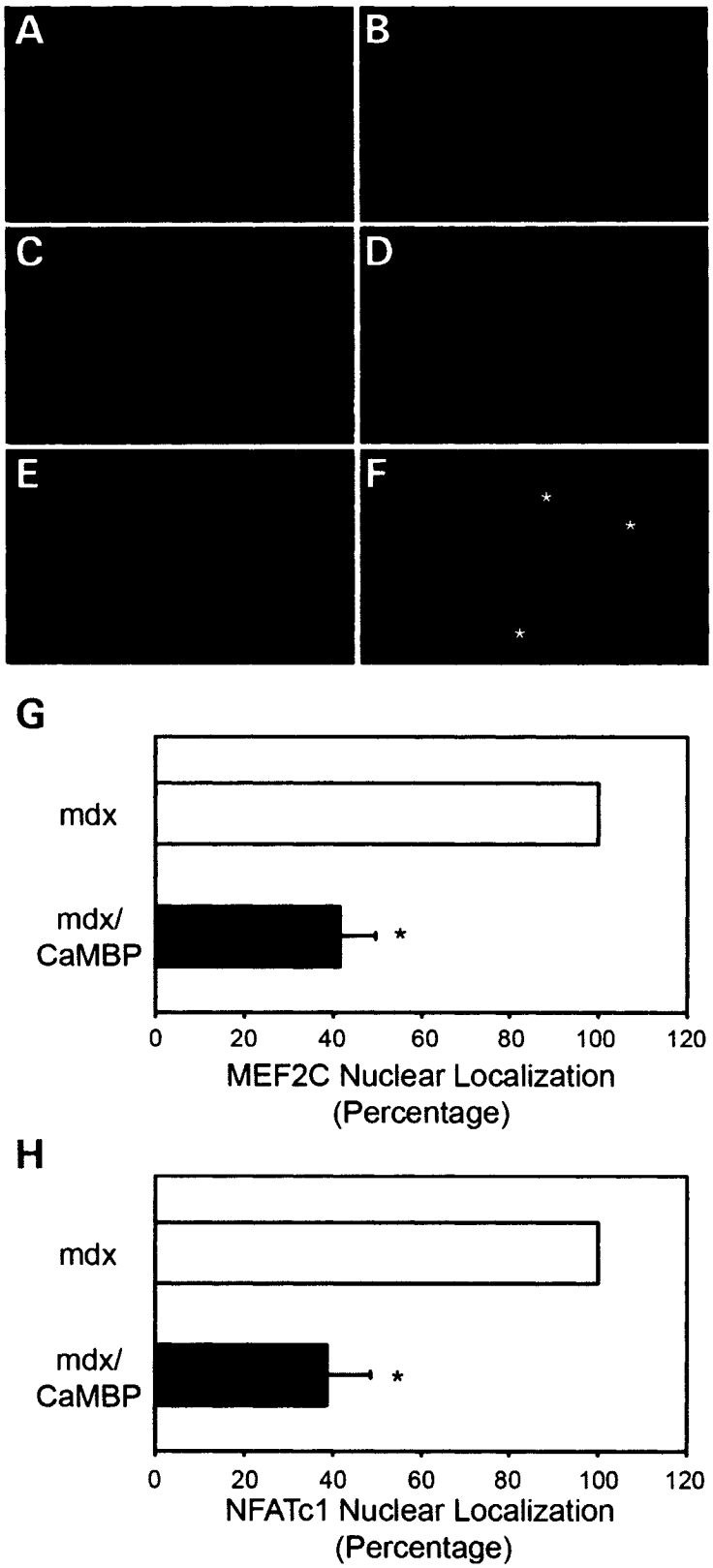
Representative photomicrographs of EtBr stained agarose gels depicting cDNA products from RT-PCR analysis of total RNA from wt, CaMBP, mdx and mdx/CaMBP soleus muscles for CaMBP mRNA. No1 and No2 refer to two separate experiments, - RT refers to RT-PCR reactions where reverse transcriptase was excluded, +ve refers to the use of DNA from CaMBP transgene positive tails, -ve refers to RT-PCR reactions where DNA was replaced with water. Note the exclusive expression of CaMBP mRNA in slow soleus muscles from transgene positive mice, without detectable levels in fast EDL muscles or muscles taken from transgene negative mice.


WT
CaMBP
mdx
mdx/
CaMBP
SOL
EDL
SOL
SOL
EDL
SOL
+ ve
No. 1
No. 2
- RT
-RT
- RT
- RT
- RT
- RT
- ve
Controls

muscles (Figure 6.1). Furthermore, the relative abundance of CaMBP expression between soleus muscles from CaMBP and mdx/CaMBP mice were comparable (Figure 6.1). This shows that the CaMBP transgene is also expressed in slow muscles in a dystrophin-deficient background.

To assess the consequences of impaired CaM signaling in slow muscle fibers from mdx/CaMBP animals, we focused our attention on two transcription factors namely, NFATc1 and MEF2C. Both the transcriptional activity and nuclear localization of NFATc1 and MEF2C have previously been shown to be regulated by the Ca^{+2}/CaM-regulated enzymes calcineurin and CaMK (36, 42-45). Immunofluorescence analysis of soleus muscles clearly demonstrated reduced nuclear staining for MEF2C (Figure 6.2A-F). Quantitative assessment of MEF2C revealed a ~ 60% ($P < 0.05$) decrease in nuclear localization of this transcription factor in soleus muscles from mdx/CaMBP mice in comparison to mdx mice (Figure 6.2G). In agreement with impaired CaM signaling, quantitative assessment of NFATc1 nuclear localization also revealed a ~ 65% ($P < 0.05$) decrease in mdx/CaMBP soleus muscles in comparison to mdx counterparts (Figure 6.2H). These effects appeared to be specific to soleus muscles, because differences in MEF2C and NFATc1 nuclear localization were not seen upon examination of fast-contracting TA muscles between mdx and mdx/CaMBP animals (data not shown). Occasionally, there appeared to be some labeling in the central portions of muscle fibers (regions occupied by contractile proteins) for MEF2C (Figure 6.2 A, E) but this likely reflects autofluorescence stemming from the fixation procedure (see for discussion 55). Therefore, expression of the CaMBP transgene in slow dystrophin-deficient muscle fibers

Figure 6.2. Reduced localization of NFATc1 and MEF2C in myonuclei of mdx/CaMBP mice. Representative photomicrographs depicting MEF2C localization in mdx and mdx/CaMBP muscle. Panels depict soleus cross-sections from mdx (A, C, E) and mdx/CaMBP (B, D, F) mice. Panels (A) and (B) show MEF2C labeling with corresponding nuclei in panels (C) and (D). Panel (E) depicts an overlay image of (A) and (C), whereas panel (F) is an overlay of image (B) and (D). Quantification of the percentage of myonuclei stained for NFATc1 and MEF2C in mdx/CaMBP soleus muscles relative to mdx controls shows that mdx/CaMBP solei contain considerably less positive nuclei for MEF2C (G) and NFATc1 (H). * denotes significant difference from mdx ($P<0.05$), n = 4 animals per group, three 20x sections from each animal. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.


A
B
C
D
E
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G
mdx
mdx/
CaMBP
0
20
40
60
80
100
120
MEF2C Nuclear Localization
(Percentage)
H
mdx
mdx/
CaMBP
0
20
40
60
80
100
120
NFATc1 Nuclear Localization
(Percentage)

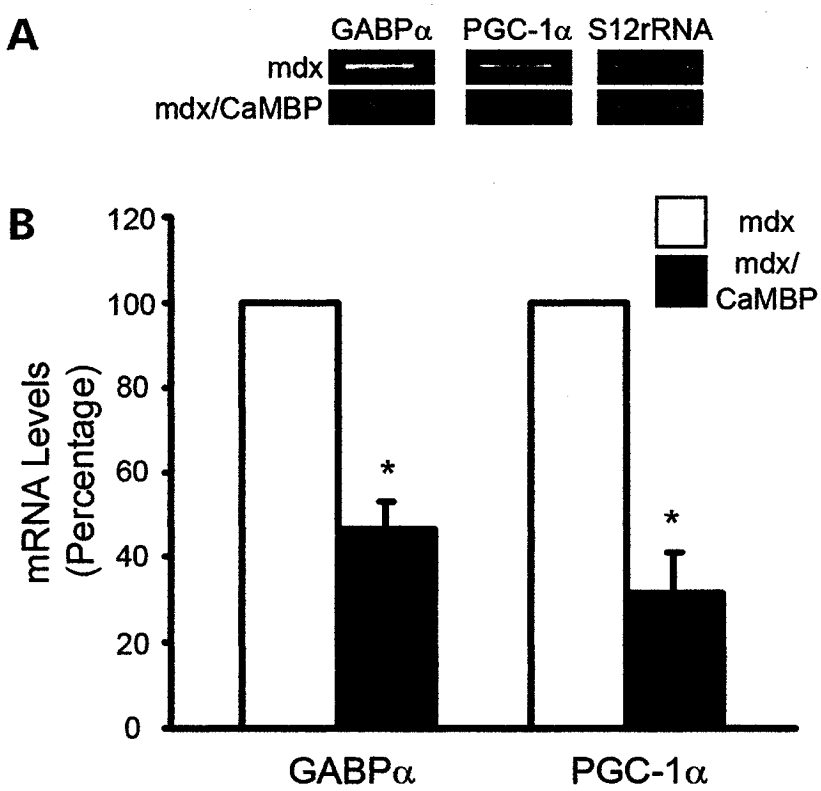
results in the impaired activity of downstream targets for CaM-based signaling including NFATc1 and MEF2C. This suggests that expression of NFATc1 and MEF2C target genes should also be impaired in mdx/CaMBP slow muscles.

Reduced Expression of PGC-1α and GABPα in mdx/CaMBP Soleus Muscles.

Recently, PGC-1α has been the focus of considerable attention due to the ability of this co-factor to regulate a variety of important physiological processes including mitochondrial biogenesis, skeletal muscle myogenesis and the specification of the slower, high oxidative, myofiber program (37, 56, 57). In this context, the Ca^{+2}/CaM-regulated transcription factors MEF2C and NFATc1 have been shown to participate in an autoregulatory transcriptional loop controlling PGC-1α expression. Considering the disruption of NFATc1 and MEF2C localization seen in mdx/CaMBP slow muscles, we next examined the levels of PGC-1α mRNA. RT-PCR analysis clearly demonstrated reductions in PGC-1α mRNA levels (Figure 6.3A). Quantitative assessment showed a ~70% ($P < 0.05$) reduction in PGC-1α mRNA levels from mdx/CaMBP soleus muscles compared to mdx counterparts (Figure 6.3B).

Over-expression of PGC-1α in skeletal muscles has been shown to stimulate the transcriptional activity of GABPα (34, 58). As seen in Figure 6.3A, there was also a clear reduction in the levels of GABPα mRNA in mdx/CaMBP soleus muscles in comparison to mdx counterparts (Figure 6.3A). Specifically, quantitative assessment demonstrated a ~55% ($P < 0.05$) reduction in GABPα mRNA levels in mdx/CaMBP soleus muscles. This result paralleled the observed reductions in PGC-1α transcripts (Figure 6.3B). Similar analysis of fast EDL muscles in these mice demonstrated no

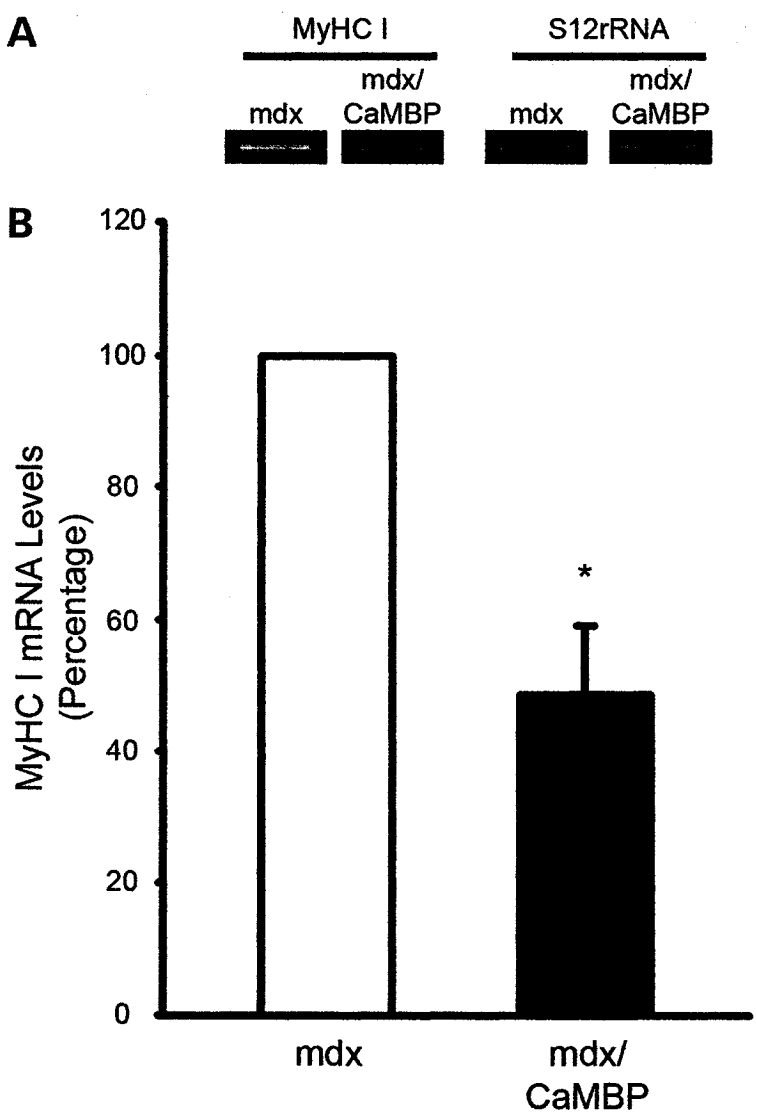
Figure 6.3. Decrease in GABPα and PGC-1α mRNA expression in mdx/CaMBP slow muscles. Representative photomicrographs of EtBr stained agarose gels depicting cDNA products from RT-PCR analysis of total RNA from mdx and mdx/CaMBP soleus muscles for GABPα, PGC-1α and S12rRNA (A). Quantification of RT-PCR products for GABPα and PGC-1α standardized to S12rRNA from soleus muscles taken from mdx and mdx/CaMBP animals (B). Note the reduced levels of GABPα and PGC-1α in soleus muscles from mdx/CaMBP mice. * denotes significant difference ($P<0.05$), 3 animals per group. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.


A
GABPα
PGC-1α
S12rRNA
mdx
mdx/CaMBP
B
mdx
mdx/
CaMBP
mRNA Levels
(Percentage)
120
100
80
60
40
20
0
*
*
GABPα
PGC-1α

significant differences in PGC-1α and GABPα mRNA levels (data not shown). This internal negative control is useful and powerful since CaMBP expression is controlled by the TnIs promoter which is not active in fast fibers (see Figure 6.1) (51-53). Collectively, these results demonstrate that impaired Ca^{+2}/CaM signaling in slow muscle fibers of mdx/CaMBP animals leads to reduced expression of the MEF2C and NFATc1 target gene PGC-1α and, by extension, GABPα.

Loss of Type I Fibers in mdx/CaMBP Soleus Muscle. NFATc1, MEF2C and PGC-1α have all been shown to participate in transcriptional regulatory networks that help stimulate the activity of genes specifying the slower, high oxidative, myofiber program (35-37, 40, 44, 56). Therefore, we next assessed whether loss of Ca^{+2}/CaM-signaling specifically in dystrophic muscle fibers expressing TnIs, and thus the CaMBP transgene, affected expression of MyHC I. Expression of MyHC I has traditionally been used as a reliable marker to identify type I, slow contracting, oxidative myofibers (59). Furthermore, calcineurin/NFAT signaling has been shown to play a prominent role in regulating the expression of MyHC I (44, 60). As seen in Figure 6.4A, there was a clear reduction in MyHC I mRNA levels in mdx/CaMBP soleus muscles. Through quantitative assessment, we observed a 50% ($P < 0.05$) reduction in the abundance of MyHC I mRNA in mdx/CaMBP soleus muscles in comparison to mdx counterparts (Figure 6.4B). The reduced expression of MyHC I mRNA in mdx/CaMBP slow muscle fibers is entirely consistent with the impaired regulation of MEF2C, NFATc1 and PGC-1α observed in these fibers.

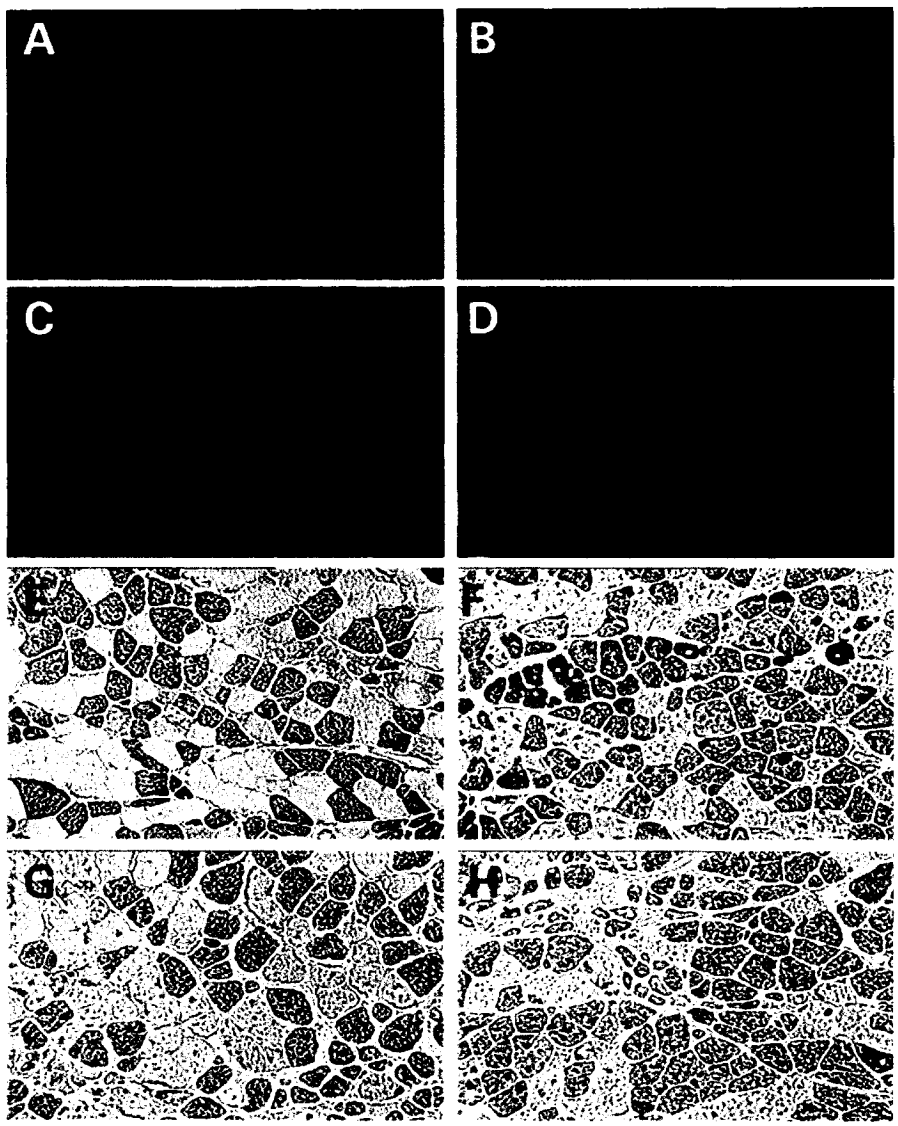
Figure 6.4. Decrease in MyHC I mRNA expression in mdx/CaMBP slow muscles. Representative photomicrographs of EtBr stained agarose gels depicting cDNA products from RT-PCR analysis of total RNA from mdx and mdx/CaMBP muscle for MyHC I and S12rRNA (A). Quantification of RT-PCR products for MyHC I standardized to S12 rRNA from soleus muscles taken from mdx and mdx/CaMBP animals (B). Note the reduced levels of MyHC I in soleus muscles from mdx/CaMBP mice. * denotes significant difference ($P<0.05$), 3 animals per group. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.


A
MyHC I
S12rRNA
mdx
mdx/
CaMBP
mdx
mdx/
CaMBP
B
120
100
80
60
40
20
0
MyHC I mRNA Levels
(Percentage)
*
mdx
mdx/
CaMBP

Next, we examined cross-sections for fiber type shifts with antibodies specific to slow MyHC I and fast MyHC IIa on soleus muscles taken from mdx and mdx/CaMBP animals. Representative photomicrographs clearly demonstrated higher numbers of MyHC I-positive fibers in mdx soleus muscles (Figure 6.5A, C) compared to mdx/CaMBP counterparts (Figure 6.5B, D). In accordance with the observed reductions in MyHC I-positive muscle fibers, we also observed in comparison to mdx soleus muscles, higher numbers of fibers staining positively for MyHC IIa in mdx/CaMBP mice(Figure 6.5 E-H). Furthermore, we determined the percentage of MyHC I-positive myofibers per field of view in soleus muscles derived from wt, CaMBP, mdx and mdx/CaMBP animals. With this analysis, we found a significant ~ 2-fold ($P < 0.05$) reduction in the number of MyHC I-positive muscle fibers in the soleus of mdx/CaMBP mice compared to mdx mice (Figure 6.5I). In contrast, we saw no significant differences ($P > 0.05$) in the muscle fiber population in wt and CaMBP animals (Figure 6.5 I, also see 48). In parallel to these observations, determination of the percentage of MyHC IIa positive muscle fibers revealed a significant ~ 1.5 fold increase ($P < 0.05$) of this parameter in mdx/CaMBP soleus muscles (Figure 6.5I). Collectively, these results suggest that fiber type shifts towards a faster contractile phenotype caused by CaMBP expression is specific to dystrophin-deficient muscle (Figure 6.5I).

Reduced A-utrophin Expression in Slow Muscle Fibers from mdx/CaMBP Mice. We have previously shown the involvement of PGC-1α and calcineurin/NFATc1 in regulating the expression of A-utrophin (26, 33, 34). Therefore, to ascertain whether A-utrophin expression is reduced in mdx/CaMBP muscle fibers expressing slow proteins,

Figure 6.5. mdx/CaMBP mice display fiber type conversions to a faster phenotype. Shown are representative photomicrographs of cross-sections from mdx (A, C, E, G) and mdx/CaMBP (B, D, F, H) soleus muscles processed to detect MyHC I (A-D) by immunofluorescence and MyHC IIa by histochemistry (E-H). Quantification revealed a significant reduction in MyHC I in mdx/CaMBP solei (I), together with significant inductions for MyHC IIa (I). * denotes significant difference from wt, CaMBP and mdx ($P<0.05$), n = 4 animals per group. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.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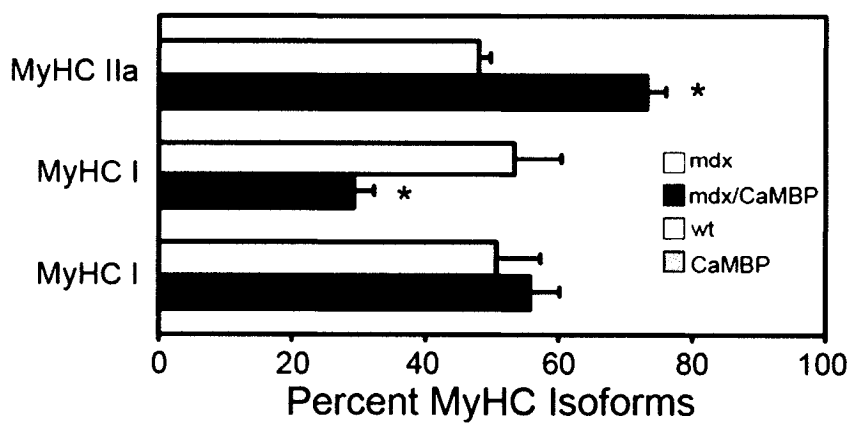

MyHC IIa
MyHC I
MyHC I
*
*
mdx
mdx/CaMBP
wt
CaMBP
0
20
40
60
80
100
Percent MyHC Isoforms

we performed RT-PCR analysis on total RNA extracted from mdx and mdx/CaMBP soleus muscles. As seen in Figure 6.6A, there was a readily apparent reduction in A-utrophin mRNA levels in the mdx/CaMBP soleus. Quantitative analysis of the abundance of A-utrophin mRNA demonstrated a ~ 2-fold ($P < 0.05$) reduction in soleus muscles from mdx/CaMBP mice in comparison to mdx mice (Figure 6.6B). As a negative control, we saw no significant differences in A-utrophin mRNA levels in the phenotypically fast EDL muscle between mdx and mdx/CaMBP mice (Figure 6.6A).

We also determined whether reductions in A-utrophin expression were evident at the sarcolemma of type I muscle fibers from mdx/CaMBP mice. As mentioned, MyHC I-positive fibers serve as a reliable marker for type I, slow oxidative, muscle fibers which in our experiments selectively express the CaMBP transgene driven by the TnI slow promoter (see Figure 6.1, 51-53). As seen in representative photomicrographs of serial sections labeled with specific antibodies recognizing A-utrophin (Figure 6.7A-D) or MyHC I (Figure 6.7A'-D'), we consistently observed the appearance of sarcolemmal A-utrophin at the periphery of MyHC I-positive muscle fibers in mdx mice (Figure 6.7 A, A'-B, B'; see also 26). In contrast, we observed a loss (or a low level) of A-utrophin at the sarcolemma of MyHC I-positive muscle fibers from mdx/CaMBP mice (Figure 6.7 C, C'-D, D'). Quantitative assessment of the percentage of type I muscle fibers with detectable levels of sarcolemmal A-utrophin revealed a ~ 70% ($P < 0.05$) reduction in the mdx/CaMBP mice in comparison to mdx counterparts (Figure 6.7E). Therefore, in mdx/CaMBP soleus muscles, we observe a combined loss of type I muscle fibers and sarcolemmal A-utrophin in remaining MyHC I-positive muscle fibers.

Figure 6.6. Decrease in A-utrophin mRNA expression in mdx/CaMBP slow muscles. (A) Representative photomicrographs of EtBr stained agarose gels depicting cDNA products for A-utrophin and S12rRNA from RT-PCR analysis of total RNA from mdx and mdx/CaMBP soleus (SOL) and EDL muscles. (B) Quantification of RT-PCR products for A-utrophin standardized to S12 rRNA from SOL muscles taken from mdx and mdx/CaMBP animals. Note the reduced levels of A-utrophin in SOL muscles from mdx/CaMBP mice. * denotes significant difference ($P<0.05$), 3 animals per group. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.

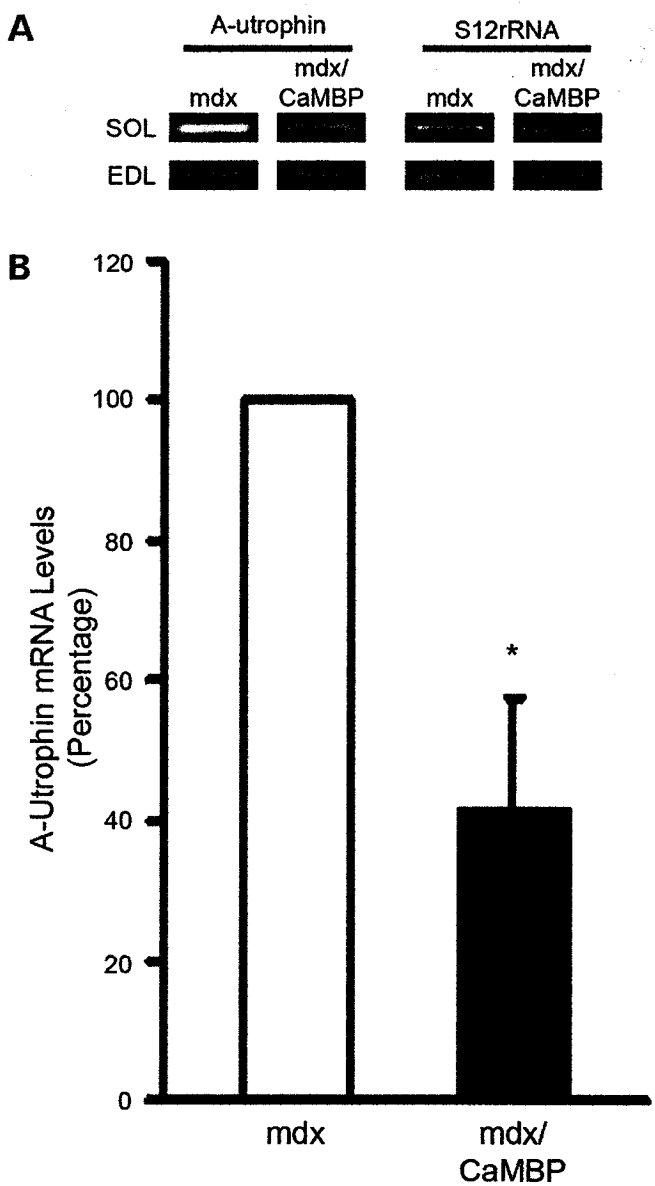
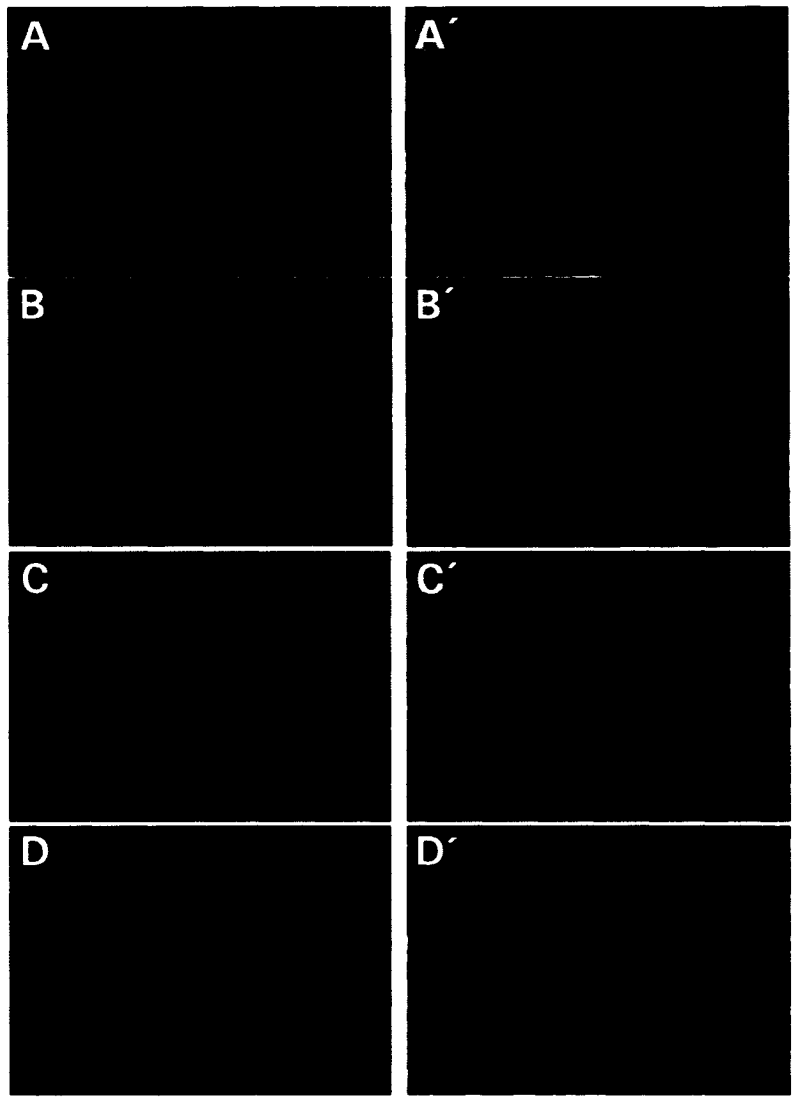

A
A-utrophin
S12rRNA
mdx
mdx/
CaMBP
mdx
mdx/
CaMBP
SOL
EDL
B
120
100
80
60
40
20
0
A-Utrophin mRNA Levels
(Percentage)
*
mdx
mdx/
CaMBP

Figure 6.7. Reductions of A-utrophin in extra synaptic compartments of type I positive fibers from mdx/CaMBP mice. Shown are representative examples of photomicrographs of serial sections from mdx (A-B') and mdx/CaMBP (C-D') processed to detect A-utrophin (A-D) and MyHC I (A'-D') by immunofluorescence. Note the lack (or low level) of utrophin staining in extra synaptic regions of type I fibers from mdx/CaMBP animals (C, C' and D,D') mice. Quantification of A-utrophin in the periphery of type I fibers revealed significant reducutions in mdx/CaMBP animals (E). * denotes significant difference from mdx ($P<0.05$), n = 4 animals per group, three 20x sections from each animal. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.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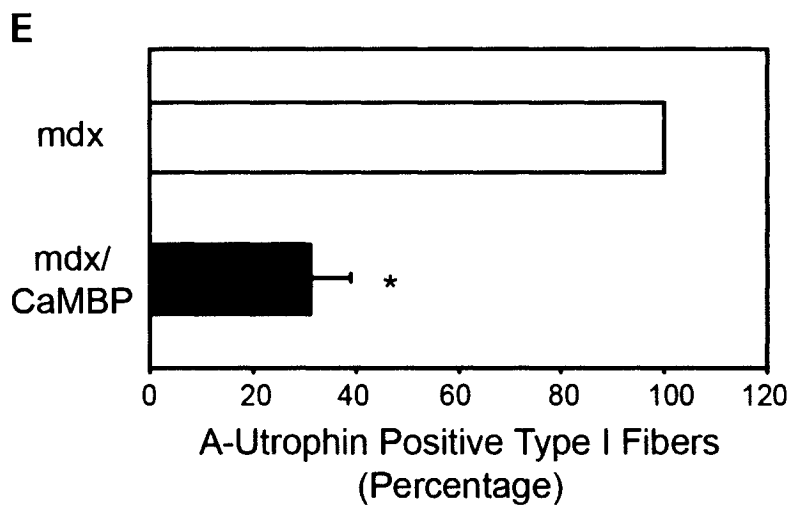
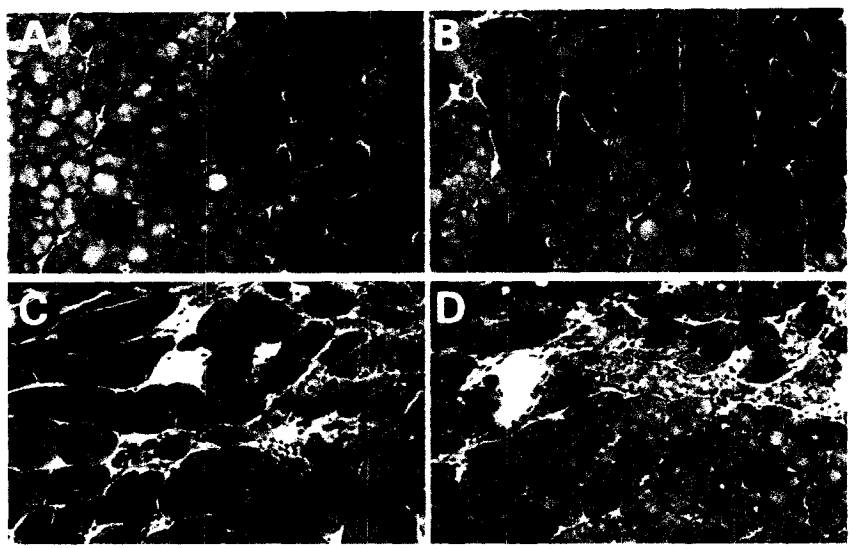

E
mdx
mdx/
CaMBP
*
0
20
40
60
80
100
120
A-Utrophin Positive Type I Fibers
(Percentage)

Both events can explain the pronounced reductions in A-utrophin expression in mdx/CaMBP slow muscles (see 26). Collectively, these events suggest that impaired Ca^{+2}/CaM signaling in mdx mice decreases A-utrophin levels due to direct mechanisms and a reduced capacity to promote the slower myofiber program.

Increased Dystrophic Pathology in mdx/CaMBP Soleus Muscles. Loss of A-utrophin expression in mdx mice has been shown to correlate with an increased severity in dystrophic pathology (61, 62). Since our mdx/CaMBP mice have reduced A-utrophin expression in slow muscle fibers, we next assessed the phenotypic consequences of this loss in soleus muscles. Comparison of hematoxylin and eosin-stained sections of soleus muscles from wt and CaMBP animals revealed no discernable differences or defects in morphological features (data not shown). In contrast, soleus muscles taken from mdx/CaMBP animals consistently showed a more severe dystrophic phenotype in comparison to mdx mice (Figure 6.8 A-D).

Healthy skeletal muscles, when examined in cross-sections, tend to display relatively uniform sizes in individual muscle fibers. Previous observations have revealed that dystrophin-deficient muscle fibers display significant increases in fiber size variability (63). Consistent with a more severe dystrophic phenotype in mdx/CaMBP muscles, we observed greater variations in muscle fiber sizes in comparison to mdx mice (Figure 6.8 A-D). Quantitative analysis of fiber size variability revealed an ~ 2-fold ($P < 0.05$) increase in mdx/CaMBP soleus muscle fibers (Figure 6.8 E). Furthermore, mdx/CaMBP soleus muscles displayed a substantial increase in the area occupied by

Figure 6.8. Increased areas of mononuclear infiltrate and fiber size variability in mdx/CaMBP slow muscles. Shown are representative photomicrographs of cross-sections from mdx (A, B) and mdx/CaMBP (C, D) muscles processed for hematoxylin and eosin staining. Quantitative analysis of fiber size variability revealed significant increases in mdx/CaMBP soleus muscles (E). Soleus muscles from mdx/CaMBP (C, D) animals also displayed significant increases in areas occupied by mononuclear infiltrate in comparison to mdx soleus (A, B). Quantitative analysis of areas occupied by mononuclear infiltrate shows a significant increase in mdx/CaMBP soleus muscles (F). * denotes significant difference from mdx ($P<0.05$), n = 4-6 animals per group from three 20x cross-sectional views per animal. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.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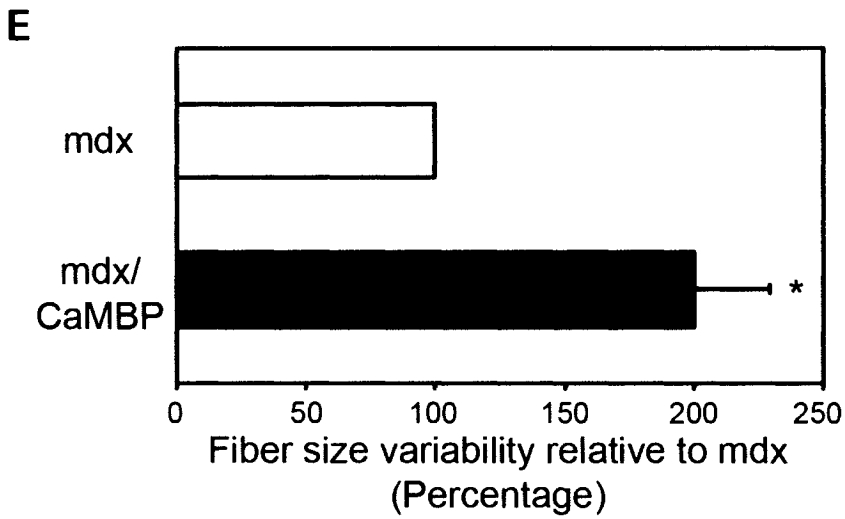
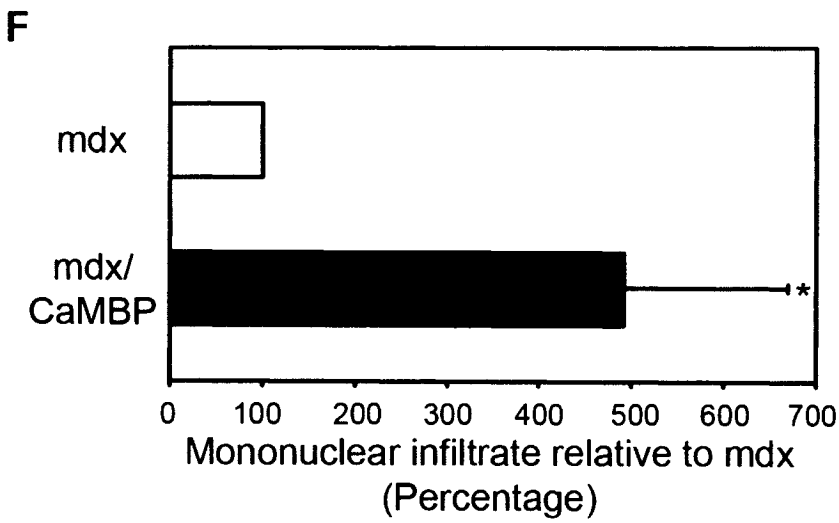

E
mdx
mdx/
CaMBP
*
0
50
100
150
200
250
Fiber size variability relative to mdx
(Percentage)

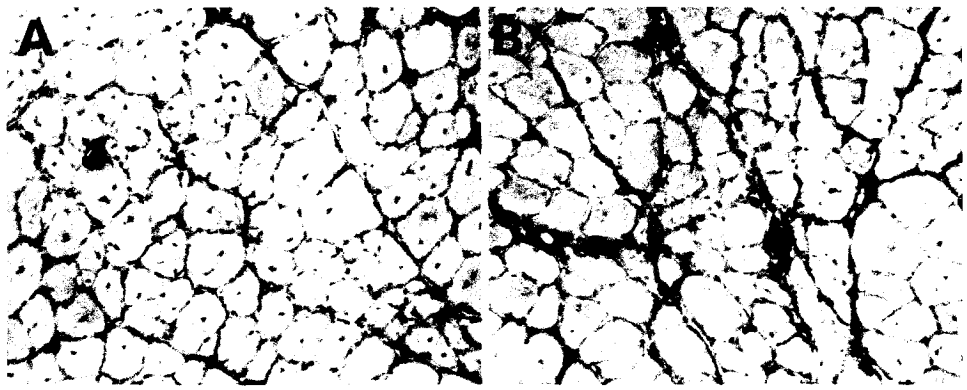

F
mdx
mdx/
CaMBP
*
0
100
200
300
400
500
600
700
Mononuclear infiltrate relative to mdx
(Percentage)

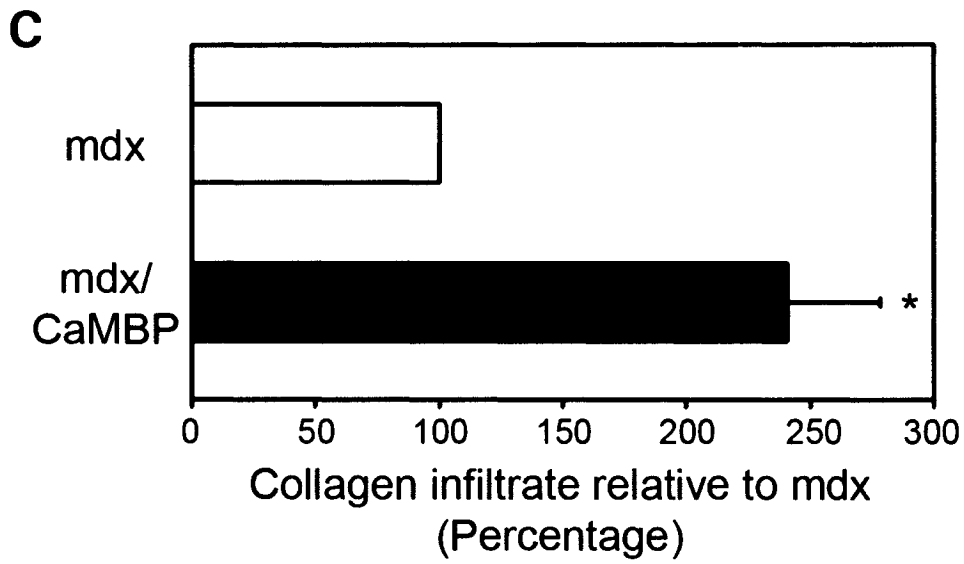
mononuclear infiltrate, an indicator of elevated muscle necrosis (64) (Figure 6.8 A-D). Quantitative analysis of the area occupied by mononuclear infiltrate in soleus muscles from mdx/CaMBP mice showed a ~ 5-fold ($P < 0.05$) increase in comparison to mdx soleus muscles (Figure 6.8 F). Counts of the percentage of central nucleation revealed no significant differences in this parameter (data not shown) consistent with previous observations of mdx/utr-/- muscles (61). The lack of a difference likely reflects the fact these muscles are significantly compromised and become infiltrated with other cell types (Figure 6.8 F see Figure 6.9C), making quantitation of central nucleation difficult especially if there is also a net loss of fibers in these muscles (see 61).

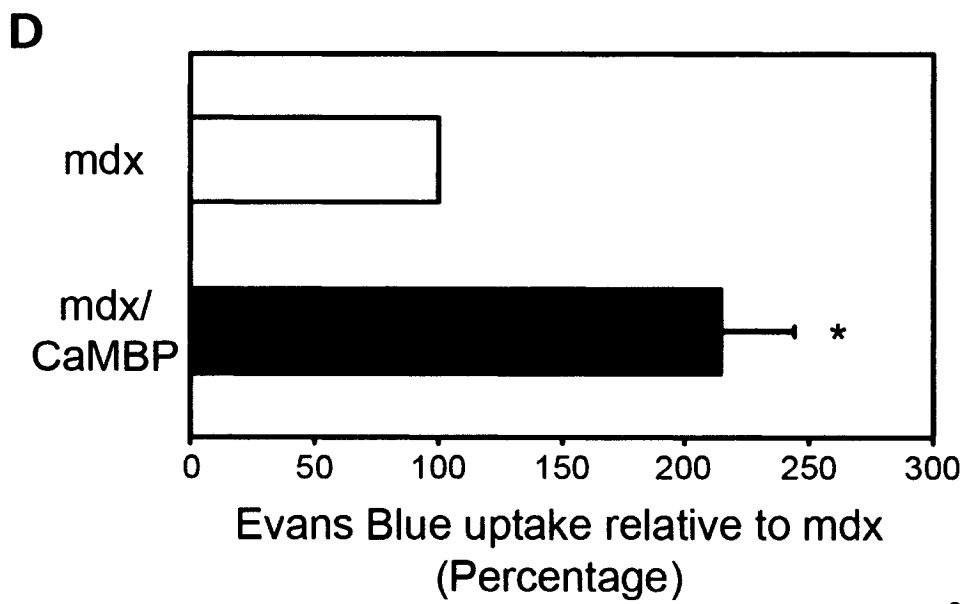
To further assess the phenotypic consequences of impaired Ca^{+2}/CaM signaling in dystrophin-deficient slow muscle, we preformed Trichrome-Masson staining on mdx/CaMBP soleus muscles. This allowed us to visualize regions of collagenous infiltrate; an additional marker of the dystrophic phenotype (65). As seen in representative photomicrographs of soleus cross-sections from mdx mice, regions of collagenous infiltrate are clearly evident as indicated by the blue coloration (Figure 6.9A). In comparison, there was a clear increase in areas occupied by collagenous infiltrate in Trichrome-Masson-stained soleus muscles from mdx/CaMBP mice (Figure 6.9B). Quantitative assessment of areas occupied by collagenous infiltrate demonstrated a ~ 3-fold ($P < 0.05$) increase in mdx/CaMBP soleus muscle in comparison to mdx mice (Figure 6.9C).

To determine whether the absence of dystrophin combined with reduced A-utrophin would further impair membrane integrity, we assessed the uptake of Evans Blue dye in soleus muscle fibers from mdx versus mdx/CaMBP mice. Evans Blue dye is a

Figure 6.9. Increased areas of collagenous infiltrate and Evans Blue uptake in mdx/CaMBP slow muscles. Shown are representative photomicrographs of cross-sections from mdx (A) and mdx/CaMBP (B) muscles processed for Trichrome-Masson staining. Blue coloration denotes regions of collagenous infiltrate. Quantitative analysis of areas of collagenous infiltrate revealed significant increases in mdx/CaMBP soleus muscles (C). The asterisk denotes significant difference from mdx ($P<0.05$), n = 4-6 animals per group from three 20x cross-sectional views per animal. (D) Assessment of the percentage Evans blue dye staining above set threshold (see Materials and Methods) in damaged mid-belly regions of mdx/CaMBP soleus muscles revealed an approximately 2-fold increase in comparison to age-matched mdx mice (C). The asterisk denotes significant difference from mdx ($P<0.05$), n = 3 animals per group. Means and SEM are shown. Statistical analysis was conducted using Student's t-tests.


A
B


C
mdx
mdx/
CaMBP
*
0
50
100
150
200
250
300
Collagen infiltrate relative to mdx
(Percentage)


D
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300
Evans Blue uptake relative to mdx
(Percentage)

small, membrane impermeable, dye normally excluded from cytosolic regions of muscle fibers except when lesions occur in the sarcolemma (7). Quantitative assessment of the percentage of muscle fibers having Evans Blue uptake above a set threshold level (see Materials and Methods) clearly showed a ~ 2.5-fold ($P < 0.05$) increase in soleus muscles from mdx/CaMBP mice (Figure 6.9D). Collectively, these results suggest that the exacerbation of the dystrophic phenotype seen in mdx/CaMBP animals occurs in part because of a decrease in membrane integrity. Furthermore, these data suggest the decrease in membrane integrity is linked to reductions in the sarcolemmal levels of A-utrophin.

Discussion

In the present study, we specifically knocked down Ca^{+2}/CaM-based signaling in dystrophic slow fibers using the small peptide inhibitor CaMBP encoded by a transgene driven by the TnIs promoter. This approach allowed us to examine specific impairments of CaM signaling in mdx slow muscle fibers without the use of drugs such as calcineurin-based immunosupressants. These drugs can interfere with other CaM-regulated processes known to affect the progression of dystrophic pathology including activation of inflammatory cells (49, 50). Expression of CaMBP in mdx slow muscle fibers inhibited Ca^{+2}/CaM-regulated pathways as exemplified by the reduced nuclear localization of the transcription factors MEF2C and NFATc1. These impairments in CaM signaling, in mdx soleus muscles, were accompanied by reduced expression of PGC-1α and GABPα. In addition, impaired CaM signaling in mdx slow muscle fibers resulted in reduced expression of A-utrophin and MyHC I together with a loss of slow/type I muscle fibers. Furthermore, mdx/CaMBP slow muscles displayed an increase in the severity of the dystrophic pathology. Collectively, these observations indicate that Ca^{+2}/CaM-regulated pathways, known to promote the slow myofiber phenotype, constitute appropriate cellular targets for pharmacological interventions aimed at improving muscle functions in DMD patients.

For decades, Ca^{+2} has been seen as a tightly regulated second messenger that can regulate numerous aspects of skeletal muscle physiology (reviewed in 66). In this context, it is now well established that Ca^{+2}, together with the enzymatic co-factor CaM, can regulate multiple pathways involved in skeletal muscle development and fiber type specification (reviewed in 35, 39, 67). Two well-characterized effectors of Ca^{+2}/CaM-

based signaling in skeletal muscle are calcineurin and CaMK. Mice genetically engineered to over-express activated variants of both calcineurin and CaMK specifically in skeletal muscle, show a conversion of myofibers toward a slower, more oxidative, phenotype (57, 68). Control of the slower, high oxidative, myofiber phenotype by both calcineurin and CaMK involves, in part, their ability to regulate transcription factors of the NFAT and MEF2 families (36, 39, 42, 44, 45). In this context, activation of calcineurin has been shown to result in the dephosphorylation of NFATc1, and to enhance the activity of the MEF2 family of transcription factors (36, 39). These events lead to elevated expression of genes typical of the slow oxidative myofiber phenotype (35, 36, 39, 44). Furthermore, activation of CaMK also regulates MEF2 transcription factors by promoting the disassociation of MEF2 from its endogenous inhibitors, i.e. the chromatin remodeling enzymes HDAC4 and 5 (45, 46, 69).

In the present study, we observed clear reductions in the nuclear localization of NFATc1 and MEF2C in mdx/CaMBP soleus muscles, a muscle displaying >50% slow fibers. These observations are consistent with the importance of CaM as an upstream co-factor regulating NFATc1 and MEF2C activity, and localization. Although the expression of MEF2C and NFATc1 were not examined in this study, it should be noted that autoregulatory loops can control the expression of both factors (70-73). Therefore, it is also possible that inhibition of calcineurin and CaMK activities could impact on the expression levels of both factors. In accordance with the aberrant regulation of transcription factors that promote the slow myofiber program, soleus muscles from mdx/CaMBP mice showed reduced expression of MyHC I mRNA and loss of type I muscle fibers. Concurrent with the loss of the type I muscle fiber population, we also

observed increases in the number of MyHC IIa positive muscle fibers in mdx/CaMBP soleus muscles. This is consistent with loss of calcineurin activity in mature innervated slow muscle fibers leading to fiber type shifts towards a faster phenotype (40, 41, 44, 60). Collectively, these observations further demonstrate the existence of a link between Ca^{+2}/CaM signaling and the specification of the slow myofiber program. In addition, these findings are coherent with impaired CaM-regulated specification of the slow myofiber program in mdx/CaMBP mice.

Calcineurin and CaMK have been shown to converge on MEF2 in order to participate in an autoregulatory transcriptional loop regulating PGC-1α levels. This loop can sustain the expression of genes characteristic of the slower, high oxidative, myofiber program (37). In a recent study, regulation of GABPα expression by PGC-1α was shown to be important in specifying a more oxidative phenotype in skeletal muscle cells (58). Furthermore, we have recently shown that PGC-1α/GABPα and calcineurin/NFATc1 signaling can stimulate the transcriptional activity of A-utrophin (26, 34). Based on these observations, we thus examined the expression of PGC-1α, GABPα and A-utrophin in mdx/CaMBP mice. In agreement with the reduced nuclear localization of MEF2C and NFATc1, we observed reduced levels of A-utrophin, PGC-1α and GABPα mRNA. Furthermore, reduced A-utrophin levels in mdx/CaMBP slow muscle fibers can be explained by both a reduced nuclear localization of MEF2C and NFATc1, and lower levels of PGC-1α and GABPα mRNAs.

Previously, we established the involvement of calcineurin/NFAT signaling in directly regulating A-utrophin transcritptional activity (26, 34). In the present study, we attempted to determine whether MEF2C directly affects the transcription of A-utrophin.

Over-expression of MEF2C in muscle cells by adenoviral delivery failed to elevate endogenous levels of A-utrophin (data not shown). In addition, we did not obtain any evidence for binding of MEF2C to the A-utrophin promoter region using chromatin immunoprecipitaion assays (CHIP) and EMSAs. Therefore, we propose that in this case, MEF2C can indirectly affect A-utrophin expression through a mechanism involving PGC-1α/GABPα signaling.

One important factor to consider is that the CaMBP peptide used in the present study is rich in amino acids that may constitute a nuclear localization signal, that can potentially interefere with mitotic processes (47). Assuming that CaMBP can localize to myonuclei of mature muscle fibers, it is not difficult, however, to still envision the peptide having the ability to inhibit both CaMK and calcineurin. Indeed, both CaMK and calicneurin have been observed to take part in nuclear events regulating the activity of both NFAT and MEF2 (45, 74-76). Furthermore, both CaMK and calcineurin display nuclear localization and activity in skeletal muscle (45, 76-78).

Previous analysis of transgenic mice in which CaMBP expression was targeted to lungs revealed important developmental defects and lethality (47, 79). This observation suggests that the CaMBP transgene may potentially have a toxic effect on the development of mdx/CaMBP muscle fibers. However, this appears highly unlikely for the following reasons: i) in the studies on lung development, the CaMBP transgene was controlled by the human surfactant protein C (SPC) promoter which induced its expression at gestational day 11.5 (47, 79); ii) in the present study, CaMBP expression is controlled by the human TnIs promoter which becomes highly active only in mature slow muscle fibers (51-53); and iii) examination of CaMBP mice revealed no defects in soleus

muscle development (48 and this study). Together, these observations indicate that the timing of expression of CaMBP is a key factor to prevent developmental defects in tissues.

In the past, there have been indications that slow muscle fibers seem to be somewhat more resistant than fast muscle fibers to the pathological consequences associated with loss of dystrophin expression (80, 81). In DMD patients for example, fast muscles undergo rounds of degeneration/regeneration before slow muscle fibers (80). Coherent with this, adult mdx fast muscles are more sensitive to damage induced by forced lengthening contractions than their slower counterparts (81). Interestingly, this sensitivity of mdx fast muscles to stretch-mediated damage is rescued by over expression of utrophin (18). Therefore, the capacity of slower muscle fibers to express higher levels of A-utrophin in extra synaptic regions (26, 32), provides this population of myofibers with an increased capacity to resist the pathological progression associated with dystrophin-deficiency.

In the present study, mdx/CaMBP soleus muscles displayed reduced A-utrophin expression due to the preferential expression of CaMBP in muscle fibers displaying a slow phenotype and reductions in the numbers of type I muscle fibers. The loss of A-utrophin expression in mdx/CaMBP slow muscle fibers correlates with an increased severity of the dystrophic phenotype including elevations in the presence of mononuclear and collagenous infiltrates, and sarcolemmal disruption. By analogy, stimulation of calcineurin activity in mdx muscles has been shown to have rescuing effects on the dystrophic pathology (33). This rescue was accompanied by shifts in muscle fiber type towards a slower phenotype and elevated A-utrophin expression (33). The present study,

together with our previous work, clearly demonstrates the role of Ca^{+2}/CaM-regulated signaling pathways in the control of A-utrophin expression. Our findings further illustrate the potential usefulness of these signaling cascades as cellular targets, for pharmacological interventions aimed at increasing A-utrophin expression in muscles from DMD patients.

Converging lines of evidence have now emerged and support the notion that promotion of the slow myofiber program is beneficial for survival of dystrophin-deficient muscle fibers. For example, treatment of mdx diaphragms with insulin-like growth factor 1 (IGF-1) was shown to promote the slower oxidative muscle fiber phenotype while also having beneficial effects on the function and survival of dystrophin-deficient muscles (82, 83), possibly through activation of calcineurin/NFATc1 and MEF2 signaling (84-86). Moreover, dystrophin-deficient progeny obtained from crossing myotonic, arrested development of righting response (adr) mice with mdx mice, constitutes another key example of a fiber type shift towards a slower phenotype that leads to significant improvements in the dystrophic pathology (87). In this context, it may be relevant to examine the potential usefulness of activating PPARδ in dystrophin-deficient muscles, especially given its known impact on promoting the slow oxidative myofiber program in normal muscle (88). Based on our recent findings (26, 33, and the present study), it seems warranted and timely to examine whether treatment of mdx mice with PPARδ agonists would result in a fiber type shift towards a slower, more oxidative, phenotype and improvements in muscle structure and function. This potential for PPARδ agonists to attenuate dystrophic pathology would, in theory, be linked to increased expression of A-utrophin.

Materials and Methods

Generation of mdx/CaMBP Mice. Generation of mdx/CaMBP animals was conducted using a previously described breeding scheme (33). Briefly, mdx females were crossed with male TnIs-CaMBP animals. The resulting male progeny demonstrate dystrophin-deficiency and resulting dystrophic pathology. Pups that express the CaMBP transgene were identified through PCR screening of genomic DNA extracted from tails (48). Mice between 10-12 weeks of age were used for all subsequent analyses.

Determination of NFATc1 and MEF2C Nuclear Localization. Assessment of MEF2C and NFATc1 nuclear localization was performed as described in detail elsewhere (33). Briefly, soleus and tibialis anterior (TA) muscles from mdx and mdx/CaMBP mice were excised mounted in OCT compound, and frozen in melting isopentane. To examine the localization of MEF2C and NFATc1, muscle cross-sections (8 μm thick) were fixed with 4% paraformaldehyde, and subsequently incubated with primary antibodies against MEF2C (E17 Santa-Cruz Biotech CA USA) or NFATc1 (Cell Signaling, USA). Following thorough washes, the sections were further incubated with Alexa-594 goat anti-rabbit secondary antibodies (Molecular Probes Eugene OR). Sections were mounted using vectashield containing DAPI (VectorLabs CA USA) and viewed with a Zeiss fluorescent microscope. Images were taken using a digital camera and processed with Northern Eclipse software. The number of myonuclei positively stained for MEF2C and NFATc1 was counted in three 20 X cross-sectional views of myofibers, from the mid-belly region of muscles, from three animals per group. For counts, the spot density tool in Northern Eclipse was used to determine the average density of labeling in individual

nuclei. A threshold average density value was established, and the percentage of nuclei with labeling above this set point was determined from different samples. The values were then averaged and compared between groups.

Fiber Typing. Immunodetection of MyHC I and IIa in muscle fibers from wt, CaMBP, mdx and mdx/CaMBP mice was conducted using previously characterized antibodies (German Collection of Microorganisms) (see 26, 33). Briefly, soleus cross-sections were incubated for 1hr with anti-MyHC I or IIa, washed and further incubated with secondary antibodies. For detection of MyHC I, soleus sections were incubated with Alexa-594 secondary antibodies (Molecular Probes Eugene OR), washed, and mounted using vectashield containing DAPI (VectorLabs CA USA). For detection of MyHC IIa, soleus sections were incubated with secondary antibodies conjugated to horseradish peroxidase (Jackson Laboratories), washed, and incubated with diaminobenzidene (DAB) media prior to mounting with permount (33). Sections were viewed using a Zeiss microscope and processed using Northern Eclipse Software. The percentage of fibers staining for MyHC I or IIa was determined from three 20 X cross-sectional views of myofibers from the mid-belly regions of soleus muscles, from three animals per group. The percentage for MyHC I or IIa-positive muscle fibers was then averaged and compared among groups (33).

A-utrophin Levels in Type I Muscle Fibers. The relative levels of sarcolemmal A-utrophin expression at the periphery of type I (MyHC I positive) fibers was examined on serial sections processed for A-utrophin and MyHC I immunofluorescence, as described

in detail elsewhere (26). Briefly, serial cross-sections from mdx and mdx/CaMBP soleus muscles were incubated with primary antibodies recognizing either A-utrophin or MyHC I, washed and further incubated with Alexa-594 appropriate secondary antibodies (Molecular Probes Eugene OR). Both sets of serial sections processed either with anti-MyHC I or anti-A-utrophin were mounted with vectashield (VectorLabs CA USA), viewed using a Zeiss fluorescent microscope and processed using Northern Eclipse Software. Upon identification of type I fibers and corresponding fibers labeled for A-utrophin, sarcolemmal levels of A-utrophin were quantified. Quantification was done by measuring the average intensity of labeling at the periphery of type I fibers using Northern Eclipse software. A threshold value was set, and the percentage of type I muscle fibers with A-utrophin levels above this threshold was determined from ~ 500 muscle fibers. Four mice were analyzed per group.

Total RNA Extraction and Quantitative RT–PCR. Total RNA was extracted from soleus and EDL muscles using TriPure (Boehringer Mannheim) as recommended by the manufacturer. Quantitative RT–PCR was carried out to determine the relative abundance of A-utrophin, MyHC I, CaMBP, GABPα, PGC-1α and S12 rRNA using previously characterized primers (26, 34, 48, 89). Cycling conditions were optimized for all targets. In all these assays, negative controls consisted of RT mixtures in which total RNA was replaced with RNase-free water. For examination of CaMBP mRNA levels, an additional control was included, whereby reverse transcriptase (RT) was excluded from the reaction to ensure lack of DNA contamination. PCR products were first visualized on 1% agarose gels containing ethidium bromide (EtBr) (Sigma-Aldrich). The labeling intensity of the

PCR product which is linearly related to the abundance of cDNAs, was quantified using Kodak digital science 1D Image analysis software. For all quantitative measurements, PCR product comparisons were done within the linear range of amplification of each primer set as described and shown in detail in our previous work (26, 33, 90). Quantitiative assessment of S12 rRNA expression in mdx/CaMBP muscles relative to mdx revealed no significant differences (data not shown). Therefore, values obtained for A-utrophin, MyHC I, GABPα and PGC-1α were standardized using S12 rRNA.

Assessment of Mononuclear Infiltrate and Muscle Fiber Size. Cross-sections of soleus muscles were stained with hematoxylin and eosin, dehydrated through a series of alcohol solutions, cleared with xylene and mounted using permount (Fisher Scientific). The sections were viewed using standard light microscopy with a Zeiss microscope. Images were captured with an analog camera and processed using Adobe photoshop 8.0. The extent of mononuclear infiltrate occurring in muscles was determined by comparing the average percent area occupied by mononucleated cells. Counts for centrally located nuclei, a marker for muscle degeneration/regeneration were conducted as described in detail elsewhere (33). Both of these analyses were conducted with Northern Eclipse Software using; three 20 X cross-sectional views of mid-belly regions of muscles, from four to six animals per group. Variability in fiber size was determined by averaging the standard deviations from three 20 X cross-sectional views of myofibers from the mid-belly regions of muscles, from four to six animals per group.

Analysis of Collagen Infiltrate. Cross-sections of soleus muscles were fixed with picric acid and subsequently stained with Trichrome Masson (Sigma-Aldrich) as described by the manufacturer. Sections were then dehydrated through a series of alcohol solutions, cleared with xylene, mounted using permount (Fisher Scientific) and viewed using standard light microscopy with a Zeiss microscope. Images were captured with an analog camera and processed using Adobe photoshop 8.0. The extent of collagenous infiltrate occurring in muscles was determined by comparing the average percent area occupied by blue stained collagen from three 20 X cross-sectional views of mid-belly regions of muscles, from four to six animals per group.

Assessment of Evans Blue Uptake. Evans blue dye injections were performed as described elsewhere (7). Briefly, 50 ml/10 mg of b.w. of Evans blue dye was injected intravenously in mouse tails. Six to 12 h later, muscles were harvested and frozen in melting isopentane. Prior to observing the sections under the microscope, muscle sections were incubated in ice-cold acetone for 10 min, washed three times for 10 min with PBS and mounted with vectashield mounting medium (Vector Labs CA USA). The presence of Evans blue dye in myofibers was observed using a Zeiss fluorescence microscope. The intensity level was determined using Northern Eclipse software by converting images to 8 bit gray scale, and the average gray intensity in cytosolic regions of myofibers was taken to measure Evans blue dye fluorescence. A threshold value for cytosolic Evans Blue fluorescence was set, based on the median average gray intensity of cytosolic labeling from a cross-section, from an mdx/CaMBP soleus. The percentage of fibers above the threshold per section was calculated, averaged and compared among

groups. Three animals per group were analyzed. Two 20 X cross-sectional views from soleus mid-belly regions for each animal were used to avoid damage that can occur in the periphery of muscle fibers, stemming from the excision procedures (9).

Statistical Analysis. Two-tailed Student's t tests were used to analyze the data. Means +/- SEM are presented throughout. All statistics were done using Analysis ToolPak Microsoft Excel.

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Chapter 7

GENERAL DISCUSSION

I. Pathways that promote the slow oxidative myofiber program regulate A-utrophin expression

Part of the objectives examined in this thesis included, deciphering the molecular mechanisms involved in maintaining higher levels of utrophin expression in extra-synaptic regions of slow muscle fibers in comparison to fast muscle fibers. Initially, through the use of antibodies and RT-PCR primers specific for A-utrophin, we determined that this isoform is expressed at high levels in slower muscle fibers relative to fast muscle fibers. Specifically, at a cellular level, we observed that A-utrophin mRNA levels positively correlate with the expression of slower MyHC isoforms. Furthermore, we also demonstrated that functional overload of fast muscles, a paradigm that promotes fiber type shifts towards a slower contractile phenotype, increased endogenous levels of utrophin, and specifically A-utrophin. These results indicate that promotion of the slow myofiber program in skeletal muscle can stimulate the expression of A-utrophin.

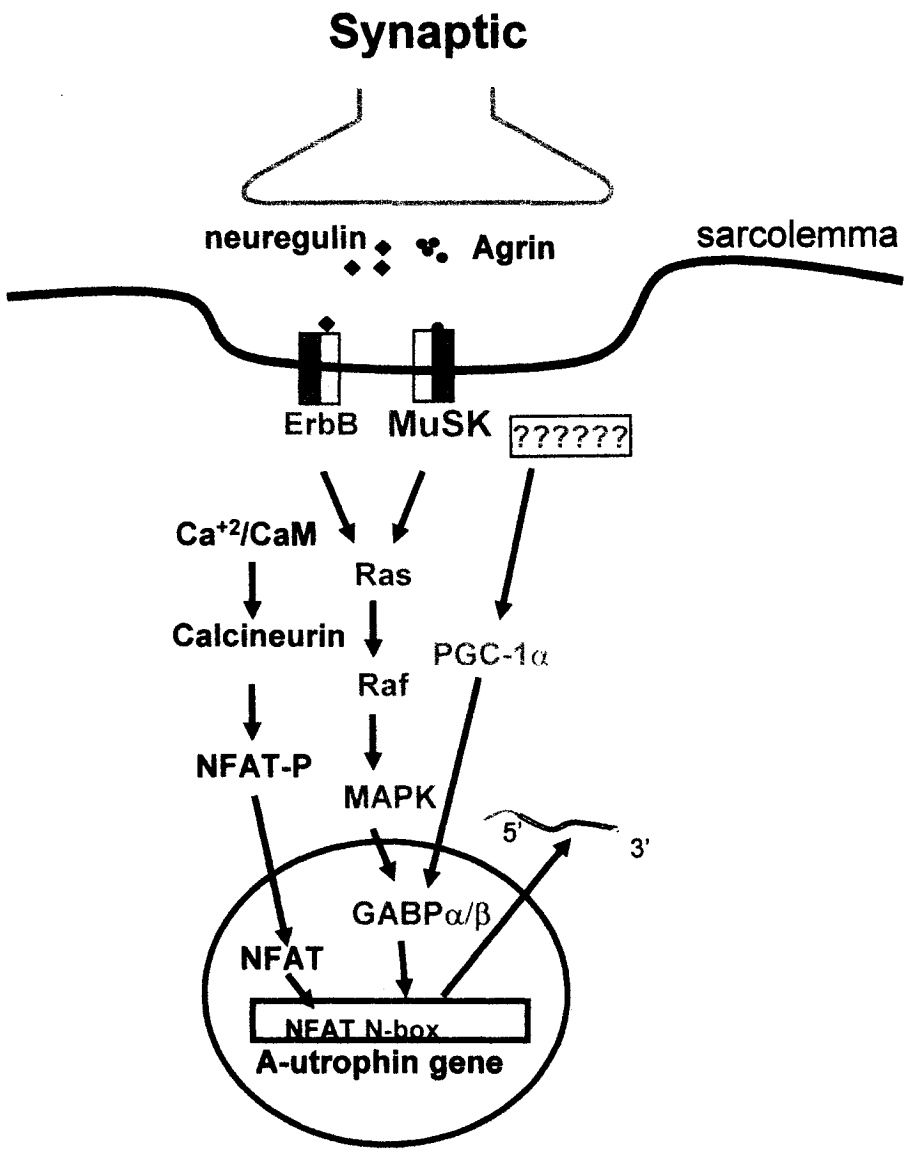
Both calcineurin/NFAT and PGC-1α/GABPα signaling are key pathways involved in the specification of the slow myofiber program (see for review Williams and Olson 2000; Lin et al., 2005). Through the use of complementary approaches, we examined the involvement of these pathways for their affects on A-utrophin expression. Both in vitro and in vivo data indicated that altered activity of both calcineurin/NFAT and PGC-1α/GABPα signaling can influence A-utrophin expression. Therefore, we

propose that these two pathways which are highly active in slow oxidative muscles are key regulators of A-utrophin expression.

In this thesis we also demonstrated that components of the calcineurin/NFAT and PGC-1α/GABPα pathways are enriched at the level of the NMJ. These observations suggest, both PGC-1α and calcineurin signaling are potentially involved in regulatory cascades that function to restrict the expression of synaptic genes to the NMJ. In support of this notion, we showed that the forced expression of PGC-1α in vivo can increase endogenous mRNA levels for AChR subunits. Other studies have also shown that calcineurin/NFAT signaling can influence the expression of additional synaptic genes (Cohen et al., 2004; Lee et al., 2004). Consistent with a role for calcineurin signaling in the formation and/or maintenance of the NMJ, treatment of myotubes with CsA and FK506 inhibits the formation of large stable agrin-induced AChR clusters (Madhavan et al., 2003). Taken together these observations indicate that both calcineurin/NFAT and PGC-1/GABPα signaling, in addition to having roles in the maintenance of the slow oxidative myofiber program, may be involved in transcriptional regulatory cascades that occur locally at the NMJ (Figure 7.1).

Due to its size and relative accessibility, in comparison to central nervous system (CNS) synapses, the NMJ is considered to be a simpler model for the study of synapse formation and maintenance (Sanes and Lichtman, 2001). Therefore, it is conceivable that PGC-1α/GABPα and calcineurin/NFAT signaling may have general roles in synapse development. In agreement with this view, it has been shown that calcineurin mediated modifications of MEF2 can affect the maturation of post-synaptic regions on dendrites (Flavell et al., 2006; Shalizi et al., 2006).

Figure 7.1. Summary of pathways with potential roles in synaptic expression of A-utrophin. In synaptic regions, neurally secreted factors such as neuregulin and agrin can act on post-synaptic receptor-tyrosine kinases ErbB and MuSK to stimulate the transcription of synaptic genes. The activation of ErbB and MuSK stimulates the Ras/MAPK pathway leading to the binding of GABPα/β to N-box motifs found in the A-utrophin promoter. Calcineurin has been shown to be enriched at the NMJ and can modulate the expression of A-utrophin. PGC-1α has been shown to be enriched at the NMJ and can stimulate the expression of synaptic genes including A-utrophin. Question marks refer to unknown factors potentially regulating PGC-1α at the NMJ.


Synaptic
neuregulin
Agrin
sarcolemma
ErbB
MuSK
??????
Ca+2/CaM
Ras
Calcineurin
PGC-1α
Raf
NFAT-P
MAPK
5'
3'
GABPα/β
NFAT
NFAT N-box
A-utrophin gene

In addition, neurons from mice null for PGC-1α display defects in neurite outgrowth and synapse formation (Lin et al., 2004). Hence, future studies on how PGC-1α and calcineurin signaling affect molecular events at the NMJ may lead to more general insights into the development of the post-synaptic apparatus in different types of synapses.

II. Calcineurin signaling regulates A-utrophin expression via transcriptional and post-transcriptional mechanisms

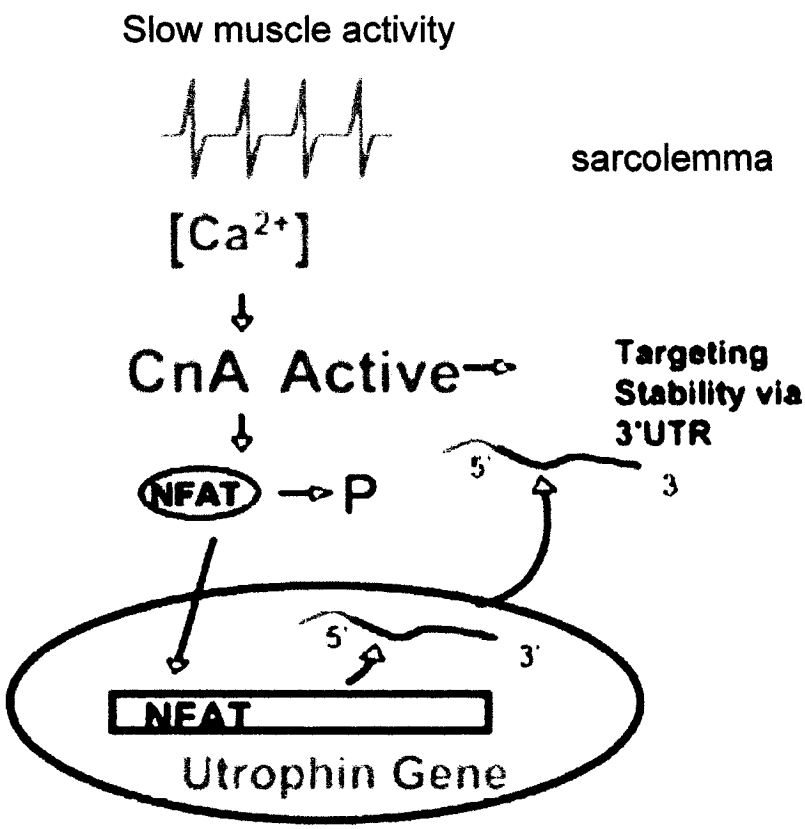
Previous studies in our laboratory implicated the involvement of post-transcriptional mechanisms, via the 3'UTR, in promoting higher levels of utrophin expression in slow versus fast muscle fibers (Gramolini, 2001). Consistent with this observation, examination of A-utrophin promoter-reporter transgenic muscles display only moderately higher levels of reporter activity in slow muscles relative to fast muscles (Stocksley, 2005). Based on these observations, in this thesis we sought to determine the molecular mechanisms whereby the utrophin 3'UTR can promote higher levels of A-utrophin mRNA expression in slow muscles relative to fast muscles.

Through the use of an in vitro stability assay, we determined that the stability of A-utrophin mRNA is greater in the presence of slow skeletal muscle protein extracts relative to fast skeletal muscle extracts. In agreement with this result, through the use of complementary approaches we also observed that reporter mRNAs fused to the utrophin 3'UTR were stabilized, and expressed at a higher level in slow muscles in comparison to fast muscles. The ability of the utrophin 3'UTR to affect the stability, and expression of

reporter mRNAs differentially in distinct muscle types was dependent upon the presence of a conserved ARE. In chapters 2 and 3 of this thesis we only observed modest changes in A-utrophin transcriptional activity upon stimulation of calcineurin signaling. Therefore, we next examined the effects of calcineurin signaling on A-utrophin mRNA stability and utrophin 3'UTR reporter activity. Through the use of an in vitro stability assay we established that A-utrophin mRNA stability is affected by calcineurin signaling. Furthermore, we also observed that the conserved ARE in the utrophin 3'UTR is responsive to calcineurin signaling. Based on these results, and the above discussion regarding the ability of calcineurin signaling to affect A-utrophin transcription; we propose that calcineurin signaling can regulate A-utrophin expression in skeletal muscle through both transcriptional and post-transcriptional mechanisms (Figure 7.2).

The ability of calcineurin to promote the slow myofiber program has been postulated to involve unidentified effectors and mechanisms, in addition to acting via the transcription factors MEF2 and NFATc1 (Parson et al., 2003; Parsons et al., 2004). In immune cells calcineurin activity can influence both the transcription and mRNA stability of cytokines (Nair et al., 1994; Hogan et al., 2003). In this context, calcineurin signaling can affect the stability of mRNAs through ARE dependent mechanisms acting on the 3'UTR (Nair et al., 1994; Bell at al., 2005). Therefore, it will be of interest to determine if calcineurin signaling can similarly influence the expression muscle fiber type specific genes through both transcriptional and post-transcriptional mechanisms. In this context, ARE-directed turnover may provide an additional mode of action, whereby calcineurin can promote the expression of genes indicative of the slow contractile phenotype.

Figure 7.2. The involvement of calcineurin/NFAT signaling in influencing the expression of utrophin. Calcineurin signaling influences utrophin expression in slow muscle fibers through both transcriptional and post-transcriptional mechanisms. Calcineurin/NFAT signaling regulates the transcription of A-utrophin by acting on specific regions in the A-utrophin promoter. Calcineurin signaling can also affect the stability of A-utrophin transcripts via the utrophin 3'UTR.


Slow muscle activity
sarcolemma
[Ca2+]
CnA Active
Targeting
Stability via
3'UTR
NFAT
P
5'
3
5'
3'
NFAT
Utrophin Gene

III. Ca+2/CaM-dependent signaling in dystrophin-deficient muscle

The finding that stimulation of calcineurin/NFAT signaling can promote the expression of A-utrophin, in extra-synaptic regions of muscle fibers, prompted us to examine this pathway in dystrophin-deficient mdx mice. Therefore, we examined utrophin expression, and the dystrophic pathology in muscles from transgenic mdx mice containing a transgene encoding for CnA*. Stimulation of calcineurin signaling in mdx skeletal muscles led to increased A-utrophin expression, together with fiber type shifts towards a slower phenotype. In accordance with the ability of A-utrophin to functionally compensate for dystrophin, we also observed in mdx/CnA* muscles the restoration of DAPs to the sarcolemma. Furthermore, increased A-utrophin expression and the restoration of DAPs to the sarcolemma correlated with improvements in the dystrophic pathology. Some of the observed improvements included decreases in fiber size variability; membrane permeability; inflammation; and degenerative/regenerative events.

In this thesis we also uncovered PGC-1α as an additional factor that through GABPα can regulate the expression of A-utrophin. Since the Ca^{+2}/CaM regulated enzymes calcineurin and CaMK can regulate the expression of PGC-1α, and calcineurin/NFAT can directly regulate A-utrophin expression, we next sought to determine the effects of knocking down CaM signaling in dystrophin-deficient muscles. Hence, we examined slow muscles from transgenic mdx mice specifically expressing CaMBP, a small peptide inhibitor for CaM, in slow muscle fibers. Consistent with impaired CaM signaling, targeted expression of CaMBP to dystrophin-deficient slow muscle fibers led to decreased expression of PGC-1α, GABPα, MyHC I, and fiber type

shifts towards a faster contractile phenotype. The observed shifts in muscle fiber type were accompanied by reductions in A-utrophin expression specifically in slow muscle fibers, where the CaMBP peptide was expressed. In addition, examination of dystrophic pathology in mdx/CaMBP slow muscles demonstrated a clear exacerbation in dystrophic pathology, as judged by increases in collagen infiltration; mononuclear infiltrate; membrane permeability; and fiber size variability. Taken together the observations made from the two mdx transgenic lines examined in this thesis, mdx/CnA* and mdx/CaMBP, support the notion that; Ca^{+2}/CaM regulated effectors calcineurin, NFATc1 and PGC-1α, may provide effective therapeutic targets to promote utrophin expression, and correspondingly alleviate dystrophic pathology in DMD patients.

IV. Pharmacological agents that stimulate calcineurin activity and the slow myofiber program for DMD.

A variety of pharmacological agents have been examined for their ability to improve the dystrophic pathology in dystrophin-deficient muscle, mostly by improving muscle regenerative capacity (see for review Chakkalakal et al., 2005). Some agents have also been examined for their ability to promote A-utrophin expression in dystrophin-deficient muscles. Treatment of mdx mice with NO donors leads to increased sarcolemmal utrophin expression, concomitant with corrections of the dystrophic pathology (Barton et al., 2005; Voisin et al., 2005). Glucocorticoid treatment has also been shown to effectively elevate utrophin expression in mdx mice (St-Pierre et al., 2004; Segalat et al., 2005). In this context, treatment of mdx mice with the glucocorticoid

deflazacort has been demonstrated to improve muscle morphology and enhance the activity of the NO pathway (Segalat et al., 2004). In addition, we have shown that treatment of mdx mice with deflazacort is capable of stimulating calcineurin/NFAT signaling and utrophin expression in skeletal muscles, with correlated improvements in dystrophic pathology (St-Pierre et al., 2004). Therefore, further characterization of deflazacort, and other compounds capable of promoting the calcineurin/NFAT pathway may lead to the elucidation of an effective pharmacological regimen for DMD patients. In theory such a regimen would promote utrophin expression, and alleviate dystrophic pathology in dystrophin-deficient muscles.

PGC-1α is a co-factor that does not contain intrinsic transcriptional activity, however can recruit transcription factors, histone acetyltransferases, and other proteins that regulate transcription (see for review Puigserver et al., 2003). Among the transcription factors PGC-1α is capable of forming interactions with in skeletal muscle includes the nuclear receptor peroxisome proliferator activated receptor δ (PPARδ) (Wang et al., 2003). PPARδ is part of a family nuclear hormone receptors initially identified for their roles in peroxisome proliferation. Subsequently, PPARs were found to be important transcriptional regulators, for genes involved in fat metabolism and oxidative respiration (see for review Gilde et al., 2003 and Lee et al., 2003). Activation of PPARs results in their translocation to nuclei, where they bind to PPAR response elements (PPRE) found in target promoters (Gilde et al., 2003 and Lee et al., 2003). Recently it has been shown that stimulation of PPARδ signaling, similar to forced expression of PGC-1α, promotes the conversion of muscle fibers to a slower more oxidative phenotype (Wang et al., 2004). For instance, the PPARδ synthetic agonist

GW501516, when fed to mice, promotes the expression of slow contractile proteins (Wang et al., 2004). Similarly, over-expression of a constitutively active variant of PPARδ, specifically in skeletal muscle, leads to the conversion of muscle fibers to a slower more oxidative phenotype (Wang et al., 2004). In the future, it would be of interest to determine if stimulation of PPARδ in dystrophin-deficient muscles can lead to fiber type conversions to a slower contractile, oxidative, phenotype. Such an approach could potentially elevate A-utrophin expression, and attenuate aspects of dystrophic pathology in dystrophin-deficient muscle.

Collectively, the observations made in this thesis suggest that pathways involved in the promotion of the slow myofiber program can potentially serve as pharmacological targets aimed at increasing endogenous utrophin expression, and correspondingly alleviate dystrophic pathology in dystrophin-deficient muscles. Therefore, an increased understanding of the ability of deflazacort to stimulate gene expression has relevance for the design of therapies that can stimulate calcineurin/NFAT signaling, and increase expression of utrophin to functionally compensate for dystrophin (St-Pierre et al., 2004). The specific PPARδ agonist GW501516 is already being used in phase II clinical trials for safety on humans with dyslipidemia (Muscat et al., 2005). Therefore, observations of the effects of GW501516 treatments on utrophin expression, and the dystrophic pathology in dystrophin-deficient animal models, may rapidly translate into the examination of GW501516 on DMD patients.

Chapter 8

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Appendices

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