IDENTIFICATION OF NOVEL GENES REGULATED BY INNERVATION AND CHARACTERIZATION OF SUBSYNAPTIC PROTEINS IN SKELETAL MUSCLE

by

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ABSTRACT

Innervation is an important regulator of neuromuscular development. At the neuromuscular junction (NMJ), localized factors released from the motoneuron terminal concentrate acetylcholine receptors (AChRs) in the postsynaptic membrane by inducing the redistribution of existing AChRs to the synaptic site and by locally activating AChR gene transcription in subsynaptic myofiber nuclei. At the same time, nerve-evoked electrical activity represses AChR gene expression in nuclei throughout the myofiber. In addition to AChRs, nearly two dozen other proteins accumulate in the postsynaptic membrane of the vertebrate NMJ, including the 43 kDa protein (rapsyn). The 43 kDa protein is a peripheral membrane protein expressed in a 1:1 stoichiometry with AChRs. Results from 43 kDa protein-mutant mice and in QT6 cells in culture demonstrate that the 43 kDa protein plays a critical role in the accumulation of AChRs at the NMJ. The first part of this thesis describes a primary muscle culture system I developed to identify sequences within the 43 kDa protein that are required for its association with the plasma membrane and with AChR clusters. I show that myristoylation is required for the 43 kDa protein to associate with AChR clusters in primary myotubes. Moreover, the amino-terminal half of the protein is insufficient to direct association of 43 kDa protein with AChR clusters. Importantly, I also show that cell types differ in their ability to cluster 43 kDa protein at the cell surface; whereas cell surface clusters of 43 kDa protein form readily in QT6 cells, such clusters form only occasionally in myoblasts and NIH3T3 fibroblasts. In addition to these studies, I also present strong evidence that dystrophin is a component of the subsynaptic membrane in *Torpedo* electrocytes.

Electrical activity is required for the regulation and maintenance of adult muscle physiology. To further understand the role of innervation in neuromuscular physiology and gene expression, we used a subtractive-hybridization and cloning approach to identify genes that are expressed differentially in innervated and denervated rat skeletal muscle. Studies in the second part of this thesis describe the partial characterization of three novel genes we identified using this technique: *INN1*, *DEN1*, and *DEN2*. We show

that *INN1* encodes three transcripts, all of which are differentially regulated by innervation; expression of the major transcripts decreases following denervation, whereas the expression of the two minor transcripts increases. These data suggest that alternative splicing of *INN1* is differentially regulated by innervation. Antibodies against INN1 react with a 22 kDa protein in skeletal muscle, and immunofluorescence studies indicate that INN1 may be a component of the sarcoplasmic reticulum. *INN1* is preferentially expressed in brain, heart, and skeletal muscle, and these results suggest that INN1 may have a role in the responsiveness of these tissues to electrical activity. Expression of the other two genes, *DEN1* and *DEN2*, is up-regulated following denervation. *DEN1* encodes a protein containing repeated motifs characteristic of muscle structural proteins such as titin, whereas *DEN2* encodes a protein with homology to αB-crystallin and a family of small heat shock proteins. In addition, we found that expression of the acute myeloid leukemia gene (*AML1*) increases dramatically following denervation. Since AML1 is a transcription factor, it may play a role in regulating the expression of other muscle genes following denervation.

Thesis Supervisor: Dr. Steven J. Burden
Title: Professor

for mom and dad

Where would I be without you?

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Chapter 2 is a collaborative effort between myself and Dr. Emma Dutton. Emma performed many of the transfections, particularly of rat primary myotubes, with the 43 kDa protein constructs. I am also grateful for Emma's wonderful, positive attitude, and her encouraging me to prepare our results for publication.

As UROP students in the Burden lab, Helen Lin and Amy Ravin assisted with screening the *Torpedo* cDNA library and isolation of the cDNAs encoding *Torpedo* dystrophin reported in Chapter 3.

Chapters 4 and 5 are collaborations with Dr. Xuejun (Joe) Zhu. Joe's contributions to Chapter 4 include the preparation of the innervated and denervated muscle-subtracted libraries as well as the original isolation of the *INN1, DEN1*, and *DEN2* cDNAs. Chapter 5 is largely Joe's work. My contributions include the characterization of the original *AML1* cDNA and the cloning and sequencing of the full-length *AML1* cDNA. It was a great pleasure to work with Joe on this project.

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ABBREVIATIONS

α-BGT	alpha-bungarotoxin
ACh	acetylcholine
AChR	acetylcholine receptor
AChE	acetylcholine esterase
AML	acute myeloid leukemia
ARIA	acetylcholine receptor inducing activity
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMD	Becker muscular dystrophy
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CBF β	core binding factor β
CGRP	calcitonin related-gene peptide
CMD	congenital muscular dystrophy
CNTF	ciliary neurotrophic factor
DGC	dystrophin glycoprotein complex
DMD	Duchenne muscular dystrophy
DPBS	Dulbecco's phosphate buffered saline
ECL	enhanced chemilluminescence
EDL	extensor digitorum longus
EGF	epidermal growth factor
EST	expressed sequence tag
GalNAc	N-acetylgalactosaminyl
GDNF	glial-cell-line-derived neurotrophic factor
HA	hemagglutinin
HeSP	heparan sulfate proteoglycan
hGH	human growth hormone
HRP	horseradish peroxidase
HSP	heat shock protein
IGF	insulin-like growth factor
kb	kilobase
kDa	kilodalton

LTP	long-term potentiation
mAb	monoclonal antibody
MAP1a	microtubule-associated protein 1a
MuSK	muscle specific kinase
MCK	muscle creatine kinase
N-CAM	neural cell adhesion molecule
NMJ	neuromuscular junction
NO	nitric oxide
NRG	neuregulin
NSP	neuroendocrine-specific protein
nt	nucleotide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PKC	protein kinase C
SR	sarcoplasmic reticulum
TMR-α-BGT	tetramethylrhodamine α-bungarotoxin
TTX	tetrodotoxin

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Chapter 1.

General Introduction

Introduction

Synapses are highly specialized structures designed for the transfer of signals from neurons to their targets. During synaptic transmission, electrical potentials are converted into chemical signals in the presynaptic nerve terminals and back to electrical potentials in the postsynaptic cell. The ability of neurons to affect this conversion rapidly and reproducibly is fundamental to the overall function of the nervous system. In addition, certain aspects of learning and memory are thought to rely on modulation of synaptic signaling, and such modulation is thought to occur through changes in synaptic biochemistry. Accordingly, understanding how synapses are formed and are regulated have become central themes in understanding how the nervous system works.

Much of what is currently known regarding synaptic structure and development has come through the study of the vertebrate neuromuscular junction (NMJ), the site of motoneuron-muscle contact. Although only one of cells at the NMJ is a neuron, the NMJ's relatively large size, accessibility, and amenability to both *in vivo* and *in vitro* manipulation have established it as a model system for understanding synaptogenesis in the central nervous system (for reviews, see Bloch and Pumplin, 1988; Hall and Sanes, 1993).

Structure of the NMJ

Skeletal muscles are innervated by motoneurons whose cell bodies reside in the spinal cord. Thus, motor axons must travel relatively long distances to reach their targets. Upon reaching the muscle, the motor axon branches to innervate up to several hundred individual muscle fibers. These axonal branches themselves branch upon reaching their target muscle fibers, producing a fine spray of terminal branches. Unlike the rest of the axon, the nerve terminals are not myelinated. Instead, each branch of the terminal is capped by Schwann cell processes, leaving the apposed surfaces of the pre- and postsynaptic membranes in close proximity.

Electron micrographs of the NMJ show that both the presynaptic nerve terminal and postsynaptic muscle membrane are highly differentiated (Engel, 1986; Ogata, 1988). Within the nerve terminals are found large numbers of 50 nm vesicles which contain the neurotransmitter, acetylcholine (ACh). Many of these synaptic vesicles are clustered adjacent to electron dense structures called active zones on the cytoplasmic side of the presynaptic membrane. During synaptic transmission, synaptic vesicles fuse with the presynaptic membrane at these active zones and release their complement of ACh into the 50 nm wide synaptic cleft which separates the pre- and postsynaptic membranes (Heuser

and Reese, 1981; Pecot-Dechavassin, 1982). Also found in nerve terminals are large, dense-core vesicles that are thought to contain neuropeptides, including the calcitonin gene-related peptide (CGRP) (Matteoli et al., 1988; 1990). Mitochondria are also very abundant in nerve terminals, providing the energy required for neurotransmitter synthesis and release and for vesicle membrane recycling.

Motoneuron terminals lie within shallow gutters on the surface of the muscle fiber. Directly underneath the nerve terminals, the postsynapatic membrane is more deeply folded, forming up to 1μm deep invaginations into the myofiber. These junctional folds are arranged such that the mouth of the folds are in precise register with the active zones in presynaptic membrane. The crests of the junctional folds appear thickened, and these thickened regions correspond to the location of acetylcholine receptors (AChRs) (Fertuck and Salpeter, 1974; Daniels and Vogel, 1975; Matthews-Bellinger and Salpeter, 1978). The density of myofiber nuclei is slightly higher under the synapse (Kelly and Zacks, 1969), and the Golgi apparatus surrounding synaptic nuclei are morphologically distinct from those in nonsynaptic regions of the muscle fiber (Jasmin et al., 1989; 1995). Moreover, synaptic nuclei are transcriptionally distinct from extrasynaptic nuclei, such that in innervated muscle, synaptic nuclei selectively transcribe genes encoding synaptic proteins (discussed below).

Lining the synaptic cleft and extending into the troughs between junctional folds is the synaptic basal lamina. Although contiguous with the basal lamina which surrounds the rest of the myofiber, the synaptic basal lamina is biochemically distinct from it. Acetylcholinesterase (AChE), which hydrolyzes and inactivates ACh, is concentrated within the synaptic basal lamina (McMahan et al., 1978), as are α3, α4, and α5 chains of collagen IV (Sanes et al., 1990; Miner and Sanes, 1994), laminin β2 (s-laminin), a synaptic form of the laminin β-chain which is thought to be important for nerve terminal differentiation (Hunter et al., 1989; Noakes et al., 1995), and factors that regulate post-synaptic development (discussed below).

Molecular components of the NMJ

Because the NMJ comprises only a small fraction (> 0.1%) of the muscle fiber, neurobiologists have had to rely on indirect means to identify molecular components of the NMJ. The most important source for the direct purification of synaptic molecules has been the electric organ of *Torpedo*, a genus of marine ray. Derived from myotube precursor cells, the electrocytes of the electric organ are innervated by cholinergic neurons over a majority of their ventral surface; therefore, electromotor synapses comprise a significantly large portion of electric tissue membranes, making the tissue a

rich source of synaptic molecules. For example, AChRs are ~1000-fold more abundant in the electric organ than in adult muscle. In addition to the electric organ, preparations of synaptosomal membranes from the central nervous system have also permitted the identification of synaptic proteins, particularly those associated with active zones in the presynaptic nerve terminal. Finally, immunocytochemistry using antibodies raised against proteins purified from other tissues has also contributed to our understanding of the molecular components of the synapse.

At present, nearly four dozen different molecules have been identified which are concentrated at the NMJ; nine concentrated in the presynaptic nerve terminal, eight in the synaptic basal lamina, and roughly two dozen in the postsynaptic muscle fiber (see Hall and Sanes, 1993 - Table 1). In the presynaptic nerve terminals, many components of synaptic vesicles have now been identified, including synaptophysin and synapsin (Torri-Tarelli et al., 1990), which may mediate synaptic vesicle transport to active zones via interactions with cytoskeletal proteins (Südhof and Jahn, 1991), and synaptotagmin (Bixby and Reichardt, 1985), which is important for synaptic vesicle docking and fusion with the presynaptic membrane (DeBello et al., 1993). Components of active zones have also been identified, and these include voltage-gated calcium (Ca^{2+}) channels (Cohen et al., 1991), syntaxin, a putative vesicle docking protein (Bennett et al., 1992), and α-latrotoxin receptor/neurexin, which has also been implicated in synaptic vesicle exocytosis (Valtorta et al., 1984; Ushkaryov et al., 1992). As the list of presynaptic proteins grows, it has become increasingly clear that the mechanisms mediating synaptic vesicle exocytosis are strikingly similar biochemically to those which mediate exocytosis in other cell types (for reviews, see Pevsner and Scheller, 1994; Sollner and Rothman, 1994).

In muscle fibers, the postsynaptic membrane is biochemically different from extrasynaptic regions of the sarcolemma. Further, the apical crests of the junctional folds are biochemically distinct from the trough regions between the folds. For example, AChRs are concentrated in the apical surfaces of the postjunctional folds, while voltage-gated sodium channels are limited to the troughs (Flucher and Daniels, 1989). Several cytoskeletal elements are also restricted to specific domains of the junctional folds. Both a 43 kilodalton (kDa) protein (rapsyn) (Sealock et al., 1984), and utrophin (dystrophin-related protein) (Bewick et al., 1992) are localized specifically with AChRs at the crests. In contrast, ankyrin is concentrated in the trough membranes (Flucher and Daniels, 1989). α7A and α7B integrins are also expressed specifically at the NMJ, but whether their distributions are further restricted to either the tops or the bottoms of the folds remains unknown (Martin et al., 1996). Other transmembrane proteins including N-CAM

(Covault and Sanes, 1986), ß1 integrin (Bozyczko et al., 1989), components of the dystrophin glycoprotein complex (DGC) (Ohlendieck et al., 1991; Peters et al. 1994), and over a dozen cytoskeletal proteins, including vinculin and filamin (Bloch and Hall, 1983), talin (Sealock et al., 1986), dystrophin (Sealock et al., 1991; Yeadon et al., 1991), and ß-spectrin (Bloch and Morrow, 1989) are also concentrated in, although not limited to, the postsynaptic membrane.

AChRs

The AChR was the first ligand-gated ion channel to be purified, and remains the most thoroughly understood neurotransmitter receptor both biochemically and structurally. The AChR is a pentameric protein comprised of four distinct, yet homologous subunits, α, β, δ, and γ, which range in molecular mass from 40 to 65 kDa. The five subunits of the pentamer form a central cation-conducting channel which opens in response to ACh binding to the α-subunit (Weill et al., 1974; Karlin et al., 1983). The stoichiometry of subunits within the AChR pentamer is $\alpha_2\beta\delta\gamma$ in both *Torpedo* electrocytes and vertebrate fetal muscle (Raftery et al., 1980); however, at mature vertebrate synapses, the γ-subunit is replaced with a fifth subunit, ε, resulting in channels with greater conductances and shorter mean open times (Mishina et al., 1986; Gu and Hall, 1988).

Oligonucleotides based on the protein sequences from purified AChR subunits were used to isolate cDNAs encoding the α, β, δ, and γ subunits from *Torpedo* (Noda et al., 1983). Subsequently, mammalian homologs for each subunit, including the ε subunit, which is not expressed in *Torpedo*, were also cloned (Mishina et al., 1986; Takai et al., 1985). To confirm that the cloned subunits could form a functional AChR channel, synthetic RNAs transcribed *in vitro* from the cloned AChR subunit cDNAs were injected into *Xenopus* oocytes to express the proteins. Electrophysiological measurements of the expressed AChRs confirmed that four subunits were required for expression of functional channels, and that substitution of ε for γ results in expression of channels with characteristics of adult AChRs (Mishina et al., 1984; 1986). Comparisons between the deduced protein sequences of the AChR subunits reveal a high degree of similarity between them, which suggests that the genes encoding the AChR subunits evolved from a single ancestral gene (Noda et al., 1983).

Interactions between AChRs and the subsynaptic cytoskeleton

AChRs are concentrated to a density of roughly 10,000/μm^2 at the apices of the junctional folds, whereas in the troughs, the density of AChRs falls to >1,000/μm^2

(Fertuck and Salpeter, 1976; Salpeter and Harris, 1983). In extrasynaptic regions of the innervated myofiber, there are fewer than 10 AChRs/μm^2 (Fertuck and Salpeter, 1976; Salpeter et al., 1988). As mentioned above, many cytoskeletal elements are concentrated at the postsynaptic membrane, and electron microscopic and deep-etch rotary shading studies of the NMJ show that a meshwork of thin fibers directly underlies AChR domains (Ellisman et al., 1976; Heuser and Salpeter, 1979; Hirokawa and Heuser, 1982). In addition, alkaline treatment of AChR-enriched membranes, which removes membrane-associated cytoskeletal elements (Neubig et al., 1979), increases the mobility of AChRs within the plane of the membrane (Lo et al., 1980; Rousselet et al., 1982) and disrupts AChR organization (Barrantes et al., 1980; Cartaud et al., 1981; Bloch and Froehner, 1987). Together, these observations suggest that AChRs are restricted to the postsynaptic membrane, in part, by interactions with the underlying postsynaptic cytoskeleton.

A crucial link between the AChR and the subsynaptic cytoskeleton appears to be the 43 kDa protein. Purified originally from AChR-enriched membranes of the electrocyte, the 43 kDa protein is a peripheral membrane protein found on the cytoplasmic face of the postsynaptic membrane (Sobel et al., 1977; Wennogle and Changeux, 1980; St. John et al., 1982). Immunocytochemistry shows that the 43 kDa protein precisely colocalizes with AChRs in both the electrocyte and the vertebrate NMJ, and at AChR clusters on muscle cells in culture (Froehner et al., 1981; Sealock et al., 1984; Burden, 1985; Flucher and Daniels, 1989). Moreover, the 43 kDa protein and AChRs are present at approximately equimolar concentrations in the postsynaptic membrane (Burden et al., 1983; LaRochelle and Froehner, 1986).

Direct biochemical evidence demonstrating interaction between the 43 kDa protein and the AChR is lacking, but a number of studies suggest that such interactions occur. First, 43 kDa protein is one of the proteins extracted from AChR-enriched membrane domains by alkaline and LiS treatments which alter AChR mobility and organization within the membrane (Neubig et al., 1979). Second, electron microscopy and X-ray diffraction studies suggest that the 43 kDa protein lies in close proximity to the AChR (Mitra et al., 1989). Third, studies have demonstrated that 43 kDa protein can be cross-linked to the AChR β-subunit (Burden et al., 1983). Fourth, AChRs fail to concentrate at the NMJs of 43 kDa protein-deficient mice, and muscle cell cultures derived from these mutant mice also fail to cluster AChRs in response to the AChR clustering factor, agrin (Gautam et al., 1995). Finally, co-expression of 43 kDa protein with AChRs in non-muscle cells can promote AChR clustering (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992). These studies showed that whereas AChRs are distributed diffusely in the plasma membrane of cells transfected with AChR expression plasmids,

co-expression of 43 kDa protein and AChRs results in the clustering of both molecules in the plasma membrane. Interestingly, when 43 kDa protein is expressed in the absence of AChR in these non-muscle cells, it is clustered on the cell surface. Although these experiments suggest that 43 kDa protein can self-organize, the 43 kDa protein may not be sufficient to direct 43 kDa protein and AChR clustering. Myoblasts and myotubes in culture express nearly equivalent levels of 43 kDa protein (Frail et al., 1989), but the 43 kDa protein clusters only in myotubes. Thus, clustering of the 43 kDa protein is regulated during myogenesis. These results also raise the possibility that the mechanisms responsible for 43 kDa protein/AChR clustering in muscle cells may differ from the mechanisms mediating clustering of the transfected 43 kDa protein in the non-muscle systems mentioned above. Understanding the relationship between 43 kDa protein clustering mechanisms in muscle and non-muscle cells is important in order to interpret results using non-muscle systems to dissect functional domains of the 43 kDa protein important for AChR clustering. In Chapter 2 of this thesis, I describe a primary muscle culture system which I developed in collaboration with Emma Dutton, a postdoctoral fellow in the lab, which allowed us to begin to identify sequences in the 43 kDa protein that are required for association of 43 kDa protein with the cell surface and with AChR clusters in myotubes.

The spectrin-based cytoskeleton of the erythrocyte is often cited as a model for understanding the skeleton of specialized cells. In the erythrocyte, spectrin tetramers and short actin oligomers are linked in a lattice-like meshwork, and are tethered to an anion transporter in the plasma membrane via ankyrin (Bennett, 1985). Such an actin/spectrin cytoskeleton also underlies AChR domains in the postsynaptic membranes. Both β-spectrin and non-filamentous actin are colocalized with AChRs in the postsynaptic membrane and at substrate-attached AChR clusters in rat myotubes in culture (Hall et al., 1981; Bloch, 1986; Bloch and Morrow, 1989), and quick-freeze, deep-etch, rotary-shadowing electron microscopy of *in vitro* AChR clusters reveals a polygonal lattice on the cytoplasmic surface of AChR membrane domains that is similar to that of the erythrocyte submembrane skeleton (Pumplin, 1989). Further, when actin alone or actin and β-spectrin together are selectively extracted from isolated, substrate-attached AChR clusters, the organization of the AChR clusters is disrupted (Bloch, 1986; Bloch and Morrow, 1989). These results suggest that actin and β-spectrin are necessary to maintain the integrity of these *in vitro* AChR clusters.

In addition to actin and β-spectrin, dystrophin and the dystrophin-related protein, utrophin, may have roles in organizing the postsynaptic membrane. Both dystrophin and utrophin are large (~400 kDa), filamentous proteins. Both proteins have central rod-like

domains comprised of spectrin-like coiled-coil repeats (Koenig et al., 1988; Tinsley et al., 1992) and amino-terminal actin-binding domains (Hemmings et al., 1992; Winder et al., 1995). As described in Chapter 3 of this thesis, dystrophin is expressed in Torpedo electrocytes, where it is localized to the postsynaptic membrane, and is concentrated at vertebrate NMJs and at AChR clusters in rat myotubes in culture (see also Chang et al., 1989; Jasmin et al., 1990; Sealock et al., 1991). Further, ultrastructural studies show that dystrophin is incorporated into the spectrin-rich cytoskeleton underlying AChR clusters in rat myotubes (Dmytrenko et al., 1993), and is also present in the postsynaptic membranes of cortical neurons in the CNS (Lidov et al., 1990). Like, dystrophin, utrophin is concentrated at the vertebrate NMJ and at AChR clusters in rat myotubes (Ohlendieck et al., 1991; Phillips et al., 1993); however, whereas dystrophin is also associated with the extrajunctional sarcolemma in adult muscle, utrophin is limited to the NMJ. Moreover, high resolution light microscopy of rat NMJs suggests that utrophin is colocalized with AChRs at the crests of the junctional folds, where as dystrophin is more concentrated in the troughs (Sealock et al., 1991; Byers et al., 1991; Bewick et al, 1992). These data suggest that utrophin has a specific role in organizing AChRs in the postsynaptic membrane, while dystrophin may be important in organizing molecules, such as sodium channels, in the troughs.

Dystrophin is attached to the sarcolemma via interactions between its carboxy-terminal region and a group of membrane glycoproteins termed the dystrophin glycoprotein complex (DGC) (Campbell and Kahl, 1989; Ervasti et al., 1990; Suzuki et al., 1994). Like dystrophin, these proteins are concentrated at, but are not restricted to, the NMJ (Ohlendieck et al., 1991; Bewick et al., 1992; Matsumura et al., 1992). Utrophin has been shown to interact with a complex of sarcolemmal proteins identical to, or at least antigenically similar to, components of the DGC (Matsumura et al., 1992), and at least one dystrophin associated protein, β2-syntrophin, is selectively localized to the postsynaptic membrane (Peters et al., 1994). Recently, α-dystroglycan, one of the components of the dystrophin glycoprotein complex, has been shown to bind the extracellular matrix molecules laminin and merosin (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993), as well as agrin, an extracellular matrix protein which induces AChR clustering in myotubes in culture (see below) (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). In addition, α-dystroglycan is also colocalized with 43 kDa protein in the cell membrane of transfected quail fibroblasts when the two proteins are co-expressed (Apel et al., 1995). These results suggest that dystrophin, utrophin, and the dystrophin glycoprotein complex are

important links connecting the extracellular matrix to the cytoskeleton, and may have crucial regulatory roles in organizing the postsynaptic membrane.

Formation of the NMJ

The neuromuscular junction develops through multiple stages over a long period of time. For example, in the developing rat, the period of neuromuscular synaptogenesis lasts from approximately one week before birth (embryonic days 13-14) through the end of the second postnatal week (Kelly and Zacks, 1976; Dennis et al., 1981). The steps in NMJ formation are similar in mammals, chick, and frog, although each species displays variations in the timing of the developmental events and the final structure of the developing synapse.

Early nerve-muscle contact

Muscle differentiation occurs concomitantly with motoneuron outgrowth. Motoneuron axons reach the differentiating mesenchymal tissues at roughly the same time as myoblast precursors are fusing to from multinucleated myotubes (rat embryonic day 13-14; chick embryonic stage 24). Although nerve and muscle membranes are in close proximity at this stage, their apposed membranes remain unspecialized, and the space between them lacks a defined basal lamina (Kelly and Zacks, 1976). Nevertheless, these rudimentary synapses are functional, since miniature endplate potentials are detected in the embryonic muscle at the earliest stages of nerve-muscle contact (Diamond and Miledi, 1962; Kullberg et al., 1977; Dennis et al., 1981). Synaptic vesicles are scattered in the nerve terminal, but, active zones are not distinguishable in the early presynaptic membrane. Taken together, these observations suggest that quantal release of ACh does not require fully differentiated active zones.

In the developing muscle, expression of AChRs begins at the onset of myotube differentiation. Studies using radiolabelled α-bungarotoxin (α-BGT), which binds tightly to the AChR α-subunit, reveal that AChRs are distributed diffusely and uniformly over the surface of the muscle fiber, reaching a density of 100-200/μm^2 (Bevan and Steinbach, 1977). Beginning at embryonic day 15 (E15) in the rat, clusters of AChRs appear in the postsynaptic region (Bevan and Steinbach, 1977; Braithwaite and Harris, 1979), eventually coalescing into a more uniform 'plaque' (Steinbach, 1981; Slater, 1982a). Concomitant with the onset of AChR clustering, the amplitude of miniature endplate potentials and the rate of rise of endplate potentials increase (Dennis et al., 1981; Harris, 1981b; Ziskind-Conhaim and Dennis, 1981). In addition, elements of the synaptic basal lamina begin to accumulate at E15 (Chiu and Sanes, 1984), followed a day later by

the accumulation of AChE (Bennett and Pettigrew, 1974; Bevan and Steinbach, 1977; Harris, 1981a; Chiu and Sanes, 1984).

Between E16 and birth, myofibers become innervated by multiple motoneurons at the same synaptic site (Redfern, 1970; Bennett and Pettigrew, 1974; Brown et al., 1976). The density of AChR in the postsynaptic membrane approaches that in the adult synaptic membrane (~10,000/μm^2) (Matthews-Bellinger and Salpeter, 1983), whereas the number of AChRs in the extrasynaptic membrane declines to < 10/μm^2 (Bevan and Steinbach, 1977). Similarly, the level of AChE in the synaptic basal lamina increases, and its distribution comes to approximate more closely the boundaries of the nerve terminal (Harris, 1981b; Steinbach, 1981). The decrease in extrajunctional AChR expression is due primarily to the repression of AChR subunit gene transcription in extrasynaptic nuclei, and is regulated by nerve-stimulated electrical activity (discussed below).

Postnatal synapse maturation

The final stages of NMJ maturation occur postnatally, concluding 2-3 weeks after birth. Thereafter, the NMJ continues to enlarge in proportion to the overall growth of the muscle fiber (Steinbach, 1981; Slater, 1982a; Matthews-Bellinger and Salpeter, 1983). Beginning just prior to birth and continuing in the postnatal period, junctional AChRs stabilize. In both embryonic and fetal rat muscle, the half-life of AChRs is ~1 day (Reiness and Weinberg, 1981), but beginning at E18, the half-life of junctional AChRs increases to ~10 days . In contrast, the half-life of extrajunctional AChRs remains unchanged (Berg and Hall, 1975b; Steinbach et al., 1979; Reiness and Weinberg, 1981; Steinbach, 1981). In chick muscle, a similar increase in the stability of junctional AChRs occurs, but not until ~3 weeks after hatching (Burden, 1977b). This change in the stability of junctional AChRs is regulated by innervation-induced electrical activity, since the half-life of junctional AChRs decreases following denervation or blockade of synaptic transmission with tetrodotoxin (TTX) (Loring and Salpeter, 1980; Bevan and Steinbach, 1983; Fumagalli et al., 1990). Concurrent with the increase in their metabolic stability, junctional AChR aggregates also become resistant to dissociation induced by denervation (Slater, 1982b), low Ca^{2+}, high KCl, or the AChR agonist, carbachol (Bloch and Steinbach, 1981). Treatment of isolated muscle fibers with collagenase also disrupts junctional AChR clusters and accelerates the turnover of the AChRs (Bloch et al., 1986). Since the synaptic basal lamina increases in biochemical complexity during the postnatal period (Chiu and Sanes, 1985), these data suggest that the resistance of synaptic AChRs to dispersing treatments may be due to increased direct or indirect interactions with basal lamina components.

The channel properties of the junctional AChRs also change during the perinatal period, as junctional AChRs acquire a greater conductance and a shorter mean open time (Sakmann and Brenner, 1978; Fischbach and Schuetze, 1980; Michler and Sakmann, 1980). As noted above, this change stems from the expression of the AChR ε-subunit and its substitution for the γ-subunit in newly synthesized AChR pentamers (Mishina et al., 1986; Schuetze and Role, 1987; Gu and Hall, 1988). The initial induction of adult (ε-type) AChRs is nerve-dependent, and requires both electrical activity- and motoneuron-derived factors (Brenner and Sakmann, 1983; Brenner et al., 1987; Martinou and Merlie, 1991). Once established, however, the continued AChR ε-subunit expression becomes both activity- and nerve-independent (Brenner et al., 1990; Martinou and Merlie, 1991), and signaling molecules associated with the synaptic basal lamina are sufficient to stimulate AChR ε-subunit mRNA in synaptic nuclei (see below).

Junctional folds begin differentiating after the first postnatal week, and the AChR aggregates become restricted to the crests of these folds (Matthews-Bellinger and Salpeter, 1983). At the same time, the number of synaptic inputs each muscle fiber receives is reduced until each muscle fiber is innervated by only a single motoneuron (Redfern, 1970; Brown et al., 1976; Slater, 1982a). This reduction in the number of innervating motoneurons is called synapse elimination.

Synapse elimination

Synapse elimination at the NMJ is thought to result from competition between the innervating motoneurons (for review, see Coleman and Lichtman, 1993), but the biochemical nature of this competition remains unclear. Evidence suggests that muscle activity influences some aspects of this competition. In both developing chick and rat muscle, blocking synaptic transmission using pharmacological agents such as curare, botulinum toxin, TTX, or α-BGT prevents synapse elimination (Srihari and Vrbova, 1978; Thompson et al., 1979; Brown et al., 1981; Duxson, 1982; Ding et al., 1983). In contrast, direct electrical stimulation of postnatal rat muscle accelerates the rate at which polyneuronal innervation is lost (O'Brien et al., 1978).

Direct observations of developing NMJs undergoing synapse elimination *in vivo* also support the idea that the postsynaptic muscle plays an active role in synaptic competition (Balice-Gordon and Lichtman, 1993). In this study, the developing presynaptic nerve terminals and the postsynaptic AChRs were visualized with the mitochondrial dye 4-Di-2-ASP and rhodamine-conjugated α-bungarotoxin, respectively. Observations of the same NMJ made over several days showed that in synapses destined for elimination, AChRs are lost from the postsynaptic membrane prior to obvious

changes in the overlying nerve terminal. A similar early loss of AChRs beneath nerve terminals is also seen during synapse elimination in denervated/reinnervated adult muscle, a process which mimics synapse elimination during development (Rich and Lichtman, 1989). These data suggest that changes in the postsynaptic membrane may trigger the removal of the overlying nerve terminals; these data do not preclude the possibility, however, that presynaptic changes occur prior to the loss of postsynaptic AChR.

One attractive model to explain the role of muscle activity in synaptic competition is a Hebbian mechanism in which synchronous depolarization of the nerve terminal and the muscle stabilizes, whereas asynchronous depolarization of the two destabilizes their synaptic connection. Evidence in support of such a model has come from *in vitro* studies using *Xenopus* myocytes and spinal neurons in coculture (Lo and Poo, 1991; Dan and Poo, 1992). In these experiments, synaptic potentials in myocytes were measured after stimulating each of two innervating neurons. One neuron was then given a tetanic stimulus, and the synaptic potentials elicited by each neuron were re-measured. These experiments showed that tetanic stimulus of the one neuron induces a rapid and prolonged suppression of the synaptic response generated by the unstimulated neuron. Further, asynchronous stimulation of the two neurons results in the repression of one or both of their synaptic responses, whereas synchronously applied tetanic stimuli to both neurons produces little effect. In singly innervated myocytes, repeated application of ACh to the synaptic site in the absence of nerve stimulation or asynchronously with stimulation also results in synaptic suppression, whereas synchronous application of ACh with stimulation does not. Thus, muscle stimulation is sufficient to produce synaptic suppression in the absence of a competing neuron. Quantal analysis indicates that the synaptic suppression induced in all of these experiments results from a reduction in the amount of ACh released by the stimulated nerve, rather than changes in the responsiveness of the myocyte. Interestingly, preloading the myocyte with BAPTA, which buffers changes in cytosolic Ca^{2+}, prevents the synaptic suppression seen in these studies. These data argue that synaptic repression is mediated by a short-lived retrograde signal released by muscle in response to increases in intracellular Ca^{2+} which follow AChR activation.

One candidate retrograde signaling molecule is nitric oxide (NO). NO is a free radical implicated in several forms of synaptic plasticity in the CNS (for review, see Schuman and Madison, 1994), and recent studies have shown that NO donors can suppress evoked synaptic currents in *Xenopus* nerve and myocyte cocultures (Wang et al., 1995). Further, synaptic suppression induced by asynchronous pre- and postsynaptic

depolarization is prevented by bathing the cultures in the NO-binding protein hemoglobin, or in inhibitors of NO synthase. These studies suggest that NO released from the postsynaptic muscle following nerve-evoked depolarization may induce the suppression of competing synaptic inputs. Interestingly, an isoform of NO synthase is expressed in fast skeletal muscle (Kobzik et al., 1994) and associates with the sarcolemma via interactions with α1-syntrophin, a component of the DGC (Brenman et al., 1996). Moreover, immunofluorescence studies using antibodies raised against NO synthase show that NO synthase, like dystrophin, is enriched at, although is not restricted to, the NMJ (Brenman et al., 1996). Thus, NO synthase is placed appropriately for NO to act as a retrograde signal in modulating NMJ development. The relationship between synaptic suppression and synaptic competition, however, is unclear. Since the effects of synaptic suppression on the morphological characteristics of the individual synapses were not examined in the *in vitro* synaptic suppression experiments described above, it is not known whether the induced suppression ultimately leads to synapse elimination.

Trophic factors have also been proposed as retrograde mediators of synaptic competition. In this scenario, trophic factors released from the stimulated muscle stabilize the stimulating nerve terminal by promoting its survival. Indeed, one recent study demonstrated that exogenous application of ciliary neurotrophic factor (CNTF) or basic fibroblast growth factor (bFGF) near developing neuromuscular synapses extends the period of polyneuronal innervation in rats (English and Schwartz, 1995). Although mice which lack a functional CNTF gene appear normal with respect to the number and morphology of motoneurons during the period of synapse elimination (Masu et al., 1993), studies of individual synapses were not reported. Thus, it remains unknown whether either of these trophic factors have any role in synapse elimination *in vivo* .

Multiple mechanisms regulate AChR clustering at the NMJ

The appearance of AChR clusters in the postsynaptic muscle is one of first morphological changes to occur during NMJ differentiation. As described above, the concentration of AChRs beneath the nerve terminal reaches a density of ~10,000/μm^2 in adult muscle. In contrast, the density of AChRs in the extrasynaptic sarcolemma is <10/μm^2. How AChR clusters are formed and how their density is maintained in the postsynaptic membrane has been the subject of intensive investigation, and it is now clear that at least three independent mechanisms are involved (for review, see Jennings and Burden, 1993; Hall and Sanes, 1993). First, pre-existing AChRs in the muscle membrane are redistributed to the postsynaptic membrane following contact with the motoneuron. This process is thought to be mediated by agrin, a component of the synaptic basal

lamina. Second, AChR gene transcription is induced in subsynaptic nuclei by a signal released from the motoneuron. Finally, nerve-evoked electrical activity in the muscle represses AChR expression in extrasynaptic nuclei. Each of these mechanisms is described in greater detail below.

Agrin-mediated AChR clustering

Some of the earliest insights into the regulation of AChR clustering in muscle were gained from studies of nerve and muscle cocultures *in vitro.* These studies took advantage of the fact that AChR clusters form in myotubes cultured in the absence of neurons (Fischbach and Cohen, 1973; Anderson et al., 1977) and showed that rather than innervating sites of pre-existing AChR clusters, motoneurons induce the formation of new AChR aggregates at the site of contact (Frank and Fischbach, 1979; Anderson and Cohen, 1977). Further, prelabelling of AChRs prior to nerve contact revealed that the new receptor clusters form, in part, from a redistribution of pre-existing AChRs on the muscle surface (Anderson and Cohen, 1977; Ziskind-Conhaim et al., 1984). These studies suggest that the motoneuron releases a localized factor that induces the redistribution of pre-existing AChRs to the synaptic site.

Studies in regenerating frog muscle suggested that such a clustering factor is deposited in the synaptic basal lamina (Burden et al., 1979; McMahan and Slater, 1984). In these studies, muscles were damaged and allowed to regenerate in the absence of the nerve. When muscle fibers degenerate, their cellular contents are engulfed and removed by macrophages, but the basal lamina surrounding each muscle fiber remains intact. Myoblast-like satellite cells which lie alongside the muscle fibers proliferate and subsequently differentiate to regenerate a new myofiber within the former fiber's basal lamina sheath. These studies showed that AChRs in the regenerated muscle reappear at former synaptic sites even in the absence of the nerve, and this suggests that an AChR clustering factor is locally deposited in the synaptic basal lamina.

The search for an AChR aggregating activity in synaptic basal lamina led to the purification of agrin from the electric organ of *Torpedo* (Godfrey et al., 1984; Nitkin et al., 1987). Agrin fits many of the requirements demanded of the AChR aggregating factor active at developing NMJs. First, soluble agrin induces the aggregation of AChRs in myotubes in culture by a post-translational mechanism without increasing receptor number. Moreover, when applied locally, agrin induces the clustering of AChRs at the site of application (Campanelli et al., 1991). Second, agrin immunoreactivity is concentrated in the synaptic basal lamina of adult muscle (Reist et al., 1987). Third, agrin proteins are expressed in motoneurons, and are transported anterogradely down

motor axons (Magill-Solc and McMahan, 1988; 1990). These results suggest strongly that agrin mediates AChR clustering at developing NMJs *in vivo*. Surprisingly, agrin is expressed by several other tissues, including skeletal muscle (Godfrey et al., 1988; Godfrey, 1991; Rupp et al., 1991). Moreover, in chick, agrin immunoreactivity associates with AChR clusters in aneural myotubes *in vivo* (Fallon and Gelfman, 1989), and muscle-derived agrin is found at both nerve-induced and *Torpedo* agrin-induced AChR clusters *in vitro* (Leith et al., 1992; Lieth and Fallon, 1993). These results raised questions about the specificity of agrin in clustering AChRs at the NMJ during development. Experiments in chimeric nerve-muscle cocultures, however, demonstrated that the muscle form of agrin does not participate in nerve-induced AChR clustering (Reist et al., 1992). Chick agrin-specific antibodies effectively block AChR clustering in chick nerve/rat muscle cocultures; however, these same antibodies fail to prevent AChR clustering when rat nerve and chick muscle are cocultured. These results demonstrate that the neural form of agrin has the primary role in stimulating AChR clustering.

Agrin has been cloned from several species including *Torpedo*, rat, chick, and mouse (Rupp et al., 1991, 1992; Tsim et al., 1992), and the deduced protein sequences have multiple domains with similarity to those from several classes of proteins. The amino-terminal half of the protein is comprised of nine domains with homology to protease inhibitors, plus a domain homologous to the laminin domain III located between the eighth and nine protease inhibitor domains. The carboxy-terminal half of agrin contains three regions with similarity to laminin G domains interspersed between four epidermal growth factor (EGF)-like repeats commonly found in signaling molecules. The protease inhibitor and laminin III domains of the amino-terminal half of agrin are not required for AChR clustering, since truncated proteins containing only the carboxy-terminal half of the protein are as effective as full-length agrin at clustering AChRs (Tsim et al., 1992; Ferns et al., 1993; Hoch et al, 1994; Gesemann et al., 1995). Specific functions for the protease inhibitor and laminin III domains in agrin have not been assigned, but it is worth noting that C-terminal fragments of agrin which lack these domains are more soluble than the full-length protein (Ferns et al., 1993). Thus, it seems likely that one or more of these amino-terminal domains may mediate agrin attachment to the extracellular matrix or to the presynaptic nerve terminal.

Multiple isoforms of agrin are generated by alternative splicing at three distinct sites, denoted x, y, and z, in the carboxy-terminal portion of the molecule (Rupp et al, 1991; Ruegg et al., 1992; Ferns et al., 1992). Whereas splicing at the y-site (4 a.a insert) has only a modest affect on the biological activity of agrin, splicing at the z-site has a profound affect (Ruegg et al., 1992; Tsim et al., 1992; Ferns et al., 1992, 1993). One or

both of two separate exons, encoding 8 and 11 a.a. respectively, may be included at the z-site, giving inserts of 8, 11 and 19 a.a. Agrin isoforms containing either the 8 a.a. or the 11 a.a. exon can cluster AChRs in myotubes, but those containing the 8 a.a. exon (8 or 19 a.a.) are the most active, having ~1000-fold greater activity than agrin which contains neither insert (Ferns et al., 1993). Differences in the activity of agrin isoforms, however, vary depending upon the conditions of the assay. For example, primary rat or mouse C2C12 myotubes respond to all rat agrin isoforms, whereas chick myotubes only respond to those containing the 8 or 11 a.a. exons at the z-site (Ferns et al., 1992, 1993). In these assays, the agrin is presented to the myotubes on the surface of agrin expressing COS cells. In contrast, when soluble chick agrin is used in similar assays, differences between chick and C2C12 myotubes are not observed; neither respond to agrin which lacks z-site inserts (Gesemann et al., 1995). These data suggests that the manner in which the lower activity agrin isoforms are presented to the myotubes can affect their activity. The most active agrin isoform, that which contains the 4 a.a. y-site insert and the 19 a.a z-site insert, is only expressed in neural tissue (Ruegg et al., 1992; Hoch et al., 1993). In skeletal muscle cells, only isoforms lacking inserts at the z-site are expressed. Although the experiments with interspecies nerve and muscle cocultures suggest that muscle agrin is neither required nor sufficient to initiate AChR clustering *in vitro*, the ability of these isoforms to cluster AChRs in rat and C2C12 cells in the cell-attached agrin assay suggests that they may function during synaptogenesis *in vivo*.

The biochemical mechanisms responsible for agrin-induced AChR clustering remain largely uncharacterized; however, several studies indicate a role for phosphorylation. For example, activation of protein kinase C with phorbol esters inhibits agrin-induced AChR aggregation in chick myotubes (Wallace, 1988). Chick myotubes transformed by infection with Rous sarcoma virus also fail to cluster AChRs either spontaneously or in response to electric organ extracts, and these affects are dependent on activation of the *src* tyrosine kinase (Anthony et al., 1984). These transformed myotubes were subsequently shown to lack expression of tropomyosin 2, a cytoskeletal component which is enriched at the NMJ, and microinjection of monoclonal antibodies against this protein also inhibits the formation of AChR clusters (Anthony et al., 1988; Marazzi et al., 1989). These data suggest a link between tyrosine phosphorylation, cytoskeletal proteins and AChR clustering. Other evidence suggests that phosphorylation of the AChRs themselves may be required. In both chick and C2 myotubes, agrin treatment results in rapid tyrosine phosphorylation of the AChR β-subunit, and this increase in β-subunit phosphorylation precedes agrin-induced AChR clustering (Wallace et al., 1991; Ferns et al., 1996). Moreover, both herbimycin and staurosporine, two inhibitors of tyrosine

kinases, effectively block both agrin-induced tyrosine phosphorylation of the β-subunit and agrin-induced AChR clustering (Wallace, 1994; Ferns et al., 1996). Since herbimycin and staurosporine undoubtedly inhibit the phosphorylation of numerous substrates, these studies do not address directly the role of AChR β-subunit phosphorylation in receptor clustering. Further, phosphorylation of the AChR β-subunit is not required for AChR clustering induced by co-expression of AChRs with the 43 kDa protein in COS cells (Yu and Hall, 1994). Thus, the role of AChR phosphorylation in AChR clustering remains unclear.

Proteoglycans have also been implicated in agrin-induced AChR clustering. Heparin and heparan sulfate inhibit both nerve- and agrin-induced AChR clustering in myotubes in culture (Hirano and Kidokoro, 1989; Wallace, 1990; Saito et al., 1993). Further, genetic variants of C2 muscle cells which are deficient in proteoglycan synthesis are significantly less responsive to exogenous agrin (Ferns et al., 1992, 1993). Treatment of myotubes with β-N-acetylhexosaminidase, which removes N-acetylgalactosaminyl (GalNAc)-terminated saccharides from proteins, or incubation with GalNAc-conjugated bovine serum albumin (BSA) also inhibits clustering, suggesting that agrin-induced AChR clustering involves a GalNAc-dependent step (Martin and Sanes, 1995).

Interestingly, heparan sulfate proteoglycans (HeSPs) also aggregate in response to agrin (Wallace, 1989), even when aggregation of AChRs is blocked with treatment of anti-AChR antibodies (Nitkin and Rothschild, 1990). Unlike agrin-induced AChR clustering, agrin-induced HeSP clustering requires ongoing protein synthesis, and does not occur through a redistribution of existing HeSPs (Wallace, 1989). These data suggest that agrin may direct the clustering of synaptic molecules via multiple mechanisms; however, agrin-induced HeSP clustering may also occur as a consequence of agrin-induced redistribution of pre-existing HeSP binding proteins in a manner analogous to agrin-induced redistribution of AChRs.

Recent efforts to identify the agrin receptor have suggested a role for α-dystroglycan, a component of the dystrophin glycoprotein complex which links dystrophin and utrophin to the sarcolemma. α-dystroglycan binds agrin with high affinity and is the principal agrin binding protein in both muscle and electrocyte membranes (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Moreover, α-dystroglycan is colocalized with AChRs at agrin-induced AChR clusters, and, like agrin-induced clustering activity, agrin binding to α-dystroglycan is Ca^{2+}-dependent, and can be inhibited by heparin (Wallace, 1988, 1990; Campanelli et al., 1994; Gee et al., 1994). Despite these results, studies suggest that α-dystroglycan is not a functional agrin receptor. First, attempts to use an anti-α-dystroglycan monoclonal

antibody, IIH6, to inhibit agrin-induced AChR clustering have produced conflicting results; in one study, IIH6 completely blocked agrin-induced AChR clustering in C2 myotubes in a concentration-dependent manner (Gee et al., 1994), whereas in other studies, IIH6 had little or no effect on agrin-induced AChR clusters (Campanelli et al., 1994; Sugiyama et al., 1994). Second, treatment with β-N-acetylhexosaminidase does not alter the eletrophoretic mobility of α-dystroglycan, nor do GalNAc-specific lectins bind α-dystroglycan (Martin and Sanes, 1995). In addition, a carboxy-terminal agrin fragment which retains some AChR clustering activity fails to bind α-dystroglycan *in vitro* , and pretreatment of muscle cells with inactive agrin (0_y, 0_z), which binds α-dystroglycan with high affinity, does not prevent AChR clustering induced by active agrin (4_y, 8_z) (Gesemann et al., 1996). Finally, estimates of the concentration of agrin necessary for half-maximal binding are over 100-fold greater than that necessary for half-maximal clustering activity (Nitkin et al., 1987; Nastuk et al., 1991; Ma et al., 1993), and this result suggests that agrin-induced clustering is amplified by downstream events. Since neither α-dystroglycan nor other characterized proteins of the dystrophin glycoprotein complex possess kinase domains common to receptors for signaling molecules, it is unclear how agrin binding to α-dystroglycan would lead to amplification of the agrin signal. Thus, it appears that agrin binding to α-dystroglycan is not sufficient to initiate AChR clustering; however, α-dystroglycan may enhance agrin-induced AChR clustering by providing additional agrin binding sites to increase the local concentration of agrin molecules at the postsynaptic membrane.

Recent evidence suggests that MuSK, a muscle-specific kinase, is involved in agrin signaling. MuSK is a transmembrane receptor tyrosine kinase with similarity to the *trk* family of receptors. It is expressed very early during embryonic myogenesis, and in adult rat muscle, MuSK becomes precisely colocalized with AChRs at the NMJ (Valenzuela et al., 1995). MuSK homologues are also expressed in both *Torpedo* electric organ and skeletal muscle (Jennings et al., 1993), and in muscle, MuSK expression is dramatically induced throughout the myofiber following denervation (Valenzuela et al., 1995). A critical role for MuSK in NMJ formation was revealed by the generation of MuSK-deficient mice (DeChiara et al., 1996). These mice are generally immobile, fail to breathe at birth, and subsequently die. An examination of the muscles from these MuSK-deficient mice revealed a complete absence of NMJs. Indeed, no evidence of any postsynaptic differentiation (e.g. AChR clusters, AChE deposits) could be found in these muscles, and these data suggest that MuSK expression is essential for NMJ differentiation *in vivo.* A similar, although slightly less severe phenotype is also seen in mice which lack agrin (Gautam et al., 1996), and this suggests that MuSK is a component

of the agrin signaling pathway. Consistent with this idea, primary myotubes prepared form MuSK-deficient muscle fail to cluster AChRs in response to agrin (Glass et al., 1996). Further, studies in myotubes in culture show that MuSK is specifically and rapidly tyrosine phosphorylated in response to active, but not inactive (no z-site insertions) forms of agrin (Glass et al., 1996). On the other hand, agrin binding to the extracellular domain of MuSK has not been detected, suggesting that MuSK does not directly interact with agrin. Thus, MuSK may be part of a multi-component agrin receptor analogous to receptors for bFGF (Yayon et al., 1991; Rapraeger et al., 1991), CNTF (Davis et al., 1993; Stahl and Yancopoulos, 1993), and glial-cell-line-derived neurotrophic factor (GDNF) (Jing et al., 1996; Treanor et al., 1996); in all three of these examples, binding of ligand to non-kinase components of the receptor complex is required for signal transduction. Consistent with this idea, binding of a soluble form of the MuSK extracellular domain to the surface of myotubes occurs only if agrin is included in the medium (Glass et al., 1996), suggesting that MuSK interacts with a component on the myotube surface in the presence of agrin. Interestingly, agrin-dependent MuSK binding in this assay only occurs on myotubes. Moreover, studies in which mouse C2C12 myoblasts were transfected with a chicken MuSK expression construct showed that agrin-induced phosphorylation of the chicken MuSK occurs only following myotube differentiation (Glass et al., 1996). Thus, the factors required for agrin-dependent MuSK binding and agrin-induced MuSK phosphorylation are regulated during myogenesis. The identity of such myotube-specific MuSK activation factors remains unknown, but α-dystroglycan is an unlikely candidate since it is expressed in both myoblasts and myotubes.

Two other tyrosine kinases, *fyn* and *fyk* have been shown to coimmunoprecipitate with AChRs in preparations of *Torpedo* electric organ membranes (Swope and Huganir, 1993). While neither protein has been implicated directly in AChR clustering or NMJ formation, it is tempting to speculate that one of these molecules may mediate AChR phosphorylation in response to agrin.

Local AChR synthesis and synapse-specific transcription

AChRs at mature NMJs are not stable. Throughout the life of the animal, junctional AChRs continue to turnover, yet their density at the NMJ remains high (Berg and Hall, 1975b; Chang and Huang, 1975; Salpeter and Harris, 1983). These observations suggest that local synthesis of AChRs in the synaptic region may contribute to the maintenance of high AChR density at the NMJ. Indeed, both direct measurement of AChR RNA levels from synapse-enriched and synapse-poor regions of muscle and *in situ* hybridization

studies show that the mRNAs encoding the AChR α, β, δ, and ε subunits are more abundant in synaptic than in extrasynaptic regions of the muscle fiber (Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990). Several mechanisms could account for this synaptic accumulation of AChR mRNA. For example, AChR genes could be selectively transcribed in synaptic nuclei. Alternatively, AChR genes could be transcribed in all myofiber nuclei, and the mRNA subsequently transported to, or selectively stabilized in the synaptic region. To distinguish between these alternatives, transgenic mice have been generated in which regulatory regions of either the AChR δ or ε subunit genes control the expression of reporter genes (Sanes et al., 1991; Simon et al., 1992). In both studies, the products of the reporter genes accumulated near synaptic nuclei, whereas reporter gene products were widely distributed when regulatory regions from other muscle specific genes (e.g. muscle creatine kinase (MCK)) were used to direct their expression. These studies demonstrate that selective transcription of AChR subunit genes in synaptic nuclei is responsible, at least in part, for the accumulation of AChR mRNA at synaptic sites.

Like AChR clustering, stimulation of synaptic transcription depends on one or more signals deposited in the synaptic basal lamina. This conclusion is the result of several studies on the expression of either endogenous AChR or AChR promoter-controlled reporter genes in muscles allowed to regenerated in the absence of innervation (Goldman et al., 1991; Jo and Burden, 1992; Brenner et al., 1992). In each study, AChR mRNA or reporter gene products reappeared in regions underneath former synaptic sites. Since in the latter two of these studies Schwann cells and perisynaptic fibroblast-like cells were destroyed in addition to the muscle, only signals associated with the synaptic basal lamina could account for the induction of synaptic transcription.

Although it is associated with the synaptic basal lamina, agrin probably does not mediate synapse-specific transcription, since treatment of myotubes in culture with crude agrin preparations does not alter AChR metabolism (Godfrey et al., 1984), nor does agrin induce expression of an AChR ε reporter gene in muscle cultures from transgenic mice (Chu et al., 1995). On the other hand, the absence of agrin-induced AChR transcription seen in these assays may reflect the inability of soluble agrin to stimulate AChR expression. Unfortunately, the ability of substrate- or cell-attached agrin to stimulate AChR expression has not been reported.

Several molecules which do increase AChR synthesis have been identified in neural tissues. These include ascorbic acid (Knaack et al., 1986; Horovitz et al., 1989), acetylcholine receptor-inducing activity (ARIA) (Usdin and Fischbach, 1986), CGRP (New and Mudge, 1986; Fontaine et al., 1986), and sciatin/transferrin (Markelonis et al.,

1982; Oh and Markelonis, 1982). Of these, CGRP and ARIA have received the most attention.

CGRP is a small neuropeptide expressed throughout the nervous system and in several peripheral tissues. CGRP is synthesized in motoneurons, is stored in dense-core vesicles in the nerve terminal, and is released following stimulation (Matteoli et al., 1988, 1990; Uchida et al., 1990). Incubation of chick primary myotubes in culture with CGRP results in increases in both AChR mRNA (3-fold) and protein levels (1.5-fold) (New and Mudge, 1986; Fontaine et al., 1986), however, CGRP treatment has no effect on AChR subunit mRNA levels in primary mouse myotube cultures (Martinou et al., 1991).

ARIA is a 42 kDa protein initially purified from chick brain extracts which also stimulates AChR synthesis in chick myotube cultures (Usdin and Fischbach, 1986). Subsequent cloning of ARIA cDNAs (Falls et al., 1993) revealed that ARIA is one of a family of ligands which includes Neu differentiation factor (Peles et al., 1992; Wen et al., 1992), glial growth factor (Marchionni et al., 1993) and heregulin (Holmes et al., 1992, for review, see Peles and Yarden, 1993). Since all of these ligands are derived from alternatively spliced transcripts of a single gene, the name *neuregulin* (NRG) has been adopted to describe both the gene and its products.

Neuregulins are the most promising candidates for a motoneuron-derived inducer of AChR expression. Although both CGRP and neuregulin induce AChR α-subunit synthesis in chick aneural myotubes, neuregulin induces a more robust response (10-fold vs. 3-fold for CGRP) (Harris et al., 1988), and unlike CGRP, neuregulin also induces expression of AChR subunit genes in mouse muscle cell cultures (Martinou et al., 1991; Chu et al., 1995; Jo et al. , 1995). The AChR ε-subunit exhibits the most dramatic response to ARIA, increasing 7- to 10-fold following treatment (Martinou et al., 1991; Chu et al., 1995). As stated above, expression of the ε-subunit is induced significantly by motoneuron innervation during perinatal development (Martinou and Merlie, 1991), and unlike the AChR α-, β-, γ-, and δ-subunits, which are expressed throughout the myofiber both prior to innervation and following denervation, the ε-subunit is expressed exclusively in the synaptic region (Brenner et al., 1990; Kues et al., 1995). Thus, induction of AChR ε-subunit expression is one of the properties expected of the motoneuron-derived signal which mediates synapse-specific gene expression. Consistent with the idea that neuregulin is this signaling molecule, neuregulin immunoreactivity is detected at synapses (Jo et al., 1995; Sandrock et al., 1995). Importantly, neuregulin immunoreactivity remains associated with the synaptic basal lamina following nerve and muscle degeneration (Jo et al., 1995). Moreover, RNAs encoding the erbB tyrosine kinases, the putative receptors for neuregulins, are expressed in skeletal muscle cells, and

antibodies specific for erbB2, erbB3, or erbB4 all label the postsynaptic membrane (Jo et al., 1995; Moscoso et al., 1995; Zhu et al., 1995). Together, these data strongly suggest that neuregulin is a signal which mediates synapse-specific gene expression in skeletal muscle *in vivo*.

Electrical activity-dependent regulation of gene transcription

While expression of synaptic AChRs continues following innervation, expression of extrasynaptic AChRs falls dramatically (Diamond and Miledi, 1962; Bevan and Steinbach, 1977). This developmental decline in extrasynaptic AChR density can be prevented by pharmacological blockade of synaptic transmission *in vivo* (Burden, 1977a; Braithwaite and Harris; 1979; Harris, 1981b). In addition, extrasynaptic AChRs reappear following either denervation or pharmacological paralysis of adult skeletal muscle (Axelsson and Thesleff, 1959; Hartzell and Fambrough, 1972; Berg and Hall, 1975a), and direct electrical stimulation of denervated muscle reduces AChR levels back to those observed in innervated muscle (Lømo and Rosenthal, 1972; Lømo and Westgaard, 1975). These data suggest that nerve-evoked electrical activity is primarily responsible for the regulation of extrasynaptic AChR density. Further, since the increase in extrasynaptic AChRs following denervation results from the synthesis of new AChRs rather than the redistribution of junctional AChRs (Brockes and Hall, 1975; Linden and Fambrough, 1979), these data suggest that electrical activity actively represses AChR biosynthesis.

The mechanism of electrical activity-dependent repression of extrasynaptic AChRs is transcriptional. AChR α-, β-, γ- and δ-subunit mRNA levels increase following denervation (Merlie et al., 1984; Goldman et al., 1988; Evans et al., 1987), and nuclear run on assays demonstrate that these elevated levels of AChR mRNAs are due, at least in part, to increased rates of transcription (Tsay and Schmidt, 1989). In addition, AChR subunit promoters confer electrical activity responsiveness to reporter genes in both transgenic mice (Merlie and Kornhauser, 1989; Merlie et al., 1994; Simon et al., 1992) and in transfected cells in culture (Chahine et al., 1992; Dutton et al., 1993). Studies using these AChR promoter/reporter gene fusions, have begun to identify the electrical activity-responsive regulatory sequences within the AChR promoters. For example, sequences within the 181 bp 5' flanking DNA of the mouse AChR δ-subunit gene are sufficient to confer electrical activity dependent regulation on a human growth hormone reporter gene both in transfected cells in culture (Dutton et al., 1993) and in skeletal muscle of transgenic mice (Tang et al., 1994). This latter study also showed that mutation of an E-box, a binding site for myogenic basic helix-loop-helix (bHLH) proteins, near the transcriptional start site effectively eliminates the increase in reporter

gene transcription in response to denervation. Similar results have also been reported for a mutation in an E-box in the chick AChR α_1-subunit promoter (Bessereau et al., 1994). These data suggest that E-box binding proteins may mediate electrical activity-dependent gene regulation. Consistent with this idea, members of the MyoD family of transcriptional activators, a family of E-box binding proteins that are involved in the induction of many muscle-specific genes during myogenesis, are regulated by electrical activity in parallel with AChR expression (Duclert et al., 1991; Eftimie et al., 1991; Witzemann and Sakmann, 1991; Neville et al., 1992; Weis, 1994). E-boxes alone, however, are not sufficient to confer electrical activity-dependent responsiveness, since other muscle specific genes which contain E-boxes in their promoter regions, such as MCK, are not regulated by electrical activity (Merlie and Kornhauser, 1989; Asher et al., 1992; Dutton et al., 1993). One explanation for the failure of E-box containing genes such as MCK to respond electrical activity may be the presence of other, dominant promoter elements which render these genes insensitive to electrical activity-induce changes in bHLH protein activity. Alternatively, additional transcriptional regulators acting via as yet uncharacterized electrical activity-responsive promoter sequences may be required, in conjunction with E-boxes, to regulate electrical activity-responsive gene expression.

In an effort to identify potential transcriptional regulators of electrical activity-dependent gene expression, as well as genes which may mediate other physiological responses of muscle to innervation, Xuejun Zhu, a graduate student in the lab, developed a PCR-based subtractive-hybridization and cloning technique to identify mRNAs that are differentially-expressed in innervated and denervated rat skeletal muscle. Using this technique, Xuejun and I identified three novel genes whose expression is differentially regulated in innervated and denervated muscle. Two of these genes, *DEN1* and *DEN2* are up-regulated following denervation. The third gene, *INN1*, is expressed at roughly equal levels in innervated and denervated muscle; alternative splicing of the *INN1* transcripts, however, is altered following denervation such that the levels of two transcripts increase while the level of a third decreases. The initial characterization of these three genes is described in Chapter 4 of this thesis. Additionally, our screen revealed that the rat homolog of the acute myeloid leukemia 1 (*AML1*) gene is significantly induced in denervated muscle. Since *AML1* encodes a potential transcription factor, we also investigated more thoroughly its expression in skeletal muscle. These results are reported in Chapter 5, and they suggest that *AML1* may act as a regulator of electrical activity-dependent gene expression in muscle.

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Chapter 2.

Membrane Targeting of the 43 kDa Protein (Rapsyn) in Muscle and Non-muscle Cells

SUMMARY

The 43 kDa protein is a peripheral membrane protein that is expressed in skeletal muscle, where it is associated with acetylcholine receptors (AChRs) in the postsynaptic membrane. It has been suggested that the 43 kDa protein may be involved in clustering AChRs in the postsynaptic membrane and in crosslinking AChRs with the underlying cytoskeleton. We developed a primary muscle culture system to identify sequences in the 43 kDa protein that are required for its association with the cell surface and with AChR clusters in myotubes. A plasmid directing expression of the mouse 43 kDa protein, tagged with an epitope from influenza hemagglutinin (HA), was transfected into myoblasts, and the distribution of the HA-tagged 43 kDa (43 kDa-HA) protein was analyzed in both myoblasts and myotubes. We show here that the 43kDa-HA protein is targeted to the myotube cell surface and is associated with AChR clusters. In addition we show that both wild-type 43 kDa protein and 43 kDa-HA protein form clusters in the quail fibroblast-like QT6 cell line. In contrast, 43kDa-HA protein expressed in myoblasts and NIH3T3 fibroblasts is clustered only rarely on the cell surface and is usually found intracellularly. These results indicate that the 43 kDa protein is targeted efficiently to the cell surface in myotubes and QT6 cells, but not in myoblasts or fibroblasts.

We studied the distribution of mutant 43kDa-HA proteins in primary myotubes. We found that mutant 43kDa-HA proteins containing only amino-terminal portions of the 43 kDa protein form clusters in the periphery of the myotube, some possibly on the myotube surface, but these clusters are not associated with AChR clusters. Mutation of an amino-terminal myristoylation addition site blocks association of the 43 kDa protein with AChR clusters and causes the 43 kDa protein to accumulate in nuclei.

Our results demonstrate that cell types differ in their ability to direct the 43 kDa protein to the cell surface, and that N-terminal myristoylation of the 43 kDa protein is required, but is not sufficient, for cell surface targeting and association with AChR clusters in myotubes.

INTRODUCTION

Accumulation of AChRs in the postsynaptic membrane is an early and essential event in neuromuscular synapse formation. The mechanisms that regulate postsynaptic differentiation have been studied extensively in cell culture, and evidence suggests a role for both extracellular matrix and cytoskeletal proteins in AChR clustering (for reviews see

Phillips and Merlie, 1992; Froehner, 1993; Hall and Sanes, 1993; Jennings and Burden, 1993). In studies designed to analyze the mechanism of AChR clustering, considerable attention has been given to an intracellular, peripheral membrane protein, the 43 kDa protein (rapsyn). The 43 kDa protein is expressed in 1:1 stoichiometry with AChRs (Burden et al., 1983; LaRochelle and Froehner, 1987), and is codistributed precisely with AChRs at synaptic sites in both *Torpedo* electric organ and skeletal muscle (Froehner et al., 1981; Sealock et al., 1984; Burden, 1985). The 43 kDa protein becomes concentrated at synapses early during synapse formation (Burden, 1985; Noakes et al., 1993), and it is clustered together with AChRs in muscle cells grown in cell culture in the absence of innervation (Burden, 1985; Peng and Froehner, 1985; Bloch and Froehner, 1987; Daniels, 1990).

Direct biochemical evidence demonstrating interaction between the 43 kDa protein and AChRs is lacking; however, numerous studies suggest that 43 kDa protein/AChR interactions play an important role in the formation and/or maintenance of AChR clusters. Electron microscopy and X-ray diffraction studies suggest that the 43 kDa protein and AChRs lie in close proximity (Mitra et al., 1989), and cross-linking studies have shown that the 43 kDa protein is closely associated with the AChR β subunit (Burden et al., 1983). In addition, treatments which remove the 43 kDa protein and other peripheral membrane proteins from preparations of AChR-enriched membranes disrupt the organization of AChRs (Barrantes et al., 1980; Cartaud et al., 1981; Bloch and Froehner, 1987) and increase the rotational and lateral mobility of AChRs within the membrane (Lo et al., 1982; Rousselet et al., 1982). More recently, studies have shown that 43 kDa protein-mutant mice fail to cluster AChRs at their NMJs, and muscle cell cultures derived from these mutant mice fail to cluster AChRs in response to the nerve-derived AChR clustering factor, agrin (Gautam et al., 1995). These results show that the 43 kDa protein is required for normal synaptogenesis and AChR clustering in vertebrate skeletal muscle.

Efforts to reconstitute the AChR clustering pathway in non-muscle cells show that 43 kDa protein can promote AChR clustering. Expression of AChRs in *Xenopus* oocytes (Froehner et al., 1990), quail QT6 cells (Phillips et al., 1991) and COS cells (Brennan et al., 1992) leads to AChRs that are distributed diffusely on the cell surface; however, co-expression of AChRs and the 43 kDa protein leads to co-clustering of AChRs and the 43 kDa protein (Froehner et al., 1990; Phillips et al., 1990; Phillips et al., 1991; Brennan, et al., 1992). Importantly, even in the absence of AChR expression, the 43 kDa protein is clustered in these cells (Froehner et al., 1990; Phillips et al., 1990). These results suggest that the 43 kDa protein can self-organize, and that AChR clusters induced by coexpression of 43 kDa protein form via association with the 43 kDa protein. More recently, similar

studies have suggested that 43 kDa protein may have a broader role in NMJ development than merely organizing AChR domains. These studies showed that in addition to clustering AChRs, 43 kDa protein also induces clustering of α-dystroglycan (Apel et al., 1995) and MuSK, an agrin and laminin binding protein (Gillespie et al., 1996), when these proteins are coexpressed with 43 kDa protein in QT6 cells. Moreover, clustering of MuSK by 43 kDa protein activates the MuSK kinase domain, and co-clustering of 43 kDa protein, MuSK, and AChRs induces the tyrosine phosphorylation of the AChR β-subunit (Gillespie et al., 1996), the same AChR subunit that is phosphorylated during agrin-induced AChR clustering in myotubes (Wallace, 1991; Ferns et al., 1996). These results suggest that the 43 kDa protein may cluster other subsynaptic proteins at the NMJ and may play a role in the MuSK signalling pathway.

Myoblasts and myotubes express similar levels of the 43 kDa protein (Frail et al., 1989); the 43 kDa protein, however, is clustered only in myotubes. These results indicate that expression of the 43 kDa protein is not sufficient for clustering in myoblasts and suggest that clustering of the 43 kDa protein is regulated during myogenesis. Moreover, these results also raise the possibility that the mechanisms that direct clustering of the 43 kDa protein in muscle cells may differ from the mechanisms that mediate clustering of the transfected 43 kDa protein in QT6 cells, COS cells and *Xenopus* oocytes. In order to study the mechanisms that regulate clustering of the 43 kDa protein during myogenesis Emma Dutton, a post-doctoral fellow in the lab, and I transfected mammalian myoblasts and fibroblasts with expression vectors encoding the 43 kDa protein tagged with an epitope from influenza hemagglutinin (HA) and determined the distribution of the transfected protein in myoblasts, fibroblasts and myotubes. In this chapter, we demonstrate that clusters of the transfected 43 kDa protein are found frequently on the cell surface of myotubes but not on the cell surface of the other cell types. These results indicate that the cellular distribution of the endogenous and transfected 43 kDa proteins are regulated similarly during myogenesis.

The primary sequences of 43 kDa protein from *Torpedo* electric organ (Carr et al., 1987; Frail et al., 1987), mouse (Frail et al., 1988) and *Xenopus* skeletal muscle (Baldwin et al., 1988) share 70-80% amino acid identity throughout their length, and possess several conserved regions which may be important for AChR clustering (Froehner, 1991). These include a highly conserved myristoylation addition site at the amino-terminus, a sequence of four leucine heptad repeats characteristic of leucine-zipper containing proteins, and a cysteine-rich sequence near the carboxy-terminus which has homology to the regulatory region of protein kinase C and which may fold into a zinc-finger-like structure. The carboxy-terminus also contains potential sites for phosphorylation by cAMP-dependent

protein kinase or protein kinase C (PKC). In order to identify which domains in the 43 kDa protein are necessary for targeting the protein to the cell surface of myotubes and for mediating the association of the 43 kDa protein with endogenous AChR clusters, we transfected myoblasts with expression plasmids encoding mutant 43 kDa proteins and analyzed the distribution of the expressed 43 kDa proteins in differentiated myotubes. Our results show that N-terminal myristoylation is required for targeting the 43 kDa protein to the myotube surface. We also show that sequences within the amino-terminal 227 amino acids of the 43 kDa protein are not sufficient for mediating the association of the protein with AChR clusters in myotubes, and that phosphorylation of serine residues near the carboxy-terminus of 43 kDa protein is not required for association of the protein with AChR clusters in the myotube membrane.

MATERIALS AND METHODS

Plasmid construction

A 1.5 kb cDNA encoding the full-length murine 43 kDa protein (Frail et al., 1988) was digested with BglI or NcoI to create deletion constructs encoding the N-terminal 113 or 227 amino acids of the 43 kDa protein, respectively. Oligonucleotides with appropriate overhanging ends were used to introduce the influenza hemagglutinin (HA) epitope YPYDVPDYA (Wilson et al., 1984) at the C-terminus of the truncated 43 kDa proteins. An aspartic acid was included in the C-terminus of the epitope tag since charged amino acids at the C-terminus have been shown to stabilize recombinant protein in prokaryotes (Parsell et al., 1990). In the HA-tagged, wild-type 43 kDa (43kDa-HA) protein construct, the wild-type stop codon was removed and a unique BamHI site was introduced by PCR to permit introduction of the HA tag; this modification resulted in the addition of two amino acids, glycine and serine, between the normal 43 kDa protein C-terminus and the HA tag. The 5'-flanking sequence upstream of the initiator methionine was removed by PCR. Amino acid substitutions in the 43 kDa protein were introduced by site-directed mutagenesis (Taylor et al., 1985). All 43 kDa-HA constructs were subcloned into the pJ4 expression vector (Morgenstern and Land, 1990) and sequenced to confirm the intended mutations. Wild-type and mutant constructs are shown in schematic form in Figure 2.1.

Tissue culture, transfection and microscopy

Primary rat myoblast and myotube cultures were prepared essentially as described (Dutton and Olek, 1990). QT6 quail fibroblasts (American Type Culture Collection, CRL-

1708), NIH3T3 fibroblasts and C2C12 myoblasts were cultured as described previously (Moscovici et al., 1977; Simon and Burden, 1993). Cells were plated in 35 mm tissue culture dishes, transiently transfected using calcium phosphate precipitation (Maniatis et al., 1982) at approximately 50% confluence and glycerol shocked 6-24 hours later. One to two days following transfection, the cultures were rinsed with Dulbecco's phosphate buffered saline (DPBS) and fixed with 2% paraformaldehyde in DPBS. After rinsing with DPBS, the cells were permeablized with 0.1% Triton X-100 (in DPBS) for 7 minutes, rinsed once with 0.1 M glycine, and incubated with 10% bovine serum albumin or 10% normal goat serum (in DPBS) for 30 minutes. The expressed 43 kDa-HA protein was detected using a monoclonal antibody (12CA5; Kolodziej and Young, 1991) against the HA tag and a fluorescein-conjugated secondary antibody. For some experiments, untagged 43 kDa protein was detected using a polyclonal rabbit antiserum against a glutathione-S-transferase/mouse 43 kDa fusion protein (Dyer and Burden, unpublished results), followed by a fluorescein-conjugated secondary antibody. Expression of the 43 kDa-HA protein was analyzed in myotubes that had differentiated from transfected myoblasts. In these experiments, the AChRs were labeled with tetramethylrhodamine-α-bungarotoxin (TMR-α-BGT) (50 nM) prior to fixation as described previously (Dutton and Olek, 1990). Cells were viewed with a Zeiss microscope equipped with filters selective for either rhodamine or fluorescein. Dorsal and ventral (substrate attached) surfaces of myotubes were distinguished by focusing through the myofiber (Olek et al., 1983; Bursztajn et al., 1984; Wallace, 1988; Dutton and Olek, 1990).

RESULTS

To determine whether the HA epitope tag interferes with targeting of 43 kDa protein to the cell surface, we transiently transfected quail QT6 fibroblasts and analyzed the distribution of wild-type and epitope-tagged (43 kDa-HA) proteins by immunofluorescence. The full-length, wild-type 43 kDa protein is targeted to the cell surface and forms clusters in QT6 cells (Phillips et al., 1991), and we found that the distribution of the wild-type and 43 kDa-HA protein was similar in QT6 cells (Fig. 2.2A, B). Cell surface clusters of the 43 kDa protein are detected in approximately 70% of cells expressing the transfected wild-type or the 43 kDa-HA protein. Smaller aggregates of the 43 kDa protein are observed in approximately 90% (wild-type) and 80% (epitope-tagged) of transiently expressing cells (Fig. 2.2B). It is our impression that most of these aggregates are cytoplasmic. Diffuse staining is also detected occasionally throughout the

cytoplasm (21% and 11% in wild-type 43 kDa and 43 kDa-HA expressing cells, respectively) and within the nucleus (54% and 82%, respectively) (Fig. 2.2A, B). Since both wild-type and 43kDa-HA protein have similar patterns of expression, we conclude that the HA epitope tag does not interfere with normal protein folding or cellular localization of the 43 kDa protein.

43 kDa protein is targeted to the cell surface and is colocalized with AChR clusters in primary rat myotubes

The 43 kDa protein is colocalized with AChRs in AChR-rich membrane domains in *Xenopus* myocytes (Peng and Froehner, 1985) and rat primary myotubes (Bloch and Froehner, 1987) grown in cell culture. To determine whether the 43 kDa-HA protein is targeted to the cell surface and is associated with endogenous AChR clusters in primary rat myotubes, we transfected primary rat myoblasts with the 43 kDa-HA expression plasmid, allowed the myoblasts to fuse to form myotubes, and examined the distribution of the HA-tagged protein. Our results show that the 43 kDa protein is colocalized with endogenous AChR clusters on the ventral (substrate attached) surface of the myotube (Fig. 2.3A, B). In addition, the 43 kDa protein is associated with small (1-10 μm) AChR clusters on the dorsal surface of myotubes (Fig. 2.3C, D). Because AChR clusters usually are not found on the dorsal surface of primary rat myotubes (Olek et al., 1983; Dutton and Olek, 1990), our results raise the possiblity that the transfected 43 kDa protein induces the formation of AChR clusters on the dorsal surface. Although it has been reported that overexpression of 43 kDa protein in stably transfected myotubes can disrupt AChR clustering (Yoshihara and Hall, 1993), AChR clusters on the ventral surface appear similar in both transfected and untransfected myotubes. This probably reflects lower levels of 43 kDa protein expression in transiently transfected than in stably transfected myotubes.

43 kDa protein forms cell surface clusters in QT6 cells, but not in primary rat myoblasts or fibroblasts, C2 myoblasts or NIH3T3 fibroblasts

In contrast to the cell surface clustering of 43 kDa protein observed in myotubes, the 43 kDa protein in mononucleated cells is usually found in small (1-5 μm) cytoplasmic aggregates which are concentrated in the perinuclear region of transfected cells (Fig. 2.2C, 4A). Surface clusters of the 43 kDa protein are found in only a minority (6%) of the cells that expressed the transfected protein. The distribution of the 43 kDa-HA protein in primary rat myoblasts and fibroblasts is clearly different from that observed in QT6 cells, where a majority (70%) of the transfected cells have surface clusters of the 43 kDa protein (Fig. 2.2). To determine whether the absence of surface clusters of the 43 kDa protein in

myoblasts and fibroblasts was unique to primary cells, we transfected C2C12 myoblasts and NIH3T3 fibroblasts. We found that the distribution of the 43 kDa protein in these cell lines is similar to that observed in primary rat myoblasts and fibroblasts. Thus, cell surface clusters of 43 kDa protein are detected readily in QT6 cells and primary myotubes, but not in primary rat myoblasts and fibroblasts, C2 myoblasts or 3T3 fibroblasts. These results indicate that the 43 kDa protein does not cluster efficiently at the surface in all cell types.

Targeting of mutant 43 kDa-HA proteins in myotubes

Recently, studies in QT6 cells (Phillips et al., 1991) and *Xenopus* oocytes (Scotland et al., 1993) using co-expression of mutant 43 kDa protein and AChR have identified regions of the 43 kDa protein which are necessary for clustering of the 43 kDa protein on the cell surface and for mediating 43 kDa protein interactions with AChR. These regions include an amino-terminal myristoylation consensus sequence, which is required for targeting the 43 kDa protein to the cell surface of QT6 cells (Phillips et al., 1991), and a zinc-finger motif (a.a. 363-403) which has a role in determining the size of 43 kDa protein clusters in *Xenopus* oocytes, but is not required for association of the protein with AChR (Scotland et al., 1993). These studies also showed that a leucine zipper domain (a.a. 82-110) is not required for clustering of the 43 kDa protein in QT6 cells (Phillips et al., 1991), and that a putative serine phosphorylation site (Ser_{406}) (Carr et al., 1987; Froehner, 1991) is not required for either clustering the 43 kDa protein at the cell surface or for association with AChRs in *Xenopus* oocytes (Scotland et al., 1993). Our results with the wild-type 43 kDa protein raise the possibility that the mechanisms used to cluster the 43 kDa protein in these non-muscle cells might differ from those in myotubes. Therefore, we determined whether the domains that are necessary for targeting the 43 kDa protein to the cell surface of non-muscle cells are also necessary for targeting the 43 kDa protein to the cell surface of myotubes.

To determine whether myristoylation is required for targeting the 43 kDa protein to the plasma membrane in myotubes, we mutated the myristoylation addition site (Gly_2 to Glu_2 substitution) and studied expression of the mutant protein in both primary rat myoblasts and myotubes. The mutated 43 kDa protein is not found associated with surface AChR clusters in myotubes; rather, most staining is confined to the nucleus (Fig. 2.4). In myoblasts also the mutated protein is detected primarily in the nucleus. Diffuse cytoplasmic staining also is detected in some cells (42%). Similar results have been reported for the distribution of a myristoylation site mutant in QT6 cells (Phillips et al., 1991). These results indicate that myristoylation is important for targeting 43 kDa protein to the cell surface in both QT6 cells and myotubes.

Deletion mutants were constructed which expressed either the amino-terminal 113 or 227 amino acids of the 43 kDa protein. These mutants, which lack both the zinc finger domain and the putative phosphorylation site, were transfected into primary rat myoblasts, and the distribution of the mutant proteins in myotubes was determined by immunofluorescence. When expressed in QT6 cells or myoblasts, the mutant 43 kDa proteins forms small aggregates (1-5 μm) distributed throughout the cell (Fig. 2.5A, B). In myotubes, the mutant 43 kDa proteins forms clusters (1-10 μm) in the periphery of the myotube, but these clusters are not colocalized with AChR clusters (Fig. 2.5C, D). Due to the abundance and small size of the clusters, however, we can not determine conclusively how many, if any, of these clusters are associated with the cell surface, but the distribution of these truncated 43 kDa protein clusters is clearly different from the distribution of wild-type 43 kDa protein in transfected myotubes (compare Fig. 2.3 with 2.5C, D) These results suggest that the amino-terminal portion of the 43 kDa protein is not sufficient to direct the association of the 43 kDa protein with AChR clusters in muscle.

The 43 kDa protein from *Torpedo* electric organ contains phosphoserine, and peptide mapping indicates that the phosphorylated serine(s) are contained in a single tryptic peptide (Hill et al., 1991). Ser_{406} is within consensus sequences for phosphorylation by either cAMP-dependent protein kinase or protein kinase C. In order to determine whether Ser_{406} has a role in targeting the 43 kDa protein to the cell surface and to AChR clusters in myotubes, we mutated Ser_{406} to Ala_{406}, and expressed the mutated protein in rat primary myoblasts and myotubes. The distribution of the mutant 43 kDa protein in myotubes is similar to that observed for the wild-type 43 kDa protein. Since amino acid 405 is also serine, we were concerned that Ser_{405} might be phosphorylated in the Ala_{406} mutant construct. The distribution of 43 kDa protein with an Ala_{405}/Ala_{406} double mutation, however, is similar to that observed for the wild-type protein in myotubes (Fig. 2.6). These results demonstrate that phosphorylation of Ser_{406} and Ser_{405} is not required for targeting the 43 kDa protein to the cell surface or for interaction of the 43 kDa protein with AChR clusters in myotubes. Although 43 kDa protein clusters are detected more frequently in C2 myoblasts expressing the 43 kDa protein containing a Ser to Ala mutation at Ser_{405} and/or Ser_{406} (14% of cells expressing Ala_{405}/Ala_{406}, 6% of cells expressing Ala_{405} and 8% of cells expressing Ala_{406}) than in myoblasts expressing the wild-type (2% of expressing cells), these results suggest that phosphorylation of 43 kDa protein at Ser_{406} cannot account alone for the absence of 43 kDa protein clusters at the cell surface of myoblasts.

DISCUSSION

We show here that cell types differ in their ability to cluster the 43 kDa protein at the cell surface. While cell surface clusters of the 43 kDa protein are found frequently in transiently transfected QT6 cells and rat primary myotubes, such clusters are found rarely at the surface of transfected C2 myoblasts, primary myoblasts and fibroblasts and 3T3 fibroblasts. Our results do not support results reported by Phillips and colleagues in C2 myoblasts (Phillips et al., 1993). In C2 myoblasts transfected with expression vectors encoding AChR subunits and 43 kDa protein, they reported that 91% of cells expressing both AChR and 43 kDa protein displayed surface domains in which both proteins were colocalized. In contrast, in our experiments, surface domains of 43 kDa-HA protein were observed in only ~2% of transfected C2 myoblasts expressing 43 kDa-HA protein. Moreover, we observed few surface clusters of 43 kDa-HA protein in transfected rat primary myoblasts and fibroblasts and in NIH3T3 fibroblasts, although these frequencies were not rigorously quantitated. Perhaps the coexpression of AChRs with 43 kDa protein in transfected C2 myoblasts might stabilize 43 kDa protein clusters in the membrane and prevent their aggregation in an intracellular compartment. Unfortunately, Phillips et al. do not report the localization of 43 kDa protein in C2 myoblasts transfected with 43 kDa protein expression vector alone.

Previous studies have demonstrated that the 43 kDa protein can cluster in the plasma membrane of QT6 cells (Phillips et al., 1991), COS cells (Brennan et al., 1992) and *Xenopus* oocytes (Froehner et al., 1990; Scotland et al., 1993). Moreover, the 43 kDa protein can cause clustering of AChRs in these cells. We show here that 43 kDa-HA protein is colocalized with AChR clusters on the ventral (substrate attached) surface of transiently transfected myotubes. Because the 43 kDa protein is associated with small AChR clusters on the dorsal surface of transfected myotubes, where AChR clusters rarely are found (Dutton and Olek, 1990; Olek et al., 1983), we favor that the 43 kDa protein can induce the formation of AChR clusters in myotubes.

The primary sequence of the 43 kDa protein contains several conserved structural features, including sites for N-terminal myristoylation and serine phosphorylation, a zinc finger domain, and a leucine zipper motif (see Froehner, 1991). Using heterologous cell culture systems, others have begun to define regions of the 43 kDa protein that are necessary to promote membrane targeting and AChR clustering (Phillips et al., 1991; Scotland et al., 1993). Their results demonstrate that N-terminal myristoylation is required for targeting of the 43 kDa protein to the plasma membrane (Phillips et al., 1991), and that mutation of the zinc finger region did not prevent association of the recombinant 43 kDa protein with AChRs, but did reduce the size of 43 kDa protein induced clusters in *Xenopus*

oocytes (Scotland et al., 1993), indicating a role for the zinc finger domain in 43 kDa protein clustering. Neither deletion of the leucine zipper (Phillips et al., 1991) nor mutation of the potential phosphorylation site (Scotland et al., 1993) prevented clustering of the 43 kDa protein in the plasma membrane, but deletion of the leucine zipper did reduce the ability of the 43 kDa protein to associate with AChRs. Since it is possible that the mechanisms which regulate 43 kDa protein clustering and 43 kDa protein/AChR association in these heterologous cell systems may differ from those in muscle, we have studied the localization of HA-tagged mutated 43 kDa protein directly in muscle cells in culture. Similar to those reported by others using heterologous cell systems, our results show that N-terminal myristoylation is required to target 43 kDa protein to the cell membrane in myotubes and that mutation of the potential serine phosphorylation site did not prevent association of the recombinant 43 kDa protein with endogenous AChR clusters. Previously, Phillips et al. demonstrated that 43 kDa protein containing only the N-terminal 254 amino acids was not targeted to the membrane and was not able to associate with AChRs in QT6 cells (Phillips et al., 1991). Consistent with these results, we found that HA-tagged recombinant 43 kDa protein containing only the N-terminal 113 or 227 amino acids failed to cluster in the membrane of QT6 cells, and did not associate with endogenous AChR clusters in myotubes. In summary, our results are consistent with those reported in heterologous cell culture systems, and suggest that mechanisms regulating 43 kDa protein induced AChR clustering in these systems reflect those in myotubes.

The endogenous 43 kDa protein is expressed at similar levels in myoblasts and myotubes but is only clustered in myotubes (Frail et al., 1989). The mechanisms that regulate clustering of the 43 kDa protein during muscle differentiation are not known. It is possible that negative factors, which are present in myoblasts but not in myotubes, prevent the 43 kDa protein from clustering in myoblasts. Alternatively, it is possible that positive factors in myotubes are required for clustering the 43 kDa protein. Because the 43 kDa protein can cluster in QT6 cells, these cells, like myotubes, would appear to either lack negative factors or contain positive factors for clustering the 43 kDa protein. Interestingly, expression of 43 kDa protein alone in QT6 cells induces the tyrosine phosphorylation of a large number of proteins (Gillespie et al., 1996), and this suggests that endogenously expressed molecules in the transfected QT6 cells interact with the 43 kDa protein and may be important for its clustering in these cells. Clusters of 43 kDa and AChRs fail to form spontaneously in a C2 muscle cell variant that is defective in glycosaminoglycan synthesis (Gordon et al., 1993). Moreover, these cells are also less responsive to agrin (Ferns et al., 1992,1993), and these studies suggest a role for glycosaminoglycans in AChR clustering. In addition, utrophin is present at the earliest stages of AChR clustering at the developing

NMJ and at AChR-rich domains in C2 myotubes (Phillips et al., 1993), and more recently, 43 kDa protein was found to induce clustering of α-dystroglycan (Apel et al., 1995). These studies suggest that utrophin and the dystrophin glycoprotein complex (DGC) may be important for the formation of 43 kDa protein/AChR clusters and that 43 kDa protein may link AChRs to the cytoskeleton via interactions with the DGC. At the present time, it is not known whether glycosaminoglycans play a role in 43 kDa protein/AChR clustering in QT6 cells, nor whether utrophin and members of the DGC are expressed in QT6 cells. Similarly, differences in glycosaminoglycan synthesis and differences in the expression of many of the DGC components in myoblasts and myotubes have not been thoroughly investigated. The differential expression of any of these molecules in myotubes and QT6 cells but not in myoblasts and 3T3 fibroblasts would be consistent with the idea that they act as positive factors which support clustering of the 43 kDa protein.

Since QT6 cell are derived from a tumor that was chemically induced by intramuscular injection (Moscovici et al., 1977), it is possible that QT6 cells are derived from mononucleated satellite cells which are present in adult muscle. In support of this idea, subclones of QT6 cells have been isolated which can fuse to form myotube-like syncitia and which express several muscle specific proteins (Antin and Ordahl, 1991). Moreover, these QT6-derived cells also express low levels of the AChR α-subunit and agrin when grown in low-serum containing medium (J. Fallon, personal communication). A myogenic origin for QT6 cells might account for their ability to cluster the 43 kDa protein with an efficiency comparable to that of myotubes.

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Figure 2.1. Summary of 43 kDa-HA fusion proteins. Schematic representations of the wild-type 43 kDa protein and HA-tagged wild type and mutant 43 kDa proteins are shown. The amino terminus of each protein is on the left. The positions of the N-myristoylation addition site (Gly_2) and the potential phosphorylation site (Ser_{406}) are indicated by arrows. The HA epitope tag is represented by a solid black box. Gray-shaded and hatched boxes indicate the locations of the leucine zipper (L-zip) and zinc finger (Zn) domains, respectively. Solid vertical bars indicate the positions of amino acid point mutations. Amino acids (parentheses) and point mutations contained within the mutant 43 kDa-HA fusion proteins (boxes) are given.

43 kDa-HA FUSION PROTEINS

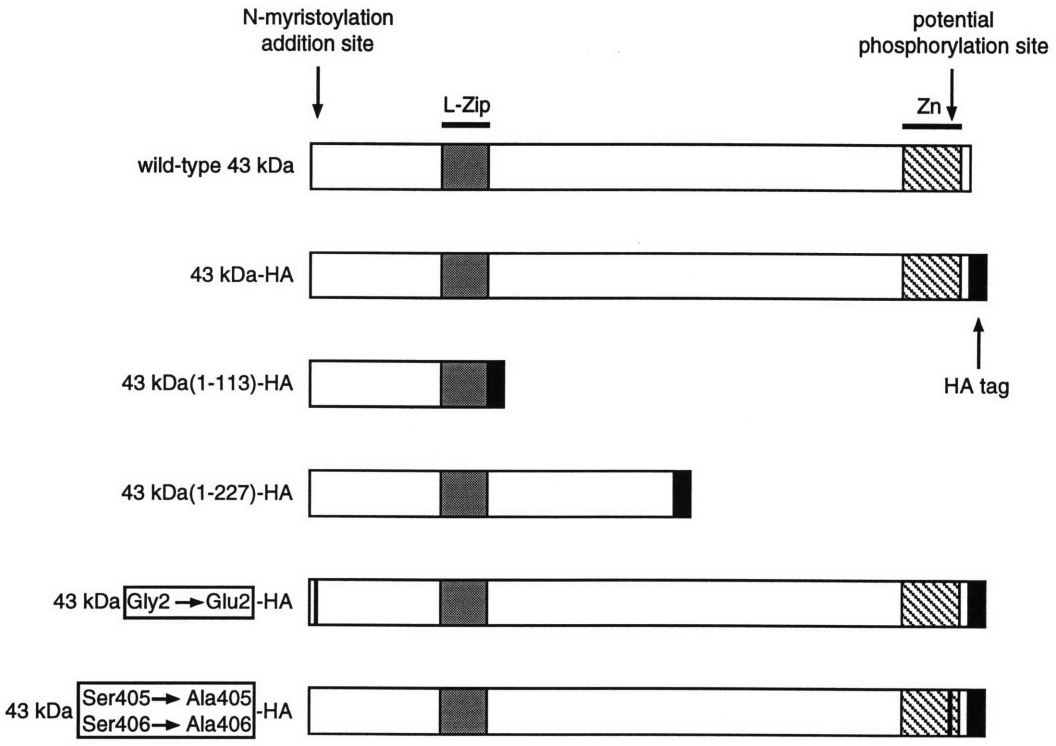
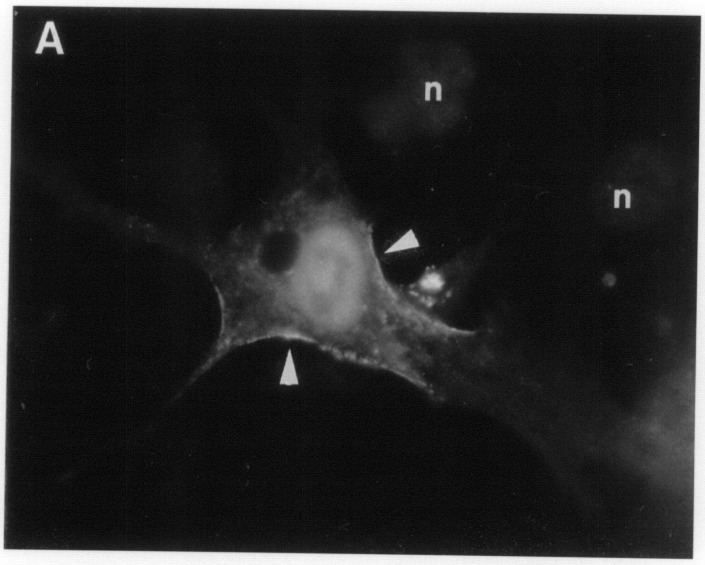


Figure 2.2. Wild-type and 43 kDa-HA protein clusters on the cell surface of transiently transfected QT6 cells but not in myoblasts. Transiently transfected QT6 cells (A,B) and C2 myoblasts (C) expressing the 43 kDa protein (A) or HA-epitope tagged 43 kDa protein (B,C) were fixed, permeablized and stained either with affinity purified rabbit anti-43 kDa protein antibodies (A) or the 12CA5 anti-HA monoclonal antibody (B,C) followed by appropriate FITC-conjugated secondary antibodies. In QT6 cells, immunoreactivity is frequently detected on the edge of cells transfected with either 43 kDa protein or 43 kDa-HA protein (arrowheads in A and B). We interpret such staining on the edge of cells as evidence for cell surface 43 kDa protein clusters. Smaller aggregates are present in most stained cells (arrows in B) and in some transfected cells which lack staining at the cell surface (not shown). Nuclei in most transfected cells also stain weakly. (Compare nucleus of transfected cell in A with nuclei of surrounding untransfected cells (n)). In myoblasts, aggregates of the 43 kDa-HA protein are concentrated in the perinuclear region of transfected cells. (see also Fig 2.4A). Aggregates of the 43 kDa-HA protein are seen rarely at the cell surface (see text). Calibration bar (A, B; C)= 10 μm.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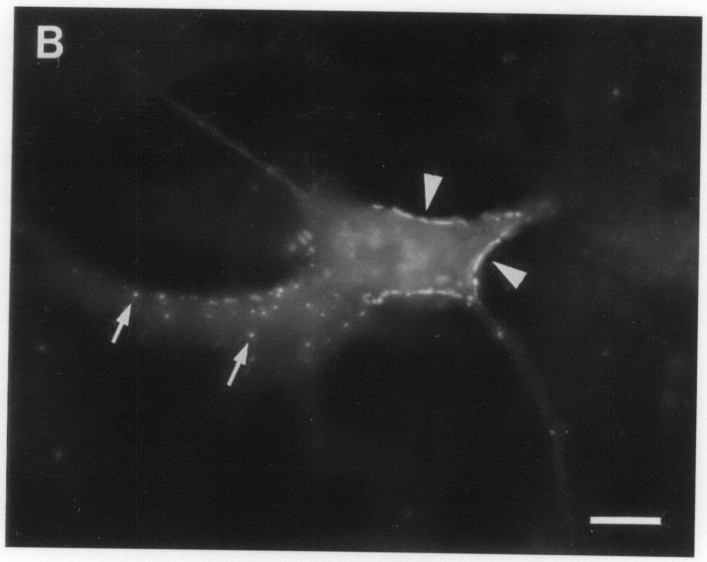
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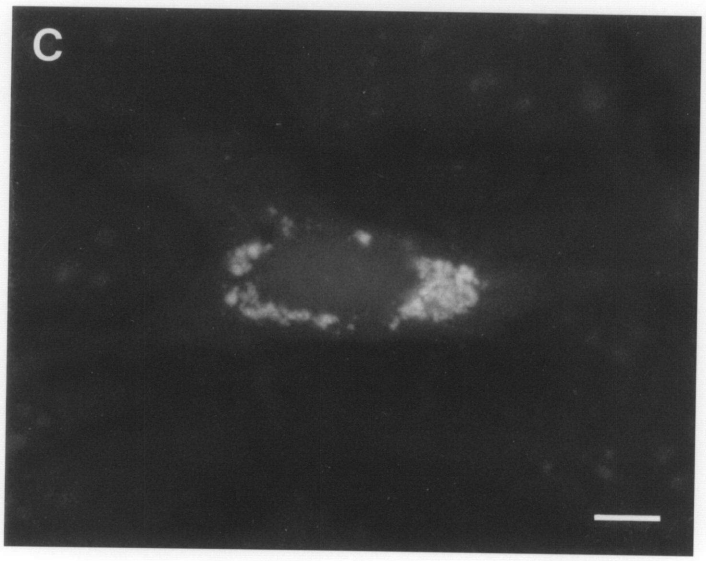
C

Figure 2.3. 43 kDa-HA protein is localized to AChR clusters in transiently transfected rat myotubes.

Transiently transfected primary rat myoblasts expressing 43 kDa-HA protein were allowed to differentiate into myotubes. Prior to fixation, dishes were incubated with TMR-α-BGT to label AChRs. Cells were fixed and stained with anti-HA monoclonal antibody (12CA5) as described (Materials and Methods). 43 kDa-HA protein (A) is coincident with AChR clusters (B) on the ventral (substrate attached) surface of the myotubes. Coincident aggregates of 43 kDa-HA protein (C) and AChR (D) also are found on the dorsal surface of the myotube. The appearance of AChR clusters (e.g. number and size) is similar in both transfected and non-transfected myotubes. Diffuse immunoreactivity is occasionally detected in the nucleus of transfected myotubes (out of focus in C). Calibration bar = 10 μm.

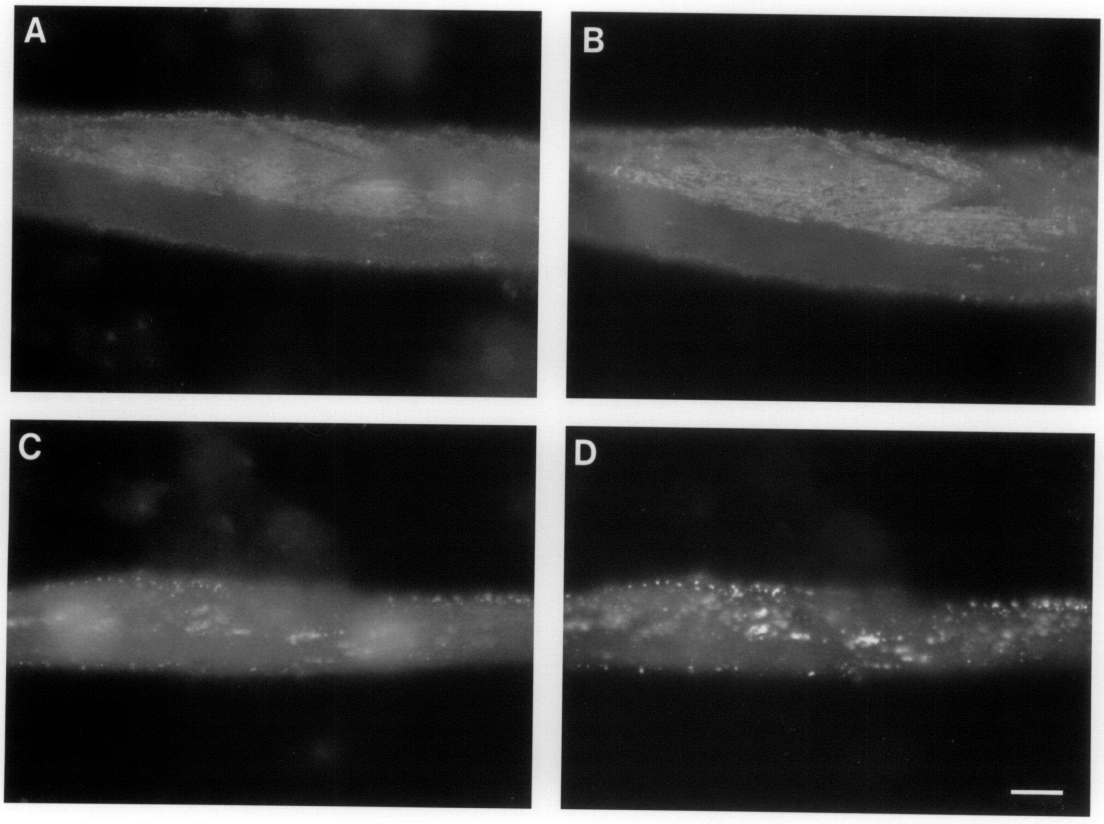
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Figure 2.4. Mutation of the myristoylation addition site prevents targeting of 43 kDa protein to the cell surface.

Transiently transfected C2 myoblasts (A, B) and rat primary myotubes (C, D) expressing either 43 kDa-HA protein (A, C) or 43 kDa-HA protein containing a mutated myristoylation site (Gly_2 to Glu_2) (B,D) were stained for HA immunoreactivity as described (Materials and Methods). In both C2 myoblasts (B) and myotubes (D) the mutant 43 kDa protein is detected primarily in the nucleus. The intensity of the nuclear immunoreactivity in both myoblasts (B) and myotubes (D) expressing this mutant 43 kDa protein appears brighter than in myoblasts (A) and myotubes (C) expressing wild type 43 kDa-HA protein . AChR clusters are detected readily in myotubes expressing the mutant 43 kDa protein (not shown), indicating that the mutant 43 kDa protein is not acting as a dominant-negative suppressor of AChR clustering in these myotubes. Calibration bar = 10 μm.

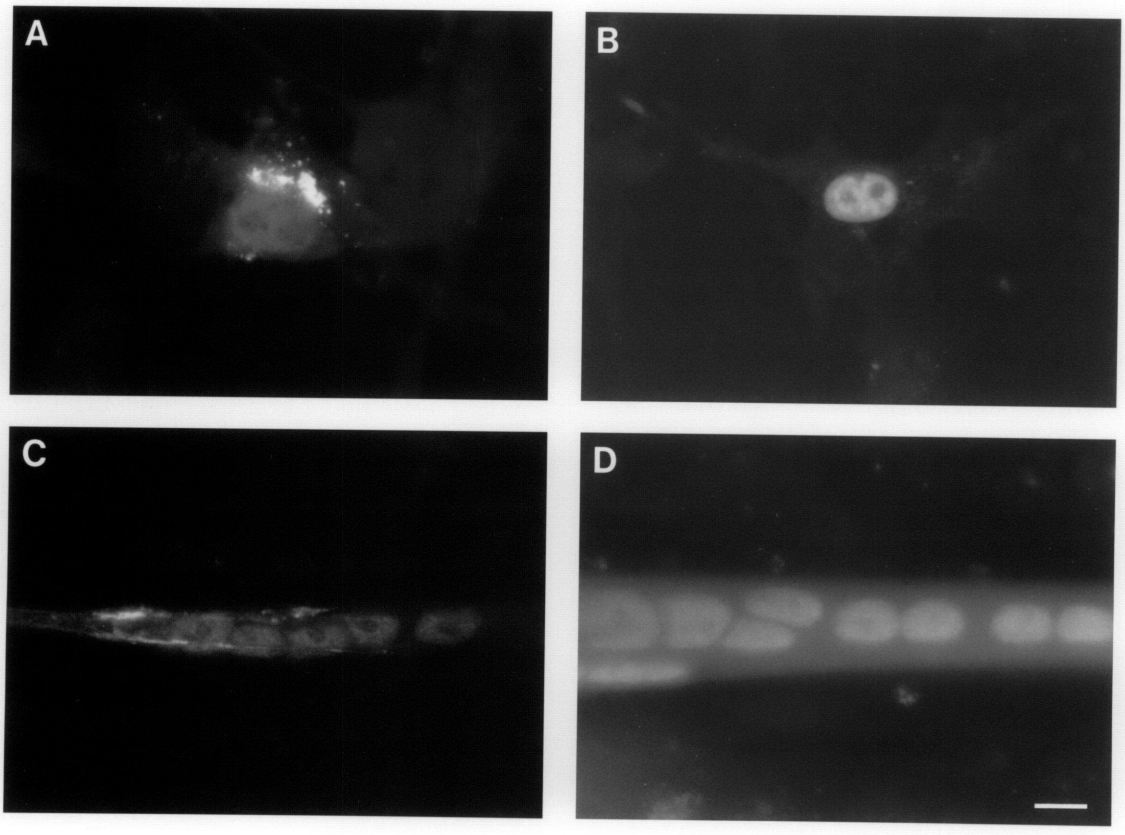
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Figure 2.5. C-terminal truncation of the 43 kDa protein promotes intracellular aggregation of 43 kDa protein and prevents association of 43 kDa protein with AChR clusters in myotubes.

Truncated, 43 kDa-HA proteins containing either the amino-terminal 113 or 227 amino acids were transiently expressed in QT6 cells, C2 myoblasts and primary rat myoblasts; the latter were allowed to differentiate into myotubes. Myotubes were treated with TMR-α-BGT just prior to fixation to label AChRs. Cells were fixed, permeablized and stained for HA as described (Materials and Methods). The HA-tagged 227 amino acid 43 kDa proteins is found in small aggregates (0.5 -1 μm) throughout the cytoplasm of both transfected QT6 cells (A) and C2 myoblasts (B). Similar results are observed in transfected cells expressing the mutant 113 amino acid 43 kDa protein (not shown). The 113 amino acid (C) and 227 amino acid (not shown) 43 kDa-HA protein also aggregate in transfected rat primary myotubes, but these aggregates are not associated with AChR clusters (D; C and D at same plane of focus). Although aggregates of these mutant 43 kDa proteins seems distributed throughout the cytoplasm of transfected cells, we cannot exclude the possiblity that some of these aggregates are associated with the cell surface. Calibration bar (A, B; C, D)= 10 μm.

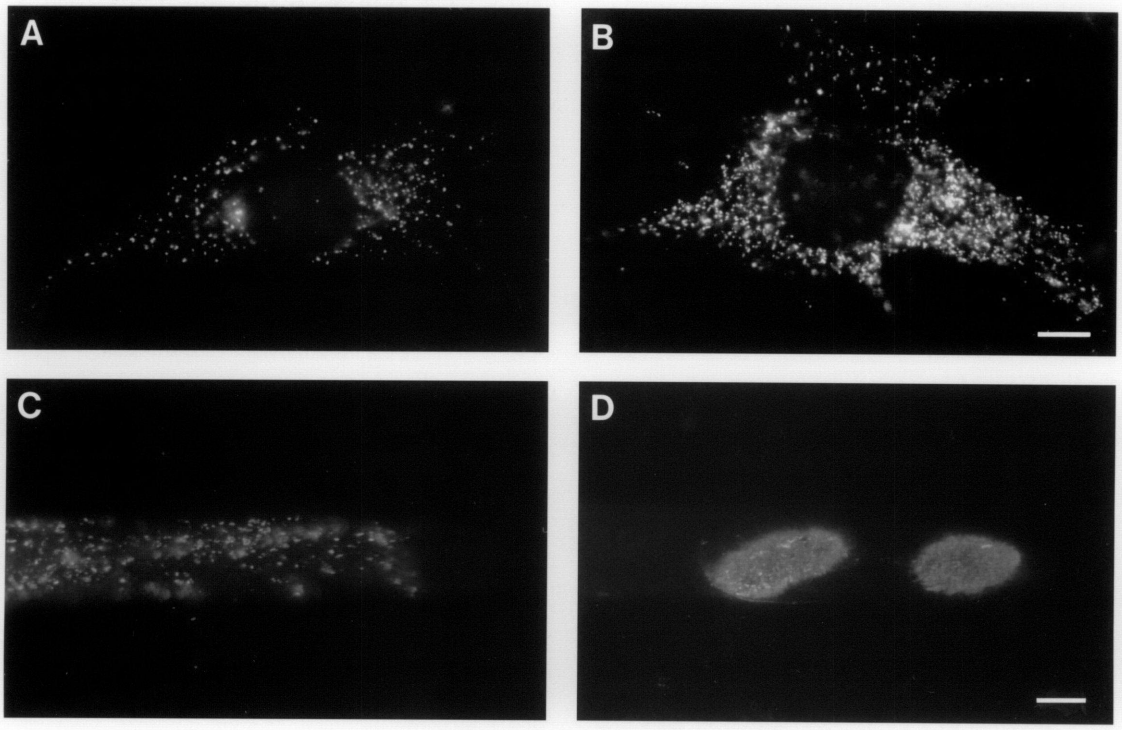
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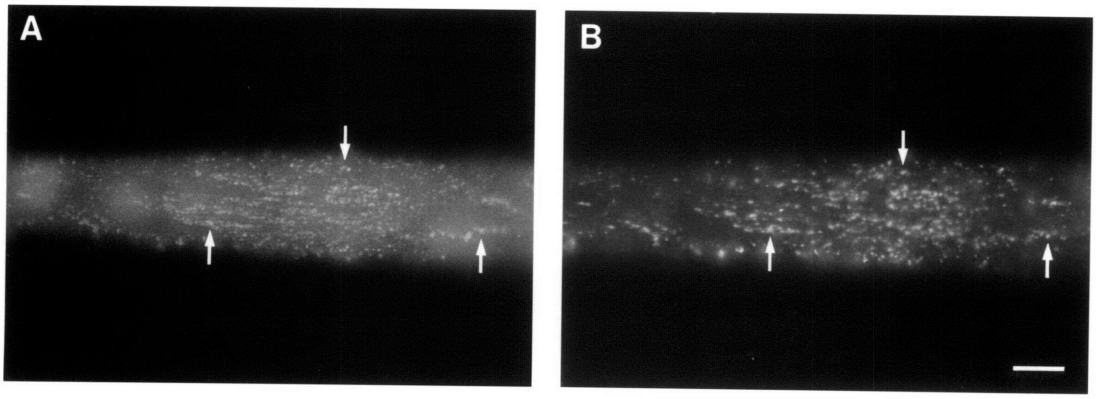
Figure 2.6. Mutation of a potential phosphorylation site does not prevent the 43 kDa protein from associating with AChR clusters in rat primary myotubes.

Transiently transfected rat primary myotubes expressing 43 kDa-HA proteins containing a double mutation (Ser_{405}, Ser_{406} to Ala_{405}, Ala_{406}) were allowed to differentiate and were stained for HA as described (Materials and Methods). Surface AChR clusters were labeled with TMR-α-BGT prior to fixation as described (Materials and Methods). Mutant 43 kDa protein (A) associates with AChR clusters (B) at the surface of transfected myotubes, suggesting that mutation of this potential phosphorylation site does not affect the ability of the mutant 43 kDa protein to associate with AChR clusters at the cell surface. Arrows are included for reference points. Calibration bar = 10 μm.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Chapter 3.

Dystrophin is a Component of the Subsynaptic Membrane

The work described in this chapter has been published in:
Yeadon, J. E., Lin, H., Dyer, S. M., and Burden S. J. (1991). Dystrophin is a component of the subsynaptic membrane. *J. Cell Biol.* **115**, 1069-1076.

Ravin, A. J., Dyer, S. M., Yeadon, J. E., and Burden, S. J. (1991). Multiple dystrophin isoforms are associated with the postsynaptic membrane of Torpedo electric organ. *J. Physiol. Paris,* **85**, 131-133.

SUMMARY

A subsynaptic protein of M_r ~300 kDa is a major component of *Torpedo* electric organ postsynaptic membranes and copurifies with the AChR and the 43 kDa subsynaptic protein. Monoclonal antibodies against this protein react with neuromuscular synapses in higher vertebrates, but not at synapses in dystrophic muscle. The *Torpedo* 300 kDa protein comigrates in SDS-PAGE with murine dystrophin and reacts with antibodies against murine dystrophin. The sequence of a partial cDNA isolated by screening an expression library with monoclonal antibodies against the *Torpedo* 300 kDa protein shows striking homology to mammalian dystrophin, and in particular to the *b* isoform. A partial cDNA encoding the *a* isoform of *Torpedo* dystrophin has also been isolated. These results indicate that dystrophin is a component of the postsynaptic membrane in *Torpedo* electric organ and at neuromuscular synapses and raise the possibility that loss of dystrophin from synapses in dystrophic muscle may have consequences that contribute to muscular dystrophy.

INTRODUCTION

Mutations in the gene encoding dystrophin can result in X-linked myopathies termed Duchenne or Becker muscular dystrophy (Hoffman and Kunkel, 1989). Duchenne muscular dystrophy (DMD), the most severe of these myopathies, is characterized by progressive and severe muscle wasting resulting in death of the patient usually before the age of twenty. Becker muscular dystrophy (BMD) is much less common than DMD (1 in 30,000 vs. 1 in 3500 boys), and BMD patients exhibit a less severe phenotype and have a much longer survival rate. In DMD, mutations of the dystrophin gene usually result in the complete absence of dystrophin protein, whereas in BMD, mutations are often in frame deletions which result in either mildly altered forms of dystrophin, lower levels of the protein or both (Monaco et al., 1988; Koenig et al., 1989).

Dystrophin is a 427 kDa cytoskeletal protein composed of four distinct domains (Koenig et al., 1988; Koenig and Kunkel, 1990). The amino terminal domain of dystrophin bears similarity to the actin-binding domain of α-actinin, and, when expressed in bacteria, can bind f-actin *in vitro* (Hemmings et al., 1992; Way et al., 1992). The large, central domain of the molecule is comprised of twenty-four α-helical coiled-coil, spectrin-like repeats and four putative 'hinge' domains which give the protein an extended, yet

flexible, rod-like structure. Rotary-shadowed electronmicroscopic images of purified dystrophin confirm a 100 nm rod-like shape, and also suggest that dystrophin monomers combine to form staggered tetramers which can in turn form end-to-end oligomers (Pons et al., 1990; Sato et al., 1992). Downstream of the central rod domain is cysteine-rich domain, resembling the amino-terminal domain of α-actinin, followed by a leucine-rich, globular carboxy-terminal domain . These later domains are vital for the normal function of dystrophin since mutations within the cysteine-rich domain and the first half of the carboxy-terminal domain often result in severe DMD phenotypes (Koenig et al, 1989; Roberts et al., 1992).

Expression of full-length dystrophin is restricted to muscle and nervous tissue (Chamberlain et al., 1988; Hoffman et al., 1988; Nudel et al., 1988). The brain and muscle isoforms of dystrophin are regulated by separate promoters which produce transcripts which differ only in the first exon of their transcribed mRNAs (Nudel et al., 1989; Feener et al., 1989). A third promoter encoding a unique first exon has also been identified which regulates dystrophin expression in cerebellar Purkinje neurons (Gorecki et al., 1992). In addition, three smaller dystrophin-derived transcripts, expressed from two different promoters, have been isolated which encode only the 3' end of the *DMD* locus (for reviews, see Ahn and Kunkel, 1993; Tinsley et al., 1993). Alternative splicing of dystrophin transcripts produces multiple dystrophin isoforms which differ in their carboxy-terminal domains (Feener et al., 1989). Although functional differences between these isoforms have not been established, tissue and developmental-stage specific splicing patterns suggest that such differences may exist (Bies et al., 1992).

In skeletal muscle, dystrophin is found at the intracellular surface of the sarcolemma (Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Bonilla et al., 1988; Sugita et al., 1988). Dystrophin constitutes roughly 5% of cytoskeletal proteins associated with the sarcolemma (Ohlendieck and Campbell, 1991a), and is therefore a major constituent of the subsarcolemmal cytoskeleton. Dystrophin associates with the sarcolemma via interactions with an oligomeric complex of proteins termed the dystrophin glycoprotein complex (DGC) (Campbell and Kahl, 1989; Ervasti et al., 1990; Yoshida and Ozawa, 1990). The DGC is comprised of at least six distinct proteins (for review, see Tinsley et al., 1993), which include the transmembrane proteins β-dystroglycan (Ibraghimov-Beskrovnaya et al., 1992), α- (adhalin) (Roberds et al.., 1993), β- (Bönnemann et al., 1995; Lim et al., 1995), and γ-sarcoglycan (Noguchi et al., 1995), the extracellular protein α-dystroglycan (Ibraghimov-Beskrovnaya et al., 1992), and members of the syntrophin family of cytoplasmic proteins (Adams et al., 1993). Recently, fusion proteins containing both the cysteine-rich and first half of the carboxy-terminal domains of dystrophin have been shown

to bind to β-dystroglycan *in vitro* (Suzuki et al., 1992, 1994). Similar studies have also revealed binding sites for both α1 and β1-syntrophin in the distal half of the carboxy-terminal domain of dystrophin (Ahn and Kunkel, 1995; Suzuki et al., 1994, 1995). Thus, interaction between carboxy-terminal domains of dystrophin and the DGC appear to mediate the linkage of dystrophin to the sarcolemma. Since α-dystroglycan can interact extracellularly with laminins (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993), dystrophin and the DGC may be important linkages connecting the sarcoplasmic cytoskeleton with the extracellular basal lamina.

It is not clear how loss of dystrophin results in myopathies. Increased Ca^{2+} leak channel activity (Fong et al., 1990) and Ca^{2+} influx (Turner et al., 1991) have been reported in dystrophic muscle. These changes in internal Ca^{2+} concentration correlate with changes in Ca^{2+}-sensitive protease activity (Spenser and Tidball, 1992) and increased protein degradation and turnover (Turner et al., 1988; MacLennan and Edwards, 1990), and suggest a causal relationship between dystrophin deficiency-induced changes in Ca^{2+} regulation and muscle necrosis. The changes in Ca^{2+} metabolism in dystrophic muscle may reflect a direct role for dystrophin in Ca^{2+} regulation across the sarcolemma. Alternatively, these changes may be important secondary consequences of dystrophin deficiency-induced changes in the integrity of the sarcolemma. Consistent with this idea, immunohistochemical analysis shows that the levels of all DGC proteins are drastically reduced in muscle from DMD patients and dystrophin-deficient *mdx* mice (Ervasti et al., 1990; Ohlendieck and Campbell, 1991b; Ohlendieck et al., 1993). These data suggest that dystrophin may organize and/or stabilize the DGC. Since muscle fibers from *mdx* mice are more susceptible to contraction-induced rupture than are muscle fibers from normal mouse muscle (Petrof et al., 1993), loss of dystrophin and/or disruption of the dystrophin/DGC linkage may affect the overall stability of the sarcolemma, making the muscle more vulnerable to damage during repeated cycles of muscular contraction. Recent studies have demonstrated that certain severe childhood autosomal-recessive muscular dystrophies, a class of myopathies clinically similar to DMD or severe BMD, results from defects in the genes encoding any one of the sarcoglycans (Roberds et al., 1994; Ljunggren et al., 1995; Bönnemann et al., 1995; Lim et al., 1995; Noguchi et al., 1995). In addition, a form of congenital muscular dystrophy (CMD) in humans, as well as the CMD-like dystrophia muscularis myopathy in mouse, have been linked recently to deficiencies in the gene encoding the laminin α2-chain (merosin M-chain) (Sunada et al., 1994; Xu et al., 1994; Helbling-Leclerc et al., 1995). These data highlight the importance of the dystrophin/DGC/extracellular matrix linkage in normal muscle physiology, and suggest that

abnormalities in the expression of any one its components may result in muscular dystrophy.

In addition to its association with the sarcolemma, dystrophin immunoreactivity is also concentrated at neuromuscular synapses (Chang et al., 1989; Jasmin et al., 1990; Fardeau et al., 1990). Because antibodies against dystrophin can cross-react with other proteins that contain homologous domains (Hoffman et al., 1989a, b), however, it was difficult to ascertain whether the cross-reacting molecule at the synapse was dystrophin rather than another molecule which shares epitopes with dystrophin. Indeed, one study showed that dystrophin-like immunoreactivity persists at synaptic sites in dystrophic muscle (Fardeau et al., 1990), and subsequent studies revealed that utrophin, a protein closely related to dystrophin, is specifically expressed with AChRs and 43 kDa protein at the NMJ (Nguyen thi Man et al., 1991; Ohlendieck et al., 1991), specifically at the crests of postjunctional folds (Bewick et al., 1992). Therefore, it remained unresolved whether dystrophin itself was present at neuromuscular synapses. In this chapter, I provide strong evidence that dystrophin is present at synaptic sites. The work presented here was done in collaboration with Helen Lin and Amy Ravin, two UROP students in the Burden lab.

We showed previously that a peripheral membrane protein of M_r ~300 kDa copurifies with postsynaptic membranes isolated from *Torpedo* electric organ and appears concentrated at neuromuscular synapses (Burden et al., 1983; Woodruff et al., 1987). We have now isolated a cDNA by screening an expression library with monoclonal antibodies against the *Torpedo* 300 kDa protein, and show that the amino acid sequence encoded by this partial cDNA is homologous to dystrophin. The cDNA encodes a protein with repeat units that have 50-70% amino acid sequence identity with the repeat units in human dystrophin and a carboxy-terminal region with 90% homology to the dystrophin *b* isoform. A second partial cDNA has also been isolated which is homologous to the *a* isoform of dystrophin. Moreover, antibodies against murine dystrophin react with a *Torpedo* 300 kDa protein, which comigrates with murine dystrophin in SDS-PAGE, and monoclonal antibodies against the *Torpedo* 300 kDa protein react with neuromuscular synapses in normal, but not dystrophic, mouse muscle. These results provide strong evidence that dystrophin is present at neuromuscular synapses and raise the possibility that the absence of dystrophin from synapses in dystrophic muscle may perturb the structure and/or function of the synapse and contribute to muscular dystrophy.

MATERIALS AND METHODS

Isolation of AChR-rich membranes and Western blotting

AChR-rich and AChR-poor membranes were isolated from *Torpedo* electric organ as described previously (Burden et al., 1983). The M_r of 300 kDa protein was determined in 6% polyacrylamide SDS gels with human erythrocyte spectrin and rabbit macrophage actin-binding protein as molecular weight standards (Woodruff et al., 1987). Peripheral proteins from AChR-rich membranes were fractionated by two-dimensional gels as described previously (Burden, 1985). Western blots were probed with monoclonal antibody (mAb) hybridoma supernatant, affinity-purified antibodies against a trpE+60 kDa mouse dystrophin fusion protein (encoding the first four repeats in murine dystrophin, Hoffman et al., 1987) diluted 1/2000, and affinity-purified antibodies against a trpE fusion protein containing the carboxy-terminal region of human dystrophin (antibody 11, Koenig and Kunkel, 1990) diluted 1/500, followed by alkaline phosphatase-coupled secondary antibodies.

Immunohistochemistry

Staining of unfixed frozen sections from *Torpedo* electric organ was performed as described previously (Woodruff et al., 1987). Affinity-purified antibodies against a trpE+60 kDa murine dystrophin fusion protein dystrophin (Hoffman et al., 1987) were used at 1/2000, and hybridoma supernatant containing mAb 602 was used undiluted. Frozen sections (8 um) from unfixed intercostal muscles were stained with biotinylated mAb 601 (identical results were obtained with a different mAb against the *Torpedo* 300 kDa protein, mAb 607) or with antibodies against the trpE+60 kDa dystrophin fusion protein (Hoffman et al., 1987) diluted 1/2000 for 1 hour at room temperature. Following incubation with either fluorescein-labeled avidin or fluorescein-labeled goat-anti-sheep IgG and TMR-BGT, sections were visualized with optics selective for either rhodamine or fluorescein (Woodruff et al., 1987).

Rat myotubes were fixed (1% paraformaldehyde in PBS) for 15 minutes, washed (in PBS), permeabilized with 0.1% NP-40 (in PBS), incubated with hybridoma supernatant containing mAb 601 (2 hours at room temperature) and subsequently with fluorescein-labeled goat-anti-mouse IgG and TMR-BGT. AChR clusters in the murine C2 muscle cell line are labeled with mAb 601 as well (J. A. Theriot and S. J. Burden, unpublished results).

Molecular biological methods.

300,000 recombinant phage from a λgt11 *Torpedo* electric organ cDNA library (Baldwin et al., 1988b) were screened with four different mAbs (601, 602, 603, 604) that react exclusively with the *Torpedo* electric organ 300 kDa protein (Woodruff et al., 1987). One positive phage (Tordys1) was detected with mAb 602, and was found to contain a 4.1 kb cDNA insert. Our other monoclonal antibodies against the *Torpedo* 300 kDa protein do not react with the fusion protein, and these antibodies presumably react with regions of the *Torpedo* 300 kDa protein that are not encoded by the partial cDNA. Affinity purified antibodies against the carboxy-terminal region of human dystrophin (antibody 10, Koenig and Kunkel, 1990) also react with the fusion protein and with the *Torpedo* 300 kDa protein (data not presented). cDNA from the purified phage was mapped with restriction endonucleases and sequenced (Sanger et al., 1977; Baldwin et al., 1988a). The cDNA clone encodes an additional ~1.5 kb beyond the termination codon and ends with a 44 bp poly A tract, which presumably represents the 3' end of the mRNA. The deduced amino acid sequences of *Torpedo* 300 kDa protein and human dystrophin were aligned (Wilbur and Lipmann, 1983).

An additional seven cDNAs were isolated by screening 80,000 recombinant phage with an 915 bp probe derived from the 4.1 kb cDNA (nt 22 - 937 in Fig. 3.7; hybridization in 5X SSC, 50°C, washed in 0.2X SSC, 56 °C) (Ravin et al., 1991). Thus, the relative abundance of cDNAs in this library encoding dystrophin (0.9/10,000), 43 kDa protein (4.5/10,000) (Baldwin et al., 1988b) and AChR alpha subunit (11/10,000) (Baldwin et al., 1988a) is similar to the relative abundance of the corresponding proteins (1:10:20; Burden et al., 1983).

^{32}P-labeled, random-primed probe derived from the 4.1 kb cDNA encoding the *Torpedo* 300 kDa protein was hybridized to a Northern blot of total RNA (~27 μg) isolated from *Torpedo* electric organ. The blot was hybridized overnight (in 5X SSPE) at 55°C and washed (in 0.2X SSC, 0.1% SDS) at 57°C (Baldwin et al., 1988a). An RNA ladder of 9.5, 7.5, 4.4, 2.4, 1.4, 0.2 kb was used to estimate the size of the *Torpedo* transcript.

RESULTS

The *Torpedo* 300 kDa protein and dystrophin share epitopes.

Antibodies against the first four repeats in murine dystrophin react with a protein concentrated in AChR-rich membranes isolated from *Torpedo* electric organ (Fig 3.1A).

The cross-reacting protein comigrates in SDS-PAGE with the 300 kDa subsynaptic protein that we identified previously as a component of the postsynaptic membrane in *Torpedo* electric organ and in skeletal myofibers (Fig. 3.1A) (Burden et al., 1983; Woodruff et al., 1987).

Because several proteins could comigrate at 300 kDa, proteins from AChR-rich membranes were resolved further by isoelectric focusing, and blots from two dimensional gels were probed either with antibodies against human dystrophin or with a monoclonal antibody (mAb) against the *Torpedo* 300 kDa protein. Figure 3.1B shows that the different antibodies react with the same protein, since the 300 kDa protein, as well as the same set of proteolytic fragments, are labeled with both antibodies. Thus, antibodies against dystrophin react with the 300 kDa protein which we identified previously. Further, all of our mAbs against the 300 kDa protein (Woodruff et al., 1987), and antibodies against murine dystrophin (Hoffman et al., 1987), react *in situ* with the postsynaptic membrane of the electrocyte (Fig. 3.1C; see also Chang et al., 1989).

mAbs against the *Torpedo* 300 kDa protein react with synaptic sites in normal, but not dystrophic, muscle.

Among the mAbs that we produced against the *Torpedo* 300 kDa protein, mAb 601 cross-reacts with neuromuscular synapses in both amphibian and mammalian muscle (Woodruff et al., 1987). We used mAb 601 to determine whether the molecule recognized by this antibody is absent from synaptic sites in dystrophic (*mdx*) mouse muscle (Bullfield et al., 1984). Figure 3.2 demonstrates that mAb 601 reacts with synaptic sites in normal, but not in dystrophic, mouse muscle. Similarly, antibodies against murine dystrophin react with synaptic sites in normal (Fig. 3.2; see also Chang et al., 1989), but not dystrophic, muscle (Fig. 3.2). Extrasynaptic staining is also detectable with both antibodies in normal, but not dystrophic, mouse muscle (Fig. 3.2). Thus, a protein in normal mouse muscle that reacts with mAb 601 is absent from dystrophic muscle.

Utrophin (dystrophin-related protein, DRP) is the product of an autosomal gene, and has striking homology to the product of the X-linked dystrophin gene (Love et al., 1989; Tinsley et al., 1992). Since utrophin is retained in muscle from *mdx* mice (Khurana et al., 1990; Ohlendieck et al., 1991), however, neither mAb 601 nor the antibodies against murine dystrophin used in this study react with utrophin.

Clusters of AChRs occur at synaptic sites in innervated skeletal muscle and can also form in the absence of innervation in cultured embryonic myotubes (Vogel et al., 1972; Fischbach and Cohen, 1973). We examined whether the molecule recognized by mAb 601 is concentrated at AChR clusters in myotube cultures as well as at synaptic sites. Figure

3.3 demonstrates that mAb 601 staining is present throughout the myotube, but is concentrated at AChR clusters. Moreover, there is an intricate arrangement of AChRs within a cluster, and a similar, although not identical arrangement is seen with mAb 601 (see also Sealock et al., 1991). Since mAb 601 reactivity is concentrated at AChR clusters in non-innervated embryonic myotubes and at synapses in newborn rats (data not presented), the association of the 300 kDa subsynaptic protein with the postsynaptic membrane is likely to be an early event during synaptogenesis. Further, since folding of the plasma membrane at AChR clusters in cultured myotubes is rare (Vogel and Daniels, 1976), the accumulation of the 300 kDa protein at these sites cannot be attributed entirely to an increase in membrane folding.

Dystrophin and the *Torpedo* 300 kDa protein

In our previous studies we used SDS-PAGE to estimate a M_r of 300 kDa for the *Torpedo* electric organ subsynaptic protein (Woodruff et al., 1987). The molecular mass of human dystrophin, calculated from the amino sequence deduced from cDNA, is 427 kDa (Koenig et al., 1988). Figure 3.4 demonstrates that the *Torpedo* electric organ 300 kDa protein and murine dystrophin comigrate in SDS-PAGE, since antibodies against murine dystrophin react with a protein of identical size in *Torpedo* AChR-rich membranes and in normal but not *mdx* mouse muscle.

The central domain of dystrophin is composed of 24 repeats (Koenig and Kunkel, 1990) which have structural similarity to repeats in spectrin and α-actinin (Davison and Critchley, 1988; Hammond, 1987; Koenig et al., 1988). Each repeat is thought to be organized into three alpha helices which form coiled-coil interactions, and this common structural feature can be detected with antibodies raised against dystrophin, which can cross-react with α-actinin (Hoffman et al., 1989). Antibodies against α-actinin (Bloch and Hall, 1983) and β-spectrin (Bloch and Morrow, 1989), as well as antibodies against dystrophin (Fig. 3.2; see also Chang et al., 1989), react with synaptic sites in skeletal muscle; however, identification of the molecule(s) recognized by these antibodies is complicated by the possibility of cross-reactivity due to the conserved structural feature described above.

Although the lack of reactivity of mAb 601 in dystrophic muscle strongly suggests that the antibody recognizes dystrophin, it remains possible, however, that the mAb cross-reacts with another molecule whose loss is an indirect consequence of the absence of dystrophin. Indeed, it is clear that other proteins, in addition to dystrophin, are absent from skeletal muscle in the *mdx* mouse (Ervasti et al., 1990; Ohlendieck and Campbell, 1991b) and in individuals with Duchenne muscular dystrophy (Ohlendieck et al., 1993).

Thus, we sought to isolate cDNAs encoding the 300 kDa subsynaptic protein to more firmly establish its relationship to dystrophin.

The sequence of a *Torpedo* electric organ cDNA is homologous to dystrophin.

We used mAbs against the *Torpedo* 300 kDa subsynaptic protein to screen an expression library from *Torpedo* electric organ, and isolated a cDNA (Tordys1) which encodes a protein that reacts with one of these mAbs (602). Since the isolated cDNA is 4.1 kb and hybridizes to RNA which is ~14 kb in length (Fig. 3.5), the sequence encoded by the Tordys1 cDNA is incomplete. Sequencing of the protein-coding portion of the Tordys1 cDNA revealed that the amino acid sequence deduced from this *Torpedo* cDNA is strikingly homologous to human dystrophin (Fig. 3.6 and Fig. 3.7).

The amino acid sequence deduced from the 5' end of the cDNA has 59% and 72% homology, respectively, with the 23rd and 24th repeat in human dystrophin (Koenig and Kunkel, 1990; Davison and Critchley, 1988). This same repeat region has more limited sequence homology with spectrins (~20%), α-actinin (28%) and myosins (~20%). Moreover, alignment of the sequences encoded by the Tordys1 cDNA and dystrophin requires no gaps, whereas alignment of either the Tordys1 cDNA or dystrophin with spectrin requires two single amino acid gaps within each repeat (Koenig and Kunkel, 1990; Davison and Critchley, 1988).

Additional sequence from this region of the *Torpedo* 300 kDa protein was obtained by rescreening our *Torpedo* cDNA library with a 915 bp fragment from the 5' end of the Tordys1 cDNA. One cDNA (Tordys2) isolated from this screen begins ~2.15 kb further 5' to the Tordys1 cDNA, and has good homology with mammalian dystrophin in the region encoding the 18th through 20th repeats (Fig. 3.6 and Fig. 3.8). The remainder of this cDNA, except for the very 3' end, has not been sequenced (see Fig. 3.6). The homology between the 18th repeat in human dystrophin and the sequence encoded by the Tordys2 cDNA is 53%, and gapping is not required. The homology between the 19th repeat in human dystrophin and the sequence encoded by the Tordys2 cDNA is 48%, however this alignment requires two gaps. One gap occurs near the beginning of the 19th repeat where mammalian dystrophin has seven amino acids that are not encoded by the Tordys2 cDNA (Fig. 3.8B). Interestingly, these very same seven amino acids are also absent from the same region of chicken dystrophin (Lemaire et al., 1988). A second gap of two amino acid has also been introduced at the end of the 19th repeat unit, and extends an additional four amino acids into the 3rd hinge region which precedes the 20th repeat unit. This 3rd hinge region encoded by the Tordys2 cDNA is 51% similar to the corresponding region of human dystrophin. Together these results show that the region of sequence homology between the

300 kDa protein encoded by the Tordys1 and Tordys2 cDNAs and human dystrophin includes a region of dystrophin that is thought to be fundamental for establishing its tertiary structure (Koenig and Kunkel, 1990; Davison and Critchley, 1988).

Since the dystrophin sequence which follows the repeat units is unrelated to spectrin and vertebrate α-actinin, comparison within this region is a more stringent criterion for establishing structural similarity to dystrophin (Koenig et al., 1988; Feener et al., 1989). Figures 3.6 and 3.7 show that there is 97% homology between the fourth hinge region of dystrophin and the proline-rich sequence following the predicted 24th repeat encoded by the Tordys1 cDNA. This degree of conservation suggests that the fourth hinge region of dystrophin has a critical functional role, and that this domain has an importance in addition to disruption of the alpha helical organization. Moreover, beyond the hinge region of dystrophin is a cysteine-rich region followed by the carboxy-terminal domain. Both of these regions are highly conserved in the *Torpedo* cDNA (91% and 89% identity, respectively). Thus, the sequence encoded by the cDNA is highly homologous to dystrophin, and is only distantly related to spectrin, α-actinin and myosin.

Since utrophin is strikingly homologous to dystrophin (the carboxy-terminal region of utrophin has 80% amino acid sequence identity with the dystrophin *a* isoform) (Love et al., 1989; Tinsley et al., 1992), it seemed possible that the protein encoded by the *Torpedo* cDNAs could be the homolog of this autosomal gene, rather than of dystrophin. However, alignment of the sequence encoded by the Tordys1 cDNA with the protein product of utrophin reveals 73% identity in the cysteine-rich and carboxy-terminal domains and requires several gaps, whereas alignment with dystrophin shows 88% identity and requires no gaps. Moreover, antibodies against mammalian dystrophin (antibody 11, Koenig and Kunkel, 1990) react with the *Torpedo* 300 kDa protein, but not with mammalian utrophin (Hoffman et al., 1989). Thus, we think it likely that the protein encoded by the *Torpedo* cDNAs is *Torpedo* dystrophin.

Multiple dystrophin transcripts, which are generated by alternative splicing, yield several isoforms (Feener et al., 1989). The *b* isoform of dystrophin lacks 13 amino acids present in the carboxy-terminal region of the *a* isoform, and is generated by splicing and removal of 39 nucleotides (Feener et al., 1989). The Tordys1 *Torpedo* dystrophin cDNA corresponds to the *b* isoform, and this alignment supports further the conclusion that the protein encoded by the *Torpedo* cDNA is dystrophin. Further, a second cDNA isolated during our rescreening of the electric organ cDNA library encodes an additional 13 amino acids and corresponds to the *a* isoform (Tordys 3) (Figure 3.9). Thus, both the *a* and *b* isoforms of dystrophin are present in *Torpedo* electric organ. Moreover, since all of our monoclonal antibodies against the *Torpedo* 300 kDa protein react with the synaptic but not

the nonsynaptic membrane of the electrocyte, the *a* and *b* isoforms coexist on the postsynaptic membrane.

DISCUSSION

This study demonstrates that a protein in *Torpedo* electric organ is highly homologous to mammalian dystrophin. The extent of sequence homology with mammalian dystrophin, and the conservation of at least one alternatively spliced exon, indicates that this *Torpedo* protein is *Torpedo* dystrophin rather than a dystrophin-related protein. Consistent with this interpretation, antibodies against murine dystrophin, which do not cross-react with murine utrophin, react with the same 300 kDa electric organ protein that copurifies with the AChR-rich postsynaptic membranes, and a mAb (601) directed against the electric organ 300 kDa subsynaptic protein reacts with synaptic and nonsynaptic membranes in normal, but not dystrophic, mouse muscle.

We think it is likely that the protein encoded by the three Tordys cDNAs, which we designate *Torpedo* dystrophin, is the 300 kDa subsynaptic protein that we identified previously on the basis of co-purification with AChR-rich postsynaptic membranes from *Torpedo* electric organ. First, the cDNA encodes a protein that reacts with a mAb (602) directed against the electric organ 300 kDa subsynaptic protein. Second, affinity purified antibodies against the carboxy-terminal region of human dystrophin (antibody 10, Koenig and Kunkel, 1990) also react with a β-gal fusion protein containing the protein encoded by the Tordys1 cDNA and with the *Torpedo* 300 kDa protein (data not presented). These results support the conclusion that the 300 kDa subsynaptic protein which copurifies with electric organ postsynaptic membranes is *Torpedo* dystrophin.

Furthermore, we have rescreened our electric organ cDNA library at moderate stringency with a probe derived from the 4.1 kb Tordys1 cDNA and isolated seven additional cDNAs that encode *Torpedo* dystrophin. Thus, the abundance of dystrophin cDNAs in the electric organ library is similar to the abundance of 300 kDa protein in the electric organ (see Methods). Since no cDNAs encoding a dystrophin-like protein were isolated during our library screens, dystrophin-related sequences are likely to be present at much lower abundance than dystrophin, if at all, in the electric organ.

Studies have demonstrated that antibodies against dystrophin cross-react with a *Torpedo* electric organ protein of ~400 kDa that is enriched at the postsynaptic membrane (Chang et al., 1989; Jasmin et al., 1990). Moreover, the former study showed that antibodies against murine dystrophin cross-react with a protein at neuromuscular synapses

in normal rat muscle, and that antibodies against the *Torpedo* 400 kDa protein react with non-synaptic membrane in normal, but not dystrophic, human muscle (Chang et al., 1989). Because antibodies against dystrophin can cross-react with other proteins that contain homologous domains, as described above, and because these studies did not determine whether antibody staining was absent from synaptic sites in dystrophic muscle, it is difficult to ascertain whether the cross-reacting molecule at the synapse is dystrophin, rather than another molecule which shares epitopes with dystrophin. Indeed additional studies demonstrated that some antibodies against dystrophin do react with synaptic sites in dystrophic muscle, and these authors suggest that a dystrophin-related protein is present at synaptic sites in normal muscle and persists in dystrophic muscle (Fardeau et al., 1990; Pons et al., 1991). The residual anti-dystrophin immunoreactivity seen in these studies is likely due to the cross-reactivity of these antibodies with utrophin, since subsequent studies showed that utrophin is specifically concentrated in the postsynaptic membrane of the NMJ (Nguyen thi Man et al., 1991; Ohlendieck et al., 1991; Bewick et al., 1992). Our study demonstrates that there is extensive homology between the amino acid sequences for mammalian dystrophin and the *Torpedo* 300 kDa subsynaptic protein, and that antibodies against the *Torpedo* subsynaptic protein react with synaptic sites in normal but not dystrophic muscle. Thus, we conclude that dystrophin itself is present in the electric organ and at synaptic sites in skeletal muscle.

Consistent with the observations of others, our data suggests that in skeletal muscle dystrophin is found in the both synaptic and nonsynaptic myofiber membrane, but is more concentrated at synaptic sites (Chang et al., 1989; Fardeau et al., 1990). Unfortunately, we can not determine from these data to what extent the increase in membrane surface area at the synaptic site, due to the postsynaptic folds, contributes to the higher dystrophin concentration observed by conventional immunofluorescence microscopy. Immunoelectronmicroscopy of the mouse NMJ using gold-labeled antibodies against dystrophin also suggests that dystrophin is slightly more abundant in the postsynaptic membrane than in the remainder of the sarcolemma (Byers et al., 1991); however, these results have yet to be confirmed by careful quantitative analysis.

The presence of dystrophin in the electrocyte, a cell which is specialized for synaptic transmission and does not contract, suggests that dystrophin can have a role other than stabilizing the plasma membrane from structural distortion during contraction. Further, the location of dystrophin exclusively at the postsynaptic membrane of the electrocyte suggests that dystrophin's function in this cell is associated with synaptic structure and/or function. This idea is further strengthened by studies showing that dystrophin immunoreactivity is concentrated at postsynaptic membrane specializations in cortical neurons of the central

nervous system (Lidov et al., 1990; Kim et al., 1992). Since AChRs are concentrated at synaptic sites in dystrophic muscle (Fig. 3.2), it seems clear that dystrophin is not required for the formation of AChR clusters. Indeed, dystrophin also appears to be absent from many AChR-rich domains at AChR clusters in cultured myotubes (Sealock et al., 1991; see also Fig. 3.4). Both high resolution fluorescence microscopy (Sealock et al., 1991) and immunoelectronmicroscopy studies (Byers et al., 1991) suggest that dystrophin is more specifically associated with the troughs of the junctional folds, and is only infrequently seen at the crests. These results suggest a role for dystrophin in organizing the structure of the postsynaptic membrane, and in particular the postjunctional folds. Consistent with this idea, electron microscopic studies of dystrophic muscle have illustrated simplification of the synapse, notably a reduction in the number of postjunctional folds (Jerusalem et al., 1974; Torres and Duchen, 1987; Nagel et al., 1990). However, these changes may reflect properties of degenerating or regenerating muscle and may only be an indirect consequence of loss of dystrophin.

Recent studies suggest that utrophin, in contrast to dystrophin, associates specifically with AChR domains at the crests of the postjunctional folds (Bewick et al., 1992), and utrophin immunoreactivity is also coincident at AChR clusters in developing muscle and at AChR clusters in cultured muscle cells (Phillips et al., 1993). These studies suggest that utrophin may be involved in clustering AChRs and/or organizing AChR-rich domains in the postsynaptic membrane, and that its function at the postsynaptic membrane is different from that of dystrophin. Alternatively, dystrophin and utrophin may be functionally identical. Consistent with this idea, high resolution immunofluorescence microscopy of the electric organ reveals dystrophin immunoreactivity throughout the postsynaptic membrane of the electric organ, including AChR-rich domains (Sealock et al., 1991). Moreover, utrophin-specific antibodies react only weakly with the postsynaptic membrane of the electric organ, whereas antibodies specific for dystrophin produce a more robust signal (Cartaud et al., 1992; see also Fig. 3.1C). These data suggest that utrophin is expressed at a much lower level that dystrophin in the electric organ, and that dystrophin substitutes functionally for utrophin in organizing the postsynaptic membrane in this tissue. The differential distributions of dystrophin and utrophin in the postsynaptic membrane of muscle may simply reflect a higher affinity of one or the other for a differentially distributed component of the DGC. Recently, β2 -syntrophin has been shown to be expressed exclusively at the NMJ in skeletal muscle (Peters et al., 1994), but it remains unknown whether its distribution is further restricted to the tops or bottoms of the postjunctional folds.

We do not know how the loss of dystrophin from skeletal muscle results in myopathies, but it has been proposed that may affect the stability of the muscle plasma membrane resulting in damage to the muscle during muscular contraction. Although the NMJs of DMD patients appear normal with respect to AChR distribution, AChR content, and the frequency and amplitude of miniature endplate potentials (Sakakibara et al., 1977), loss dystrophin from the NMJ nevertheless may have consequences which subtly contribute to muscular dystrophy. Current ideas for therapeutic treatment of patients with muscular dystrophy include gene replacement therapy (Acsadi et al., 1991; Ragot et al., 1993), and injections of normal myoblasts into dystrophic muscle to promote formation of chimeric myotubes containing normal nuclei (Partridge et al., 1989). Since the synaptic region of a skeletal myofiber is less than 1% of the myofiber volume, and dystrophin may not diffuse freely in a myofiber, reconstitution of synaptic dystrophin may require that normal nuclei be situated in the synaptic region. If dystrophin has an important role at the synapse, successful therapeutic treatment may require a substantial increase in the efficiency of normal myoblast incorporation into chimeric myotubes.

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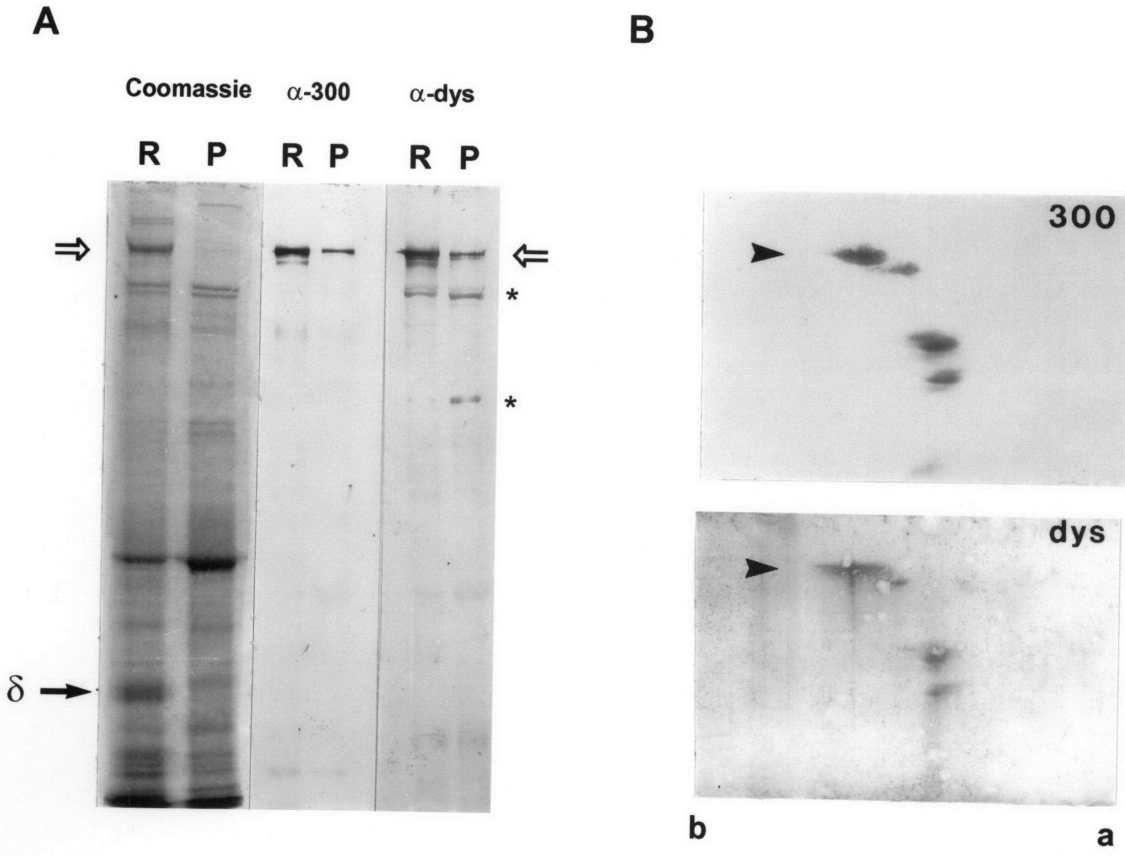
Figure 3.1. Antibodies against dystrophin cross-react with the *Torpedo* 300 kDa subsynaptic protein, which copurifies with AChR-rich membranes. A) Postsynaptic membranes from *Torpedo* electric organ were fractionated in an equilibrium density sucrose gradient and the protein composition was analyzed by SDS-PAGE. Proteins in AChR-rich (R) and AChR-poor (P) membranes were fractionated by SDS-PAGE (6% polyacrylamide) and either stained with Coomassie Brilliant Blue or transferred to nitrocellulose and probed with mAb (602) to the *Torpedo* 300 kDa protein or antibodies to murine dystrophin (trpE+60 kDa). Both antibodies react with the 300 kDa protein (open arrow), which is more abundant in AChR-rich than in AChR-poor membranes. Labeling of the protein band just beneath the 300 kDa protein with mAbs against the 300 kDa protein is variable, and is likely due to partial proteolysis of the 300 kDa protein. Antibodies against dystrophin, but not mAbs against the *Torpedo* 300 kDa protein, react with several proteins (∗) that are enriched in AChR-poor membrane fractions and are thus not likely to be proteolytic fragments of dystrophin; these proteins are not labeled with secondary antibody alone, and therefore cross-react with the antibodies against murine dystrophin. B) Peripheral membrane proteins from AChR-rich were resolved by two dimensional electrophoresis, transferred to nitrocellulose and probed either with mAb (602) to the *Torpedo* 300 kDa protein or with affinity purified antibodies to the carboxy terminal region of human dystrophin (antibody 11). The different antibodies react with the same protein, since the 300 kDa protein (arrowhead) as well as the same set of proteolytic fragments are labeled with both antibodies. The basic (b) and acidic (a) directions are indicated. C) The innervated surface of the *Torpedo* electrocyte is labeled with mAbs against the *Torpedo* 300 kDa protein and antibodies against dystrophin. Single frozen sections of *Torpedo* electric organ were labeled with tetramethylrhodamine-labeled α-bungarotoxin (BGT) (b, d ,f) and with either mAb 602 (a), antibodies against dystrophin (c), or mAb against a *Torpedo* intermediate filament protein (e) (Burden, 1982). The magnification is 400x.


A
Coomassie
α-300
α-dys
R P R P R P
δ
B
300
dys
b
a

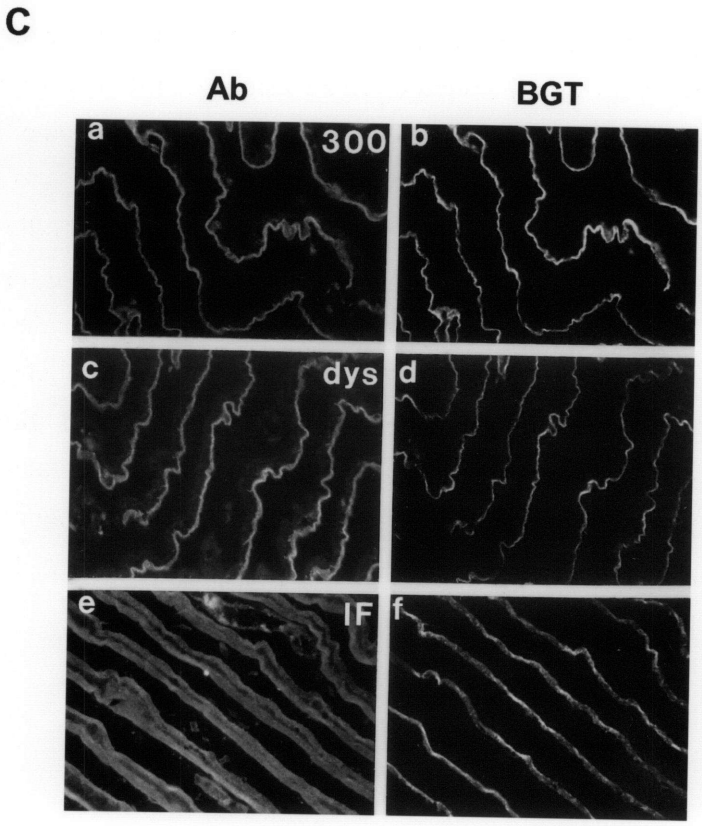

C
Ab
BGT
a
300
b
c
dys
d
e
IF
f

Figure 3.2. Both antibodies against murine dystrophin and mAb against the *Torpedo* 300 kDa protein label synaptic sites in normal, but not in dystrophic, mouse muscle.

Frozen sections of normal (a-d) and *mdx* (e-h) mouse muscle were labeled with biotinylated mAb 601 (a, e), or anti-dystrophin antibodies (trpE+60) (c, g), followed by appropriate fluorescein-labeled secondary reagents and TMR-BGT (b, d, f, h). We have not investigated whether the number or size of endplates is altered in *mdx* muscle. The bar is 20 μm.

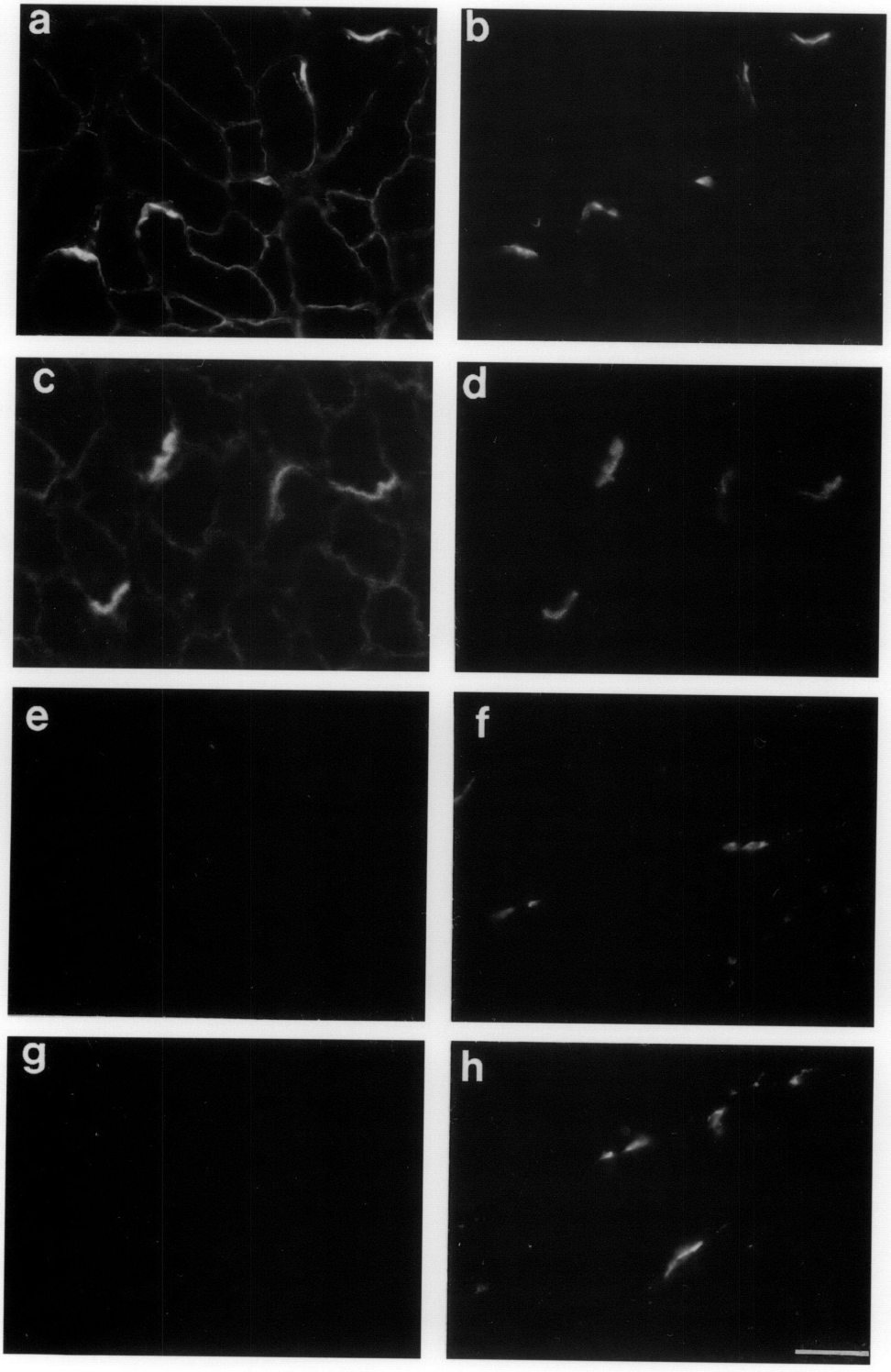

a
b
c
d
e
f
g
h

Figure 3.3. AChR clusters in primary rat myotubes are labeled with mAb against the *Torpedo* 300 kDa protein.

Rat myotubes were labeled with mAb 601 (a, c), followed by fluorescein-labeled goat-anti-mouse IgG and TMR-BGT (b, d). Antibody labeling is present throughout the myofiber, but is concentrated at AChR clusters. Within AChR clusters, a similar pattern of labeling is seen with TMR-BGT and mAb 601. The bar is 10 μm.

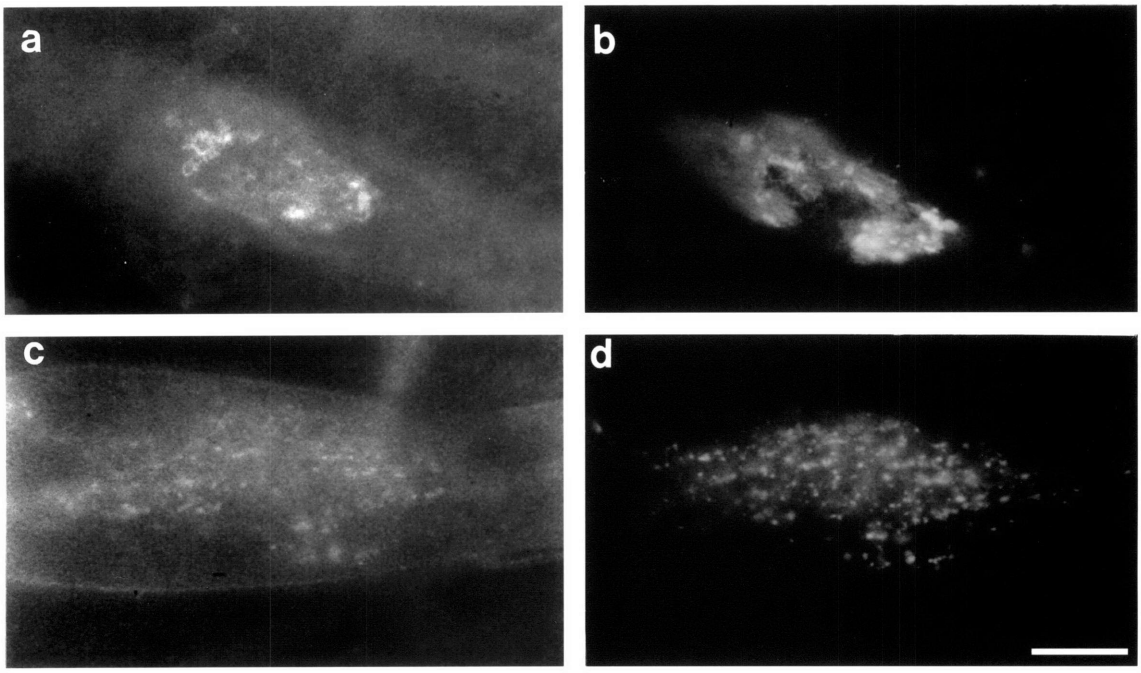

a
b
c
d

Figure 3.4. The *Torpedo* 300 kDa protein and murine dystrophin comigrate in SDS-PAGE.

Proteins from *Torpedo* electric organ AChR-rich membranes and total protein (~70 mg) from normal and *mdx* mouse muscle were fractionated by SDS-PAGE (6% polyacrylamide), transferred to nitrocellulose and probed with antibodies to murine dystrophin (trpE+60 kDa; Hoffman et al., 1987). The *Torpedo* electric organ 300 kDa protein and murine dystrophin comigrate in SDS-PAGE (arrowhead), since antibodies against murine dystrophin react with a protein of identical size in *Torpedo* AChR-rich membranes (R) and in normal (nor), but not *mdx* (mdx) mouse muscle. Labeling of skeletal muscle protein(s) that migrate more slowly than dystrophin is non-specific, since labeling is detected with secondary antibody alone (control); the anti-dystrophin antibodies, however, cross-react weakly with myosin (arrow), which is present at similar levels in normal and *mdx* muscle.

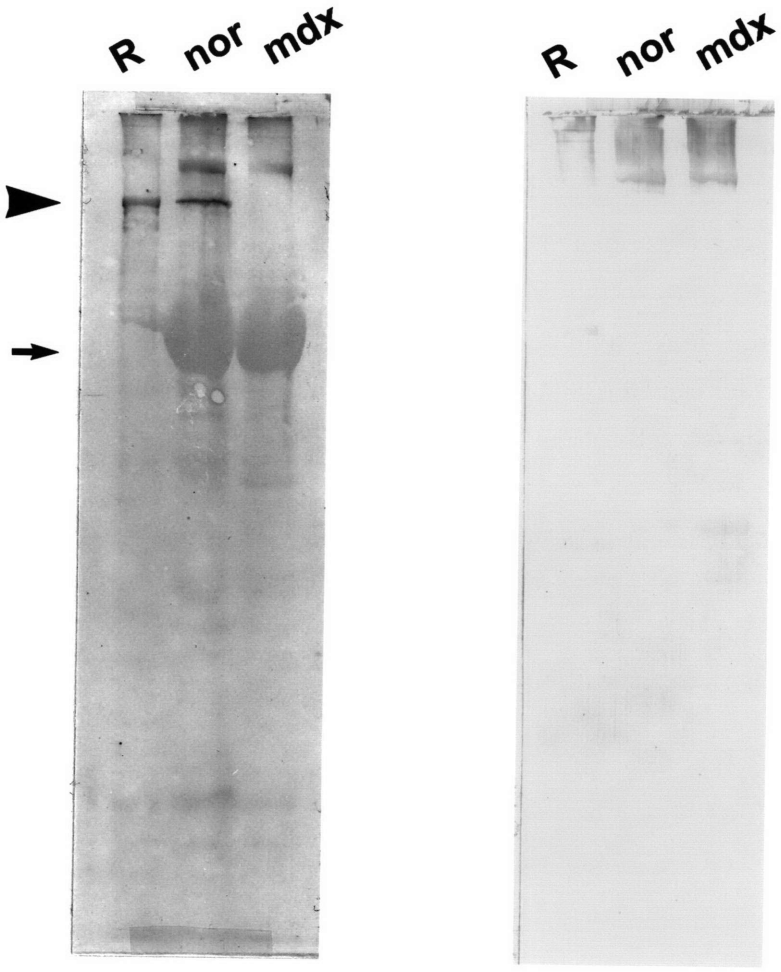

α-dys
control
R
nor
mdx
R
nor
mdx

Figure 3.5. The *Torpedo* 300 kDa protein is encoded by a 14 kb mRNA. The cDNA encoding the *Torpedo* 300 kDa protein hybridizes to a ~14 kb RNA (arrowhead) in *Torpedo* electric organ. The positions of RNA size markers are indicated. The filter was exposed to x-ray film with an intensifying screen for 3 days at -70°C.

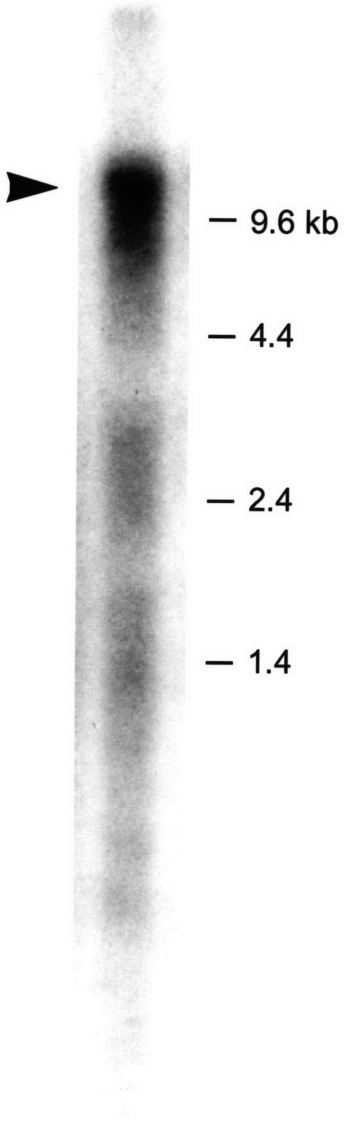
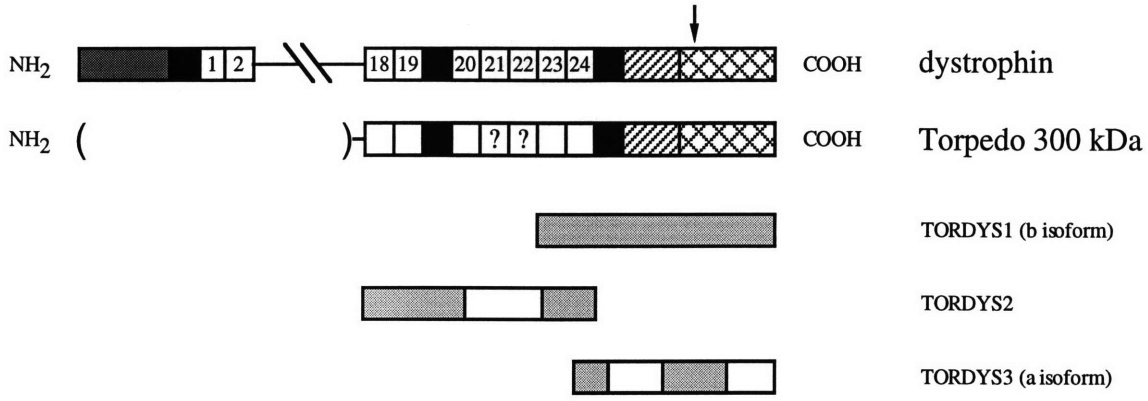

9.6 kb
4.4
2.4
1.4

Figure 3.6. The domain structure of dystrophin and the domain organization of the *Torpedo* 300 kDa protein is identical.

A.) The cartoon illustrates the location of the amino terminal region (shaded box), repeat units (open boxes), hinge regions (black boxes), cysteine-rich region (hatched box) and carboxy-terminal domain (cross-hatched box) in dystrophin (Koenig and Kunkel, 1990). Homologous regions within the *Torpedo* 300 kDa protein are labeled similarly. Repeat units 3-17 and the 2nd hinge region of dystrophin are indicated by the broken line. Parentheses illustrate the region of the 300 kDa protein that is not encoded by any of our 300 kDa protein cDNAs. The putative 21st and 22nd repeats of the 300 kDa protein (question marks) have not been sequenced, but are presumably included in the Tordys2 cDNA. The approximate location of the thirteen amino acid alternatively spliced exon which is absent in the dystrophin *b* isoform is indicated by the arrow. The three cDNAs described in this study are shown below the regions of the *Torpedo* 300 kDa protein they encode. Tordys1 is the original *Torpedo* 300 kDa protein cDNA identified as the dystrophin *b* isoform. Tordys2 is the cDNA (2.4 kb) that encodes sequence further 5' to Tordys1. Tordys3 encodes the *a* isoform of *Torpedo* dystrophin, as it has the thirteen amino acids (residues 3410-3422 in human dystrophin) which are characteristic of the dystrophin *a* isoform. Light gray shaded regions represent the approximate locations of sequenced portions of the cDNAs. B) Homology between human dystrophin and the *Torpedo* 300 kDa protein is indicated separately for each domain: 18th repeat, amino acids 2209-2318; 19th repeat, amino acids 2319-2423; 3rd hinge, amino acids 2424-2470; 23rd repeat, amino acids 2803-2931; 24th repeat, 2932-3040; 4th hinge, 3041-3112; cysteine-rich, 3113-3360 and carboxy-terminal, 3361-3685 (Koenig and Kunkel, 1990; Koenig et al., 1988).

A



B

Domain	Amino Acid Identity (%)
18th repeat	53
19th repeat	48
3rd hinge	51
:	:
23rd repeat	59
24th repeat	72
4th hinge	97
Cys-rich	91
Carboxy terminal	89

Figure 3.7. The amino acid sequences of the *Torpedo* 300 kDa subsynaptic protein and human dystrophin are similar.

A) The nucleic acid and amino acid sequence of the protein coding portion of the 4.1 kb Tordys1 cDNA are shown. B) The protein coding portion of the Tordys1 cDNA encoding the 300 kDa protein (TORDYS) is aligned with the human dystrophin (HUMDYS) sequence that extends from amino acid 2803 to the carboxy-terminus of the *a* isoform. Identical residues in the HUMDYS sequence are indicated with a dash (-) and amino acid substitutions are shown by the one-letter amino acid notation. A gap in the TORDYS sequence, which is indicated by asterisks (*), aligns with amino acids 3409-3421 in HUMDYS, and signifies the *b* isoform of dystrophin; homology between the human dystrophin *b* isoform and the portion of the *Torpedo* 300 kDa protein encoded by the Tordys1 cDNA is 84%. The boundaries between specific domains in human dystrophin are shown below the aligned sequences (R - repeat unit; 4^{th} H - 4^{th} hinge domain; Cys - cysteine-rich domain; C-term - carboxy-terminal domain).

A

```
AGTGGTGAGCAGTGGAAGCGGCTGCAGATCTCCCTCCAGGACTTCCTGACGTGGATGAATTTGAAGAACGATGAACTAAGGAGGCAAATGCCCATTGGTGGTGATGCTCCCACAGTCTGT  120
 S  G  E  Q  W  K  R  L  Q  I  S  L  Q  D  F  L  T  W  M  N  L  K  N  D  E  L  R  R  Q  M  P  I  G  G  D  A  P  T  V  C  (40)

CAGCAGAACGATGTTCACCGAATTTTCAAGCGAGAGTTGAAGGCAAAGGAACCAGTGGTCATGAGCGCTTTGGACACAGTGCATTTGTTCTTGGCTGATCCAGCAATCAGAGCTCCTGAA  240
 Q  Q  N  D  V  H  R  I  F  K  R  E  L  K  A  K  E  P  V  V  M  S  A  L  D  T  V  H  L  F  L  A  D  P  A  I  R  A  P  E  (80)

AGTCTTTTAACTGGACCAAGAGAGAAAATACCTGAAGAGAACATCCAAAATGTTGCGAAACGCATTCGGAAGTATGCCGAGGAGGTGAAAGTGGAATGGGATAAGCTGAGCAATCGCTCT  360
 S  L  L  T  G  P  R  E  K  I  P  E  E  N  I  Q  N  V  A  K  R  I  R  K  Y  A  E  E  V  K  V  E  W  D  K  L  S  N  R  S  (120)

GTTGATTGGCAGAAGCGTATAGATGAGGCCTTGAAGAGACTACTGGAATTGCAAGATTCAATGGATGAATTGAACCTCAAATTGAGACAGGCTGAAGCTATCAAAGATACATGGCAACCT  480
 V  D  W  Q  K  R  I  D  E  A  L  K  R  L  L  E  L  Q  D  S  M  D  E  L  N  L  K  L  R  Q  A  E  A  I  K  D  T  W  Q  P  (160)

GTCGGGGATCTACTAATAGACTCATTGCAGGATCACATTGAAAAAGTCAAGGTTTTTCGAGCAGAAATTGCTCCCATGAAGGAAAATGTGACTCACATGAATGATCTCGCTTCCCAGTTC  600
 V  G  D  L  L  I  D  S  L  Q  D  H  I  E  K  V  K  V  F  R  A  E  I  A  P  M  K  E  N  V  T  H  M  N  D  L  A  S  Q  F  (200)

ACACCACCTGATATCCAATTATCCCCGTACAATCTAAACCAGTTGGAGGATCTGAACACACGGTGGAAACTCTTGCAGGTGTCTATAGATGAGCTTCTGAAGCAGCTGCATGAGGCTCAT  720
 T  P  P  D  I  Q  L  S  P  Y  N  L  N  Q  L  E  D  L  N  T  R  W  K  L  L  Q  V  S  I  D  E  L  L  K  Q  L  H  E  A  H  (240)

AGAGATTTTGGACCAACATCCCAGCACTTCCTATCAACTTCTGTCCAAGGTCCTTGGGAGCGAGCAATATCACCCAACAAGGTTCCCTACTACATTAACCACCAAACTCAGACCACCTGC  840
 R  D  F  G  P  T  S  Q  H  F  L  S  T  S  V  Q  G  P  W  E  R  A  I  S  P  N  K  V  P  Y  Y  I  N  H  Q  T  Q  T  T  C  (280)

TGGGATCACCCCAAGATGACGGAGCTCTATCAGTCACTAGCCGATCTAAATAATGTCAGGTTTTCTGCATATAGAACTGCCATGAAACTGCGAAGACTGCAGAAAGCTCTTTGCTTGGAT  960
 W  D  H  P  K  M  T  E  L  Y  Q  S  L  A  D  L  N  N  V  R  F  S  A  Y  R  T  A  M  K  L  R  R  L  Q  K  A  L  C  L  D  (320)

CTTCTCTCCCTGCCAAGTGCTTGTGAGGCCTTTGATCAGCACAACCTGAAACAGAACGATCAACTGTTGGATATTTTAGAAATAATTAACTGCTTGACTTCAATCTATGATCGGCTGGAG 1080
 L  L  S  L  P  S  A  C  E  A  F  D  Q  H  N  L  K  Q  N  D  Q  L  L  D  I  L  E  I  I  N  C  L  T  S  I  Y  D  R  L  E  (360)

CAGGAACACAGCAACCTTGTCAACGTGCCTCTTTGTGTGGACATGTGTTTAAACTGGCTGCTCAACGTCTATGACACTGGTCGAACAGGGAAGATCCGTGTCCTGTCCTTTAAAAGTGGA 1200
 Q  E  H  S  N  L  V  N  V  P  L  C  V  D  M  C  L  N  W  L  L  N  V  Y  D  T  G  R  T  G  K  I  R  V  L  S  F  K  S  G  (400)

ATCATGTCTATGTGCAAAGCACACCTGGAGGACAAATACCGTTATCTGTTCAAGCAAGTGGCGAGTCCCACTGGATTCTGTGATCAGCGCAGGCTTGGGCTTCTACTGCATGAGGCGATT 1320
 I  M  S  M  C  K  A  H  L  E  D  K  Y  R  Y  L  F  K  Q  V  A  S  P  T  G  F  C  D  Q  R  R  L  G  L  L  L  H  E  A  I  (440)

CAGATTCCACGCCAGCTGGGAGAGGTTGCATCCTTTGGAGGCAGCAACATAGAACCCAGTGTTCGCAGCTGCTTCCAGTTTGCCAATAACAAGCCGGAGATCGAGGCAGCTTTGTTCCTG 1440
 Q  I  P  R  Q  L  G  E  V  A  S  F  G  G  S  N  I  E  P  S  V  R  S  C  F  Q  F  A  N  N  K  P  E  I  E  A  A  L  F  L  (480)

GATTGGATGAGGCTGGAGCCTCAGTCCTTGGTCTGGATGCCAGTCCTCCACAGAGTGGCAGCAGCTGAGACAGCCAAACACCAGGCCAAGTGCAATATATGCAAAGAATGTCCCATCATT 1560
 D  W  M  R  L  E  P  Q  S  L  V  W  M  P  V  L  H  R  V  A  A  A  E  T  A  K  H  Q  A  K  C  N  I  C  K  E  C  P  I  I  (520)

GGATTCAGATACCGGAGCTTAAAGCACTTCAATTATGATGTCTGCCAAAGTTGCTTCTTTTCTGGTCGAACAGCAAAAGGTCATAAAATGCATTACCCAATGGTGGAATATTGTACACCG 1680
 G  F  R  Y  R  S  L  K  H  F  N  Y  D  V  C  Q  S  C  F  F  S  G  R  T  A  K  G  H  K  M  H  Y  P  M  V  E  Y  C  T  P  (560)

ACAACATCAGGAGAAGATGTCCGAGATTTTGCCAAGGTATTAAAAAACAAGTTCCGAACAAAGAGGTACTTTGCAAAACATCCCCGGATGGGATATCTGCCAGTGCAGACAGTTCTGGAA 1800
 T  T  S  G  E  D  V  R  D  F  A  K  V  L  K  N  K  F  R  T  K  R  Y  F  A  K  H  P  R  M  G  Y  L  P  V  Q  T  V  L  E  (600)

GGGGATAACTTAGAAACACCTGCCTCCTCCCCTCAGTTGTCACATGATGATACTCATTCACGAATTGAACATTATCTGAGCAGGCTAGCAGAAATGGAAAACAGAAATGGATCTTACTTG 1920
 G  D  N  L  E  T  P  A  S  S  P  Q  L  S  H  D  D  T  H  S  R  I  E  H  Y  L  S  R  L  A  E  M  E  N  R  N  G  S  Y  L  (640)

AATGACAGCATCTCACCCAATGAGAGCATAGATGATGAGCATCTGCTGATTCAGCATTACTGCCAGAGCCTGAACCAGGAGTCGCCACTGAGTCAGCCTCGAAGCCCTGCTCAAATTCTC 2040
 N  D  S  I  S  P  N  E  S  I  D  D  E  H  L  L  I  Q  H  Y  C  Q  S  L  N  Q  E  S  P  L  S  Q  P  R  S  P  A  Q  I  L  (680)

ATCTCCTTGGAGAGTGAAGAGAGGGGAGAGTTGGAAAAGAATCCTCGCTGACCTGGAGGATGAAAACAGAAACCTTCAGTCTGAGTATGAGAAGCTAAAGCAGCAACATGATCACAAGGGT 2160
 I  S  L  E  S  E  E  R  G  E  L  E  R  I  L  A  D  L  E  D  E  N  R  N  L  Q  S  E  Y  E  K  L  K  Q  Q  H  D  H  K  G  (720)

CTGTCTCCTTTGCCGTCCCCTCCTGAGATGATGCCCATTTCACCGCAGAGTCCCAGGGATGCCGAGCTCATCGCTGAAGCCAAACTGCTTCGTCAGCACAAAGGTCGCCTGGAGGCAAGG 2280
 L  S  P  L  P  S  P  P  E  M  M  P  I  S  P  Q  S  P  R  D  A  E  L  I  A  E  A  K  L  L  R  Q  H  K  G  R  L  E  A  R  (760)

ATGCAGATCCTGGAAGATCACAACAAGCAGTTGGAATCGCAGCTCCACAGACTGAGGCAGCTGCTGGAGCAGCCACAGGCGGAGGTCAGAGTCAATGGTACTTCGGTTTCCTCACCCTCG 2400
 M  Q  I  L  E  D  H  N  K  Q  L  E  S  Q  L  H  R  L  R  Q  L  L  E  Q  P  Q  A  E  V  R  V  N  G  T  S  V  S  S  P  S  (800)

ACTTCCTCACAAAGATCAGACAGCAGCCAGCCAGTCCTACTGCATGGAGTTGGCAGTCAAACTTCAGGAATACTGGGTGAGGATGATTTGTTGAGTGCTCCCCAAAGTACCAGCAGTGAA 2520
 T  S  S  Q  R  S  D  S  S  Q  P  V  L  L  H  G  V  G  S  Q  T  S  G  I  L  G  E  D  D  L  L  S  A  P  Q  S  T  S  S  E  (840)

TTGGAAGATGTAATGGAGCAACTCAGCAGTTCCTTTCCAAGCTCTCAACGGAGGGATACTCCTGGAAAGAAAATGAAAGAGGTACCAATGTAG                          2613
 L  E  D  V  M  E  Q  L  S  S  S  F  P  S  S  Q  R  R  D  T  P  G  K  K  M  K  E  V  P  M  .                          (870)
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B

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TORDYS   SGEQWKRLQISLQDFLTWMNLKNDELRRQMPIGGDAPTVCQQNDVHRIFKRELKAKEPVVMSALDTVHLFLADPAIRAPESLLTGPREKIPEENIQNVAKRIRKYAEEVKVEWDKLSNRS   120
HUMDYS   -SD-----HL---EL-V-LQ--D---S--A-----F-A-QK------A------T----I--T-ET--I--TEQPLEGL-K-YQE---LP---RA---TRLL--Q----NT--E--NLH-  2922

TORDYS   VDWQKRIDEALKRLLELQDSMDELNLKLRQAEAIKDTWQPVGDLLIDSLQDHIEKVKVFRAEIAPMKENVTHMNDLASQFTPPDIQLSPYNLNQLEDLNTRWKLLQVSIDELLKQLHEAH   240
HUMDYS   A---RK---T-E--Q---EAT---D-------V--GS--------------L----AL-G----L----S-V----R-L-TLG--------ST-------------AVEDRVR------  3042
                  23R  24R                                                                                                24R  4thH
TORDYS   RDFGPTSQHFLSTSVQGPWERAISPNKVPYYINHQTQTTCWDHPKMTELYQSLADLNNVRFSAYRTAMKLRRLQKALCLDLLSLPSACEAFDQHNLKQNDQLLDILEIINCLTSIYDRLE   360
HUMDYS   -----A--------------------------E-------------------------------------------------SA--D-L----------PM---Q------T------  3162
                                                                  4thH  Cys
TORDYS   QEHSNLVNVPLCVDMCLNWLLNVYDTGRTGKIRVLSFKSGIMSMCKAHLEDKYRYLFKQVASPTGFCDQRRLGLLLHEAIQIPRQLGEVASFGGSNIEPSVRSCFQFANNKPEIEAALFL   480
HUMDYS   ---N--------------------------R-------T--I-L-----------------S--------------DS-----------------------------------------  3282

TORDYS   DWMRLEPQSLVWMPVLHRVAAAETAKHQAKCNICKECPIIGFRYRSLKHFNYDVCQSCFFSGRTAKGHKMHYPMVEYCTPTTSGEDVRDFAKVLKNKFRTKRYFAKHPRMGYLPVQTVLE   600
HUMDYS   ---------M--L-------------------------------------------I---------V-----------------------------------------------------  3402
                                                                                 Cys  C-term
TORDYS   GDNLET**********PASSPQLSHDDTHSRIEHYLSRLAEMENRNGSYLNDSISPNESIDDEHLLIQHYCQSLNQESPLSQPRSPAQILISLESEERGELERILADLEDENRNLQS   707
HUMDYS   ---M--PVTLINFWPVDSA--------------------A--------S-------------------------------D-------------------------------E------A  3522

TORDYS   EYEKLKQQHDHKGLSPLPSPPEMMPISPQSPRDAELIAEAKLLRQHKGRLEARMQILEDHNKQLESQLHRLRQLLEQPQAEVRVNGTSVSSPSTSSQRSDSSQPVLLHGVGSQTSGILGE   827
HUMDYS   --DR-----E---------------T-------------------------------------------------------AK----T-------L--------M--RV------DSM--  3642

TORDYS   DDLLSAPQSTSSELEDVMEQLSSSFPSSQRRDTPGKKMKEVPM   870
HUMDYS   E----P--D--TG--E-----NN-----RG-N----P-R-DT-  3685
```

Figure 3.8. The amino acid sequence of upstream regions of the *Torpedo* 300 kDa protein also show similarity to dystrophin.

A) The partial nucleotide and amino acid sequence encoded by the 5' end of the Tordys2 cDNA is shown. This cDNA was isolated by rescreening the *Torpedo* electric organ cDNA library with a 5' fragment of the Tordys1 cDNA. The 3' end of the Tordys2 cDNA corresponds to nucleotide 742 in the Tordys1 cDNA (see Fig 3.6), confirming that the Tordys1 and Tordys2 cDNAs encode overlapping regions of the same RNA. B) The amino acid sequence encoded by the Tordys2 cDNA (TORDYS) is aligned with amino acids 2221-2522 of human dystrophin (HUMDYS). Identical residues in the HUMDYS sequence are indicated with a dash (-) and amino acid substitutions are shown by the one-letter amino acid notation. Gaps have been introduced to optimize alignment and are indicated by asterisks (*). Boundaries between specific domains in the human dystrophin sequence are indicated beneath the aligned sequences (R - repeat unit; 3^{rd} H - 3^{rd} hinge). The 7 amino acids in human dystrophin (amino acid 2325-2331) which span the gap introduced in the 19^{th} repeat of the TORDYS sequence are also absent from the corresponding region of chick dystrophin. The homology between human dystrophin and the region of the 300 kDa protein shown is 56%.

A

```
GAATTCCCCCATTGGCTAGAAGATGCAGTGAACACTCTGGGATTAGTACCTGACCCTGGAAATGAGCAGCAGCTG     75
 E  F  P  H  W  L  E  D  A  V  N  T  L  G  L  V  P  D  P  G  N  E  Q  Q  L    (25)

AAAGATTCACTGGAGAGCGTCAAGTTGCGAGTGGAGCAATTACCCACACGGAAAGGGATATTGAAGTGTTTAAAT    150
 K  D  S  L  E  S  V  K  L  R  V  E  Q  L  P  T  R  K  G  I  L  K  C  L  N    (50)

GATAGAGGAGCATCAGCATTCCAAAGCAAGTCTCTGGTCCCAGAACAACGCTTTAAATTGGAAACACATCTCCTG    225
 K  R  G  A  S  A  F  Q  S  K  S  L  V  P  E  Q  R  F  K  L  E  T  H  L  L    (75)

CAGGGTAATAATCGCTGGACAAAGGTATCAAAAGATCTGCCTGAGAAACAAAAAGAAATCGAAGAGTCTTTGAAG    300
 Q  G  N  N  R  W  T  K  V  S  K  D  L  P  E  K  Q  K  E  I  E  E  S  L  K   (100)

GACTTTGCACTATTTCAGCAGCAGCTCAACCTGCTGACTCTCTGGATCTCCACAACAAAGCAGCAGTTGGAATCA    375
 D  F  A  L  F  Q  Q  Q  L  N  L  L  T  L  W  I  S  T  T  K  Q  Q  L  E  S   (125)

TACACGCAAGCACCATTGCCAGGAATCTTTAATATTCAGGAGACAGAAGAAAGAGTACTAGCCAAACAGCCTGAT    450
 Y  T  Q  A  P  L  P  G  I  F  N  I  Q  E  T  E  E  R  V  L  A  K  Q  P  D   (150)

GTGGAAGACATATTTTCCAAGGCACAGCATTTCTATCAGGATAAACCGATTTCTCAACCATTAAAGGGAAAAATT    525
 V  E  D  I  F  S  K  A  Q  H  F  Y  Q  D  K  P  I  S  Q  P  L  K  G  K  I   (175)

GACAACCTGAATTCTGACTGGAAGATAATAAACAAGTTATTGCATGACCTGAAACAAAGTCATCAACCTGGTGTT    600
 D  N  L  N  S  D  W  K  I  I  N  K  L  L  H  D  L  K  Q  S  H  Q  P  G  V   (200)

GCTTCATCCACACTGCAAACAGTATCCGTGGTGGCACAGATGCCCGTTACCACGGAAACATCCTTCACAAAGTTA    675
 A  S  S  T  L  Q  T  V  S  V  V  A  Q  M  P  V  T  T  E  T  S  F  T  K  L   (225)

GACATGCCATCCTCTTTGCTCATGGATGTCCCAGCTCTGGCTGACTTCAATAAGGCCTGGGTGGAACTGACAGAC    750
 D  M  P  S  S  L  L  M  D  V  P  A  L  A  D  F  N  K  A  W  V  E  L  T  D   (250)

TGGCTCTCACTGCTGGATCGTGTGATCAAGTCTCAGCTTGTGACTGTGGGAGACGTTGAAGAAATCAATGACATG    825
 W  L  S  L  L  D  R  V  I  K  S  Q  L  V  T  V  G  D  V  E  E  I  N  D  M   (275)

ATCATAAAACAGAAGGCCACTCTGCAAGACTTGGATCAAA...                                     865
 I  I  K  Q  K  A  T  L  Q  D  L  D  Q  ...                                    (288)
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B

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TORDYS   EFPHWLEDAVNTLGLVPDPGNEQQLKDSLESVKLRVEQLPTRKGILKCLN       50
HUMDYS   --VL---E-D-IASIPLE--K-----EK--Q---L--E--L-Q----Q--     2271

TORDYS   DRGASAFQSKSLVPEQRFKLETHLLQGNNRWTKVSKDLPEKQKEIEESLK      100
HUMDYS   ET-GPVLV-APIS--EQD---NK-K-T-LQ-I---RA-----G-I-AQI-     2321
                                                    18R  19R
TORDYS   DFA*******LFQQQLNLLTLWISTTKQQLESYTQAPLPGIFNIQETEER      143
HUMDYS   -LGQLEKKLEDLEE---H-L--L-PIRN---I-N-PNQE-P-DV----IA     2371

TORDYS   VLAKQPDVEDIFSKAQHFYQDKPISQPLKGKIDNLNSDWKIINKLLHDLK      193
HUMDYS   -Q-K-------L--GQ-L-KE--AT--V-R-LED-S-E--AV-R--QE-R     2421

TORDYS   *******QSHQPGVASSTLQTVSVVAQMPVTTETSFTKLDMPSSLLMDVP      236
HUMDYS   AKQPDLAPGLTTIG--P-*---TL-T-PV--K--AIS--E-----MLE--     2471
         19R  3rd H                                      3rd H
TORDYS   ALADFNKAWVELTDWLSLLDRVIKSQLVTVGDVEEINDMIIKQKATLQDLDQ    288
HUMDYS   ------R--T----------Q-----R-M---L-D--E--------M---E-   2522
          20R
```
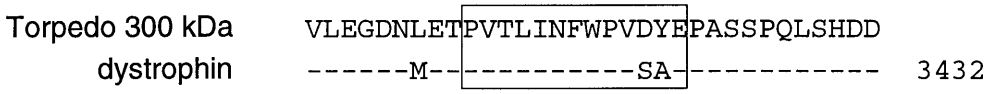

Figure 3.9. The dystrophin *a* isoform is present in *Torpedo* electric organ. The region of human dystrophin (amino acids 3400-3432) that includes the thirteen amino acids which are characteristic of the *a* isoform (boxed amino acids) is aligned with the corresponding region of a *Torpedo* dystrophin cDNA (Tordys3 in Fig. 3.6). Amino acids residues of human dystrophin that are identical to those in the *Torpedo* 300 kDa protein are illustrated by dashes (-), otherwise the one letter amino acid notation is used.


Torpedo 300 kDa
VLEGDNLETPVTLINFWPVDYEPASSPQLSHDD
dystrophin
------M----------SA----------
3432

Chapter 4.

Identification and Partial Characterization of *INN1*, *DEN1*, and *DEN2*: Three Novel Genes That are Regulated by Innervation

SUMMARY

Innervation is an important regulator of neuromuscular development and muscle physiology. Upon innervation, localized factors released from the nerve terminal concentrate acetylcholine receptors (AChRs) in the postsynaptic membrane by inducing both a redistribution of existing AChRs, and by activating AChR gene expression in nuclei near the synaptic site. Simultaneously, electrical stimulation of the muscle fiber by the motoneuron inactivates AChR gene expression in all myofiber nuclei, while it activates expression of acetylcholine esterase (AChE). In addition, nerve-evoked electrical activity in muscle has been implicated in secondary fiber myogenesis, motoneuron survival and polyneuronal synapse elimination. To further understand how innervation affects neuromuscular physiology and gene expression, we used a subtractive-hybridization and cloning approach to identify three novel genes, *INN1*, *DEN1*, and *DEN2*, whose expression are differentially regulated in innervated and denervated rat skeletal muscle. In RNA blots, three distinct *INN1* RNAs are detected in adult muscle (1.7, 2.3, and 5.2 kb). Expression of the 1.7 kb major *INN1* transcript is down-regulated ~5-fold following denervation, whereas expression of the 2.3 and 5.2 kb minor transcripts are up-regulated 2- to 3-fold and ~5-fold, respectively. Thus, while the total level of *INN1* RNA does not change dramatically following denervation, the levels of individual *INN1* transcripts change differentially, and these results suggest that splicing of *INN1* transcripts is regulated by innervation. A cDNA corresponding to the denervation down-regulated 1.7 kb *INN1* transcript was isolated, and sequencing showed that this transcript encodes a 22 kDa protein with 66% identity to a protein encoded by *NSP1*, a gene which has been cloned from both rat brain and human small cell lung carcinoma. Tissue distribution studies reveal that *INN1* is expressed at low levels in many tissues, but it is expressed at much higher levels in brain, heart and skeletal muscle, suggesting a role of INN1 in the response of these tissues to electrical stimulation. Consistent with this idea, immunolocalization of INN1 in skeletal muscle suggests that INN1 is a component of the sarcoplasmic reticulum and may be involved in the regulation of sarcoplasmic calcium levels in response to electrical stimulation. In contrast to *INN1*, *DEN1* and *DEN2* encode single transcripts (~9 and 2 kb, respectively) which are up-regulated following denervation (>50-fold and 3- to 5-fold, respectively). Sequencing of a partial *DEN1* cDNA reveals that DEN1 contains multiple immunoglobulin C2 and fibronectin type III motifs similar to those found in muscle structural proteins such as titin, while DEN2 has similarity to the family of small heat

shock proteins which include αB-crystallin and HSP27. In light of these findings, potential functions of DEN1 and DEN2 in muscle physiology are discussed.

INTRODUCTION

Denervation of skeletal muscle results in profound atrophy of myofibers correlated with changes in electrophysiological properties (Thesleff, 1974), sarcomeric morphology (Engel and Stonnington, 1974), an overall increase in proteolysis (Furano et al., 1990) and the loss of contractile strength (Finol et al., 1981). Similar effects are observed also when muscle is rendered electrically inactive by pharmacological blockade of neuronal stimulation (Drachman and Johnston, 1975; Brown et al., 1982; Spector, 1985a). Direct electrical stimulation of denervated muscle lessens the effects of denervation on the properties of the myofiber (see Lømo, 1976), and these results point to the importance of nerve-evoked electrical activity in the maintenance of skeletal muscle physiology.

Nerve-induced muscle activity has been implicated also in many aspects of neuromuscular development, including myogenesis, fiber-type differentiation, synapse formation, motoneuron survival, and poly-neuronal synapse elimination. During skeletal muscle myogenesis, two populations of myotubes, termed primary and secondary, form during two distinct waves of proliferation and fusion (Kelley and Zacks, 1969; Ross et al., 1987a). Primary myotube fusion occurs independent of innervation, since initial myotube formation proceeds normally in both aneural and electrically inactive muscle (Harris, 1981; McLennan, 1983; Ross et al., 1987b). In contrast, secondary myotubes fail to form under these conditions, and these results indicate that formation of secondary myotubes is dependent upon electrical activity. Since the numbers of mononucleated cells are also reduced in aneural or partially denervated muscle, the regulation of secondary myotube formation by electrical activity may be mediated through the activation of myoblast proliferation (Ross et al., 1987b).

The frequency of the nerve-evoked electrical stimulation also affects the differentiation of muscle fiber types. Mature muscle fibers can be broadly classified by their contractile and biochemical properties as either fast of slow. These classifications also reflect the nature of the motoneurons which innervate them in that fast and slow muscles are innervated by neurons which provide either high or low frequency stimulation, respectively. Many years ago, Buller et al. demonstrated that when the nerve from a slow twitch muscle is redirected to innervate a fast twitch muscle, the myofibers acquire properties of slow-twitch muscle. Similarly, a slow muscle can be transformed

into a fast muscle by cross-innervation with a fast-twitch nerve (Buller et al., 1960). Subsequent studies showed that simply altering the firing patterns of the innervating motoneurons is sufficient to transform muscle fibers from one type to the other (for review, see Pette and Vrbová, 1985). Moreover, in denervated rat soleus (a slow muscle) which is reinnervated by both the regenerated slow motoneuron and ectopically by a fast-twitch nerve, fast muscle myosin heavy chain is induced in a localized area around the ectopic endplate (Salviati et al., 1986). Taken together, these studies convincingly demonstrate that motoneuron activity influences the expression of muscle fiber type-specific genes.

During synapse formation, muscle activity has been implicated in maturation of the postsynaptic membrane. In muscle paralyzed during the period of synapse formation by treatment with α-bungarotoxin, folding of the post-synaptic membrane is simpler when compared to untreated controls (Duxson, 1982). In addition, synaptic AChE is greatly reduced in paralyzed muscle both *in vivo* and *in vitro* (Giacobini et al., 1973; Rubin et al., 1980), but direct electrical stimulation of the paralyzed muscle can counteract this effect (Rubin et al., 1980). Further, studies using ectopically innervated muscle have demonstrated that while contact between the muscle and foreign nerve is required to determine the location of the ectopic synapse, the continued presence of the ectopic nerve is not required; direct stimulation of the muscle induces AChE expression and folding of the postjunctional membrane at ectopic synapses even after the degeneration of the ectopically innervating motor axon (Lømo and Slater, 1980; Brenner et al., 1983). These results strongly indicate that electrical activity is required for both synapse maturation and AChE expression in muscle.

In contrast, muscle activity has a repressive effect on AChR expression. In adult muscle, AChRs are approximately 1000- to 5000-fold more concentrated in the postsynaptic than in the extrasynaptic membrane (Fertuck and Salpeter, 1976). Following denervation, however, the expression of extrajunctional AChRs increases dramatically (Axelsson and Thesleff, 1959; Hartzell and Fambrough, 1972), and this increase in extrasynaptic AChRs can be reversed by direct stimulation of the muscle (Lømo and Rosenthal, 1972; Lømo and Westgaard, 1975). Thus, while electrical activity induces the appearance of AChE, it simultaneously suppresses the expression of extrasynaptic AChR.

The increase in extrasynaptic AChR expression following denervation results from synthesis of new receptors and not simply redistribution of junctional AChRs (Brockes and Hall, 1975; Linden and Fambrough, 1979; Merlie et al., 1984). More recently, electrical activity has been shown to down-regulate the levels of AChR subunit mRNAs

in myofiber nuclei *in vivo* (Goldman, et al., 1988; Merlie and Kornhauser, 1989; Tsay and Schmidt, 1989; Simon et al., 1992), and in myotubes in culture (Klarsfeld and Changeux, 1985; Dutton et al., 1993). Thus, inactivation of extrasynaptic AChR expression in innervated muscle is regulated at the level of transcription. Members of the MyoD family of transcriptional activators, which are involved in the induction of many muscle specific genes during myogenesis, are regulated by electrical activity in parallel with AChR genes (Duclert et al., 1991; Eftimie et al., 1991; Witzemann and Sakmann, 1991; Neville et al., 1992; Weis, 1994). Moreover, recent studies have demonstrated that an E-box near the transcription start site of the AChR δ regulatory region is critical for regulation of the gene by electrical activity (Tang et al., 1994), and similar results have been reported for the regulation of the AChR α-subunit following denervation (Bessereau et al., 1994). Since the expression of other muscle genes with E-boxes in their regulatory regions, such as muscle creatine kinase, is relatively unaffected by denervation (Asher et al., 1992), E-boxes alone are not sufficient to confer activity-dependent regulation. These results raise the possiblity that additional transcriptional regulators, in conjuction with E-box binding proteins, regulate electrical activity-responsive gene expression.

Electrical activity regulates AChR levels post-translationally as well as transcriptionally. By three weeks following denervation, the half-life of pre-existing junctional AChRs decreases roughly 4-fold, from ~10 days to ~3 days (Loring and Salpeter, 1980; Bevan and Steinbach, 1983; Fumagalli et al., 1990). Since treatment of innervated muscle with TTX mimics the effect of denervation on AChR stability, whereas direct stimulation of denervated muscle prevents the change in synaptic AChR half-life (Fumagalli et al., 1990), junctional AChR stability is regulated by electrical activity.

The mechanism of activity-dependent AChR stabilization appears to involve changes in cytosolic calcium and/or cyclic AMP (cAMP). Treatment of chronically denervated muscle in organ culture with calcium ionophore stabilizes synaptic AChRs, while blockade of dihydropyridine sensitive Ca^{2+} channels in electrically stimulated muscle prevents stabilization of endplate AChRs (Rotzler et al., 1991). Curiously, Ca^{2+} influx through the plasma membrane seems to be required for maintenance of junctional receptor stability since increasing internal calcium levels through release of Ca^{2+} from the sarcoplasmic reticulum does not stabilize junctional AChRs in denervated muscle (Rotzler et al., 1991). Treatment of muscles in organ culture with agents which elevate cytoplasmic cAMP levels also can reverse the denervation-dependent changes in the half-life of junctional AChRs (Shyng, et al., 1991); it is not clear, however, whether the cAMP and Ca^{2+} mediated pathways affecting AChR stability share the same mechanism.

In addition to its effects on muscle development and physiology, muscle activity also affects the development of motoneurons. Concomitant with synapse formation, a large fraction of motoneurons degenerate and die. If muscle fiber activity is blocked either presynapticly or postsynapticly during this period, however, a greater percentage of motoneurons survive (Pittman and Oppenheim, 1978; 1979; Laing and Prestige, 1978). On the other hand, electrical stimulation of the muscle increases the number of motoneurons which die (Oppenheim and Núnez, 1982). These studies argue that muscle activity has a role in motoneuron survival.

Similar studies have also demonstrated the role of muscle activity in the elimination of polyneuronal innervation at motor endplates. During the period of synaptogenesis, myofibers become innervated by multiple motoneurons, usually at a single synaptic site. Later in development, the number of motoneurons innervating any single myofiber are culled until each myofiber is innervated by a single motoneuron (Redfern, 1970; Brown et al., 1976). Studies in both developing rat and chick demonstrated that pharmacological paralysis of muscle prevents the loss of multiple innervation (Thompson et al., 1979; Brown et al., 1981; Ding et al., 1983). In contrast, direct electrical stimulation of muscle enhances the rate of synapse elimination (O'Brien et al., 1978).

Additional studies showed that high frequency stimulation of muscle is more effective at accelerating synaptic loss than low frequency stimulation (Thompson, 1983). Moreover, in singly innervated myofibers, local application of α-bungarotoxin to a small region of the endplate results in the loss of postsynaptic AChRs and the withdrawal of that portion of the nerve terminal overlying the region of treated endplate (Balice-Gordon and Lichtman, 1994). These studies suggest that differences in the activity of synaptic sites determine which synapses are lost during synapse elimination. Efforts to test this hypothesis directly in developing muscle, however, have led to conflicting results; in one case the more active input at the endplate was favored, while in another, endplates of the least active input were preserved (Betz et al., 1990; Callaway et al., 1987). Another hypothesis proposed to explain synapse elimination is competition between motoneurons for muscle derived neurotrophic factors. Indeed, recent studies demonstrated that exogenous application of ciliary neurotrophic factor (CNTF) or basic fibroblast growth factor (bFGF) near developing neuromuscular synapses extends the period of polyneuronal innervation in rats (English and Schwartz, 1995). In addition, injections of insulin-like growth factor IGF1 prevent the normal down-regulation of growth-associated proteins in motoneurons which normally occurs during the period of synapse elimination in adult rats (Caroni and Becker, 1992); whether these molecules play any role in endogenous synapse elimination, however, remains unknown.

Nerve terminal sprouting is another phenomenon associated with muscle denervation which is affected by muscle activity. Both presynaptic and postsynaptic blockade of electrical activity induces neurite sprouting from motoneuron terminals (Duchen and Strich, 1968; Brown and Ironton, 1977; Holland and Brown, 1980), and, again, such sprouting can be blocked by direct stimulation of the denervated muscle (Brown et al., 1980). Since extracts of denervated muscle have an enhanced neurite-promoting activity *in vitro* compared with extracts of innervated muscle (Henderson et al., 1983), it is believed that denervated muscle releases one or more diffusable factors which stimulate nerve terminal sprouting. Electrical activity presumably inhibits synthesis of these factors, or, as with AChRs, restricts their expression to the motor endplate region of the innervated muscle fiber. Indeed, studies in partially denervated muscle have suggested that release of sprouting factors may be coupled to AChR insertion into the sarcolemma, since sprouting from terminals adjacent to extrasynaptic regions of the denervated muscle fibers is delayed with respect to sprouting from terminals closer to the denervated endplate (Pockett and Slack, 1982).

Nerve terminal sprouting and synapse elimination may be closely linked mechanisticly. CNTF, bFGF, and IGFs, in addition to affecting synapse elimination, all promote neurite outgrowth *in vitro* and *in vivo* (Gurney et al., 1992; Caroni and Grandes, 1990). Consistent with a role for IGFs in the regulation of neurite sprouting, increases in the levels of IGF1 mRNA and protein in skeletal muscle are detectable within 12 hours following denervation or paralysis, and remain at elevated levels for many days thereafter (Caroni and Schneider, 1994). Similarly, IGF2 mRNA levels are up-regulated in denervated muscle, but with a much slower time course, reaching a maximum (~4-fold increase) at 10 days following denervation (Ishii, 1989; Glazner and Ishii, 1995). Moreover, adult rat motoneurons express IGF1 receptors (Caroni and Becker, 1992), and IGF binding proteins delivered to paralyzed muscle effectively prevent nerve sprouting *in vivo* (Caroni et al., 1994). These studies all suggest a role for IGFs in the regulation of motoneuron physiology by muscle.

In an effort to further understand the affects of innervation and electrical activity on muscle physiology and gene expression, Xuejun Zhu, a graduate student in the lab, developed a PCR-based subtractive-hybridization and cloning technique to identify mRNAs that are differentially-expressed in innervated and denervated rat skeletal muscle. This chapter describes the partial characterization of three novel genes in skeletal muscle, *INN1, DEN1,* and *DEN2*, that Xuejun and I identified using this technique. Our results show that the expression of both *DEN1* and *DEN2* are up-regulated following denervation. In contrast, the regulation of *INN1* expression response to denervation is

more complex; whereas the expression of one transcript decreases, the expression of two other transcript increases following denervation. In addition, our subtractive-hybridization screen identified several known genes whose expression in muscle was not previously known to be regulated by innervation. One of these, the acute myeloid leukemia gene *AML1*, encodes a DNA binding protein with homology to the protein encoded by the *Drosophila* pair-ruled gene *runt*, and disruptions or translocations of the *AML1* gene can result in a form of acute myeloid leukemia. A paper describing the expression of *AML1* in innervated, denervated and developing rat muscle has been published (Zhu et al., 1994) and is included in the following chapter.

MATERIALS AND METHODS

Denervation of rats and preparation of RNA

Adult Sprague-Dawley rats were anesthetized by interperitoneal injection (20 mg/ml ketamine (KetaSet), 1 mg/ml xylazine (Xylaject) in 0.9% NaCl), and the lower leg was denervated by removing ~2 mm of sciatic nerve. RNA from the denervated (4 - 5 days) and the contralateral innervated lower leg muscle was isolated as described (Chomczynski and Sacchi, 1987), and stored at -80°C. Poly $(A)^+$ RNA was purified with oligo(dT) cellulose (Aviv and Leder, 1972).

Preparation of subtraction libraries

Oligo$(dT)_{12\text{-}18}$ (Pharmacia) was used to prime the first strand cDNA synthesis from 2 μg of poly $(A)^+$ RNA. The second strand was synthesized as described (Gubler and Hoffman, 1983). The average size of the cDNA was about 2 kilobases. Innervated and denervated muscle cDNAs were digested with AluI and RsaI separately and pooled to produce short fragments appropriate for PCR amplification. Linkers bearing EcoRI sites were added to the blunt-ended cDNAs, and cDNAs in the range of 150 bp to 1 kb were gel-purified and pooled. PCR amplification, photo-biotinylation of driver DNA, hybridization and removal of biotinylated driver DNA were done as described (Wang and Brown, 1992), except that only 2 μg of target DNA and 40 μg of driver DNA were included in the hybridization reactions. Four rounds each of long and short hybridizations were carried-out and the subtraction efficiency was evaluated by probing subtracted innervated and denervated cDNA with denervated cDNA probe. Denervated subtracted cDNA hybridized 40x stronger than the innervated cDNA, indicating that there is minimal shared sequences between the two subtracted libraries. The subtracted DNAs

were digested with EcoRI and cloned into the EcoR1 site of pBluescript II SK+ (Stratagene) to generate the subtracted libraries. The libraries were screened with random-primed ^{32}P-labeled subtracted probe, and positive colonies were then subjected to further analysis. DNA was sequenced with Sequenase, according to the manufacturer's (USB) instructions.

Initially, 20 colonies from the denervated-minus-innervated subtracted library were screened; 13 colonies contained cDNA encoding αB-crystallin, and 7 colonies contained cDNA encoding either MAP1a, filamin, or AML1. A second denervated subtracted library was prepared by subtracting αB-crystallin sequences from the original library. Eighteen colonies from this library contained cDNAs encoding mRNAs that are expressed at higher levels in denervated than in innervated skeletal muscle; these included cDNAs encoding AChR subunits, N-CAM, filamin, DEN1 and DEN2. At present, only 2 colonies from the innervated-minus-denervated subtracted library have been screened.

Muscle cDNA library construction

Innervated and denervated muscle cDNA libraries were constructed for the isolation of full-length sequences using the isolated cDNA fragments from the subtracted libraries as probes. Muscle mRNA was isolated as described above. First strand of cDNA was primed with an oligo(dT) primer containing a SalI site at the 5' end. The second strand was synthesized as described (Gubler and Hoffman, 1983). The cDNAs were blunt-ended with T4 polymerase and EcoR1 adapters were ligated to the ends. The cDNAs were size selected, digested with SalI, and cloned between the SalI and EcoRI sites of pBluescript II SK+. The average size of the cloned inserts was approximately 1.5 kb.

RNase protection assay

RNase protection assays were performed as described (Simon et al., 1992). Briefly, antisense RNA probes were radiolabelled during transcription *in vitro* from linearized plasmids containing short cDNA inserts, and were hybridized to total RNA in hybridization buffer overnight at 42°C. The reactions were treated with RNases A and T1 to remove unhybridized RNA, digested with proteinase K, and extracted using phenol/chloroform. Protected RNA was precipitated with ethanol, and fractionated in 5% denaturing polyacrylamide gels. Protected RNAs were quantitated by Phosphoimager (Bio-Rad).

Northern blots

Total RNA was fractionated by electrophoresis in 1.1% agarose/formaldehyde gels and transferred to Nytran membrane (Schleicher and Schuell) by downward alkaline capillary transfer (Chomczynski, P., 1992). DNA probes were labeled by random-oligonucleotide priming (High Prime Labeling Kit - Boehringer-Mannheim), and hybridized to membranes overnight in 6X SSPE, 5X Denhardt's solution, 0.5% SDS, and 100 μg/ml herring sperm DNA at 68°C. Membrane were washed in 0.1% SDS, 0.1X SSPE for 1-2 hours at 68°C and exposed with an intensifying screen for up to 8 days. In some experiments, radiolabelled probe was removed prior to rehybridization with a second probe by boiling the membrane in 0.1X SSPE, 0.5% SDS for 10 min.

Immunohistochemistry

Frozen sections (~10 μm) from unfixed rat soleus muscle were stained with a rabbit polyclonal antiserum raised against the N-terminal INN1 peptide (MDGQKKHWKDKVVD) coupled to a MAP-carrier (Research Genetics, Huntsville, AL). Sections were stained overnight at 4°C, followed by incubation with fluorescein-conjugated goat-anti-rabbit IgG (Cappel) (1:200). Sections were mounted in 100 μg/ml paraphenylenediamine, 90% glycerol, 100 mM Tris, pH 9.5, and visualized using optics selective for fluorescein. Images were captured and digitized using a CCD camera (Princeton Instruments), and image processing software software (Metamorph; Universal Imaging Corp.)

Preparation of protein extracts and fractionation.

Muscle from 5-day denervated and contralateral innervated rat lower leg muscle was harvested and quickly frozen in liquid nitrogen. The muscle was pulverized using mortar and pestle under liquid nitrogen and homogenized using a Polytron homogenizer (Brinkman Scientific) in RIPA buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1mM EDTA, plus protease inhibitors: 2 μg/ml aprotinin, 2μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM pefabloc (Boehringer-Mannheim Pharmaceuticals)). The lysates were cleared by centrifugation, aliquoted, and stored at -80°C.

For fractionation, pulverized tissue was homogenized in 20mM Tris maleate pH 7, 0.3 M sucrose, 0.5 mM EDTA, 0.1 mM DTT, 1 mM iodoacetamide, 1 mM benzamidine, 2 μg/ml aprotinin, 2μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM pefabloc using a glass/Teflon homogenizer on ice. The homogenate was centrifuged at 15,000xg for 15 min. at 4°C to remove unbroken cells, nuclei, and connective tissue. KCl to 0.6M was added to the supernatant and the mixture was incubate 1 hr. at 4°C on a rocker platform.

The KCl treated lysate was centrifuged at 100,000xg to pellet membranes. The supernatant containing soluble proteins was saved, and the pellet resuspended in Tris maleate/0.1% Triton X-100 buffer. Aliquots of both soluble and membrane protein fractions were flash frozen in liquid nitrogen, and stored at -80°C.

Protein concentrations were estimated using the *DC* Protein Assay (Bio-Rad).

Western blots

SDS sample buffer was added to whole muscle or fractionated muscle lysates to 1X (62.5 mM Tris pH 6.8, 10% glycerol, 0.1 M DTT, 1% SDS, 2.5 μg/ml bromophenol blue), the samples heated for 5 min. at 65°C, and the proteins fractionated by SDS-PAGE on 12% gels. Following electrophoresis, proteins were transferred to nitrocellulose filters, and stored at 4°C. Protein blots were probed with either the anti-INN1 polyclonal antiserum (1:2000) or monoclonal antibody IXE11$_2$ (1:500) against the rabbit TS28 transverse-tubule antigen (Jorgensen et al., 1990). Horseradish peroxidase (HRP) coupled antibodies were used to detect bound primary antibody, and were visualized by enhanced chemilluminescence (ECL, DuPont).

RESULTS

Identification of mRNAs that are regulated by innervation

We used a PCR-based subtractive-hybridization and cloning method (Wang and Brown, 1991) to identify genes which are regulated by innervation in rat skeletal muscle. Approximately twenty or so genes are known to be regulated by electrical activity and/or innervation (see Table 4.1 legend). cDNAs encoding transcripts from four of these genes (AChR α and δ subunits, N-CAM, and αB-crystallin), whose expression increases following denervation, were detected in a denervated-minus-innervated library screened with a subtracted probe. cDNAs encoding three characterized proteins (MAP1a, filamin, AML1) whose expression was not known to be regulated by electrical activity were also identified in this screen. In addition, we identified cDNAs encoding two novel proteins (*DEN1*, *DEN2*) whose expression increases following denervation and a novel protein (*INN1*) whose expression decreases following denervation. The results of the subtractive-hibridization and cloning screen are summarized in Table 4.1. I selected three of these genes, *INN1*, *DEN1* and *DEN2* for further study.

INN1 mRNA expression is down-regulated following denervation.

To confirm that *INN1* expression in rat muscle is down-regulated following denervation, the levels of *INN1* RNA in both innervated and 5 day denervated rat skeletal muscle were quantified by RNase protection. Figure 4.1 shows that *INN1* mRNA levels decrease approximately 5- to 7-fold following denervation.

Since the expression of several genes are restricted to subsets of muscle fibers (i.e. fast and slow fibers) (see Gunning and Hardeman, 1991), I also examined the expression of *INN1* mRNA in muscles comprised primarily of either fast (extensor digitorum longus, EDL) or slow (soleus) fibers. Figure 4.1 shows that *INN1* mRNA is expressed in both fast (EDL) and slow (soleus) muscle fibers. The level of *INN1* mRNA in innervated fast muscle was slightly higher (approximately 30%) than in innervated slow muscle. *INN1* mRNA, however, is down-regulated similarly (5- to 7- fold) in both fast and slow muscles.

RNase protection assays were also used in a time course experiment to study the rate at which *INN1* mRNA levels fall following denervation. This study reveals that *INN1* mRNA levels decrease gradually following denervation (Fig. 4.2).

In innervated skeletal muscle, mRNAs from several genes are concentrated at synaptic sites owing to selective expression of these genes in subsynaptic nuclei (Merlie and Sanes, 1985; Goldman and Staple, 1989; Brenner et al., 1990). Several non-muscle cell types, including Schwann cells and fibroblast-like cells (Conner and McMahan, 1987; Gatchalian et al., 1989; Weis et al., 1991), are also found near synaptic sites and respond to denervation. Thus, these perisynaptic cells could be the source of *INN1* expression in muscle. Since synaptic sites are usually confined to a restricted area of mammalian muscles, the synapse-enriched and synapse-poor regions of the rat soleus muscle were dissected, and the level of *INN1* mRNA in each fraction was measure by an RNase protection assay. These experiments show that equal amounts of *INN1* RNA are expressed in both synapse-enriched and synapse-poor regions (Fig. 4.3). Thus, *INN1* expression is not restricted to synaptic sites, but is expressed throughout the muscle, presumeably in myofibers. These studies, however, do not exclude the possibility that *INN1* mRNA is expressed in Schwann cells and synaptic fibroblasts in addition to skeletal muscle.

In order to determine whether *INN1* is expressed in tissues other than skeletal muscle, RNase protection assays were performed using RNA isolated from a variety of rat tissues. These studies demonstrate that low levels of *INN1* mRNA can be detected in almost all rat tissues assayed. Significantly higher levels of *INN1* RNA (~10- to 75-fold), however, are expressed in brain, cerebellum, heart, and innervated skeletal muscle (Fig. 4.4). Since brain, heart and skeletal muscle are all electrically active tissues, the

increased expression of *INN1* in these tissues suggests that *INN1* protein may have a function related to responsiveness of these tissues to electrical stimulation (see Discussion).

Cloning of a full-length cDNA encoding *INN1*

The *INN1* cDNA isolated from the library enriched for sequences that are up-regulated in innervated muscle encodes only 217 nucleotides. In order to obtain a full-length *INN1* cDNA, the 217 bp fragment was used to screen an innervated rat muscle cDNA library. A 1650 bp cDNA was isolated from this screen, and sequencing revealed an open reading frame encoding a 199 amino acid protein with a predicted molecular weight of approximately 22 kDa (Fig. 4.5). An ATG start codon at nucleotide (nt) 129 from the 5' end of the insert, is preceded by an in frame stop codon at nt 87 and is flanked by nucleotides with similarity to the consensus sequence for vertebrate translation initiation (Cavener and Ray, 1991). A polyadenylation addition site at nt 1633 is followed by a 20 nucleotide poly A sequence at the 3' end of the 1650 bp cDNA insert. These results suggest that the 1650 bp cDNA represents a full-length *INN1* transcript.

A hydropathy analysis of the INN1 primary sequence reveals two large hydrophobic regions within the INN1 protein (amino acid 24 - 59, and 126 - 151) (Kyte and Doolittle, 1982) (Fig. 4.6A), and this suggests that *INN1* may encode a transmembrane protein; the INN1 protein, however, lacks an amino terminal signal peptide. The INN1 protein contains two N-glycosylation consensus sequences (i.e. Asn-X-Ser/Thr) at residues 106-108 and 177-179, as well as sites for potential phosphorylation by protein kinase C (PKC) (Ser_{60}, Ser_{125}, and Ser_{179}), and tyrosine kinase (Tyr_{12}), but whether such modifications of the INN1 protein occur remains unknown.

INN1 is related to *NSP1*.

A search of protein databases reveals a striking similarity between INN1 and proteins encoded by the neuroendocrine specific protein gene (*NSP1*) from human small cell lung carcinoma cell lines (Roebroek et al., 1993) The human *NSP1* gene encodes three alternatively spliced mRNAs, (~3.4 kb, 2.1-2.3 kb and 1.8 kb), encoding NSP1A, 1B and 1C proteins respectively, and cDNAs encoding each of these transcripts have been isolated (Roebroek et al., 1993). In addition, a rat homologue of NSP1, which is identical to human NSP1C at all but two amino acids (98% identity) has been identified from a rat brain library (Wieczorek and Hughes, 1991). The primary sequences of INN1 and NSP1C, are 66% identical (Fig. 4.6B), thus, the *INN1* cDNA does not encode rat

NSP1. Rather, *INN1* is related to *NSP1* and may be a new member of a larger gene family.

Alternative splicing of *INN1* is regulated by innervation.

To confirm that the 1650 bp INN1 cDNA encodes the full-length INN1 transcript, this fragment was used to probe blots of total RNA isolated from innervated and 5-day denervated rat muscle. Figure 4.7 shows that this probe hybridizes to a major transcript of approximately 1.7 kb whose expression is down-regulated in denervated muscle in accordance with the 5- to 7-fold decrease in *INN1* RNA levels measured by an RNase protection assay. Two minor transcripts of ~2.4 kb and ~5.2 kb were also detected in these experiments, and this result suggests *INN1* encodes multiple transcripts derived by alternative splicing. Further, whereas expression of the 1.7 kb transcript is down-regulated following denervation, expression of the 2.4 and 5.2 kb transcripts is up-regulated, and these results suggest that these splicing events are regulated by innervation.

Since the denervation-induced decrease in 1.7 kb *INN1* transcript levels parallels the decrease in *INN1* RNA measured by RNase protection assays, it seems likely that the probe used in the RNase protection assays described above only recognizes the 1.7 kb *INN1* transcript. If so, then the change in the overall level of *INN1* RNA following denervation may be less significant than those measured by RNase protection, since the 2.4 kb and 5.2 kb *INN1* transcripts are up-regulated following denervation. The sequence of the 217 bp *INN1* cDNA fragment isolated from the innervated-minus-denervated subtractive library is identical to the 5' end of the 1.65 kb *INN1* cDNA (nt 1 - 217). In order to investigate the possibility that this probe only recognizes the 1.7 kb *INN1* transcript, the RNase protection assay was repeated using a probe from an overlapping region of the 1.65 kb *INN1* cDNA (nt 169 - 410). In contrast to the previous results, no difference in the levels of *INN1* RNA in innervated and denervated muscle was detected (Fig. 4.8A). These results suggest that while alternative splicing of *INN1* transcripts responds to denervation, the total amount of *INN1* mRNA remains unchanged. Moreover, these results suggest that at least one additional *INN1* transcript shares the same 3' sequence with the 1.7 kb *INN1* transcript.

Since I have not isolated cDNAs encoding the 2.4 and 5.2 kb *INN1* mRNAs, the sequences of the proteins encoded by these transcripts remains unknown. Comparison with the human *NSP1* gene, however, may provide some insight into the proteins that are likely to be encoded by the alternatively spliced *INN1* mRNAs. *NSP1* encodes at least three proteins which are produced by alternative splicing of the *NSP1* transcript such that

the three proteins share the same 188 carboxy terminal amino acids. If splice sites are conserved between *INN1* and *NSP1*, the different *INN1* transcripts should share the same 3' sequences and should have unique 5' ends. This idea is consistent with the results from the RNase protection assays described above. Moreover, when the sequences of *INN1* and *NSP1C* are aligned, the position of the *NSP1C* splice site aligns with the *INN1* sequence just upstream of the region shared by the two *INN1* RNase protection assay probes (Fig. 4.8B). These results are consistent with the idea that *INN1* is alternatively spliced and that the location of the splice sites in *INN1* and *NSP1* are conserved such that at least two of the three *INN1* transcripts share the same 3' sequences. Further, since the location of this splice site (nt 162) is between Lys_{11} and Val_{12} in the INN1 sequence, it is possible that the INN1 proteins contain unique amino termini.

A 22 kDa protein encoded by *INN1* is expressed in skeletal muscle.

In order to determine the subcellular distribution of INN1 protein in skeletal muscle, I produced a polyclonal antiserum against a peptide encoding the amino-terminal 14 amino acids of INN1. Immunoblotting of extracts from lower leg muscle showed that the INN1 antiserum recognizes a 22 kDa protein (Fig. 4.9), which is consistent with the size of the INN1 protein predicted from the 1.65 kb cDNA sequence. Binding of antibodies to the 22 kDa protein is specific since it can be blocked by preincubation of the antiserum with the appropriate peptide (Fig. 4.9), and the protein is not recognized by pre-immune serum (see Fig. 4.13). The antiserum also recognizes a second protein at 42 kDa, and binding to this protein is also specific since it is not observed with pre-immune serum and is competed with peptide. The 42 kDa protein may represent the protein product of one of the larger *INN1* transcripts. Indeed, if *INN1* transcripts are alternatively spliced and if the splice sites in *INN1* and *NSP1* are conserved as described above, the last three amino acids in the 14 amino acid immunizing peptide should be shared by all INN1 proteins. Thus, binding of the INN1 antiserum to multiple proteins is not entirely unexpected. Alternatively, the 42 kDa protein could be a protein unrelated to INN1 which nevertheless cross-reacts with the INN1 antiserum. Figure 4.9 also shows that roughly equivalent levels of both the 22 kDa and 42 kDa proteins are expressed in innervated and 5-day denervated muscle. Thus, the differential regulation of *INN1* transcripts in innervated and denervated muscle is not reflected in a similar regulation of the level of the INN1 protein at 5 days after denervation. Perhaps longer denervations will be required to detect changes in the level of INN1 protein.

Since the protein encoded by the 1.65 kb *INN1* cDNA contains hydrophobic regions characteristic of membrane proteins, crude membrane fractions from innervated rat

muscle were prepared, and the abundance of INN1 protein was assayed by immunoblotting. Figure 4.10 demonstrates that the 22 kDa INN1 protein is enriched in membrane fractions of rat muscle extracts, and this result is consistent with the idea that INN1 is a membrane protein, tightly associated with membranes, or sequestered within a membrane-bound organelle. Surprisingly, the 42 kDa protein appears to be enriched in the supernatant fraction from these preparations, suggesting that it is a soluble protein. If the splicing of *INN1* and *NSP1* transcripts is conserved, the carboxy-termini of all INN1 proteins should contain two large hydrophobic regions which have been proposed as transmembrane domains (see above). Thus, if the 42 kDa protein is a protein encoded by *INN1*, these hydrophobic regions may not be transmembrane domains. Further, these results would suggest that differences in the amino-terminal portions of the 22 kDa and 42 kDa *INN1* proteins would be responsible for their differential fractionation.

I stained frozen sections of skeletal muscle with the INN1 antiserum to determine the distribution of INN1 protein within skeletal myofibers. In cross-sections of skeletal muscle, anti-INN1 staining reveals a honeycomb-like, reticulated pattern within each myofiber (Fig. 4.11). This staining is specific since it is not observed with pre-immune serum and is competed with preincubation of the antiserum with the peptide. In longitudinal sections of muscle, stripes of α-INN1 staining are observed in register with the sarcomeric I bands (Fig. 4.12). Similar immunostaining patterns have been reported for proteins found in the sarcoplasmic reticulum and in transverse tubules (Jorgensen et al., 1979; Jorgensen et al., 1990). These results suggest that INN1 proteins may be localized to the SR and/or transverse tubules as well. In order to determine whether INN1 is localized to transverse tubules, preparations of KCl-washed microsomal membranes and purified transverse tubule membranes from skeletal muscle were obtained, and the presence of INN1 was determined by immunoblotting. These experiments show that INN1 protein is present in the microsomal membrane preparations, but not in purified transverse tubules which are enriched for the transverse tubule protein TS28 (Fig. 4.13). Thus, INN1 is membrane associated, but is not localized to the transverse tubule membranes of skeletal muscle.

DEN 1 and *DEN2* are up-regulated following denervation.

Two novel genes, *DEN1* and *DEN2*, which are up-regulated in denervated muscle, were identified by subtractive-hybridization and cloning. RNase protection assays show that *DEN1* and *DEN2* are up-regulated 50- to 100-fold and 3- to 5-fold, respectively, after 5-days of denervation (Fig. 4.14). To determine how rapidly *DEN1* and *DEN2* RNA levels increase following denervation I measured their expression levels by an RNase

protection assay and found that increases in *DEN1* and *DEN2* RNA levels are first seen two days following denervation (Fig. 4.15). Between 2 and 5 days following denervation, *DEN2* RNA levels increase only slightly, whereas *DEN1* RNA levels increase substantially (25- to 50-fold) (Fig. 4.15). The time course of the increase in *DEN1* RNA levels in response to denervation is similar to that observed for AChR subunit RNAs (Fig. 4.15).

Northern blots of total RNA from innervated and 5-day denervated rat skeletal muscle show that *DEN1* and *DEN2* encode single transcripts of approximately 9 kb and 2 kb, respectively (Fig. 4.16). These RNA blots confirm the results from RNase protection assays that the levels of *DEN1* and *DEN2* RNA increase approximately 50- and 5-fold, respectively, by 5-days following denervation.

DEN1 encodes a protein with fibronectin type III and immunoglobulin-like repeats.

The 198 bp *DEN1* cDNA isolated by subtraction hybridization cloning was used to probe a cDNA library made from 5-day denervated rat skeletal muscle RNA, and several overlapping cDNAs were isolated. The longest of these, a 3318 bp cDNA, contains a large open reading frame encoding 929 amino acids (Fig. 4.17). The open reading frame begins at the 5' end of the 3.3 kb cDNA and extends to a stop codon at nt 2790. This stop codon was followed by 529 bp of additional DNA, including a polyadenylation addition site (ATTAAA) at nt 3293 and 25 bp of poly A sequence. Since the open reading frame begins at the very 5' end of the 3.3 kb *DEN1* cDNA, and since RNA blots reveal that the full-length *DEN1* cDNA is approximately 9 kb, this 3.3 kb cDNA encodes only the C-terminal region of the *DEN1* protein.

Database searches revealed that DEN1 contains domains which are similar to fibronectin type III motifs (FnIII, F) and immunoglobulin C2 domains (Ig, I). Both FnIII and Ig domains are common to a variety of thick filament-associated structural proteins found in vertebrate and invertebrate muscle, including titin (Labeit et al., 1990; Labeit and Kolmerer, 1995), C-protein (Einheber and Fischman, 1990), M-protein (Noguchi et al., 1992), twitchin (Benian et al., 1989), and smooth muscle myosin light chain kinase (Olson et al., 1990). Beginning with Met 141 in the DEN1 sequence, each motif is repeated four times within the C-terminal region of DEN1. The arrangement of the FnIII and Ig repeats with respect to one another is illustrated in Figure 4.18.

For the most part, the ~100 amino acid FnIII and Ig repeats in the DEN1 C-terminal region follow one right after the other, with no intervening sequence between domains. However, FnIII domain Fd and Ig domain Id are separated by a 12 amino acid stretch containing a KSP motif. Multiple KSP motifs are tandemly arrayed in the interdomain

insertion sequence between Ig domains 5 and 6 in the C-terminus of human cardiac titin (Gautel et al., 1993), and studies have demonstrated that these KSP motifs in titin can be phosphorylated in myoblast-derived cell extracts by cdc2 kinase (Gautel et al., 1993). It is possible that DEN1 may be phosphorylated in a similar manner. Since phosphorylation of KSP motifs in neurofilaments inhibits the ability of neurofilaments to association with microtubules (Hisanaga et al., 1991), KSP motifs may similarly regulate the ability of titin and/or DEN1 to interact with other muscle structural proteins.

Database searches showed that the 5' most 140 amino acids preceding the first DEN1 FnIII domain bear little similarity with any known proteins. However, this region is enriched in charged amino acids (45 of 140, 32%), suggesting that this region of the protein may interact with other hydrophilic moieties. Alternatively, this region of the *DEN1* protein may be an interdomain insertion sequence separating the first FnIII motif (Ia) of the C-terminal region with other, as yet unsequenced, upstream FnIII and Ig motifs. Since the largest interdomain insertion sequence in the C-terminal region of titin is 490 bp (Gautel et al., 1993), finding additional FnIII and Ig domains further 5' would not be unexpected. Isolation of cDNAs encoding more 5' *DEN1* sequence should resolve this issue, and may provide greater insight into DEN1 function.

DEN2 encodes a heat shock-related protein.

The *DEN2* cDNA fragment isolated from the subtractive-hybridization screen was used likewise to screen a 5-day denervated rat muscle cDNA library, and a 1.7 kb cDNA was isolated. Since RNA blots demonstrate that *DEN2* encodes a single transcript approximately 2 kb in length, it is likely that the 1.7 kb cDNA encodes the full-length *DEN2* protein. Using convenient restriction sites to create several deletion constructs, greater than 90% of this 1.7 kb cDNA has been sequenced (Fig. 4.19A). Searches of nucleotide databases reveal a region of the *DEN2* cDNA that is identical to an expressed sequence tag (EST) (EST111753) isolated from an NGF-treated rat PC-12 cell library. Regions of the *DEN2* cDNA also share considerable homology (71% to 90%) to EST sequences isolated from diverse species and tissues, including mouse liver/spleen, and human placenta and eye, and the high degree of similarity between these sequences and the *DEN2* sequence suggests that these ESTs may be derived from homologs of *DEN2*.

Searches of protein databases using ORFs from the sequenced portions of the 1.7 kb cDNA reveal an ORF with similarity to a family of small, 20 - 28 kDa heat shock proteins, one of which, HSP27, has been cloned from rat (Uoshima et al., 1993). αB-crystallin, whose expression also increases in skeletal muscle following denervation (see Table 4.1, but see Inaguma et al., 1993), is also related to these small heat shock proteins,

and recent studies have shown that both αB-crystallin and HSP27 proteins can serve as molecular chaperones to prevent aggregation of unfolded proteins during heat shock (Jakob et al., 1993). Sequence comparison shows that DEN2 is 41% (80/196 a.a.) similar to rat HSP27 and 31% similar (60/196 a.a.) to rat αB-crystallin (Fig 19B). (HSP27 and αB-crystallin are 36% (74/205 a.a.) identical.) Taking conserved amino acid substitutions into consideration, similarities between DEN2/HSP27 and DEN2/aB-crystallin rise to approximately 62% and 57%, respectively. Thus, *DEN2* appears to encode another member of the family of small heat shock proteins with slightly greater similarity to HSP27 than to αB-crystallin.

DISCUSSION

We have used subtractive-hybridization cloning to identify three novel genes - *INN1*, *DEN1* and *DEN2* - whose expression is differentially regulated in innervated and denervated rat skeletal muscle. RNase protection assays and RNA blots confirmed that *INN1* is down-regulated, while *DEN1* and *DEN2* are up-regulated following denervation. Using the original isolates as probes, cDNAs encoding full-length *INN1* and *DEN2* transcripts and a partial *DEN1* transcript were isolated, and sequencing of these showed that all three genes encode proteins which bear significant similarity to different, known proteins.

INN1 is a novel gene which is related to *NSP1*. Sequence analysis showed that the 1.65 kb *INN1* cDNA encodes a 22 kDa protein which is 66% identical to NSP1C. Further, both genes encode multiple transcripts, and RNase protection assays using probes from two adjacent regions of the *INN1* sequence suggest that *INN1* transcripts, like *NSP1* transcripts, share the same 3' sequences, and that the location of at least one splice site may be conserved between *NSP1* and *INN1*. *NSP1* has been cloned from both human small cell lung carcinoma cells (Roebroek et al., 1993) and rat brain (Wieczorek and Hughes, 1991), and its expression is limited to tissues of neuroendocrine origin. In rat, tissue distribution studies showed that *NSP1* is expressed only in brain. Since *NSP1* expression was detected in cultured neurons, but not in cultured glia, the *NSP1* expression detected in brain is probably neurally derived (Wieczorek and Hughes, 1991). Results reported here show that *INN1*, unlike *NSP1*, is expressed at low levels in nearly all tissues, however, expression is substantially higher in brain, heart and skeletal muscle. Thus, although *INN1* and *NSP1* are expressed in different subsets of rat tissues, both are expressed to the greatest extent in electrically excitable tissues.

The functions of the *INN1*- and *NSP1*-encoded proteins remain unknown, but, the fact that both genes are expressed preferentially in electrically stimulated tissues, suggests that they may play rolls in the responsiveness of these tissues to electrical stimulation. Primary sequence analysis did not reveal any functional domains which might provide clues as to the roles INN1 and NSP1 play in electrically excitable cells, but both proteins contain two large hydrophobic regions which suggests that they are transmembrane proteins. Indeed, immunoblotting experiments using an antiserum raised against the amino terminal peptide of INN1 showed that INN1 is enriched in the high-speed pellet of fractionated skeletal muscle, and this is consistent with the idea that INN1 is associated with membranes. Immunofluorescence studies using the INN1 antiserum revealed a polygonal staining pattern within each individual myofiber in cross-sections of skeletal muscle, while staining of longitudinal sections revealed INN1 immunoreactivity associated with the Iband region of the sarcomere Similar polygonal patterns and I-band associated immunofluorescence have also been observed in muscle sections stained with antibodies directed against proteins of the sarcoplasmic reticulum (SR) and transverse tubules (Jorgensen et al., 1979; Jorgensen et al., 1990), and these results suggest that INN1 may be associated with one of these intracellular membrane compartments. Systematic immunoblotting studies to determine the subcellular distribution of INN1 have yet to be performed, however, I found that INN1 immunoreactivity is not enriched in immunoblots of muscle membrane fractions enriched in transverse tubular proteins. Therefore, INN1 may be associated with SR rather than transverse tubules.

Despite the absence of INN1 in preparations enriched in transverse tubules, a role for INN1 in excitation/contraction coupling, remains intriguing. One of the functions of the SR is to regulate intracellular Ca^{2+} concentration during excitation-contraction coupling (for review see Rüegg, 1988). Electrical stimulation of muscle induces Ca^{2+} ion release from the SR, resulting in an increase in intracellular Ca^{2+} concentrations and the initiation of contraction. Ca^{2+} is then rapidly resequestered into the SR, causing the contractile apparatus to return to the resting state.. Local changes in Ca^{2+} concentrations have also been observed in dendritic spines of hippocampal neurons following stimulation (Guthrie et al., 1991; Müller and Connor, 1991; Yuste and Denk, 1995), and precise regulation of intracellular Ca^{2+} concentrations within dendritic spines is thought to be important in neuronal input integration and synaptic plasticity. During long-term potentiation (LTP), the most commonly studied model for synaptic plasticity, most of the stimulus-induced rise in internal Ca^{2+} within post-synaptic dendrites has been attributed to Ca^{2+} flux through NMDA receptors (for review, see Malenka, 1992), but recent studies have suggested that Ca^{2+}-induced Ca^{2+} release from internal Ca^{2+} stores can occur

following repeated presynaptic stimulation (Alford et al., 1993; Llano et al., 1994). In addition, thapsigargin, an inhibitor of Ca^{2+} release for internal stores, interferes with the induction of LTP (Harvey and Collingridge, 1992). Thus, regulation of Ca^{2+} release from intracellular compartments may also play a role in synaptic modulation in the CNS. The apparent enrichment of INN1 immunoreactivity in muscle membrane fractions, its intracellular distribution in stained muscle sections, and the preferential expression of INN1 in muscle and neural tissues, are all consistent with the a role for INN1 (and by analogy, NSP1) in the release and/or uptake of intracellular Ca^{2+} ions. Localization of INN1 in both heart and brain and more precise immuno-ultrastructural localization in muscle should provide further insight in the role of INN1 in electrically excitable cells.

My analysis of *INN1* transcripts in muscle shows that *INN1* encodes three transcripts, and that these transcripts are differentially regulated by innervation. Further, comparison with *NSP1* suggests that the multiple *INN1* transcripts are generated by alternative splicing, and this implies that alternative splicing of *INN1* is regulated by innervation. Although cDNAs encoding the two larger *INN1* transcripts have not been cloned, my data shows that two probes derived from adjacent regions of the *INN1* cDNA yield divergent results in RNase protection assays of innervated and denervated muscle, and these results are consistent with the idea that splicing of INN1 is regulated by innervation. Studies have shown that NCAM transcripts are differentially regulated in innervated and denervated muscle (Covault et al., 1986), and that expression of fast muscle troponin T isoforms is altered following denervation of adult muscle (Matsuda et al., 1984; Shimuza and Shimada, 1985). Since the different isoforms of both N-CAM and troponin T are encoded by a single gene (Wilkinson et al., 1984; Breitbart et al., 1985), these studies demonstrate that alternative splicing can be a target for neural regulation in muscle. The biochemistry of alternative splicing mechanisms has been studied extensively in the rat troponin T and α-tropomyosin genes (for reviews, see Nadal-Ginard, 1990 and Nadal-Ginard et al., 1991), and these studies suggest that both cis- and tissue-specific trans-acting factors are important for splicing decisions. Although splicing factors may be activated in response to denervation by post-translational mechanisms, trans-acting splicing factors may be a targets for differential transcriptional regulation by innervation. Subtraction-hybridization strategies similar to the one reported in this study may prove useful in the isolation and characterization of such splicing factors.

We isolated a partial cDNA encoding the C-terminus of the putative DEN1 protein, and sequencing analysis showed that the C-terminal end of DEN1 is composed of four copies of each of FnIII and Ig motifs. Although identified originally in the sequences of

extracellular proteins, both FnIII and Ig motifs are commonly found in a variety of filamentous, thick filament-associated, sarcomeric proteins in both vertebrate and invertebrate muscle. These include: titin (Labeit et al., 1990; Labeit and Kolmerer, 1995), skelemin (Price and Gomer, 1993), C-protein (Einheber and Fischman, 1990), H-protein (Vaughn et al., 1993) M-protein (Noguchi et al., 1992), twitchin (Benian et al., 1989) and projectin (Ayme-Southgate et al., 1991). FnIII and Ig motifs are found also in smooth muscle myosin light chain kinase (Olson et al., 1990). In extracellular proteins, FnIII and Ig motifs are thought to mediate cell-cell and cell-substrate interactions through both homophilic interactions between like domains and heterophilic interactions with cell-surface receptors (Cunningham et al., 1987 ; Williams and Barclay, 1989). Similarly, in sarcomeric proteins, these domains are thought to mediate interactions among thick filament-associated proteins, and between these proteins and myosin. For example, titin is capable of binding both myosin and C-protein (Soteriou et al., 1993; Labeit et al., 1992), and can cause synthetic myosin filaments to aggregate *in vitro* (Maruyama et al., 1988). Further, the myosin binding domain of C-protein has been localized to its C-terminal Ig motif (Okagaki et al., 1993). When compared with those of other proteins, the FnIII and Ig repeats in DEN1 most resemble those in titin, but their arrangement in the C-terminus of DEN1 (I-I-F-F-F-I-F-I), clearly distinguishes *DEN1* from any previously cloned muscle structural gene. Thus, *DEN1* encodes a novel protein with structural similarity to thick-filament-associated proteins in muscle sarcomeres. At present, antibodies against DEN1 protein are not available, and the distribution of DEN1 in denervated and/or innervated muscle remains unknown, yet the similarity between DEN1 protein and these sarcomeric proteins suggests that DEN1 can interact with components of the thick filament assembly.

The C-terminal region of DEN1 also contains a KSP sequence within the short 12 amino acid sequence separating the last FnIII and Ig repeats. Large, tandem arrays of KSP motifs have been characterized in the multi-phosphorylation repeat in the C-terminal tail domain of neurofilament-H and -M subunits (Myers et al., 1987; Lees et al., 1988), and studies have shown that phosphorylation of this KSP containing domain by cdc2 kinase abolishes interactions between neurofilaments and microtubules (Hisanaga et al., 1991). Thus, the regulation neurofilament interactions with other proteins is mediated by phosphorylation of these KSP sequences. Four such KSP motifs are present also in an interdomain insertion sequence in the C-terminus of cardiac titin, and studies have shown that the Ser residues on all four KSPs motifs are phosphorylated *in vitro* by neonatal muscle extracts (Gautel et al., 1993). Further, these authors found that KSP phosphorylation is two orders of magnitude lower in extracts prepared from adult muscle,

and that KSP kinase activity falls dramatically following differentiation of myotubes in culture. These data suggest that titin phosphorylation is under developmental control, and that regulation of titin phosphorylation is important in maturation of the sarcomere. The presence of a KSP motif in the C-terminus of DEN1 raises the possibility that phosphorylation of DEN1 is regulated in a similar fashion; however, I have not investigated whether DEN1, like titin, is developmentally regulated, nor whether phosphorylation of the KSP motif in DEN1 occurs. Studies directed at addressing these issues, in conjunction with the preparation of antibodies to determine the distribution of DEN1 protein in muscle, will certainly aid in understanding DEN1 function.

DEN2, the third novel cDNA isolated from our subtracted muscle libraries, encodes a member of the family of small heat shock proteins which include HSP27 and αB-crystallin. Both HSP27 and αB-crystallin have been cloned from rat (Uoshima et al., 1993; Atomi et al., 1991), and studies have shown that significant levels of HSP27 and αB-crystallin protein are expressed in rat skeletal muscle (Kato et al., 1991; Inaguma et al., 1993) Curiously, the levels of both proteins are considerably higher in slow-twitch, than in fast-twitch muscle (Kato et al., 1991; Inaguma et al., 1993), and, at least in the case of αB-crystallin, the higher level of protein in soleus is reflected in significantly higher levels of RNA (Atomi et al., 1991). Moreover, studies in denervated muscle have demonstrated that the levels of αB-crystallin RNA and protein increase dramatically in fast-twitch muscle by 3 days following denervation, but decrease in slow-twitch muscle (Atomi et al, 1991; Inaguma et al., 1993). Changes in the level of HSP27 protein in fast and slow muscle in response to denervation parallel those seen for αB-crystallin protein, but the decrease in HSP27 protein in denervated slow muscle is less dramatic than that observed for αB-crystallin (Inaguma et al., 1993). Thus, expression of both αB-crystallin and HSP27 and their response to denervation are regulated by fiber type. Our studies show that *DEN2* expression increases 3- to 5-fold by 5 days following denervation; however, we have not studied the fiber type specificity of *DEN2* expression, nor whether the increased expression of *DEN2* in response to denervation is restricted to either fast or slow muscle. It may be that denervation-induced changes in *DEN2* gene expression in certain fiber-types may be masked by minimal, or even inverse changes in other fiber types. Thus, as was done for *INN1*, it will be necessary to study both the fiber-type specificity, and whether changes in expression following denervation are restricted to certain fiber types for all of the cDNAs isolated from our subtraction hybridization libraries.

HSP27 and αB-crystallin are expressed in a variety of rat tissues in addition to skeletal muscle (Kato et al., 1991; Inaguma et al., 1993). Although we have not studied

the distribution of DEN2 in different rat tissues, ESTs encoding what appear to be *DEN2* homologues have been isolated from cDNA libraries derived from several different tissues from various species, including liver/spleen (mouse), placenta (human), and eye (human). Thus, it seems likely that *DEN2* is expressed in other tissues in addition to skeletal muscle, and this suggests a more ubiquitous role for *DEN2* in cellular physiology.

Biological functions for the small heat shock proteins remain unclear. Studies have shown that both αB-crystallin and HSP27 proteins are induced by heat shock (Klemenz et al., 1991), and that both proteins can act as molecular chaperones preventing aggregation of unfolding proteins during heat shock (Jakob et al., 1993). HSP27 mRNA and protein levels are also induced in estrogen stimulated cells (Adams et al., 1983; Fuqua et al., 1989), and a recent study has implicated HSP27 in the increase of endothelial cell growth rates in response to estrogen (Piotrowicz et al., 1995). Other studies have implicated HSP27 in the regulation of microfilament dynamics. In mouse fibroblasts, HSP27 is localized in lamellapodia, and overexpression of HSP27 in these cells increases both the concentration of cortical F-actin and the rate of pinocytotic activity, and prevents actin stress-fiber depolymerization induced by heat shock or cytochalasin D treatment (Lavoie et al., 1993a; 1993b). Moreover, the HSP27-mediated affects on actin filament dynamics appear to be dependent on phosphorylation of HSP27 (Lavoie et al., 1993b; 1995), and this result suggests that HSP27 may have a role in signal transduction between mitogens and actin filaments. Interestingly, an avian homologue of HSP27 blocks actin polymerization *in vitro* (Miron et al., 1988; 1991), and the inhibitory affect of this protein on actin polymerization is reduced also by phosphorylation (Benndorf et al., 1994).

Denervation of skeletal muscle is characterized by atrophy of the myofibrillary apparatus featuring an early accelerated loss of myosin thick filaments relative to actin thin filaments (Jakubiec-Puka et al., 1981), and changes in the dimensions of mitochondria and the SR/transverse-tubule membrane systems (Engel and Stonnington, 1974). Denervated muscle also manifests increased endocytotic activity, particularly at the motor endplate (Libelius and Tågerud, 1984), which appears to be coupled to an increased rate of exocytotic activity (Vult von Steyern et al., 1993). Thus, it is tempting to speculate that DEN2 and perhaps other members of the small HSPs play a role in modulating the muscle cytoskeleton, including stabilizing actin filaments in the sarcomere. Our finding that RNAs for the cytoskeletal proteins filamin and MAP1a are also enriched in denervated muscle may be indicative also of the degree of cytoskeletal remodeling occurring in denervated muscle. Again, immunohistochemical studies on the

localization of DEN2 and the other small HSPs in may shed some light on their role in skeletal muscle physiology.

The mechanisms regulating the innervation-dependent changes in muscle gene expression are unknown, but at least two pathways are indicated: first, expression might be regulated by myotrophic factors released from the nerve terminal, and second, expression is influenced by nerve-induced electrical stimulation.

Evidence for the first comes from studies demonstrating that functional and morphological changes in immobilized or paralyzed muscle are less severe than in denervated muscle (Spector, 1985b). Further, the administration of extracts from peripheral nerve can partially ameliorate denervation-induced atrophy of skeletal muscle (Davis and Kiernan, 1981; Heck and Davis, 1988). In this model, denervation and subsequent degeneration of the nerve results in the removal of such factors from the muscle milieu. The proposed myotrophic factor could have either positive or negative effects on muscle gene expression in innervated muscle; for example, either maintaining the expression the 1.7 kb *INN1* transcript or repressing transcription of genes such as *DEN1* whose expression is very low in innervated muscle.

No specific myotrophic factors have been identified, however, a molecule from peripheral nerve extracts with myotrophic activity in denervated muscle has been partially purified (Davis et al., 1985), and more recently, ciliary neurotrophic factor (CNTF) has been shown to have a similar trophic effect on denervated muscle (Helgren et al., 1994). Since CNTF is expressed abundantly in myelin-related Schwann cells in sciatic nerve (Rende et al., 1992; Friedman et al., 1992), and expression of receptors for CNTF are rapidly up-regulated following denervation of muscle (Davis et al., 1993), CNTF seems a likely myotrophic factor *in vivo*. On the other hand, mice which lack a functional CNTF gene are viable, and only exhibit signs of muscle weakness many months after birth at a time when motoneurons in these mice have begun to degenerate (Masu et al., 1993). In any case, such myotrophic signals are clearly different from the molecule which has been implicated in gene activation in synaptic nuclei, since this signal persists in the synaptic basal lamina following nerve lesion and degeneration (Jo and Burden, 1992).

The ability of electrical activity to regulate muscle genes expression is well established; studies have shown that exogenous stimulation of denervated muscle via implanted electrodes prevents the expression of extrasynaptic AChRs which normally occurs following denervation, whereas extrasynaptic AChR expression is enhanced in muscle which has been rendered electrically inactive either by pharmacological paralysis or blockade of synaptic transmission (Lømo and Rosenthal, 1972; Lømo and Westergard,

1975). Interestingly, the mechanisms linking electrical stimulation to gene transcription appear to differ among species. In rat myotubes in culture, suppression of AChR expression by electrical activity can be reversed by increasing intracellular cAMP levels, which suggests that muscle activity and AChR expression are coupled via a cAMP-dependent pathway (Chahine et al., 1993). Consistent with this notion, both denervation and TTX paralysis of muscle increase cAMP and protein kinase A activity in muscle (Chahine et al., 1993). In contrast, the down-regulation of AChR expression by muscle activity in chick appears to involve a PKC-dependent pathway, since inhibitors of PKC or depletion of PKC activity by prolonged exposure to phorbol esters increases expression of AChR α subunit in myotube cultures (Klarsfeld et al., 1989) and prolongs AChR subunit gene expression in electrically stimulated, denervated muscle *in vivo* (Huang et al., 1992). Moreover, PKC activity is rapidly increased in electrically stimulated, denervated chick muscle, and this is consistent with PKC's role in negative regulation of muscle gene expression in response to electrical stimulation. (Huang et al., 1992). In both organisms, however, calcium appears to play a central role, since treatment of both chick and rat myotubes in culture with calcium ionophore blocks TTX-induced increases in AChR mRNA (Klarsfeld et al., 1993; Walke et al., 1994), and in chick, treatment of myotube cultures with the calcium channel blocker verapamil increases the levels of AChR α subunit RNA and protein (Klarsfeld et al., 1989).

One of the targets for stimulation-evoked kinase activity may be one of the myogenic factors. Studies in transgenic mice have demonstrated that mutation of an E-box near the transcriptional start site in a gene fusion between the mouse AChR δ subunit promoter and human growth hormone (hGH) reduces the amount of hGH expressed in denervated muscle compared to a gene fusion with the wild-type AChR δ promoter (Tang et al., 1994). In addition, mRNAs for several myogenic factors increase following denervation (Duclert et al., 1991; Eftimie et al., 1991; Witzemann and Sakmann, 1991; Weis, 1994), and this is consistent with a possible role for these myogenic factors in electrical activity-dependent regulation. Since phosphorylation of myogenin by PKC can abolish its DNA-binding activity (Li et al., 1992; Mendelzon et al., 1994), it has been proposed that stimulation-dependent activation of PKC results in phosphorylation of myogenin, and abolishes myogenin's ability to activate target genes (Mendelzon et al., 1994).

While myogenic factors may be important for regulating transcription of muscle genes in response to electrical activity, not all genes containing E-boxes in their regulatory regions are affected by stimulation. E-box containing genes which do not respond to electrical activity may be regulated by factors indifferent to electrical activity.

These factors may dominate the affect of bHLH proteins on the expression of these genes in adult muscle, rendering them insensitive to electrical activity-induced changes in bHLH protein activity. Alternatively, additional trans-acting factors, acting via as yet uncharacterized electrically responsive cis-elements, may exist which are required, in conjunction with E-boxes, to regulate electrical activity-dependent gene expression. If additional factors are induced in denervated muscle, we may be able to identify them using the same subtractivehybridization libraries we used to isolate cDNAs for *DEN1* and *DEN2*. Since the denervation-induced increase in the expression of these factors may precede that of their target genes, as has been observed for myogenin, subtraction-hybridization libraries using RNA prepared from 1-2 day denervated muscle may be more useful in identifying additional denervation-responsive factors.

Interestingly, one of the cDNAs isolated from our subtractive-hybridization libraries encoded rat *AML1*, and RNase protection assays revealed that *AML1* transcripts increase ~100-fold following denervation. Since *AML1* encodes a DNA binding protein with similarity to the protein encoded by the *Drosophila* pair-ruled gene *runt*, our results suggest that AML1 is a potential mediator of denervation-dependent gene expression. We went on to characterize the expression of *AML1* more thoroughly in rat muscle, and these results are reported in the following chapter.

Our screens of the innervated- and denervated-subtractive libraries were not exhaustive (see Materials and Methods), since we isolated only four of the twenty genes known to be induced by denervation. These libraries have also proven useful in identifying genes (e.g. *INN1*) in which splicing, rather than gene expression, changes in response to denervation. Additional rounds of subtraction to remove sequences identified from previous screens may facilitate identification of additional cDNAs which may be represented in lower abundance in these libraries.

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Table 4.1. mRNAs identified by subtractive-hybridization cloning

cDNA	RNA level (Den/Inn)	Abundance (%) in innervated muscle
AChR α subunit	15	0.001
AChR δ subunit	15	0.001
N-CAM	40	0.001
crystallin (αB)	6	0.02
MAP1a	12	0.001
filamin	5	0.04
AML1	100	0.0001
DEN1. Novel. Contains Ig and Fn III repeats characteristic of muscle structural proteins	50-100	0.001
DEN2. Novel. Similarity to αB crystallin and small heat shock proteins.	3-5	0.05
INN1. Novel. Similarity to NSP1C.	0.2	0.05

The following mRNAs are known to be up-regulated (>3-fold) following denervation of skeletal muscle: αB crystallin (Atomi et al., 1991), AChR subunits (Evans et al., 1987), muscle specific kinase (Valenzuela et al., 1995), N-CAM (Covault et al., 1986), sodium channels (SkM2 subtype) (Yang et al., 1991), myogenin and MyoD (Duclert et al., 1991; Eftimie et al., 1991; Witzemann and Sakmann, 1991), jun proto-oncogenes (Bessereau et al., 1990), muscle LIM protein (Arber et al., 1994), glucose transporter (GLUT-1) (Block et al., 1991), N-cadherin (Hahn and Covault, 1992), CNTF receptor subunits (Helgren et al., 1994), carbonic anhydrase III (Milot et al., 1991), and decorin (Brandan et al., 1992).

In contrast, the following RNAs are down-regulated (>3-fold) following denervation: AChE (Michel et al., 1994), glucose transporter (GLUT-4).(Block et al., 1991), and sarcoplasmic reticulum Ca^{2+} pump (Schulte et al., 1994).

Figure 4.1. *INN1* is down-regulated following denervation.
Levels of *INN1* RNA decrease ~5-fold by 5 days following denervation in both fast and slow muscle. Total RNA was prepared from innervated (I) and 5-day denervated (D) rat EDL and soleus (Sol) muscles, and levels of *INN1* and GAPDH RNA were measured by an RNase protection assay. ~10 μg or ~5 μg of total RNA were used for the INN1 and GAPDH assays, respectively. In most experiments, we observed a decrease (<50%) in GAPDH RNA:total RNA ratio following denervation. Quantitation by Phosphoimager showed that approximately equal ratios of *INN1* RNA relative to GAPDH are present in EDL and soleus.

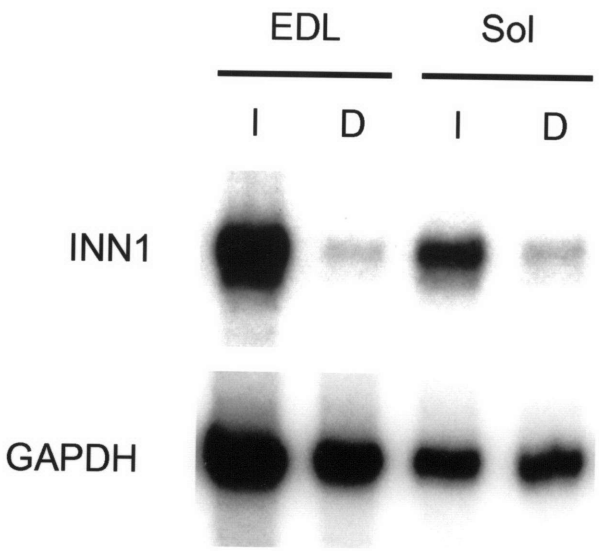

EDL
Sol
I
D
I
D
INN1
GAPDH

Figure 4.2. *INN1* RNA decreases gradually following denervation.
Total RNA was prepared from innervated (Inn) rat lower leg muscle and muscle denervated for 1, 2 and 5 days, and the levels of *INN1*, AChR δ-subunit and GAPDH RNA were measured by an RNase protection assay. The GAPDH control suggests that the amount of RNA used in the 1-day denervated muscle lanes is ~50% lower than that used in 2-day denervated muscle lane.

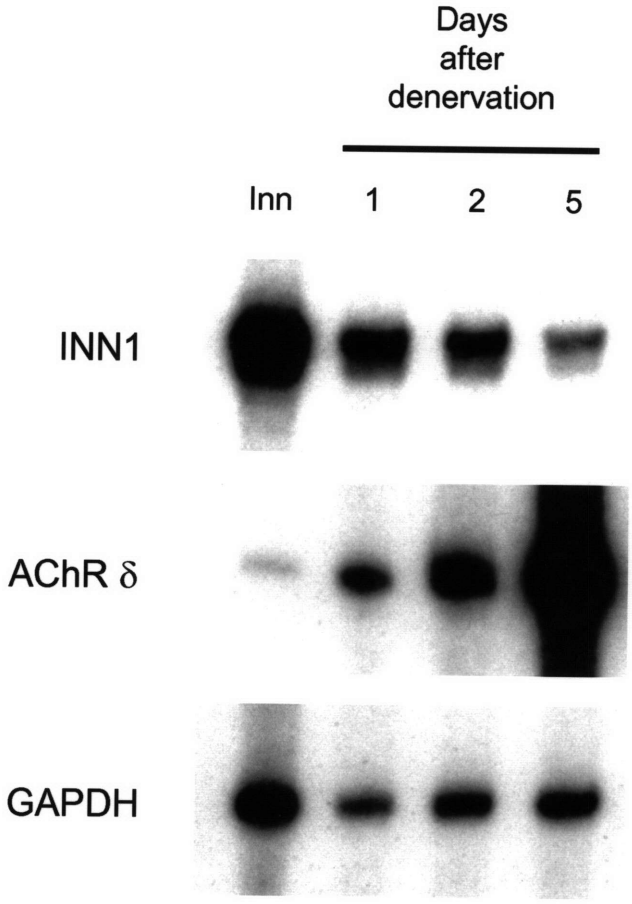

Days
after
denervation
Inn
1
2
5
INN1
AChR δ
GAPDH

Figure 4.3. *INN1* RNA is not enriched at the motor endplate.
Rat soleus muscle was dissected into synapse-enriched (ep^+) and synapse-free (ep^-) regions, total RNA was prepared, and the level of *INN1* RNA was measured by an RNase protection assay. 10 μg and 1 μg of total RNA were used in the INN1 and GAPDH assays, respectively. The levels of both *INN1* and GAPDH RNA are similar in both the synapse-enriched and synapse-free fractions. In contrast, the level of AChR ε-subunit RNA is approximately 12-fold higher in the synapse-enriched fraction than in the synapse-poor fraction, demonstrating that the dissection enriches for synaptic nuclei.

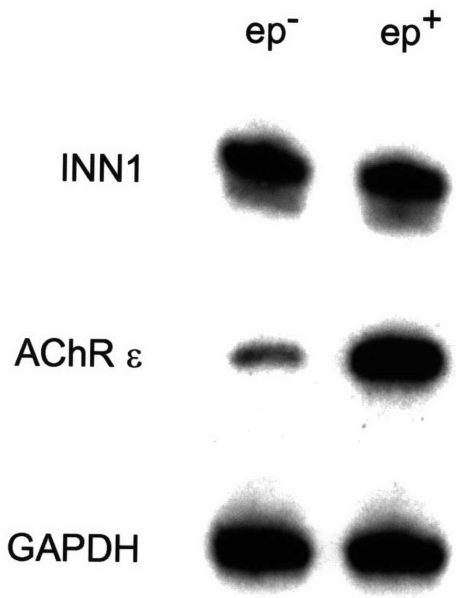

ep-
ep+
INN1
AChR ε
GAPDH

Figure 4.4. Tissue distribution of *INN1* RNA.

Total RNA was prepared from the indicated rat tissues, and the levels of *INN1* RNA were measured by an RNase protection assay (20 μg RNA/assay). Low levels of *INN1* RNA were detected in kidney, lung, stomach and testes. Significantly higher levels of *INN1* RNA were detected in brain, cerebellum, heart, and skeletal muscle. Little, if any, *INN1* RNA was detected in liver, but GAPDH RNA levels were also substantially lower, suggesting that the concentration of liver RNA used in the assays was much lower than expected. *INN1* RNA levels are ~10-fold higher in cerebellum and ~30-fold higher in brain than in stomach and kidney (normalized to GAPDH). GAPDH is reported to be expressed at higher levels in skeletal and heart muscle than in non-muscle rat tissues (2 - 5-fold) (Stauffer et al., 1993). Therefore, the levels of *INN1* RNA in heart and innervated skeletal muscle are on the order of 20- to 75-fold higher relative to that found in kidney.

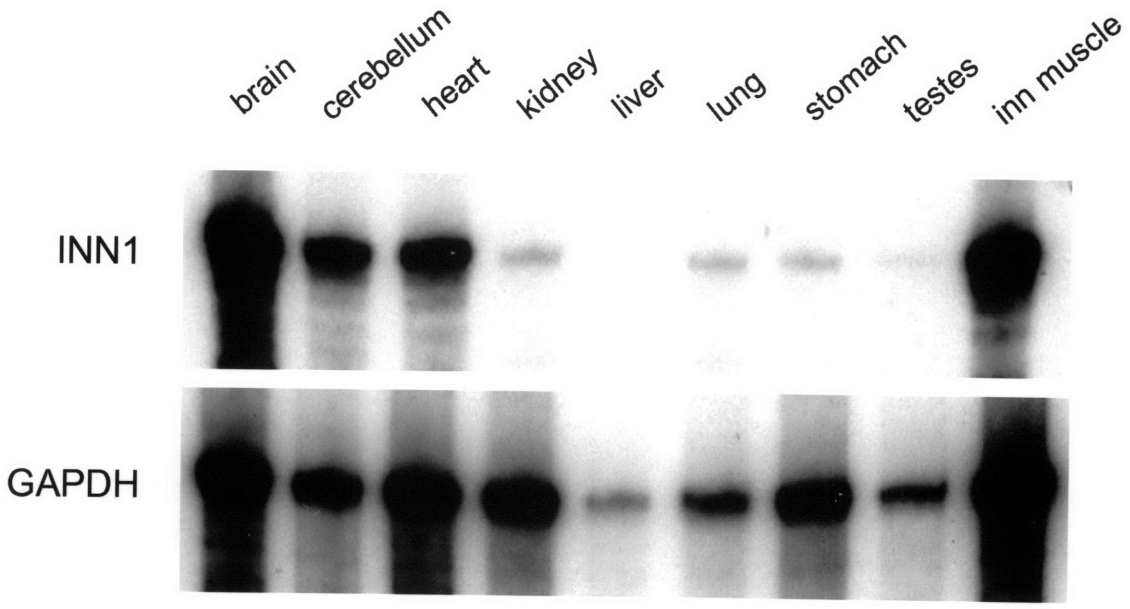

brain
cerebellum
heart
kidney
liver
lung
stomach
testes
inn muscle
INN1
GAPDH

Figure 4.5. Complete nucleotide sequence of the *INN1* cDNA.

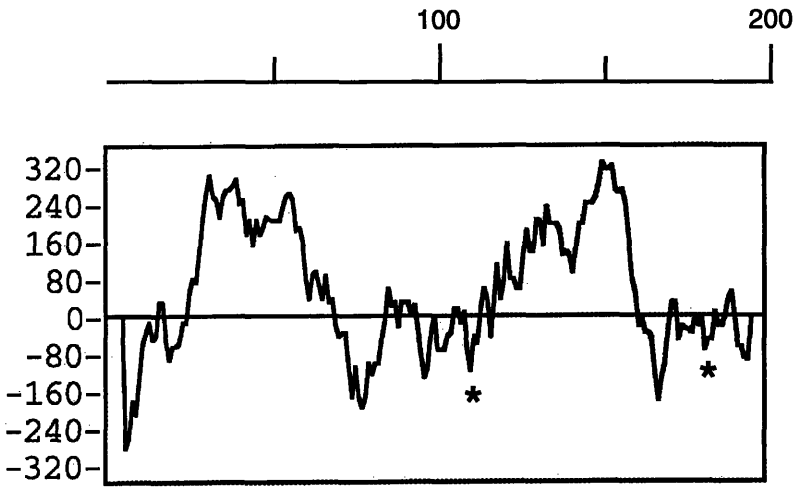
The complete nucleotide and encoded amino acid sequence of the 1.65 kb INN1 cDNA is shown. A TGA stop codon is indicated by an asterisk. Sites for potential N-linked glycosylation are boxed, and a site for polyadenylation addition is underlined. Nucleotide and amino acid positions (parentheses) are given at right.

```
5'   GAATTCGGACTGCAGGCTTAGTCTGGGGAAGCGGGTGTTTCATGTCTCAGGGAGAATTTT        60
     GCAGTTTACAGCGTTTCTGTTGGTATGCATAATTTGTAATTGCTGCTGGAGGGCAGATCG       120
     TGGCAAGAA   ATG GAC GGA CAG AAG AAA CAT TGG AAG GAC AAG GTT GTT     168
                  M   D   G   Q   K   K   H   W   K   D   K   V   V    (13)

     GAC CTC CTC TAC TGG AGA GAC ATT AAG AAG ACT GGA GTG GTG TTT GGT     216
      D   L   L   Y   W   R   D   I   K   K   T   G   V   V   F   G    (29)

     GCC AGC TTA TTC CTG CTG CTG TCT CTG ACA GTG TTC AGC ATT GTC AGT     264
      A   S   L   F   L   L   L   S   L   T   V   F   S   I   V   S    (45)

     GTA ACG GCC TAC ATT GCC TTG GCC CTG CTC TCG GTG ACT ATC AGC TTT     312
      V   N   A   Y   I   A   L   A   L   L   S   V   T   I   S   F    (61)

     AGG ATA TAT AAG GGC GTG ATC CAG GCT ATC CAG AAA TCA GAT GAA GGC     360
      R   I   Y   K   G   V   I   Q   A   I   Q   K   S   D   E   G    (77)

     CAC CCA TTC AGG GCA TAT TTA GAA TCT GAA GTT GCT ATA TCA GAG GAA     408
      H   P   F   R   A   Y   L   E   S   E   V   A   I   S   E   E    (93)

     TTG GTT CAG AAA TAC AGT AAT TCT GCT CTT GGT CAT GTG AAC AGC ACA     456
      L   V   Q   K   Y   S   N   S   A   L   G   H   V  [N   S   T]  (109)

     ATA AAA GAA CTG AGG CGG CTT TTC TTA GTT GAT GAT TTA GTT GAT TCC     504
      I   K   E   L   R   R   L   F   L   V   D   D   L   V   D   S   (125)

     CTG AAG TTT GCA GTG TTG ATG TGG GTG TTT ACT TAT GTT GGT GCC TTG     552
      L   K   F   A   V   L   M   W   V   F   T   Y   V   G   A   L   (141)

     TTC AAT GGT CTG ACA CTA CTG ATT TTA GCT CTG ATC TCA CTC TTC AGT     600
      F   N   G   L   T   L   L   I   L   A   L   I   S   L   F   S   (157)

     ATT CCT GTT ATT TAT GAA CGG CAT CAG GTG CAG ATA GAT CAT TAT CTA     648
      I   P   V   I   Y   E   R   H   Q   V   Q   I   D   H   Y   L   (173)

     GGA CTT GCA AAC AAG AGT GTT AAG GAT GCC ATG GCC AAA ATC CAA GCA     796
      G   L   A  [N   K   S]  V   K   D   A   M   A   K   I   Q   A   (189)

     AAA ATC CCT GGA TTG AAG CGC AAA GCA GAT TGA AAAAGCCCCAAACAGA        745
      K   I   P   G   L   K   R   K   A   D   *                      (199)

     AGTTCATCTTTAAAGGGGACACTCACTTGATTACGGGGGTGGGAGGGTCAGGGGTGAGCC       805
     CTTGGTGGCCGTGCGGTTTCAGCTCTTTATTTTTAGCAGTGCACTGTTTGAGGAAAAATT       865
     ACCTGTCTTGACTTCCTGTGTTTATCATCTTAAGTATTGTAAGCTGCTGTGTATGGATCT       925
     CATTGTAGTCACACTTGTCTTCCCCAATGAGGCGCCTGGTGAATAAAGGACTCGGGGAAA       985
     GCTGTGCATTGTATCTGCTGCAGGGTAGTCTAGCTGTATGCAGAGAGTTGTAAAGAAGGC      1045
     AAATCTGGGGGCAGGGAAAACCCTTTTCACAGTGTACTGTGTTTGGTCAGTGTAAAACTG      1105
     ATGCAGATTTTTCTGAAATGAAATGTTTAGATGAGAGCATACTACTAAAGCAGAGTGGAA      1165
     AACTCTGTCTTTATGGTGTGTTCTAGGTGTATTGTGAATTTACTGTTATATTGCCAATAT      1225
     AAGTAAATATAGACCTAATCTATATATAGTGTTTCACAAAGCTTAGATCTTTAACCTTGC      1285
     AGCTGCCCCACAGTGCTTGACCTCTGAGTCATTGGTTATGCAGTGTAGTCCCAAGCACAT      1345
     AAACTAGGAAGAGAAATGTATTTGTAGGAGTGCTACCTACCACCTGTTTTCAAGAAAATA      1405
     TAGAACTCCAACAAAAATATAGAATGTCATTTCAAAGACTTACTGTATGTATAGTTAATT      1465
     TTGTCACAGACTCTGAAATTCTATGGACTGAATTTCATGCTTCCAAATGTTTGCAGTTAT      1525
     CAAACATTGTTATGCAAGAAATCATAAAATGAAGACTTATACCATTGTGGTTTAAGCCGT      1585
     ACTGAATTATCTGTGGAATGCATTGTGAACTGTAAAAGCAAAGTATCAATAAAGCTTATA      1645
     GACTT(A)20  3'                                                    1650
```

Figure 4.6. Hydrophobicity plot of *INN1* protein and comparison of INN1 and NSP1C.

A. A Kyte-Doolittle hydrophobicity plot of INN1 shows that the protein contains two regions of hydrophobicity. The sites for potential N-linked glycosylation relative to these hydrophobic regions are indicated (*). B. Comparison of *INN1* and *NSP1C* protein. Conserved amino acids between the two proteins are indicated by dashes in the NSP1C sequence. Overall, the INN1 and NSP1C are 66% identical, however, many of the amino acid substitutions are conserved with respect to charge, resulting in an overall similarity of 82% between the two proteins. Sites for potential N-linked glycosylation in INN1 are indicated (*).

A.



B.

```
INN1    MDGQKKHWKDKVVDLLYWRDIKKTGVVFGASLFLLLSLTVFSIVSVNAYI   50
NSP1C   --CVWSN--SQAI---------Q--I---SF-L--F---Q--V---V--L

        ALALLSVTISFRIYKGVIQAIQKSDEGHPFRAYLESEVAISEELVQKYSN  100
        ---A--A--------S-L--V--T------K----L-ITL-Q-QI---TD

        SALGHVNSTIKELRRLFLVDDLVDSLKFAVLMWVFTYVGALFNGLTLLIL  150
        CLQLY----L---------Q-------------LL-------------LM

        ALISLFSIPVIYERHQVQIDHYLGLANKSVKDAMAKIQAKIPGLKRKAD   199
        -VV-M-TL--V-VK--A-V-Q----VRTHINAVV---------A--H-E
```

Figure 4.7. *INN1* encodes multiple transcripts.

15 μg of total RNA from innervated (I) and 5-day denervated (D) rat lower leg muscle was fractionated by formaldehyde/agarose gel electrophoresis, and transferred to Nytran membrane. The blot was hybridized with radiolabeled 1.65 kb *INN1* cDNA and exposed for 5 days with an intensifying screen. Consistent with RNase protection data, a major transcript at ~1.7 kb is ~5-fold more abundant in innervated muscle than in 5-day denervated muscle. Additional transcripts were detected at 2.3 and 5.2 kb. In contrast to the 1.7 kb transcript which is more abundant in innervated muscle, the 5.2 kb transcript is ~5-fold more abundant in denervated muscle. The 2.3 kb transcript appears ~2-fold enriched in denervated muscle as well. The blot was stripped and rehybridized with a GAPDH cDNA probe (below).

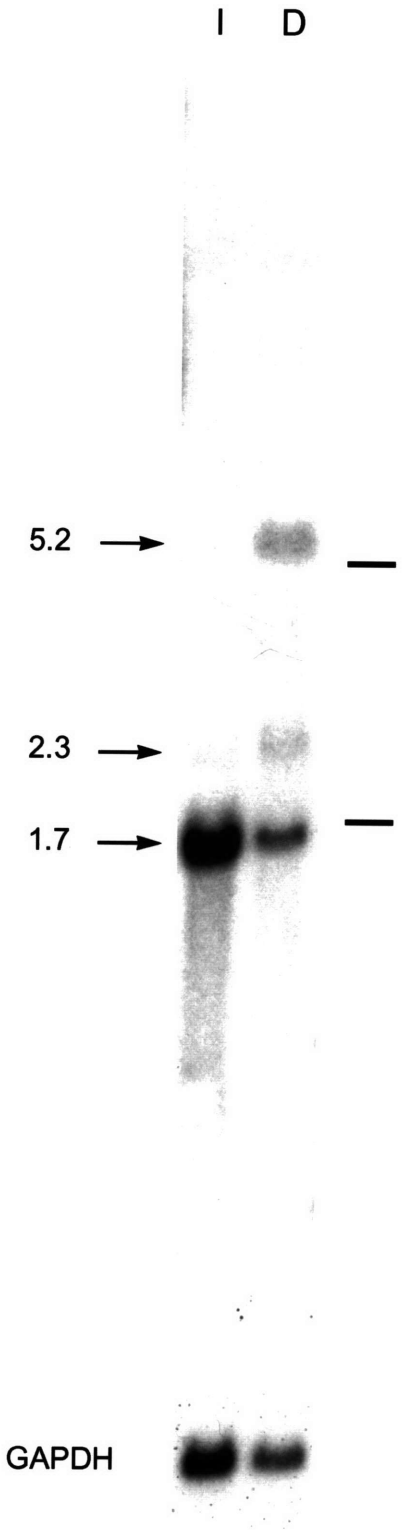
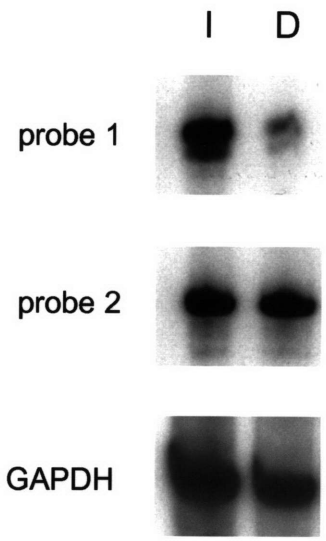

I
D
5.2
2.3
1.7
GAPDH

Figure 4.8. Different levels of *INN1* RNA in denervated muscle are detected by probes from adjacent regions of the *INN1* cDNA.

A. The levels of *INN1* RNA in innervated (I) and 5-day denervated (D) muscle were measured by RNase protection using two probes from overlapping regions of the *INN1* cDNA. Probe 1 is the original *INN1* cDNA isolated by subtractive-hybridization, and encodes the 5' 217 bp of the 1.65 kb cDNA. Probe 2 encodes nucleotides 169-410 of the 1.65 kb *INN1* cDNA (see part B; also Fig. 4.6). Using probe 1, approximately 5-fold less *INN1* RNA is detected in denervated muscle than innervated muscle. In contrast, no difference in the level of *INN1* RNA was seen using probe 2. These data suggest that the multiple INN1 transcripts are derived from alternative splicing, and that transcripts containing only the 5' sequence of the 1.65 kb *INN1* cDNA are down-regulated following denervation. The assays were done at the same time, using the same RNA preparations (10 μg/assay), and are reproducible. A GAPDH probe indicates approximately equivalent levels of RNA were used in each assay (5 μg RNA/assay). B. The cartoon shows the positions of the probe 1 and 2 sequences relative to one another in the *INN1* 1.65 cDNA and relative to the alternatively spliced domain in *NSP1C* (open box). This splice site aligns with nucleotide 162 in the *INN1* sequence.

A.


I
D
probe 1
probe 2
GAPDH

B.

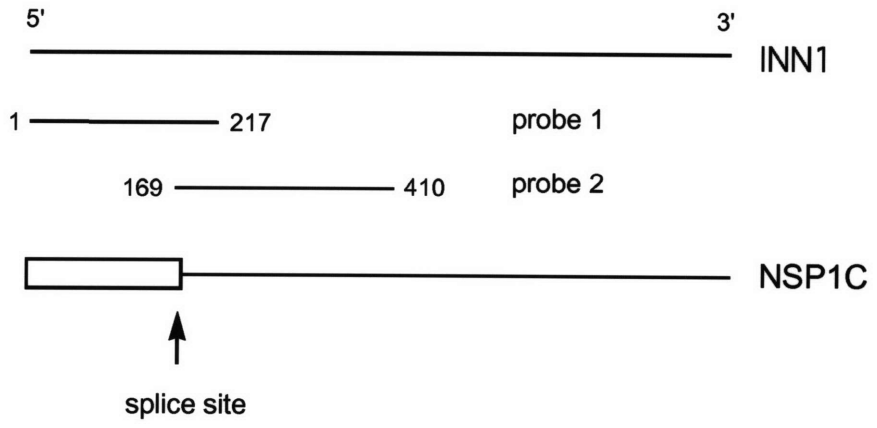

5'
3'
INN1
1
217
probe 1
169
410
probe 2
NSP1C
splice site

Figure 4.9. INN1 protein is expressed in both innervated and denervated muscle. Crude lysates from innervated and 5-day denervated muscle were prepared, and proteins were separated by SDS-PAGE (12% gel, 50 μg protein/lane). Proteins were transferred to nitrocellulose and blotted with an INN1 antiserum (1:2000) (see Materials and Methods), followed by a horseradish peroxidase conjugated secondary antibody. The INN1 antiserum binds to a major protein at ~22 kDa, consistent with the size of the INN1 protein predicted from the cDNA sequence. The antiserum also reacts with a second antigen at ~42 kDa which may be the product of one of the larger alternatively spliced *INN1* transcripts or an unrelated, cross-reacting protein. Both antigens react with the INN1 antiserum specifically since they can be competed with peptide and are not seen in blots stained with preimmune serum (not shown, but see Fig. 4.13). The levels of the two proteins appear to be equivalent in both innervated and denervated muscle, suggesting that although the level of 1.7 kb INN1 transcript decreases 5- to 7-fold the level of protein is unaffected 5 days following denervation. Two lanes from the same gel and stained with Coomassie stain (Coo), are shown to compare the levels of protein loaded for each extract; the position of size markers is indicated.

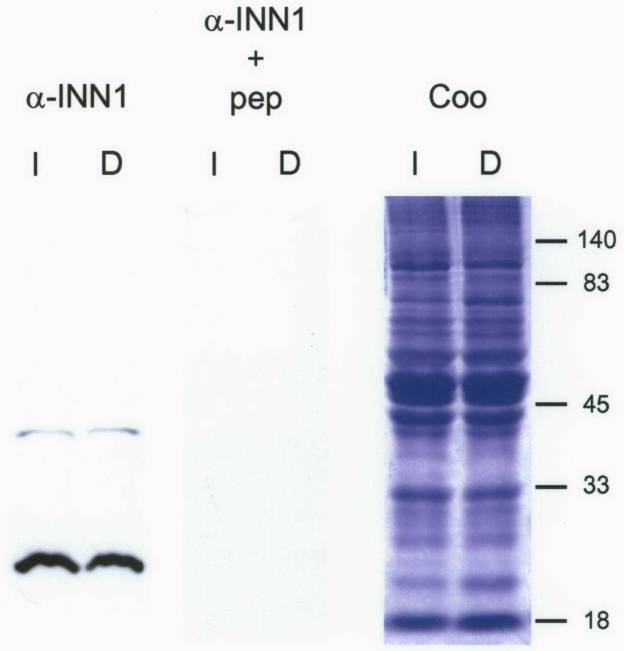

α-INN1
α-INN1
+
pep
Coo
I
D
I
D
I
D
140
83
45
33
18

Figure 4.10. INN1 is enriched in membrane fractions of rat skeletal muscle. Lysates of innervated rat skeletal muscle were fractionated by centrifugation as described in Materials and Methods. Equivalent volumes of high-speed supernatant (S) and pellet (P) fractions (30 μg and 4 μg, respectively) were loaded onto each lane, and the proteins separated by SDS-PAGE in a 12% gel. Nitrocellulose blots of the separated proteins were incubated with INN1 antiserum (1:2000), followed by a horseradish peroxidase conjugated secondary antibody. The 22 kDa INN1 protein is enriched in the membrane-containing pellet fraction compared to the soluble protein-containing supernatant fraction. Low levels of the 42 kDa protein (see Fig. 4.9 and Fig. 4.13) are detected in the supernatant fraction, suggesting that this protein is cytosolic. Preincubation of the antiserum with the immunizing peptide blocks antibody staining of both the 22 kDa and 42 kDa antigens. Two lanes from the same gel and stained with Coomassie stain (Coo) are shown to compare the levels of protein loaded for each fraction; the position of size markers is indicated.

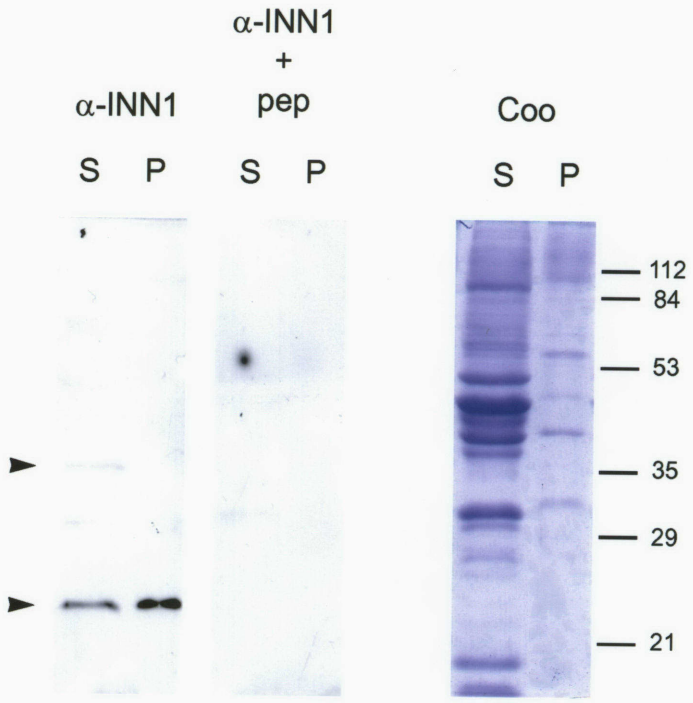
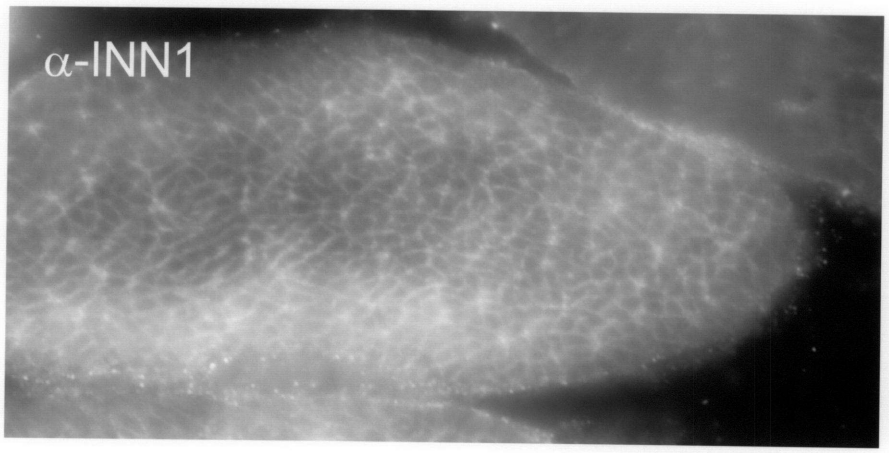
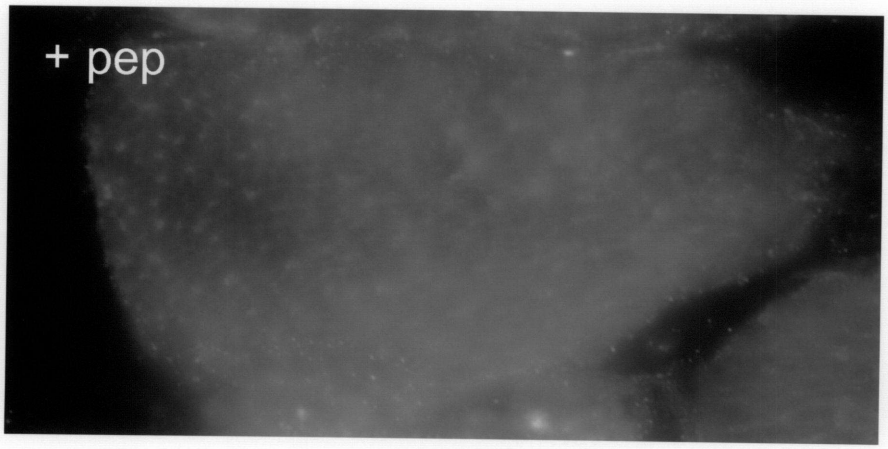

α-INN1
α-INN1
+
pep
Coo
S P S P S P
112
84
53
35
29
21

Figure 4.11. Localization of INN1 in cross-sections of skeletal muscle.
Frozen transverse sections of innervated rat soleus muscle were stained with INN1 antiserum (1:500) (α-INN1), INN1 antiserum preincubated with peptide (+ pep), or pre-immune serum (1:500) (pre) followed by a fluorescein-conjugated secondary antibody as described in Materials and Methods. A reticulated pattern can be seen in cross-sections of muscle fibers stained with the INN1 antiserum. This staining pattern is eliminated by preincubation of the INN1 antiserum with peptide (+pep), and is absent in sections stained with preimmune serum (pre).


α-INN1


+ pep

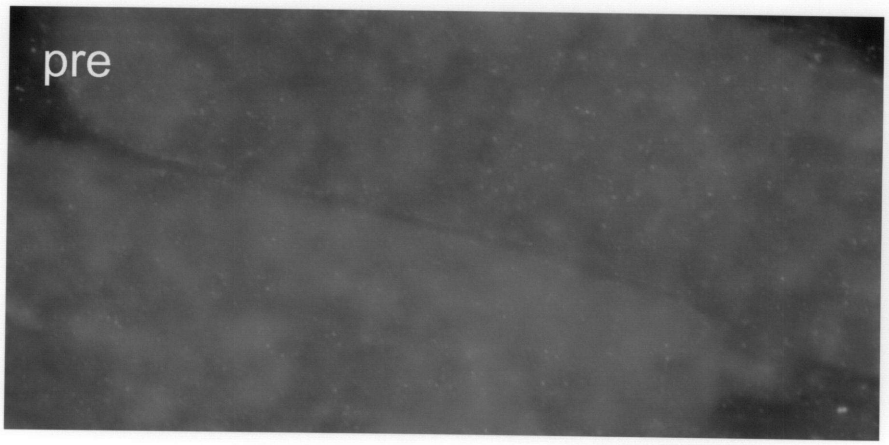

pre

Figure 4.12. Localization of INN1 in longitudinal sections of skeletal muscle.
A. A longitudinal frozen section of innervated rat soleus muscle was stained with INN1 antiserum (1:500) followed by a fluorescein-conjugated secondary antibody (1:200). B. A phase-contrast image of the the same field in panel A only inverted with respect to vertical to permit alignment of anti-INN1 staining with sarcomeres. Stripes of INN1 staining (arrowheads) align with the I band regions of each sarcomere. The A and I bands of three sarcomeres are indicated. The length of each sarcomere (distance between the midlines of consecutive I bands) is approximately 2.2 μm.

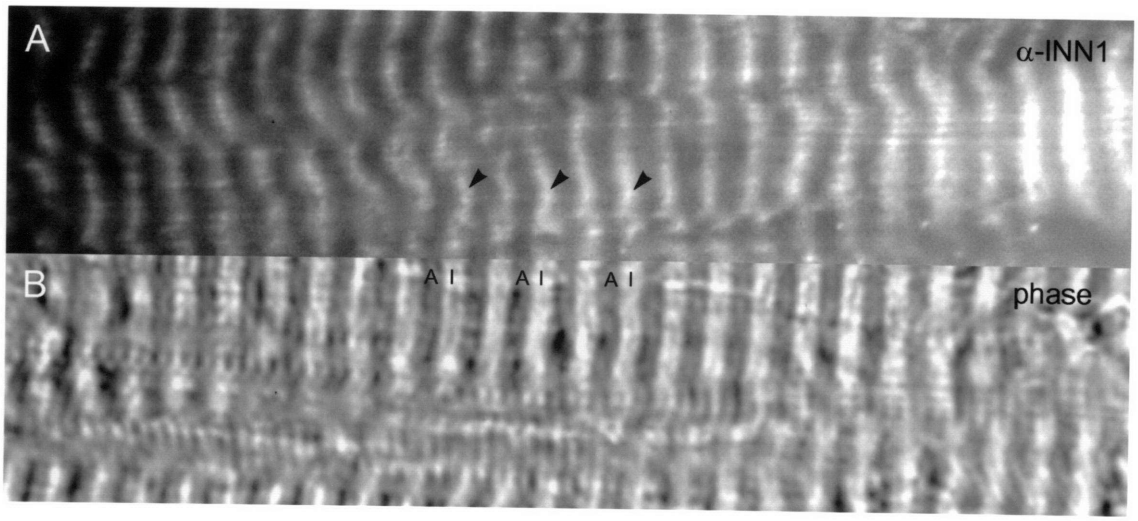

A
α-INN1
B
A I
A I
A I
phase

Figure 4.13. INN1 is not enriched in transverse tubules.

Rat skeletal muscle total extract (Rt. ext. - 50 μg), rabbit microsomes (μs - 75 μg), and rabbit T-system (TT - 20 μg) preparations were separated by SDS-PAGE (12% gel) and transferred to nitrocellulose. Blots from the same gel were then stained with INN1 antiserum (1:2000), pre-immune serum (pre), or IXE11$_2$ (1:500) monoclonal antibody against TS28, a protein enriched in transverse tubule preparations (Jorgensen et al., 1990) (α-TS28), followed by appropriate HRP-conjugated secondary antibodies. The INN1 antiserum recognizes 22 and 42 kDa proteins in both rat muscle extracts and rabbit microsomes, but neither protein is enriched in rabbit T-system preparations. Molecular weight size markers are indicated.

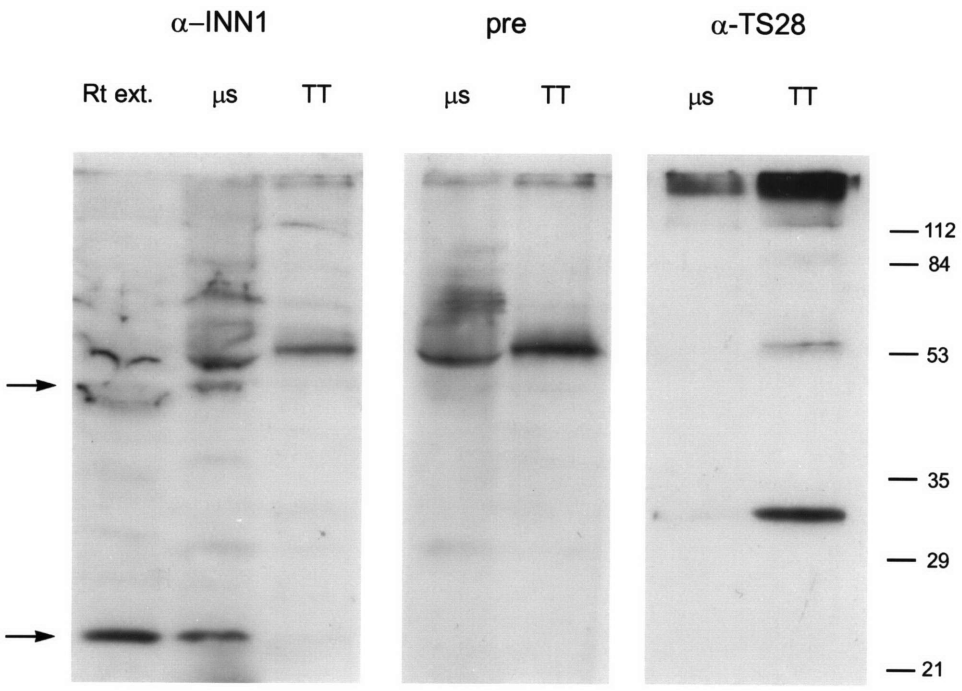

α–INN1
pre
α-TS28
Rt ext.
μs
TT
μs
TT
μs
TT
112
84
53
35
29
21

Figure 4.14. *DEN1* and *DEN2* are up-regulated in denervated muscle.
Total RNA was prepared from innervated (I) and 5-day denervated (D) rat lower leg muscle, and the levels of *DEN1, DEN2,* and GAPDH RNA were measured by an RNase protection assay. Approximately 10 μg (DEN1 and DEN2 lanes) or 1 μg (GAPDH lanes) of total RNA were used in each lane. Quantitation of these results showed that *DEN1* is ~100-fold and *DEN2* is ~4-fold more abundant in denervated muscle than in innervated muscle.

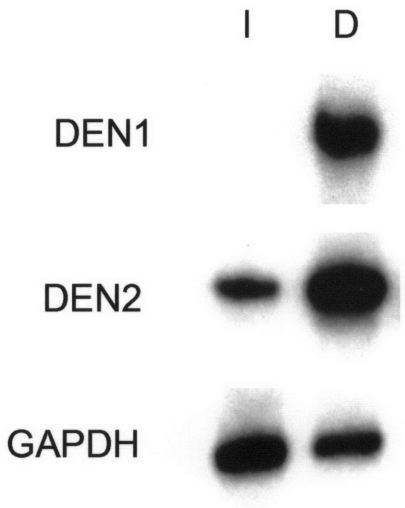

I
D
DEN1
DEN2
GAPDH

Figure 4.15. Expression of *DEN1* and *DEN2* increases with a slow time course following denervation.

Total RNA was prepared from innervated rat lower leg muscle (Inn) or lower leg muscle denervated for 1, 2, or 5 days, and the levels of *DEN1*, *DEN2*, AChR δ, and skeletal actin were measured by an RNase protection assay. 10 μg (DEN1, DEN2, AChR δ lanes) or 1 μg (actin lanes) of total RNA were in each assay.

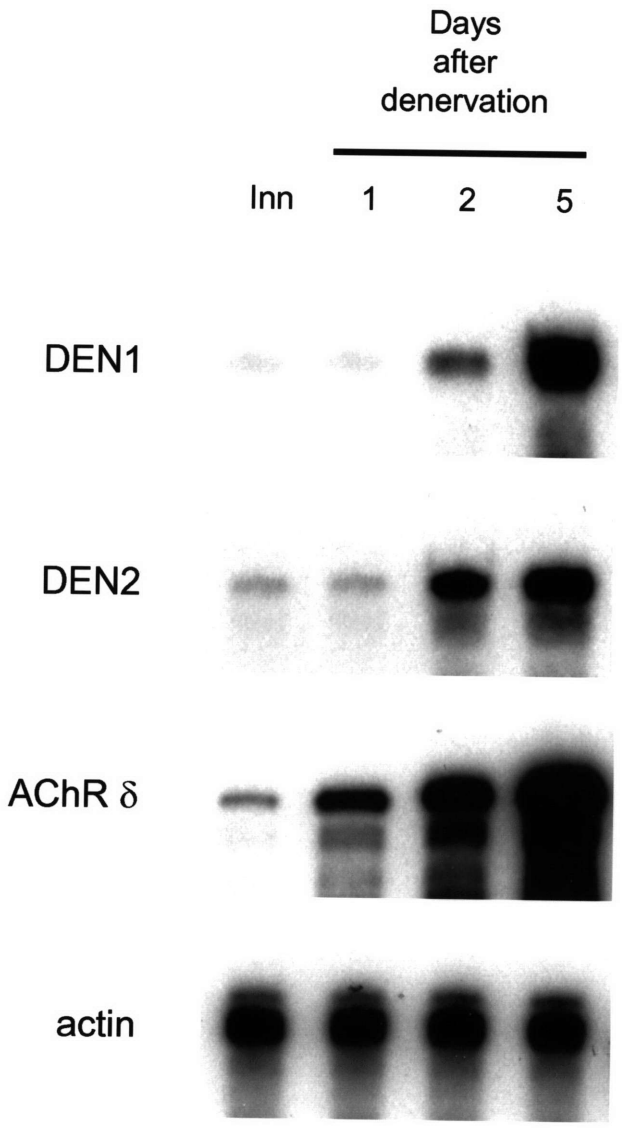

Days
after
denervation
Inn
1
2
5
DEN1
DEN2
AChR δ
actin

Figure 4.16. Both *DEN1* and *DEN2* encode single transcripts.
Total RNA from innervated (I) and 5-day denervated (D) rat lower leg muscle were separated in 1.1% formaldehyde/agarose gels, and transferred to nitrocellulose membranes (15 μg/lane (DEN1 lanes); 20 μg/lane (DEN2 lanes)). The RNA blots were hybridized with either *DEN1* or *DEN2* probes, washed and exposed to film with an intensifying screen. Both probes detect single transcripts (arrows) which are up-regulated following denervation. The blots were then stripped and re-hybridized with a GAPDH probe (shown below each blot). The migration of 28S and 18S ribosomal RNA in each gel is shown.

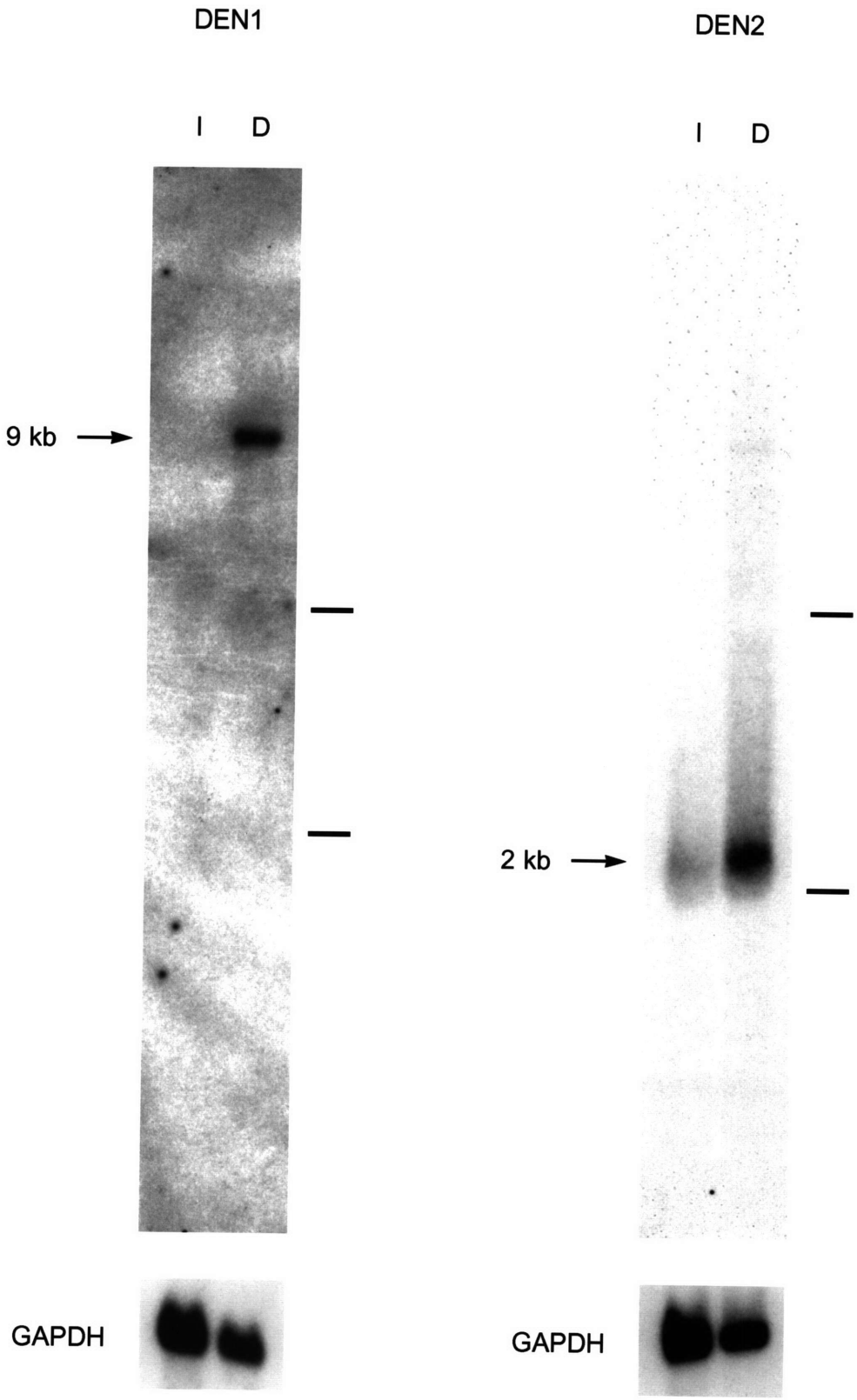

DEN1
I
D
9 kb
GAPDH
DEN2
I
D
2 kb
GAPDH

Figure 4.17. Sequence of a partial cDNA encoding *DEN1*.

The complete nucleotide and encoded amino acid sequence of the 3.3 kb *DEN1* cDNA is shown. Each fibronectin type III repeat (F) and immunoglobulin C2 motif (I) is underlined and labeled. The KSP sequence between motif Fd and Id is indicated (^). The TAG stop codon is indicated by an asterisk (*), and a polyadenylation addition site in the 3' untranslated sequence is boxed.

```
...GAATTCGGAAGGCCGAGACTGACAAGGTCCAAGGATCCAGGACCTCAATG      50
      I  R  K  A  E  T  K  D  V  Q  G  S  R  T  S  M      (16)

AAGTCTGAAAGTCAGGAAGGGAAAGACTCTGGCTGGTCAGGCAGGAAGCCTGGCCTCTGTGGAAGTGGGCATCAG      125
 K  S  E  S  Q  E  G  K  D  S  G  W  S  G  R  K  P  G  L  C  G  S  G  H  Q      (41)

GTGCAGGCTGGGACTGAAGTTGGATCAGCAAAGAGAGGGAGCACAGAGGAGGCCGGAGGTCTGGGGACTTGGACT      200
 V  Q  A  G  T  E  V  G  S  A  K  R  G  S  T  E  E  A  G  G  L  G  T  W  T      (66)

GGCAATGAGGAGAGAGGGTCTCTGAGAGAACCTTGGAGTGAAGACAGGAGGCGAGGCCCCCACAGGCATCTTGGC      275
 G  N  E  E  R  G  S  L  R  E  P  W  S  E  D  R  R  R  G  P  H  R  H  L  G      (91)

AGCAGGAGAGATACCCAAGAGGGCAGGTCAGATGTCTATGGCCAGGCACAAGATGCTACCCAGAGCCCCAGATCC      350
 S  R  R  D  T  Q  E  G  R  S  D  V  Y  G  Q  A  Q  D  A  T  Q  S  P  R  S      (116)

AGATACAAGCCTGGCCCTGGCTGCTCTTCTTCGGAGGCCCGAGGCTCCATGGACCACTTCTCTCAGGGTCTGACT      425
 R  Y  K  P  G  P  G  C  S  S  S  E  A  R  G  S  M  D  H  F  S  Q  G  L  T      (141)
                                                 Ia

GATACAGAGGTACAACTGGGGGAAGCTGCCATACTCTCCTGTACCCTTTCCAGTGACCTGGGACCTGGCACCTGG      500
 D  T  E  V  Q  L  G  E  A  A  I  L  S  C  T  L  S  S  D  L  G  P  G  T  W      (166)

TTCAAGGATGGCGTCAAGCTCACTGCCCAAGATGGGGTCATCTTTGAGCAAGATGGGCTCACACACAGACTGATC      575
 F  K  D  G  V  K  L  T  A  Q  D  G  V  I  F  E  Q  D  G  L  T  H  R  L  I      (191)

CTCACCCACGTGGAGGGGACCCAAGCTGGGAGATACACTTTTGTAGCCGGCCGCCAGCACAGTGAGGCCAGTCTG      650
 L  T  H  V  E  G  T  Q  A  G  R  Y  T  F  V  A  G  R  Q  H  S  E  A  S  L      (216)

ACCGTTCAGGATCCCCCCACCATTGCTCCAGATGTGACCGAGAAACTGAGAGAGCCACTGGTGTTCAAGGCTGGG      725
 T  V  Q  D  P  P  T  I  A  P  D  V  T  E  K  L  R  E  P  L  V  F  K  A  G      (241)
                           Ib

AAGCCGGTCACTGTGAAAATACCTTTCCAGAGCCGCCTCCCTGTCCAGGCTGCCTGGAGGAAAGATGGGAAGGAA      800
 K  P  V  T  V  K  I  P  F  Q  S  R  L  P  V  Q  A  A  W  R  K  D  G  K  E      (266)

GTGGCGGGCAGCAACCACAAGGGCATCCAACTTGCTCTGGGAGATGGCTACACCCGGCTGTGCCTCCCCAGTGTG      875
 V  A  G  S  N  H  K  G  I  Q  L  A  L  G  D  G  Y  T  R  L  C  L  P  S  V      (291)

TGCAGGAAGGACAGTGGCCGGTACAGCGTGAGGCTGAAGAGTGAGGGAGGCTGTGTGGAGGCTGAGTTCACCCTG      950
 C  R  K  D  S  G  R  Y  S  V  R  L  K  S  E  G  G  C  V  E  A  E  F  T  L      (316)

CAAGTTATAGATAAGCCTCGGCCCCCACAAGGACCCCTGGAGGTTCAGGCTTGCCACAGAGCAGGTGTCTGCCTC     1025
 Q  V  I  D  K  P  R  P  P  Q  G  P  L  E  V  Q  A  C  H  R  A  G  V  C  L      (341)
                Fa

CGCTGGTTACCCCCGAGGGACGATGGGGGACAGGTCATACAACACTATGTGGTGGAGAGGCGACAGGCTGGAAGG     1100
 R  W  L  P  P  R  D  D  G  G  Q  V  I  Q  H  Y  V  V  E  R  R  Q  A  G  R      (366)

AGCACTTGGCTGAAGGTGGGAGAGCCCCCACCAGACAGTACCAGCTTCACGGATGTCATCGTGGAGCAGGGCAGG     1175
 S  T  W  L  K  V  G  E  P  P  P  D  S  T  S  F  T  D  V  I  V  E  Q  G  R      (391)

AAGTATGCCTTCCGTGTTCGGGCCGTGACCTCAGAGGGAGCCGGGGATGCCCTGGAGTCTGAGGAGGTGCTGATA     1250
 K  Y  A  F  R  V  R  A  V  T  S  E  G  A  G  D  A  L  E  S  E  E  V  L  I      (416)

GCTCCTGAGGCTCTCCCGGGGCCCCCTTCTGCTCCAGCAATCCTGTCAGCCTCCAGCCAGAGCATCACTCTCACG     1325
 A  P  E  A  L  P  G  P  P  S  A  P  A  I  L  S  A  S  S  Q  S  I  T  L  T      (441)
          Fb

TGGGGAGCACCACAGGGCCCTGGCAGTACCCACATCTTGGGCTACCTGGTTGAGAAGCGCAAGAAGGGGAGCAGC     1400
 W  G  A  P  Q  G  P  G  S  T  H  I  L  G  Y  L  V  E  K  R  K  K  G  S  S      (466)

ACCTGGATGGCTGTGAATGAGCAGCCCGTGTCTGAAAGGAGGTACACTGTGGTGGATCTGCGACAGGGTTGCCAG     1475
 T  W  M  A  V  N  E  Q  P  V  S  E  R  R  Y  T  V  V  D  L  R  Q  G  C  Q      (491)

TATGAGTTCAGGGTCATGGCTGTGACTCTGTCGGGCCCTGGAGAGCCTGGGCCTCCATCCGATGCTGTCTTTGCC     1550
 Y  E  F  R  V  M  A  V  T  L  S  G  P  G  E  P  G  P  P  S  D  A  V  F  A      (516)
```

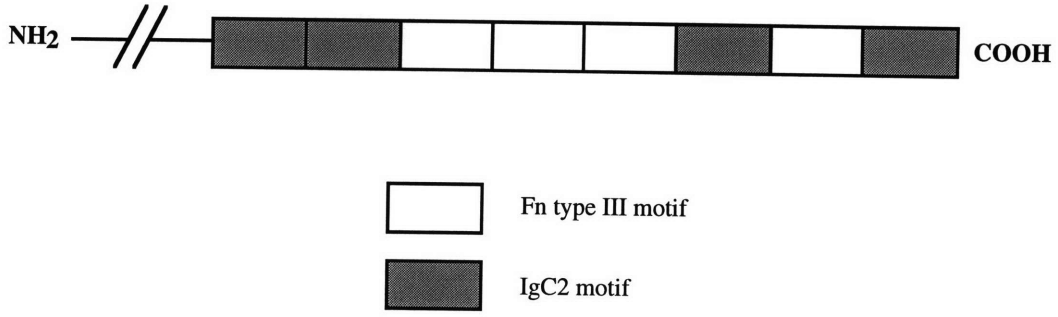
```
AGAGATCCCATGAGACCTCCAGGGCCTGTGCGGGATCTCCAGGTTACAGACACATCAGACACTAGCATCACCGTG   1625
 R  D  P  M  R  P  P  G  P  V  R  D  L  Q  V  T  D  T  S  D  T  S  I  T  V     (541)
             Fc
AGCTGGATGGTGCCAGATGCCCAGGATGCTGATGAAGCTCAGGGATACATTGTCGAGCTGTGTGGTTCAGACAGC   1700
 S  W  M  V  P  D  A  Q  D  A  D  E  A  Q  G  Y  I  V  E  L  C  G  S  D  S     (566)

CATCAGTGGAGCCCATGCCACATGGGCACTGTGCCAGGCACCATCTTCACAGCCAAGGGGCTTCGGCCACAAGAA   1775
 H  Q  W  S  P  C  H  M  G  T  V  P  G  T  I  F  T  A  K  G  L  R  P  Q  E     (591)

GGCTACTTTGTACGAGTGACAGCGGTGAATGATGGGGGCCGGAGTCCGGCCATGTCCCTGGACACATTGGTTCAT   1850
 G  Y  F  V  R  V  T  A  V  N  D  G  G  R  S  P  A  M  S  L  D  T  L  V  H     (616)

GCCATGCCTGCCACTGTCTGTCCCAGGTTCCTCATGGATTCCAGCACAAAGGATACACTGATGGTCAGGGTTGGG   1925
 A  M  P  A  T  V  C  P  R  F  L  M  D  S  S  T  K  D  T  L  M  V  R  V  G     (641)
                          Ic
GACAGCATTCGAGTTCCTGTCCCGTTCGAAGCTGCCCCCATGCCTGAGGTGACCTGGTTGAAGGACGGTTTGCCT   2000
 D  S  I  R  V  P  V  P  F  E  A  A  P  M  P  E  V  T  W  L  K  D  G  L  P     (666)

TTGCCCAAAAGAAGTGTGACCACTGTGAAGGATGGCCTCACCCAGCTTCTGATTCCTGTAGCTAGCCTCTCAGAC   2075
 L  P  K  R  S  V  T  T  V  K  D  G  L  T  Q  L  L  I  P  V  A  S  L  S  D     (691)

TGTGGCCGATACACGGTGAGGCTGAGGAACCTACAGGGAAAGGAAGCCACCTACAGCTTCTTCATCAGTGTGGCA   2150
 C  G  R  Y  T  V  R  L  R  N  L  Q  G  K  E  A  T  Y  S  F  F  I  S  V  A     (716)

GCATGCCCACAGGCACCGGGTTCCATCTATCTTCAGGAGAATGTGCCTGGGACAGTGACGGTCCAATGGGAACCC   2225
 A  C  P  Q  A  P  G  S  I  Y  L  Q  E  N  V  P  G  T  V  T  V  Q  W  E  P     (741)
    Fd
TCTCCGGATGAGGCTCAGGGCACCCCCTTGCATTATACAGTGTTGATGCGGTCCTCATCTCACGGATCCTGGCAT   2300
 S  P  D  E  A  Q  G  T  P  L  H  Y  T  V  L  M  R  S  S  S  H  G  S  W  H     (766)

GAGGTGGCCGACTATGTCCGCACCAACCGCTTTACCCTCCTAGGGGTCCTCCCTGGCCATGAGTATCACTTCCGG   2375
 E  V  A  D  Y  V  R  T  N  R  F  T  L  L  G  V  L  P  G  H  E  Y  H  F  R     (791)

GTGCTGGCCAAGAATGAGCTGGGGGCCAGCAAACCTTCAGACACCAGCCAACCCTGGTGTATCCCCAGGCAGAGA   2450
 V  L  A  K  N  E  L  G  A  S  K  P  S  D  T  S  Q  P  W  C  I  P  R  Q  R     (816)

GACAGGTTCTCAGTGAAGTCTCCAACCTACCGGGAACCTGACCTGAGCCAGAAACCCCGCTTCCTGGTGGGTCTG   2525
 D  R  F  S  V  K  S  P  T  Y  R  E  P  D  L  S  Q  K  P  R  F  L  V  G  L     (841)
               ^  ^  ^                                Id
CGGGCTCACCTTCTGCCCCAGGGCTGCGAGTGCCGCATGACCTGCGCGGTGCAGGGCTCACCCCAGCCCCAAGTC   2600
 R  A  H  L  L  P  Q  G  C  E  C  R  M  T  C  A  V  Q  G  S  P  Q  P  Q  V     (866)

ACCTGGTTCAAGAACGACCAAAGCTTGGACAGCAACCCTGCGATGTACAGCACCGACTTGCTGGGTGTGTGCTCC   2675
 T  W  F  K  N  D  Q  S  L  D  S  N  P  A  M  Y  S  T  D  L  L  G  V  C  S     (891)

CTCATCATCCCCAGCGTGTCCCCTAAGGACAGTGGCGAATACAAGGCCGTGGCCAAAAACCCACTCGGACAAGCC   2750
 L  I  I  P  S  V  S  P  K  D  S  G  E  Y  K  A  V  A  K  N  P  L  G  Q  A     (916)

ATCAGCACAGACACCCTCATCGTCACAGAATACAACTCCTAGTCCACTGGATCTGCAATGCTAGCCTTGCCCCAC   2825
 I  S  T  D  T  L  I  V  T  E  Y  N  S  *                                     (929)

CCTTCAGCTATGAGATGGATTGCAGACCTCTGAAGCTTTGCTCCTGATACCCACACTGACCTGGGGAGCAGTCCA   2900
GCATGACAGGAAATGTTCCAGGGTGAGACAGGAACAAGGGGGCTTCTGGTGAGAGTGACCAGAAGGGCTGAAGTC   2975
CTTGGAGAAGAAATAGAAGAAGAGTGGAGGATGTAGCCTGGAGCTGAATGAAGGCTTGGAGTGACCCTGTTCCTG   3150
TTCTTTGTTCTGCACCTCTGGAGGAGCTCCAGAGAGAGTGGTCCACAGGGTGGAAACTGAGCCCACAGTGACCCC   3225
AGGAATGGAAAGTCATAGAGCAGAGGGCCTGGGTGAGGGCACCAAGGGGAGGCACTGTCTGTACTTCATTAAAAT   3300
GACAAGCAACTGAAGGTG(A)N                                                        3318
```

Figure 4.18. Organization and alignment of DEN1 repeats.

A. The arrangement of fibronectin type III (open boxes) and immunoglobulin C2 (shaded boxes) motifs in the carboxy terminus of DEN1 is illustrated. B. The alignment of DEN1 FnIII and Ig repeats with respect to each other and the FnIII and Ig consensus sequences from titin and twitchin are shown. Amino acids which are shared in three of the four DEN1 repeats, or amino acids which are shared with the repeat consensus sequences in titin and twitchin, are shaded. Gaps have been introduced to optimize alignments, and dashes in the titin and twitchin consensus sequences represent non-conserved amino acids.

Among the DEN1 FnIII and Ig repeats, amino acid identities range from 32% (Fa versus Fb) to 21% (Fa versus Fc and Fc versus Fd) among the FnIII motifs and from 27% (Ib versus Ic) to 16% (Ia versus Ic) among the Ig motifs. Within each class of DEN1 domain, several amino acids are conserved in all four copes of the motifs, and often these same amino acids are conserved also within the FnIII and Ig consensus sequences of other muscle structural proteins. The FnIII and Ig repeats in the DEN1 C-terminus are more similar to FnIII and Ig repeats of other thick filament associated muscle proteins, most notably titin, than with non-muscle proteins containing similar motifs (e.g. immunoglobins, N-CAM), and the degree of amino acid identity between the DEN1 repeats and individual FnIII and Ig repeats in titin ranged from 22% (Ib) to 38% (Fb).

A. ORGANIZATION OF DEN1 REPEATS



B. ALIGNMENT OF DEN1 REPEATS

Fibronectin Type III motifs

```
Fa:            KPRPPQQGPLEVQACHRAGVCLRWLPPRDDGGQVIQHYVVERRQAGRSTWLKVGEPPPDSTSFTDVIVEQGRKYAFRVRAVTSEGAGDALESEEVLIAPE        (321-419)
Fb:            ALPGPPSAPAILSASSQSITLTWGAPQQPGSTHILGYLVEKRKKGSSTWMAVNEQPVSERRYTVVDLRQGCQYEFRVMAVTLSGPGEPGPPSDAVFARDPM      (420-520)
Fc:            RPPGPVRDLQVTDTSDTSITVSWMVPDAQDADEAQGYLVELCGSDSHQWSPCHMGTVPGTIFTAKGLRPQEGYFVRVTAVNDGGRSPAMSLDTLVHAMPATVCP  (521-624)
Fd:            CPQAPGSIYLQENVPGT VTVQWEPSPDEAQGTPLHITVLMRSSSHGSWHEVADY VRTNRFTLLGVLPGHEYHFRVLAKNELGASKPSDTSQPWCIPRQRDRF  (718-819)
titin con:     D-PGPP----V--VT--SV-I-W--P--DGGS-I-GYIVEKRD-----W--------------VT-I-EG-EY-FRV-AVN-AG-G-I------V---D-----
twitchin con:  D-PG-R--PEV-DV--D--TI-W-PR-DEGGAPK--MVVEM-D---G-W--V-------T---V-GL-EG-EYEFRV-AVN-AG-SDES------AK-P
```

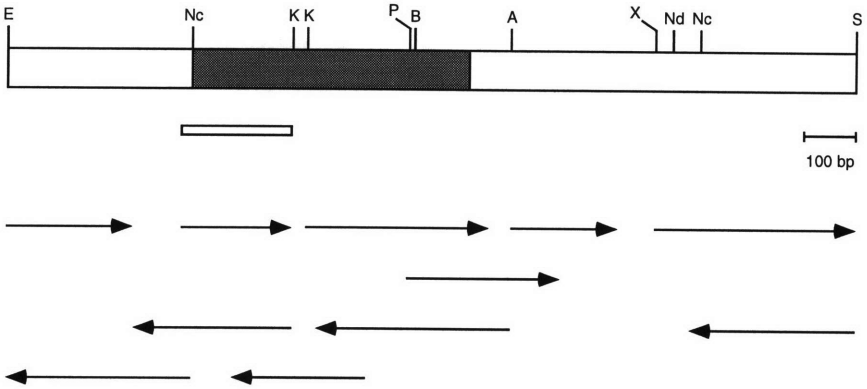
IgC2 motifs

```
Ia:            MDHFSQGL TDTEVQLGEAAILSCTLSSDLGP GTWFKDGVKLTAQDGVIFEQDGLTH   RLILTHVEGTQAGRYTFVAGRQHSEASL  TVQDPPTIA    (133-225)
Ib:            PDVTEKLR EPLVFKAGKPVTVKIPFQSRLPVQAAWRKDGKEV AGSNHKGIQLALGDGYTRLCLPSVCRKDSGRYSVRLKSEGGCVEAEFTLQVID      (226-320)
Ic:            MDSSTKDT   LMVRVGDSIRVPVPFEAAPMPEVTWLKDGLPLPKRSVTTVKD      GLTQLLIPVASLSDCGRYTVRLRNLQGKEATYSFEISVAA      (628-717)
Id:            KPRFLVGLRAHLLPQ GCECRMTCAVQGSPQPQVTWFKNDQSLDSNPAMYSTD   LLGVCSLIIPSVSPKDSGEYKAVAKNPLGQAISTDTLIVTEYNS    (834-929)
titin con:     PG--LD-----L-V-AG----F-VPI-GRP-P-V-W-KDG--L---TR-----ST---T-L-V------K-D-G-Y--TL-N--G-K---V-V-VLD
twitchin con:  P-I--------- VKAG----F-V-V-G-R-P-V-W-KNG--I-----K--I--------L-I--  A-R-DAG-VTI-A-N--GD--- V-I-V-
```

Figure 4.19. Restriction map of *DEN2* cDNA, and comparison of DEN2, HSP27 and αB-crystallin proteins.

A. Restriction map of the DEN2 1.7 kb cDNA. The length of sequences determined from internal deletions and subcloning of DEN2 fragments are represented by arrows. The position of the DEN2 ORF is shaded, and the position of the 217 bp DEN2 cDNA isolated from the subtractive-hybridization library is shown below the map (thin open box). Restriction sites: Acc1 (A); BamH1 (B); EcoR1 (E); Kpn1 (K); Nco1 (Nc); Nde1 (Nd); Pst1 (P); Sal1 (S); Xba1 (X). B. Comparison of DEN2 amino acid sequence with HSP27 (top) and αB-crystallin (CRY - bottom). Amino acids shared between DEN2 and HSP27 or DEN2 and αB-crystallin are shaded. Gaps were introduced to optimize the alignment, and are indicated with dashes.

A.



B.

```
HSP27    MTERRVPF--SLLRSPSWEPFRDWYPAHSRLFDQAFGVPRFPDE----WSQW----FSSAGW
DEN2     MADGQLPFPCSYPSRLRRDPFRDS-PLSSRLPDDGFGMDPFPDDLTAPWPEWALPRLSSA-W
CRY      -MDIAIHHP---WIRRPFFPFH----SPSRLFDQFFGEHLLESDLF-----STATSLS----

HSP27    PGYVRPLPAATAEGPAAVTLARPFSRALNRQLSSGVSEIRQTADRWRVSLDVNHFAPEELTVK
DEN2     PGTLRSGMVPR--GPTAT--ARFGVPAEGRNPPPFPGEP------WKVCVNVHSFKPEELMVK
CRY      PFYLRPPS---------------FLRAPSW-IDTGLSEMRMEKDRFSVNLDVKHFSPEELKVK

HSP27    TKEGVV-EITGKHEERQDEHGYISRCFTRKYTLPPGVDPTLVSSSLSPEGTLTVEAPLPKAVTQ
DEN2     TKDGYV-EVSGKHEEKQQEGGIVSKNFTKKIQLPAEVDPVTVFASLSPEGLLIIEAPQVPPYSP
CRY      -VLGDVIEVHGKHEERQDEHGFISREFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRKQASGP

HSP27    SAEITIPVTFEARAQIGGPESEQSGAK
DEN2     FGESSFNNELPQDNQEVTCS
CRY      --ERTIPITREEKPA-VTAAPKK
```

Chapter 5.

AML1 Is Expressed in Skeletal Muscle and Is Regulated by Innervation

The work described in this chapter has been published in:
Zhu, X., Yeadon, J. E., and Burden, S. J. (1994) *AML1* is expressed in skeletal muscle and is regulated by innervation. *Mol. Cell. Biol.* **14**, 8051-8057.

SUMMARY

Although most skeletal muscle genes are expressed at similar levels in electrically active, innervated muscle and in electrically inactive, denervated muscle, a small number of genes, including those encoding the acetylcholine receptor, N-CAM and myogenin, are expressed at significantly higher levels in denervated than in innervated muscle. The mechanisms that mediate electrical activity-dependent gene regulation are not understood, but these mechanisms are likely to be responsible, at least in part, for the changes in muscle structure and function that accompany a decrease in myofiber electrical activity. To understand how muscle activity regulates muscle structure and function, I used a subtractive-hybridization and cloning strategy to identify and isolate genes that are expressed preferentially in innervated or denervated muscle. One of the genes which we found to be regulated by electrical activity is the recently discovered acute myeloid leukemia 1 (AML1) gene. Disruption and translocation of the human *AML1* gene is responsible for a form of acute myeloid leukemia. AML1 is a DNA binding protein, but its normal function is not known and its expression and regulation in skeletal muscle was not previously appreciated. Because of its potential role as a transcriptional mediator of electrical activity, we characterized expression of the *AML1* gene in innervated, denervated and developing skeletal muscle. We show that *AML1* is expressed at low levels in innervated skeletal muscle and at 50 to 100-fold greater levels in denervated muscle. Four *AML1* transcripts are expressed in denervated muscle, and the abundance of each transcript increases after denervation. We transfected C2 muscle cells with an expression vector encoding AML1, tagged with an epitope from hemagglutinin, and we show that AML1 is a nuclear protein in muscle. AML1 dimerizes with core binding factor β (CBF β), and we show that CBF β is expressed at high levels both in innervated and denervated skeletal muscle. PEBP2α, which is structurally related to AML1 and which also dimerizes with CBF β, is expressed at low levels in skeletal muscle and is up-regulated only weakly by denervation. These results are consistent with the idea that AML1 may have a role in regulating gene expression in skeletal muscle.

INTRODUCTION

Vertebrate neuromuscular synapses arise as a result of complex interactions between motor neurons and developing skeletal muscle cells (Hall and Sanes, 1993). One of the more striking specializations of the neuromuscular synapse is the remarkable concentration of acetylcholine receptors (AChRs) in the postsynaptic muscle membrane

(Hall and Sanes, 1993; Jennings and Burden, 1993). Experiments designed to determine how AChR expression is regulated have revealed that a combination of three separate mechanisms mediate the clustering of AChRs at synaptic sites. First, motor neurons synthesize and deposit agrin in the synaptic basal lamina, where it induces a redistribution of non-synaptic AChRs to the newly formed synapse (Anderson and Cohen, 1977; McMahan, 1990; Nastuk and Fallon, 1993). Second, an unknown factor, which like agrin is deposited into the synaptic basal lamina and which is presumably synthesized by motor neurons, activates a signaling pathway leading to transcription of AChR genes selectively in nuclei near the synaptic site (Burden, 1993). Finally, nerve-induced muscle electrical activity represses AChR transcription throughout the muscle fiber, decreasing the number of AChRs expressed in the non-synaptic region (Hall and Sanes, 1993; Jennings and Burden, 1993).

The molecular mechanisms that mediate electrical activity-dependent regulation of AChR genes are unknown, but a clearer view of the electrical activity-dependent signaling pathway is beginning to emerge (Huang et. al, 1992; Laufer and Changeux, 1989; Tang et al., 1994). Recent studies demonstrate that a binding site (E-box) for myogenic basic helix-loop-helix (bHLH) proteins in the AChR δ subunit gene is critical for activity-dependent gene regulation, and these results suggest that an E-box binding protein(s) is directly involved in electrical activity-dependent regulation of the δ subunit gene (Tang et al., 1994). Because the level of mRNAs encoding myogenic bHLH proteins rises substantially following denervation (Duclert et al., 1991; Eftimie, et al., 1991; Neville et al., 1992; Voytik et al., 1993; Witzemann and Sakmann, 1991), these proteins may directly mediate the increase in AChR expression in denervated muscle by binding to the AChR regulatory region (Jennings and Burden, 1993). Nevertheless, electrical activity-dependent regulation of the AChR subunit genes may be controlled by transcription factors in addition to E-box binding proteins, and expression of other genes that are regulated by electrical activity may be controlled by other pathways and transcription factors. Thus, it remains unclear whether E-box binding proteins are the only transcriptional mediators of electrical activity-dependent regulation.

In addition to its role in repressing AChR gene expression, nerve-induced muscle activity has been implicated as a regulator of other steps in the formation and maturation of synapses. For example, motor neuron survival, motor neuron growth and editing of initial synaptic connections are affected by nerve-induced muscle activity (Hall and Sanes, 1993). To understand how muscle activity regulates muscle structure and function, we used a subtractive-hybridization and cloning strategy to identify and isolate genes that are expressed preferentially in innervated or denervated muscle. We

identified several genes that are expressed preferentially in denervated muscle and one gene that is expressed preferentially in innervated muscle. One of the genes that we found to be regulated by electrical activity is the recently discovered acute myeloid leukemia 1 (AML1) gene (Miyoshi et al., 1993).

Disruption and translocation of the human *AML1* gene is responsible for a form of acute myeloid leukemia (Erickson et al., 1992; Miyoshi et al., 1993). AML1 is a DNA-binding protein, containing a DNA-binding region homologous to the Drosophila runt protein (Bae et al., 1994; Daga et al., 1992; Erickson et al, 1992; Kania et al., 1990). The normal function of AML1, however, is not known and its expression and regulation in skeletal muscle was not previously appreciated. AML1 is thought to dimerize with core binding factor β (CBF β) (Ogawa et al., 1993a; Ogawa et al, 1993b; Wang et al., 1993), and translocations in the β subunit gene likewise cause acute myeloid leukemia (Liu et al., 1993). The AML1/CBF β heterodimer was purified independently on the basis of its ability to bind core sequences (Speck and Baltimore, 1987; Wang and Speck, 1992), which are important for viral pathogenesis, in murine leukemia virus enhancers (Hallberg et al, 1991; Speck et al., 1990). Because of its potential role as a transcriptional mediator of electrical activity, we characterized expression of the *AML1* gene in innervated, denervated and developing skeletal muscle.

MATERIALS AND METHODS

RNA analysis

RNase protection assays were performed as described previously (Wang et al., 1993). Briefly, antisense RNA was hybridized with total RNA (Chomczynski and Sacchi, 1987) in hybridization buffer overnight at 42°C. Non-hybridized RNA was digested with RNase (A and T1) for 1 hour at 30°C. Following proteinase K digestion and phenol extraction, the protected RNA was precipitated by ethanol and fractionated in 5% denaturing polyacrylamide gels.

mRNA was purified by oligo-dT cellulose chromatography (Aviv and Leder, 1972), electrophoresed in a 1% formaldehyde agarose gel and transferred to a GeneScreen (Du Pont) membrane. DNA probes were labelled by random-hexamer-priming. The membranes were hybridized overnight in 5XSSPE, 5X Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA at 68°C. The filter was washed in 0.1% SDS, 0.1XSSPE for 1 hour at 68°C and exposed for 2 days with an intensifying

screen. Northern blots and RNase protection assays were quantitated with a PhosphorImager.

Epitope-tagging of AML1

The hemagglutinin (HA) epitope-tag was added to the carboxyl terminus of AML1 by PCR. The HA-tagged *AML1* cDNA was inserted into a myosin light chain vector (MDAF, kindly provided by Dr. J. P. Merlie). Mouse C2 cells were grown and transfected as described previously (Simon and Burden, 1993). Pooled stably transfected cells were stained with a monoclonal antibody (9E10) against the HA epitope; the 9E10 hybridoma cell line was obtained from ATCC.

Site-directed mutagenesis

The AChR δ subunit 5' regulatory sequence, -840/+25, was mutagenized by site-directed mutagenesis (Simon and Burden, 1993). The mutations were confirmed by sequencing, and the mutated regulatory sequence was subcloned into p0hGH (Selden et al., 1986). AChR δ-hGH and pSV2-CAT plasmids were co-transfected into C2 myoblasts, the amount of hGH secreted from myotubes was determined by a radioimmunoassay 4 or 5 days after transfection and the amount of secreted hGH was normalized to the level of CAT activity (Simon and Burden, 1993). Duplicate dishes were included in each experiment; expression from duplicate dishes varied by less than 20%.

Electrophoretic mobility-shift assay

AML1-binding to wild-type and mutated sequences was assayed by an electrophoretic mobility-shift assay (Wang et al., 1993). Oligonucleotides were end-labelled and purified with a Biospin-3 column (Biorad), and AML1/CBF β proteins were translated in reticulocyte lysates (Promega).

The GenBank/EMBL accession number for the rat *AML1* sequence is L35271.

RESULTS

AML1 expression increases following denervation.

We isolated several cDNA fragments encoding the rat homologue of human AML1 from our denervated minus innervated muscle library, and we used the cDNA fragment to isolate a cDNA encoding full-length AML1 protein from a rat skeletal

muscle library. The 1.98 kb cDNA contains an ORF of 1.35 kb that encodes a 48.5 kDa protein (Fig. 5.1) which is highly homologous to human (99%) and mouse (99%) AML1 (Bae et al., 1993) and less homologous (82%) to the related mouse protein, PEBP2α (Ogawa et al., 1993b). The rat cDNA encodes an AML1 protein that is identical in length to the mouse AML1 protein but longer than the human AML1 protein.

To confirm that *AML1* expression is regulated by innervation, we measured the level of *AML1* mRNA in innervated and denervated muscle by an RNase protection assay. Figure 5.2 shows that *AML1* is expressed in rat skeletal muscle and that *AML1* expression increases substantially (50- to 100-fold) following denervation.

Because Schwann cells and other perisynaptic cells respond to denervation (Brocks, 1984; Gatchalian, et al., 1989; Reynolds and Woolf, 1992), we considered the possibility that perisynaptic cells, rather than muscle cells, accounted for the increased *AML1* expression in denervated muscle. Muscle can be conveniently dissected into a synaptic region, containing perisynaptic cells, and a non-synaptic region, lacking perisynaptic cells (Merlie and Sanes, 1985). We dissected the synaptic and non-synaptic regions of innervated and denervated skeletal muscle and measured the level of *AML1* expression in each region. Figure 5.3 shows that *AML1* expression in the non-synaptic region increases 50 to 100-fold following denervation. Because non-synaptic regions of muscle lack Schwann cells and other perisynaptic cells, this result indicates that muscle fibers are the predominant source of *AML1* RNA in denervated muscle.

Although *AML1* is expressed at similar levels in synaptic and non-synaptic regions of innervated muscle, *AML1* is expressed preferentially in the synaptic region of denervated muscle (Fig. 5.3). We do not know the cellular source of the preferential *AML1* expression in the synaptic region of denervated muscle; *AML1* expression may be greater in the synaptic than the non-synaptic region of denervated muscle fibers and/or *AML1* expression may also increase in Schwann cells or other perisynaptic cells following denervation.

The distribution of *AML1* expression in different tissues has received little attention. Both B-cell lines and T-cell lines express multiple *AML1* transcripts (2.1, 4.3, 5.4, 8.2 kb), although the rank order of their abundance differs among different cell lines (Bae et al., 1993; Miyoshi et al., 1991). Although little is known about the different transcripts, there is evidence that the different transcripts encode different proteins (Bae et al., 1994). Four *AML1* transcripts, which are similar in size to those in B-cell lines and T-cell lines, are expressed in denervated skeletal muscle, and the abundance of each transcript increases following denervation (Fig. 5.4). The predominant *AML1* transcripts in denervated skeletal muscle are ~7.0 and 6.3 kb long.

We measured the time course of *AML1* expression following denervation using an RNase protection assay. A two-fold increase in *AML1* expression is detectable by 1 day after denervation, and a 50- to 100-fold increase is detectable by 5 days after denervation (Fig. 5.4). The increases in AChR and N-CAM expression following denervation follow a similar time course (Covault et al., 1987; Merlie et al., 1984), whereas the increase in myogenin expression precedes that of the other genes (Duclert et al., 1991; Eftimie et al., 1991; Neville et al., 1992; Voytik et al., 1993; Wilson et al., 1984).

Although most skeletal muscle genes are expressed at similar levels in fast and slow muscle fibers, a subset of muscle genes are expressed preferentially in certain fiber types (Atomi et al., 1991; Donoghue et al., 1991; Hughes et al., 1993; Pette and Staron, 1990; Schiaffino et al., 1989; Voytik, et al., 1993). To determine whether *AML1* is expressed preferentially in fast or slow myofibers, we measured the abundance of *AML1* RNA in innervated and denervated rat soleus (slow) and extensor digitorum longus (EDL) (fast) muscles. Figure 5.6 shows that *AML1* is expressed at similar levels in rat soleus and EDL muscles and that denervation causes a 50- to 100-fold increase in *AML1* expression in both slow and fast muscles. Thus, the regulation of *AML1* expression is similar in slow and fast muscles.

Many genes that are expressed in skeletal muscle are expressed at low levels in myoblasts and at significantly higher levels following myoblast fusion and muscle differentiation. To determine whether *AML1* is induced during muscle differentiation, we measured *AML1* expression in C2 myoblasts and myotubes. Figure 5.7 shows that expression of *AML1* is not up-regulated during muscle differentiation. Indeed, the level of *AML1* expression is similar in myoblasts, embryonic myotubes and denervated adult muscle (Fig. 5.7).

CBF β and PEBP2α mRNAs are expressed in skeletal muscle.

AML1 is thought to complex with a second protein, termed CBF β, and the heterodimer, CBF, has a higher affinity for an AML1 binding site (see below) than AML1 alone (Ogawa et al., 1993a; Ogawa et al., 1993b; Wang et al., 1993). Therefore, we determined whether CBF β is expressed in skeletal muscle. Figure 5.8 shows that CBF β RNA is expressed in skeletal muscle (Wang et al., 1993). CBF β is more abundant than AML1 in innervated muscle, but CBF β expression increases little following denervation. As a consequence, CBF β and AML1 are expressed at similar levels in denervated muscle.

PEBP2α is structurally related to AML1 and contains a runt homology region (Ogawa et al., 1993b), but its expression is thought to be restricted to T cells, where it can function as a transcriptional activator (Ogawa et al., 1993b). To determine whether PEBP2α is expressed in skeletal muscle, we measured the level of PEBP2α RNA in innervated and denervated muscle. Figure 5.8 shows that PEBP2α is expressed at low levels in innervated skeletal muscle and at ~4-fold greater levels following denervation.

CBF was purified from thymus nuclear extracts on the basis of its ability to bind core sites in viral enhancers (Kamachi et al., 1990; Wang and Speck, 1993). To determine whether AML1 is a nuclear protein in skeletal muscle, we transfected an expression vector encoding AML1, tagged with an epitope from HA, into C2 muscle cells and located the epitope-tagged protein by immunofluorescence. Figure 5.9 shows that *AML1* expression is restricted to the nuclei of myoblasts and myotubes. Thus, AML1 is a nuclear protein in muscle.

The cis-acting region of the AChR δ subunit gene that confers regulation by electrical activity lacks a functional binding site for AML1/CBF β.

A consensus binding site for CBF has been determined by site-selection assays (Melnikova et al., 1993). The regulatory region of the AChR δ subunit gene, which confers innervation-dependent regulation (Tang et al., 1994), lacks this consensus sequence but contains a sequence which deviates from the consensus sequence by a single nucleotide.

We used recombinant AML1/CBF β, synthesized *in vitro*, to determine whether this variant sequence binds AML1. Figure 5.10 shows that AML1 and AML1/CBF β bind the variant sequence in the δ subunit regulatory region but with a 30-fold lower affinity than the consensus CBF target sequence.

To determine whether the weak AML1-binding sequence is required for δ subunit gene expression, the AML1-binding site (AACCACC) in the AChR δ-subunit regulatory region (positions -31 to -25) was mutagenized to generate either a nonfunctional (AACGTCC) or a consensus (AACCACA) binding sequence for AML1 (Melnikova et al, 1993). Gene fusions between hGH and wild-type or mutated AChR δ-subunit sequences (nucleotides -840 to +25) were transfected into C2 myoblasts, and hGH expression was measured as described in Materials and Methods. We found that a mutation which prevents AML1/CBF β binding has little or no effect on δ-subunit expression in cultured myotubes. Moreover, mutation of the weak AML1-binding sequence to a consensus AML1-binding sequence has little or no effect on δ subunit

expression. These results indicate that the weak AML1-binding sequence is not a target for AML1-mediated transcriptional activation in myotubes grown in cell culture.

DISCUSSION

We show that *AML1* is expressed in skeletal muscle and is regulated by electrical activity. Although previous studies had shown that *AML1* is expressed in 3T3 cells, B-cell lines and T-cell lines, expression of *AML1* in other cell types and tissues had not been examined. Our results demonstrate that *AML1* expression is not restricted to the hematopoetic lineage; rather, *AML1* is expressed in skeletal muscle where it is regulated by physiological signals.

Alternative splicing of *AML1* RNA results in four transcripts of different sizes. We found that the level of all four *AML1* transcripts increases in response to denervation. Although little is known about the different transcripts, the different transcripts encode different proteins which may have different functions (Bae et al., 1994; Bae et al., 1993; Meyers et al., 1993; Miyoshi et al., 1991). Indeed, others have speculated that certain isoforms of AML1 may bind DNA and activate transcription whereas other isoforms may bind DNA but repress gene expression (Meyers et al., 1993).

Since translocations involving either *AML1* or the CBF β subunit gene lead to leukemia, one might predict that AML1 might participate in regulation of cell growth or differentiation. Our data, however, does not support the idea that *AML1* regulates proliferation during muscle differentiation, since *AML1* is expressed at similar levels in both proliferating myoblasts and in differentiated myotubes grown in cell culture. Our studies, however, do not address the possibility that AML1 might be regulated post-transcriptionally during muscle differentiation or that AML1 might regulate muscle growth later in development.

It is not clear how the t(8;21) DNA rearrangement, which results in a fusion between the *AML1* and *ETO* genes, causes leukemia. Since AML1 can bind to target sequences in the T-cell receptor β enhancer and activate transcription (Bae et al., 1994), it is possible that the gene fusion disrupts the normal transcriptional activity of *AML1* and that the function of wild-type AML1 is inhibited by the mutant AML1 protein in a dominant negative manner (Bae et al., 1994; Miyoshi et al., 1993). Alternatively, the gene fusion could result in a novel transcriptional activity, which in turn leads to aberrant gene expression and cell proliferation.

Some of the muscle genes whose expression is repressed by electrical activity, such as AChR subunit genes and N-CAM, are also locally expressed or concentrated in the synaptic region. At present, we do not know the distribution of AML1 protein or mRNA within the muscle fibers. Our experiment to examine the localization of *AML1* mRNA by measuring its abundance in dissected synapse-rich and synapse-free muscle fractions does not provide evidence for the enrichment of *AML1* mRNA in the synaptic region. However, our dissection was relatively crude, the synaptic-rich region we obtained still contains a vast number of non-synaptic nuclei; therefore, even a very low level of *AML1* expression in the non-synaptic region would mask the mRNA concentration gradient. A more careful study with in situ hybridization will be necessary to determine the distribution of *AML1* mRNA in muscle.

Muscle genes that are regulated by electrical activity, including those that regulate muscle atrophy, motor neuron survival, motor neuron growth and editing of initial synaptic connections are potential targets for AML1. Our data, however, demonstrate that the AChR δ subunit gene is not directly regulated by AML1 in developing muscle, and these results suggest that AML1 does not directly regulate expression of the AChR δ subunit gene in response to changes in muscle fiber electrical activity. If AML1 has a role in electrical activity-dependent regulation of the AChR δ subunit gene, then AML1 is likely to act indirectly. The target genes for AML1 and the consequences of increased *AML1* expression in muscle may be best examined by increasing *AML1* expression in innervated muscle or decreasing *AML1* expression in denervated muscle.

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Figure 5.1. The complete nucleotide sequence of the rat *AML1* cDNA.
The complete nucleotide and encoded amino acid sequence of the 1.98 kb *AML1* cDNA is shown. A TGA stop codon at nt 1726 is indicated by an asterisk. Amino acids are represented by their single letter abbreviations below the codons encoding them. A polyadenylation addition site at position 1837 in the 3' untranslated sequence is boxed.

```
                                                  5'     GAATTCTCCAAATCTAGTTTGTTTT     25
TTTTTCTGGTCATTTTTTTTAATTCTGCGCATAAATATTTTAGGACGCATACGGGAATTTTGCCTCCGGGACCGT    100
TTGTAATAGCCAAAGACTGAACTCCAAACTCTGAAAGCGAGGCTCCTTGGGGCATTTGACTTTGAATTCTGGGTT    175
GCCCACTTTTCAATGCACTAAGCGGCCAGTTGCTAACCCTGCCTGGGTGTAAGGCCGATCCGCGTAACTAGATCT    250
CAGTCCTGCTACCCCCACAACCCTCCGGTAGTAATAAAGGCTCCTGAACTTGTATGTTGGTCTCCCGGGAGCAGC    325
TTGCAGAAGATCCGAGTCCCTGTCGCCGTCTAGTAGGAGCTGTTTTCAGGGTCCTTACTCAATCGGCTTGTTGTG    400
ATGCGTATCCCCGTAGATGCCAGCACGAGCCGCCGCTTCACGCCGCCTTCCACTGCGCTGAGCCCCGGCAAGATG    475
 M  R  I  P  V  D  A  S  T  S  R  R  F  T  P  P  S  T  A  L  S  P  G  K  M     (25)

AGCGAGGCGCTGCCGCTGGGCGCCCCGGATGGCGGCGCCGCCCTGGCCAGCAAGCTGAGGAGCGGCGACCGCAGC    550
 S  E  A  L  P  L  G  A  P  D  G  G  A  A  L  A  S  K  L  R  S  G  D  R  S     (50)

ATGGTGGAGGTACTAGCTGACCACCCCGGCGAGCTAGTCCGCACCGACAGCCCCAACTTCCTCTGCTCCGTGCTG    625
 M  V  E  V  L  A  D  H  P  G  E  L  V  R  T  D  S  P  N  F  L  C  S  V  L     (75)

CCCACTCACTGGCGCTGCAACAAGACCCTGCCCATCGCTTTCAAGGTGGTAGCGCTGGGCGACGTTCCGGATGGC    700
 P  T  H  W  R  C  N  K  T  L  P  I  A  F  K  V  V  A  L  G  D  V  P  D  G     (100)

ACTCTGGTCACCGTCATGGCGGGCAATGACGAAAACTACTCGGCGGAGCTGAGAAACGCCACCGCGGCCATGAAG    775
 T  L  V  T  V  M  A  G  N  D  E  N  Y  S  A  E  L  R  N  A  T  A  A  M  K     (125)

AACCAAGTGGCGAGATTCAACGACCTCAGGTTTGTCGGTCGGAGCGGCAGAGGCAAGAGCTTCACTCTGACCATC    850
 N  Q  V  A  R  F  N  D  L  R  F  V  G  R  S  G  R  G  K  S  F  T  L  T  I     (150)

ACCGTCTTTACAAACCCCCCACAAGTTGCCACCTACCATAGAGCCATCAAAATCACAGTGGACGGCCCCCGAGAA    925
 T  V  F  T  N  P  P  Q  V  A  T  Y  H  R  A  I  K  I  T  V  D  G  P  R  E     (175)

CCCCGAAGACATCGGCAGAAACTAGATGATCAGACCAAGCCCGGGAGTTTGTCCTTTTCCGAGCGGCTCAGTGAA   1000
 P  R  R  H  R  Q  K  L  D  D  Q  T  K  P  G  S  L  S  F  S  E  R  L  S  E     (200)

CTGGAGCAGCTGCGGCGCACGGCCATGAGGGTCAGCCCGCATCACCCAGCCCCCACGCCCAACCCTCGGGCCTCC   1075
 L  E  Q  L  R  R  T  A  M  R  V  S  P  H  H  P  A  P  T  P  N  P  R  A  S     (225)

TTGAACCACTCCACTGCCTTTAACCCTCAGCCTCAAAGTCAGATGCAGGATGCCAGGCAGATCCAGCCGTCCCCA   1150
 L  N  H  S  T  A  F  N  P  Q  P  Q  S  Q  M  Q  D  A  R  Q  I  Q  P  S  P     (250)

CCGTGGTCCTATGACCAGTCTTACCAGTACCTGGGGTCCATCACCTCTTCTGTGCACCCGGCGACACCCATTTCT   1225
 P  W  S  Y  D  Q  S  Y  Q  Y  L  G  S  I  T  S  S  V  H  P  A  T  P  I  S     (275)

CCCGGCCGAGCCAGTGGCATGACCAGCCTCTCAGCGGAAACTTTCCAGTCGACTCTCAACAGCCCCGGACCTGACA   1300
 P  G  R  A  S  G  M  T  S  L  S  A  E  L  S  S  R  L  S  T  A  P  D  L  T     (300)

GCCTTCGGCGACCCGCGCCAGTTCCCCACGCTGCCGTCCATCTCCGACCCGCGCATGCACTACCCAGGCGCCTTC   1375
 A  F  G  D  P  R  Q  F  P  T  L  P  S  I  S  D  P  R  M  H  Y  P  G  A  F     (325)

ACCTACTCGCCTCCTGTCACATCGGGCATCGGCATTGGCATGTCAGCCATGAGCTCGACCTCTCGCTACCACACC   1425
 T  Y  S  P  P  V  T  S  G  I  G  I  G  M  S  A  M  S  S  T  S  R  Y  H  T     (350)

TACCTGCCGCCGCCCTACCCAGGCTCCTCGCAGGCGCAGGCCGGGCCCTTCCAGACCGGCTCGCCCTCCTACCAC   1500
 Y  L  P  P  P  Y  P  G  S  S  Q  A  Q  A  G  P  F  Q  T  G  S  P  S  Y  H     (375)

CTGTACTACGGCACCTCGGCAGGCTCCTACCAATTCTCCATGGTGGGCGGCGAGCGATCACCCCCGCGCATCCTG   1575
 L  Y  Y  G  T  S  A  G  S  Y  Q  F  S  M  V  G  G  E  R  S  P  P  R  I  L     (400)

CCGCCCTGCACCAACGCGTCCACCGGAGCCGCGCTGCTCAACCCCAGCCTCCCCAGCCAGAGCGACGTGGTGGAA   1650
 P  P  C  T  N  A  S  T  G  A  A  L  L  N  P  S  L  P  S  Q  S  D  V  V  E     (425)

ACCGAGGGCAGCCATAGCAACTCGCCCACCAACATGCCACCCGCGCGCCTGGAGGAGGCTGTCTGGCGGCCCTAC   1725
 T  E  G  S  H  S  N  S  P  T  N  M  P  P  A  R  L  E  E  A  V  W  R  P  Y     (450)

TGAGCGCCTTCGGGGGACTGAGCCCGCCGTCCATGCACAGACCCCGCCAGGAGAGCCCTTGGAGGCCAACAGGAG   1800
 *

GGAAACTGTGAATGCTTCTGATTTAGTAATGCTGTGAATAAAAGAAAGATTTTATACCCTTGACTTCACTTTTTA   1875
ACCACGTTGTTTATTCCAAAGAGTGTGGAATGTTTTCGGTTCCGGGTGGGGAAGACGCAGCCCATCCTGTTTGGC   1950
ATCTATTTCTTTTCCGCACCTTATTGATTGC(A)10      3'                                    1981
```

Figure 5.2. *AML1* expression increases 50 to 100-fold following denervation. RNA was extracted from 5-day denervated (Den) or contralateral innervated (Inn) rat lower leg muscle, and the levels of *AML1* and actin mRNAs were measured by RNase protection. The level of *AML1* mRNA increases approximately 50- to 100-fold after denervation. Actin mRNA level remains unchanged.

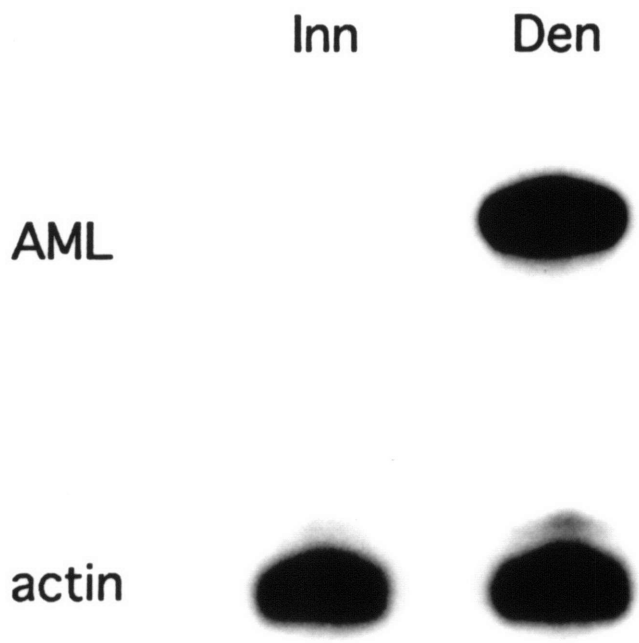
Inn
Den
AML
actin

Figure 5.3. *AML1* expression increases in non-synaptic regions of rat soleus muscle following denervation.

Innervated (Inn) and 5-day denervated (Den) soleus muscle were dissected into synaptic (ep+) and non-synaptic (ep-) regions, and the levels of *AML1* and actin mRNAs in each region were measured by RNase protection. *AML1*, normalized to actin, is expressed at similar levels in synaptic and non-synaptic regions of innervated muscle; the synaptic region contains 10-fold more AChR ε subunit mRNA than the non-synaptic region. *AML1* is expressed at 5-fold higher levels in the synaptic than non-synaptic region of denervated muscle.

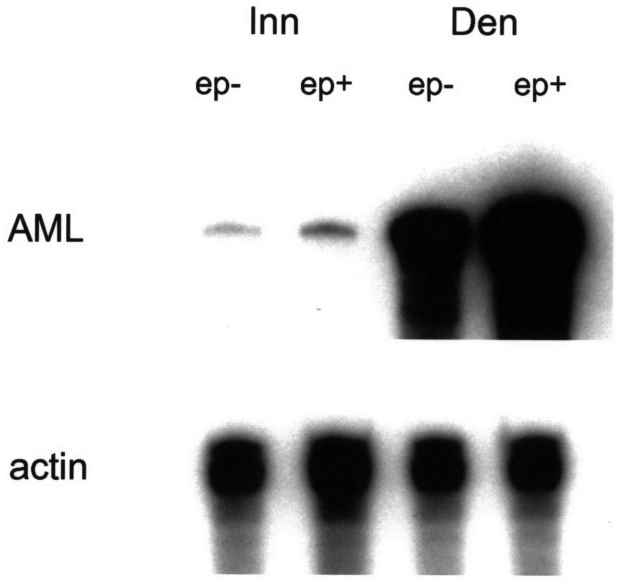
Inn
Den
ep-
ep+
ep-
ep+
AML
actin

Figure 5.4. The level of four *AML1* transcripts increases following denervation. Innervated and denervated rat muscle mRNA (3 μg) were fractionated by gel electrophoresis and transferred to a GeneScreen membrane. The filter was hybridized with a full-length *AML1* cDNA probe and exposed for 2 days with an intensifying screen. Four transcripts (arrowheads), 7.0, 6.3, 3.9 and 3.4 kb in size, are detected in denervated muscle but not innervated muscle. The level of actin mRNA in innervated and denervated muscle was similar.

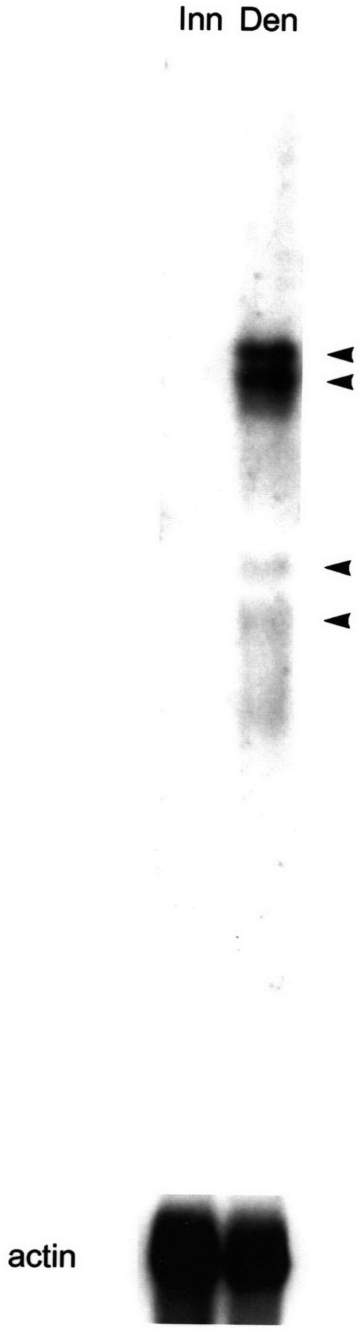
Inn Den
actin

Figure 5.5. Expression of *AML1* and AChR genes increase with a similar time course after denervation.

Rat lower leg muscles were denervated for 1, 2 or 5 days, and the levels of AML1, AChR δ subunit, myogenin and actin mRNAs were measured by RNase protection. *AML1* expression increases approximately 2-fold one day after denervation, and increases 50- to 100-fold by 5 days after denervation. AChR δ subunit expression increases in parallel following denervation. Myogenin expression, in contrast, reaches its highest level after 2 days and therefore, precedes that of *AML1* and the AChR δ subunit gene.

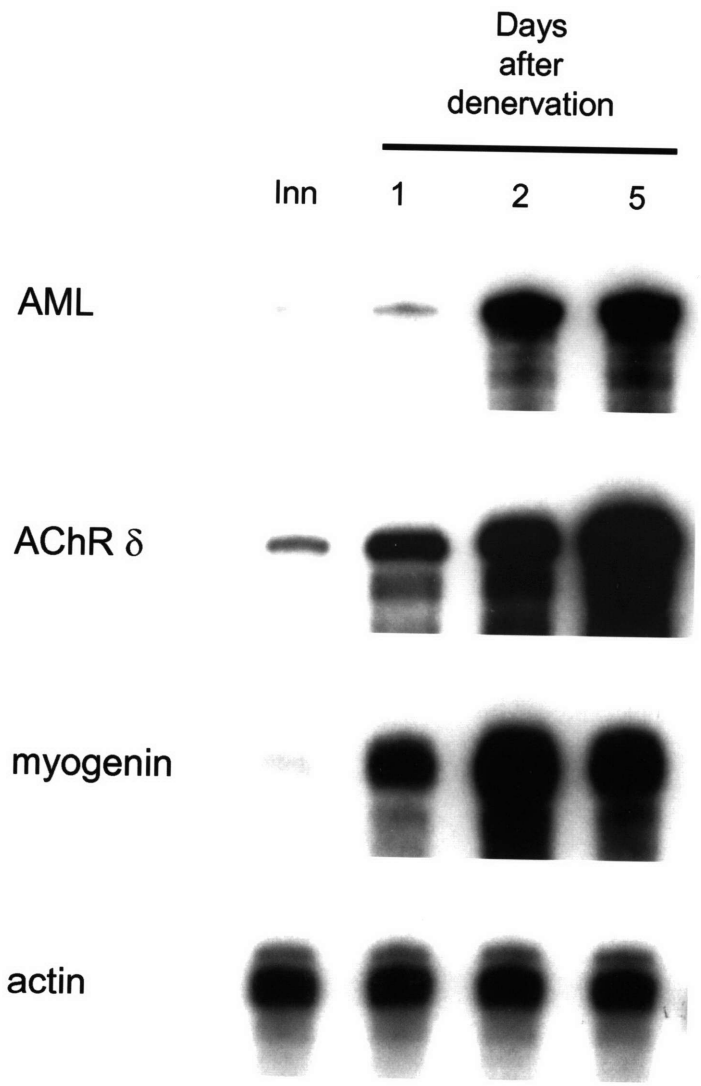
Days
after
denervation
Inn
1
2
5
AML
AChR δ
myogenin
actin

Figure 5.6. *AML1* expression increases in both slow (soleus) and fast (EDL) muscle after denervation.

The level of *AML1* and actin mRNA in innervated and denervated soleus and EDL muscles were quantitated by RNase protection. The level of *AML1* expression is comparable in denervated slow (soleus) and fast (EDL) muscles.

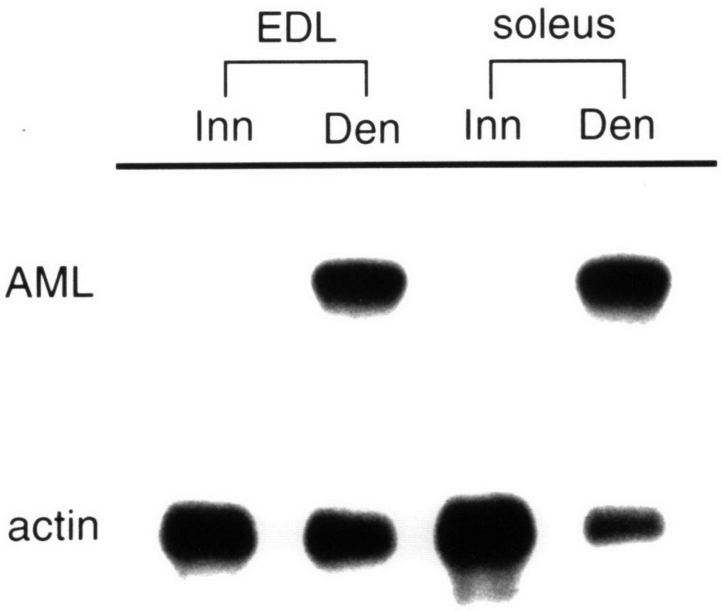
EDL
soleus
Inn
Den
Inn
Den
AML
actin

Figure 5.7. *AML1* is expressed at similar levels in cultured myoblasts and myotubes. Total RNA was extracted from cultured C2 myoblasts (MB) and myotubes (MT), and the level of *AML1* and actin mRNAs was quantitated by RNase protection. The level of *AML1* mRNA is comparable in C2 myoblast and myotubes.

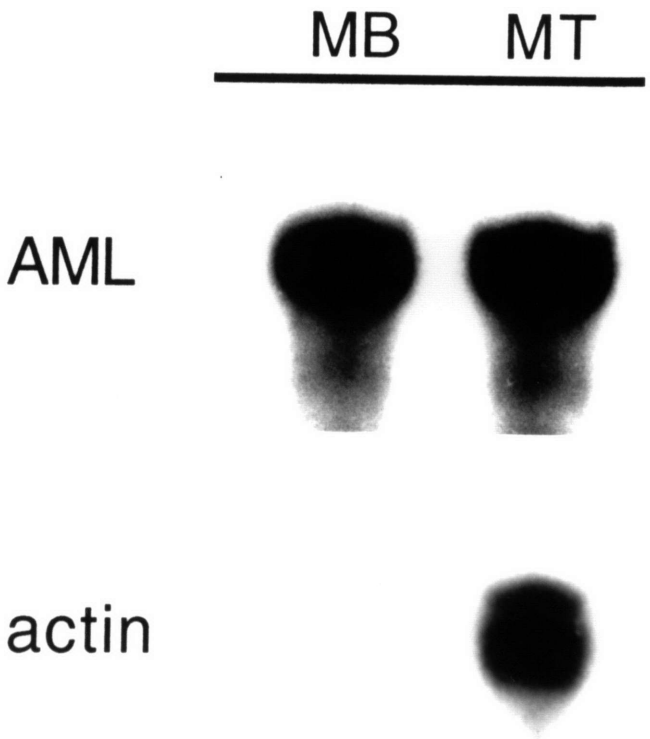
MB
MT
AML
actin

Figure 5.8. CBF β and PEBP2α are expressed in skeletal muscle.
CBF β expression increases ~2-fold and PEBP2α expression increases ~4-fold following denervation. Total RNA was extracted from innervated and denervated (4-day) mouse lower leg muscle, and the level of CBF β and PEBP2α mRNAs were measured by RNase protection.

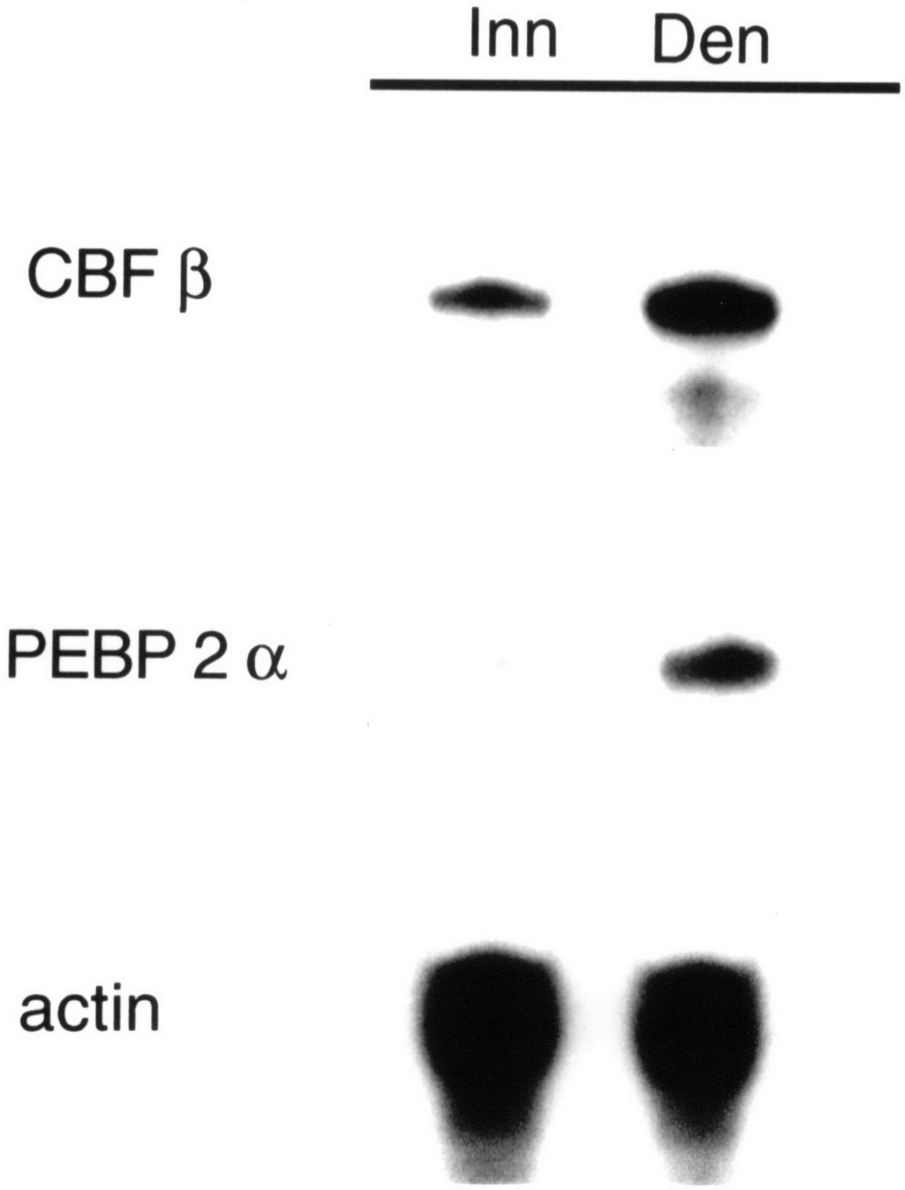
Inn
Den
CBF β
PEBP 2 α
actin

Figure 5.9. AML1 is a nuclear protein in muscle.

HA-tagged AML1 and pSV2 neo plasmid DNAs were co-transfected into C2 myoblasts, and stably transfected cells were selected with G418 (750 μg/ml). Resistant cells were pooled, induced to differentiate into myotubes and stained with a monoclonal antibody (9E10) against the HA epitope (a). Nontransfected C2 myotubes are not stained by the monoclonal antibody 12CA5 (b).

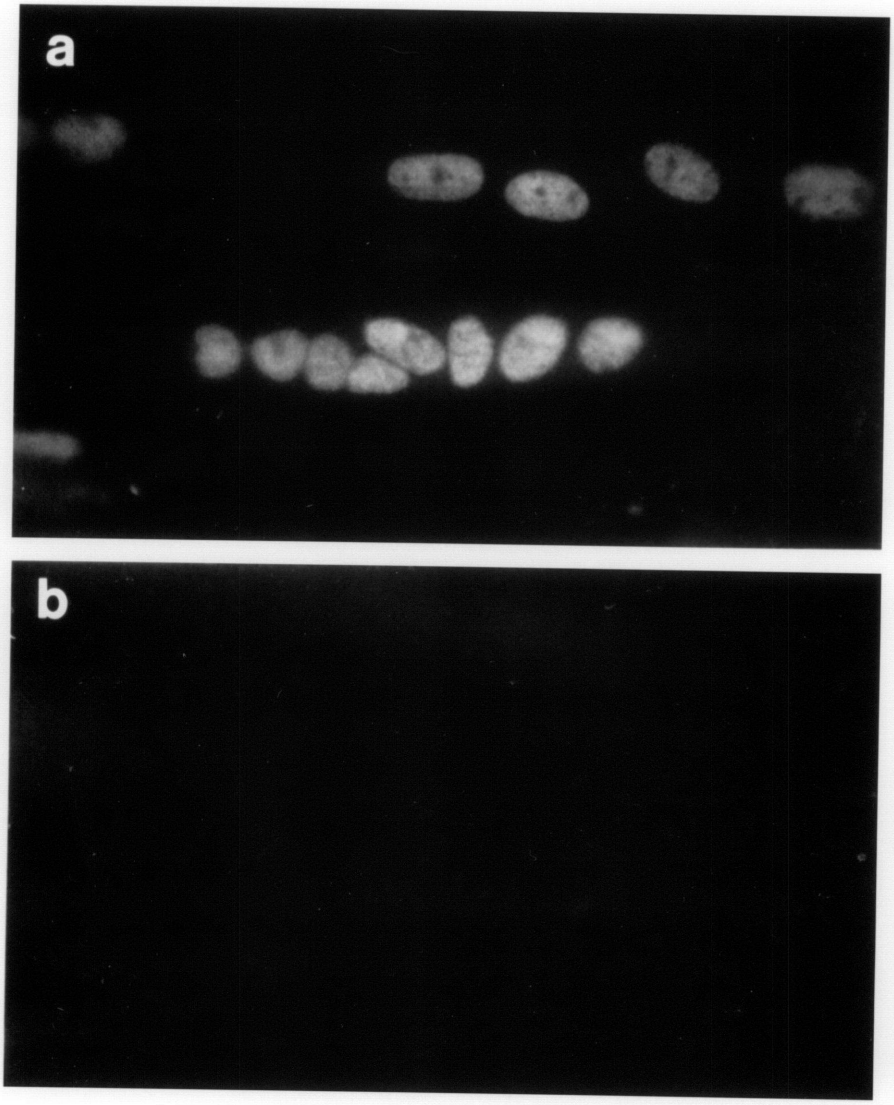
a
b

Figure 5.10. AML1/CBF β binds weakly to a sequence in the AChR δ subunit regulatory region.

AML1/CBF β proteins were translated *in vitro* and incubated with a consensus binding sequence for AML1 (AACCACA) or with a similar sequence (AACCACC) present in the AChR δ subunit regulatory region (-31/-25). 100-fold excess of unlabelled oligonucleotides were used in competition experiments. The consensus sequence has a 30-fold higher affinity for AML1/CBF β than the -31/-25 sequence in the AChR δ subunit regulatory region.

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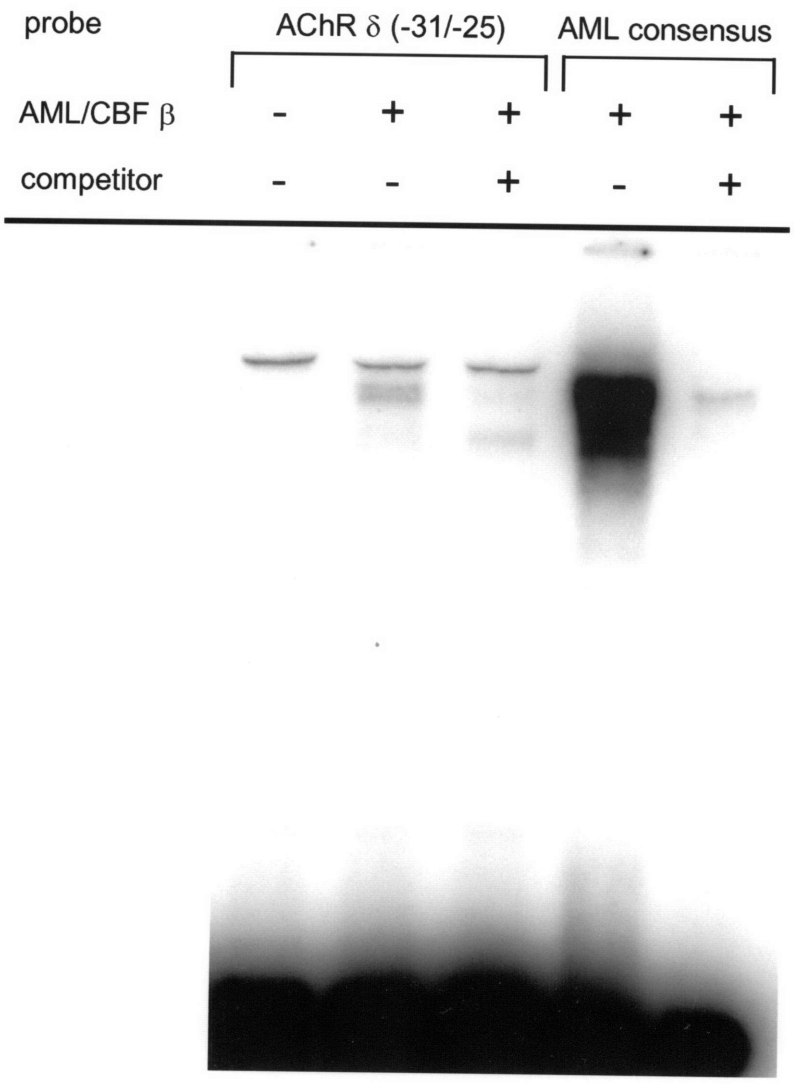
probe
AChR δ (-31/-25)
AML consensus
AML/CBF β
- + + + +
competitor
- - + - +