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Function and Genetics of Dystrophin and Dystrophin-Related Proteins in Muscle

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Blake, Derek J., Andrew Weir, Sarah E. Newey, and Kay E. Davies. Function and Genetics of Dystrophin and Dystrophin-Related Proteins in Muscle. *Physiol Rev* 82: 291–329, 2002; 10.1152/physrev.00028.2001.—The X-linked muscle-wasting disease Duchenne muscular dystrophy is caused by mutations in the gene encoding dystrophin. There is currently no effective treatment for the disease; however, the complex molecular pathology of this disorder is now being unravelled. Dystrophin is located at the muscle sarcolemma in a membrane-spanning protein complex that connects the cytoskeleton to the basal lamina. Mutations in many components of the dystrophin protein complex cause other forms of autosomally inherited muscular dystrophy, indicating the importance of this complex in normal muscle function. Although the precise function of dystrophin is unknown, the lack of protein causes

membrane destabilization and the activation of multiple pathophysiological processes, many of which converge on alterations in intracellular calcium handling. Dystrophin is also the prototype of a family of dystrophin-related proteins, many of which are found in muscle. This family includes utrophin and α-dystrobrevin, which are involved in the maintenance of the neuromuscular junction architecture and in muscle homeostasis. New insights into the pathophysiology of dystrophic muscle, the identification of compensating proteins, and the discovery of new binding partners are paving the way for novel therapeutic strategies to treat this fatal muscle disease. This review discusses the role of the dystrophin complex and protein family in muscle and describes the physiological processes that are affected in Duchenne muscular dystrophy.

I. INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe X-linked recessive, progressive muscle-wasting disease affecting ~1 in 3,500 boys (146). Patients are usually confined to a wheelchair before the age of 12 and die in their late teens or early twenties usually of respiratory failure. A milder form of the disease, Becker muscular dystrophy (BMD), has a later onset and a much longer survival. Both disorders are caused by mutations in the DMD gene that encodes a 427-kDa cytoskeletal protein called dystrophin. The vast majority of DMD mutations result in the complete absence of dystrophin, whereas the presence of low levels of a truncated protein is seen in BMD patients. In addition to these diseases, mutations in the genes encoding many components of the dystrophin-associated protein complex (see below) cause other forms of muscular dystrophy such as the limb-girdle muscular dystrophies and congenital muscular dystrophy.

There is currently no effective therapy for DMD, although various strategies are being developed driven by the increasing understanding of the molecular processes involved in the progression of the muscle weakness. This review summarizes the current knowledge of the gene and protein as well as the disease process and also illustrates how these studies have led to a broader understanding of muscle function.

II. DUCHENNE MUSCULAR DYSTROPHY

A. Clinical Progression of Duchenne and Becker Muscular Dystrophies

Typically, DMD patients are clinically normal at birth, although serum levels of the muscle isoform of creatine kinase are elevated. The first symptoms of DMD are generally observed between the ages of 2 and 5 years (135, 259), with the child presenting with a waddling gait or difficulty in climbing stairs. There is often a delay in the achievement of motor milestones, including a delay in walking, unsteadiness, and difficulty in running. Subsequently, the onset of pseudohypertrophy of the calf muscles, proximal limb muscle weakness, and Gowers' sign (the use of the child's arms to climb up his body when going from a lying to standing position) suggest DMD (188). Eventually, decreased lower-limb muscle strength and joint contractures result in wheelchair dependence, usually by the age of 12 (146). Weakness of the arms occurs later along with progressive kyphoscoliosis. Most patients die in their early twenties as a result of respiratory complications due to intercostal muscle weakness and respiratory infection. Death can also be the result of cardiac dysfunction with cardiomyopathy and/or cardiac conduction abnormalities observed in some patients (146).

In individuals affected by BMD (24), the clinical course is similar to that of DMD, although the onset of symptoms and the rate of progression are delayed. More than 90% of patients are still alive in their twenties, with some patients remaining mobile until old age (146). There is a continuous clinical spectrum between a mildly affected BMD patient and a severely affected DMD patient. BMD and DMD patients also present with mild cognitive impairment, indicating that brain function is also abnormal in these disorders (reviewed in Refs. 42, 335).

B. Histological Features

Normal skeletal muscle consists of muscle fibers that are evenly spaced, angular, and of a relatively uniform size. Muscle, being a syncytium, is multinucleated with nuclei located at the periphery of the fiber. Fetal DMD muscle is histologically normal except for occasional eosinophilic hypercontracted fibers (34, 145, 304). Necrotic or degenerating muscle fibers are characteristically seen in all postnatal DMD muscle biopsies even before muscle weakness is clinically observed. Degenerating fibers are often seen in clusters (grouped necrosis), and studies of longitudinal and serial transverse muscle sections show this process is often confined to segments of the muscle fiber (186, 438). These necrotic fibers are subject to phagocytosis, and muscle biopsies from DMD patients reveal the presence of inflammatory cells at perimysial and endomysial sites (12, 13). These cells are predominantly macrophages and CD4+ lymphocytes (330). A secondary sign of muscle fiber necrosis, at least in the early stages of the dystrophinopathies, is the active regeneration of muscle to replace or repair lost or damaged fibers (438). Early regenerating fibers are recognized by virtue of their small diameter, basophilic RNA-rich cytoplasm, and large, centrally placed myonuclei (29, 56, 438). Even-

tually, the regenerative capacity of the muscles is lost and muscle fibers are gradually replaced by adipose and fibrous connective tissue, giving rise to the clinical appearance of pseudohypertrophy followed by atrophy (reviewed in Ref. 146). The combination of progressive fibrosis and muscle fiber loss results in muscle wasting and ultimately muscle weakness.

III. DYSTROPHIN: GENE AND PROTEIN

A. Gene Sequence

The identification of the DMD gene on the X chromosome was the first triumph of positional cloning and opened up a new era in DMD research (280, 354). The gene was localized to Xp21 by studies of rare female DMD patients with balanced X;autosome translocations with the translocation breakpoint in Xp21 (54). This localization was confirmed using DNA markers (123), and the disease was shown to be allelic with a milder disease of similar clinical course, BMD (273). The gene was eventually identified by taking advantage of a patient with a large deletion who suffered from four X-linked phenotypes including DMD (162). The DMD gene is the largest described, spanning ~2.5 Mb of genomic sequence (Fig. 1) (98, 355) and is composed of 79 exons (98, 355, 417). The full-length 14-kb mRNA transcribed from the DMD locus was found to be predominantly expressed in skeletal and cardiac muscle with smaller amounts in brain and covered a large genomic region (280, 351, 354). The protein product encoded by this transcript was named dystrophin since the lack of it causes dystrophy (280).

B. Tissue-Specific Promoters

Expression of the full-length dystrophin transcript is controlled by three independently regulated promoters.

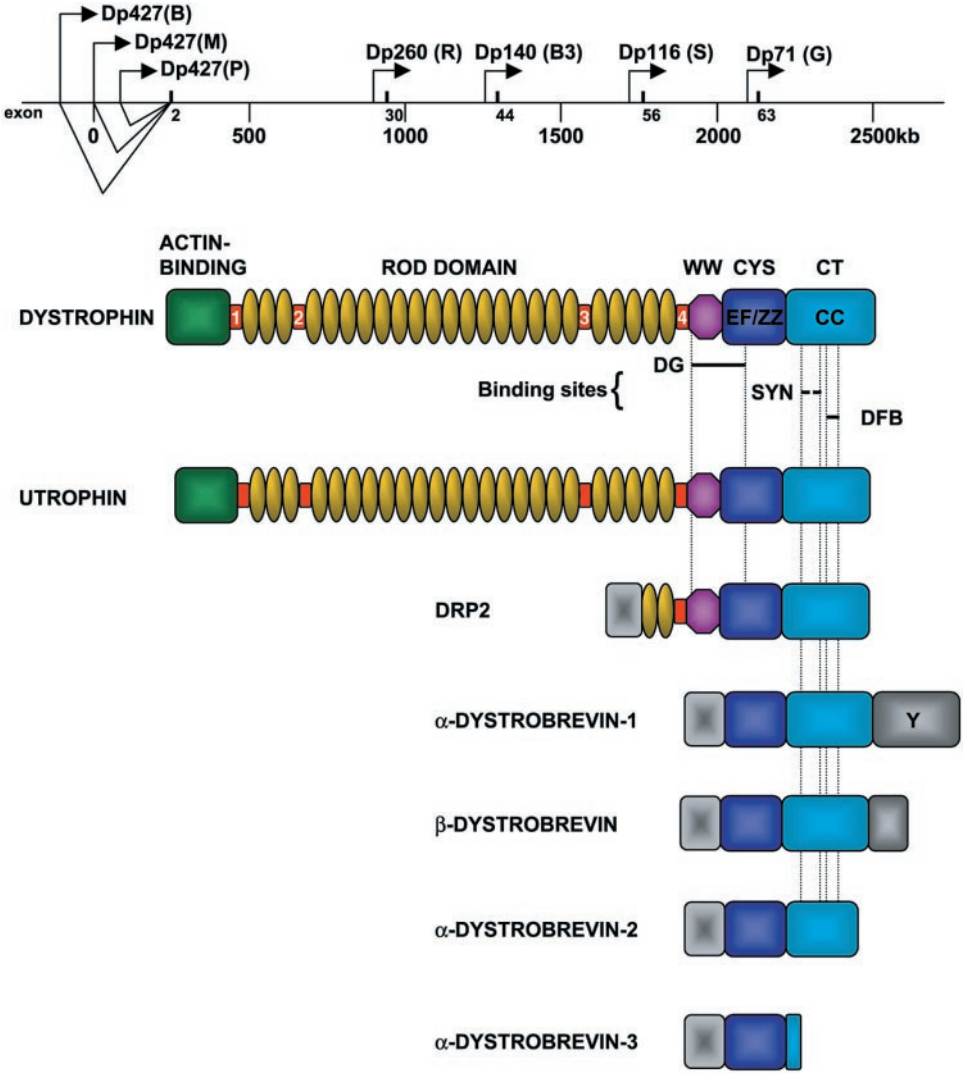

FIG. 1. Schematic showing the organization of the human Duchenne muscular dystrophy (DMD) gene and the dystrophin-related protein family. The DMD gene is 2.5 Mb and encodes 7 different protein isoforms. The "full-length" dystrophin transcripts are transcribed from promoters (depicted by arrows) in the 5′-end of the gene. Each mRNA encodes a 427-kDa protein that only differs in its NH_2-terminal sequences. The three products are designated Dp427 (B), Dp427 (M), and Dp427 (P) to reflect their tissue-specific expression pattern; B is in the brain, M in muscle, and P in cerebellar Purkinje cells. The smaller isoforms are produced from distally located promoters expressed in the retina (R: Dp260), brain (B3: Dp140), Schwann cells (S: Dp116) or are general (G: Dp71) ubiquitously expressed. The identifiable domains in the cysteine-rich (CR) region and COOH terminus (CYS) of dystrophin are identified. These are the WW domain, the EF hands, the ZZ domain, and the paired coiled-coil (CC). The four proline-rich hinge regions are designated 1–4. The binding sites for β-dystroglycan (DG), syntrophin (SYN), and the dystrophin family binding site (DFB) are shown for each protein (dotted lines). The organization of the utrophin protein shows that it is very similar to dystrophin, whereas the DRP2 and the dystrobrevins proteins only have sequence similarity to the COOH-terminal regions of dystrophin as shown. Three α-dystrobrevin isoforms are expressed in muscle representing successive COOH-terminal truncations. The α-dystrobrevin-1 isoform has additional COOH-terminal sequence that contains the sites for tyrosine phosphorylation (Y). β-Dystrobrevin is not expressed in muscle and is most similar to α-dystrobrevin-1 but lacks the sites for tyrosine phosphorylation.

The brain (B), muscle (M), and Purkinje (P) promoters consist of unique first exons spliced to a common set of 78 exons (Fig. 1) (53, 93, 185, 274, 315, 374). The names of these promoters reflect the major site of dystrophin expression. The B promoter drives expression primarily in cortical neurons and the hippocampus of the brain (19, 93, 185), while the P promoter is expressed in the cerebellar Purkinje cells and also skeletal muscle (185, 231). The M promoter results in high levels of expression in skeletal muscles and cardiomyocytes and also at low levels in some glial cells in the brain (19, 93). These three promoters are situated within a large genomic interval of ~400 kb (Fig. 1) (53).

C. Dystrophin Isoforms and Splice Variants

The DMD gene also has at least four internal promoters that give rise to shorter dystrophin transcripts that encode truncated COOH-terminal isoforms. These internal promoters can be referred to as retinal (R), brain-3 (B3), Schwann cell (S), and general (G). Each of these promoters utilizes a unique first exon that splices in to exons 30, 45, 56, and 63, respectively, to generate protein products of 260 kDa (Dp260) (134a), 140 kDa (Dp140) (295), 116 kDa (Dp116) (72), and 71 kDa (Dp71) (43, 241, 291). Dp71 is detected in most nonmuscle tissues including brain, kidney, liver, and lung (43, 237, 238, 241, 291, 436, 439) while the remaining short isoforms are primarily expressed in the central and peripheral nervous system (72, 134a, 295, 439). Dp140 has also been implicated in the development of the kidney (142). These COOH-terminal isoforms contain the necessary binding sites for a number of dystrophin-associated proteins (see sect. VI, *E* and *F*), and although the molecular and cellular function of these isoforms has not been elucidated, they are thought to be involved in the stabilization and function of nonmuscle dystrophin-like protein complexes.

Alternative splicing at the 3′-end of the dystrophin gene generates an even greater number of isoforms (40, 152). These splice variants not only affect full-length dystrophin but are also found in the shorter isoforms such as Dp71. This differential splicing may regulate the binding of dystrophin to dystrophin-associated proteins at the membrane (114).

D. The Dystrophin Protein

Dystrophin is 427-kDa cytoskeletal protein that is a member of the β-spectrin/α-actinin protein family (282). This family is characterized by an NH_2-terminal actin-binding domain followed by a variable number of repeating units known as spectrin-like repeats. Dystrophin can be organized into four separate regions based on sequence homologies and protein-binding capabilities (Fig. 1). These are the actin-binding domain at the NH_2 terminus, the central rod domain, the cysteine-rich domain, and the COOH-terminal domain. The NH_2 terminus and a region in the rod domain of dystrophin bind directly to but do not cross-link cytoskeletal actin (reviewed in Refs. 425, 512). The rod domain is composed of 24 repeating units that are similar to the triple helical repeats of spectrin. This repeating unit accounts for the majority of the dystrophin protein and is thought to give the molecule a flexible rodlike structure similar to β-spectrin. These α-helical coiled-coil repeats are interrupted by four proline-rich hinge regions (281).

At the end of the 24th repeat is the fourth hinge region that is immediately followed by the WW domain. The WW domain is a recently described protein-binding module found in several signaling and regulatory molecules (50). The WW domain binds to proline-rich substrates in an analogous manner to the src homology-3 (SH3) domain (313). Although a specific ligand for the WW domain of dystrophin has not been determined, this region mediates the interaction between β-dystroglycan and dystrophin, since the cytoplasmic domain of β-dystroglycan is proline rich (see below). However, the entire WW domain of dystrophin does not appear to be required for the interaction with dystroglycan because Dp71, a dystrophin isoform that contains only part of the WW domain, is reported to bind to β-dystroglycan (421). Interestingly, transgenic mice overexpressing Dp71 in dystrophin-deficient muscle restore β-dystroglycan and the DPC at the membrane but do not prevent muscle degeneration (113, 202).

The WW domain separates the rod domain from the cysteine-rich and COOH-terminal domains. The cysteine-rich domain contains two EF-hand motifs that are similar to those in α-actinin and that could bind intracellular Ca^{2+} (282). The ZZ domain is also part of the cysteine-rich domain and contains a number of conserved cysteine residues that are predicted to form the coordination sites for divalent metal cations such as Zn^{2+} (395). The ZZ domain is similar to many types of zinc finger and is found both in nuclear and cytoplasmic proteins. The ZZ domain of dystrophin binds to calmodulin in a Ca^{2+}-dependent manner (11). Thus the ZZ domain may represent a functional calmodulin-binding site and may have implications for calmodulin binding to other dystrophin-related proteins. The ZZ domain does not appear to be required for the interaction between dystrophin and β-dystroglycan (412).

The COOH terminus of dystrophin contains two polypeptide stretches that are predicted to form α-helical coiled coils similar to those in the rod domain (47). Each coiled coil has a conserved repeating heptad $(a,b,c,d,e,f,g)_n$ similar to those found in leucine zippers where leucine predominates at the "d" position (reviewed in Refs. 68, 310). This domain has been named the CC

(coiled coil) domain. Approximately 3–5% of proteins have coiled-coil regions. Coiled coils are well-characterized protein interaction domains. The CC region of dystrophin forms the binding site for dystrobrevin and may modulate the interaction between syntrophin and other dystrophin-associated proteins (see sect. VI) (47, 430).

E. Mutations in DMD

The frequency of DMD coupled with a high new mutation rate (1×10^{-4} genes/generation) has led to the characterization of hundreds of independent mutations. Mutations that cause DMD generally result in the absence, or much reduced levels, of dystrophin protein while BMD patients generally make some partially functional protein. There is some correlation between mutations in the DMD gene and the resulting phenotype. The study of such mutations has revealed the importance of a number of the structural domains of dystrophin and facilitated the design of dystrophin "mini-genes" for gene therapy approaches (reviewed in Ref. 9).

Approximately 65% of DMD and BMD patients have gross deletions of the DMD gene (279, 353). After the characterization of many such mutations, it became apparent that the size and position of the deletion within the DMD gene often did not correlate with the clinical phenotype observed. This observation can be largely explained by the reading frame theory of Monaco et al. (352). This argues that if a deletion leads to the expression of an internally truncated transcript without shifting the normal open reading frame, then a smaller, but functional version of dystrophin could be produced. This scenario would be consistent with a BMD phenotype. If, on the other hand, the deletion creates a translational frameshift, then premature termination of translation will result in the synthesis of a truncated protein. This latter scenario is often associated with extremely low levels of dystrophin expression due to mRNA or protein instability and results in a DMD phenotype. With the use of this reading frame theory and the knowledge of exon structure of the DMD gene, it has been possible in many cases to predict whether a young male is likely to develop BMD or DMD (279). However, there are exceptions to this reading frame rule (22, 316, 514), and there are cases in which complete dystrophin deficiency may be associated with a relatively benign phenotype (216).

The vast majority of large deletions detected in BMD and DMD cluster around two mutation "hot spots" (279, 281), although the reasons for this are unclear. It is possible, however, that the chromatin structure in Xp21 influences the occurrence of deletion or recombinant hotspots. Deletion cluster region I spans exons 45–53 (25) and removes part of the rod domain, while deletion cluster region II spans exons 2–20 and removes some or all of the actin-binding sites together with part of the rod domain (296). Most of the breakpoints occurring in cluster region II occur in the large introns 1 and 7. Most of these large deletions can be detected using a simple multiplex PCR test that screens the exons most commonly deleted and allows accurate genetic counseling in the majority of affected families via DNA-based diagnostics (26, 85).

One-third of DMD cases are caused by very small deletions and point mutations, most of which introduce premature stop codons (293, 419). Unlike the large deletions that cluster in two regions of the DMD gene, small deletions and point mutations appear to be evenly distributed throughout the gene (169, 398, 419). Although it might be predicted that such mutations would give rise to normal amounts of truncated protein, usually very little or no protein is detected, indicating that the corresponding transcripts or the truncated proteins are unstable (228). This has disappointing implications for the functional dissection of the dystrophin protein, since many mutations do not generate any information regarding the importance of a particular domain. Despite this setback, a small number of useful mutations have been identified that generate a mutated or truncated protein and convey information regarding the functional importance of the different dystrophin domains.

At the NH_2 terminus of dystrophin, the importance of the actin-binding domain was demonstrated by the identification of missense mutation (Arg for Leu-54) that resulted in a DMD phenotype associated with reduced amounts of protein (398). Furthermore, DMD patients have been described with in-frame deletions of exons 3–25 and produce normal amounts of truncated protein (488).

The rod domain of dystrophin has been found to accommodate large in-frame deletions without serious clinical consequences. The most notable example was the discovery of a patient with an in-frame deletion of 46% of the dystrophin coding sequence which resulted in only a mild case of BMD (deletions of exons 17–48) (147). This observation suggests that the rod domain acts as a spacer between the actin binding domain and the cysteine-rich and COOH-terminal domains of dystrophin, and truncation of this region merely shortens the bridge between these two functional regions without adversely affecting the function of the protein. Indeed, this deletion has been the basis of a dystrophin mini-gene that was incorporated into expression plasmids as well as retroviral and adenoviral vectors for transfer to muscle fibers in vivo (1, 139, 407). Furthermore, this mini-dystrophin was able to restore the normal muscle phenotype in transgenic *mdx* mice (391, 504). Other large deletions of the rod domain have also been observed in BMD patients (305, 514).

Although few missense mutations have been described in DMD patients, two informative substitutions have been identified in the cysteine-rich domain. The substitution of a

conserved cysteine residue with a tyrosine at position 3340 results in reduced but detectable levels of dystrophin. This mutation alters one of the coordinating residues in the ZZ domain (Fig. 1 and sect. III*D*) that is thought to interfere with the binding of the dystrophin-associated protein β-dystroglycan (294). Another reported substitution of an aspartate residue to a histidine residue at position 3335 is also thought to affect the β-dystroglycan binding site, and although there was normal localization and amounts of dystrophin detected, a severe phenotype resulted (184). Interestingly, the cysteine-rich domain is never deleted in BMD patients, suggesting that this domain is critical for dystrophin function (402).

A small number of cases have been reported in which an abnormally truncated protein that is deleted for the COOH terminus is synthesized and localized at the sarcolemma. A DMD patient was found to have a deletion that removed almost the entire cysteine-rich and COOH-terminal domain (39, 229) (Fig. 1 and sect. III*D*). The abnormal protein was normally localized but resulted in a severe clinical phenotype. Another DMD patient has been reported to be deleted for everything 3′ of exon 50 but again generates a truncated protein that is localized to the sarcolemma (222). These examples illustrate the functional importance of the cysteine-rich and COOH-terminal domains of dystrophin that presumably reflects their interactions with other dystrophin-associated proteins (see sect. VI, *E* and *F*).

Finally, cases of X-linked cardiomyopathy are caused by mutations in the DMD gene that abolish the cardiac gene expression of dystrophin, while retaining expression in skeletal muscle. This condition involves ventricular wall dysfunction, dilated cardiomyopathy, and cardiac failure in the absence of skeletal myopathy (153). Mutations in the muscle-specific M-promoter selectively abolish expression in the heart.

IV. THE *MDX* MOUSE AND OTHER DYSTROPHIN-DEFICIENT ANIMALS

The discovery of dystrophin allowed the recognition of other animals with lesions in their orthologous genes. Dystrophin-deficient mice, dogs, and cats (which arose by spontaneous mutation) and more recently nematodes [in which the DMD gene has undergone targeted disruption (35)] play a number of important roles in research into the functions of dystrophin. To a greater or lesser extent they provide models of DMD and allow study of the pathophysiological processes at work. The ease with which the murine genome can be manipulated has made the *mdx* mouse particularly useful in testing functional hypotheses. These animals also allow initial testing of putative treatments for DMD and indeed have been used in screening strategies for such treatments (8, 200).

This section aims to describe the phenotypes of the known dystrophin-deficient vertebrates.

A. The Dystrophin-Deficient *mdx* Mouse

The *mdx* mouse was initially identified because of raised serum creatine kinase levels (an enzyme released from damaged muscle) and was then found to have muscle pathology (67). It lacks full-length dystrophin (228) because of a point mutation in exon 23 of the DMD gene, which forms a premature stop codon (443). The *mdx* mouse retains expression of some COOH-terminal dystrophin isoforms, but mice lacking these too have been generated by ethyl-nitroso-urea induced and insertional mutagenesis (90, 112, 246, 505). These animals are phenotypically similar to the *mdx* mouse, arguing that full-length dystrophin is the functionally significant isoform in muscle.

Obvious weakness is not a feature, and the life span of *mdx* mice is not grossly reduced (311, 383). It has therefore been suggested that this mutant is not a helpful model of DMD (122). However, it is clear that simple in vivo tests can demonstrate muscle dysfunction (79, 403). True muscle hypertrophy is an important feature of *mdx* muscle (unlike DMD), but normalized force production and power output are significantly reduced (311). Muscle fiber necrosis occurs and is particularly frequent during a crisis period at 3–4 wk (469). There is a vigorous regenerative response as evidenced by frequent expression by fibers of the fetal myosin heavy chain isoform, and the majority of fibers become centrally nucleated, as occurs in muscle regeneration after nonspecific insults (109, 132, 211). After the crisis period, central nucleation remains frequent, although expression of fetal myosin heavy chain declines. Degeneration and regeneration continue; however, *mdx* muscle in which regeneration has been blocked by γ-irradiation shows a decline in total fiber numbers and does so as fast at 15–21 wk as at 2–8 wk (378). Further satellite cells (the undifferentiated muscle precursor cell which proliferates in regeneration) continue to express markers of activation (260). In the diaphragm (in which pathology appears most marked), muscle fiber loss and collagen deposition are significant (456). Atrophy and fibrosis are also features in limb muscles of older *mdx* mice (382). It is clear then that the *mdx* mice show many features of DMD but at later times relative to life span than patients. Why this should be is not clear but may relate to differences in the murine biology of muscle regeneration (186). Despite this, the *mdx* mouse has been a key resource in the exploration of dystrophic pathophysiology.

B. The Dystrophin-Deficient Dog

Several dystrophin-deficient dogs have been identified and the causative genetic lesion defined in at least

three (186, 437, 441, 509). The best-characterized phenotype is the golden retriever (the GRMD dog) (104). Muscle weakness becomes apparent at 2 mo and progresses; life span is significantly reduced (491). Histologically muscle shows necrosis, fibrosis, and regeneration (489). The GRMD dog shows perhaps the closest similarity to DMD and has been used to test potential treatments (21).

C. The Dystrophin-Deficient Cat

Hypertrophic feline muscular dystrophy (HFMD) occurs in cats harboring a deletion of the dystrophin muscle and Purkinje promoters; muscle levels of dystrophin are therefore much reduced though nonzero (171, 510). Animals have an abnormal gait and histologically necrosis is present but fibrosis is not seen and hypertrophy is very marked. This later feature causes death in some individuals. Although this odd phenotype could be due to the particular mutation, a previous less well-characterized dystrophin-deficient cat also showed prominent hypertrophy, suggesting that this may be a feature of feline pathophysiology (81). Clinically, therefore, the HFMD cat seems a poor model of DMD.

V. PATHOPHYSIOLOGY OF DYSTROPHIN-DEFICIENT MUSCLE

This section describes the pathophysiological features of dystrophin-deficient muscle and the possible relationships between them. For the purposes of this review, we have divided data about dystrophin deficiency into two sets. One set of results flowed very directly from the discovery of dystrophin; biochemical and genetic techniques have then allowed the identification of binding partners and homologs. Investigation of the changes that occur in the expression of these molecules in dystrophin-deficient muscle has been a fruitful task, and this set of results is discussed in sections VI and VII. The second set of data in contrast have come from lines of investigation that could at least in principle have been carried out without detailed knowledge of dystrophin. These results are discussed in this section.

A. Abnormalities of the Muscle Cell

1. Membrane structure and function

In 1975 Mokri and Engel used electron microscopy to describe the ultrastructural features of DMD muscle (349). They noted absent or disrupted sections of sarcolemma overlying wedge-shaped areas of abnormal cytoplasm, the so-called delta lesions. This observation, subsequently confirmed, together with the high levels of several cytosolic proteins in the blood of patients with DMD, gave rise to the theory that the primary pathology of DMD muscle might be an abnormal fragility and leakiness of the cell membrane (349, 422). Although no equivalent to the delta lesion has been found in the *mdx* mouse (120, 481) or GRMD dog (489), there is good evidence that dystrophin-deficient muscle is characterized by increased permeability to macromolecules flowing in and out of the cell and that this abnormal permeability is made worse by mechanical stress.

DMD and *mdx* muscle contain an increased number of fibers that stain positively for endogenous extracellular proteins (albumin, IgG, IgM) (34, 95, 460). For example, Clarke et al. (95) examined the triceps of 12-wk-old mice and found that 25% of fibers stained for albumin in the *mdx* muscle and only 4% in normal muscle. A similar pattern can be seen using exogenous vital dyes that are normally excluded from muscle cells. *mdx* mice to whom Procion orange or Evans blue (which binds tightly to albumin) has been administered show an increased number of fibers containing the dye (55, 327, 460). Recently, an albumin targeted contrast agent has been developed that allows visualization of these changes in vivo by magnetic resonance imaging (457). To demonstrate that these differences reflect an increased permeability of some dystrophin-deficient muscle cells and not just an increased number of necrotic cells (which do take up these dyes), it is important that the dyes can be shown to accumulate in nonnecrotic cells. Several studies of, for example, Evans blue do demonstrate this (107, 460), but some others have not (319, 457).

These dyes are not taken up uniformly between or within muscles; typically groups of dye-positive fibers are seen and at widely different frequencies in different muscles (460). When animals are exercised on a treadmill, the number of dye-positive fibers increases in both normal and *mdx* but remains much higher in *mdx* muscle (65, 95).

An increased number of permeable fibers which increases further with mechanical stress can also be demonstrated in isolated muscle preparations. These also allow more precise control of applied stress than in live animal studies (255, 347, 390, 503). For example, Petrof et al. (390) applied a variety of mechanical stress/electrical stimulation protocols to isolated normal and *mdx* muscles and then counted fibers that had taken up Procion orange for the fluid in which the muscle was bathed. There were about fivefold more dye-positive fibers in *mdx* muscle under all the stress/stimulation protocols (including when no stress/stimulation had been applied). The most dye-positive fibers were seen after the application of "eccentric" contractions when a stimulated muscle is lengthened. The number of dye-positive fibers after different protocols was correlated with the peak mechanical stress but not the number of electrical stimulations (390).

Does an equivalent phenomenon occur in single fibers or cultured myotubes? Menke and Jockusch (339,

340) have subjected myotubes to hyposmolar stress before assessing their uptake of horseradish peroxidase and their release of several endogenous proteins. They concluded that *mdx* myotubes leak more (340).

There is thus good evidence that dystrophin-deficient muscle contains fibers that allow ingress of molecules normally excluded from the cytoplasm and that this tendency is enhanced after muscle has been put under mechanical stress. Why should this be? There is evidence that cells and especially muscle cells experience frequent transient cell membrane disruptions that are repaired by active resealing mechanisms (333). These disruptions are more frequent after mechanical stress (332). Does the absence of dystrophin render muscle cell membranes more susceptible to these disruptions? The costameres (a rectilinear array of proteins including vinculin and β-spectrin which lies just under the sarcolemma in register with the sarcomeres) are deranged in *mdx* muscle (380, 506). Cytoskeletal γ-actin is normally tightly bound to the sarcolemma but is not in *mdx* muscle (427). There are therefore structural and functional deficits within the sarcolemmal cytoskeleton that could plausibly leave the membrane vulnerable to mechanical damage. Several attempts have been made to define biophysical abnormalities of the dystrophin-deficient sarcolemma and supporting cytoskeleton. Results are not conclusive. Several studies have measured the pressure which, when applied via a patch clamp, ruptures the membrane of myotubes. *mdx* and control myotubes do not differ (163, 164, 243), nor could a difference be found in the stress, strain, or energy required to rupture isolated muscles (289). In contrast, the stiffness of the subsarcolemmal cytoskeleton is decreased fourfold in *mdx* myotubes (381). The biophysical correlate of the enhanced permeability of dystrophin-deficient muscle cells therefore remains rather obscure. A clearer view may come from a more sophisticated theory of how membrane and sarcolemmal cytoskeleton behave under stress (242). Another possibility is that dystrophin plays a role in the resealing mechanisms mentioned above (333).

Two observations should be mentioned that may perhaps be linked to the above phenomena. First, there is evidence that the rate of progression of the pathological process (assessed histologically) may be altered by manipulating the levels of activity of *mdx* mice. Immobilizing a limb by splinting or neurotomy reduces pathology (265, 345, 348). Second, the tension that *mdx* muscle can develop drops faster than in normal muscle as it is subjected to repeated eccentric contractions (64, 347, 428). It may be that this is due to accumulating membrane "damage." An alternative explanation might invoke changes in fiber type composition and therefore in the isoforms of sarcomeric proteins expressed (which are known to occur in *mdx* muscle; Refs. 101, 390). However, single fibers isolated from normal and *mdx* muscle and subjected to chemical membrane disruption do not differ in the rate at which a force deficit develops during eccentric contractions (312). The contrast between this finding and the results in whole muscle may imply a causative role for the membrane.

2. Calcium homeostasis

Calcium homeostasis is critical to many aspects of muscle function (31), and early suggestions that it might be perturbed in dystrophin-deficient muscle stemmed from several observations. Hypercontracted fibers are the earliest morphological abnormality of DMD and were ascribed to persistently raised intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) (119). DMD muscle biopsies showed an increase in the number of fibers positive for a histochemical calcium stain (49). It was hypothesized therefore that [Ca^{2+}]$_i$ is raised in dystrophin-deficient muscle and that this is an important cause of the pathophysiological processes leading to cell death (138). This speculation has spawned much investigation.

Spectroscopic studies demonstrate that the total calcium content of DMD muscle is raised even at an early stage (33, 34, 324). Examination of *mdx* and GRMD muscle broadly agrees (140, 409, 490). However, these studies could not distinguish the intracellular component of the total; this had to await a methodological advance.

A) [Ca^{2+}]$_i$. Some fluorescent calcium chelators (e.g., fura 2) have different excitation/emission spectra in their bound and unbound states. When introduced into cells, therefore, and after appropriate calibration, they allow determination of [Ca^{2+}]$_i$ (466). These techniques can be applied to muscle fibers or myotubes (but not intact animals). In 1988, two groups reported the use of this technique to show that the [Ca^{2+}]$_i$ of DMD myotubes and *mdx* myofibers was about double that of controls (356, 484). The technique has been widely taken up and applied using seemingly similar protocols, but reported data are in conflict. Steinhardt and co-workers (160, 236, 482, 483) confirmed and extended their original observations in dystrophin-deficient myotubes and fibers, and an independent group confirmed a doubling of [Ca^{2+}]$_i$ over controls in *mdx* myotubes (17). However, others have found no change (102, 166, 220, 292, 397, 413). One of these groups in the course of a further study found a small (20%) but statistically significant increase in [Ca^{2+}]$_i$ from *mdx* fibers over controls (485).

How can this conflict be explained? Part of the difficulty may be methodological, and the issues of calibration, altered handling of the dye by dystrophin-deficient cells, variable subcellular compartmentalization of different dyes, and techniques for introducing the dye into cells have been raised (182, 183, 236). Another variable may be the history of the cells used. Some studies prepared myofibers using an enzymatic disassociation step; others used

purely mechanical steps. In addition, after fusion of myoblasts has been induced, myotubes show spontaneous contractions only after some days have elapsed. Given the role that mechanical stresses have been postulated to play in dystrophin-deficient cells, this may be an important factor. One of the above groups found no differences from controls in $[Ca^{2+}]_i$ in noncontracting *mdx* myotubes but large increases when tubes were cultured using conditions that promote spontaneous contractions. Stopping the contractions with tetrodotoxin reduced *mdx* $[Ca^{2+}]_i$ back to control values (248, 413). Steinhardt and coworkers (236) too have reported that chronic but not acute treatment with tetrodotoxin reduces $[Ca^{2+}]_i$ in *mdx* myotubes back to control values.

Investigation of the changes in $[Ca^{2+}]_i$ after electrical or K^+-induced depolarization have also not achieved unanimity. Several found a normal peak value but a slower return to baseline in dystrophin-deficient preparations (102, 250, 356, 484, 485), but some found no change at all (220) and some a higher peak and slower decline (248). The considerations set out above may explain some of this variation.

Some of these investigators have used these techniques to examine how the absence of dystrophin alters changes in $[Ca^{2+}]_i$ when myotubes or fibers are challenged by increased external calcium concentrations and/or hyposmotic shock. Here there is agreement that larger rises in $[Ca^{2+}]_i$ occur in dystrophin-deficient cells (128, 249, 292, 397, 399, 482, 484).

The data so far apply to values for $[Ca^{2+}]_i$ averaged over the whole of the cytoplasm of the cell. Are there differences in regional $[Ca^{2+}]$ between cells with and without dystrophin that could be missed because of this? In *mdx* myofibers challenged by raised external $[Ca^{2+}]$, Turner et al. (482) saw regional $[Ca^{2+}]_i$ rise more close to the sarcolemma than deep within the fiber. However, they could not confirm this finding in myotubes, and further characterization of subcellular variation was beyond achievable resolution. Two more recent studies have however addressed the issue using different techniques. Allard and colleagues (317) (who found no difference from controls in whole cell $[Ca^{2+}]_i$ in *mdx* fibers) used patch-clamp measurements in estimate subsarcolemmal $[Ca^{2+}]_i$ in fibers. By measuring characteristics of calcium-activated K^+ channels with the patch clamp in both the cell-attached and inside-out configurations, they estimated that $[Ca^{2+}]_i$ at the sarcolemma was threefold greater in *mdx* than wild-type fibers (102, 317). In the other study, myotubes were transfected with various DNA constructs that express a calcium-sensitive photoprotein tagged with different signal proteins that target to different subcellular regions (415). $[Ca^{2+}]_i$ at the sarcoplasmic reticulum (SR) was almost 50% greater in *mdx* than control myotubes. No differences could be demonstrated in cytoplasmic $[Ca^{2+}]_i$, although the authors caution that the photoprotein signal is insensitive in the relevant range. The peak of the depolarization-induced transient was raised above control in mitochondria but not in bulk cytoplasm or subsarcolemma (at least in younger cultures; in 11-day myotubes the peak was greater in all three regions). The authors interpret their findings as consistent with an increase in cytoplasmic $[Ca^{2+}]_i$, which is amplified in the SR.

In summary, data exist showing an increase in $[Ca^{2+}]_i$ in dystrophin-deficient myofibers and myotubes (especially after a challenge to calcium homeostasis) and also higher levels of calcium in the SR. It appears that consensus has been reached that conflicting data can largely be understood on the basis of methodological considerations (423). It should be remembered that all these are in vitro data; we are ignorant of $[Ca^{2+}]_i$ changes in intact animals.

B) CALCIUM FLUXES. An increase in $[Ca^{2+}]_i$ in dystrophin-deficient cells might arise from abnormal fluxes of calcium into the cytoplasm from outside the cell or from within the SR. What evidence is there for such calcium flows?

C) FLOWS OF CALCIUM INTO THE CELL. Different approaches to recording the rate of calcium entry into a cell are available. One uses the phenomenon of manganese quenching of the fluorescence of calcium-sensitive dyes like fura 2. If it is assumed that the divalent ions Mn^{2+} and Ca^{2+} enter a cell in the same way, then the rate of signal quenching after Mn^{2+} are introduced extracellularly gives a measure of calcium influx. Using this technique, two groups have demonstrated that the calcium entry in *mdx* myotubes and fibers is about double that in normal controls (236, 485). However, there was disagreement about the pharmacological features of the flow. Hopf et al. (236) found that nifedipine doubled the quenching rate, whereas Tutdibi et al. (485) found no change.

Another approach is to use patch-clamp techniques to study calcium channels. Franco and Lansman (163) have described abnormalities in mechanosensitive calcium channels. They found a calcium channel activity in normal myotubes that had a low opening probability and was activated by stretching. In *mdx* myotubes they also found a calcium channel activity that had a high opening probability and was inactivated by stretch. This activity was not found in control myotubes, and it was suggested that it might be responsible for extra calcium influx into *mdx* myotubes. Although this second channel activity could not be shown to occur in *mdx* myofibers, the authors showed that in this situation the open probability of the first kind of mechanosensitive channel was greater in *mdx* than control fibers (164, 217).

However, Steinhardt and co-workers (160) have described abnormalities in a different calcium channel activity in myotubes. They demonstrated a leak channel (i.e., voltage independent) activity in normal myotubes

which in *mdx* myotubes had a threefold greater open probability. Nifedipine (an antagonist of L-type voltage-dependant calcium channels) increased the activity ascribed to this channel. The channel was also shown to be calcium selective (482).

These two groups agree that they are describing different phenomena (160, 164, 482). Franco-Obregon and Lansman (164) speculate that the leak type activity is an artifact of degenerating cultures. However, a leak channel activity increase in *mdx* myotubes has been confirmed independently (80). Moreover, Steinhardt and colleagues (236) managed to extend their original observations from myotubes to myofibers where again increased activity of calcium leak channels in dystrophin-deficient cells was seen (although the quantitative electrophysiological features of the channel were different in myofibers and tubes). In a separate study by this group in normal muscle, it was demonstrated that this channel had the properties of a capacitance current (i.e., was responsive to the state of intracellular calcium stores). Pharmacological antagonists of the activity were also described (235). However, the molecular correlate of this activity is unknown. That this activity is causally related to the rise in $[Ca^{2+}]_i$ in dystrophin-deficient muscle cells is evidenced by the ability of a leak channel antagonist to return $[Ca^{2+}]_i$ to normal (484). Data relevant to the cause of the increased calcium leak channel activity is considered in the section considering the role of proteolysis in dystrophin deficiency.

Carlson and Officer (76, 78, 80) have offered an alternative explanation for calcium leak channel activity and its increase in dystrophin deficiency. Using patch-clamp recordings from myotubes, they distinguished two types of channel activity: one a calcium leak channel and one attributed to acetylcholine receptor activity. These activities did not occur in single patches as independent events, and in *mdx* patches studied over long periods their relative frequencies changed. This prompted the speculation that calcium leak channel activity might be associated with acetylcholine receptors that had altered in some way and that this alteration was occurring more frequently in the context of a dystrophin-deficient membrane. The nature of this change in acetylcholine receptors has not yet been further defined.

D) FLUXES INTO THE SR. As mentioned above, some groups have found that the transient rise in $[Ca^{2+}]_i$ after depolarization is exaggerated or abnormally prolonged in dystrophin-deficient muscle preparations. This slowing of sequestration could be due to dysfunction of the SR Ca^{2+}-ATPase or secondary to increased calcium levels with in the SR. Attempts have been made to directly examine SR Ca^{2+}-ATPase activity, but the results are in conflict. Two studies of the tensions developed in *mdx* myofiber after manipulations that cause the SR to empty and refill concluded that calcium uptake by the SR was normal (269, 465). However, a study of the Ca^{2+}-ATPase activity of SR vesicle preparations demonstrated almost a halving of the maximum uptake rate in *mdx* muscle. Turner et al. (482) have presented data that do not suggest an intrinsic problem of the SR calcium pump (482). Lowering the calcium concentration external to a *mdx* myotube brings its $[Ca^{2+}]_i$ back down to normal levels. Under these circumstances, the kinetics of the $[Ca^{2+}]_i$ transient also become normal.

3. Proteolysis

Abnormal levels of several proteases are a feature of a wide variety of muscle diseases (224, 287, 385, 493). Changes in protease expression or activity in DMD or *mdx* muscle may therefore be nonspecific features, causally far removed from the primary pathological process (264, 286). However, there are data indicating that proteases and in particular calpains may have an important role in the pathophysiology of dystrophin deficiency. Protein degradation rates in isolated normal muscle (as assessed by tyrosine release) can be raised or lowered by manipulations that raise or lower $[Ca^{2+}]_i$ (165, 523). Turner et al. (484) having found a raised $[Ca^{2+}]_i$ in *mdx* myofibers therefore studied tyrosine release rates in isolated *mdx* muscle. Proteinolysis occurred 80% faster than in normal muscle, but this difference could be abolished by lower extracellular calcium concentrations (and perhaps therefore normalizing $[Ca^{2+}]_i$). This result was subsequently confirmed, and the effect was shown to be blocked by leupeptin (a thiol protease inhibitor) (314). Steinhardt's group (482) went on to show that leupeptin not only blocked the extra proteolysis of *mdx* myotubes but also normalized their $[Ca^{2+}]_i$ and the open probability of their calcium leak channels (483). The exaggerated increase in $[Ca^{2+}]_i$ seen in *mdx* myotubes after hyposmolar shock is also abolished by a protease inhibitor (292). These are not the results that would have been expected if the abnormalities of calcium influx were a direct result of dystrophin deficiency. An alternative hypothesis was therefore put forward in which transient membrane ruptures allow an influx of calcium. This then causes local activation of proteases which modify calcium leak channels to cause further calcium ingress. Thus a vicious circle might be established in which calcium homeostasis becomes deranged. Two further studies in support of this notion have been performed (7, 328). McCarter and Steinhardt (328) simulated the initial steps in this process by using a patch clamp to rupture the membrane of a normal myotube. The patch clamp was then reattached either close to (<5 μm) or far from (50 μm) the rupture, and the calcium leak channel activity was measured. Channels close to the lesion had fourfold increased open probability. Incubating with leupeptin abolished this effect (328). Alderton and Steinhardt (7) used a more direct technique

than tyrosine release to assess proteolysis in myotubes: hydrolysis of a fluorogenic calpain substrate. They confirmed that proteolysis occurs faster in *mdx* myotubes than controls and that this can be stopped by lowering external calcium concentration and by an antagonist of calcium leak channel activity. A variety of proteolysis inhibitors showed that most of the extra proteolysis was not due to lysosomal or proteosomal pathways (7). Candidates for this proteolytic activity include m- and μ-calpain (75). Evidence to specifically implicate calpains in the pathology of dystrophin-deficient muscle has also been presented (452). A difficulty here is that the regulation of calpain activity is complex and controversial. In particular, equating active calpain with the product of its autolytic lysis may not be justified (75). Direct evidence for the role of calpain activity in the pathophysiology of DMD is therefore lacking.

4. Oxidative damage

The hypothesis that the primary abnormality of dystrophin-deficient muscle is vulnerability to oxidative damage arose initially from two sorts of observation. First, DMD and *mdx* muscle show biochemical hallmarks of oxidative damage (363). Of course, this could be a nonspecific secondary feature (161). Second (but no more specifically), muscle diseases in which oxidative damage may play a primary role show features in common with DMD (338). However, there is now stronger evidence implicating oxidative damage early in the dystrophic process.

In the *mdx* mouse, there is very little necrosis before the wave of degeneration that occurs at around 3 wk, and during this time serum creatinine kinase levels are normal (327). Disatnik et al. (134) assayed the muscles of such very young mice for a marker of lipid peroxidation and for expression of several genes encoding antioxidants. They found that these were increased in *mdx* muscle at 2 wk. The same investigators studied the resistance of *mdx* and control myoutubes in culture to damage from a range of toxins; some were classed as pro-oxidants (e.g., hydrogen peroxide) and some nonoxidants (e.g., staurosporine which promotes apoptosis by inhibiting a range of protein kinases) (408). The *mdx* myotubes were more vulnerable to the pro-oxidants than controls. There was no difference with nonoxidants. The *mdx* and control myoblasts (before the expression of dystrophin) did not show differential toxicity with the pro-oxidants.

5. Apoptosis

Necrotic myofibers are a feature of dystrophic muscle. Several investigators have looked for the features of myofibers undergoing apoptosis or programmed cell death in dystrophin-deficient muscle (4, 433). In *mdx* muscle, myonuclei showing the internucleosomal DNA fragmentation characteristic of apoptosis can be found (110, 319, 434, 473). They are present at 2 wk when necrosis is not a feature (473), and their numbers decline thereafter (433). The search for apoptotic myonuclei in DMD has been less clear cut. Some studies have found none (27, 251, 342), another found that that 10% of intact myofibers showed signs of DNA fragmentation and another that apoptotic nuclei were present but most were in satellite cells and macrophages. The reason for these differences is not clear.

What significance does the occurrence of apoptosis in (at least) *mdx* muscle have? The distinction between necrosis and apoptosis may not be rigid, and different intensities of a cellular insult may cause apoptosis and necrosis (372). It would be unsafe then to infer from the occurrence of apoptosis and necrosis in dystrophin-deficient muscle that different pathological processes must be at work.

Finally, Sandri et al. (435) compared the effect of *cis*-platinum (an inducer of apoptosis) on cultured *mdx* and control myotubes. They demonstrated more apoptotic myotubes in the *mdx* cells. *Cis*-platinum may achieve some of its effect by the generation of free radicals, so this result is consistent with the increased vulnerability of *mdx* myotubes to oxidative damage (318, 408).

B. Abnormalities of the Muscle Tissue

1. Vascular problems

In DMD muscle (and its animal models), necrotic fibers often occur in clusters. An explanation put forward to explain this "grouped necrosis" highlights a role for vascular dysfunction (leading to focal areas of ischemia). To support this, microembolization was found to produce pathology in rabbit muscle reminiscent of DMD (338). However, this model was subsequently criticized (57, 205), and structural studies revealed no striking vascular abnormality (155, 278, 343).

More recent work has focused on nitric oxide (NO) and its roles in muscle. NO is a vasodilator and a key modulator of vascular tone (157). In skeletal muscle, NO is produced by endothelial cells and by muscle fibers themselves which express neuronal-type nitric oxide synthase (nNOS) (277, 365). In DMD and *mdx* muscle however, nNOS disappears from its normal position at the sarcolemma, becoming cytoplasmic (60, 88). Could it be that loss of nNOS causes disregulation of vascular tone, ischemia, and the pathology of DMD? This seems not to be the case because mice in whom the nNOS gene has been disrupted do not have muscle disease (89, 239). Nor does it seem that the relocalization of nNOS from sarcolemma to cytoplasm (where it could conceivably have deleterious effects) contributes to the pathology; *mdx*

mice crossed with nNOS-deficient mice have a phenotype indistinguishable from *mdx* (118).

However, evidence is available that the lack of nNOS in dystrophin-deficient muscle may still play a part in the pathological process. Sympathetic nervous input to muscle vasculature causes vasoconstriction. However, the relationship between vascular tone and sympathetic input differs in resting and exercising muscle. For a given increase in sympathetic input, vascular tone increases more in resting than in exercising muscle. The mechanisms responsible for this metabolic modulation of sympathetic vasoconstriction seem to depend on NO and nNOS because the effect is abolished by NOS inhibitors or in nNOS-deficient mice. It has now been demonstrated that this metabolic modulation is also much reduced in children with DMD and in *mdx* mice (432, 471). It is possible therefore that this deficit could cause functional ischemia of areas of muscle during exercise; although not in itself sufficient to cause disease, this might exacerbate some other pathological process (115).

2. Inflammation and fibrosis

Once necrosis starts, DMD and *mdx* muscle contain an increased number of a variety of inflammatory cells (14, 330, 364, 481). In the *mdx* mouse, the time course of the increase in CD4 and CD8 T lymphocytes mirrors that of the necrosis, peaking at 4–8 wk before declining. Are these cells reactive, and do they themselves contribute to cell death or some other pathological feature? This question has been addressed by a number of investigators using genetic or other manipulations to remove specific sets of inflammatory cells or mediators (454). Preliminary reports of *mdx* mice deficient in either mast cells or macrophages saw no change in histology at 4 wk (186), while *mdx* mice unable to produce tumor necrosis factor (TNF; a T cell-derived cytokine) developed in some muscles rather worse pathology (453) than *mdx*. The *mdx* mice missing perforin (a cytotoxic molecule secreted by T lymphocytes) have also been analyzed (455). In these some reduction in apoptotic and necrotic fibers was seen at the time point examined. In another model, antibody-mediated depletion of either CD4 or CD8 T cells was found to reduce pathology as assessed by a "histopathological index" (454). The contribution of T lymphocytes to the progressive fibrosis seen particularly in the *mdx* diaphragm has also been studied. Crosses of *mdx* with nude mice (that lack T cells) show some reduction in fibrosis at 12 and 24 wk (361). Transforming growth factor-β1 (TGF-β1) has been muted as a mediator of fibrosis in DMD (32, 516), but this has not yet been directly tested.

3. Regeneration

Muscle from normal mice and humans is capable of regeneration after extensive damage. That this process is occurring too in *mdx* mice is clear from experiments in which regeneration has been inhibited. The effect of γ-irradiation to make plain the importance of ongoing regeneration in the *mdx* phenotype has been referred to above (378). Similarly, *mdx* mice that also carry mutations in genes important in muscle regeneration (for example, fibroblast growth factor-6, *Mnf,* and *MyoD)* develop very severe muscle disease (158, 170, 334). However, both in patients with DMD and *mdx* mice regeneration eventually fails to keep up with ongoing necrosis so that atrophy occurs. Studies that have compared regeneration of normal and *mdx* muscle after damage by toxins or the like seem to confirm that *mdx* muscle especially in older animals regenerates less well than normal (252, 410, 522). Why should this be?

Myofibers themselves are postmitotic, but skeletal muscle contains a population of mononuclear muscle precursor cells within the basement membrane of the fibers (325). These satellite cells proliferate and fuse during regeneration (362). Although it has been demonstrated recently that populations of cells exist in other tissues that can gain access to muscle via the circulation and contribute to muscle regeneration, satellite cells are responsible for the predominant part of muscle regeneration (154). Is there some defect in satellite cells in dystrophin-deficient muscle? Studies from patients with DMD largely show an increase in satellite cell numbers (497, 500). However, muscle precursor cells isolated from DMD muscle are capable of fewer replications in vitro than age-matched controls (411, 502). This may reflect the larger number of replications they have already undergone in vivo (at least as assessed by decreasing telomere length) (124). These data seem to support the notion that because of a greater rate of turnover the satellite cell population becomes exhausted in dystrophin-deficient muscle. However, this work has been criticized on the grounds that the extraction of precursor cells from muscle is highly inefficient, retaining only a small fraction of the in vivo population (375, 411). This small fraction may not be representative. Indeed, it has become apparent that satellite cells are heterogeneous and contain functionally distinct subpopulations (23, 400). Renault et al. (411) were able to demonstrate a population of radiation-resistant precursor cells that was absent in *mdx* muscle. However, analysis of whole fiber cultures (which may allow study of a more representative pool of satellite cells) provided no evidence for a progressive general exhaustion of myogenic potential (48). The extent to which sustained high satellite cell turnover is responsible for impaired muscle regeneration is therefore uncertain. An alternative possibility is that some feature of the muscle environment (for example, factors secreted by fibroblasts or muscle fibers themselves) becomes inimical to regeneration. There is some evidence that TGF-β1 and insulin-

like growth factor binding protein-5 may play such a role, but this hypothesis is yet to be tested in vivo (336, 337).

C. Summary

The changes that occur in dystrophin-deficient muscle are complex, and unpicking the causal relationships between them is not straightforward. The difficulty is compounded because the results described above relate to model systems at several different levels: intact animals, isolated whole muscles, single muscle fibers, and cultured myoblasts and myotubes. Finally, the absence of dystrophin may cause pathology by more than a single distinct mechanism.

Thus abnormalities of NO modulation of vascular tone may well contribute to pathology but cannot alone explain it. The same may be true of the inflammatory, fibrotic, and regenerative processes in dystrophin-deficient muscle. Further insights into these processes may come from microarray and other technologies that allow examination of changes in many mRNA levels in dystrophin-deficient tissues and thus reveal groups of up- or downregulated genes (86, 94, 151, 478). For example, Hoffman and colleagues (94) used high-density oligonucleotide arrays to compare the abundance of 6,000 mRNA species between normal, dystrophin-deficient, and α-sarcoglycan-deficient muscle.

Important abnormalities of dystrophin-deficient muscle cells have been demonstrated in three areas: calcium homeostasis, an increased susceptibility to oxidative toxins, and increased (and stress enhancable) membrane permeability. Confirmation that the absence of dystrophin is indeed responsible for these abnormalities comes from experiments in which dystrophin has been restored. This has been achieved by making *mdx* mice transgenic for a construct consisting of a muscle and heart-specific promoter and a full-length dystrophin cDNA (111). This mouse makes dystrophin at supraphysiological levels. Comparison of *mdx* with normal mice has shown that myotube calcium homeostasis and susceptibility to oxidative stress (111, 130, 133) become normal. How are these various abnormalities related? One possible scheme (7) is outlined in Figure 2. It highlights the abnormal permeability of mechanically stressed muscle cells as the primary problem and links this through changes in protease and calcium channel activity to explain how a cell with badly deranged calcium homeostasis could result. This could in turn trigger necrosis or apoptosis. It is the case, however, that details of several of these steps are missing, for example, the molecular identity of the abnormal calcium channel and the biophysical nature of the membrane deficit. Other schemes have been suggested (77), and it should be recognized that the hierarchy of physiological derangements at play in dystrophin-deficient muscle remains uncertain.

VI. DYSTROPHIN-ASSOCIATED PROTEIN COMPLEX

The dystrophin-associated protein complex (DPC) was identified because dystrophin was found to be enriched in muscle membrane fractions eluted from a wheat germ agglutinin (WGA) column (74, 149, 519). WGA is a plant lectin that has high affinity for *N*-acetylglucosamine,

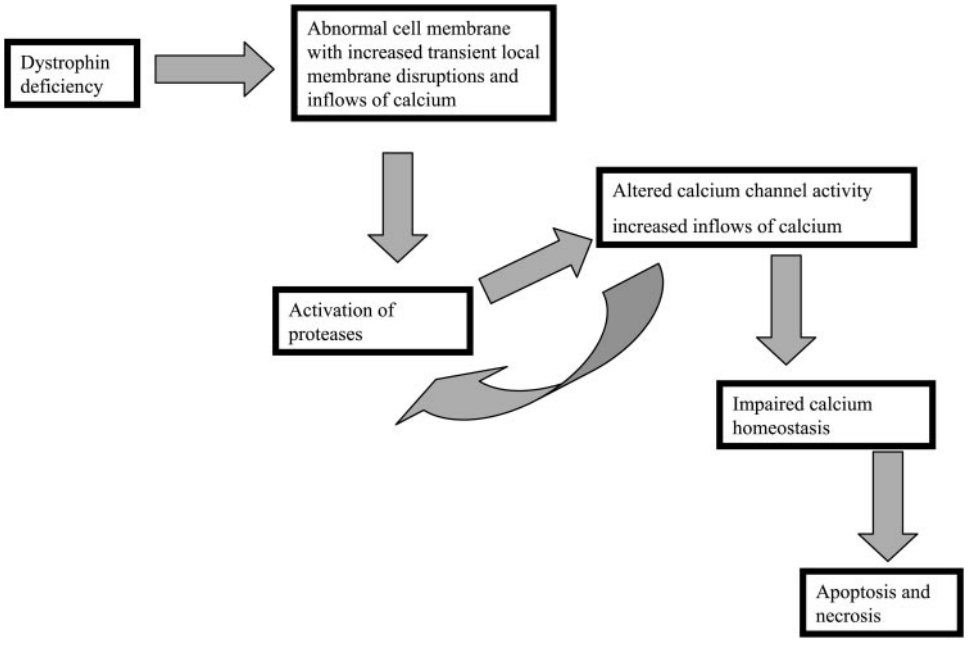

FIG. 2. The pathophysiology of dystrophin deficiency. This diagram illustrates the scheme described by Steinhardt and others. For references, see text.

a common constituent found in the glycans of some glycoproteins. WGA-affinity chromatography was subsequently used to purify a complex of dystrophin-associated proteins and glycoproteins from rabbit skeletal (149, 519).

The consensus view of the DPC stoichiometry is that dystrophin is linked to the sarcolemma of normal muscle by a protein complex composed of at least 10 different proteins (Fig. 3 and Table 1). In contrast to spectrin that appears to be a functional heterodimer, the dystrophin complex is monomeric (426). This complex spans the membrane and links the actin-based cytoskeleton to the muscle basal lamina. Thus the DPC can be thought of as a scaffold connecting the inside of a muscle fiber to the outside.

The DPC can be divided into several separate subcomplexes based on their location within the cell and their physical association with each other. Using detergent extraction and two-dimensional gel electrophoresis, Yoshida et al. (520) showed that the DPC could be dissociated into three distinct complexes. These complexes are the *1*) the dystroglycan complex, *2*) the sarcoglycan:sarcospan complex, and *3*) the cytoplasmic, dystrophin-containing complex. Each of these subcomplexes is considered in detail below.

A. Dystroglycan and the Dystroglycan Complex

Dystroglycan was the first component of the DPC to be cloned (244). The single dystroglycan gene produces a precursor protein that is processed by an unidentified protease to produce α- and β-dystroglycan. The dystroglycan gene is composed of only two exons, and there is no evidence of alternative splicing, although several glycoforms are produced (245). The relative molecular weights of α-dystroglycan differ in different tissues as a result of the aforementioned differential glycosylation (see below). In muscle, α-dystroglycan has a molecular mass of 156 kDa, whereas β-dystroglycan is 43 kDa. In brain, α-dystroglycan has a molecular mass of 120 kDa and was independently identified as a protein called cranin (447, 448).

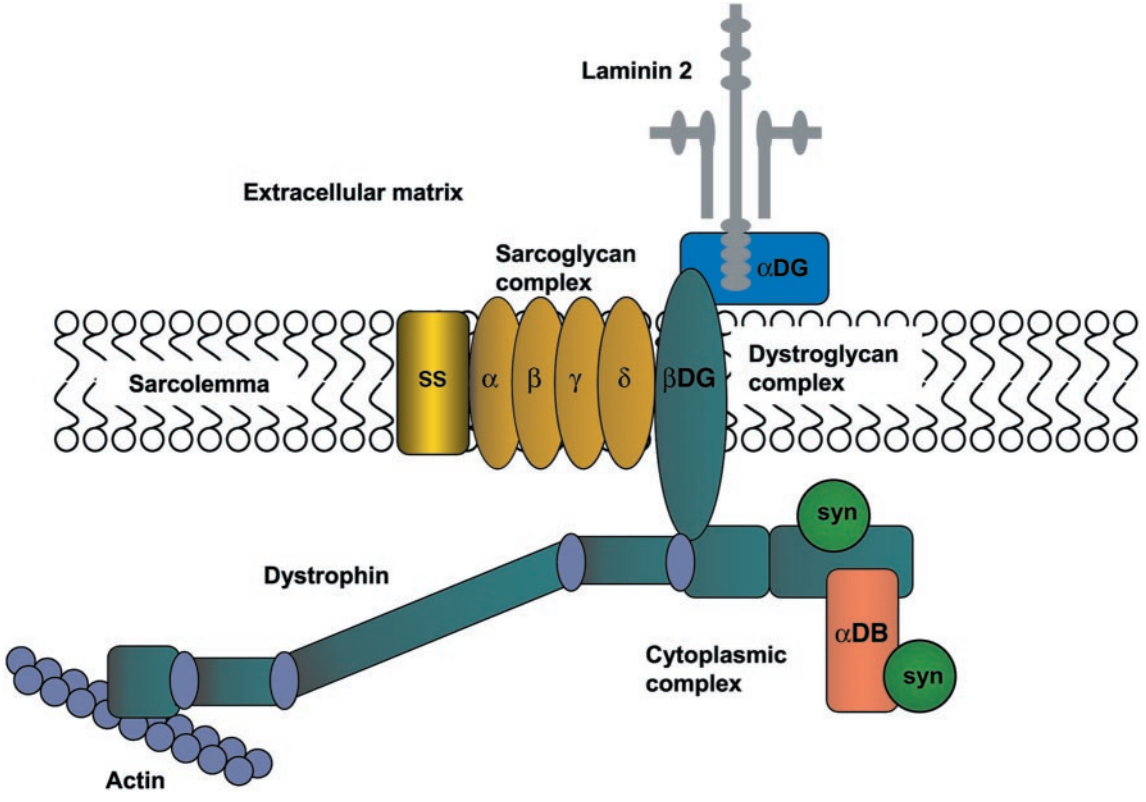

FIG. 3. The dystrophin-associated protein complex (DPC) in skeletal muscle. Dystrophin binds to cytoskeletal actin at its NH_2 terminus. At its COOH terminus, dystrophin is associated with a number of integral and peripheral membrane proteins that can be classified as the dystroglycan subcomplex, the sarcoglycan-sarcospan subcomplex, and the cytoplasmic subcomplex. The cytoplasmic subcomplex includes the syntrophins (syn) and α-dystrobrevin (αDB). The sarcoglycan-sarcopsan subcomplex comprises the sarcoglycans (α, β, γ, δ) and sarcospan. The extracellular component of the dystroglycan complex, α-dystroglycan (αDG), binds to laminin-2 in the extracellular matrix and β-dystroglycan (βDG) in the sarcolemma. In turn, β-dystroglycan binds to the dystrophin, thus completing the link between the actin-based cytoskeleton and the extracellular matrix. Additional DPC binding partners are omitted for clarity, but a full list of the proteins can be found in Table 1.

TABLE 1. *Knockouts of components of the dystrophin-associated protein complex*

Protein	Muscle	NMJ	Comments	Reference Nos.
Dystrophin	MD	Psm abnormal	CNS and retinal abnormalities	67, 443
Utrophin	Normal	Psm abnormal	mdx:utrn −/− mutant dies after 10 wk	125, 126, 190, 191
Dystroglycan	MD in chimera	Psm abnormal in chimera	Ko embryonic lethal due to bm abnormalities	108, 507
α-Sarcoglycan	MD	ND		137
β-Sarcoglycan	MD	ND		15, 141
γ-Sarcoglycan	MD	ND	Apoptotic changes in muscle	207, 209
δ-Sarcoglycan	MD	ND	Dilated cardiomyopathy	105
Sarcospan	Normal	ND		290
α-Syntrophin	Normal	Psm abnormal	Reduced nNOS at membrane and utrophin at the NMJ	3, 263
β_1-Syntrophin	ND	ND		
β_2-Syntrophin	ND	ND		
α-Dystrobrevin	Mild MD	Psm abnormal	DPC intact, reduced nNOS at membrane, lower cGMP	189, 192

MD, muscular dystrophy; ND, not done; ko, knockout; psm, postsynaptic membrane; CNS, central nervous system; nNOS, neuronal nitric oxide synthase; NMJ, neuromuscular junction; DPC, dystrophin-associated protein complex.

β-Dystroglycan has a single transmembrane domain and is inserted into the muscle plasma membrane with the COOH terminus on the cytoplasmic side. In contrast, α-dystroglycan is located in the extracellular matrix where it is thought to be directly associated with β-dystroglycan through multiple covalent interactions. The extreme COOH terminus of β-dystroglycan contains several proline residues that are required for dystroglycan binding to dystrophin (261, 412, 463, 464). The last 15 amino acids of β-dystroglycan appear to bind directly to the cysteine-rich region of dystrophin. This region of β-dystroglycan is proline rich and contains a site for tyrosine phosphorylation (258). Recently, the crystal structure of β-dystroglycan bound to dystrophin has been determined (240). The structure of this region of dystrophin shows that dystroglycan forms contacts with both the WW domain and EF hands of dystrophin, emphasizing the functional importance of both of these domains to the dystrophin family of related proteins.

The COOH terminus of β-dystroglycan also binds to the adaptor protein Grb2 (517) (Table 2). This interaction is mediated by the SH3 domain of Grb2 that binds to proline-rich sequences in the cyoplasmic tail of β-dystroglycan. This interaction raises the possibility that β-dystroglycan may participate in the transduction of extracellular-mediated signals to the muscle cytoskeleton (517).

TABLE 2. *DPC-associated proteins*

DPC Component	Partner	Method of Identification	Reference Nos.
Dystrophin	Aciculin	Coimmunoprecipitation	28
	Calmodulin	In vitro binding	11
	α-Actinin-2	Y2H	213
α-Syntrophin	nNOS	Y2H	59, 60
	SAPK3	Y2H	214
	Voltage-gated sodium channels*	Y2H	172, 440
	Calmodulin	In vitro binding	254, 367
β_2-Syntrophin	MAST205/SAST	Y2H	308
	ErbB4	Y2H	168
β-Dystroglycan	Grb2	In vitro binding	517
	Rapsyn	Protein cross-linking	83
	Caveolin-3	In vitro binding	451
α-Dystroglycan	Agrin	In vitro binding	51, 73, 173, 461
	Laminin	Ligand blotting	244
	Perlecan	In vitro binding	386, 468
	Biglycan	Biochemical purification	52
γ- and δ-sarcoglycan	Filamin-2	Y2H	472
α-Dystrobrevin	Syncoilin	Y2H	370
	Dysbindin	Y2H	30
	Desmuslin	Y2H	346

A number of proteins have been identified that associate with different members of the DPC but are not components of the core DPC described by the Campbell and Ozawa groups. SAPK3, stress-activated protein kinase-3; MAST205, microtubule-associated serine-threonine kinase; Y2H, yeast two hybrid system (156). * Sodium channels also interact with other members of the syntrophin protein family (172).

Interestingly, in vitro studies show that dystrophin inhibits the interaction between Grb2 and β-dystroglycan, suggesting that Grb2 is only bound when β-dystroglycan is not associated with dystrophin. These data could reflect the use of that same binding site (the last 20 amino acids of β-dystroglycan). Alternatively, Grb2 may regulate the dynamic interaction between β-dystroglycan and the DPC (424).

Caveolin-3 is a recently described binding partner for β-dystroglycan (451) (Table 2). The caveolins are a family of transmembrane proteins that form microdomains in the plasma membrane that are able to recruit different signaling molecules. Caveolin-3 is specifically expressed in muscle (449, 470, 501). Caveolin-3 also contains a divergent WW domain that is required for β-dystroglycan binding (451). Caveolin-3 mutations cause autosomal dominant limb girdle muscular dystrophy type 1C (344). This disorder is often associated with a reduction in the levels of membrane-associated dystroglycan. Loss of caveolin-3 therefore affects components of the DPC, providing further evidence linking caveolin-3 to the dystrophin protein complex (226) (Table 3).

Caveolin-3 and dystrophin appear to compete for the same binding site at the COOH terminus of β-dystroglycan that includes the tetrapeptide PPPY (451). Overexpression of caveolin-3 in muscle also causes muscular dystrophy (167). The overexpression of caveolin-3 in this model is associated with a reduction in the levels of dystrophin and β-dystroglycan (167). These data support the suggestion that caveolin-3 may compete directly for the dystrophin/dystroglycan binding site in muscle and that the overexpression of caveolin-3 results in a disruption of the dystrophin/β-dystroglycan interface that is critical for normal muscle function. In addition to caveloin-3 and Grb2, rapsyn, a protein essential for neuromuscular junction formation (83), also binds to β-dystroglycan (Table 2). This interaction has important implications for the role of both α- and β-dystroglycan in neuromuscular junction formation (see below).

α-Dystroglycan is a dumbbell-shaped protein that has a central mucin-like region flanked by globular domains (58). Dystroglycan binds to the laminin G (LG) domains in laminins (α_1-chain and α_2-chain), agrin, and perlecan with varying affinities. These interactions are calcium dependent, and calcium is found bound to the edge of the LG5 interaction face of laminin α_2-chain (230). The LG domain is also required for heparin binding but does not antagonize the interaction of laminin-2 and α-dystroglycan (329, 379). It has been suggested that the interaction between α-dystroglycan and laminin-2 is dependent on the presence of anionic oligosaccharides on α-dystrolgycan (148, 230). Mutations in at least three different genes, fukutin, fukutin-related protein, and LARGE (Table 3), have been shown to cause muscular dystrophy with abnormal α-dystroglycan processing (16, 62, 63, 204, 219, 276). Thus it is tempting to hypothesize that the muscle disease in these patients is in part caused by the disruption of the laminin-2:α-dystroglycan interaction.

Dystroglycan is involved in an increasing variety of cellular processes (see Refs. 84, 225, 511 for review). These include epithelial development and viral adherence/infection and neuromuscular junction formation. The organization of the extracellular matrix appears to be a consistent feature of dystroglycan function. Mice lacking dystroglycan die at the preimplantation stage due to a

TABLE 3. *Proteins that potentially modify the disease state in muscular dystrophy*

Protein	Evidence	Reference Nos.
α_7-Integrin	Mutated in congenital muscular dystrophy	218
	Mice lacking develop α_7-integrin develop MD	326
	Overexpression rescues mdx:utrn −/− mice	69
Caveolin-3	Mutated in muscular dystrophy	344
	Mice lacking caveolin-3 develop MD	210
	Binds directly to β-dystroglycan	451
	Dystroglycan complex dissociates in the absence of caveolin-3	226
	Overexpression of causes a MD phenotype	167
Agrin	Binds to α-dystroglycan	51, 73, 173, 461
	Can replace laminin-2 in the *dy/dy* mouse model of MD	350
Utrophin	Can functionally replace dystrophin restoring the DPC to the sarcolemma	474, 477
Fukutin	Mutated in FCMD	276
	Putative phosphoryl-ligand transferase	16
	Associated with abnormal α-dystroglycan glycosylation	219
Fukutin-related protein	Mutated in MDC1C and LGMD2I; putative phosphoryl-ligand transferase; associated with abnormal α-dystroglycan glycosylation	62, 63
LARGE	Putative glycosyltransferase associated with abnormal α-dystroglycan glycosylation; mutated in the *myd* mouse	204

Presented is a list of proteins whose expression can alter the composition or function of the DPC. In the case of α_7-integrin, caveolin-3, and agrin, these proteins appear to function at the interface between the basal lamina and sarcolemma, whereas utrophin can directly replace dystrophin. The remaining proteins could play a role in the posttranslational modification of α-dystroglycan. MD, muscular dystrophy; FCMD, Fukuyama congenital muscular dystrophy; MDC1C, congenital muscular dystrophy type 1C LGMD2I.

failure of an embryonic membrane known as Rechiert's membrane to form (507).

Dystroglycan, dystrophin, and utrophin have all been implicated in the process of neuromuscular synaptogenesis. α-Dystroglycan has been shown to bind directly to the secreted glycoprotein agrin in the basal lamina of the neuromuscular junction (51, 73, 173). These initial findings lead to the proposal that α-dystroglycan was a functional receptor for agrin and that the agrin-induced changes that occur during synapse formation were orchestrated by signaling proteins linked to dystroglycan. This view has been challenged because deletion of the α-dystroglycan binding site on agrin has no effect on agrin-induced acetylcholine receptor (AChR) clustering (174). It now seems likely that agrin-mediated signaling occurs via a receptor tyrosine kinase called muscle-specific kinase (MuSK) that is part of a protein complex at the neuromuscular junction (reviewed in Ref. 275). The function of the DPC in synaptogenesis may be in the stabilization of AChR clusters rather than in promoting receptor clustering. This hypothesis is supported by the findings of Campanelli et al. (73), who showed that components of the DPC, including utrophin and α-sarcoglycan, are recruited to receptor clusters after agrin induction.

Myotubes derived from dystroglycan-deficient embryonic stem cells respond to agrin but produce abnormal AChR clusters. These clusters are larger than normal AChR clusters but contain a reduced density of AChR. In the same cultures, the extracellular matrix molecules perlecan, laminin, and acetylcholinesterase fail to cocluster with the AChR, whereas rapsyn and agrin are found associated with the receptors. Thus dystroglycan is required for the stabilization of the AChR clusters and for the formation of the specialized extrajunctional sarcolemma (256). A recent study by Grady et al. (192) showed marked differences to the data obtained by Jacobson et al. (256). Grady et al. (192) showed that dystroglycan-deficient myotubes produced normal numbers of AChR clusters in response to agrin treatment. These receptor clusters differed from the normal clusters because they contained micro-aggregates of AChRs (192). Taken together, these data suggest that agrin is at least partially (or completely, according to Grady et al., Ref. 192) dispensable for agrin-induced AChR clustering.

Recent studies on muscle from chimeric mice that have an absence of dystroglycan in muscle have shown that dystroglycan is not essential for the formation of the extracellular matrix, at least in striated muscle (108). Although the extracellular matrix is apparently normal, muscle from these chimeras degenerates in response to activity-dependent mechanical injury. Immmunocytochemical studies on these muscles showed that dystrophin and α-sarcoglycan immunoreactivities were severely reduced both at the sarcolemma and neuromuscular junction. These data confirm the functional importance of the interaction of dystroglycan with dystrophin for maintaining the DPC at the sarcolemma. Synapse formation in chimeric mouse muscle was also abnormal and was characterized by an alteration in junctional morphology and a severe reduction in the levels of acetylcholinesterase. No alteration in any component of the extracellular matrix was observed in the chimeric mice, suggesting that there is little evidence for functional compensation among members of the extracellular matrix that can bind to laminin such as the integrins.

B. Other Extracellular Matrix Proteins

Several components of the muscle basal lamina and extracellular matrix bind directly to α-dystroglycan. Laminin-2 is the best-characterized dystroglycan binding protein and can be considered to be part of the DPC. Mutations in the gene encoding the laminin α_2-chain (merosin) cause merosin-negative congenital muscular dystrophy (CMD). There is also a mouse model of this form of muscular dystrophy called the *dy* (*dystrophia muscularis*) mouse (462, 515). Two different strains of *dy* mouse exist: the severely affected *dy/dy* strain and the milder dy^{2J}. The dy^{2J} allele produces a protein that is defective in polymerization and has a low affinity for heparin (103). Patients with merosin-negative muscular dystrophy have normal immunostaining for the intracellular components of the DPC including dystrophin (221). However, the muscles of these patients show signs of severe muscular dystrophy. Thus, although α-dystroglycan is a major laminin-binding protein in muscle, the connection between laminin in the basal lamina and dystroglycan is not essential for the preservation of the DPC at the membrane. One reason why laminin mutations result in muscular dystrophy while preserving the DPC could be due to the presence of other α-dystroglycan-binding proteins in the basal lamina.

The interdependence of different extracellular matrix molecules for the maintenance of the muscle basal lamina was recently demonstrated by an innovative experiment described by Moll et al. (350). Expression of an agrin minigene in *dy/dy* muscle (see above) causes the amelioration of muscular dystrophy, restoration of the normal structure of the muscle basal lamina, and lowering in the serum creatine kinase levels (350). This reversal is associated with an increase in the levels and stability of α-dystroglycan and laminin α_4- and α_5-chains (350). These data demonstrate that agrin can functionally replace laminin α_2 in muscle, reestablishing the link between α-dystroglycan and an intact basal lamina.

$\alpha_1\beta_7$-Integrin is another major laminin binding protein in muscle that provides an additional transmembrane link between the myofibrillar cytoskeleton and the extra-

cellular matrix (450, 494). In some patients with CMD caused by mutations in the laminin α_2-chain, there is a secondary reduction in the levels of α_{7B}-integrin at the sarcolemma (100). Interestingly, mice lacking α_7-integrin and humans with mutations in the α_7-integrin gene also have muscular dystrophy (218, 326). These data suggest muscular dystrophy caused by mutations in laminin-α2 chain probably occurs primarily by disrupting the laminin-integrin interface preserving the DPC.

These data are partially supported by the recent findings of Burkin et al. (69) who have shown that overexpression of $\alpha_7\beta_1$-integrin rescues muscular dystrophy in dystrophic mice (69). In this study, the α_7-chain of integrin was expressed as a transgene in the muscles of mice lacking both dystrophin and utrophin (see sect. VII*H*). These mice have a severe phenotype and, in contrast to *mdx* mice, die prematurely (125, 191). Transgenic expression of α_7-integrin upregulates its heterodimeric partner β_{1D}-integrin and ameliorates the muscular dystrophy in these mice increasing their longevity (69). Although this "rescue mechanism" is not fully understood, it is possible that the enhanced expression of $\alpha_7\beta_1$-integrin in muscle increases the interaction between the extracellular matrix and myofibrillar cytoskeleton through the sarcolemma.

C. Sarcoglycan Complex

In skeletal and cardiac muscle, the sarcoglycan complex is composed of four transmembrane glycoproteins α-, β-, γ-, and δ-sarcoglycan and a member of the tetraspan family of proteins called sarcospan (reviewed in Refs. 116, 297). α-Sarcoglycan is a type I membrane protein, whereas β-, γ-, and δ-sarcoglycans are type II membrane proteins. The distribution of α-sarcoglycan appears to be restricted to skeletal and cardiac muscle, whereas β-, δ-, and γ-sarcoglycan are also expressed in smooth muscle (20, 414, 459). Recently, ϵ-sarcoglycan, a transmembrane glycoprotein related to α-sarcoglycan, has been identified that has a broad tissue distribution and replaces α-sarcoglycan in smooth muscle sarcoglycan-sarcospan complexes (150, 331, 459). The molecular association of the sarcoglycan-sarcospan components with each other and with other components of the DPC is currently unclear. Vainzof et al. (486) suggest that α-, β-, and δ-sarcoglycan might be closely associated with each other and that γ-sarcoglycan may interact with dystrophin. In contrast, in vitro studies using myotubes suggested that β-, γ-, and δ-sarcoglycan are more closely associated with one another than α-sarcoglycan and that δ-sarcoglycan binds tightly to dystroglycan (87).

The sequence of the sarcoglycans provides little information about their functional roles in muscle. The γ- and δ-sarcoglycan are paralogs, so it may be expected that each protein could have a similar function such as shared binding partners. Several studies on sarcoglycan function suggest that they may play a role in intracellular signal transduction. The cytoplasmic domain of γ-sarcoglycan has five tyrosine residues, and studies suggest bidirectional signaling with integrins (521). α-Sarcoglycan has been reported to have ecto-ATPase activity (36). Yeast two-hybrid studies have identified filamin-2 as a γ- and δ-sarcoglycan interacting protein (472). Filamin family members are involved in actin reorganization and signal transduction cascades associated with cell migration, adhesion, differentiation, force transduction, and survival (functions reviewed in Refs. 300, 499). The identification of filamin as a sarcoglycan interacting protein supports the concept that the DPC does not merely function as structural support but rather that this complex has a signaling role in maintaining skeletal muscle integrity. Identifying the extracellular and intracellular ligands that bind to the subunits of the sarcoglycans will be important in defining any potential interactions with the cytoskeleton or signaling cascades.

D. Sarcoglycanopathies and Their Animal Models

Mutations in the α-, β-, γ-, and δ-sarcoglycan genes have been found to be primary defects in some forms of human autosomal-recessive limb-girdle muscular dystrophy (LGMD2) (reviewed in Refs. 71, 297). Interestingly, mutations in the ϵ-sarcoglycan gene cause the movement disorder myoclonus-dystonia syndrome, identifying an additional role for DPC-like proteins in the central nervous system (525). In this review we only discuss the sarcoglycanopathies that affect skeletal and cardiac muscle.

One feature of the sarcoglycanopathies is that the absence of one sarcoglycan has important consequences for the stability of the other remaining sarcoglycan components at the plasma membrane. Typically, the loss of one sarcoglycan results in the absence or severe reduction in the remaining components of the sarcoglycan complex, although recent studies of patient muscle biopsies have demonstrated a variation in the pattern of sarcoglycan complex disruption (reviewed in Ref. 71). For example, LGMD-2C patients with mutations in γ-sarcoglycan frequently retain α-sarcoglycan at the sarcolemma (487). In vitro experiments using expression of sarcoglycans in heterologous cells suggest that the correct assembly and trafficking of the sarcoglycan complex to the membrane appears to be dependent on the cosynthesis of all four components (α-, β-, γ-, and δ-sarcoglycan) (87, 232). Mutant sarcoglycans are thought to block complex formation and insertion of the sarcoglycans into the plasma membrane. This has led to the proposal that the molecular defect in the sarcoglycanopathies is due to aberrant sarcoglycan complex assembly and trafficking, which results in the absence or reduction of the complex at the sarco-

lemma (87, 232). This hypothesis is supported by the finding that overexpression of γ-sarcoglycan in muscle causes muscular dystrophy (524). The overexpression of γ-sarcoglycan in these mice is associated with an increase in the levels of α- and β-sarcoglycan (524). These data suggest that either alterations in the stoichiometry of the sarcoglycan complex or its mislocalization in muscle are sufficient to cause muscular dystrophy.

Recently, mouse models of LGMD have been generated by targeted disruption of the α-sarcoglycan gene (137), the β-sarcoglycan gene (15, 141), the γ-sarcoglycan gene (209), and the δ-sarcoglycan gene (105, 208; for review, see Ref. 8). Before the development of these mouse models, the BIO 14.6 hamster was extensively used as an animal model of autosomal recessive cardiomyopathy and muscular dystrophy (234). The primary genetic defect in this hamster has been shown to be a large deletion in the δ-sarcoglycan gene (373, 377, 431). All sarcoglycan-deficient animal models develop a progressive muscular dystrophy of variable severity and are reported to show a loss of sarcolemmal integrity, as assessed by the uptake of Evans blue. However, it is not clear from these models whether the observed changes in the plasma membrane integrity are the cause of muscle degeneration or simply the result of it (since the dye does not accumulate in nonnecrotic cells) (137, 207, 209). In addition, there is significant secondary reduction in the expression of the other members of the sarcoglycan-sarcospan complex as well as some variable degree of disruption of other components of the DPC.

Surprisingly, although sarcospan expression is affected consistently by loss of the sarcoglycan subcomplex in sarcoglycan-deficient animal models, sarcospan null mice do not present with muscle pathology (290). These mice maintain the expression of all sarcoglycan proteins at the sarcolemma together with components of DPC. Sarcolemma integrity is also preserved in sarcospan-deficient muscle, as is normal force and power generation capacities. Sarcospan is also lost in LGMD patients with primary mutations in the α-, β-, or γ-sarcoglycan genes, but to date no mutations in the sarcospan gene have been detected (117). These data suggest that sarcospan is not required for the normal function of the DPC and is not crucial in the formation and stabilization of the sarcoglycan complex, but it may be the case that another protein, perhaps another tetraspan protein, can compensate for the loss of sarcospan (290).

There is now considerable evidence supporting the conclusion that the sarcoglycan-sarcospan complex is important for anchoring or stabilizing the dystroglycan complex in the sarcolemma. In the α-sarcoglycan-null mouse, the association of α-dystroglycan with the membrane is disrupted as assessed by immunofluorescence and Western blot analysis of membrane preparations from normal and mutant skeletal muscle (137). Furthermore, the BIO 14.6 hamster demonstrates depleted levels of α-dystroglycan at the sarcolemma, despite the normal localization of dystrophin (253, 414, 458). Laminin, dystrophin, and α- and β-dystroglycan are all normally localized at the sarcolemma of the β-sarcoglycan-deficient mouse, although this complex was also found to be unstable in the absence of the sarcoglycan-sarcospan complex (15, 141). The γ-sarcoglycan-null mouse is also reported to have normally localized laminin, β-dystroglycan, and dystrophin, although the stability of this complex and the presence of α-dystroglycan at the membrane were not examined (209). Taken together, these results suggest that one function of the sarcoglycan-sarcospan complex may be to strengthen the dystrophin-dystroglycan axis connecting the basement membrane with the cytoskeleton.

The molecular composition of the sarcoglycan-sarcospan complexes in skeletal and smooth muscle has been investigated using the range of sarcoglycan-deficient animal models. The α-sarcoglycan null mouse demonstrates a concomitant reduction of β-, γ-, and δ-sarcoglycan together with sarcospan at the sarcolemma, although the expression and localization of ε-sarcoglycan is unaffected (137). This observation is explained by the existence of a separate ε-sarcoglycan-containing sarcoglycan complex in both skeletal and smooth muscle (141, 299, 459). The existence of two separate sarcoglycan-sarcospan complexes has been demonstrated in C2C12 myotubes whereby α- and ε-sarcoglycan form separate membrane complexes with β-, γ-, and δ-sarcoglycans (299). It is proposed that ε-sarcoglycan serves a function similar to that of α-sarcoglycan and that residual β-, γ-, and δ-sarcoglycan seen at the sarcolemma in α-sarcoglycan null mice is due to its association with ε-sarcoglycan (299).

The identification of the ε-sarcoglycan-containing sarcoglycan complex in smooth muscle has led to significant progress in the understanding of the pathogenic mechanisms that contribute to muscular dystrophy and also cardiomyopathy. Mice that are disrupted in the β- or δ-sarcoglycan genes develop severe muscular dystrophy and also cardiomyopathy (15, 105, 141). Biochemical and immunohistochemical analysis reveals that the sarcoglycan-sarcospan complex is completely disrupted in the vascular smooth muscle in these mice (105, 141). In contrast, α-sarcoglycan-null mice demonstrate a progressive muscular dystrophy but no cardiomyopathy (105, 137). Because α-sarcoglycan is not expressed in smooth muscle, the smooth muscle expression of the sarcoglycan-sarcospan complex is unaffected (459). Further examination of vascular smooth muscle in β- and δ-sarcoglycan-deficient mice revealed that the missing sarcoglycan-sarcospan complex perturbs vascular function as illustrated by vascular constrictions in the coronary arteries (105). It is suggested that these changes in vascular function initiate cardiomyopathy and exacerbate the muscular dystrophy phenotype (105, 141). As predicted, no

abnormalities in vascular function were observed in the α-sarcoglycan-deficient mice (141). Thus disruption of the sarcoglycan-sarcospan complex in vascular smooth muscle perturbs vascular function and induces ischemic injury in cardiac and skeletal muscle. Recently, Cohn et al. (99) have demonstrated that long-term treatment of β- and γ-sarcoglycan-null mice with verapamil, a calcium channel blocker with vasodilator properties, prevented the development of cardiomyopathy. In contrast, verapamil did not prevent the cardiac muscle pathology observed in *mdx* mice that do not demonstrate either a disrupted smooth muscle sarcoglycan complex or vascular abnormalities. The authors suggest that verapamil acts to prevent the onset of cardiomyopathy in β- and γ-sarcoglycan null mice by alleviating vascular constrictions and protecting the cardiac muscles from ischemic damage.

Although it has been noted that all the sarcoglycan-deficient animal models share a number of features, including muscular dystrophy and a secondary reduction in the localization of other components of the sarcoglycan complex, experiments by Hack and colleagues (207, 208) have demonstrated that loss of an individual sarcoglycan can have apparently different mechanical consequences for the muscle fibers. Like *mdx* muscle, δ-sarcoglycan-deficient muscle exhibits a significant drop in force generation as a result of eccentric muscle contraction (207, 208). In contrast, similar studies using isolated muscles lacking γ-sarcoglycan showed normal resistance to mechanical strain induced by eccentric muscle contraction and minimal uptake of Procion orange dye (an indicator of membrane damage). Thus the apparent lack of contraction-induced injury in γ-sarcoglycan-deficient muscle implies that "nonmechanical" pathways, perhaps involving unknown signaling cascades, could contribute to muscle degeneration. Because sarcoglycan loss is also a feature of DMD, the same nonmechanical defect may also contribute to the pathology of dystrophin-deficient muscular dystrophy.

E. Syntrophins

The syntrophin family of proteins is composed of five members, α-syntrophin, β_1- and β_2-syntrophin, and γ_1- and γ_2-syntrophin (2, 5, 6, 393). The syntrophins all have a similar domain structure consisting of a split PH (pleckstrin homology) domain and intact PH domain, a PDZ domain, and the syntrophin unique region at the COOH terminus. The syntrophins are differentially distributed in muscle. α-Syntrophin is present at the sarcolemma of all fibers, β_1-syntrophin is found predominantly in fast-twitch muscle fibers, whereas β_2-syntrophin is concentrated at the neuromuscular junction. The syntrophins bind directly to dystrophin and dystrobrevin to adjacent to the first coiled coil of both proteins. The PDZ domain of the syntrophins is known to be involved in the interaction with other proteins (Table 2).

The syntrophins are potentially involved in a number of cellular functions and could be considered adaptor proteins that link membrane-associated proteins to the DPC or DPC-like complexes in nonmuscle tissue. Recently, it has been shown that there are two syntrophin-binding sites in the COOH terminus of dystrophin and an additional two sites on α-dystrobrevin (368). These sites are formed by two homologous adjacent α-helices (203, 368). Thus four syntrophin molecules can be anchored to the DPC in muscle (Fig. 4). The second syntrophin binding site is located in the alternatively spliced vr3 sequence of dystrobrevin. Similarly, the second syntrophin binding site on dystrophin is encoded by exon 72, which is also alternatively spliced in fetal tissue. Thus the common spliced variant of dystrophin missing exons 71–74 lacks both COOH-terminal syntrophin-binding sites.

The syntrophins have been shown to bind to a variety

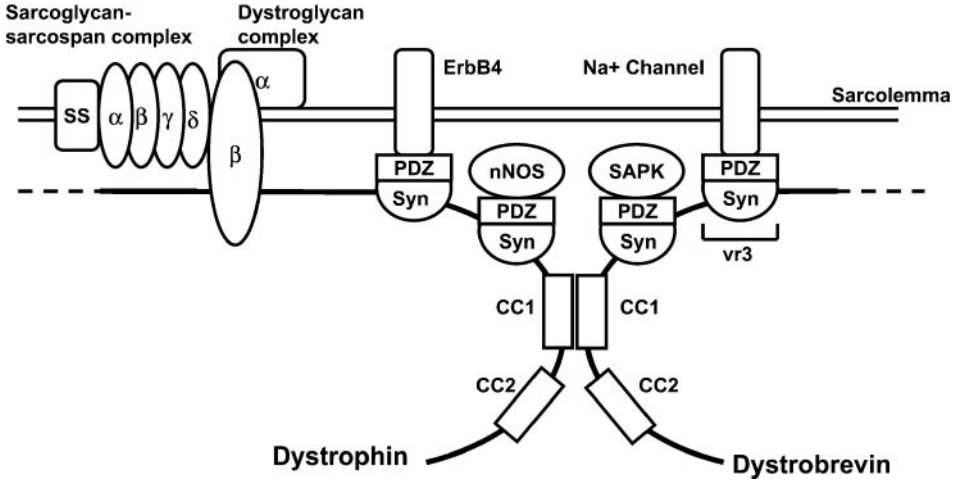

FIG. 4. Protein interactions at the COOH terminus of dystrophin. The figure illustrates a revised model of the detailed interactions occurring at the COOH terminus of dystrophin. The existence of two tandem syntrophin binding motifs in both dystrophin and α-dystrobrevin generates four possible syntrophin binding sites in the DPC. Four potential syntrophin binding proteins are shown (ErbB4 receptor tyrosine kinase, voltage-gated sodium channel, nNOS, and SAPK) are shown that interact with the syntrophin PDZ domain. Syn, syntrophin; nNOS, neuronal nitric oxide synthase; SAPK, stress-activated protein kinase-3; PDZ, PSD-95, Dlg, and Zo-1 domain; CC, coiled-coil domain.

of different molecules in muscle and brain (Fig. 4 and Table 2). Most of these interactions utilize the PDZ domain of syntrophin and the COOH-terminal tails of a number of transmembrane proteins. The exception to this rule is the interaction between nNOS and α-syntrophin that is mediated by a β-hairpin finger of nNOS binding to the peptide groove of the PDZ domain in α-syntrophin (227, 479). Reduced levels of sarcolemmal-associated nNOS are found in patients with DMD and BMD and the *mdx* mouse (89). However, the correlation between nNOS localization and muscular dystrophy is unclear as is its role in pathogenesis of muscular dystrophy. nNOS is also associated with syntrophin and the synaptic scaffolding protein PSD-95 in the brain (59, 215). These interactions could tether syntrophin and the DPC to the postsynaptic membrane of neurons and could contribute to the cognitive impairment in many patients with DMD (42, 59, 215).

α-Syntrophin-deficient mice show no evidence of a myopathy, although these animals do have abnormal neuromuscular junctions (3, 263). Interestingly, α-syntrophin-deficient mice show reduced levels of nNOS at the sarcolemma and also show a specific reduction in utrophin immunoreactivity at the neuromuscular junction (3, 263). It will be interesting to observe the phenotypes of the mice null for the other isoforms of syntrophin.

F. Dystrobrevin

Dystrobrevin was initially identified as an 87-kDa protein that copurified with the acetylcholine receptor from the electric organ of *Torpedo californica* (82). These studies showed that the 87-kDa protein copurified with a number of non-AChR-associated proteins and was enriched at the neuromuscular junction but also found at the sarcolemma (495). Cloning of the *Torpedo* ortholog of dystrobrevin (the 87-kDa postsynaptic protein) showed that dystrobrevin was a dystrophin-related protein that had significant protein sequence homology to the COOH terminus of dystrophin (495). *Torpedo* dystrobrevin is also a major phosphotyrosine-containing protein in the *Torpedo* electric organ (18, 495, 496). The dystrobrevin family of protein isoforms bind directly to dystrophin family of proteins in muscle, brain, and other tissues (Figs. 1 and 4). The dystrobrevin family of proteins is encoded by two different genes. The α-dystrobrevin gene is located on human chromosome 18 (mouse chromosome 18) and encodes at least five different protein isoforms (45, 429). The β-dystrobrevin is encoded by a gene on human chromosome 2 (mouse chromosome 12) that produces a number of COOH-terminal alternatively spliced variants (44, 301, 388). The complexity of the α-dystrobrevin protein isoforms is mirrored by the organization of the three promoters in the dystrobrevin gene (233). However, there is no correlation between the expression of the individual isoforms and the promoter usage. Instead, the dystrobrevin promoters drive the expression of each dystrobrevin isoform in different tissues. Interestingly, this strategy is employed by the DMD gene to produce the different Dp427 isoforms in brain and muscle (Fig. 1).

The distribution of the different dystrobrevin isoforms has been determined in muscle (366, 369, 389). With the use of isoform-specific antibodies, α-dystrobrevin-1 and -2 were found to be concentrated at the neuromuscular junction (366, 389). Both isoforms are also localized to the sarcolemma where α-dystrobrevin-2 is more abundant than α-dystrobrevin-1. In the absence of dystrophin, this sarcolemmal localization is largely lost (341). These isoforms remain at the neuromuscular junction in *mdx* mice, suggesting that the mechanism for anchoring these complexes at the neuromuscular junction is different from that at the sarcolemma. At the neuromuscular junction they may be associated with the dystrophin-related protein utrophin (see sect. VI). α-Dystrobrevin-3 is clearly a component of skeletal and cardiac muscle, but its localization is unclear. It is unlikely to be associated with the DPC via its NH_2 terminus, since this isoform lacks the coiled-coil domain shown to mediate the direct interaction of α-dystrobrevin-1 and -2 with dystrophin and the proposed syntrophin binding site. More recent experiments suggest that the α-dystrobrevins are associated with the sarcoglycan complex (518). This association is thought to be mediated by the NH_2-terminal region of α-dystrobrevin common to all isoforms, anchoring all three dystrobrevin isoforms to the DPC.

Protein tyrosine phosphoryation plays a crucial role during in vitro synaptogenesis. The presence of the muscle-specific kinase (MuSK) at the neuromuscular junction and the action of agrin during synapse formation suggest that α-dystrobrevin could be a downstream substrate for tyrosine phosphorylation. Agrin induces the tyrosine phosphorylation of the β-subunit of the AChR indirectly and also causes MuSK autophosphorylation. Although α-dystrobrevin-1 is phosphorylated in muscle, it is not phosphorylated in response to agrin (366). It is possible that src family kinases could phosphorylate dystrobrevin, since this protein family has also been implicated in signal transduction at the maturing neuromuscular junction. Indeed, some anti-src antibodies cross-react with α-dystrobrevin-1, suggesting that they share similar phosphorylated epitopes (495).

The precise role of the dystrobrevins in relation to the DPC at the sarcolemma is unknown, but they are also proposed to play a role in intracellular signal transduction in this context. α-Dystrobrevin binds directly to dystrophin and thereby recruits further syntrophin proteins to the membrane. Analysis of mice lacking α-dystrobrevin has revealed a dual role for α-dystrobrevin in the pathogenesis of muscular dystrophy and in AChR-cluster stabi-

lization at the neuromuscular junction (189, 192). Mice lacking α-dystrobrevin uniquely develop mild muscular dystrophy without perturbing the assembly of the other components of the DPC at the sarcolemma. Although these mice have residual low levels of dystrobrevin cross-reactive proteins at the neuromuscular junction and sarcolemma, they develop a milder form of muscular dystrophy than the *mdx* mouse. Although similar in pathology, very few muscle fibers appear damaged in the dystrobrevin-deficient mouse compared with the *mdx* mouse that has many more damaged membranes. The only immunocytochemical abnormality in these mice is the reduction of nNOS at the sarcolemma. In addition, the levels of intracellular cGMP are also reduced in the α-dystrobrevin mutant. Comparison of the levels of cGMP in normal, α-dystrobrevin-deficient, and nNOS-deficient mice showed that the α-dystrobrevin mouse had no significant increase in the levels of cGMP in resting compared with electrically stimulated muscle. Similar findings are observed in *mdx* muscle and muscle lacking nNOS. nNOS inhibits vasoconstriction during exercise by stimulating the production of cGMP.

The role of α-dystrobrevin in intracellular signal transduction or other cellular functions via associated proteins will eventually be elucidated through the study of its binding partners. Three dystrobrevin binding partners have recently been identified: dysbindin (30), syncoilin (370), and desmuslin (346). Syncoilin and desmuslin are both predicted to be intermediate filament proteins (346, 370). While syncoilin appears to be a novel protein, desmuslin is very similar to the intermediate filament protein synemin (201), suggesting that they are orthologs. Syncoilin and α-dystrobrevin-1 are both concentrated at the neuromuscular junction of normal skeletal muscle (370). In contrast, desmuslin is expressed predominantly at the muscle sarcolemma (346). The identification of these proteins suggests that α-dystrobrevin may provide a link between the DPC and the intermediate filament cytoskeletal network in muscle. The interaction between α-dystrobrevin and dysbindin is not understood because dysbindin contains no identifiable protein domains (30). Dysbindin has a coiled-coil domain at the NH_2 terminus that may play a role in recruiting as yet unidentified proteins to the DPC (30). However, in common with syncoilin, dysbindin is also upregulated at the sarcolemma of *mdx* mouse muscle (30, 346, 370). Dysbindin may have additional functions because it is widely expressed and binds to the dystrobrevins in nonmuscle tissues (30).

Recent studies have shown that transgenic *mdx* mice expressing dystrophin lacking both the syntrophin and dystrobrevin binding domains (deleted for exons 71–78) display normal muscle function (114). This suggests that both dystrobrevin and syntrophin can bind to the DPC independently from dystrophin and that this is sufficient to prevent the development of a myopathy. It is therefore possible that dystrobrevin and syntrophin bind to additional sites on dystrophin or are anchored to the muscle sarcolemma by an interaction with another component of the DPC (518) or an as yet unidentified protein.

β-Dystrobrevin is expressed in many nonmuscle tissues and forms specific complexes with dystrophin, Dp71, and utrophin (so-called DPC-like complexes) (41, 303). β-Dystrobrevin is also associated with the different syntrophins. In a recent study, Loh et al. (303) identified several different DPC-like complexes in the kidney. β-Dystrobrevin is also expressed in epithelial cells where it may play a role along with syntrophin in cellular polarization (262). β-Dystrobrevin-deficient mice are viable but fail to accumulate DPC-like complexes at the membrane of renal tubules and hepatic sinusoids (302). However, an important member of the dystrophin protein family, β-dystrobrevin, is not expressed in muscle and will not be considered further in this review.

Similarly, dystrophin-related protein-2 (DRP2, Fig. 1) is another member of the dystrophin-related protein family that is not expressed in skeletal or cardiac muscle (418). DRP2 is similar in size and domain organization to the dystrophin isoform Dp116 (72) and the utrophin isoform G-utrophin (46). DRP2 is expressed in the brain where it is associated with postsynaptic densities and cholinergic neurons (420). A recent study by Sherman et al. (442) has demonstrated a novel and exciting role for DRP2 in peripheral nerves. DRP2 binds directly to L-periaxin, a PDZ domain containing protein expressed in peripheral nerve (180). L-Periaxin-deficient mice have a severe demyelinating neuropathy (181). Loss of L-periaxin causes the selective reduction of DRP2 and disruption of the DRP2:dystroglycan complex (442). Interestingly, mutations in laminin α_2-chain (in patients with merosin-negative CMD and the *dy/dy* mouse) are associated with defective myelination (322). These data demonstrate that DPC-like complexes have important roles in nonmuscle tissues.

VII. THE DYSTROPIN PARALOG UTROPHIN

A. The Utrophin Gene

Two years after the discovery of dystrophin a fragment of cDNA derived from fetal muscle was described that was similar to but distinct from the COOH terminus of the DMD gene (306). This cDNA derives from an autosomal gene (chromosome 6 in humans, 10 in mice) (66). The full-length cDNA is 13 kb long and encodes a protein with 3,433 amino acids and a predicted molecular mass of 395 kDa (equivalent parameters for dystrophin are 14 kb, 3,678 amino acids, and 427 kDa) (475). The predicted primary structure is similar to dystrophin throughout its

full length; the NH_2 and COOH termini are especially alike (Fig. 1). Because of its structural kinship with dystrophin and its ubiquitous tissue distribution, this protein was called utrophin (43, 270, 307).

The similarities between utrophin and dystrophin extend beyond their primary structures. Utrophin too is encoded by multiple small exons arranged over a very large genomic region (~1 Mb) (384). The transcript is transcribed from several promoters (70, 131). A variety of shorter COOH-terminal isoforms have been described as have variable splicing patterns (46, 309, 508). Utrophin and dystrophin are very likely paralogs that arose by duplication early in vertebrate evolution (416).

B. Utrophin Localization

Utrophin is more widely expressed than dystrophin and occurs not only in skeletal, cardiac (394), and smooth muscle (371) cells but also in, for example, vascular endothelia (321), retinal glial cells (96), platelets (143), Schwann cells of the peripheral nerves (323), and several cell types within the kidney (303, 401). Within skeletal muscle tissue, the majority of utrophin protein and mRNA is found associated with nervous and vascular structures (272, 492). In muscle fibers themselves, utrophin is distributed very differently to dystrophin. In adult healthy muscle, utrophin is confined to the neuromuscular and myotendinous junctions (371, 376). In contrast, in developing muscle, utrophin is found all along the sarcolemma, a distribution also seen in muscle regenerating after injury (97, 193, 288, 298, 467). Extrajunctional sarcolemmal utrophin (and indeed an increase in the total amount of utrophin) is also found in various myopathies (Fig. 5). This may in some cases be secondary to regeneration, but in dystrophin-deficient muscle (and also inflammatory myositis) it is a feature of fibers that appear not to be regenerating (223, 266).

At the neuromuscular junction, utrophin is found at the crests of the junctional folds, whereas dystrophin occurs mainly in the troughs (37). Likewise, AChR concentrate at the crests (159), and this close colocalization of AChR clusters and utrophin is also found in developing muscle and in muscle culture (38, 73, 392). In the myasthenias (a heterogeneous group of neuromuscular junction diseases in which AChR are lost) utrophin too is lost from the neuromuscular junction whether the pathology is induced by antibodies directed against AChR or by mutations of AChR subunits (445, 446).

C. Functional Domains and Binding Partners: Interactions With Actin

The NH_2 terminus of utrophin is similar to actin binding regions identified in dystrophin, spectrin, and α-actinin (475, 513). These consist of ~240 amino acids which form a pair of calponin homology domains; these structures have been solved crystallographically for utrophin and dystrophin and are similar (267, 268, 357). In vitro studies have shown that this region binds strongly to F-actin (with higher affinity for cytoskeletal than for sarcomeric isoforms) (513). However, some differences between utrophin and dystrophin actin binding have been identified. On the one hand, utrophin lacks the additional actin-binding activity associated with the dystrophin rod

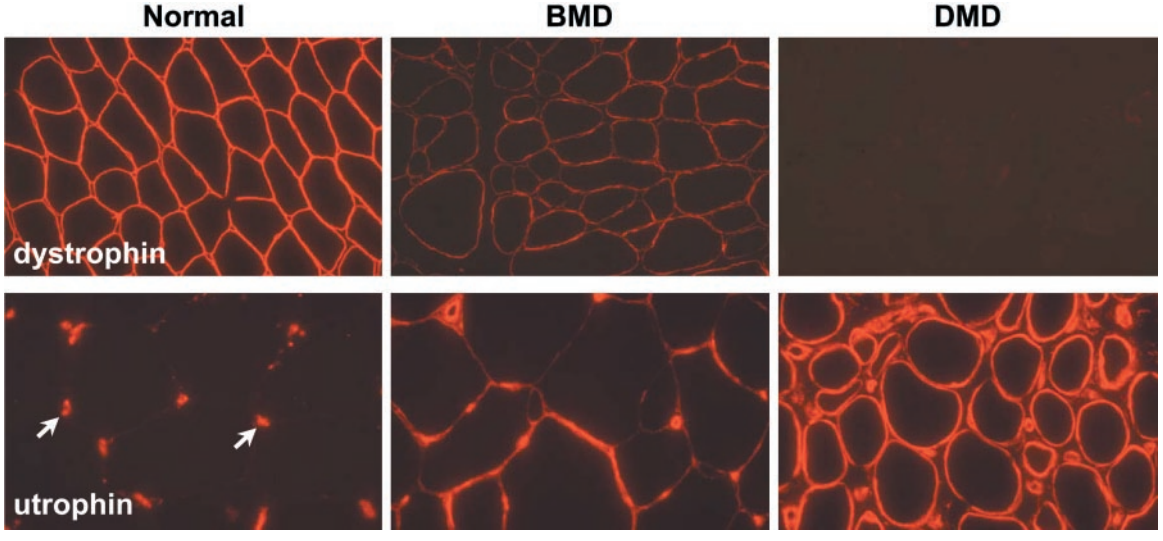

FIG. 5. Utrophin in normal and dystrophic muscle. Immunohistochemistry to visualize dystrophin (top row) and utrophin (bottom row) in human muscle taken from healthy controls (first column) and patients with Becker's (middle column) and Duchenne's muscular dystrophy (third column). The arrows identify the endomyosial capillaries that strongly express utrophin.

domain (10). On the other hand, the NH_2 terminus of utrophin contains a short extension not found in dystrophin which contributes to its affinity for actin; utrophin NH_2-terminal fragments thus bind cytoskeletal β-actin more strongly in vitro than dystrophin (358, 360, 512).

D. Functional Domains and Binding Partners: Interactions of the COOH Terminus of Utrophin

The primary structures of the COOH termini of utrophin and dystrophin are also very similar, and this suggested that utrophin too might be able to bind members of the DPC (475). There is much in vitro and in vivo evidence to show that utrophin can bind β-dystroglycan (320, 480), α-dystrobrevin-1 (389), and the syntrophins (285, 387) and also that it can form part of a complex that includes the sarcoglycans (320). It is however technically difficult to identify with certainty the elements of a utrophin-associated complex at a particular cellular or subcellular location. Utrophin and other potential complex members have a wider distribution than muscle fibers even within muscle tissue (247), and there may be a varied pattern of different utrophin/dystrophin-associated complexes within tissues (303, 368). In the case of the syntrophins for example, the junctional location of β_2-syntrophin and the results of coimmunoprecipitation studies of muscle protein extracts initially suggested it as a binding partner for utrophin at the neuromuscular junction (387). However, higher resolution study shows that β_2-syntrophin is concentrated at the troughs of synaptic folds and that α-syntrophin (which colocalizes with utrophin to the crests) may be a more likely binding partner (284). This is consistent with the loss of utrophin from the neuromuscular junctions of α-syntrophin knockout mice (3).

E. Regulation of Expression

The similarities of structure and binding partners between dystrophin and utrophin have raised the possibility of some functional redundancy between the two proteins. This idea is supported by functional studies described below. In particular, it has been suggested that a manipulation which could raise muscle fiber utrophin levels in patients with DMD might be of therapeutic value (476). In this context, the regulation of utrophin assumes particular interest.

The concentration of utrophin protein at the neuromuscular junction is found too at the RNA level (though to a lesser degree) (198, 492). A mechanism of enhanced synaptic transcription of utrophin, shared by subunits of the AChR and by acetylcholinesterase, has been described that may account for this. One of the utrophin promoters confers a degree of synaptic expression on a reporter gene injected into muscle (198). This activity is associated with a 6-bp motif within the promoter called the N box (283). This element binds transcription factors growth-associated binding protein (GABP)α/β, and in turn the activity of this factor could be increased at the synapse via nerve-associated neuregulin signaling (61, 194, 197, 271).

The expression pattern of utrophin mRNA may also be influenced by posttranscriptional processes; the 3′-untranslated region of utrophin message may help determine both its subcellular location and stability (195, 196). However, processes operating at the protein level are likely to have an important role too. Tagged COOH-terminal utrophin constructs, transcribed by viral (nonsynapse specific) promoters and lacking the 3′-untranslated region, still target to the neuromuscular junction (206, 309). Likewise, the increased levels of utrophin seen in regenerating, dystrophin-deficient, or inflamed muscle seems to occur without a corresponding increase in RNA (199). The nature of these regulatory mechanisms is obscure. They could involve known or unidentified binding partners (3), competition for binding partners with dystrophin (266), or regulation of utrophin binding sites by phosphorylation or calmodulin (258).

F. Functional Studies: Utrophin Transgenes

The hypothesis that utrophin is sufficiently like dystrophin to substitute for it and ameliorate the pathology of dystrophin deficiency has been tested by generating several lines of *mdx* mice harboring utrophin transgenes (474, 477). These transgenes consist of a muscle-specific promoter driving the expression of either truncated utrophin (missing a section of the rod domain) or full-length utrophin. In these mice utrophin is localized throughout the sarcolemma, and the elements of the DPC are reconstituted (474, 477) (Fig. 6). These studies show that increased muscle fiber utrophin significantly reduces pathology as judged histologically or by measures of muscle function (187, 474, 477). Abnormalities of calcium homeostasis and membrane permeability are also corrected (128, 474). There is evidence for a dose-response relationship between levels of muscle utrophin and degree of improvement (474). Similar results have been obtained using viral vectors to deliver utrophin (144, 178, 179, 498). These studies have prompted a search for ways to increase muscle utrophin that could be extended to patients with DMD (91, 92, 106).

G. Functional Studies: Null Mouse Mutants

The close association of utrophin with the AChR and its localization synaptically in adult muscle fibers have suggested a role for utrophin at the neuromuscular junction. Studies of the phenotype of several independent

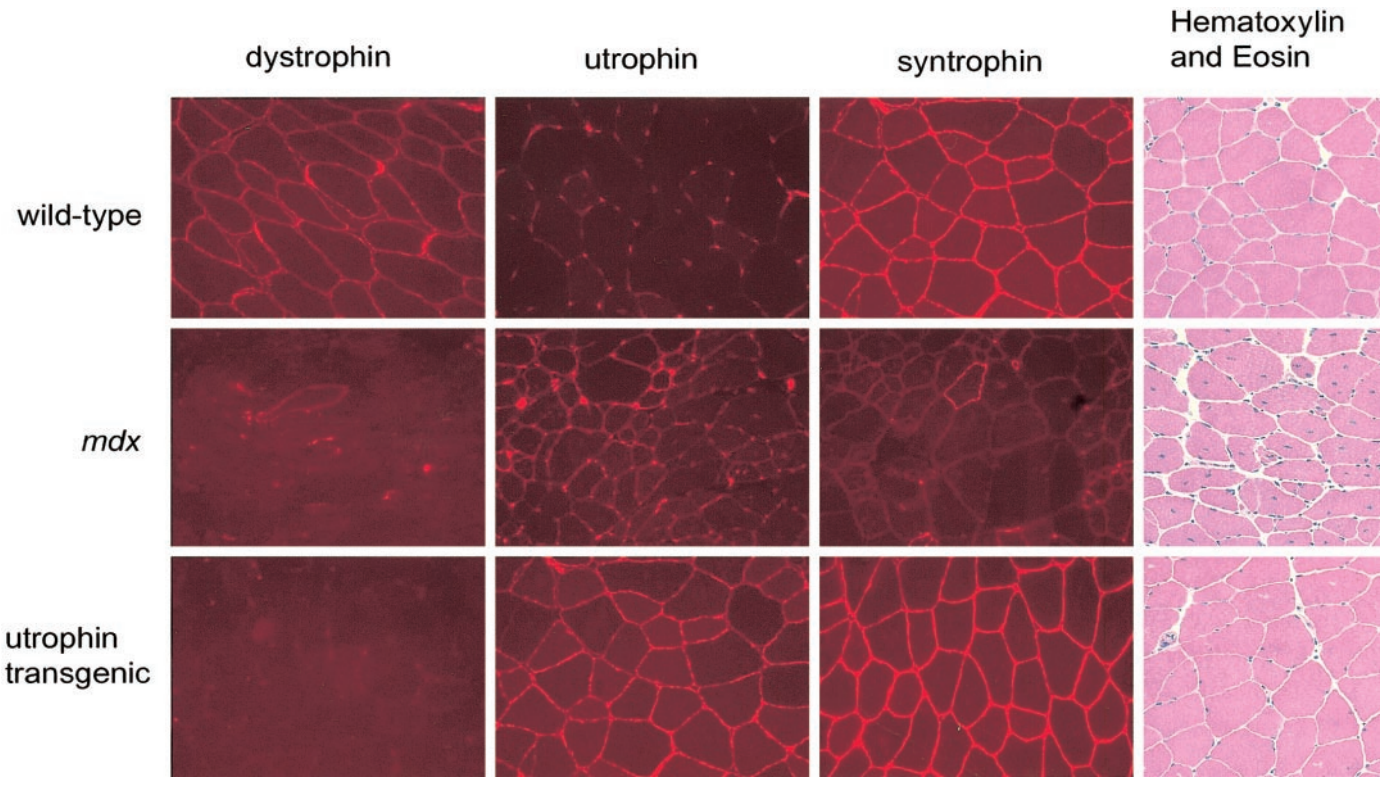

FIG. 6. Induced expression of utrophin restores elements of the DPC and normalizes histology in the *mdx* mouse. Tibialis anterior muscle from 1-yr-old wild-type (top row), *mdx* (middle row), and utrophin transgenic (bottom row) mice is shown. Immunohistochemistry was performed with antibodies against dystrophin (first column), utrophin (second column), and α_1-syntrophin (third column). In the utrophin transgenic, α1-syntrophin is restored. The histology of the muscle (fourth column; hematoxylin and eosin) is also normal in the utrophin transgenic (note the loss of the central nuclei seen in *mdx*).

lines of mice in which the utrophin gene has been disrupted by homologous recombination have provided limited support for this notion (125, 190). These mice appear normal, have a normal life span, and breed normally. No abnormalities outside skeletal muscle have been described, and within that tissue abnormalities are limited to the neuromuscular junction; there is no muscle fiber necrosis. Neuromuscular junctions are normal at birth but develop a reduced amount of folding; perhaps as a consequence the density of AChR is reduced. The function of the neuromuscular junction does not however appear to be impaired. Because of possible functional redundancy between utrophin and dystrophin, evidence of changes in dystrophin expression was sought but none was found.

H. Functional Studies: Dystrophin/Utrophin Null Mutants

Breeding schemes using utrophin-deficient and dystrophin-deficient mice allow production of mice in which both proteins are absent (125, 191). These mice present a much more dramatic phenotype than do mice missing either protein in isolation. They lose weight, develop spinal deformities (also a feature of DMD), and die very prematurely. Muscle disease seems to account for this because expression of a utrophin transgene in skeletal muscle alone prevents it (404). Indeed, pathology outside skeletal and cardiac muscle has not been identified even in mice also lacking all the COOH-terminal dystrophin isoforms (406). Although the morphology of the neuromuscular junction appears more abnormal in double knockout than in either single knockout, this does not seem to impair their electrophysiological properties (191, 405). However, fiber necrosis starts earlier in doubly deficient animals than *mdx* (125, 191). The extraocular muscles (which do not become necrotic in *mdx* mice) are involved when utrophin too is absent (396). The tendency to abnormal activity induced increases in membrane permeability characteristic of dystrophin-deficient muscle is further exaggerated in utrophin/dystrophin null animals (127). [The calcium homeostasis and vulnerability to oxidative stress (see sect. v) of doubly deficient myotubes have not yet been assessed.] These data seem consistent with the idea that sufficient functional redundancy exits between utrophin and dystrophin for the small amount of sarcolemmal utrophin in dystrophin-deficient muscle to

partially compensate for the absence of dystrophin; this compensation is lost when utrophin too is missing, resulting in a more severe phenotype.

I. Summary

Clear functional roles for utrophin remain unclear, and there may be more information to be gained from examination of these various knockout mice for more subtle phenotypes. Utrophin does seem to play a role in establishing the fully morphologically mature neuromuscular junction. There is good evidence that utrophin and dystrophin can perform similar functions at the sarcolemma.

VIII. MOLECULAR PHYSIOLOGY OF MODEL ORGANISMS

The genetic analysis of simpler model organisms may be one way in which researchers can determine the true function of dystrophin and its associated proteins. The complete sequences of the *Drosophila melanogaster* and *Caenorhabditis elegans* genomes are now freely available and can be used to design experiments for studying the function of the DPC in these organisms. Although little is known about the identity and organization of DPC-like complexes in *D. melanogaster*, Laurent Segalat and colleagues (35, 175-177) have undertaken the genetic analysis of the DPC and the function of this complex in *C. elegans*. The dystrophin-like protein in *C. elegans*, DYS-1, is a protein of 3,674 amino acids that probably represents the *C. elegans* ortholog of dystrophin rather than utrophin (35). Mutations in the *dys-1* gene do not result in muscle degeneration but cause hyperactivity in mutant worms. The *dys-1* mutants are also sensitive to acetylcholine and aldicarb, an inhibitor of acetylcholinesterase. These data suggest that one role for the dystrophin ortholog in *C. elegans* is in cholinergic neurotransmission. Interestingly, almost identical findings are observed in *C. elegans* strains that lack the DYB-1 protein encoding the ortholog of dystrobrevin (175, 177).

A major advantage in using simple model organisms to study gene function is that they can be used to screen for suppressor mutations. As the name suggests, this strategy looks for mutations in other genes that reverse or ameliorate the phenotype under study. A good example of this is the dystrophin-dependent locomotive dysfunction in *C. elegans*. To augment the locomotive phenotype of the *dys-1* mutant, a double mutant was made by placing the dystrophin mutant on a mild allele of the *C. elegans* homolog of MyoD called *hlh-1* (176). In mice, the dystrophin-deficient, MyoD-deficient double mutant produces a more severe muscular dystrophy compared with the *mdx* phenotype (334). The *dys-1*:*hlh-1* phenotype in *C. elegans* is characterized by impaired locomotion and egg laying and progressive muscle degeneration (176). Geisler et al. (176) used this mutant to identify suppressors of the phenotype by overexpressing different genes. This screen identified the *dyc-1* gene as a suppressor of the locomotive abnormality in mutant worms. Interestingly, mutations in the *dyc-1* gene have the same phenotype as the *dys-1* mutant and the *dyb-1* mutant (176). Dyc-1 encodes the *C. elegans* homolog of CAPON, a protein that regulates the interaction of PSD-95 and nNOS in neurons (257). Given the interaction between syntrophin and nNOS in muscle and PSD-95 and nNOS in neurons, a possible role for this protein may be to regulate the association of nNOS with the DPC-like complex in *C. elegans* (59, 60). However, the *C. elegans* genome lacks an obvious nNOS homolog. Thus the ancestral role of the dyc-1 gene in *C. elegans* could involve the regulation of homotypic PDZ-dependent interactions.

Whereas dystrophin is an absolute requirement for normal muscle function, its precise role is unknown. Although dystrophin is clearly required to maintain the structural integrity of the muscle fiber, how this is achieved remains unresolved. One clue as to the function of dystrophin may come from the analysis of related proteins in model organisms. As mention earlier, dystrophin is a member of the α-actinin/β-spectrin superfamily of proteins. Three genes encoding different spectrin isoforms exist in *C. elegans* and *D. melanogaster*. Recently, each of these genes has been either mutated or gene function has been suppressed by RNA interference techniques (136, 212, 359). These studies have shed light on the ancestral roles of the spectrins in simpler organisms and have implications for the cellular function of the dystrophin protein family. The structural hypothesis of dystrophin functions is based largely on its similarity with spectrin. In humans, spectrin mutations are associated with membrane abnormalities in erythrocytes producing the diseases hereditary elliptocytosis and sphereocytosis. Before the studies in model organisms, spectrin was thought to play a mechanical role at the erythrocyte membrane. It now appears that one of the ancestral roles for the spectrins is in the assembly of membrane-associated protein complexes. It is also interesting to note that in common with dystrophin, the spectrins bind to a large variety of different proteins (reviewed in Ref. 129).

IX. CONCLUSIONS

The identification of mutations in the dystrophin gene as the cause of DMD led the way for the positional cloning of many other genes responsible for single gene disorders. Since then, the study of DMD has also led to insights into other muscular dystrophies and to a better understanding of the function of normal muscle. The

cloning of the gene was a triumph for molecular genetics in the mid 1980s. The work reviewed here demonstrates that the study of dystrophin, dystrophin-associated proteins, and dystrophin-related proteins has provided insights not only into important structural components of skeletal muscle but also into intracellular communication. The animal models harboring mutations in the genes encoding these proteins produced over the last few years will provide a valuable resource for the next stages of investigation as well as for the development of effective therapies.

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