

**ABSTRACT:** Muscular dystrophies represent a heterogeneous group of disorders, which have been largely classified by clinical phenotype. In the last 10 years, identification of novel skeletal muscle genes including extracellular matrix, sarcolemmal, cytoskeletal, cytosolic, and nuclear membrane proteins has changed the phenotype-based classification and shed new light on the molecular pathogenesis of these disorders. A large number of genes involved in muscular dystrophy encode components of the dystrophin-glycoprotein complex (DGC) which normally links the intracellular cytoskeleton to the extracellular matrix. Mutations in components of this complex are thought to lead to loss of sarcolemmal integrity and render muscle fibers more susceptible to damage. Recent evidence suggests the involvement of vascular smooth muscle DGC in skeletal and cardiac muscle pathology in some forms of sarcoglycan-deficient limb-girdle muscular dystrophy. Intriguingly, two other forms of limb-girdle muscular dystrophy are possibly caused by perturbation of sarcolemma repair mechanisms. The complete clarification of these various pathways will lead to further insights into the pathogenesis of this heterogeneous group of muscle disorders.

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## MOLECULAR BASIS OF MUSCULAR DYSTROPHIES

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The term muscular dystrophy covers a diverse group of inherited disorders characterized by progressive muscle weakness and wasting, in which the primary defect is believed to be in skeletal muscle. The mode of inheritance, the age of onset, the involvement of particular skeletal muscle types and the overall progression have been used to classify different forms of muscular dystrophy. Marked intrafamilial phenotypical heterogeneity in patients with muscular dystrophy made it very difficult, however, to definitively classify these diseases. During the past 10 years, an increasing number of genes have been

identified to cause different forms of muscular dystrophy (Table 1). These findings have led to a profound change in the classification of muscular dystrophy, with a new focus on the molecular genetic basis rather than on clinical symptoms only.

The identification of dystrophin<sup>75</sup> and the subsequent characterization of the dystrophin-glycoprotein complex (DGC),<sup>26,50</sup> with its integral and peripheral components in skeletal muscle (Fig. 1), was a major breakthrough and the first step taken towards clarification of the molecular pathogenesis of muscular dystrophy. The DGC is a multisubunit complex<sup>47,136</sup> of proteins which connects the extracellular matrix with the cytoskeleton.<sup>69</sup> Disruption of this link, caused by mutations of dystrophin or the sarcoglycans, causes sarcolemmal instability, which in turn may render the muscle fibers susceptible to necrosis, the major event in muscular dystrophies.<sup>41</sup> Subsequent studies of various forms of muscular dystrophy have identified novel genes encoding proteins with subcellular localization different from the DGC.

The first part of the present review focuses on the discovery of novel proteins responsible for the devel-

**Abbreviations:** ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; AD-EMD, autosomal dominant EMD; BMD, Becker muscular dystrophy; CMD, congenital muscular dystrophy; DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; EMD, Emery-Dreifuss muscular dystrophy; FCMD, Fukuyama-type congenital muscular dystrophy; ITGA7, alpha7 integrin subunit; LAP 2, lamina-associated protein 2; LGMD, limb-girdle muscular dystrophy; LMNA, lamin A/C gene; MM, Myoshi myopathy; MRI, magnetic resonance imaging; nNOS, neuronal nitric oxide synthase; PFK, phosphofructokinase; SG-SSPN, sarcoglycan-sarcospan complex; TCAP, telethonin; TMD, tibial muscular dystrophy; X-EMD, X-linked EMD

**Key words:** cardiomyopathy; dystrophin-glycoprotein complex; laminin; muscular dystrophy; sarcoglycan

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**Table 1.** Muscular dystrophies and gene locations.\*

Disease	Mode of inheritance	Gene locus	Gene product
X-linked muscular dystrophies			
Duchenne/Becker muscular dystrophy	XR	Xp21	Dystrophin
Emery-Dreifuss muscular dystrophy	XR	Xq28	Emerin
Emery-Dreifuss muscular dystrophy	AD	1q11	Lamin A/C
Limb-girdle muscular dystrophy			
LGMD 1A	AD	5q22–q34 <sup>12</sup>	
LGMD 1B	AD	1q11–21 <sup>20,128</sup>	Lamin A/C
LGMD 1C	AD	3p25	Caveolin-3
LGMD 1D	AD	6q22 <sup>94</sup>	
LGMD 1E	AD	7	
LGMD 2A	AR	15q15	Calpain-3
LGMD 2B	AR	2p13	Dysferlin
LGMD 2C	AR	13q12	γ-Sarcoglycan
LGMD 2D	AR	17q12–q21	α-Sarcoglycan
LGMD 2E	AR	4q12	β-Sarcoglycan
LGMD 2F	AR	5q33–q34	δ-Sarcoglycan
LGMD 2G	AR	17q11–q12	Telethonin (TCAP)
LGMD 2H	AR	9q3–q34	
Distal muscular dystrophy			
Miyoshi myopathy	AR	2p13	Dysferlin
Tibial muscular dystrophy	AD	2q31	Titin?
Congenital muscular dystrophy (CMD)			
“Classic” or “pure” CMD	AR	6q22	Laminin α2
Fukuyama CMD	AR	9q31–q33	Fukutin
α7 integrin congenital myopathy	AR	12q13	α7 Integrin
Rigid spine CMD	AR	1p35–36	
Muscle-eye–brain disease	AR	1p32–p34	
Other forms of muscular dystrophy			
Bethlem myopathy	AD	21q22	Collagen VI α1
	AD	21q22	Collagen VI α2
	AD	2q37	Collagen VI α3
Epidermolysis bullosa and MD	AR	8q24–qter <sup>115</sup>	Plectin
Oculopharyngeal muscular dystrophy	AD	14q11.2–q13 <sup>22</sup>	Poly A binding protein 2
Facioscapulohumeral muscular dystrophy	AD	4q35 <sup>133</sup>	
Myotonic dystrophy	AD	19q13 <sup>24</sup>	Myotonin-protein kinase?

AD, autosomal dominant; AR, autosomal recessive; LGMD, limb-girdle muscular dystrophy; XD, X-linked dominant; XR, X-linked recessive.  
\*This table includes disorders which have been mapped to a chromosome.

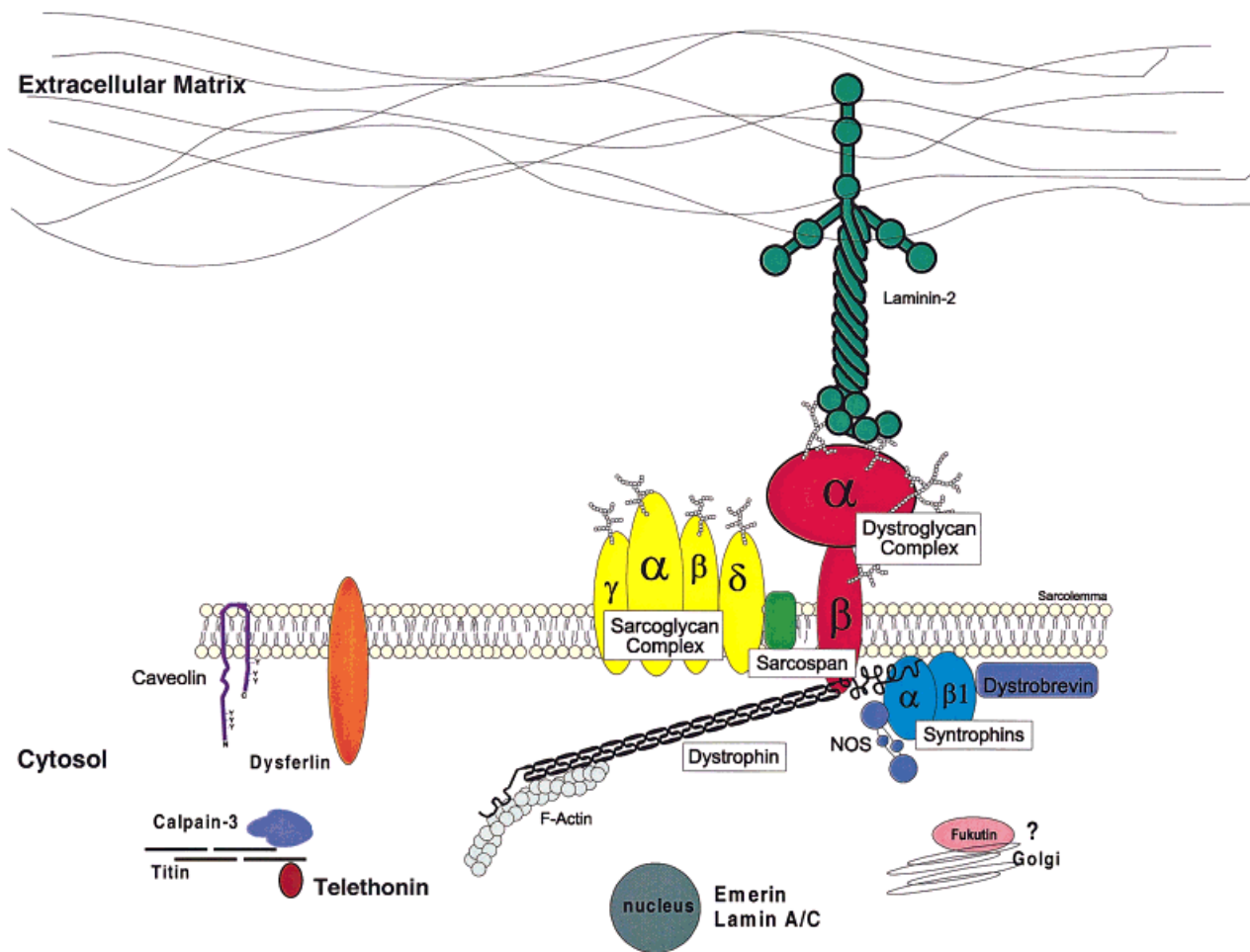
opment of muscular dystrophy. This part lists these proteins with respect to their different subcellular localization and function, including proteins which are localized in the nucleus, the cytosol, the cytoskeleton, sarcolemma, and the extracellular matrix (Table 2). The second part will describe new insights into the molecular pathogenesis of muscular dystrophies and cardiomyopathies associated with mutations within the DGC.

### MUSCULAR DYSTROPHY ASSOCIATED WITH MUTATIONS OF NUCLEAR MEMBRANE

**Emerin and Emery-Dreifuss Muscular Dystrophy.** A triad of symptoms characterizes Emery-Dreifuss muscular dystrophy (EMD): early-onset contractures, progressive weakness in humero-peroneal muscles, and cardiomyopathy with conduction block.<sup>46</sup> The

heritability of the disease was first described as X-linked recessive (X-EMD) but an autosomal dominant form (AD-EMD) has also been reported.<sup>95</sup> Recently, both genes involved in these clinically strikingly similar diseases have been discovered. The X-EMD gene encodes a protein, emerin, which shows limited sequence similarity in the amino- and carboxy-terminal regions to the lamina-associated protein 2 (LAP2).<sup>17</sup> Emerin was localized to the inner nuclear membrane<sup>27,102</sup> in skeletal, cardiac, and smooth muscle cells as well as other tissues. Most X-EMD patients reveal a specific loss of emerin at the nuclear membrane, whereas other neuromuscular disorders do not exhibit any alterations in emerin expression.<sup>84,18</sup>

Patients with AD-EMD were identified by their clinical symptoms resembling X-EMD and by the



**FIGURE 1.** The molecular organization of integral and peripheral components of the DGC and novel proteins involved in muscular dystrophy in skeletal muscle.

presence of emerin in skeletal muscle biopsies. Bonne et al.<sup>20</sup> mapped the locus for AD-EMD to an 8-cM interval on chromosome 1q11–q23 in a large French pedigree, and found that the EMD phenotype in another four small families was potentially linked to this locus. This region contains the lamin A/C gene (LMNA), a candidate gene encoding two proteins of the nuclear lamina, lamins A and C, produced by alternative splicing. Immunostaining of nuclei in these patients revealed that both emerin and lamin A/C were present, suggesting that AD-EMD is caused by haploinsufficiency of lamin A/C.<sup>20</sup> Furthermore, Fatkin et al.<sup>53</sup> reported that missense mutations in the rod domain of the lamin A/C gene can cause dilated cardiomyopathy and conduction system disease. It has been suggested that an emerin-nuclear protein complex (composed of emerin, lamin A and B, and nuclear actin) exists at the nuclear envelope and that one of its primary roles is to sta-

bilize the nuclear membrane against the mechanical stresses that are generated in muscle cells during contraction.<sup>52</sup>

#### **MUSCULAR DYSTROPHIES ASSOCIATED WITH MUTATIONS IN CYTOSOLIC PROTEINS**

**Calpain and LGMD 2A.** LGMD 2A was first studied in isolated populations from La Réunion Island.<sup>14</sup> The age of onset of the disease is extremely variable with a majority ranging from 8–15 years, though a range from 2–40 years has been reported. The disease is predominantly symmetrical and atrophic, with prominent calves seen in only a minority of cases. Pelvic girdle weakness is present and symptomatic from the onset but, strikingly, the hip abductors are spared. Linkage analysis mapped the disease locus to the region of chromosome 15q15.1–q15.3. Using a positional cloning approach, several mutations in the gene encoding calpain-3 were identified in patients from families who had been linked to chro-

**Table 2.** The various proteins involved in different types of muscular dystrophy and their subcellular localization.\*

Subcellular localization	Protein	Type of muscular dystrophy
Nucleus	Emerin	X-linked Emery-Dreifuss muscular dystrophy
	Lamin A/C	Autosomal dominant Emery-Dreifuss muscular dystrophy
Cytosol	Calpain-3	Limb-girdle muscular dystrophy type 2A
	Fukutin (?)*	Fukuyama congenital muscular dystrophy
Cytoskeleton	Dystrophin	Duchenne/Becker muscular dystrophy
	Telethonin	Limb-girdle muscular dystrophy 2G
Sarcolemma	$\alpha$ -, $\beta$ -, $\gamma$ -, $\delta$ -Sarcoglycan	Limb-girdle muscular dystrophy 2C-F
	Caveolin-3	Limb-girdle muscular dystrophy 1C
	$\alpha$ 7 Integrin	Congenital myopathy
	Dysferlin	Limb-girdle muscular dystrophy 2B/Miyoshi myopathy
Extracellular matrix	Laminin $\alpha$ 2	Congenital muscular dystrophy
	Collagen VI	Bethlem myopathy
	Fukutin (?)*	Fukuyama congenital muscular dystrophy

\*The precise localization of fukutin, the protein involved in Fukuyama congenital muscular dystrophy, has not yet been determined.

mosome 15q15.1–q15.3 and some additional isolated cases.<sup>109</sup>

Recently Anderson et al.<sup>5</sup> characterized three different monoclonal antibodies against calpain-3 for Western blot analysis. Patients with a homozygous null mutation showed the expected lack of the protein. Interestingly, patients with missense mutations showed variable levels of full-length calpain-3 protein expression ranging from normal to very low amounts. Calpain-3 is a member of a family of calcium activated proteases. An interaction with titin (connectin) has been shown via the muscle-specific IS2 region of calpain, which also contains a nucleus signal-like sequence.<sup>116</sup> It has been suggested that calpain-3 may play a role in the control of expression of muscle-specific transcription factors and thereby play a role in the upregulation of muscle differentiation.<sup>73</sup>

In this regard, a recent study demonstrated an intriguing hypothesis for the pathogenesis of LGMD 2A.<sup>10</sup> The authors show that calpain-3 deficiency is associated with myonuclear apoptosis and profound perturbation of the I $\kappa$ B $\alpha$ /NF- $\kappa$ B pathway, a pathway which is important for apoptosis. Although the significance of these findings still needs to be established, this could account, at least in part, for the progressive loss of skeletal muscle cells in LGMD 2A. An alternative hypothesis comes from studies in transected myelinated mammalian axons and severed giant axons, which revealed that calpain isoforms are involved in the process of membrane resealing and repair.<sup>57,68</sup> It is therefore tempting to speculate that calpain-3 might be involved in the process of membrane resealing and repair of damaged muscle fibers.

**Fukutin and Fukuyama Congenital Muscular Dystrophy.** Fukuyama-type congenital muscular dystrophy (FCMD), one of the most common autosomal

recessive disorders in Japan (incidence is 0.7–1.2 per 10,000 births), is characterized by congenital muscular dystrophy associated with brain malformation (polymicrogyria) due to a defect in the migration of neurons.<sup>54</sup> Hayashi et al.<sup>64</sup> reported a partial laminin  $\alpha$ 2 chain deficiency in FCMD patients as a secondary phenomenon. It is interesting to note that primary and secondary alterations in the laminin  $\alpha$ 2 chain are associated with congenital forms of muscular dystrophy implying an essential impact of laminin and basement membrane function in skeletal muscle development. In addition, some FCMD cases show disruption of DGC proteins at the sarcolemma.<sup>87</sup>

Recent data demonstrated a retrotransposal insertion of tandemly repeated sequences within the candidate gene which has been named fukutin.<sup>74</sup> This gene is expressed in various tissues in normal individuals, but is absent in FCMD patients who carry the insertion.<sup>74</sup> A systematic analysis of the FCMD gene in 107 unrelated patients identified four novel non-founder mutations in five of them.<sup>76</sup> The discovery of the protein primarily responsible for FCMD opens up a new avenue of research directed towards the molecular pathogenesis of this unique type of muscular dystrophy as well as its functional role in neuronal migration and neuromuscular disease in general. Abnormalities in basal lamina in FCMD muscle and brain have been seen by electron microscopy.<sup>70,103</sup> In light of these observations, it has been suggested that fukutin may be located in the extracellular matrix, where it interacts with and reinforces a large complex encompassing the outside and inside of muscle membranes. However, in vitro experiments suggested that fukutin could also be localized to the Golgi and secretory granules.<sup>74</sup> A detailed computer analysis of the fukutin protein sequence resulted in the prediction that it is an enzyme that

might modify cell-surface glycoproteins and/or glycolipids.<sup>8</sup> These findings, along with the observation of the perturbed expression of some DGC components in FCMD, might imply that fukutin modifies sugar residues of dystroglycan and other glycoproteins within the extracellular matrix and/or sarcolemma. However, so far no antibodies against fukutin are available in order to characterize its subcellular distribution, which is a prerequisite for establishing possible pathogenetic mechanism for this complex disease.

#### **MUSCULAR DYSTROPHIES ASSOCIATED WITH ALTERATIONS IN SARCOMERIC PROTEINS**

**Telethonin and LGMD 2G.** The autosomal recessive form of LGMD type 2G, which clinically resembles Kugelberg-Welander disease has been mapped to chromosome 17q11–12 in a Brazilian family.<sup>100</sup> The clinical appearance is mostly characterized by a predominant weakness of the distal muscles and a rather late onset of the disease (10 years and older). In addition, some patients have been reported to have cardiac problems. Recently, mutations in the gene encoding a sarcomeric protein, telethonin (TCAP) have been identified in affected members of these families.<sup>101</sup> Telethonin is a sarcomeric protein of 19 kDa which is expressed exclusively in skeletal and cardiac muscle.<sup>127</sup> Telethonin is a substrate of titin kinase, which phosphorylates the carboxy terminal of telethonin<sup>59</sup> and it is thought to provide binding sites for other sarcomeric proteins, which is important for the sarcomere assembly.<sup>88</sup> The mutations reported in the LGMD 2G patients cause disruption of the carboxy terminal region and could therefore lead to alterations in sarcomeric structure and assembly. It is interesting to note that two proteins causing clinically different forms of limb-girdle muscular dystrophy, the sarcomeric protein telethonin and the cytosolic protein calpain-3, both interact with titin.

#### **MUSCULAR DYSTROPHIES ASSOCIATED WITH ALTERATIONS IN THE SARCOLEMMA**

**Caveolin-3 and LGMD.** Caveolin, a 21–24-kDa integral membrane protein, is a major component of the caveolae membrane, which are microdomains of the plasma membrane that have been implicated in signal transduction. Recently, mutations in the caveolin-3 gene on chromosome 3p25 were detected in two families with autosomal dominant limb-girdle muscular dystrophy 1C.<sup>97</sup> These patients had normal milestones and onset of disease at around 5 years of age, with cramping muscle pains after exercise, calf hypertrophy, and mild to moderate proximal weak-

ness.<sup>97</sup> In addition, a missense mutation in the caveolin-3 gene has also been reported in a patient with a suggested autosomal recessive inheritance.<sup>91</sup> It is possible that these mutations interfere with caveolin-3 oligomerization and may disrupt caveolae formation at the sarcolemma of skeletal muscle. Galibati et al.<sup>55</sup> showed that LGMD 1C mutations lead to formation of unstable high molecular mass aggregates of caveolin-3 that are retained within the Golgi complex and are not targeted to the plasma membrane. Consistent with its autosomal dominant mode of transmission, the group demonstrated that LGMD 1C mutants of caveolin-3 behave in a dominant-negative fashion, causing the retention of wild type caveolin-3 at the level of the Golgi complex.

Biochemical analysis has shown that caveolin-3 is not an integral component of the DGC, although small quantities of caveolin-3 can be detected in partially purified DGC preparations.<sup>38</sup> In this regard, it has been hypothesized that an increased number of caveolae at the sarcolemma can be detected in DMD.<sup>108</sup> The muscle isoform of phosphofruktokinase (PFK), the rate-limiting enzyme in glycolysis, is a caveolin-3 binding protein. This interaction is modulated by extracellular glucose and by intracellular allosteric effectors of PFK,<sup>113</sup> suggesting that regulation of glycolysis by caveolin-3 could play an important role in muscle pathophysiology.

**Alpha7 Integrin and Congenital Myopathy.** The integrins are a large family of heterodimeric transmembrane cellular receptors, which mediate the association between the extracellular matrix and cytoskeletal proteins. The  $\alpha 7\beta 1$  integrin is a major laminin-binding integrin in skeletal and cardiac muscle and is thought to be involved in myogenic differentiation and migration processes. Targeted deletion of the gene for the alpha7 integrin subunit (ITGA7) in mice leads to a mild form of muscular dystrophy.<sup>89</sup> Interestingly, ultrastructural analysis revealed that myotendinous junctions of alpha7 integrin-deficient myofibers lose their interdigitations and the myofilaments retract from the sarcolemmal membrane, whereas the lateral side of the myofibers remains morphologically normal.<sup>98</sup> In contrast, *mdx* mice have normal myotendinous junctions, with a matrix protein pattern also found in wild-type mice, but the lateral sides of the myofibers are severely damaged. It is possible that  $\alpha 7\beta 1$  integrin has a more important binding function at the myotendinous junction, whereas the DGC is more important for the lateral integrity of the cell.

Recent studies unveiled mutations of the  $\alpha 7$  integrin gene in patients with congenital myopathy.<sup>63</sup>



The patients showed delayed motor milestones, with one patient also showing mental retardation. Interestingly,  $\alpha 7$  integrin is also lost from the sarcolemma of patients and *dy/dy* mice with primary mutations of the laminin  $\alpha 2$  gene, and it is overexpressed in dystrophinopathies<sup>31,65,125</sup> (see below).

**Dysferlin and LGMD 2B and Miyoshi Myopathy.** A novel mammalian gene that is predicted to encode a product related to *Caenorhabditis elegans* spermatogenesis factor *fer-1*, has been shown to be mutated in LGMD type 2B and in Miyoshi myopathy (MM).<sup>13,83</sup> Interestingly, different muscle groups are primarily involved in these disorders with the proximal muscle groups affected in LGMD 2B and the distal muscle groups affected in MM. The 230 kDa protein encoded by this gene has been named dysferlin. Genetic studies demonstrated that an identical mutation could cause either LGMD 2B or MM within the same family. This suggests that the distinction between these two clinical phenotypes requires the identification of an additional factor(s), such as a modifier gene(s).<sup>130</sup> Interestingly, a naturally occurring animal model, the SJL mouse strain, was identified to have a deletion in the dysferlin gene associated with phenotypical features of progressive muscular dystrophy.<sup>19</sup>

Immunohistochemical studies show that dysferlin is expressed at the plasma membrane in skeletal muscle and is absent in human patients with LGMD 2B and MM.<sup>6,86</sup> In human fetal skeletal muscle, dysferlin was detected at Carnegie stage 15 or 16 (embryonic age 5–6 weeks) when limb development starts to show regional differentiation. It is tempting to speculate that the lack of dysferlin at this critical time may contribute to the pattern of muscle involvement that develops later, with the onset of a muscular dystrophy primarily affecting proximal or distal muscles.<sup>6</sup> Based on its homology with the fertilization factor *fer-1* of *C. elegans*, it is hypothesized that dysferlin might be a vesicle-associated membrane protein involved in fusion of large vesicles and membrane docking. *Fer-1* is exclusively expressed in primary spermatocytes, and homozygous mutants are infertile due to the failure of the fusion of vesicles known as membranous organelles with the plasma membrane in spermatides.<sup>1</sup> A possible pathogenetic mechanism in dysferlinopathies might thus be related to a defect in vesicular trafficking within the muscle fiber. Absence of dysferlin could lead to perturbation of membrane resealing and repair of damaged muscle fibers, which require intact vesicle trafficking. A possible pathogenic consequence would

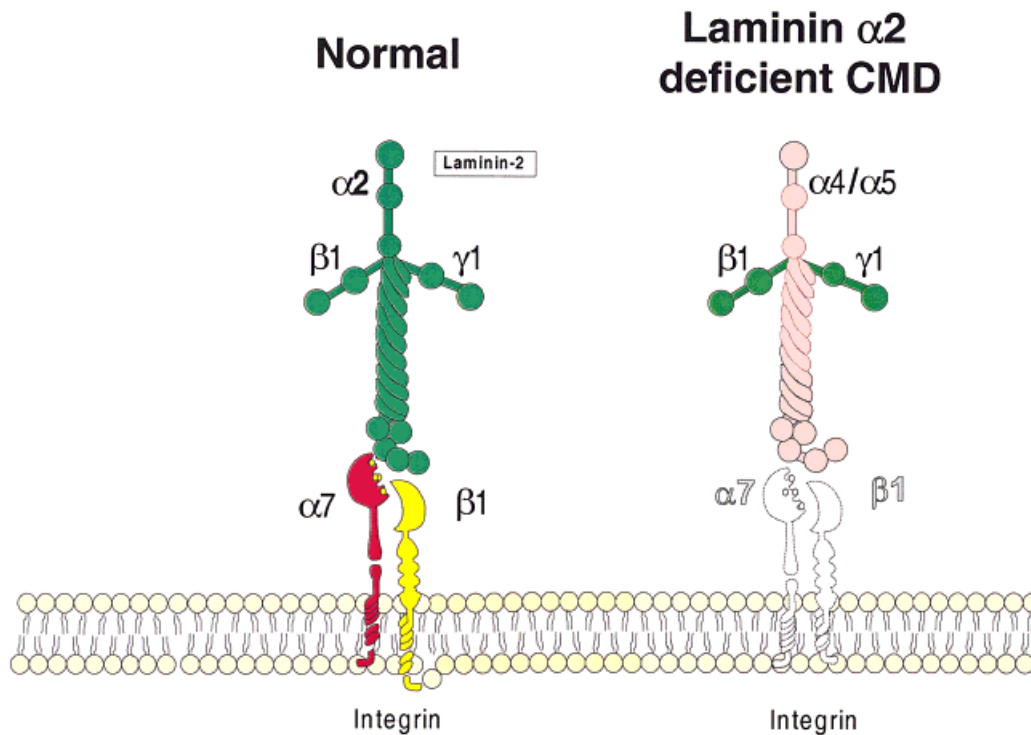
be the development of dystrophic alterations in skeletal muscle.

Just recently, a highly homologous protein named myoferlin has been identified.<sup>39</sup> Myoferlin is abundantly expressed in skeletal and cardiac muscle where it is located at both the sarcolemma and the nuclear membrane; this makes myoferlin a candidate gene for muscular dystrophy and cardiomyopathy. Interestingly, myoferlin expression is upregulated at the sarcolemma of *mdx* mice (deficient in dystrophin) and mice deficient for  $\gamma$ -sarcoglycan.<sup>60</sup> It is possible that the upregulation of myoferlin at the sarcolemma in animal models with increased muscle fiber damage might account for the compensatory yet insufficient efforts of membrane repair in these mice.

#### MUSCULAR DYSTROPHIES ASSOCIATED WITH MUTATIONS IN THE EXTRACELLULAR MATRIX

**Laminin  $\alpha 2$  and Congenital Muscular Dystrophies.** About 50% of the patients diagnosed with classic congenital muscular dystrophy (CMD) show a primary deficiency of the laminin  $\alpha 2$  chain (reviewed elsewhere<sup>122,129</sup>). However, recent studies have shown that, in particular, partial absence of the NH<sub>2</sub>-terminus of the laminin  $\alpha 2$  chain can be associated with milder forms of muscular dystrophy with later onset as compared to the classic form of CMD.<sup>2,29,114,120</sup> An interesting finding is the occurrence of white matter changes on brain magnetic resonance imaging (MRI) in the merosin-deficient patients,<sup>40,42,120</sup> but the mechanism responsible for the development of these alterations is not fully understood.

The laminin  $\alpha 2$  chain together with laminin  $\beta 1$  and  $\gamma 1$  forms the laminin-2, which is the specific laminin for basement membranes of the adult skeletal muscle fibers, Schwann cells, and trophoblasts.<sup>80</sup> Together with collagen IV, nidogen and perlecan, the laminins form the structural backbone of basement membranes. In skeletal muscle laminin-2 interacts with  $\alpha$ -dystroglycan, a component of the DGC, and with the  $\alpha 7\beta 1$  integrin complex. The primary absence of laminin  $\alpha 2$  chain leads to several secondary changes in the protein composition of the sarcolemma and the extracellular matrix (ECM) (see Fig. 2). One interesting and still inexplicable finding is the overexpression of the laminin  $\alpha 5$  chain at the basement membrane of laminin  $\alpha 2$  chain negative skeletal muscles.<sup>29,96,121,123</sup> In addition, a strong upregulation of the laminin  $\alpha 4$  chain in basement membranes of blood vessels, the endoneurium of intramuscular nerves, and of isolated regenerating muscle fibers was detected in the *dy/dy* mice, a natu-



**FIGURE 2.** Composition of the laminin-integrin complex in normal skeletal muscle and merosin-deficient congenital muscular dystrophy (CMD). The primary absence of laminin  $\alpha 2$  chain is associated with an upregulation of laminin  $\alpha 4/\alpha 5$  and loss of the integrin  $\alpha 7\beta 1$  complex.

ral occurring animal model for laminin  $\alpha 2$ -deficient CMD.<sup>110</sup> However, it seems that upregulation of the alternative laminin  $\alpha$ -chains do not seem to have a functional compensating influence on the severe phenotype observed in human patients and mice. Interestingly, another laminin chain, laminin  $\beta 2$ , has been reported to be deficient in laminin  $\alpha 2$  chain deficient CMD.<sup>29,30</sup>

Previous studies demonstrated that mouse laminin-1 binding to  $\alpha$ -dystroglycan was inhibited dramatically by heparin,<sup>48</sup> whereas laminin  $\alpha 2$  binding to dystroglycan was only marginally blocked.<sup>104</sup> McDearmon et al.<sup>90</sup> demonstrated a quantitative decrease in the expression of laminin  $\beta$  and  $\gamma$  chain immunoreactivity in *dy/dy* mice. Furthermore,  $\alpha$ -dystroglycan binding activity was reduced in laminin extracts from laminin  $\alpha 2$  chain deficient *dy/dy* mice and  $\alpha$ -dystroglycan binding activity to the residual laminins was dramatically inhibited by heparin. Taken together, these findings suggest that heparan sulfate proteoglycans perturb  $\alpha$ -dystroglycan binding to the laminin variants present in laminin  $\alpha 2$  deficient skeletal muscle, arguing in favor of a specific and functionally important interaction between  $\alpha$ -dystroglycan and laminin-2 in adult skeletal muscle.

In addition to secondary alterations observed within the ECM, secondary loss of  $\alpha 7$  integrin, a transmembrane protein, has been described in laminin  $\alpha 2$  deficient patients and the *dy/dy* mice.<sup>31,65,125</sup> Furthermore, a selective loss of  $\alpha 7$  integrin at the neuromuscular junction in laminin  $\beta 2$ -deficient mice has been reported,<sup>85</sup> indicating that the laminin  $\alpha 2$  chain, the laminin  $\beta 2$  chain, and  $\alpha 7$  integrin subunits interact functionally and structurally. The interaction appears essential for normal muscle cell function and stability. Taken together, these data demonstrate that in skeletal muscle the  $\alpha 7\beta 1$  integrin serves as an important transmembrane functional link between the ECM and the cytoskeleton. Consequently, it appears that myofibers may necessitate at least two separate but parallel attachment systems for their anchorage-dependent stability and survival: first, the basement membrane-DGC anchorage system, and second, the  $\alpha 7\beta 1$  integrin-laminin-2 anchorage system. It also appears that both confer mechanophysical stability and ECM cell-survival signaling.

**Collagen VI and Bethlem Myopathy.** Bethlem myopathy is a mild, dominantly inherited disorder characterized by early childhood onset of generalized

muscle weakness and wasting and, commonly, contractures of multiple joints.<sup>9,15</sup> Mutations resulting in Bethlem myopathy have recently been identified in three genes, *COL6A1*, *COL6A2*, and *COL6A3*, that code for subunits of the extracellular matrix protein collagen VI on chromosome 2q.<sup>71,77,105</sup> The microfibrillar type VI collagen is believed to play a role in bridging cells with the extracellular matrix. In Bethlem myopathy, it is suggested that reduced collagen VI microfibrillar network can no longer adequately anchor the muscle cells to the surrounding connective tissue.<sup>78</sup> Interestingly, some cases with Bethlem myopathy do show a secondary decrease in expression of the laminin  $\beta$ 1 chain in skeletal muscle but not in blood vessels,<sup>93</sup> suggesting a functional correlation between collagen VI and laminin  $\beta$ 1.

**Other Forms of Muscular Dystrophy.** Untiring efforts in many different laboratories have achieved further progress in characterizing new chromosomes and genes which are responsible for various muscular dystrophies, currently being classified as clinical entities. Subsequently, a new gene for autosomal recessive LGMD in Manitoba Hutterites maps to chromosome region 9q31–q33, and was recently named as *LGMD 2H*.<sup>131</sup> Speer et al.<sup>117</sup> identified a new autosomal dominant muscular dystrophy locus on chromosome 7. Identification of a new locus on chromosome 1p35–36 has been found in a peculiar form of CMD with early rigidity of the spine, scoliosis, and reduced vital capacity.<sup>99</sup> The muscle-eye-brain disease gene has been assigned to 1p32–p34 by linkage analysis and homozygosity mapping.<sup>33</sup> Recent immunohistochemical analysis demonstrated a secondary deficiency of laminin  $\alpha$ 2 chain in this disease, suggesting that this new gene may again encode for a novel protein localized in the extracellular matrix.<sup>62</sup> A genome scan with highly polymorphic markers has established linkage for tibial muscular dystrophy (TMD), a recently described late-onset distal myopathy, to a novel myopathy locus on chromosome 2q31.<sup>124</sup> There is one superior candidate gene on the 2q31 locus, the gene encoding a giant protein titin, specifically expressed in heart and skeletal muscle.

#### **NOVEL INSIGHTS INTO THE PATHOGENESIS OF MUSCULAR DYSTROPHIES ASSOCIATED WITH MUTATIONS WITHIN THE DGC**

**Dystrophin-Glycoprotein Complex.** The dystrophin-glycoprotein complex<sup>26,49,135</sup> is a multisubunit complex comprised of peripheral and integral membrane proteins which form a structural linkage between the F-actin cytoskeleton and the extracellular

matrix. The proteins that comprise the DGC are structurally organized into three distinct subcomplexes. These are the cytoskeletal proteins, dystrophin and the syntrophins; the sarcolemmal localized dystroglycans ( $\alpha$  and  $\beta$  subunits); and the sarcoglycans ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  subunits) and sarcospan.<sup>35</sup> Several forms of muscular dystrophy arise from primary mutations in genes encoding components of this complex (see below).<sup>25</sup> Interactions between subcomplexes are evidently important for targeting to the sarcolemma, as well as for membrane stabilization.<sup>36</sup>

Dystrophin, a 427 kDa protein which is absent or reduced in Duchenne/Becker muscular dystrophy (DMD/BMD) is located on chromosome Xp21.<sup>75</sup> It has been shown that the NH<sub>2</sub> terminus of dystrophin interacts directly with F-actin in an extended, lateral fashion, similar to many actin side-binding proteins.<sup>4,111,112</sup> Furthermore, it was demonstrated that a cluster of basic repeats in the dystrophin rod domain binds F-actin through an electrostatic interaction.<sup>4</sup> Interestingly, the autosomal homologue of dystrophin, utrophin, lacks this rod domain binding activity.<sup>3</sup> Dystrophin interacts with other DGC subcomplexes through its COOH terminal domain, which directly binds to the COOH terminus of  $\beta$ -dystroglycan, an integral membrane protein with a single transmembrane helix.<sup>72</sup> The  $\beta$ -dystroglycan, in turn, binds  $\alpha$ -dystroglycan, anchoring it to the extracellular surface of the sarcolemma. In turn,  $\alpha$ -dystroglycan serves as a laminin-2 receptor, thereby completing the structural connection between the actin cytoskeleton and the extracellular matrix.<sup>48</sup> The significance of the DGC for structural integrity has recently also been shown in other tissues. Reduced glomerular expression of dystroglycan was shown in kidney basement membranes diagnosed with minimal-change nephrosis.<sup>107</sup>

An important role of the DGC for muscle function and stability is to provide mechanical support to the plasma membrane during myofiber contraction.<sup>106,132</sup> The complete loss of dystrophin from the sarcolemma of patients with DMD perturbs the structural composition of the DGC. In addition to dystrophin, the dystroglycan complex and the sarcoglycan complex along with sarcospan<sup>36</sup> are reduced at the sarcolemma in these patients. Likewise, a nonsense mutation in the murine dystrophin gene (*mdx*) eliminates expression of dystrophin and, consequently, the DGC proteins are also reduced at the sarcolemma. Patients with BMD have reduced expression of dystrophin and a milder clinical phenotype than DMD patients. Interestingly, the expression of the DGC proteins is only moderately



perturbed, which favors the belief that the precise expression of the DGC is necessary to protect the muscle fiber membrane from contraction-induced damage. This is evidenced by increased permeability of muscle fibers to intravenously administered Evans blue dye<sup>67,119</sup> as well as leakage of muscle-specific enzymes into the serum. However, the mechanism by which absence of dystrophin leads to increased muscle fiber degeneration, necrosis, and fibrosis is not completely understood. The importance of dystroglycan for muscle function and physiology has recently been demonstrated in chimaeric mice deficient for dystroglycan who display morphological abnormalities of skeletal muscle.<sup>34</sup>

In addition to the mechanical and structural function, recent findings suggest that the DGC might play a role in cellular communication, as highlighted by the interaction of the DGC with known signaling molecules such as nitric oxide synthase or Grb2.<sup>23,58,134</sup> This idea is further supported by results obtained in mice deficient for  $\alpha$ -dystrobrevin, a possible cytoplasmic protein of the DGC. While expression of DGC components is not affected in the absence of  $\alpha$ -dystrobrevin, a DGC-associated signaling protein, neuronal nitric oxide synthase (nNOS), is displaced from the membrane and nitric-oxide-mediated signaling is impaired.<sup>58</sup> However, skeletal muscle from nNOS dystrophin-null mice maintains the dystrophic features observed in dystrophin-deficient *mdx* mice.<sup>37</sup> These data demonstrate that relocalization of nNOS to the cytosol does not contribute significantly to pathogenesis of muscular dystrophy in *mdx* mice.

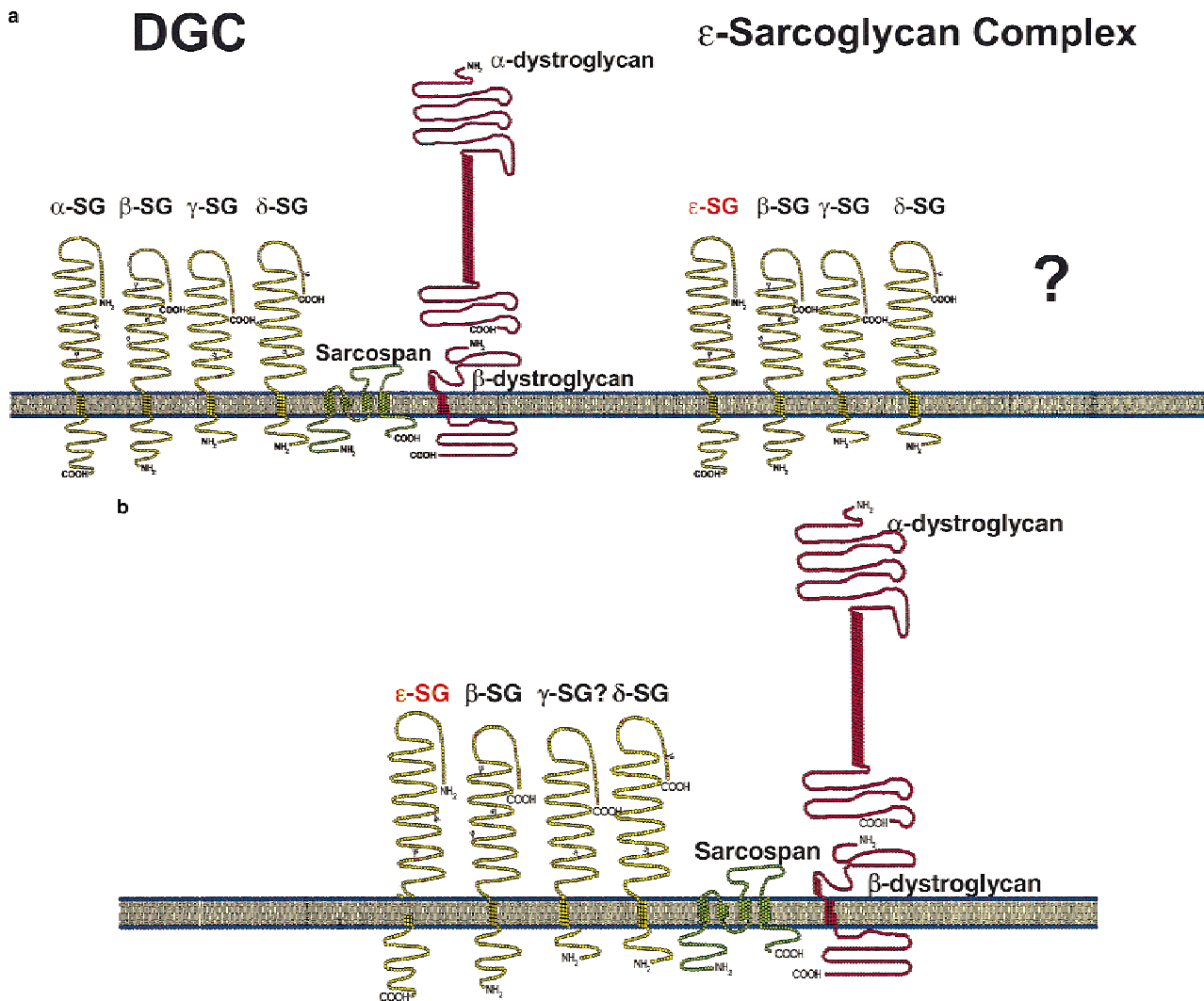
**Sarcoglycanopathies and the Sarcoglycan-Sarcospan Complex.** The involvement of the sarcoglycan complex in patients with clinically characterized autosomal recessive limb-girdle muscular dystrophy (LGMD2) has become increasingly clear. The existence of this group as a separate pathophysiological entity had been questioned for a long time because of the overlap of symptomatology with BMD, manifesting carriers of DMD mutations, and patients with spinal muscular atrophy, and mitochondrial and metabolic disorders. Among LGMD2 are four distinct subtypes caused by mutations in sarcoglycan glycoproteins (reviewed elsewhere<sup>21,81</sup>): LGMD2D ( $\alpha$ -sarcoglycan), LGMD2E ( $\beta$ -sarcoglycan), LGMD2C ( $\gamma$ -sarcoglycan), and LGMD2F ( $\delta$ -sarcoglycan). We will refer to this group of disorders as sarcoglycanopathies rather than to LGMD, because of the variable clinical features presented by patients affected with sarcoglycan mutations. No mutations in the human sarcospan gene have been detected so

far. Interestingly, mice deficient for sarcospan maintain expression of all sarcoglycan proteins at the sarcolemma and do not develop muscular dystrophy.<sup>79</sup>

Little is known about the functional role of the sarcoglycan complex, other than that its integrity is critical for normal muscle physiology. A shared feature of the four types of sarcoglycanopathies is that mutations in a single sarcoglycan gene result in abnormal expression of all sarcoglycans at the sarcolemma. Experimental studies in a heterologous cell system demonstrated that the sarcoglycans are glycosylated when assembled into a complex that resides in the plasma membrane and that complex assembly was dependent on the simultaneous synthesis of all four sarcoglycans.<sup>66</sup> Mutant sarcoglycans blocked complex formation and insertion of the sarcoglycans into the plasma membrane, supporting the idea that the molecular defect in sarcoglycanopathies is because of aberrant sarcoglycan complex assembly and trafficking, which leads to the absence of the complex from the sarcolemma.<sup>66</sup>

A new avenue of research, opened up by studying the different tissue distribution of the sarcoglycan-sarcospan complex (SG-SSPN), which revealed new insights into the pathogenesis of sarcoglycanopathies. In skeletal and cardiac muscle the SG-SSPN complex is composed of  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan and sarcospan. Recently,  $\epsilon$ -sarcoglycan, a transmembrane glycoprotein showing 43% amino acid identity with  $\alpha$ -sarcoglycan has been identified<sup>51,92</sup> (Fig. 3a). In contrast to  $\epsilon$ -sarcoglycan which has a broad tissue distribution,  $\alpha$ -sarcoglycan expression is restricted to skeletal and cardiac muscle.<sup>44,118</sup> Biochemical fractionation studies demonstrated that  $\epsilon$ -sarcoglycan replaces  $\alpha$ -sarcoglycan in smooth muscle as an integral component of a unique SG-SSPN complex composed of  $\epsilon$ -,  $\beta$ -, and  $\delta$ -sarcoglycan and sarcospan (Fig. 3b). The expression pattern of  $\gamma$ -sarcoglycan in smooth muscle needs further genetic and biochemical studies; western blot analysis of smooth muscle clearly demonstrated the presence of  $\gamma$ -sarcoglycan, but immunohistochemical studies have so far failed to detect  $\gamma$ -sarcoglycan in smooth muscle<sup>44,45</sup> (Barresi and Campbell, unpublished data).

The significant impact of the distinct tissue distribution of the sarcoglycans on pathogenic mechanisms has been demonstrated in animal models deficient for various sarcoglycan proteins. Targeted disruption of  $\alpha$ -sarcoglycan leads to progressive muscular dystrophy and to a concomitant deficiency of  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan along with sarcospan in skeletal and cardiac muscle.<sup>43</sup> Interestingly,  $\epsilon$ -sarcoglycan expression is still preserved in skeletal and car-

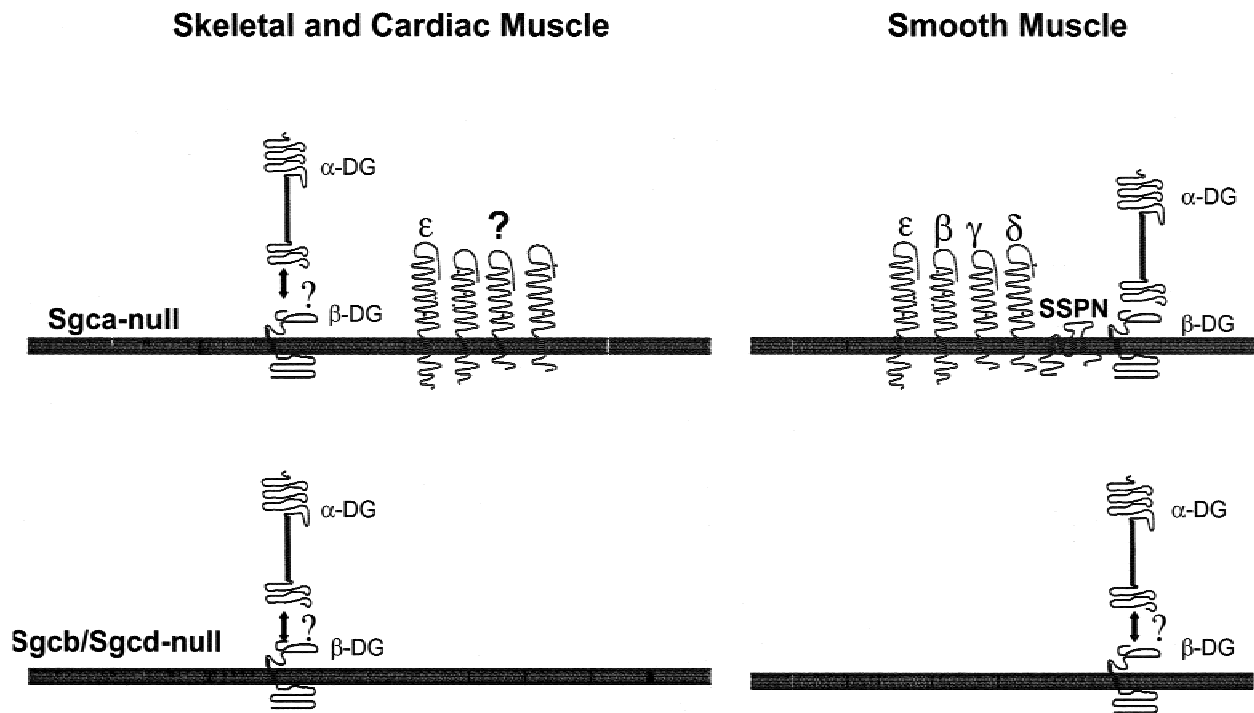


**FIGURE 3. (a)** Integral membrane components of the “traditional” DGC and an additional  $\epsilon$ -sarcoglycan–containing complex in striated muscle. The binding partners of the  $\epsilon$ -sarcoglycan complex will need to be determined in the future. **(b)** Integral membrane components of the DGC in smooth muscle. In smooth muscle  $\epsilon$ -sarcoglycan replaces  $\alpha$ -SG as an integral component of the sarcoglycan complex.

diac muscle of these mice. Further biochemical analysis of skeletal muscle from  $\alpha$ -sarcoglycan deficient mice revealed that  $\alpha$ -dystroglycan binding to the sarcolemma was greatly destabilized by the loss of the sarcoglycan-sarcospan complex, suggesting that the SG-SSPN complex anchors  $\alpha$ -dystroglycan to the membrane.<sup>120</sup> Interestingly, although the SG-SSPN complex is absent in cardiac muscle,  $\alpha$ -sarcoglycan deficient mice do not develop cardiomyopathy. In contrast, targeted disruption of  $\beta$ - or  $\delta$ -sarcoglycan (animal models for LGMD 2E and 2F) develop severe muscular dystrophy associated with cardiomyopathy.<sup>7,32,45</sup> They have the histological hallmark of focal areas of necrosis in skeletal and cardiac muscle.<sup>32,45</sup> Immunohistochemical and biochemical studies of vascular smooth muscle and other smooth

muscle types revealed disruption of the SG-SSPN complex in these mice.<sup>44,118</sup> In contrast, smooth muscle expression of the SG-SSPN complex in  $\alpha$ -sarcoglycan deficient mice was not affected (Fig. 4).

Further analysis of vascular smooth muscle in  $\beta$ - and  $\delta$ -sarcoglycan deficient mice demonstrated that the absence of the SG-SSPN complex perturbed vascular function as demonstrated by multiple microvascular constrictions in arteries of the heart, diaphragm, and kidney.<sup>45</sup> The data indicate that vascular dysfunction initiates the cardiomyopathy and exacerbates the muscular dystrophy phenotype.<sup>32,45</sup> Interestingly, no abnormalities in vascular perfusion of these same tissues were observed in mice deficient for  $\alpha$ -sarcoglycan including the diaphragm, ruling out the possibility that alterations



**FIGURE 4.** Alterations of the sarcoglycan-sarcospan complex in skeletal, cardiac and smooth muscle of  $\alpha$ ,  $\beta$ , and  $\delta$ -sarcoglycan deficient murine muscular dystrophy models (*Sgca*, *Sgcb*, *Sgcd*, respectively). In *Sgca*-null mice, the  $\epsilon$ -sarcoglycan complex in striated muscle as well as the smooth muscle sarcoglycan complex are still preserved. In contrast, both complexes are disrupted in *Sgcb*- and *Sgcd*-null mice.

within the muscle itself lead to secondary perturbation of vascular function.

Another interesting observation in  $\beta$ - and  $\delta$ -sarcoglycan deficient skeletal and cardiac muscle is the concomitant loss of  $\epsilon$ -sarcoglycan, arguing in favor of a second sarcoglycan complex in skeletal and cardiac muscle<sup>45</sup> (Fig. 3a). Liu and Engvall<sup>83</sup> have made similar observations *in vivo* and *in vitro*. Their study demonstrated that  $\epsilon$ -sarcoglycan is complexed with  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycans in both wild type and  $\alpha$ -sarcoglycan deficient mice and that  $\alpha$ - and  $\epsilon$ -sarcoglycans form separate complexes with  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycans. A complex pathogenetic mechanism can be suggested for LGMD2E and F: mutations in either  $\beta$ - or  $\delta$ -sarcoglycan gene lead to a disruption of the SG-SSPN complex containing  $\alpha$ -sarcoglycan in striated muscle and that there is a second sarcoglycan complex containing  $\epsilon$ -sarcoglycan in vascular, skeletal, and cardiac muscle. This eventually leads to skeletal and cardiac muscle membrane instabilities that render the muscle cells more susceptible to intermittent ischemia due to microvascular constrictions.

In this regard, it is important to note that positron emission tomography studies in several sarcoglycan deficient patients (one patient with a known mutation in the  $\beta$ -sarcoglycan gene) revealed

blunted coronary vasodilator reserve suggesting dysfunction of coronary artery smooth muscle.<sup>56</sup> The significant contribution of the vascular smooth muscle to the phenotype observed in  $\beta$ - and  $\delta$ -sarcoglycan deficient mice demonstrates that future novel pharmaceutical and genetic therapeutic strategies should be directed not only toward skeletal and cardiac muscle but also toward vascular smooth muscle.

Mice deficient for  $\gamma$ -sarcoglycan also exhibit cardiomyopathy in addition to skeletal muscle myopathy.<sup>60</sup> The reason for this phenomenon might become clearer with further characterization of the expression pattern of  $\gamma$ -sarcoglycan in tissues such as smooth muscle. Recently, *in vivo* and *in vitro* assessment of physiological function in skeletal muscle of  $\gamma$ -sarcoglycan deficient mice revealed that contraction-induced muscle injury and mechanical weakness are not required for the development of dystrophic alterations in skeletal muscle.<sup>61</sup> Thus, it is possible that some signaling pathways play an important role in the pathogenesis of muscular dystrophy of  $\gamma$ -sarcoglycan deficient mice. The results are in contrast to *in vitro* studies in  $\alpha$ - and  $\delta$ -sarcoglycan deficient mice where a decrease in specific force developed by the extensor digitorum longus muscle can be observed<sup>43</sup> (Coral and Campbell, unpub-

lished observation). The concept that different sarcoglycans may exhibit different functional roles is further supported by the hypothesis that  $\alpha$ -sarcoglycan may have an ecto-adenosine triphosphatase (ATPase) activity and that elevated extracellular adenosine triphosphate (ATP) may contribute to calcium overload and muscle fiber death.<sup>16</sup> Taken together, the analyses of animal models deficient for various sarcoglycans demonstrate that not only variations in tissue distribution but also differences in the functional role among sarcoglycan proteins within the same complex may have an impact on the pathogenetic mechanism and the resulting phenotype.

Extensive clinical, genetic, and immunohistochemical studies in human patients with sarcoglycan deficient muscular dystrophy during the last few years has unveiled a more complex situation. Several studies obtained from single biopsies in human patients showed that the primary mutations in one sarcoglycan is not necessarily associated with the complete loss of the entire sarcoglycan complex.<sup>11,126</sup> In regard to these findings, Vainzof et al.<sup>126</sup> suggested that  $\alpha$ -,  $\beta$ - and  $\delta$ -sarcoglycan might be more closely associated with each other and that  $\gamma$ -sarcoglycan might interact more directly with dystrophin. In contrast, *in vitro* studies in myotubes by Chan et al.<sup>28</sup> suggested that  $\beta$ -,  $\gamma$ -, and  $\delta$ - sarcoglycan are more closely associated to one another than  $\alpha$ -sarcoglycan and that  $\delta$ -sarcoglycan tightly binds to dystroglycan. Future experiments combining the findings obtained in human patients with the various available animal models is needed to fully elucidate the interaction of the sarcoglycans with each and with other components of the DGC and the ECM. This will consequently expand insights into the functional role of each single sarcoglycan as well as the SG-SSPN complex in normal and diseased skeletal, cardiac and smooth muscle.

### CONCLUDING COMMENTS

The discovery of novel genes encoding proteins with different subcellular localization, which cause various types of muscular dystrophy, has shed new light on the pathogenetic mechanisms of this heterogeneous group of disorders. Perturbation of the components of the DGC leads to disruption of the linkage between the extracellular matrix and the cytoskeleton. The loss of sarcolemmal integrity renders muscle fibers more susceptible to membrane damage due to a variety of reasons such as mechanical force, signaling events, and/or intermittent ischemia. Pathogenetic mechanisms of other forms of muscular dystrophy such as the limb-girdle muscular dystrophies due to dysferlin or calpain-3 mutations

might be related to impaired membrane repair and resealing of damaged muscle fibers instead of increased occurrence of membrane damage itself. It will be the future challenge to focus not only on the discovery of new genes involved in muscular dystrophies but also to concentrate on the discovery of molecular mechanisms and how they lead to the wide variety of clinical phenotypes observed. This knowledge will improve future efforts to develop genetic and pharmacological therapeutic interventions.

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