

Structure and function of basement membranes

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ABSTRACT The importance of basement membranes in development and adult tissue function has been inferred from a number of observations. Cells migrate along basement membranes during development, basement membranes are required for the polarization of cells in both the embryo and the adult, and basement membranes serve as substrates for cell adhesion and migration during wound healing and nerve regeneration. The importance of basement membranes in adult tissue function has been directly demonstrated by the genetic diseases caused by mutations in the genes for structural basement membrane components. Examples of such diseases are Alport syndrome and junctional epidermolysis bullosa. Recently, defects in the major laminin variant in muscle, merosin, has been shown to be correlated with muscular dystrophies in man and animals. We are using the dystrophic mutant mouse *dy*, which lacks laminin-2, to analyze the function of laminin-2 in different tissues. Studies of laminin defects in animals and humans are expected to give new information on the function of basement membrane in general and on laminin in particular. Such information may give directions for future diagnosis and treatment of diseases involving basement membranes.

KEY WORDS: *basement membranes, laminins, growth factors, muscular dystrophy, cell adhesion*

Background

Our current research program originated from the hypothesis that basement membranes are spatially and temporally unique. This may seem obvious today, but 10 years ago it was still just a hypothesis. At that time, the structural aspects of extracellular matrices and basement membranes were emphasized over any functional aspects, and so were commonalities over differences. Thus, it was often stated that "basement membranes contain laminin, type IV collagen and heparan sulfate proteoglycan". Today, it is no surprise that laminin and type IV collagen, like so many other extracellular matrix proteins, belong to families of proteins, and that each family member has its distinct expression pattern in tissues. The current assumption is that the expression patterns have a purpose and that each family member has its unique function in the life of an animal. Another thing that has changed dramatically in recent years is the definition of a basement membrane. The definition used to be very strict and was based on the appearance of the basement membrane in the electron microscope as a distinct structure with a lamina lucida and a lamina densa. However, with the realization that the morphology of the basement membrane as observed after strong fixation may be an artefact, the distinction between what a basement membrane is and what is not has become less strict. There is no doubt that the basement membrane zone is a highly specialized extracellular matrix with its unique components, but perhaps it is more variable than we thought. In particular, proteins that are not tradi-

tionally thought of as basement membrane components may be selectively incorporated into basement membranes and confer biologically important qualities to the membranes. Therefore, such "extrinsic" molecules need to be considered in the study of basement membrane structure and function.

Lynn Sakai and I started on a joint effort to discover new molecules of extracellular matrices and basement membranes using monoclonal antibody technology (Hessle *et al.*, 1984). Our projects were indeed successful and led to the identification and characterization of a number of new proteins, in particular fibrillin (Sakai *et al.*, 1986) and merosin/M-laminin/laminin-2 (Leivo and Engvall, 1988)

The importance of basement membranes

The importance of basement membranes in development and adult tissue function has been inferred from a number of observations. For example, cells migrate along basement membranes during development, basement membranes are required for the polarization of cells in both the embryo and the adult, and basement membranes serve as substrates for cell adhesion and migration during wound healing and nerve regeneration (Streuli *et al.*, 1991; Paulsson, 1992; Ekblom, 1993; Kleinman and Schnaper, 1993).

Abbreviations used in this paper: FGF, fibroblast growth factor; HBGAM, heparin-binding growth-associated molecule.

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The importance of basement membranes in adult tissue function has been directly demonstrated by the genetic diseases caused by mutations in the genes for structural basement membrane components. In junctional epidermolysis bullosa (Epstein, 1992; Uitto and Christiano, 1992), patients lack a laminin variant normally present in the skin basement membrane (Verrando *et al.*, 1991; Aberdam *et al.*, 1994; Pulkkinen *et al.*, 1994). Patients with Alport syndrome lack a collagen type IV variant in the kidney (Barker *et al.*, 1990). More recently, defects in the major laminin variant in muscle, merosin, has been shown to be correlated with muscular dystrophies in man and animals (Hayashi *et al.*, 1993; Sunada *et al.*, 1994; Tomé *et al.*, 1994; Xu *et al.*, 1994a,b). Mutations in basement membrane proteins in species other than mammals, including *Drosophila* and *C. elegans*, have also contributed to the concept that basement membranes as structures are essential for organogenesis and/or for the mature function of an organ (Volk *et al.*, 1990; Ishii *et al.*, 1992; Rogalski *et al.*, 1993).

A large body of work *in vitro* with isolated basement membrane components and extracts of basement membranes has shown that the behavior of cells is greatly influenced by these components. Basement membrane components promote cell adhesion via integrins, proteoglycans and lectins, and modulate the *in vitro* phenotype of the cells (Cooper *et al.*, 1991; Mecham, 1991; Ruoslahti, 1991; Hynes 1992; Kibbey *et al.*, 1993). Basement membranes also serve as depositories of growth factors, and may thereby modulate access to, and activity of, such growth factors (Klagsbrun, 1990; Schubert and Kimura, 1991; Thiery and Boyer, 1992; Haralson, 1993). Most recently, several groups have shown that disruption of cell adhesion to extracellular matrix *in vitro* may induce programmed cell death, apoptosis, in the cells (Meredith *et al.*, 1993; Frisch and Francis, 1994; Ruoslahti and Reed, 1994).

Although all the accumulated *in vitro* data have told us that basement membranes have profound effects on cells, it is not possible yet to translate these effects into *in vivo* functions for any of the individual components.

Composition of basement membranes

Intrinsic, structural basement membrane components.

The major structural components of basement membranes are: 1) members of the laminin family; 2) members of the type IV collagen family; and 3) heparan sulfate proteoglycan(s). One should perhaps make the distinction between families of different gene products and families of different splice variants originating from one gene. The variation in the basement membrane components seems to be due predominantly to the expression of different genes. The heparan sulfate proteoglycan, perlecan, is a unique protein (Iozzo, 1994). However, our understanding of this may change. In *C. elegans*, *unc-52*, has been proposed to be the nematode homolog of the mammalian perlecan. However, *unc-52* is only partially homologous to perlecan. The C-terminal domains in perlecan and *unc-52* are different. In perlecan, the C-terminus has repeats in common with laminin α chains, while in *unc-52* the C-terminus is unique and without homology to any other protein known. The genes for perlecan and *unc-52* may have diverged before the divergence of nematodes and mammals. If that is the case, mammals may have a homolog of *unc-*

52 in addition to perlecan. A precedent for such a case has been recently described (Serafini *et al.*, 1994). The product of the *C. elegans* gene, *unc-6*, is a protein which is partially homologous to the laminin β chains. However, the true homolog of *unc-6* in mammals is the protein netrin, which is, like *unc-6*, involved in the guidance of neurons during development.

Extrinsic basement membrane components

Basement membranes are also "banks" (depositories) for extrinsic, sometimes very active molecules such as growth and differentiation factors (Klagsbrun, 1990; Schubert and Kimura, 1991; Thiery and Boyer, 1992; Haralson, 1993). The capture of locally or transiently produced factors by basement membranes could be a very efficient way of rapid regulation of basement membrane activity in development and regeneration. Potentially, all molecules that can bind to heparan sulfate could also bind to perlecan in the basement membrane. In addition there may be molecules that could bind specifically to laminin or type IV collagen. The binding of growth factors to basement membranes has been shown most clearly for FGFs. In the future, it is expected that other growth factors will be found to bind to basement membranes.

We became interested in studying the potential function of small molecular weight heparin binding growth factors as basement membrane components. We decided to work on HBGAM, a novel heparin-binding, growth-associated molecule, which is developmentally regulated (Rauvala, 1989; Merenmies and Rauvala, 1990). HBGAM is an 18 kDa protein which is relatively abundant in the central nervous system at late stages of development. It has been given a number of names, including the name pleiotrophin, referring to its supposedly pleiotrophic effects on cells (Li *et al.*, 1990). HBGAM has been proposed to have growth promoting activity *in vitro* and *in vivo* (Fang *et al.*, 1992; Chauhan *et al.*, 1993). However, we and others have not been able to demonstrate a direct growth promoting effect of HBGAM on cells. Instead, HBGAM may regulate the activity of growth factors in a manner similar to that recently described for platelet factor 4 (Watson *et al.*, 1994). Ironically, HBGAM appears to be expressed predominantly at sites where there are no or few structurally identifiable basement membranes. We have therefore been unable yet to test the hypothesis of a basement membrane-associated function for this heparin binding growth factor.

Cell surface receptors for basement membrane components may also qualify as belonging to the basement membrane zone, e.g. the integrin $\alpha 6 \beta 4$. Integrin $\alpha 6 \beta 4$ was one of the potential tissue-specific basement membrane components that we identified (Hessle *et al.*, 1984). In skin $\alpha 6 \beta 4$ is strictly localized to the basement membrane, i.e. the apical surface of the basal keratinocytes. Other keratinocyte integrins are localized also to cell-cell junctions and to suprabasal layers of more differentiated keratinocytes.

Structure and properties of laminins

Laminins are a family of proteins composed of a heavy chain, now called α (for new nomenclature on laminins see Burgeson *et al.*, 1994) and two similar but not identical lighter chains, β and γ (Sasaki *et al.*, 1988; Engel, 1992; Engvall, 1993). The "classical" laminin from the mouse EHS tumor has the subunit compo-

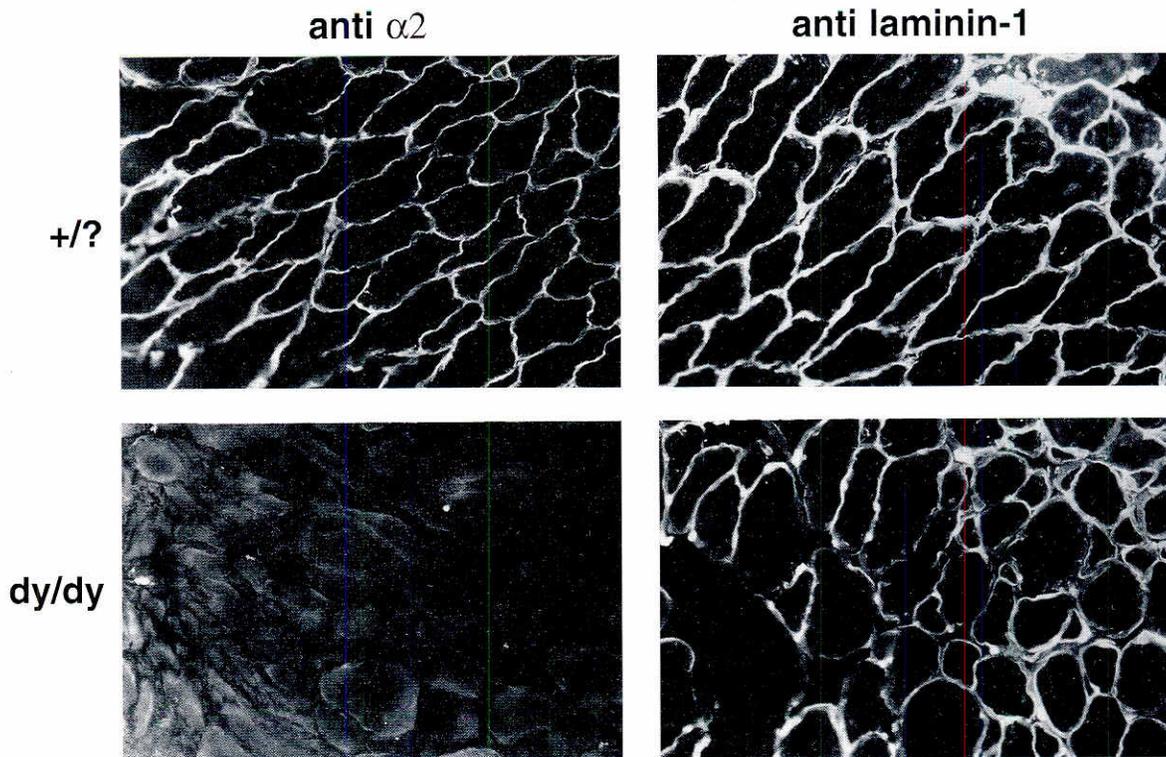


Fig. 1. Detection of laminin in skeletal muscle from normal and dystrophic *dy* mice by indirect immunofluorescence. Laminin-2 cannot be detected in muscle of homozygous *dy/dy* mice when using antibodies specific for the laminin $\alpha 2$ chain. A polyspecific antiserum reacting with several laminin variants detects an unknown variant of laminin in the *dy* muscle basement membrane.

sition $\alpha 1-\beta 1-\gamma 1$. Several additional subunits of the laminin family have been discovered from cDNA cloning or characterization of extracellular matrix from tissues or cultured cells. In our search for tissue-specific basement membrane components, we discovered the $\alpha 2$ chain, which is a homolog of the classical $\alpha 1$ chain and the heavy chain of merosin. Merosin is then the collective name for all laminins with an $\alpha 2$ chain (Leivo and Engvall, 1988; Ehrig *et al.*, 1990). The $\alpha 2$ chain is most prominent in placenta, striated muscle and peripheral nerve. Sanes' laboratory (Hunter *et al.*, 1989) identified the laminin "S" chain ($\beta 2$) as a polypeptide associated with synapses in skeletal muscle. K-laminin (laminin-6) and kalinin (laminin-5) were discovered as proteins characteristic of skin basement membranes and synthesized by keratinocytes (Carter *et al.*, 1991; Kallunki *et al.*, 1992; Marinkovich *et al.*, 1992). The various subunits in the family are expressed in different cell types and at different times during development. Since laminins are heterotrimeric molecules, a number of different laminins may be assembled from the already known subunits and perhaps more subunits and combinations may become known in the future.

The different subunits in laminins are all homologous, except that the heavy chains have an extra domain at the C-terminus, named the G domain. The G domain in laminin $\alpha 1$ chain consists of five repeats of 150-200 amino acids. The G type repeats were first identified in the laminin $\alpha 1$ chain (Sasaki *et al.*, 1988) and subsequently served to recognize "merosin" (M-laminin/laminin-2) as a laminin $\alpha 2$ chain homolog (Ehrig *et al.*, 1990). The G

motif is also present in several other proteins, including perlecan, agrin, neurexins, and the two small plasma proteins, sex hormone binding protein and protein S (Rupp *et al.*, 1991; Joseph and Baker, 1992; Kallunki and Tryggvason, 1992; Patthy, 1992; Ushkaryov *et al.*, 1992). It may be interesting to note that the different α chains vary in the G domain. The last two G repeats are easily cleaved off in both the $\alpha 1$ and $\alpha 2$ chains, generating from the $\alpha 1$ chain the well characterized E3 fragment. In the $\alpha 2$ chain, the G repeats 3-5 are found in tissues as a fragment under apparent physiological condition. The $\alpha 3$ chain of laminin-5 is similarly processed. It is not clear yet what the variation in the G domain of different α chains means in terms of the function of the different laminins. The globular domains at the ends of the three short arms of laminin, which are the N-termini of the three subunits, are involved in the assembly of laminin in basement membranes (Yurchenko and Schittny, 1990). The short arms are truncated in laminin-5, which may then not incorporate into a basement membrane by the same mechanism as the other laminin variants.

Laminin is the basement membrane component with the most striking effects on cells *in vitro*. Binding of cells to laminin promotes their migration or neurite outgrowth, and changes their pattern of gene expression and state of differentiation (Reichardt and Tomaselli, 1991; Streuli *et al.*, 1991; Ekblom, 1993; Kleinman and Schnaper, 1993). It can be expected that these activities of laminin could be utilized in the development of therapeutically active compounds. In this regard, a number of labo-

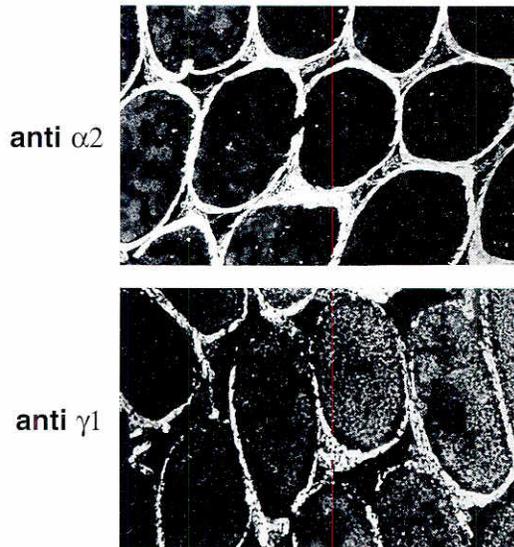


Fig. 2. Presence of laminin-2 in the seminiferous basement membrane of the adult mouse testis. Sections have been stained by indirect immunofluorescence with antibodies specific for laminin chains $\alpha 2$ and $\gamma 1$.

ratories have reported on synthetic peptides mimicking one or another laminin activity. However, the physiological importance of the sites in laminin that these peptides represent and the utility of the peptides is not clear.

Basement membranes are thought to have a very slow turnover. However, this may not be true for all basement membranes and/or for all basement membrane components. The transient expression of merosin in the rat liver during development and regeneration (Wewer *et al.*, 1992) shows that this laminin may have a quite rapid turnover. In fact, one of the functions of merosin may be to allow for more plasticity of a basement membrane by being metabolized more rapidly. This flexibility of the basement membrane may be important in active tissues such as skeletal and heart muscle. On the other hand, a consequence of rapid turnover of merosin may be that any reduction in the amounts of laminin synthesized may make muscle basement membrane more sensitive to force-generated injuries.

Laminin-2 is reduced in patients with Fukuyama congenital muscular dystrophy and absent in certain other muscular dystrophies

A study on the composition of basement membrane components in the muscle of patients with various myopathies showed that patients with the Fukuyama congenital muscular dystrophy (FCMD) had greatly reduced levels of the laminin $\alpha 2$ chain in their skeletal muscle and peripheral nerve as detected by indirect immunofluorescence (Hayashi *et al.*, 1993). Toda *et al.* (1993) performed linkage analysis in FCMD patients and located the mutated gene to chromosome 9. However, the $\alpha 2$ chain gene is located on chromosome 6 (Vuolteenaho *et al.*, 1994). Therefore, the defect in laminin $\alpha 2$ may not be the primary defect in FCMD. The low levels of merosin may be the consequence of

the lack of another protein, perhaps one which is involved in the expression, assembly, localization, and/or attachment to merosin. The identity of the chromosome 9 FCMD gene is likely to be known in the near future, and it will then be possible to study the association of its product with merosin.

There is a correlation of merosin deficiency with muscular dystrophy also in other types of muscular dystrophies. In one study, merosin was completely absent from the muscle basement membrane in 13 out of 30 cases of congenital muscular dystrophy (CMD) as measured by immunofluorescence (Tomé *et al.*, 1994) and this finding is being confirmed by several groups. It is possible that laminin defects may characterize a large group of previously unclassified muscular dystrophies. Indeed, the autosomal recessive nature of CMDs suggests that mutations in the laminin $\alpha 2$ chain gene may not be uncommon.

Laminin $\alpha 2$ chain is absent in the dystrophic mouse *dy* and is truncated in *dy*²

We have recently shown that mutations in the $\alpha 2$ chain of merosin may indeed be the direct cause of muscular dystrophy. We analyzed two spontaneous mutations in mice and were able to identify a mutation in the laminin $\alpha 2$ gene in one of them. The first mouse mutant, *dy*, appeared spontaneously in a colony of 129/ReJ mice in the 50s (Michelson *et al.*, 1955). The mutation has been mapped to mouse chromosome 10. The *dy* mouse has a severe form of progressive muscular dystrophy with symptoms noticeable at around 3 weeks of age. The *dy* mouse was used as a model of human muscular dystrophy, particularly before the molecular defect in the most common Duchenne/Becker muscular dystrophies in human became known as defects in the cytoskeletal protein dystrophin (Hoffman *et al.*, 1987). The *dy* mouse was then largely replaced by the dystrophin-deficient *mdx* mouse as a model (Sicinski *et al.*, 1989). Now it appears that the *dy* mouse will again be useful as an animal model for CMDs where merosin is defective.

The adult homozygous *dy/dy* mouse lacks the $\alpha 2$ chain of merosin in skeletal muscle (Fig. 1), heart muscle, peripheral nerve, and in the intestinal crypts as measured by immunofluorescence and immunoblotting (Simon-Assman *et al.*, 1994; Xu *et al.*, 1994a,b). We could detect a laminin $\alpha 2$ chain message of normal size in muscle, but the message was present at very low levels. It is possible that a stop mutation in the coding region of the gene affects the stability of the mRNA. Another possibility is that a mutation in a promoter region affects the level of expression of mRNA. We could not detect any gross abnormality in the $\alpha 2$ chain gene by Southern blotting after digestion of genomic DNA with a number of restriction enzymes. Although we were not able to identify the mutation in the *dy* mouse, it seemed likely that the *dy* mutation is a mutation in the $\alpha 2$ chain gene. In fact, the *dy* mutation maps closely to the utrophin gene on mouse chromosome 10 (Buckle *et al.*, 1990). This region of mouse chromosome 10 is homologous to human chromosome 6, where the genes for $\alpha 2$ chain and utrophin also map very closely (Buckle *et al.*, 1990; Vuolteenaho *et al.*, 1994). Sunada *et al.* (1994) have independently made similar observations.

Analysis of another independent but allelic spontaneous mutation in the mouse, *dy*², provided the proofs for the direct

causal relationship between mutations in laminin $\alpha 2$ and muscular dystrophy (Xu *et al.*, 1994 b). The dy^2 mouse has a slightly milder phenotype than the dy mouse and expressed a truncated laminin $\alpha 2$ chain. The truncation is caused by abnormal splicing of mRNA and affects the N-terminal region of the $\alpha 2$ chain. The mutation is not expected to affect the synthesis and secretion of merosin, but may impair the assembly of the mutated merosin into basement membranes and/or make the protein more sensitive to proteolysis.

Defects in muscle attachment proteins may result in abnormalities outside the muscle, depending on the normal tissue distribution of the proteins. For example, there are indications that patients with Duchenne muscular dystrophy are slightly mentally retarded, perhaps as a consequence of dystrophin having an important function in the central nervous system. Similarly, the merosin deficient mice dy and dy^2 have a number of extramuscular phenotypes that may be the result of the lack of merosin at sites where laminin-2 is normally expressed (Leivo and Engvall, 1988; Engvall *et al.*, 1990; Sanes *et al.*, 1990; Vuolteenaho *et al.*, 1994). An example may be the correlation between the normal presence of merosin in the mature testis and the poor breeding performance of the $\alpha 2$ chain-deficient dy/dy mice (Fig. 2). Interestingly, the dy^2 mouse mutant is able to breed. This suggests that the truncated $\alpha 2$ chain in the dy^2 mouse, although poorly functional in muscle, may be at least partially functional in the testis.

Applications of laminin in human medicine

The immediate application of the recent findings on merosin in muscular dystrophy may be the possibility of diagnosis including prenatal diagnosis.

Molecular classifications of muscular dystrophies

Currently, only the Duchenne/Becker X-linked dystrophy can be identified molecularly and genetically as a subgroup among an unknown number of different types of muscular dystrophies. It would be valuable to be able to classify these dystrophies based on the identity of the protein affected. Such classification could have diagnostic and prognostic value. The findings of merosin deficiencies in muscular dystrophy will allow us to identify such cases by simple immunohistochemistry with antibodies to the $\alpha 2$ chain. This will then allow for the follow-up of this particular subgroup of CMD. Data from such studies may inspire new directions for therapy. Furthermore, among the CMDs not showing merosin defects, there may be cases caused by defects in other attachment proteins along the pathway that may link merosin extracellularly to dystrophin intracellularly. Perhaps all CMDs are somehow related to defects in the merosin to dystrophin pathway, similar to epidermolysis bullosa being caused by defects in the kalinin/K-laminin to keratin pathway (Epstein, 1992; Uitto and Christiano, 1992; Aberdam *et al.*, 1994; Pulkkinen *et al.*, 1994).

Prenatal diagnosis of muscular dystrophy

Merosin is expressed very early in human placenta, while it may be expressed later in other tissues. The early expression in placenta makes it possible to detect any defects in merosin at an early stage of pregnancy. Families who have already had a child

with merosin deficiency may thus be offered the possibility of prenatal testing to prevent the birth of another affected baby. It is possible that such prenatal diagnosis could be used for other muscular dystrophies, as long as the protein which is defective in these diseases is normally expressed in placenta or in fetal membranes.

Future prospects in research on basement membrane structure and function

The discovery of the merosin-deficient mouse (Xu *et al.*, 1994a) has resulted in the first animal model in which one may study various aspects of laminin function *in vivo*. The dy mouse has a number of extramuscular phenotypes that may depend on the absence of merosin in tissues where it normally occurs. As mentioned above, the homozygous dy/dy mice do not reproduce. This may depend less on muscle weakness than on the requirement for merosin in the normal seminiferous basement membrane of the testis of the sexually mature mouse. The dy homozygous mouse is frail and dies early from unknown causes. Merosin is normally present in the thymus, pituitary, thyroid, and intestine (Beaulieu and Vachon, 1994; Chang *et al.*, 1993; Farnoud *et al.*, 1994; Simon-Assman *et al.*, 1994), and the lack of merosin in these tissues in the dy mouse could be a cause of extramuscular organ malfunction and contribute to its early death.

We expect that work on spontaneous and experimental mutant mice will tell us a great deal about the function of the laminins and other basement membrane proteins in the future. A limitation of these studies is that one may primarily study the defect that gives the predominant phenotype, as is demonstrated by the dy mouse, which has degenerating muscle as its most prominent problem. Problems in other organs can only be studied if the dominant phenotype is corrected. This may be accomplished by creating transgenic mice in which the mutation is corrected in one or a few organs. These are some of the many questions about laminin function in general and function of merosin in particular that can now be addressed.

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