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A Role for Dystroglycan in Basement Membrane Assembly

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Summary

Basement membranes are composed of ordered arrays of characteristic extracellular matrix proteins, but little is known about the assembly of these structures in vivo. We have investigated the function of dystroglycan, a cell-surface laminin receptor expressed by cells contacting basement membranes in developing and adult tissues. We find that dystroglycan is required for the formation of a basement membrane in embryoid bodies. Our results further indicate that dystroglycan–laminin interactions are prerequisite for the deposition of other basement membrane proteins. Dystroglycan may exert its influence on basement membrane assembly by binding soluble laminin and organizing it on the cell surface. These data establish a role for dystroglycan in the assembly of basement membranes and suggest fundamental mechanisms underlying this process.

Introduction

Basement membranes, or basal laminae, are specialized extracellular matrices that are fundamental elements of metazoan tissues. They are composed, in part, of a characteristic set of large extracellular glycoproteins—laminins, entactin/nidogen, perlecan, and type IV collagen (for review, Timpl and Brown, 1996)—which lie in direct contact with cell surfaces. These molecules are typically arranged in an ordered fashion, exhibiting an analogous, though not identical, sheet-like ultrastructure in tissues (Vracko, 1974). The diverse and dynamic physiological roles of basement membranes are determined by the nature of their molecular constituents, their architecture, and cellular interactions with these elements (see references in Yurchenco, 1994; Ekblom et al., 1996; Timpl and Brown, 1996). A number of basement membrane proteins interact with one another. Most notably, at least some isoforms of type IV collagen (Yurchenco and Furthmayr, 1984) and laminin (Yurchenco et al., 1985; Cheng et al., 1997) can self-assemble into lattice-like polymeric networks in physiological buffers, which resemble laminin and type IV collagen matrices in vivo (for review, Yurchenco, 1994). Entactin/nidogen is able to link laminin-1 and type IV collagen into a ternary complex (Fox et al., 1991), and perlecan binds to entactin/nidogen and laminin (Battaglia et al., 1992).

Although interactions among these key proteins are likely to be important factors governing basement membrane assembly, alone they do not easily explain how this process occurs in vivo. Since basement membrane proteins are secreted and thus may diffuse into the extracellular milieu, mechanisms must be in place to regulate local concentrations of basement membrane proteins. Moreover, during development basement membranes form specifically beneath epithelial cells, but in some cases, their constituent proteins may be synthesized by either the epithelial or mesenchymal compartments (Thomas and Dziadek, 1993; Ekblom et al., 1994). Also, in *Caenorhabditis elegans*, type IV collagen assembles on tissues that do not express it (Graham et al., 1997). These spatial disparities between the sites of basement membrane protein synthesis and ultimate deposition argue strongly that basement membrane assembly is mediated by cell-surface receptors.

We have been studying dystroglycan (DG), a cell-surface laminin receptor. DG was first biochemically isolated from, and characterized in, skeletal muscle where it is a central component of the dystrophin–glycoprotein complex (Ervasti et al., 1990). Within this complex, the α and β subunits of DG form, respectively, a molecular linkage between laminin-2, in the muscle basement membrane, and dystrophin, which associates with the actin cytoskeleton (Ervasti and Campbell, 1993). α-DG binds to the carboxy-terminal G domains on the laminin α1 chain (Gee et al., 1993). These domains are present and relatively well conserved in all five known laminin α chains, and thus DG has a potential binding site on all known laminin heterotrimers. Furthermore, DG binds G domain–like sequences in other extracellular matrix molecules like agrin (Gesemann et al., 1996) and possibly perlecan (Peng et al., 1998). DG is expressed in many developing and adult nonmuscle tissues, typically in cell types that adjoin basement membranes, such as epithelial and neural tissue (Matsumura et al., 1993; Durbeej et al., 1998). Earlier work has shown that DG is involved in kidney epithelial morphogenesis (Durbeej et al., 1995). As a part of our continuing efforts to understand DG function, we disrupted its gene in the mouse (Williamson et al., 1997). DG-null embryos failed to progress beyond the early egg cylinder stage of development and were characterized by structural and functional perturbations of Reichert's membrane, one of the earliest basement membranes that form in the rodent embryo. These data indicated that DG is necessary for the development of Reichert's membrane and suggested that it might generally be important for the assembly of basement membranes.

Here, we have further characterized the function of DG using a genetic approach. In DG-null embryoid bodies, there was a dramatic and severe disruption of the prominent subendodermal basement membrane as demonstrated by light and electron microscopy analyses. Further evidence that DG promotes the assembly of a laminin matrix comes from the analysis of embryonic stem (ES) cells where it is required for the deposition of laminin on their surfaces. Finally, the mutant phenotypes observed in both the DG-null embryoid bodies and ES

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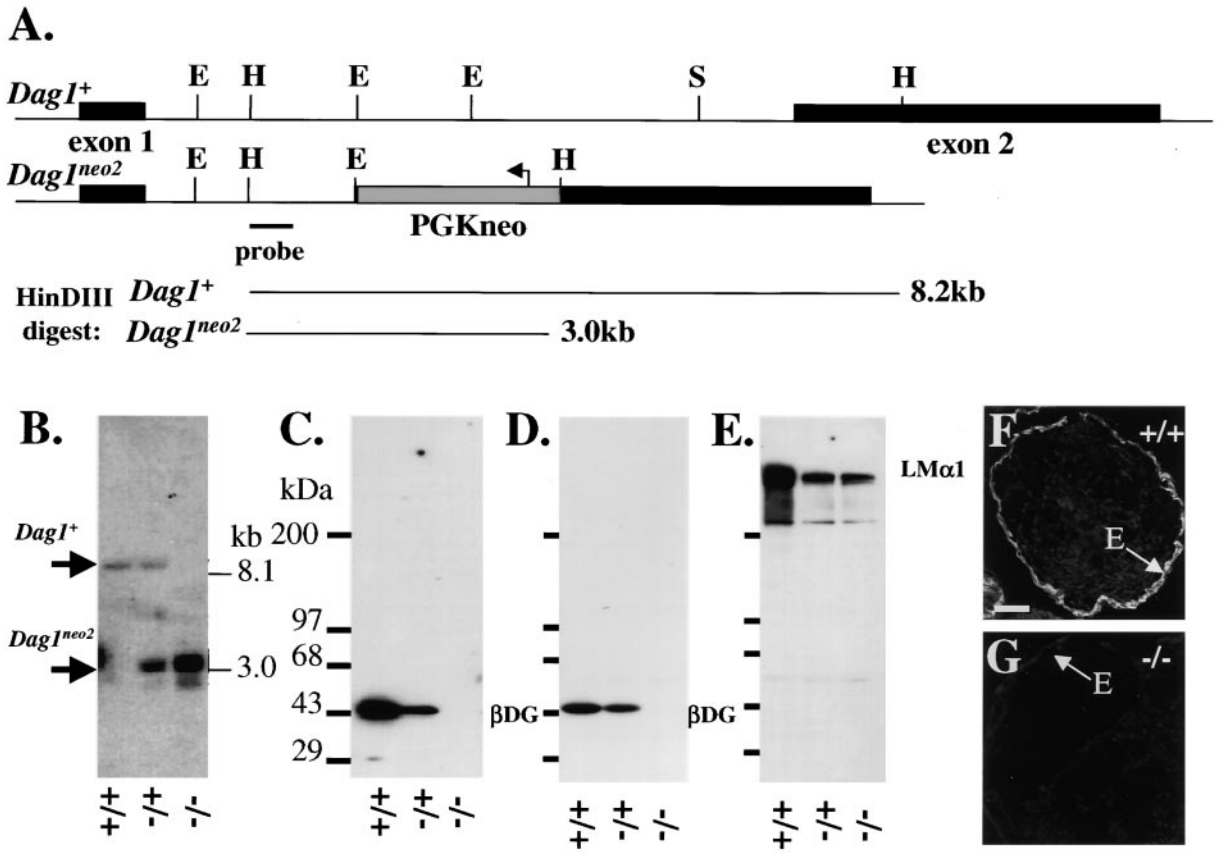

Figure 1. Generation of DG$^{-/-}$ ES Cells and Embryoid Bodies

(A) Schematic map of the wild-type (*Dag1*$^{+}$) and DG-null mutant (*Dag1*^{neo2}) alleles and the predicted fragment sizes from each after HindIII digestion. E, EcoRI; H, HindIII; S, SalI. (B) Representative Southern blot analysis of HindIII-digested DNA from wild-type (+/+), heterozygous (+/−), and homozygous-null (−/−) mutant ES cell lines showing loss of the 8.2 kb wild-type fragment in a DG$^{-/-}$ cell line. Western blot analysis of ES cell (C) and embryoid body (D) extracts probed for β-DG, and embryoid body extracts probed for laminin α1 (E). MW standards are indicated at the left of each blot. α-DG is localized to the outer endodermal layer (denoted by "E") in wild-type (F), but not DG$^{-/-}$ embryoid bodies (G). Bar, 50 μm.

cells are rescued by restoration of DG expression. Taken together, these results demonstrate that DG is necessary for the formation of a basement membrane and that it fulfills this role by, perhaps, nucleating the assembly of a laminin matrix on the cell surface.

Results

Generation of Dystroglycan-Null Embryonic Stem Cells and Embryoid Bodies

In an effort to explore further the biological role of DG, we generated DG-null (DG$^{-/-}$) ES cells from existing heterozygous (DG$^{+/-}$) ES clones (Williamson et al., 1997). Figure 1 shows the gene disruption strategy and characterization of mutant ES cell lines. Despite the fact that DG is expressed in ES cells, we have observed no overt differences in the growth characteristics or cell morphology of undifferentiated DG$^{-/-}$ ES cell lines (data not shown). With these cells, we generated embryoid bodies from DG$^{-/-}$ and control ES cell lines. DG was expressed by the outer layer of endoderm (Figures 1D and 1F) and in cardiomyocytes that develop within the core of the control embryoid bodies (see Figure 2F for example), but, as expected, it was not expressed in DG$^{-/-}$ embryoid bodies (Figure 1G). DG$^{-/-}$ ES cells generated embryoid bodies that possessed several characteristic features, including an outer layer of differentiated endoderm and spontaneously contracting cardiomyocytes in their core (Doetschman et al., 1985). The overall growth characteristics of DG$^{-/-}$ embryoid bodies were similar to wild-type and DG$^{+/-}$ controls. The gross morphology and histology of DG$^{-/-}$ embryoid bodies were essentially indistinguishable from controls (Figures 2A–2D). A similar proportion (~1%) of control and DG$^{-/-}$ embryoid bodies formed large fluid-filled cysts (Figures 2C and 2D), consistent with the presence of differentiated visceral endoderm. Occasionally, we noted that the endodermal layer was separated from the underlying cells (see Figure 2C for example). Although this was observed in embryoid bodies regardless of genotype, we noted it more frequently in DG$^{-/-}$ embryoid bodies (see Figure 3K for example). Finally, DG$^{-/-}$ embryoid bodies generated spontaneously contracting cardiomyocytes, visually apparent as rhythmically beating embryoid bodies, in comparable proportions (~30%) and kinetics (by 8 days in culture) as wild-type and DG$^{+/-}$ controls. Consistent with these observations, we detected molecular markers for endoderm (Figures 2E and

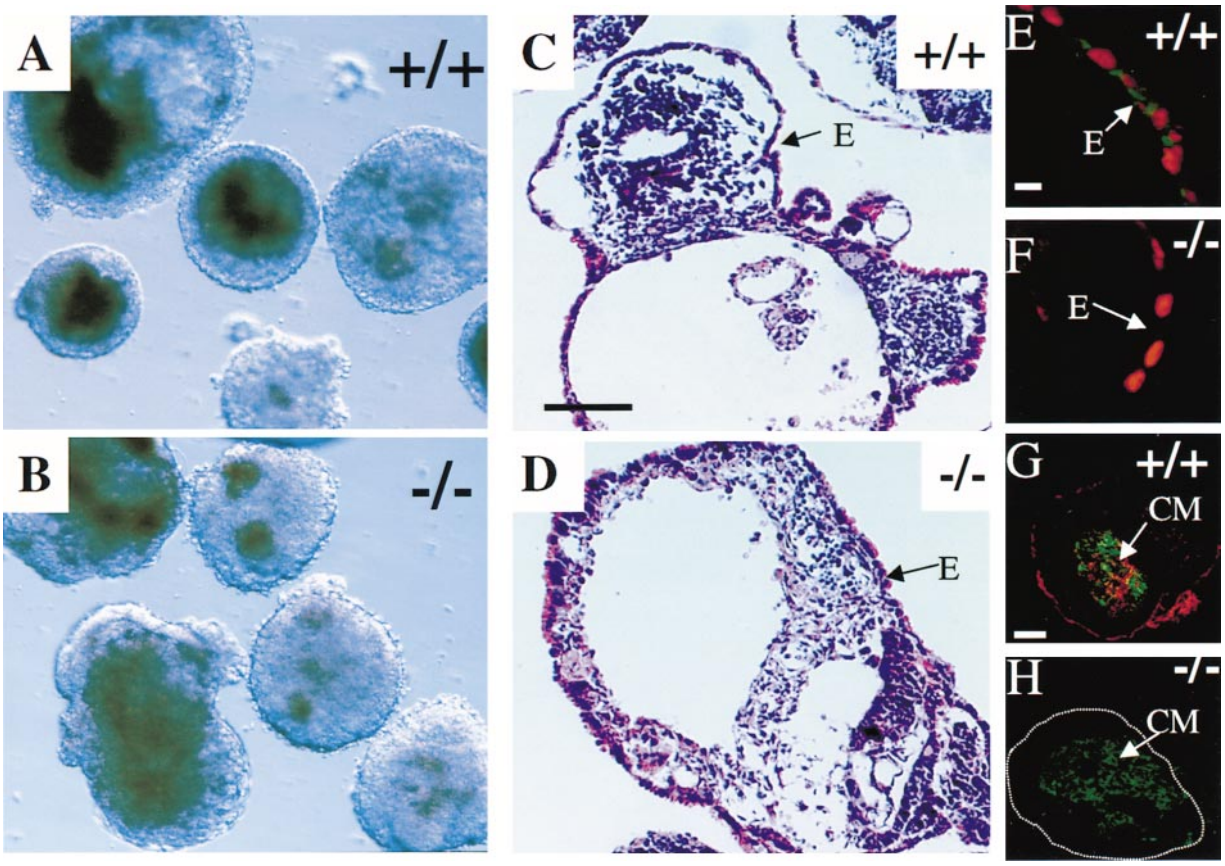

Figure 2. Morphology and Histology of Wild-Type and DG$^{-/-}$ Embryoid Bodies

(A and B) Hoffman modulated contrast images of wild-type and DG$^{-/-}$ 10-day-old embryoid bodies.

(C and D) Periodic acid-Schiff stained sections from cystic 10-day-old embryoid bodies. Endoderm is indicated by "E".

(E and F) Double-label immunofluorescence analysis of α-DG (green) and Pem (red).

(G and H) Double-label immunofluorescence analysis of β-DG (red) and cardiac tropomyosin (green). Dotted line in (H) indicates the margin of the embryoid body. Note also that α-DG (E) and β-DG (F) yield identical staining patterns in the endoderm and cardiomyocytes of embryoid bodies. Bars: (C), 100 μm for (C) and (D); (E), 10 μm for (E) and (F); (G), 50 μm for (G) and (H).

2F) and cardiomyocytes (Figures 2G and 2H) in both control and DG$^{-/-}$ embryoid bodies. Therefore, DG$^{-/-}$ ES cells are able to generate embryoid bodies containing functionally differentiated endoderm and cardiomyocytes—the two cell types in which DG is expressed in embryoid bodies.

Basement Membrane Disruption in Dystroglycan-Null Embryoid Bodies

Although certain aspects of embryoid body development appeared normal without DG expression, given our previous results with DG$^{-/-}$ mutant mouse embryos, we looked more closely at the elaboration of a particular basement membrane in the mutant embryoid bodies. Typically, embryoid bodies develop a basement membrane subjacent to the outer layer of endoderm (Martin et al., 1977; Doetschman et al., 1985). Well-formed endodermal layers comprise a morphologically distinct feature of embryoid bodies in cross section (Figures 3C, 3G, and 3K). We examined the subendodermal basement membrane in DG$^{-/-}$ and control embryoid bodies by immunostaining with antibodies directed against various basement membrane proteins. In control embryoid bodies (wild-type and DG$^{+/-}$), laminin α1 is colocalized with DG on the basal surfaces of endodermal cells, showing a typical sheet-like basement membrane pattern (Figures 3A–3H). Other laminin α1 immunoreactivity was detected in the core of the embryoid bodies as small, irregular patches, appearing inside and outside of cells, but DG was not colocalized with this patchy laminin staining except where it overlapped with cardiomyocytes. In marked contrast, the subendodermal basement membrane staining pattern of laminin α1 was grossly disrupted in DG$^{-/-}$ embryoid bodies, instead only appearing as the patchy deposits described above (Figures 3I–3L). In a minority of DG$^{-/-}$ embryoid bodies, we could detect subendodermal staining of laminin α1 similar to control cultures; however, the ultrastructural analysis described below indicates that this is not organized into a basement membrane structure. Similar results were obtained using antisera reacting with the laminin β1 and γ1 chains, arguing that the laminin α1–specific antibody detected the entire laminin-1 heterotrimer (data not shown). Interestingly, the disruption of basement membrane molecules in DG$^{-/-}$ embryoid bodies extended beyond DG's direct binding partner laminin-1. In wild-type and DG$^{+/-}$ embryoid bodies, type IV collagen and perlecan localized to the subendodermal

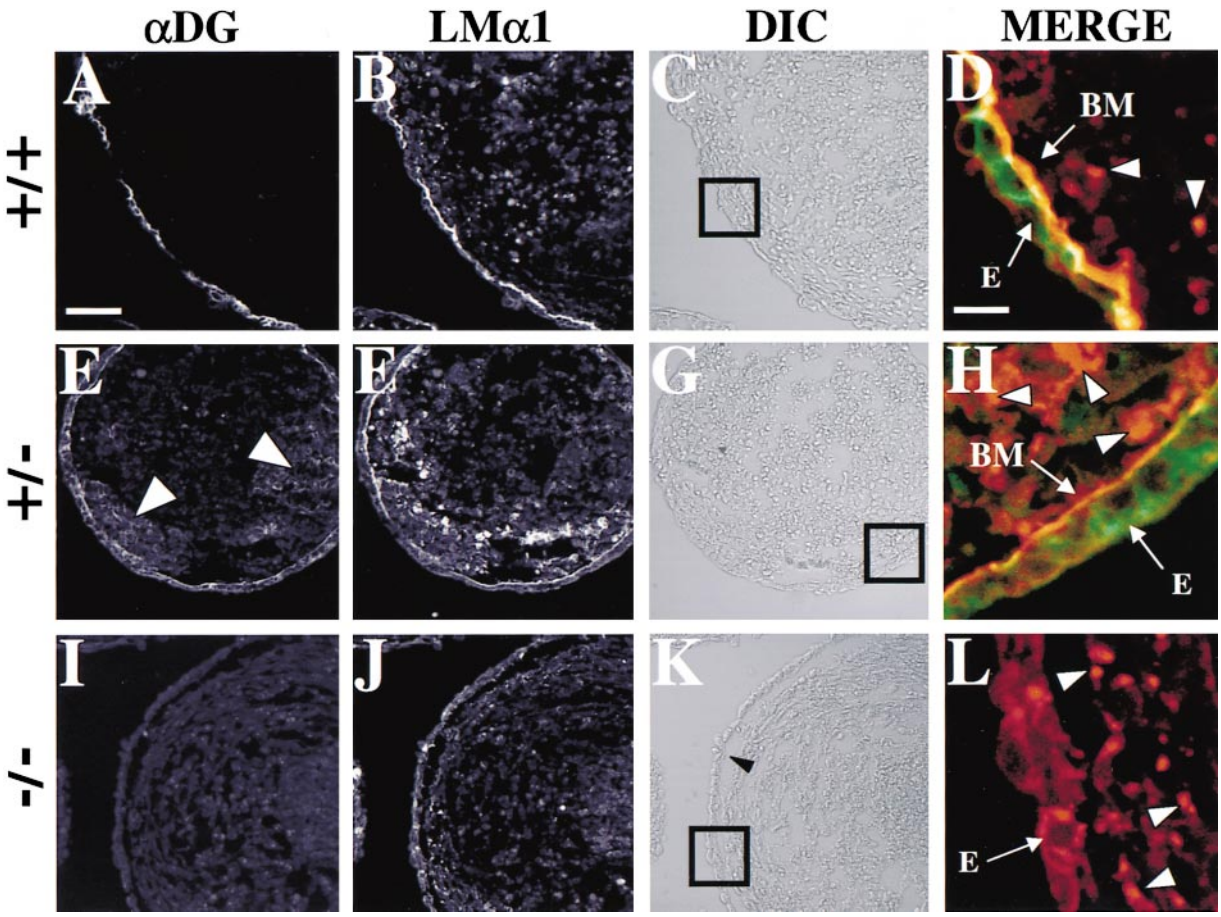

Figure 3. Disruption of Laminin-1 Localization in $DG^{-/-}$ Embryoid Bodies

Sections of wild-type, $DG^{+/-}$, and $DG^{-/-}$ 10-day-old embryoid bodies stained with antibodies specific for α-DG (αDG, green channel) and the laminin α1 chain (LMα1, red channel) are shown. Paired single-channel fluorescence images are shown in (A), (B), (E), (F), (I), and (J). Corresponding DIC images are shown in (C), (G), and (K). Boxed regions in the DIC images are shown at higher magnification as merged images (D, H, and L), respectively. Endodermal layer (E) and its subjacent basement membrane (BM) are indicated. Arrows in (D), (H), and (L) point to patches of laminin staining in the core of the embryoid bodies. Arrows in (E) indicate α-DG expression in association with cardiomyocytes in the core of the embryoid bodies. Arrow in (K) denotes separation between the endodermal layer and underlying cells. We note that the gain settings used to collect the images in (A), (B), and (D) were set lower to give comparable signal intensity to the heterozygote and DG-null images. Bars: (A), 50 μm for (A)–(C), (E)–(G), and (I)–(K); (D), 10 μm for (D), (H), and (L).

basement membrane and patchy deposits in the cores like laminin-1 (Figures 4A–4D). Similarly, the subendodermal basement membrane localization of these molecules was disrupted in $DG^{-/-}$ embryoid bodies (Figures 4E and 4F). To confirm our immunofluorescence data and demonstrate that failed basement membrane formation in $DG^{-/-}$ embryoid bodies was not a secondary consequence of a lack of expression of laminin-1, we performed Western blot analysis. This demonstrated that laminin α1 is expressed in $DG^{-/-}$ embryoid bodies (Figure 1E). The laminin α1 expression levels in the $DG^{+/-}$ and $DG^{-/-}$ embryoid bodies were lower than the wild-type control, but comparable to each other. The basis for this difference in laminin expression between wild-type and derivative embryoid bodies is not yet understood. However, despite the difference in the steady-state level of laminin α1 expression, $DG^{+/-}$ embryoid bodies elaborate well-defined subendodermal basement membranes similar to wild-type embryoid bodies. Since $DG^{+/-}$ and $DG^{-/-}$ embryoid bodies exhibited comparable levels of laminin α1, the failure of basement membrane formation in $DG^{-/-}$ embryoid bodies cannot be accounted for by reduced expression of laminin α1. Together, these results show that $DG^{-/-}$ embryoid bodies have a gross disruption in the localization pattern of several important basement membrane structural proteins.

To examine this further, we analyzed ultrastructure in the embryoid bodies by electron microscopy. Figure 5 shows electron micrographs of endodermal cells in control and $DG^{-/-}$ embryoid bodies. At lower magnifications, microvilli can be seen protruding from the apical surfaces of the endodermal cells (Figures 5A and 5E). In other sections, we could see that tight junctions had formed between endodermal cells in both control and $DG^{-/-}$ embryoid bodies (data not shown). This indicates that the endodermal cells are able to generate a polarized morphology in the absence of DG. In extracellular

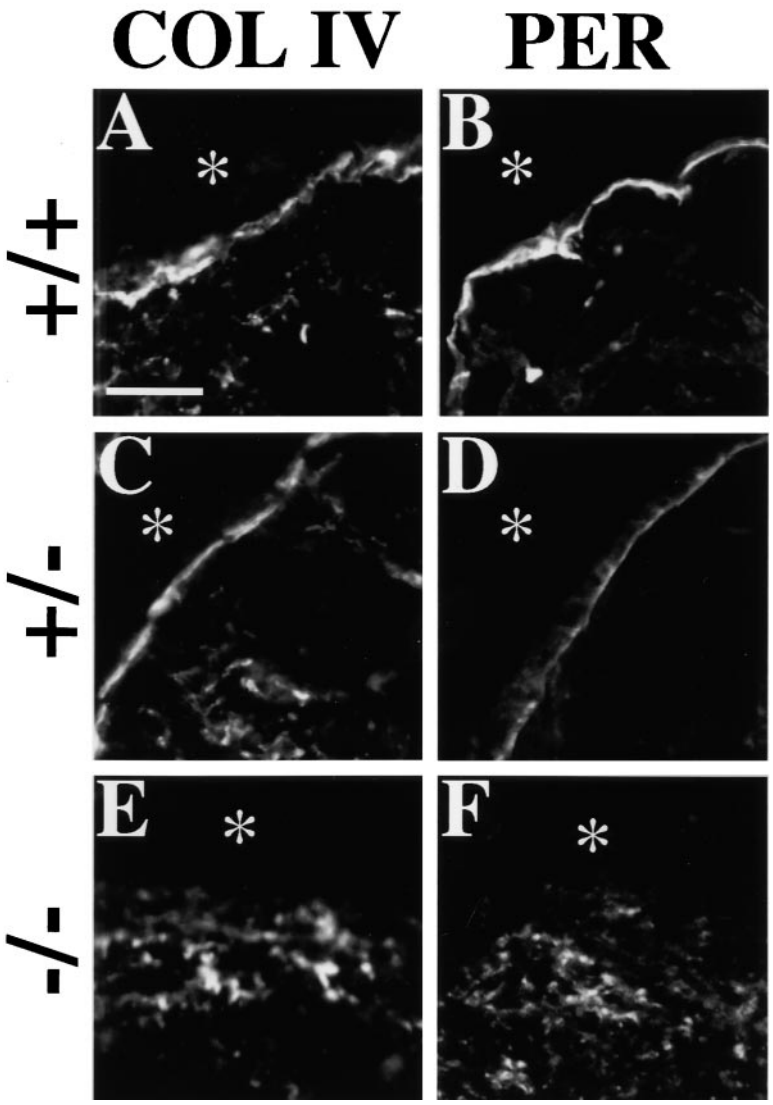

Figure 4. Disruption of Type IV Collagen and Perlecan in DG$^{-/-}$ Embryoid Bodies

Sections of 10-day-old wild-type (A and B), DG$^{+/-}$ (C and D), and DG$^{-/-}$ (E and F) embryoid bodies stained for type IV collagen (A, C, and E) and perlecan (B, D, and F). The subendodermal basement membrane is seen as the intensely stained curve running through the field in (A)–(D). The asterisk indicates the outside of the embryoid body.

spaces beneath the endodermal cell layer in control embryoid bodies, we detected basement membranes associated with the basal surfaces of the endodermal cells (Figures 5B–5D). These basement membranes displayed the characteristic lamina lucida/lamina densa morphology and ran parallel with the plasma membrane, maintaining a relatively uniform distance from it. In some sections, we could see fibrillar collagen making contact with the subendodermal basement membrane (data not shown). In contrast, inspection of the DG$^{-/-}$ embryoid bodies revealed that the subendodermal basement membranes were completely absent (Figures 5F–5H). Instead, the endodermal cells appeared to rest upon a fibrous matrix (Figure 5, black arrowheads). A similar fibrous matrix was detected underneath the basement membrane of control embryoid bodies. However, in control embryoid bodies, the basement membrane separated the endodermal plasma membrane from the fibrous matrix, whereas in DG$^{-/-}$ mutant embryoid bodies, the endodermal plasma membrane appeared to directly connect with the fibrous matrix (Figures 5F and 5G). Another feature of the subendodermal compartment in DG$^{-/-}$ embryoid bodies is that we observed punctate deposits of electron-dense material that might represent aggregated basement membrane proteins (Figure 4, white arrowheads). We did not detect subendodermal basement membranes in any of the DG$^{-/-}$ embryoid bodies examined (n = 24 from independent experiments). In sum, the ultrastructural analysis revealed that DG$^{-/-}$ endodermal cells exhibit a polarized morphology, but do not form a basement membrane on their basal surface, in agreement with our light microscopy findings.

Dystroglycan Is Required for the Formation of Laminin-1 Clusters on the Surface of Embryonic Stem Cells

The foregoing results demonstrated that DG is required for the formation of the subendodermal basement membrane in embryoid bodies, and they suggested that DG–laminin interactions are critical for the deposition of other basement membrane molecules. Thus, a DG–

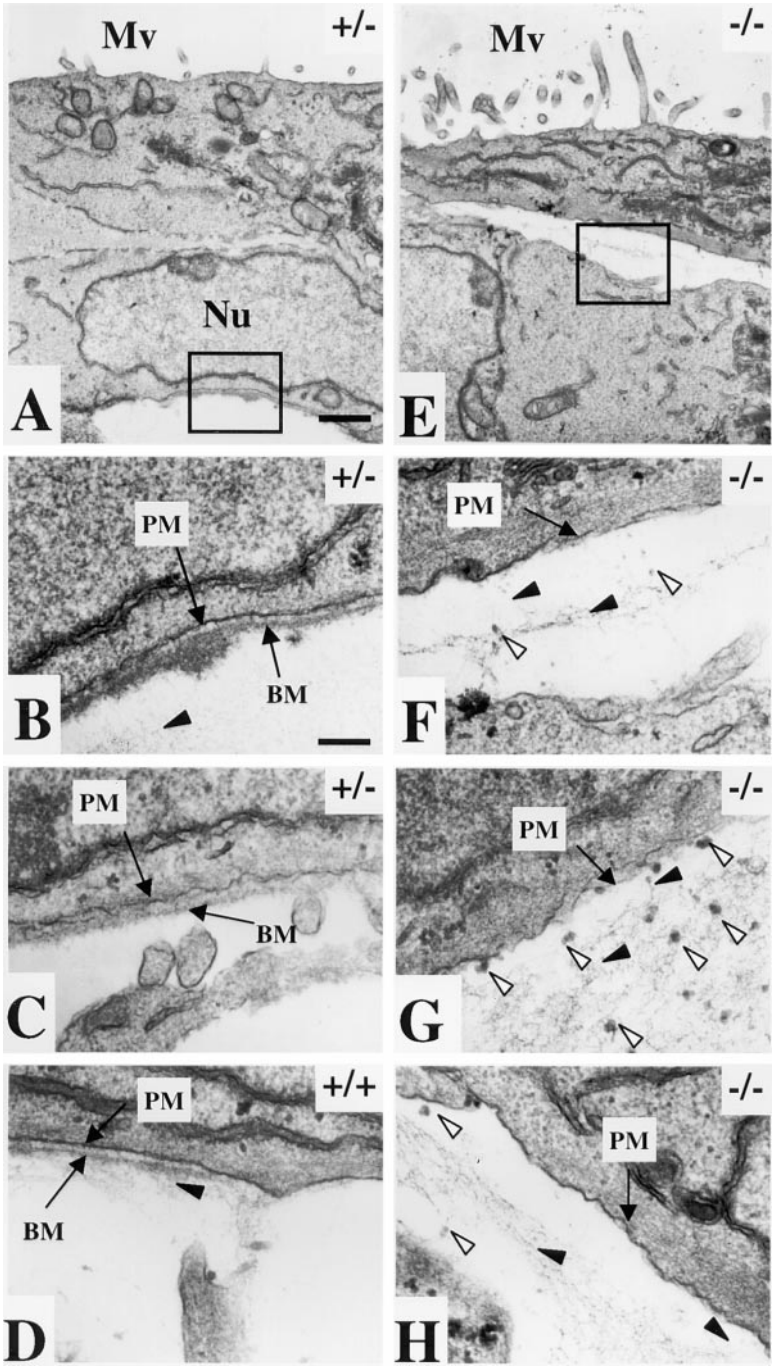

Figure 5. Ultrastructural Analysis of Embryoid Bodies

Electron micrographs of control (A–D) and $DG^{-/-}$ (E–H) embryoid bodies. (A) and (E) are lower magnification images of an endodermal cell on the surface of embryoid bodies showing apical microvilli (Mv). Boxed regions of (A) and (E) are shown at higher magnification in (B) and (F), respectively. In spaces beneath endodermal cells from control embryoid bodies, we could detect basement membranes (BM) running parallel to the plasma membrane (PM), but not in $DG^{-/-}$ embryoid bodies. Two examples from separate $DG^{+/-}$ (B and C), one from a wild-type (D), and three from separate $DG^{-/-}$ (E–G) embryoid bodies are shown. Black arrowheads indicate fibrous matrix. White arrowheads in $DG^{-/-}$ embryoid bodies indicate putative aggregated basement membrane molecules. For this analysis, we examined $16^{+/+}$, $19^{+/-}$, and $24^{-/-}$ embryoid bodies from two independent experiments. Nu, nucleus. Bars: (A), 1.25 μm for (A) and (E); (B), 250 nm for (B)–(D) and (F)–(H).

laminin complex might be involved in nucleating basement membrane assembly. An important feature of a cell-surface molecule involved in the initial formation of basement membranes would seem to be that it could interact with soluble matrix molecules, before they are assembled into an insoluble structure. To investigate this idea further, we exploited the finding that DG is expressed in undifferentiated ES cells. In those cells, DG is typically localized diffusely over the surface of the cells with some apparently concentrated localization at sites of cell–cell contact (data not shown). Undifferentiated ES cells express little, if any, laminin-1, and the scant amount of laminin-1 that is present in the cultures is apparently derived from spontaneously differentiated endodermal cells that are present at low frequency (data not shown). Therefore, we added laminin-1 to the ES cell cultures. After its addition to the culture medium, laminin-1 was deposited in clusters, colocalized with DG, on the surfaces of control ES cells, but not on $DG^{-/-}$ ES cells (Figure 6). On control ES cells, these clusters formed at laminin-1 concentrations far below the critical concentration for laminin-1 self-assembly observed in vitro (~100 nM; Yurchenco et al., 1985) and were routinely observed at concentrations of 1 nM, the lowest

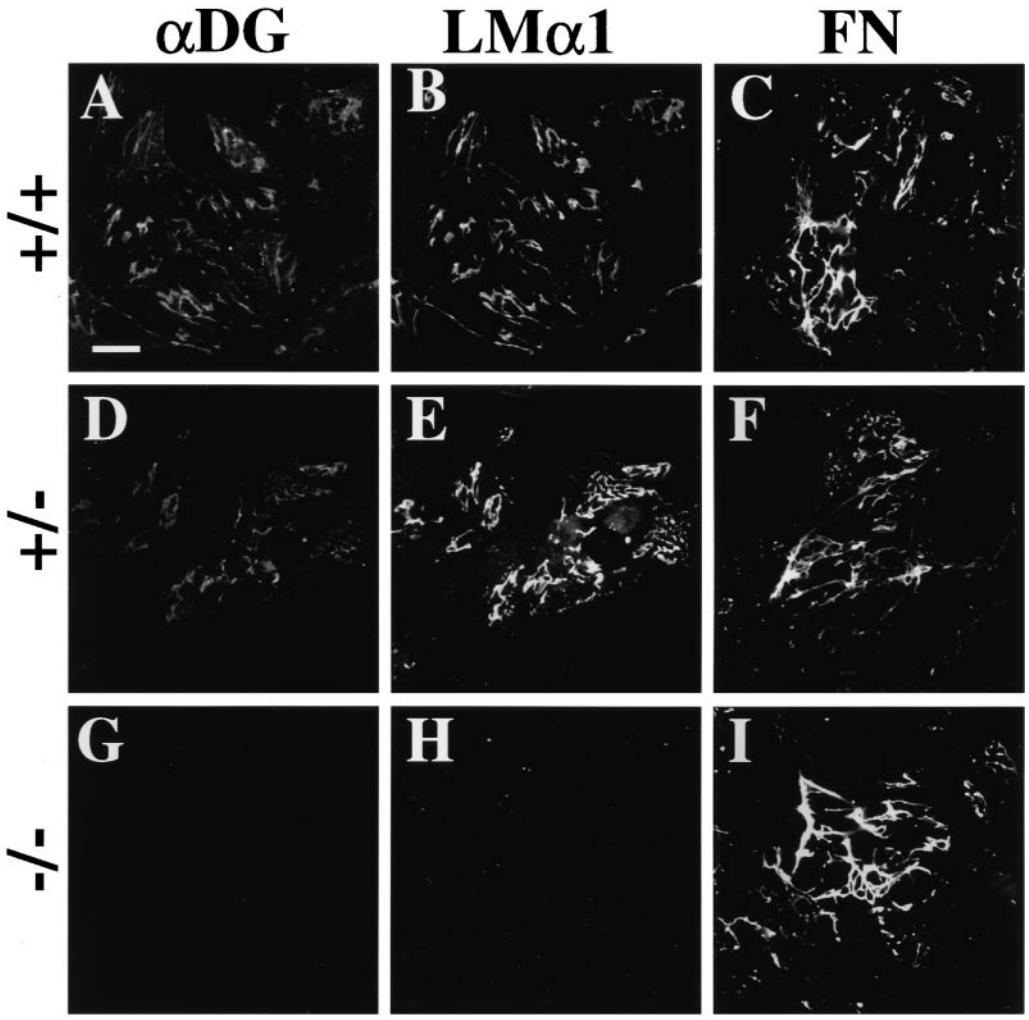

Figure 6. DG Mediates the Formation of Laminin Clusters on the Surface of ES Cells

Staining for αDG and LMα1 on wild-type (A and B), $DG^{+/-}$ (D and E), and $DG^{-/-}$ (G and H) ES cells. Paired immunofluorescence images (αDG and LMα1) were compiled from a 5 μm z-series extending from the dorsal-most surface of the ES cells in 0.5 μm steps. Staining for fibronectin (FN) on wild-type (C), $DG^{+/-}$ (F), and $DG^{-/-}$ (I) ES cells. Bar, 10 μm.

concentration tested. At 7.5 nM laminin-1, we observed only faint, punctate staining on the surface of $DG^{-/-}$ cells (Figure 6H). This punctate staining was dependent on the addition of laminin-1 to the culture, but the majority of it was of a fine particulate nature similar to that observed on the surface of the culture dish and may therefore represent aggregated laminin nonspecifically associated with the cell surface. We did not observe the formation of laminin-1 clusters on $DG^{-/-}$ ES cell lines at 120 nM, the highest concentration of laminin-1 tested. However, at this high concentration, large particles of laminin-1 were detected over the surfaces of the cells and on exposed regions of the culture dish in both control and $DG^{-/-}$ cultures (data not shown).

We tested whether $DG^{-/-}$ ES cells have a general defect in extracellular matrix assembly on their surfaces by examining the status of fibronectin. Fibronectin, either from the serum in the culture medium or produced by the ES cells, was present in a fibrillar staining pattern on the dorsal surfaces of control and $DG^{-/-}$ ES cells (Figure 6C, 6F, and 6I). The proportion of cells displaying this pattern of staining was equivalent among the cultures, suggesting that the extent of fibronectin matrix assembly is not affected in the absence of DG. Interestingly, the fibronectin staining pattern was similar, though not identical, to that of laminin-1, consistent with other reports of the codistribution on these molecules on cell surfaces (Hayman et al., 1981; Darribere et al., 1986).

Restoration of Dystroglycan Expression Rescues Phenotypes in Dystroglycan-Null Embryonic Stem Cells and Embryoid Bodies

Our results are consistent with the conclusion that DG is required for basement membrane formation in embryoid bodies and laminin clustering on ES cells. However, to address the possibility that the observed phenotypes were due to stable secondary changes in the $DG^{-/-}$ ES cell lines rather than the primary loss of DG function, we tested whether the phenotypes observed in $DG^{-/-}$

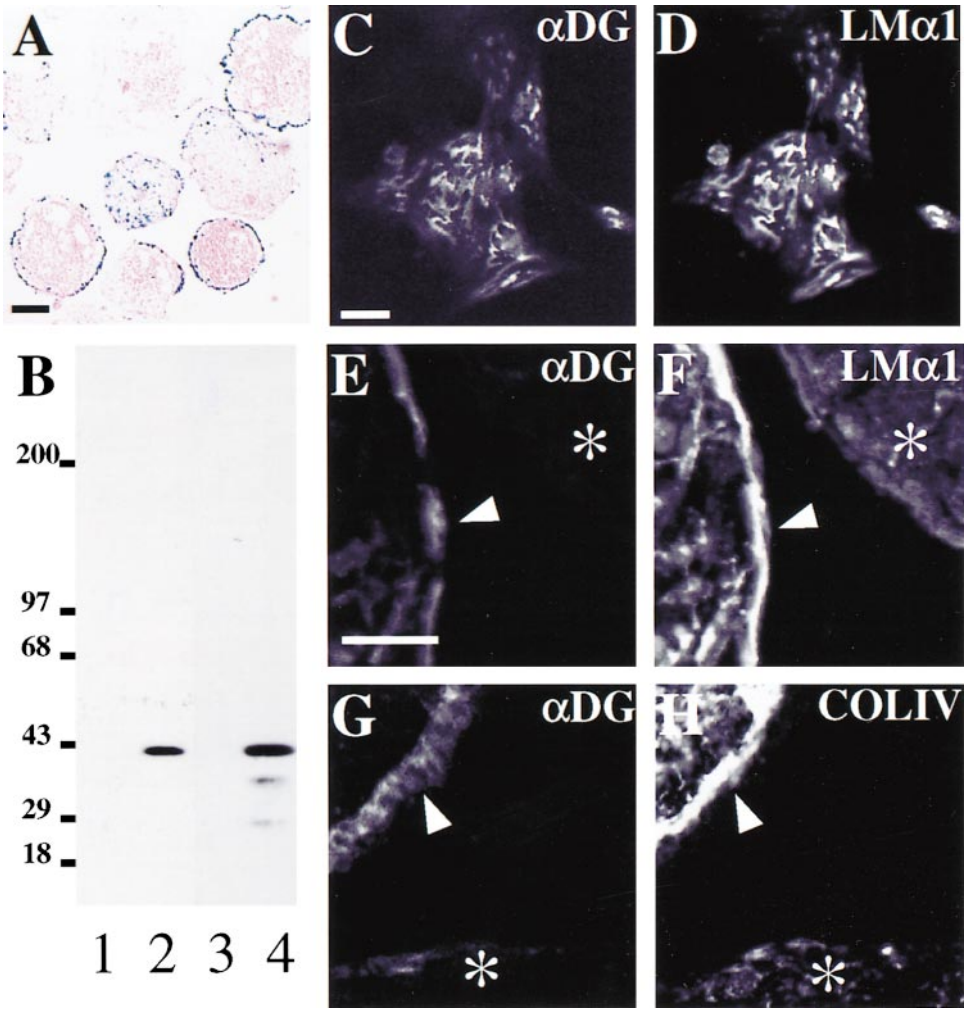

Figure 7. Adenoviral-Mediated Rescue of Mutant Phenotypes in DG$^{-/-}$ ES Cells and Embryoid Bodies

Adenoviral transfer of the *lacZ* gene to embryoid bodies (A). (B) Western blot analysis of extracts taken from DG adenovirus–infected and uninfected DG$^{-/-}$ ES cells and 10-day-old embryoid bodies. Lane 1, DG$^{-/-}$ ES cells, uninfected; lane 2, DG$^{-/-}$ ES cells, infected; lane 3, DG$^{-/-}$ embryoid bodies, uninfected; lane 4, DG$^{-/-}$ embryoid bodies, infected. (C and D, respectively) paired images of αDG and LMα1 staining in DG adenovirus–infected DG$^{-/-}$ ES cell culture. Paired images of αDG and LMα1 staining (E and F, respectively) and αDG and type IV collagen (G and H, respectively) in DG adenovirus–infected 10-day-old embryoid body cultures. Arrowhead denotes the subendodermal basement membrane that forms in embryoid bodies reexpressing DG. Note that surrounding embryoid bodies which do not show endodermal DG expression do not have a basement membrane staining pattern (asterisk). Bars: (A), 50 μm; (C), 10 μm for (C) and (D); (E), 25 μm for (E)–(H).

ES cells and embryoid bodies could be rescued by restoration of DG expression. To this end, we employed an adenovirus carrying a rabbit DG cDNA. Because adenoviruses have not been used extensively for gene transfer in ES cells or embryoid bodies, we first tested the ability of our adenoviral vector to confer reporter gene expression in these systems. We could achieve nearly 100% reporter gene transfer in ES cells with little apparent cytotoxicity (data not shown). In embryoid bodies, we found that the expression was often limited to the outer endodermal layer, though not all embryoid bodies showed expression of the reporter construct (Figure 7A). The virus may be excluded from the core of the embryoid body by the endodermal layer, or those cells might be more permissive to infection than others. This was fortuitous for our experiments because DG is normally expressed in endodermal cells. Next, we infected DG$^{-/-}$ ES cells with the DG adenovirus. By doing this, we were able to restore DG expression in both DG$^{-/-}$ ES cells and embryoid bodies (Figure 7B). After infection of DG$^{-/-}$ ES cells, we added laminin-1 to the culture medium and assayed laminin clustering. We identified cells expressing the viral construct by staining with an anti-DG antibody. In cells now expressing DG, we observed laminin clusters colocalized with DG (Figures 7C and 7D). Similarly, when we infected embryoid bodies with the DG adenovirus, we could identify those expressing DG by an immunofluorescence assay (Figures 7E and 7G). In DG$^{-/-}$ embryoid bodies showing renewed DG expression in the endodermal layer, we observed the typical basement membrane colocalization of laminin-1 with DG (Figure 7F). When we examined type IV collagen localization in DG adenovirus–infected embryoid bodies, we found that type IV collagen, too, was associated

with DG-expressing cells in a basement membrane staining pattern (Figure 7H). Control experiments utilizing an adenovirus carrying a green fluorescent protein cDNA did not rescue, or alter, the mutant phenotypes observed in $DG^{-/-}$ ES cells and embryoid bodies (data not shown).

Discussion

Cell-surface receptor-mediated mechanisms for basement membrane assembly have at least three attractive features: (1) they can specify where and when basement membranes will form, explaining, in part, their rather precise spatial and temporal development; (2) they could maintain high local concentrations of basement membrane molecules near the surfaces of cells and thus facilitate interactions among basement membrane proteins; and (3) they provide a direct means for the cell to get feedback on the status of the assembly process. In addition to mediating the assembly of basement membranes, receptors for basement membrane molecules may be involved in the maintenance and remodeling of, and cell signaling elicited by, basement membranes. Although these ideas have been around for some time, sorting out which receptors are responsible for these various functions is an ongoing process. Our results point toward an important role for DG in the assembly of basement membranes.

DG-null embryoid bodies seem normal in certain respects, including expression of a stage-specific developmental marker, elaboration of a polarized morphology, and in the function of vectoral fluid transport; however, immunofluorescence and electron microscopy revealed that a basement membrane failed to form beneath these cells. This demonstrates that DG is required for the formation of the subendodermal basement membrane. We stress that despite their disorganization, at least some basement membrane proteins are still expressed in $DG^{-/-}$ embryoid bodies, perhaps explaining why the mutant phenotype was not more severe. It seems most probable that DG is involved in the basement membrane assembly through its direct, high-affinity interaction with laminin-1. In addition to laminin-1, we found that the localization of perlecan and type IV collagen was also disrupted in DG-null embryoid bodies. Although a direct interaction with DG (Peng et al., 1998) might explain perlecan's absence from the basement membrane, DG is not known to interact directly with type IV collagen. Therefore, the localization of some basement membrane proteins might depend on prior DG–laminin complex formation, extending studies that have shown that laminin function is crucial for the deposition of other basement membrane components (Kadoya et al., 1995; De Arcangelis et al., 1996). Therefore, formation of a DG–laminin complex is likely to be an early step in the formation of a basement membrane, perhaps even nucleating basement membrane assembly. Consistent with this hypothesis, we found that DG is required for the organization of soluble laminin on the surfaces of ES cells. Although laminin-1 associated with DG on the surfaces of control ES cells does not superficially resemble the smooth, continuous distribution of laminin present in mature basement membranes, these DG–laminin complexes could be nascent structures formed during the basement membrane assembly process. Alternatively, cell-surface laminin-1 clusters might form in the absence of other molecular interactions that serve to stabilize a mature basement membrane structure. Supporting the former is the discontinuous distribution of laminin at sites of basement membrane formation in developing tissues (Darribere et al., 1986; Form et al., 1986). After the formation of nucleation sites, other basement membrane molecules may come together to establish the final structure reminiscent of other biological assembly processes. We therefore suggest that DG–laminin complexes may represent nucleation sites for basement membrane assembly.

Recent studies indicate that laminin-binding members of the β1 integrin family may be involved in basement membrane assembly (Kreidberg et al., 1996; Bloch et al., 1997; DiPersio et al., 1997; Fleischmajer et al., 1998; Sasaki et al., 1998). In one example, loss of α3β1 integrin function led to perturbations in the organization of the epidermal basement membrane (DiPersio et al., 1997). These defects were characterized by ultrastructural abnormalities in (lateral and medial discontinuities) and cell detachment from basement membranes. Disorganized basement membranes were also observed in teratomas derived from β1 integrin–null ES cells (Sasaki et al., 1998). These phenotypes suggest that α3β1 and perhaps other members of the β1-integrin family are involved in the assembly of basement membranes, but their role(s) in this process is not yet clear (see discussion in DiPersio et al., 1997). By way of contrast, in the $DG^{-/-}$ embryoid bodies we see a comparatively more severe basement membrane phenotype—no detectable basement membrane. Considering that DG and integrins are coexpressed in many tissues that form basement membranes (Durbeej et al., 1998) and the phenotypes described above, it is tempting to speculate that DG might act at a distinct, and perhaps earlier, step in basement membrane assembly than β1 integrins. Indeed, DG and laminin-binding integrins interact with distinct domains of the laminin molecule, indicating that they might collaborate in some way during the basement membrane assembly process as has already been suggested for the involvement of these two receptors in epithelial morphogenesis (Ekblom et al., 1996).

Although DG was required for the assembly of laminin-1 on the cell surface, it was not required for the assembly of fibronectin on the cell surface, consistent with integrin involvement in fibronectin matrix assembly (Wennerberg et al., 1996 and references therein). It was striking, however, that the surfaces of DG-null ES cells were devoid of any clustered laminin. This finding indicates that DG acts as a high-affinity laminin-1 receptor in vivo, which complements existing data demonstrating a direct, low nanomolar affinity interaction between soluble laminin-1 and DG by blot overlay assays (Ibraghimov-Beskrovnaya et al., 1992; Gee et al., 1993) and by affinity coelectrophoresis (A. Lander and K. C., unpublished results). Apparently, other laminin receptors did not compensate for the loss of DG function. This suggests several possibilities: (1) other laminin receptors are not expressed in DG-null ES cells; (2) expressed

laminin receptors do not bind to soluble laminin-1 at the concentration range tested; or (3) other laminin receptors bind to laminin-1, but they are in low abundance or do not cluster laminin in a way that is detectable by immunofluorescence microscopy. We have begun to investigate these possibilities. With regard to the first possibility, it is likely that laminin-binding β1 integrins are expressed in $DG^{-/-}$ ES cells, as we can detect β1 integrin and also, interestingly, we find that $DG^{-/-}$ ES cells adhere to laminin-coated substrata as well as control ES cells (M. D. H. and C. Lebakken, unpublished results). This implies that laminin-binding integrins or other laminin receptors, and not DG, may be supporting cell adhesion to laminin, again suggesting divergent functions between DG and other laminin receptors.

How might DG organize soluble laminin on the cell surface? DG has been implicated in the clustering of some of its extracellular matrix ligands on the surfaces of myotubes (Campanelli et al., 1994; Gee et al., 1994; Cohen et al., 1997; Montanaro et al., 1998; Peng et al., 1998). However, DG's role in those cells has been largely thought of in the context of formation of the neuromuscular junction. Here, we demonstrate DG's involvement in clustering laminin in a novel, nonmuscle context and tie its function to basement membrane assembly. Therefore, DG's function in developing muscle may more generally be to promote basement membrane formation, a subset of which is the specialized basement membrane that exists at the neuromuscular synapse (Hall and Sanes, 1993). Consistent with this hypothesis is the fact that laminin clustering on myotubules is MuSK independent (Sugiyama et al., 1997), whereas neuromuscular junction formation is clearly MuSK dependent (DeChiara et al., 1996). Cohen has proposed that laminin self-assembly, through the short-arm domains of laminin, is a driving force for DG-mediated clustering of laminin on muscle cells (Cohen et al., 1997). This mechanism might hold for the laminin clustering that we see on ES cells. One possibility is that DG simply concentrates laminin on the cell surface, facilitating its self-assembly reaction and explaining why we see DG-dependent laminin clustering at laminin-1 concentrations approximately two orders of magnitude lower than the critical concentration for laminin-1 self-assembly in vitro. However, other evidence suggests that the clustering mechanism is more complex. For instance, laminin isoforms that lack one or more short arm domains, such as laminin-5, neither self-assemble nor do they coassemble with assembly-competent laminin isoforms (Cheng et al., 1997), despite being localized to basement membranes. Moreover, a laminin self-assembly mechanism alone does not easily explain the linear and patterned arrays of laminin-1 that develop on ES cells over time (M. D. H., unpublished results). Therefore, by way of analogy to integrin-mediated fibronectin assembly (Wu et al., 1995; Zhang et al., 1997), receptor ligation may engage cytoskeletal changes and induce intracellular signaling that feeds back on the assembly process. DG's connection to the actin cytoskeleton through dystrophin-related molecules (Ervasti and Campbell, 1993), and interaction with known mediators of signal transduction (Yang et al., 1995) provide possible pathways to these mechanisms.

In conclusion, we show the following: (1) DG is required for the formation of the subendodermal basement membrane in embryoid bodies; (2) loss of DG function has consequences for basement membrane molecules with which it does not directly interact, indicating that DG–laminin interactions are required for the deposition of other basement membrane molecules; and (3) DG is required for the formation of laminin clusters on the surfaces of ES cells, which may represent nucleation structures for basement membrane assembly. DG's widespread expression in cells that are directly contacting basement membranes suggests that it may generally be required for the formation of basement membranes, but that remains to be tested. Finally, it will be interesting to understand how this developmental role for DG relates to its functions in mature tissues like skeletal muscle.

Experimental Procedures

Generation of Dystroglycan-Null ES Cells and Preparation of Embryoid Bodies

We isolated $DG^{-/-}$ ES cells from two independently derived parental $DG^{+/-}$ ES cell clones TD354 and TD556 (Williamson et al., 1997) by selection in high concentration of G418 (Mortensen et al., 1992) and retargeting with a hygromycin-selectable gene disruption construct (Mortensen et al., 1991). $DG^{-/-}$ ES cells derived from both strategies yielded similar results. For simplicity, only those derived by the first method are described here. Cells lines were first passaged twice off of feeder layers in ES growth medium (GM) (DMEM-high glucose, + 20% heat-inactivated fetal bovine serum [FBS, Hyclone], 2 mM L-glutamine [GIBCO BRL], 1 mM nonessential amino acids [GIBCO BRL], 10^3U/ml leukemia inhibitory factor [LIF, GIBCO BRL], and 0.001% β-mercaptoethanol [Sigma, tissue culture grade]). Selection was carried out in ES-GM + 1.0 mg/ml active G418 (GIBCO BRL). After selection, surviving colonies were picked, expanded, and analyzed by Southern blotting. We screened HindIII-digested DNA samples using a HindIII-PstI fragment from intron 1 of the mouse DG gene as a probe. From this screen, we identified one $DG^{-/-}$ clone from TD354 (354.B11) and one $DG^{-/-}$ clone from TD556 (556.E5). Most of the data presented is from 354.B11. $DG^{+/-}$ lines surviving selection were used as controls for subsequent experiments. For wild-type controls we passed the parental R1 ES cells (Nagy et al., 1993) off of feeders. All cells lines were used for experiments between 1–7 passages after removal from the feeders. We prepared embryoid bodies by plating 5×10^5 ES cells in 60 mm bacteriological grade plastic dishes (Fisher) in ES-GM without LIF. We changed this medium after 2 days and after 4 days, replaced the medium with DMEM + 10% heat-inactivated FBS and 2 mM L-glutamine, then changed the medium daily until 10 days, at which time we analyzed the embryoid bodies.

Antibodies and Protein Analysis

We used the following specific primary antibodies: mAb 8D5 against β-DG (Novocastra), affinity-purified rabbit pAb #83 against β-DG (Williamson et al., 1997), mAb IIH6-C4 against α-DG (Ervasti and Campbell, 1991), rabbit pAb #317 against the laminin α1 G domains (Durbeej et al., 1996), goat pAb (1340–01) against collagen α1,2(IV) (Southern Biotechnology Associates), rat mAb MAB1948 against perlecan (Chemicon), rabbit pAb against human fibronectin (Wennerberg et al., 1996), pAb against Pem (Lin et al., 1994), mAb CH1 against cardiac tropomyosin (Lin et al., 1985). Western blotting of embryoid body and ES cell extracts was accomplished as described (Ervasti and Campbell, 1991) except that chemiluminescent detection of secondary antibodies was used. For immunohistochemistry of embryoid bodies, we collected 10 day cultures, fixed them 15–30 min in 4% paraformaldehyde and dehydrated, cleared, and embedded them in paraffin. We then cut 7 μm sections on a rotary microtome, affixed the sections to slides, and deparaffinized and rehydrated them before staining. Hematoxylin/eosin, periodic acid-Schiff, and β-galactosidase staining were performed according to standard protocols. For immunostaining, we blocked nonspecific binding with

PBS + 1% BSA for 1 hr and incubated the sections overnight in appropriately diluted antibody solutions. Primary antibodies were detected with fluorescently tagged secondary antibodies (Jackson Immunoresearch). For the laminin clustering assay, we first plated ES cells onto coverslips coated with 5 $\mu g/cm^2$ human fibronectin (Collaborative Biomedical Products), which promoted cell spreading. After plating and overnight incubation, we replaced the culture medium with ES-GM containing varying concentrations of mouse laminin-1 (Collaborative Biomedical Products) and incubated them overnight. After incubation, coverslips were fixed in 4% paraformaldehyde in PBS for 15 min and washed and stained as described above. For all double-label immunofluorescence experiments, we performed control experiments utilizing each primary/secondary antibody combination singly, each primary antibody plus the opposite secondary antibody, and secondary antibodies alone to validate the double labeling. Sections and cells were photographed under a Nikon inverted microscope with Hoffman modulation contrast optics, a Leitz Diaplan microscope for brightfield images, or a Bio-Rad MRC 1024 laser scanning confocal microscope.

Electron Microscopy

Ten-day-old embryoid bodies were fixed with 2% paraformaldehyde/2% glutaraldehyde in PBS, rinsed three times in 0.1 M sodium cacodylate, then postfixed with a solution of 1% OsO_4 and 1.25% potassium ferrocyanide in cacodylate buffer. After three rinses in cacodylate buffer, the samples were exposed to a 1% aqueous tannic acid solution for 20 min, dehydrated through a graded series of acetones, and embedded in Eponate-12 (Ted Pelling, Inc., Redding, CA). After embedment and polymerization, individual embryoid bodies were selected, mounted on blocks, 110 nm sections were cut using a Reichert Ultracut E ultramicrotome, and these were stained with 5% uranyl acetate followed by Reynold's lead citrate. Sections were examined and photographed with a Hitachi H-600 transmission electron microscope operated at 75KV.

Adenoviral-Mediated Dystroglycan Gene Transfer

The DG adenovirus was constructed by subcloning a rabbit DG cDNA (Ibraghimov-Beskrovnaya et al., 1992) into the pAdRSVpA shuttle vector. The DG construct was incorporated into an adenovirus vector through standard methods of homologous recombination with Ad5 backbone dl309 by the University of Iowa Gene Transfer Vector Core. Recombinant viruses were purified using established methods (Graham and Eb, 1973; Davidson et al., 1994). Lysates from infected 293 cells were collected and tested for expression of DG by Western blot analysis. Recombinant virus was plaque purified, amplified, and purified by CsCl gradient centrifugation. Viruses containing *lacZ* and green fluorescent protein expression constructs were obtained from the University of Iowa Gene Transfer Vector Core. For infection of ES cells, we added 1×10^9 viral particles diluted in fresh ES growth medium to $\sim 1 \times 10^6$ ES cells grown under standard conditions. Estimating roughly 1% of total viral particles are infective, this corresponds to a multiplicity of infection of $\sim$10. Cells were cultured with the virus overnight, and fresh medium was added the next day. Cells were assayed for expression of the viral construct 48–72 hr after infection. For infection of embryoid bodies, we added 1×10^9 viral particles diluted in DMEM supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine to a single 60 mm dish of 6-day-old embryoid bodies. Fresh medium was added after overnight incubation with the virus, and the infected embryoid bodies were assayed for expression of the viral construct at 10 days.

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References

Battaglia, C., Mayer, U., Aumailley, M., and Timpl, R. (1992). Basement-membrane heparan sulfate proteoglycan binds to laminin by its heparan sulfate chains and to nidogen by sites in the protein core. Eur. J. Biochem. *208*, 359–366.

Bloch, W., Forsberg, E., Lentini, S., Brakebusch, C., Martin, K., Krell, H.W., Weidle, U.H., Addicks, K., and Fassler, R. (1997). β1 integrin is essential for teratoma growth and angiogenesis. J. Cell Biol. *139*, 265–278.

Campanelli, J.T., Roberds, S.L., Campbell, K.P., and Scheller, R.H. (1994). A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. Cell *77*, 663.

Cheng, Y.S., Champliaud, M.F., Burgeson, R.E., Marinkovich, M.P., and Yurchenco, P.D. (1997). Self-assembly of laminin isoforms. J. Biol. Chem. *272*, 31525–31532.

Cohen, M.W., Jacobson, C., Yurchenco, P.D., Morris, G.E., and Carbonetto, S. (1997). Laminin-induced clustering of dystroglycan on embryonic muscle cells: comparison with agrin-induced clustering. J. Cell Biol. *136*, 1047–1058.

Darribere, T., Riou, J.F., Shi, D.L., Delarue, M., and Boucaut, J.C. (1986). Synthesis and distribution of laminin-related polypeptides in early amphibian embryos. Cell Tissue Res. *246*, 45–51.

Davidson, B.L., Doran, S.E., Shewach, D.S., Latta, J.M., Hartman, J.W., and Roessler, B.J. (1994). Expression of Escherichia coli β-galactosidase and rat HPRT in the CNS of Macaca mulatta following adenoviral mediated gene transfer. Exp. Neurol. *125*, 258–267.

De Arcangelis, A., Neuville, P., Boukamel, R., Lefebvre, O., Kedinger, M., and Simon-Assmann, P. (1996). Inhibition of laminin α1-chain expression leads to alteration of basement membrane assembly and cell differentiation. J. Cell Biol. *133*, 417–430.

DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., et al. (1996). The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell *85*, 501–512.

DiPersio, C.M., Hodivala-Dilke, K.M., Jaenisch, R., Kreidberg, J.A., and Hynes, R.O. (1997). $\alpha 3\beta 1$ integrin is required for normal development of the epidermal basement membrane. J. Cell Biol. *137*, 729–742.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morphol. *87*, 27–45.

Durbeej, M., Larsson, E., Ibraghimov-Beskrovnaya, O., Roberds, S.L., Campbell, K.P., and Ekblom, P. (1995). Non-muscle α-dystroglycan is involved in epithelial development. J. Cell Biol. *130*, 79–91.

Durbeej, M., Fecker, L., Hjalt, T., Zhang, H.Y., Salmivirta, K., Klein, G., Timpl, R., Sorokin, L., Ebendal, T., Ekblom, P., and Ekblom, M. (1996). Expression of laminin α1, α5 and β2 chains during embryogenesis of the kidney and vasculature. Matrix Biol. *15*, 397–413.

Durbeej, M., Henry, M.D., Ferletta, M., Campbell, K.P., and Ekblom, P. (1998). Distribution of dystroglycan in normal adult mouse tissues. J. Histochem. Cytochem. *46*, 449–457.

Ekblom, P., Ekblom, M., Fecker, L., Klein, G., Zhang, H.Y., Kadoya, Y., Chu, M.L., Mayer, U., and Timpl, R. (1994). Role of mesenchymal

nidogen for epithelial morphogenesis in vitro. Development *120*, 2003–2014.

Ekblom, P., Durbeej, M., and Ekblom, M. (1996). Laminin isoforms in development. In The Laminins, P. Ekblom and R. Timpl, eds. (Amsterdam, The Netherlands: Harwood Academic Publishers), pp. 185–216.

Ervasti, J.M., and Campbell, K.P. (1991). Membrane organization of the dystrophin-glycoprotein complex. Cell *66*, 1121–1131.

Ervasti, J.M., and Campbell, K.P. (1993). A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J. Cell Biol. *122*, 809–823.

Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G., and Campbell, K.P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature *345*, 315–319.

Fleischmajer, R., Utani, A., MacDonald, E.D., Perlish, J.S., Pan, T.C., Chu, M.L., Nomizu, M., Ninomiya, Y., and Yamada, Y. (1998). Initiation of skin basement membrane formation at the epidermo-dermal interface involves assembly of laminins through binding to cell membrane receptors. J. Cell Sci. *111*, 1929–1940.

Form, D.M., Pratt, B.M., and Madri, J.A. (1986). Endothelial cell proliferation during angiogenesis. In vitro modulation by basement membrane components. Lab Invest. *55*, 521–530.

Fox, J.W., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Mann, K., Timpl, R., Krieg, T., Engel, J., et al. (1991). Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. EMBO J. *10*, 3137–3146.

Gee, S.H., Blacher, R.W., Douville, P.J., Provost, P.R., Yurchenco, P.D., and Carbonetto, S. (1993). Laminin-binding protein 120 from brain is closely related to the dystrophin-associated glycoprotein, dystroglycan, and binds with high affinity to the major heparin binding domain of laminin. J. Biol. Chem. *268*, 14972–14980.

Gee, S.H., Montanaro, F., Lindenbaum, M.H., and Carbonetto, S. (1994). Dystroglycan-α, a dystrophin-associated glycoprotein, is a functional agrin receptor. Cell *77*, 675–686.

Gesemann, M., Cavalli, V., Denzer, A.J., Brancaccio, A., Schumacher, B., and Ruegg, M.A. (1996). Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. Neuron *16*, 755–767.

Graham, F.L., and Eb, A.J.v.d. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*, 456–467.

Graham, P.L., Johnson, J.J., Wang, S., Sibley, M.H., Gupta, M.C., and Kramer, J.M. (1997). Type IV collagen is detectable in most, but not all, basement membranes of Caenorhabditis elegans and assembles on tissues that do not express it. J. Cell Biol. *137*, 1171–1183.

Hall, Z.W., and Sanes, J.R. (1993). Synaptic structure and development: the neuromuscular junction. Cell *72*, 99–121.

Hayman, E.G., Engvall, E., and Ruoslahti, E. (1981). Concomitant loss of cell surface fibronectin and laminin from transformed rat kidney cells. J. Cell Biol. *88*, 352–357.

Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W., and Campbell, K.P. (1992). Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature *355*, 696–702.

Kadoya, Y., Kadoya, K., Durbeej, M., Holmvall, K., Sorokin, L., and Ekblom, P. (1995). Antibodies against domain E3 of laminin-1 and integrin α6 subunit perturb branching epithelial morphogenesis of submandibular gland, but by different modes. J. Cell Biol. *129*, 521–534.

Kreidberg, J.A., Donovan, M.J., Goldstein, S.L., Rennke, H., Shepherd, K., Jones, R.C., and Jaenisch, R. (1996). α3β1 integrin has a crucial role in kidney and lung organogenesis. Development *122*, 3537–3547.

Lin, J.J., Chou, C.S., and Lin, J.L. (1985). Monoclonal antibodies against chicken tropomyosin isoforms: production, characterization, and application. Hybridoma *4*, 223–242.

Lin, T.P., Labosky, P.A., Grabel, L.B., Kozak, C.A., Pitman, J.L., Kleeman, J., and MacLeod, C.L. (1994). The Pem homeobox gene is X-linked and exclusively expressed in extraembryonic tissues during early murine development. Dev. Biol. *166*, 170–179.

Martin, G.R., Wiley, L.M., and Damjanov, I. (1977). The development of cystic embryoid bodies in vitro from clonal teratocarcinoma stem cells. Dev. Biol. *61*, 230–244.

Matsumura, K., Yamada, H., Shimizu, T., and Campbell, K.P. (1993). Differential expression of dystrophin, utrophin and dystrophin-associated proteins in peripheral nerve. FEBS Lett. *334*, 281–285.

Montanaro, F., Gee, S.H., Jacobson, C., Lindenbaum, M.H., Froehner, S.C., and Carbonetto, S. (1998). Laminin and alpha-dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway. J. Neurosci. *18*, 1250–1260.

Mortensen, R.M., Zubiaur, M., Neer, E.J., and Seidman, J.G. (1991). Embryonic stem cells lacking a functional inhibitory G-protein subunit (αi2) produced by gene targeting of both alleles. Proc. Natl. Acad. Sci. USA *88*, 7036–7040.

Mortensen, R.M., Conner, D.A., Chao, S., Geisterfer-Lowrance, A.A., and Seidman, J.G. (1992). Production of homozygous mutant ES cells with a single targeting construct. Mol. Cell Biol. *12*, 2391–2395.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc. Natl. Acad. Sci. USA *90*, 8424–8428.

Peng, H.B., Ali, A.A., Daggett, D.F., Rauvala, H., Hassell, J.R., and Smalheiser, N.R. (1998). The relationship between perlecan and dystroglycan and its implication in the formation of the neuromuscular junction. Cell Adhes. Commun. *5*, 475–489.

Sasaki, T., Forsberg, E., Bloch, W., Addicks, K., Fassler, R., and Timpl, R. (1998). Deficiency of β1 integrins in teratoma interferes with basement membrane assembly and laminin-1 expression. Exp. Cell Res. *238*, 70–81.

Sugiyama, J.F., Glass, D.G., Yancopoulos, G.D., and Hall, Z.W. (1997). Laminin-induced acetylcholine receptor clustering: an alternative pathway. J. Cell Biol. *139*, 181–191.

Thomas, T., and Dziadek, M. (1993). Genes coding for basement membrane glycoproteins laminin, nidogen, and collagen IV are differentially expressed in the nervous system and by epithelial, endothelial, and mesenchymal cells of the mouse embryo. Exp. Cell Res. *208*, 54–67.

Timpl, R., and Brown, J.C. (1996). Supramolecular assembly of basement membranes. Bioessays *18*, 123–132.

Vracko, R. (1974). Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure. Am. J. Pathol. *77*, 314–346.

Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S., and Fassler, R. (1996). β1 integrin-dependent and -independent polymerization of fibronectin. J. Cell Biol. *132*, 227–238.

Williamson, R.A., Henry, M.D., Daniels, K.J., Hrstka, R.F., Lee, J.C., Sunada, Y., Ibraghimov-Beskrovnaya, O., and Campbell, K.P. (1997). Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. Hum. Mol. Genet. *6*, 831–841.

Wu, C., Keivens, V.M., Te, O.T., McDonald, J.A., and Ginsberg, M.H. (1995). Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix. Cell *83*, 715–724.

Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K.P. (1995). SH3 domain-mediated interaction of dystroglycan and Grb2. J. Biol. Chem. *270*, 11711–11714.

Yurchenco, P.D. (1994). Assembly of laminin and type IV collagen into basement membrane networks. In Extracellular Matrix Assembly and Structure, P.D. Yurchenco, D.E. Birk, and R.P. Mecham, eds. (San Diego, California: Academic Press), pp. 351–388.

Yurchenco, P.D., and Furthmayr, H. (1984). Self-assembly of basement membrane collagen. Biochemistry *23*, 1839–1850.

Yurchenco, P.D., Tsilibary, E.C., Charonis, A.S., and Furthmayr, H. (1985). Laminin polymerization in vitro. Evidence for a two-step assembly with domain specificity. J. Biol. Chem. *260*, 7636–7644.

Zhang, Q., Magnusson, M.K., and Mosher, D.F. (1997). Lysophosphatidic acid and microtubule-destabilizing agents stimulate fibronectin matrix assembly through Rho-dependent actin stress fiber formation and cell contraction. Mol. Biol. Cell *8*, 1415–1425.