Basement Membrane Deposition of Nidogen 1 but Not Nidogen 2 Requires the Nidogen Binding Module of the Laminin γ1 Chain*

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The nidogen-laminin interaction is proposed to play a key role in basement membrane (BM) assembly. However, though there are similarities, the phenotypes in mice lacking nidogen 1 and 2 (nidogen double null) differ to those of mice lacking the nidogen binding module (γ1III4) of the laminin γ1 chain. This indicates different cell- and tissue-specific functions for nidogens and their interaction with laminin and poses the question of whether the phenotypes in nidogen double null mice are caused by the loss of the laminin-nidogen interaction or rather by other unknown nidogen functions. To investigate this, we analyzed BMs, in particular those in the skin of mice lacking the nidogen binding module. In contrast to nidogen double null mice, all skin BMs in γ1III4-deficient mice appeared normal. Furthermore, although nidogen 1 deposition was strongly reduced, nidogen 2 appeared unchanged. Mice with additional deletion of the laminin γ3 chain, which contains a γ1-like nidogen binding module, showed a further reduction of nidogen 1 in the dermoepidermal BM; however, this again did not affect nidogen 2. This demonstrates that in vivo only nidogen 1 deposition is critically dependent on the nidogen binding modules of the laminin γ1 and γ3 chains, whereas nidogen 2 is independently recruited either by binding to an alternative site on laminin or to other BM proteins.

In skin, the BMγ3 forms at the dermoepidermal junction, separating dermis and epidermis while providing an adhesive and dynamic interface. Hemidesmosomes are responsible for firm anchorage of basal keratinocytes to the dermoepidermal BM and are crucial for the structural and functional integrity of the skin (1–3). All BMs contain members of the laminin, nidogen, collagen IV, and proteoglycan (including perlecan) families.

The mammalian nidogen family consists of two members, nidogen 1 and nidogen 2 (4–7). Both are ubiquitous BM proteins with nidogen 2 showing clear tissue specificity in adulthood (6, 8). Based on their binding repertoire, it has been proposed that nidogens play a key role in BM assembly (6, 8–10). However, mice lacking both nidogen isoforms (showing a perinatal lethal phenotype) form ultrastructurally normal BMs in many tissues (11–13) thus demonstrating that BMs differ in their requirement for nidogens.

The binding of nidogen 1 to laminin is one of the best studied extracellular matrix interactions (14–16). The nidogen-binding site of laminin is localized to a single laminin-type epidermal growth factor-like module of the laminin γ1 chain γ1III4 (14) and is therefore present in most laminin isoforms. Both nidogens 1 and 2 bind in vitro with similar affinities to this module (6, 8). Although the γ2 chain of laminin 332 contains a homologous binding module, γ2III4, no significant nidogen binding occurs to this laminin (17). The laminin γ3 chain contains a γ1-like nidogen binding motif with only a single conservative amino acid substitution (18, 19). The γ3-nidogen binding module has been shown in vitro to bind to nidogen 1 and 2 albeit with a lower affinity than the γ1III4 module (20).

Competitive inhibition of laminin-nidogen 1 interaction by antibodies or a corresponding recombinant γ1 fragment resulted in defects of BM formation and epithelial branching morphogenesis in a number of tissues and organ cultures (21–23). Accordingly, in skin organotypic cocultures, this interference completely prevented BM deposition and assembly of hemidesmosomes (24). Similar defects were observed in coculture systems with fibroblasts lacking nidogen expression (25). However, studies in mice with genetic deletion of the nidogen binding module γ1III4 showed that although the laminin-nidogen interaction is important for the formation of certain BMs, it is not a general prerequisite for BM assembly (11, 16). For instance, development of the kidney is impaired in the absence of laminin-nidogen interaction, whereas formation of other organs appears grossly normal as in the case of the nidogen 1 and 2 double null mice. This suggests that
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nidogens and their interaction with laminin may play different roles for BM formation and function in various tissues. To understand this further, we investigated nidogen 1 and 2 deposition in BMs, particularly in the skin.

EXPERIMENTAL PROCEDURES

Generation and Genotyping of Mice Lacking the Nidogen Binding Module γ1III4 and the Laminin γ3 Chain—The mutation in the LAMC1 gene was introduced by deletion of the nidogen binding module γ1III4 as described previously. Mice homozygous for this deletion die shortly after birth due to lung failure (16). The mutation in the LAMC3 gene was generated by deletion of exon 1. Mice homozygous for the deletion of the laminin γ3 chain show no obvious phenotypes and are fertile (26). Embryos lacking the γ1III4 module were produced by breeding heterozygous mice. Mice lacking both the γ1III4 module and the laminin γ3 chain (LAMC3) were generated by breeding mice heterozygous for deletion of the γ1III4 module and homozygous for deletion of the laminin γ3 chain. All strains have been crossed back into the C57BL/6 background for at least six generations. Wild-type and mutated alleles were assessed by Southern blot hybridization and/or PCR of DNA isolated from mouse tail biopsies as described previously (16, 26). For all experiments, littermates were used as controls.

Preparation of Tissue Samples for Histological Analysis and Immunofluorescence Staining—Skin samples from embryos at embryonic day E18.5, noon of the day of the vaginal plugging being considered to be E0.5, were fixed in 4% paraformaldehyde-phosphate-buffered saline on ice for 45 min, embedded in paraffin wax, sectioned (6 μm), and stained with hematoxylin/eosin. For immunofluorescence, the skin samples were snap-frozen in optimal-cutting-temperature compound (O.C.T. Tissue Tec®). Immunostaining was performed as described previously (11, 27) and examined with a Nikon Eclipse 800 fluorescence microscope or with a Leica TCS confocal laser scanning microscope.

Immunohistochemistry, Immunofluorescence, and Immunoblot Analysis—For immunofluorescence, antigen retrieval was performed on paraffin sections either by heating in citrate buffer at 90 °C, followed by incubation with 0.1% trypsin for 10 min at 37 °C (collagen IV and integrins) or with sodium citrate (ECL; Pharmacia Biotech, Freiburg, Germany). Equal protein loading was verified by applying a mouse monoclonal actin antibody (1:6000; MP Biomedicals, Heidelberg, Germany).

Pulldown Experiment—Purified recombinant His-tagged nidogen 2 (25) was coupled to Ni-NTA-agarose beads for 1 h at 4 °C. Then, the beads were incubated with cell lysates combined with serum-free supernatants of control or γ1III4-deficient skin fibroblasts. The protein lysates had been precleared by incubation with unloaded Ni-NTA-agarose beads. After overnight incubation at 4 °C, the beads were washed twice with washing buffer (50 mM Na2HPO4, 300 mM NaCl, 20 mM imidazole, pH 8), suspended in 2× SDS sample buffer, and boiled for 5 min at 94 °C. The samples were analyzed by SDS-PAGE and immunoblotting.

Image Analysis, Morphometry, and Statistical Analysis—Light microscopic images were taken with a Leica DM 4000 B microscope and a digital camera using the DISKUS software (Hilgers, Königswinter, Germany), a semiautomatic operator-dependent imaging system (28). Fluorescence images were captured with the digital camera DXM1200F and the software Lucia G (Lucia GF-4.81). Ki67-positive cells were only counted in the interfollicular epidermis. The number of proliferating cells was quantified by counting Ki67-positive cells in 10 high power fields (40×) per section in 10 individual control and mutant mice. The average number of positive cells per high power field was calculated. Results are presented as mean values ± S.D. For comparison of means between different groups, the Student’s t test using the GraphPad Prism software was used. Differences were considered as statistically significant at values of p < 0.05 (indicated by asterisk).

Electron Microscopy—Specimens were fixed in 2.5% glutaraldehyde (in cacodylate buffer, pH 7.2), followed by 2% OsO4 stained en bloc with uranyl acetate, and processed for embedding in Epon 812-equivalent (Serva Bioproducts, Heidelberg, Germany) following previous protocols (29, 30). Ultrathin sections were counterstained with uranyl acetate and lead citrate.

RESULTS

Lack of Nidogen Binding Module γ1III4 Does Not Affect Skin Morphology and Epidermal Differentiation—Because mice deficient for the nidogen binding module of the laminin γ1 chain (γ1III4-deficient mice) die within a few hours of birth due to lung failure (16), we collected embryos at E18.5 for skin analysis. At this time point, the epidermis has developed to a fully differentiated stratified squamous epithelium (31). Histological analysis of skin sections revealed a normal
morphology, including appendages and epidermal stratification in γ1III4-deficient mice (Fig. 1, a and b). However, staining of skin sections with antibodies directed against the proliferation marker Ki67 showed significantly more Ki67-positive cells in the basal compartment of the interfollicular epidermis of mutant mice (d). The number of proliferating cells was quantified by counting Ki67-positive cells in 10 high power fields (40×) per section in 10 individual control and mutant mice (g, n = 10; p = 0.0001). White bar, controls (γ1III4+/+); black bar, mutants (γ1III4−/−). Immunohistochemistry of skin sections with antibodies against keratin K14 shows a uniform staining of the basal cell layer in control (e) and mutant (f) mice; E, epidermis; D, dermis. The hair follicle is indicated by an arrow in b. Scale bar, 100 μm (a–d) and 25 μm (e and f).

Deposition of Nidogen 1 Is Impaired in γ1III4-deficient Skin—To investigate whether the change in epidermal homeostasis in γ1III4-deficient mice might be caused by changes in BM deposition, we applied antibodies directed against major BM components. As both murine nidogens have been shown to bind with comparable affinity with the nidogen binding module γ1III4 (8, 14), we first asked whether deletion of this

FIGURE 1. Proliferation of basal keratinocytes is increased in the skin of γ1III4-deficient mice. In histological sections, embryonic skin (E18.5) of control (a) and γ1III4-deficient (b) mice show a comparable epidermal morphology, including skin appendages. Immunohistochemistry of skin sections with antibodies against Ki67 (c and d) shows statistically significant higher numbers of proliferating cells in the basal compartment of the interfollicular epidermis of mutant mice (d). The number of proliferating cells was quantified by counting Ki67-positive cells in 10 high power fields (40×) per section in 10 individual control and mutant mice (g, n = 10; p = 0.0001). White bar, controls (γ1III4+/+); black bar, mutants (γ1III4−/−). Immunohistochemistry of skin sections with antibodies against keratin K14 shows a uniform staining of the basal cell layer in control (e) and mutant (f) mice; E, epidermis; D, dermis. The hair follicle is indicated by an arrow in b. Scale bar, 100 μm (a–d) and 25 μm (e and f).

FIGURE 2. Epidermal differentiation is not altered in γ1III4-deficient mice. Immunofluorescence of control (a–d) and γ1III4-deficient (e–h) embryonic skin sections (E18.5) shows comparable epidermal differentiation patterns by staining for basal K14 (a and e, and insets), suprabasal K10 (b and f), and the late markers loricin (c and g) and filaggrin (d and h). Scale bar, 100 μm; insets, 3-fold magnification.
module led to alterations of nidogen deposition (Fig. 3A). Skin sections of control embryos revealed a continuous linear nidogen 1 staining of the dermoepidermal BM and of capillary walls (Fig. 3A, a). In contrast, in mutant skin, nidogen 1 staining was dramatically reduced and discontinuous at the dermoepidermal junction and completely lost from the vessel walls (Fig. 3A, d). Scale bar, 100 μm (d and h) and 20 μm (a–c and e–g). B, Western blots of protein extracts from γ11I14-deficient skin show strongly reduced nidogen 1 (nd1) levels when compared with control littermates (γ11I14+/−), whereas nidogen 2 (nd2) is not altered. Three individual samples of control and γ11I14-deficient mouse skin were resolved on SDS-polyacrylamide gradient gels under reducing (nd1, actin as loading control) and nonreducing (nd2, ponceau staining) conditions. C, cell lysates of γ11I14−/− (lane 1, input) or γ11I14−/− (lane 2, input) fibroblasts were incubated with His-tagged nidogen 2 bound to Ni-NTA-agarose beads (lanes 3 and 6, respectively). Cell lysates of γ11I14−/− (lane 3) and γ11I14−/− (lane 4) fibroblasts incubated with uncoupled Ni-NTA-agarose beads. After washing, the beads were boiled, and the released proteins were analyzed by Western blots with antibodies against the His tag to detect nidogen 2 (middle panel) and against the laminin γ1 chain (γ1, bottom panel). Coommassie blue staining (top panel), molecular masses (kDa) are indicated to the right.

To verify this, a mouse strain lacking both the nidogen binding module γ11I14 and the laminin γ3 chain was generated. This double knock-out strain dies perinatally, showing the same phenotypes as described in γ11I14-deficient mice (16). In skin sections of mice lacking the laminin γ3 chain (LAMC3−/−) nidogen 1 and 2 deposition is unchanged when compared with control sections (Fig. 4, a and c; compare with Fig. 3A, a and d). However, skin analysis of double knock-out mice (γ11I14−/− and laminin γ3-deficient) revealed a further overall reduction of nidogen 1 staining around hair follicles and ectopic diffuse staining in the dermis. In contrast, nidogen 2 was again not affected in these mice (Fig. 4, b and d).
This indicates that both the γ1- and γ3-nidogen binding modules are important for retaining nidogen 1 but not nidogen 2 within the dermoepidermal BM. To determine whether nidogen 2 binds to another epitope on laminin, we performed pulldown experiments with His-tagged nidogen 2 as the bait protein. As shown in Fig. 3C, nidogen 2 interacts with γ1-containing laminins in the absence of the nidogen binding module γ1III4 (lane 6).

We then asked whether distribution and deposition of other BM components are affected in γ1III4-deficient mouse skin. To detect the major laminin isoforms in skin, laminin 511 and 411, we used antibodies directed against the laminin γ1 chain. Immunofluorescence revealed the expected strong linear, continuous laminin staining at the dermoepidermal BM and the blood vessel walls in sections from γ1III4-deficient mice (Fig. 5, a and e), with similar findings observed for collagen IV and perlecan, (data not shown). Laminin 332, the main constituent of the anchoring filaments (32), was detected by antibodies against the laminin γ2 chain showing also a comparable linear staining of the dermoepidermal BM as in control skin (Fig. 5, b and f). The main laminin receptors on keratinocytes are the integrins α3β1 and α6β4 (33, 34). Antibodies against β1 decorated the plasma membrane of basal keratinocytes with slightly higher intensity at the basal surface (Fig. 5, c and g; showing also staining of fibroblasts and endothelial cells), whereas β4 localization was strongly polarized and mostly restricted to the dermoepidermal junction (Fig. 5, d and h), corresponding to its partner α6 (data not shown). Thus, there were also no obvious differences of integrin patterns between the skin of control and γ1III4-deficient mice. These results were confirmed on the protein level by immunoblotting, showing comparable amounts of laminin γ1, integrin chains β1 and β4, respectively (Fig. 5i). Similar results were obtained with skin extracts of double knock-out mice (data not shown). Altogether this indicates that addi-
Skin BMs in γ1III4-deficient Mice Appear Ultrastructurally Normal—Examination by electron microscopy confirmed the regular epidermal morphology and differentiation in mice lacking the nidogen binding module (Fig. 6, a and b). Furthermore, the ultrastructure of the dermoeipidermal BM zone was completely normal with a continuous, well-formed lamina densa and regular adhesion structures, including completely matured hemidesmosomes (Fig. 6, c and d). There was no indication of microblisters as seen in nidogen double-deficient mice (13). Also vessel walls appeared ultrastructurally normal in the γ1III4-deficient mouse skin (Fig. 6, e–h). However, the vascular BMs seemed to be somewhat less dense suggesting a minor deficit in assembly. Taken together, these results clearly demonstrate that nidogen 2 does not require laminin γ1III4 binding and/or the presence of laminin γ3 for deposition and that neither nidogen 1 nor nidogen 2 binding to the laminin module γ1III4 is required for assembly of skin BMs.

Nidogen 1 Deposition Is also Impaired in Other Tissues—To examine whether nidogen deposition is altered in other tissues, we performed immunofluorescence analysis on heart and lung sections from mice lacking the nidogen binding site (Fig. 7). This revealed the typical localization of nidogen 1 in the BM zone of the myocardium and cardiac blood vessels in control embryos at E18.5 (Fig. 7a), whereas in the mutants nidogen 1 was almost completely lost (Fig. 7b). In contrast, nidogen 2 antibodies mainly stained the capillary walls, whereas it was barely detectable in the myocardial BMs. However, there was no difference in nidogen 2 staining intensity or pattern between control and mutant hearts (Fig. 7, c and d). In contrast to the mutant hearts, which were normally developed, the mutant lungs show major morphological changes (16). They immature, with compact poorly inflated prealveolar sacculi and mesenchymal thickening between the terminal airspaces. Lung sections at birth showed in control animals comparable staining patterns for nidogen 1 and 2 (Fig. 7, e and g). In contrast, nidogen 1 staining is completely lost in the mutant lungs, whereas nidogen 2 is retained around the developing alveoli (Fig. 7, f and h).

### DISCUSSION

The nidogen-laminin interaction has been believed to be a crucial event for BM assembly. In vitro studies have shown that the two murine nidogens bind with similar affinities to the γ1III4 module of the laminin γ1 chain. Hence, as laminins have been considered as the main interaction partners for both nidogens (8), it was surprising that the phenotypes of nidogen double null mice and of mice lacking the nidogen binding module γ1III4 were not phenotypically identical (11, 12, 16). As published previously (16) and shown here, nidogen 1 deposition is impaired in all tissues in the absence of the γ1III4-binding module, whereas nidogen 2 staining is unaltered. However, in the heart and skin but not in the lung, nidogen 2 is able to rescue the phenotypes observed in nidogen double null mice (11, 16). This indicates tissue specificity of the observed changes. The differences may be explained by presently unknown, laminin-independent functions of nidogen 1 and 2 or by unidentified additional activities of the γ1III4 module. The possibility that the deletion interferes with folding and assembly of laminins has been largely excluded by biochemical and ultrastructural analysis (16, 35). To dissect these different options, we performed a detailed analysis of the skin BMs lacking the γ1III4 module and mice with the additional loss of the laminin γ3 chain, which carries a similar nidogen binding site.

At E18.5, γ1III4-deficient mice showed a normal skin morphology. However, although epidermal layering and differen-
tiation was comparable between γ11114-deficient and control mice, proliferation of basal keratinocytes was significantly increased in the mutant epidermis. Whether this hyperproliferative phenotype would persist during further skin maturation cannot be studied owing to the perinatal lethality in this mouse strain. Interestingly, skin wounding in nidogen 1-deficient mice showed a transient delay in epidermal thinning due to hyperproliferation of basal keratinocytes in the newly formed epidermis (36). Because in these wounds, nidogen 2 deposition is unaltered when compared with control wounds, this suggests a specific role for nidogen 1, which cannot be compensated by nidogen 2. It is therefore tempting to speculate that, in the absence of nidogen 1 or the nidogen binding module γ11114, nidogen 2 complexed with laminin or another BM protein may exert different functions in epidermal homeostasis than the γ11114-mediated laminin-nidogen 1 complex.

In skin, the predominant γ1 chain-containing isoform is laminin 511. This is believed to promote migration and proliferation of keratinocytes through binding to α3β1 (37, 38). In vivo laminins containing the γ1 chain are always complexed with nidogen; hence, the role of laminin 511 could be modulated by binding of either nidogen 1 or 2 through different binding sites or of both nidogens at the same time. As nidogen 2 was able to pull down the mutated laminin γ1 chain, lacking the high affinity nidogen 1 binding module γ11114, the existence of such alternative complexes is very likely. Co-localization of laminin and nidogen 1 and laminin and nidogen 2 has been shown by immunoelectron microscopy, strongly suggesting such ternary interactions (39). Furthermore, in vitro binding studies revealed that laminin 111 completely saturated with nidogen 1 still allowed nidogen 2 binding (6). Taken together with our data this strongly argues for a laminin-nidogen 2 complex formed via a laminin epitope not represented by the γ11114 module. Whether this second site represents an additional or the sole nidogen 2 binding site on laminin in vivo is not yet clear.

In contrast to nidogen double null mice, which showed ruptures of the capillary walls (13), no microhemorrhages were detected in the skin of γ11114-deficient mice and ultrastructurally, the vascular BMs appear normal. Immunofluorescence revealed the complete loss of nidogen 1 from the capillary walls in γ11114-deficient skin, whereas nidogen 2 staining was unaltered. This is in good agreement with results from skin analysis of nidogen 1-deficient mice, which also showed no microhemorrhages (27) and indicates that in γ11114-deficient skin, nidogen 2 can fully compensate the lack of nidogen 1, thereby rescuing capillary leakage (13).

There was also nidogen 1 loss from the dermoeidermal BM. However, in contrast to the capillary walls, some nidogen 1 staining was retained. This staining was further reduced after additional deletion of the laminin γ3 chain, whereas nidogen 2 was not affected. This demonstrates for the first time that laminins containing the γ3 chain also contribute to nidogen 1 recruitment to BMs in vivo. The γ3 chain is not found in capillary walls of the skin, which explains why deletion of the γ1-nidogen binding module is sufficient to completely eliminate nidogen 1 from vascular BMs.

Nidogen linking of the laminin and collagen IV networks has been considered to be the main driving force for BM assembly. However, in both nidogen double null mice (13) and γ11114-deficient mice, the dermoeidermal BM appears ultrastructurally normal. Thus, this cannot be the case for all tissues. Indeed, we demonstrate that in vivo neither nidogens nor laminin-nidogen interaction via the γ11114-module are required for the formation of this BM. This argues strongly for the existence of alternative, nidogen-independent mechanisms for BM assembly. Direct interaction of collagen IV and laminin G domain-like modules, although contributing little to collagen IV recruitment, could represent such an alternative to circumvent the necessity for laminin-nidogen interaction (40). Furthermore, collagen IV linking to laminin via the heparan sulfate side chains of perlecarn (41, 42) provides another, more likely mechanism bypassing nidogen bridging.

Taken together, our data indicate that nidogen 1 and 2, though homologous and ubiquitous BM components, play different, tissue-specific roles in BM assembly and function in vivo. These differences might be due to distinct binding repertoires of the two nidogens as we could demonstrate that only nidogen 1 recruitment to BMs depends on the nidogen binding modules of the laminin γ1 and γ3 chains, whereas nido-
gen 2 recruitment occurs independently of these most likely via an alternative epitope on the laminin trimer.

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