The Epidermal Basement Membrane Is a Composite of Separate Laminin- or Collagen IV-containing Networks Connected by Aggregated Perlecan, but Not by Nidogens*

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Background: The supramolecular assemblies of basement membranes are incompletely understood.

Results: Basement membranes are suprastructure composites of two separable, alloyed networks containing collagens IV or laminins.

Conclusion: The laminin- and collagen IV-containing networks are “spot-welded” by perlecan aggregates, but not by nidogens.

Significance: Biological properties of supramolecular assemblies, such as basement membranes, go beyond the sum of individual macromolecular attributes.

The basement membrane between the epidermis and the dermis is indispensable for normal skin functions. It connects, and functionally separates, the epidermis and the dermis. To understand the suprastructure and functional basis of these connections, heterotypic supramolecular aggregates were isolated from the dermal-epidermal junction zone of human skin. Individual suprastructures were separated and purified by immunomagnetic beads, each recognizing a specific, molecular component of the aggregates. The molecular compositions of the suprastructures were determined by immunogold electron microscopy and immunoblotting. A composite of two networks was obtained from fibril-free suspensions by immunobeads recognizing either laminin 332 or collagen IV. After removal of perlecan-containing suprastructures or after enzyme digestion of heparan sulfate chains, a distinct network with a diffuse electron-optical appearance was isolated with magnetic beads coated with antibodies to collagen IV. The second network was more finely grained and comprised laminin 332 and laminins with α5-chains. The core protein of perlecan was an exclusive component of this network whereas its heparan sulfate chains were integrated into the collagen IV-containing network. Nidogens 1 and 2 occurred in both networks but did not form strong molecular cross-bridges. Their incorporation into one network appeared to be masked after their incorporation into the other one. We conclude that the epidermal basement membrane is a composite of two structurally independent networks that are tightly connected in a spot-welding-like manner by perlecan-containing aggregates.

The dermal-epidermal junction zone of human skin represents an architectural link and a functional continuum between the epidermis and the dermis (1). This junction zone also acts as a barrier against the unhindered passage of, for example, chemicals or pathogens into or out of water or electrolytes out of the body. The structural link comprises interactions from the nuclei of keratinocytes to the dermal connective tissue and vice versa. Actin and keratin intermediate filaments in keratinocytes originate at the nuclear membrane and terminate at focal adhesions and hemidesmosomes, respectively, located at the basal intracellular face of the plasma membrane. Focal adhesions, in turn, are attached to the underlying epidermal basement membrane through aggregates of α3β1-integrin. The link of hemidesmosomes to the basement membrane consists of clustered α6β4-integrin molecules and collagen XVII-aggregates, together constituting the anchoring filaments. Finally, the basement membrane is tethered to the papillary dermal connective tissue by anchoring fibrils that intercalate with and bind tightly to dermal banded collagen fibrils (2) or anchoring plaques, i.e. basement membrane-like patches interspersed into the papillary dermis (3). Information is mediated in both directions of the dermal-epidermal junction zone and results in appropriately fine-tuned functions of the epidermis and the dermis.

The structure and assembly mechanisms of basement membranes in general have been subject to intensive investigation by morphological, developmental, genetic, and biochemical approaches (for recent reviews, see Refs. 4, 5). Basement membranes are supramolecular composites of two independent, but physically connected networks whose quantitatively major...
components are laminins and isoforms of collagen IV, respectively. Laminins constitute a family of heterotrimeric proteins with three distinct polypeptide chains, called α-, β-, and γ-chains, forming cross-shaped molecules (6). The long arm arises by an α-helical supercoil of the C-terminal portion of all three chains. The N-terminal portions of each polypeptide form the three short arms. Each polypeptide of the short arms folds into a string of several globular domains, LE- and LN-domains, which are essential for the supramolecular assembly. The C-terminal portion of the α-chain folds into five globular LG-domains which, in some cases, are truncated to three globules by proteolytic processing. The LG-domains are mainly involved in cellular interactions via integrins. In the epidermal basement membrane, laminins can self-assemble into networks, but the mode of integration of laminin 332 remains unknown because this laminin lacks most of the short arms after proteolytic processing.

Collagen IV is another major component of basement membranes. There are six genetically distinct α-chains of collagen IV, called α1(IV) through α6(IV), but only three distinct heterotrimers occur in tissues, i.e. (α1(IV))2α2(IV), α3(IV) α4(IV) α5(IV), and (α5(IV))2α6(IV). Similarly to laminins, the three collagen IV isoforms can aggregate into networks that also can be heterotypic. However, in the epidermal basement membrane, only (α1(IV))2α2(IV)- and (α5(IV))2α6(IV)-heterotrimers can be found (7).

The aggregation of suprastructural laminin networks in situ is orchestrated by clusters of cell surface (macro)molecules, such as integrins, dystroglycan, or sulfatides, that bind and, thereby, concentrate laminins at the plasma membrane. They also reduce the activation energy of self-assembly by juxtaposing interacting surfaces of the nascent suprastructures at the nanoscale. Furthermore, selective binding in each case of discrete mixtures of macromolecular components ascertain the formation of networks with appropriate tissue-specific functions. Finally, the laminin- and collagen IV-containing networks are linked to each other to form functional basement membranes. In an attempt to identify the molecular linkers, studies of direct interactions between individual basement membrane macromolecules have been performed. The results have hinted at an important role in this context of nidogens 1 and/or 2 (for reviews, see Refs. 5, 8). Mouse models have shown that the presence of certain laminin chains is required already at very early development, before implantation of the embryos into the uterine wall. Genetic ablation of other chains causes early postnatal lethality or still milder phenotypes (for review, see Ref. 6). The inactivation of the Col4a1 and Col4a2 genes encoding collagen IV also is incompatible with life, although only at later stages of gestation. In view of the network adaptor role assigned to the nidogens, the knock-out of the nidogen genes had surprisingly mild consequences in mice. The single knock-outs were viable and fertile, and even the compound ablation of both nidogen genes did not cause immediate postnatal lethality. It was concluded from the studies that, despite the severe developmental abnormalities observed in the double knock-outs, nidogens were not essential for basement membrane formation, challenging the role of nidogens as connectors between basement membrane networks (4, 9, 10).

Nanoscale interaction sites may either be created or obliterated by aggregating macromolecules. However, this simple fact often is overlooked in studies on molecular assembly of extracellular matrices. For example, collagen VII aggregates bind tightly to fibrils reconstituted from collagen I in vitro, and the same is true for anchoring fibrils and banded dermal collagen fibrils in situ (2). By contrast, soluble collagen I and VII molecules, i.e. the quantitatively major macromolecular constituents of the same suprastructures, do not interact detectably (11). Conversely, an anchorless adhesin of Staphylococcus aureus, called Eap, avidly binds to many species of extracellular matrix macromolecules, including several collagen types, or even to denatured polypeptides thereof. However, this promiscuity is entirely lost after supramolecular aggregation, and only fibronectin-containing networks remain good binding substrates for Eap (12).

Keeping in mind that basement membranes are supramolecular composites, we have investigated the mode of incorporation of perlecan and of nidogens 1 and 2 into supramolecular laminin- and collagen IV-containing networks isolated after mechanical fragmentation of the epidermal basement membrane. The focus in these studies was on the structural composites and their connections existing in epidermal basement membrane in situ rather than on the modalities of network assemblies from macromolecules. The results show that both nidogens are incorporated into laminin- and collagen IV-containing networks of the epidermal basement membrane. However, nidogens 1 and 2 lose their affinity for laminin-containing networks after incorporation into collagen IV-containing suprastructures and vice versa. This bridging function is reserved for perlecan that does so in a “spot-welding” manner, i.e. by strong interactions at relatively few sites separated by large distances. The core protein of perlecan and its heparan sulfate chains are parts of the laminin- and collagen IV-containing networks, respectively.

**EXPERIMENTAL PROCEDURES**

**Preparation of Suprastructures from Human Dermal-Epidermal Function Zone**—Human skin specimens were obtained after informed consent from patients (both sexes, age range 25–50 years) undergoing cosmetic surgery. Subcutaneous layers were mechanically removed, and the remaining tissue was either used directly or frozen at −80 °C. Pieces of about 2 cm² were subjected to artificial epidermolysis (13–15). Briefly, the epidermis was peeled off after incubating skin samples for 4 h in 0.05 M Tris-HCl, pH 7.4, containing 1 M NaCl and complete protease inhibitor mixture (Roche Applied Science). The dermis was frozen on a flat surface in liquid nitrogen, and the uppermost dermal layer of about 200 μm, containing the papillary dermis and the epidermal basement membrane zone, was shaved off with a dermatome. The tissue was then homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in 6.6 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl (PBS) and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethyl-maleimide, 5 mM benzamidine, and 100 mM ε-aminocaproic acid). After low speed centrifugation, a skin extract enriched in components of the
epidermal basement membrane (enriched extract, EE) in the supernatant was used for further isolation of authentic suprastuctures.

**Immunomagnetic Beads**—Superparamagnetic polystyrene immunobeads Dynabeads® M-280 sheep anti-mouse IgG and Dynabeads® M-280 sheep anti-rabbit IgG, used for the specific isolation of special target antigens, were purchased from Invitrogen.

**Antibodies**—Unless stated otherwise, the antibodies used in this study were from commercial sources and were ascertained for their specificity by immunoblotting. The antibodies used for the generation of specific immunomagnetic beads were: rabbit polyclonal antibody to collagen I (R1038; Acris Antibodies, Herford, Germany), rabbit polyclonal antibody to collagen IV (R1041; Acris Antibodies), mouse monoclonal antibody to collagen IV (AF5910; Acris Antibodies), mouse monoclonal antibody to domain III of human perlecain (13-4400; Invitrogen), rabbit polyclonal antibody to nidogen-1 (GTX102897; GenTex, Irvine, CA), rabbit polyclonal antibody to nidogen 2 (ab14513; abcam plc, Cambridge, UK), and mouse monoclonal antibody 6F12 to laminin 332 (laminin β3-chain) (16). These antibodies were coupled to immunomagnetic beads as described previously (2). The antibodies used for immunogold transmission electron microscopy were: mouse monoclonal antibody to collagen IV (MAB1910; Millipore), mouse monoclonal antibody to laminin 332 (laminin γ2-chain) (MAB19562; Millipore), rat monoclonal antibody to nidogen 1 (MAB1886; Millipore), rat monoclonal antibody to perlecain (MAB1948; Millipore), rabbit polyclonal antibody to nidogen 2 (APO2275SU-S; Acris Antibodies), and rabbit polyclonal antibody to the laminin α5-chain. 12-nm and 18-nm colloidal gold particles conjugated anti-mouse, anti-rat, and anti-rabbit immunoglobulins were purchased from Jackson ImmunoResearch Laboratories. The antibodies used for immunoblotting were: rabbit polyclonal antibody to collagen IV (R1041; Acris Antibodies) and rabbit polyclonal antibody to laminin 332 (ab14509; abcam plc). Peroxidase-conjugated polyclonal anti-rabbit immunoglobulins were from Sigma-Aldrich.

**Enzymes**—Heparinase I (H2519) and heparinase III (H8891) were purchased from Sigma-Aldrich. Lyophilized enzymes were restored in heparinase buffer (20 mM Tris-HCl, pH 7.5, containing 50 mM sodium chloride, 4 mM calcium chloride, and 0.01% BSA) at final concentrations of 0.1 unit/µl. Aliquots were stored at −20 °C.

**Suprastructure**—The specific isolation of authentic suprastuctural components from the enriched extract was performed with magnetic polystyrene beads covered with sheep anti-mouse and sheep anti-rabbit secondary antibodies. Primary antibodies, recognizing target antigens of interest, were covalently coupled to anti-mouse and anti-rabbit secondary antibodies of the immunobead surface as reported previously (2). In brief, 2–3 µg of the primary antibodies were used per 10^7 beads for incubation under rotation for 14 h at 4 °C. After washing the beads with PBS, pH 7.4, and 0.2 M triethanolamine, pH 8.2, primary antibodies were covalently linked by incubating the beads in 20 mM dimethyl pimelimidate dihydrochloride (Pierce/Thermo Fisher Scientific) in 0.2 M triethanolamine, pH 8.2, for 30 min at room temperature. To stop the reaction, beads were separated from the cross-linking solution and resuspended in 50 mM Tris, pH 7.5, for 15 min. Finally, beads were washed and resuspended in PBS, pH 7.4, containing 0.1% (w/v) BSA. These beads were then used for the isolation of suprastuctures from the EE.

**Removal of Collagen I-containing Dermal Fibrils**—Before collagen IV- and laminin 332-containing basement membrane networks were isolated from the EE, collagen I-containing dermal fibrils were removed by the use of magnetic immunobeads coupled with antibodies to collagen I (R1038). For this purpose, 250 µg of EE was incubated with 5 × 10^7 anti-collagen I immunobeads in a 1.5-ml protein low bind tube (Eppendorf, Hamburg, Germany) under gentle rotation for 14 h at 4 °C. To assure adequate sample mixture and isolation specificity, the reaction volume was adjusted to 500 µl with PBS, pH 7.4, 0.1% (w/v) BSA, and 0.04% (v/v) Tween 20. Magnetic separation was carried out by placing the tube for 3 min on a magnetic particle concentrator (Dynal® MPC™; Invitrogen). This isolation step was repeated three times with an incubation time of 2 h for each bead isolation to yield a postfibrillar extract.

**Isolation of Collagen IV- and Laminin 332-containing Networks**—Postfibrillar extract was incubated with 7 × 10^7 anti-collagen IV immunobeads or with 10 × 10^7 anti-laminin 332 (laminin β3-chain) immunobeads. Incubation was carried out in a 1.5-ml protein low bind tube (Eppendorf) under gentle rotation for 14 h at 4 °C. After magnetic separation the bead pellets were washed five times with PBS, pH 7.4, and resuspended in an appropriate volume of PBS. The isolation with anti-collagen IV or anti-laminin 332 (laminin β3-chain) immunobeads was repeated three times for 2 h. The separated and washed bead pellets from each step were pooled and used for further analyses.

**Isolation of Perlecain-containing Suprastructure**—The procedure for the isolation of collagen IV- and laminin 332-containing basement membrane networks was modified by isolating perlecain-containing suprastructure after the removal of dermal fibrils and prior isolation of basement membrane networks. The postfibrillar extract was incubated with 4 × 10^7 anti-perlecain immunobeads in analogy to the isolation of basement membrane networks. Four bead pellets were combined and used for further studies.

**Isolation of Nidogen 1- and Nidogen 2-containing Structures**—Analogously to the isolation of perlecain-containing suprastructure, nidogen 1- and nidogen 2-containing structures were isolated from postfibrillar extracts with 4 × 10^7 anti-nidogen 1- and 4 × 10^7 anti-nidogen 2-immunobeads.

**Digestion with Heparinases**—Postfibrillar extract was digested with heparinases I and III before isolation of collagen IV- or laminin 332-containing networks. To ensure optimal enzyme activities the initial isolation procedure was slightly modified. For the removal of dermal fibrils, the reaction volume was adjusted to 500 µl with heparinase buffer (20 mM Tris-HCl, pH 7.5, containing 50 mM sodium chloride, 4 mM calcium chloride, and 0.01% (w/v) BSA). The postfibrillar extract was incubated with 1 unit of both heparinases I and III in a 1.5-ml protein low bind tube (Eppendorf) under gen-

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4 The abbreviation used is: EE, enriched extract.
tle rotation for 14 h at 37 °C. After digestion, BSA and Tween 20 were added to final concentrations of 0.1% (w/v) and 0.04% (v/v), respectively. The supernatant was used for the following isolation of basement membrane networks.

**Immunogold Transmission Electron Microscopy**—For morphological and compositional analyses, immunogold electron microscopy (immunogold EM) was performed. 10–15 μl of isolated suprastructures attached to the immunobeads were adsorbed for 15 min to nickel EM grids previously coated with a Formvar®/carbon film. Grids were washed with PBS, pH 7.4, and blocked with 2% (w/v) BSA and 2% (v/v) normal goat serum (Dako, Glostrup, Denmark) in PBS. For immunolabeling, grids were incubated for 2 h at room temperature on drops of primary antibodies in PBS containing 2% (v/v) BSA-c (Aurion, The Netherlands) and 0.025% (v/v) Tween 20. After washing with the same solution, immunodetection was performed by secondary antibodies conjugated to gold particles. Single immunogold labeling was done with 18-nm, and double immunogold labeling with 18-nm and 12-nm gold-conjugated immunogold-labeled. After washing five times with distilled water (HPLC grade), the material was negatively stained with 2% (w/v) uranyl acetate for 7 min. In negative controls, primary antibodies were omitted. Electron micrographs were recorded on a Philips EM-410 transmission electron microscope at 80 kV.

**Gel Electrophoresis and Immunoblotting**—For biochemical analyses, bead pellets containing isolated suprastructures were separated with a magnetic particle concentrator and resuspended in SDS-PAGE loading buffer containing 5% (v/v) β-mercaptoethanol. SDS-PAGE was performed on 4–15% gradient gels following standard protocols. Separated proteins were transferred onto a nitrocellulose membrane (Whatman) by a wet blot procedure. Transferred proteins were visualized by total protein staining (Pierce/Thermo Fisher Scientific). For immunodetection, the membrane was washed with distilled water and blocked with 5% (w/v) dried skimmed milk in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl (TBS) for 1 h at room temperature. Primary antibodies were incubated in 2.5% (w/v) dried skimmed milk in TBS containing 0.04% (v/v) Tween 20 (TBS-T) for 2 h at room temperature. After washing three times for 10 min in TBS-T, the membrane was incubated with secondary peroxidase-conjugated anti-rabbit immunoglobulins in 2.5% (w/v) dried skimmed milk in TBS-T for 2 h at room temperature. After washing with TBS-T, signals were detected with a chemiluminescence substrate (ECL; Pierce/Thermo Fisher Scientific).

**RESULTS**

Fragments of extracellular matrix suprastructures (fibrils, microfibrils, network-like aggregates) were extracted from homogenates of superficial (about 200 μm) human dermis (2, 15) and were subjected to a separation protocol schematically summarized in the flow chart in Fig. 1. Briefly, the dermis was frozen after artificial epidermolysis and superficial layers, removed with a dermatome, were homogenized in PBS. After elimination of tissue debris by low speed centrifugation, a slightly turbid suspension was obtained that represented the starting material in Fig. 1, i.e. a preparation enriched in suprastructures from the epidermal basement membrane zone, EE.

Banded collagen fibrils were removed from the EE with immunomagnetic beads coated with an antibody to collagen I (2). From this collagen fibril-free suspension, designated as postfibrillar extract (Fig. 1), fragments of suprastructures from the epidermal basement membrane were adsorbed to immunomagnetic beads coated with antibodies to either laminin 332 or collagen IV (product A in Fig. 1). These suprastructures had an impenetrable electron density, but honeycomb-like structures became well discernible at the fragment boundaries (Fig. 2, A and B). Double immunogold labeling revealed the presence of both laminin 332 and collagen IV, regardless of whether the immunomagnetic beads recognized laminin 332 or collagen IV (Fig. 2, A and B). For analysis by immunoblotting, the basement membrane networks were heat-denatured and subjected to SDS-PAGE and immunoblotting. Bands migrating with an electrophoretic mobility corresponding to individual polypeptide chains of collagen IV or laminin 332 were detected with the corresponding antibodies (Fig. 3, A and B), indicating that the major molecular basement membrane components were connected to each other, directly or indirectly, within the network-like suprastructures. Taken together, our observations showed that we could isolate suprastructural composites of collagen IV- and laminin 332-containing networks from the human epidermal basement membrane. These composites were stable enough to withstand the stress generated during their isolation by mechanical fragmentation and purification.

Next, we isolated from postfibrillar extracts perlecan-containing fragments with anti-perlecan beads (product B in Fig. 1). Very small patches were obtained, exhibiting a network-like appearance similar to that of the larger fragments of the composite networks isolated from postfibrillar extracts with anti-

![FIGURE 1. Flow chart representation of isolation protocol of suprastructures from human dermal-epidermal basement membrane. To obtain a postfibrillar extract, dermal fibrils were removed with anti-collagen I immunobeads from the skin extract enriched in components of the epidermal basement membrane (EE). Networks isolated directly from the postfibrillar extract with anti-collagen IV immunobeads or anti-laminin 332 immunobeads are composites comprising both laminins and collagen IV (A). Treatment of the postfibrillar extract with anti-perlecan immunobeads or heparinase leads to the postperlecan or postheparinase extract, respectively. Perlecan-containing network fragments are obtained with anti-perlecan immunobeads (B). Distinct collagen IV- and laminin 332-containing networks (D and D') could be separated from postperlecan or postheparinase extracts by anti-collagen IV immunobeads or anti-laminin 332 immunobeads. Nidogen-containing suprastructures (E) were removed from the postfibrillar extract by anti-nidogen immunobeads. Composite networks containing laminins and collagen IV (F) were isolated from the postnidogen extract with either anti-collagen IV beads or anti-laminin 332 beads.](image-url)
To break out small patches of the composite of laminin 332- and collagen IV-containing networks that also contained perlecan. From this, we predict that the perlecan-containing patches occurred at preferred locations separated by large intervening spaces. After their removal, larger regions of the composite were left behind which were essentially free of perlecan. If the perlecan-containing patches constitute the major structural link within the suprastructural composite of basement membranes, we expect that isolation of the individual laminin- or collagen IV-containing networks should be feasible.

To test these predictions, we used the perlecan-depleted postfibrillar extract (postperlecan extract in Fig. 1) to isolate individual networks containing either collagen IV or laminin 332. As shown in Fig. 4, this separation indeed was achieved. The two suprastructures reproducibly had distinct appearances in the electron microscope after negative staining. The collagen IV networks (product C in Fig. 1) had amorphous contours similar to those seen in the crude preparations (compare Fig. 2, A and B, with Fig. 4, A, C, and E). By contrast, the laminin 332-containing structures (product D in Fig. 1) were more finely grained and honeycomb-like (Fig. 4, B, D, and F). The collagen IV networks were not labeled with antibodies to laminin 332 and vice versa (Fig. 4, A–D). These results were also confirmed by immunoblot analysis of the separated networks. Signals for collagen IV were absent in laminin 332-containing networks and, conversely, laminin 332 was not detected in the...
collagen IV-containing networks (Fig. 3, C and D). The laminin α5-chain was found in the laminin 332-containing network (Fig. 4F), indicating that at least one laminin containing a α5-chain is amalgamated into the same heterotypic network structure comprising laminin 332. The collagen IV-containing networks did not contain any of the laminin chains (Fig. 4, C and E). Importantly, immunolabeling with antibodies to the core protein of perlecan was undetectable in both networks (Fig. 5, E and F) indicating that perlecan-containing structures were effectively removed by the preceding immunobead separation. These results confirm that basement membranes are composites of two separate suprastructures with laminins and collagen IV being the respective major macromolecular components (5). Further, the results extend the current knowledge by showing that fragments of the two networks can be isolated, at least from the epidermal basement membrane, in forms retaining their authentic supramolecular organizations. Moreover, the results are consistent with the prediction that the two networks are connected by perlecan-containing patches. The removal of these patches from the composite is sufficient to render the two individual networks mechanically separable. Their distinct suprastructural assemblies are amenable to further examination.

Because perlecan is a heparan sulfate proteoglycan it is conceivable that its core protein and glycosaminoglycan chains are incorporated differentially into the two suprastructures. Battaglia et al. (17) concluded from molecular solid phase binding
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studies that the heparan sulfate chains of perlecan bind to several basement membrane proteins whereas the core protein had an affinity for nidogen(s). To examine the mode of supramolecular affiliation of perlecan within basement membranes directly, the postfibrillar extract was treated with heparinases I and III to eliminate all heparan sulfate chains. After this treatment, the two network structures also became separable. The collagen IV network (product C’ in Fig. 1) and laminin network (product D’ in Fig. 1) displayed comparable appearances in the electron microscope and a similar, mutually exclusive immunogold staining for collagen IV and γ2-chains of laminin 332, respectively (data not shown). Immunogold staining for perlecan core protein was positive in the laminin 332-containing suprastructure (Fig. 5H), but negative in the collagen IV network (Fig. 5G). We conclude that the heparan sulfate chains integrate into the collagen IV-containing network. Conversely, the perlecan core protein amalgamates into the laminin 332-containing suprastructures. This further corroborates our notion that the perlecan-containing patches constitute the major adaptor between the basement membrane networks.

It has been proposed that nidogens act as molecular adaptors between the laminin network and collagen IV network in basement membranes (18–21). Therefore, we investigated the occurrence of nidogens 1 and 2 in separated collagen IV- and laminin 332-containing suprastructures extracted from postfibrillar extracts after removal of perlecan or digestion with heparinases. All preparations reacted with antibodies specific for nidogens 1 or 2 (Fig. 5, A–D).

In analogy to perlecan patches, aggregates containing nidogens 1 and 2 were removed from the postfibrillar extracts by respective immunomagnetic beads (product E in Fig. 1). These aggregates adhered to the immunomagnetic beads and showed a positive immunogold labeling for both nidogens (data not shown). The nidogen 1- (Fig. 6, A and B) and the nidogen 2-containing patches (data not shown) were also positive for both laminin 332 and collagen IV, albeit weakly. The elimination of nidogen-containing structures was successful in that both nidogens were no longer detectable in the networks left behind after immunomagnetic bead extraction (data not shown). The attempt to separate discrete laminin 332- and collagen IV-containing networks, in analogy to the separation of the same networks in the postperlecan extract, was unsuccessful. Suprastructures adhering to immunomagnetic beads specific for either collagen IV or laminin 332 (product F in Fig. 1) showed immunogold labeling for collagen IV (Fig. 6, C and D) as well as laminin 332 (Fig. 6, E and F). Similarly, both immunomagnetic bead preparations gave positive reactions for both collagen IV and laminin 332 in immunoblots (Fig. 7, A and B).

We concluded that both nidogens had avidity for each of the networks. However, upon incorporation into one of the networks, they lose their affinity for the other one. This implies that in the suprastructural context, the nidogens do not act as direct connectors between the basement membrane networks.
FIGURE 8. Model of the suprastructural organization of epidermal basement membrane. Laminin isoforms 511 (A) and 521 (B) co-polymerize by interactions between N-terminal globular LN domains to form a laminin network (D, top view). Lateral aggregation of laminins leads to further polymerization into a heterotypic laminin suprastructure (D) also integrating laminin 332 lacking short arms (C). Formation of networks by the collagen IV isoforms (\(\alpha 1(IV)\)),\(\alpha 2(IV)\) (E) and (\(\alpha 5(IV)\)),\(\alpha 6(IV)\) (F), but not the collagen IV variant \(\alpha 3(IV)\alpha 4(IV)\alpha 5(IV)\) requires end-to-end interactions between C-terminal globular NC1 domains and interactions through N-terminal 7S domains (G, top view). Additionally, lateral aggregation and twisting of collagenous domains provide a tightly entangled collagen IV-network (G). Oligomers of the heparan sulfate proteoglycan perlecan (H) connect the two distinct basement membrane networks (J, lateral view) by integrating in a spot-welding manner its core protein into the laminin polymer and its heparan sulfate chains into the collagen IV-network. Nidogens 1 and 2 (I) are integral parts of both networks (J).
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DISCUSSION

Cytoskeletal or extracellular matrix suprastructures are large aggregates that usually consist of more than one species of (macro)molecules. Such aggregates are insoluble because of their very large molecular masses. The analysis of interactions between individual macromolecules, or even domains or peptides thereof, has often been considered necessary and sufficient for the understanding of functional relationships between suprastructures. However, this constitutes a leap of faith that is not always warranted. Several types of macromolecules can assemble into extensive aggregates that may be homogeneous macromolecular amalgamates or, in other cases, composites in which distinct regions comprise discrete molecular components. In either case, macromolecular aggregation can lead to the creation of new surfaces at the nanoscale that do not exist on individual macromolecules. Conversely, aggregation may extinguish binding sites that were accessible on individual macromolecules. Such situations are readily appreciated in the case of histochemical masking of antibody epitopes on tissue sections. However, similar considerations apply to interactions at the supramolecular level in general.

To address these concerns, we have developed novel methods to generate mixtures of distinct mechanical fragments of suprastructures from the epidermal basement membrane of human skin, followed by their immunochemical analysis. One part of these studies confirmed the existing view that laminins and the isoforms of collagen IV were integral parts of two independent and distinguishable networks. Another part showed directly that these networks were tightly connected and that this connection was not merely the result of an entanglement. The connections between the networks were strong and consisted of sparsely scattered regions throughout the epidermal basement membrane in a spot-welding manner. The connections contained perlecan with the core protein being a part of the laminin-containing suprastructure whereas, most likely, the heparan sulfate chains are integrated into the collagen IV-containing network. To address the same question, Battaglia et al. (17) performed solid phase binding assays with soluble, individual macromolecules. They concluded that networks containing laminins or collagen IV were solely connected in basement membranes by heparan sulfate chains whereas the core protein of perlecan played no role in this interaction. This interpretation contrasts with the conclusions drawn here in that the core protein of perlecan is an important component tethering the two networks together. This disagreement probably derives from the differences relating to individual proteins versus suprastructures and highlights the importance of direct studies on suprastructures rather than on individual macromolecules.

Our results also challenge the widely accepted belief that nidogens 1 and 2 are directly or indirectly involved in the structural connection between the two networks (22) (for review, see Refs. 5, 20). They are consistent, however, with the recent observations that the simultaneous genetic ablation of both nidogens causes severe abnormalities but is not incompatible, in general, with the formation of basement membranes (9, 23). Treatment with immunomagnetic beads coated with antibodies to nidogens was sufficient for a mechanical break-up of intact basement membranes and resulted in the extraction of very small network fragments containing all basement membrane macromolecules investigated, including nidogens 1 and 2. The larger remaining network fragments lacked nidogens. However, removal of nidogen-containing network patches did not allow a subsequent separation of laminin- and collagen IV-containing suprastructures. Hence, it is unlikely that the nidogens play a major structural role in the epidermal basement membrane by directly connecting networks containing laminins or collagen IV. Moreover, even after their separation, both, laminin and collagen IV networks contained nidogens 1 and 2. This is consistent with the notion that both nidogens indeed co-polymerize with both laminins and collagen IV and, possibly, with further components not investigated here (24, 25). However, integration of the nidogens into each of the networks obliterated incorporation into the other one. These features are only apparent when interaction studies are conducted at the supramolecular level.

Our revised model of the suprastructural organization of epidermal basement membrane is represented schematically in Fig. 8. This model accommodates information derived from this and our previous studies (2) as well as numerous findings reported in the literature and reviewed recently in Refs. 5, 26. Both the laminin- and the collagen IV-containing networks are highly polymeric, heterotypic aggregates with modes of lateral and end-to-end interactions between different molecular species. Both networks are comparable with macromolecular alloys. The laminin network comprises laminin 332 co-polymerized with laminin(s) containing α5-chains, such as laminins 511 and/or 521, resulting in the formation of finely structured networks observable by transmission electron microscopy. The fact that the size of the connections within the networks is larger than that of individual laminin molecules and that immunogold labeling for several chains of laminins, including α5, β1, β2, γ1, and γ2-chains, was observed within the same suprastructures necessitates lateral co-polymerization of different laminin isoforms. In a similar vein, the isoforms (α1(IV))2-α2(IV), α3(IV)α4(IV)α5(IV) and (α5(IV))2α6(IV) of collagen IV in glomerular, mesangial, and tubular basement membranes in the kidney can amalgamate into heterotypic networks with diffuse electron density (27). The modulation of the composition will provide distinct stabilities and tissue-specificities to the basement membrane networks.

Finally, the connection between the two suprastructurally distinct networks is affected by aggregated perlecan (28) in a spot-welding-like manner. Nidogens modulate the surfaces of both network types and, possibly, their macromolecular organizations and cellular interactions.

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