Review

The ultrastructural composition of basement membranes *in vivo*

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Summary. The ultrastructure of basement membranes has a homogeneous appearance. The enormous cell biological importance of basement membranes and their components for cell proliferation, migration and differentiation implies that their composition is more complex than their structure suggests. To elucidate the molecular composition of basement membranes *in vivo*, we optimised immunogold histochemistry to allow the determination of the molecular arrangement of matrix molecules.

Basically, we apply a mild fixation and embed the tissues in the hydrophilic LR-Gold®. This preserves the basement membrane with a quality similar to freeze substitution. The application of two antibodies directed toward the C- and N-terminal ends of a molecule and coupled to gold particles of different sizes allows determination of the orientation of a molecule within the basement membrane. We were able to demonstrate that the molecular orientation of the laminin-1 molecule changes in the basement membrane according to cell biological needs. We also showed that ultrastructurally identical basement membranes like the ones of the proximal and distal tubules of the kidney have a differing molecular arrangement. Integrin $\alpha 7$ influences the molecular composition of the basement membranes at the myotendinous junction. With the help of double labelling at the ultrastructural level we could show that nidogen-1 is co-localised with laminin-1 and only found in fully developed, mature basement membranes. In general, laminin-1, nidogen-1 and collagen type IV are localised over the entire width of basement membranes, with laminin-1 and nidogen-1 co-localised, in accordance with the current basement membrane models.

Incidentally, our investigations warn us, that not every matrix protein found at the light microscopic level as a linear staining pattern underneath an epithelium (basement membrane zone) is a real basement membrane component when investigated at the

ultrastructural level. Instead, one and the same molecule, e.g. endostatin, can be a basement membrane component in one organ and a matrix molecule in another.

Key words: Basement membrane ultrastructure, Laminins, Nidogens, Immunogold histochemistry

Introductory remarks

The elucidation of the biochemical composition of the basement membrane components is already far advanced and there is a wealth of *in vitro* data on their binding repertoire and cell biological activities. In contrast, the molecular organisation of these molecules in basement membranes *in vivo* is largely unknown. The ultrastructural image of basement membranes has been described, but the assembly of components to make a basement membrane has not. Whether the structure is as uniform on the molecular level as implied by its ultrastructural appearance is still not known.

The basement membrane structure is altered in a wide range of pathologies. In order to influence these alterations therapeutically in the future, it is necessary to know the normal molecular construction of basement membranes *in vivo*.

The function of basement membranes and their main components

Basement membranes are condensed, polymer-like aggregates of the extracellular matrix which fulfil numerous cell biological functions (Timpl and Brown, 1996; Timpl, 1996). They not only compartmentalize tissues, but, also, support the cell phenotype and exert morphogenetic stimuli during development (Hay, 1991; Ekblom, 1993). Basement membranes are involved in embryonic development (Leivo et al., 1980; Dziadek and Timpl, 1985; Miosge et al., 1993), wound healing (Kefalides et al., 1979), metastasis (Liotta et al., 1984) and remodelling of tissues (Ekblom et al., 1996; Streuli, 1996). They function as filtration barriers in the glomerulus of the kidney (Caulfield and Farquhar,

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1978). To name just a few, basement membrane alterations are responsible for diabetic nephropathy (Goode et al., 1995) and retinopathy (Ljubimov et al., 1998), Goodpasture (Kalluri, 1999) and Alport syndromes (Zhou et al., 1991) and glomerulonephritis (Couser, 1999) and a variety of skin diseases characterized by blisters (Bruckner-Tuderman and Bruckner, 1998).

Laminins

Laminin-1, the longest known and best characterised isoform, is a cross-shaped heterotrimer, approx. 120 nm long, that consists of three glycoprotein chains (Timpl et al., 1979; Timpl and Brown, 1994; Sasaki and Timpl, 1999). According to the classification of Burgeson et al. (1994), they are named $\alpha 1$, ß1, $\gamma 1$ chains. The long arm is a coiled-coil structure of all three arms with a globular domain at the C-terminal end (Maurer and Engel, 1996). Laminin molecules polymerise through their side arms and build networks *in vitro* (Schittny and Yurchenco, 1990; Yurchenco et al., 1992). When laminin-1 is isolated from tissues, it is found as a laminin-nidogen complex (Paulsson et al., 1987a). Proteases generate laminin fragments (Ott et al., 1982), each with its own cell biological function (Edgar et al., 1984; Goodman et al., 1987). Laminin, especially the globular domain of the long arm (Sorokin et al., 1990; Aumailley and Smyth, 1998), is a binding partner for cell matrix receptors, such as integrins, of the plasma membrane. The integrin $\alpha 7$ of the muscle cell binds to the $\alpha 2$ chains of laminin-2 and laminin-4 (Yao et al., 1996). At the present time, five α, four ß and three γ chains have been identified, forming different laminin isoforms (Koch et al., 1999).

Nidogens

Nidogen-1 (150kDa), also known as entactin, is a small molecule, approx. 30 nm long, with three globular domains (G1, G2, G3), connected via two linear rod-like structures (Fox et al., 1991; Mayer and Timpl, 1994). It has a high *in vitro* binding affinity towards laminin (Fox et al., 1991). The binding site has been identified as a single LE module (*laminin-type epidermal growth factor-like*), $\gamma 1$III4, on the $\gamma 1$ chain (Mayer et al., 1993). Nidogen-1 also binds to collagen type IV (Aumailley et al., 1989) and forms ternary complexes together with laminin (Fox et al., 1991; Mayer et al., 1995). Recently a further isoform, nidogen-2 has been described (Kohfeldt et al., 1998).

Collagen type IV

Collagen type IV is the prototypic basement membrane collagen (Hudson et al., 1993; Kühn, 1994) and its monomer consists of three chains $(2\alpha 1[IV]1\alpha 2[IV])$. It forms supramolecular networks through its 7S and NC1 domains at both ends of the molecule (Timpl et al., 1981), as well as through lateral connections (Yurchenko and Ruben, 19897). Thereby collagen type IV is able to mechanically stabilise the basement membrane (Timpl et al., 1981; Yurchenco and Ruben, 1987). Up to now, collagen type IV isoforms consisting of six different α chains have been discovered (Paulsson, 1993).

Perlecan

Perlecan consists of a core-protein, formed by globular domains (400-450kDa) with two or three terminally attached heparane sulfate side chains (Hassell et al., 1980; Paulsson et al., 1987b). Its domain IV binds nidogen-1 and the nidogen-laminin complex *in vitro* (Hopf et al., 1999). Perlecan and its fragments (Klein et al., 1988) are involved in the current-dependent filtration process in the kidney (Caulfield and Farquhar, 1978).

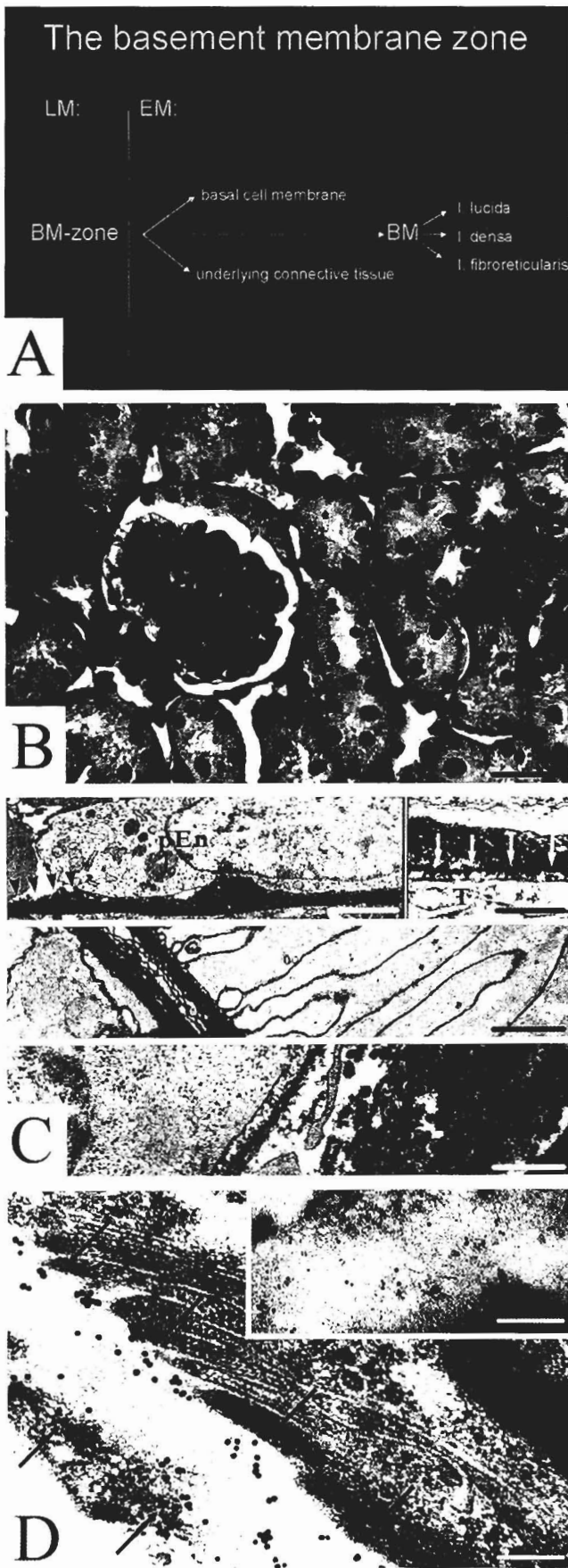## Ultrastructural localisation of basement membrane components

At the light microscopic level, matrix molecules have been localised in a linear staining pattern seen underneath epithelial cells in what we call the basement membrane zone. None the less, a basement membrane is an ultrastructure that deserves an electron microscopic approach. Only ultrastructural methods, e.g. immunogold histochemistry, can determine whether a matrix molecule is a real basement membrane component (Fig. 1).

Only few authors have applied this method to the ultrastructural localisation of basement membrane components like laminins (Herken et al., 1987; Grant and Leblond, 1988; Schittny et al., 1988; Desjardin and Bendayan, 1989; Miosge et al., 1993, 1995, 1999a), nidogen (Grant and Leblond, 1988; Schittny et al., 1988; Desjardins and Bendayan, 1989; Katz et al., 1991; Miosge et al., 1999a), collagen type IV (Grant and Leblond, 1988; Zhu et al., 1994; Miosge et al., 1999a) or perlecan (Faber et al., 1992; Akuffo et al., 1996).

Basement membrane ultrastructure and basement membrane models

Electron microscopically, the typical basement membrane displays three layers (lamina lucida, lamina densa and lamina fibroreticularis). There are also multi-layered basement membranes such as Reichert's membrane, built up of multiple laminae densae (Inoue et al., 1983; Merker, 1994). As a result of the type of fixation, a basement membrane can also have a homogeneous appearance (Goldberg and Escaig-Haye, 1986; Herken et al., 1987).

Current basement membrane models are based on biochemical *in vitro* binding studies, on the appearance of the molecules in a rotary shadowing image and the localisation of single basement membrane components in tissues. Timpl et al. (1981) described a collagen type

Fig. 1. A. Diagram of the basement membrane zone. LM: light microscopy; EM: electron microscopy; BM: basement membrane; l.: lamina. **B.** Basement membrane zones of the mouse kidney stained for nidogen-1. Bar: 25 μm. **C.** Types of basement membranes at the ultrastructural level: at the top Reichert's membrane, in the middle, tubular basement membranes of the kidney, at the bottom, basement membrane of an enterocyt. Bars: 0.3 μm. **D.** Labelling for laminin-1 of the lamina lucida and lamina fibroreticularis of a proximal tubular basement membrane of the mouse kidney. Bar: 0.25 μm; inset: same tubule, different segment, labelling for laminin-1 over the entire widths of the basement membrane. Bar: 0.3 μm. Reproduced with permission from Miosge N et al., Ultrastructural triple localization of laminin-1, nidogen-1, and collagen type IV helps to elucidate the basement membrane structure. Anat. Rec. 254:382-388 (1999).

IV network as basic scaffold of the basement membrane. Laurie et al. (1986) postulated the integration of laminin within this scaffold. Yurchenco et al. (1992) added the idea of an independent laminin network. Due to its binding activities nidogen-1 could function as the link between these two networks and thereby stabilise the basement membrane (Timpl and Brown, 1996) (Fig. 2).

The ultrastructural method

Inherent in all postembedding methods is the fixation process which is necessary for the preservation of the structure, although it does alter the antigenicity of the tissue (Hayat, 1989). A decrease in the number of the fixation steps leads to a loss of ultrastructural features of the tissues but enhances the reactivity for the immunohistochemical approach. Omission of the fixation process in order to preserve the antigenicity does not improve the overall result because one cannot correlate the reaction product with a tissue ultrastructure which is not well preserved. The method we apply is a compromise between the preservation of the ultrastructure and the antigenicity. Conventional fixation leads to a three-layered structure composed of the lamina
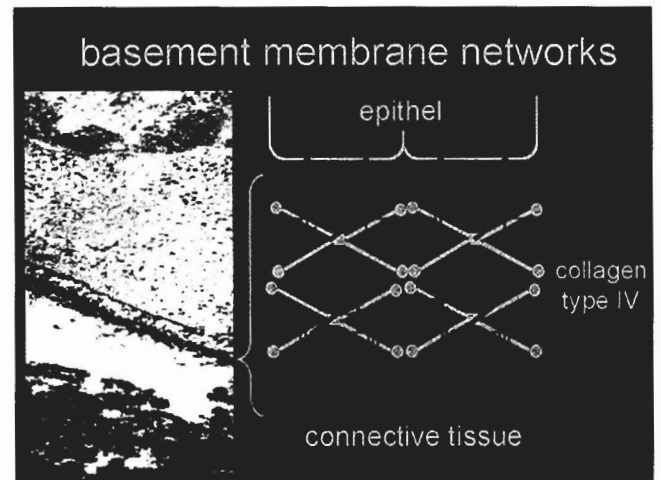

Fig. 2. Drawing of the basement membrane networks and ultrastructure of an epithelial basement membrane, not to scale and highly imaginative.

lucida, lamina densa and lamina fibroreticularis (Inoue et al., 1983; Merker, 1994), while freeze substitution generates a homogeneous basement membrane (Goldberg and Escaig-Haye, 1986). The mild fixation applied by our group also results in a homogeneous basement membrane, as does freeze substitution, thereby staying close to the situation in living tissues.

Even if a protein is a basement membrane component *in vivo*, our method can still fail to detect it. This might be due to masking of the epitope by other interacting proteins or protein folding, so that the antibody cannot bind. If we do not detect a matrix protein within the basement membrane, but instead can localise it in adjacent tissue structures, we feel confident in stating that the particular matrix protein is not a real basement membrane component, but rather a component of the adjacent extracellular matrix.

Basement membranes during early embryonic development

Even though basement membrane components are present in the morulae, our ultrastructural investigation has shown that there are no basement membranes visible at this time point. The first extracellular material is deposited outside the mural trophoblast in the early blastocyst. Also ultrastructural lectin histochemistry shows glycoconjugates in the morula, but the first stained basement membranes are again found at the blastocyst stage (Miosge et al., 1997). After implantation, the inner cell mass develops into ecto- and endoderm and the first extra-embryonic basement
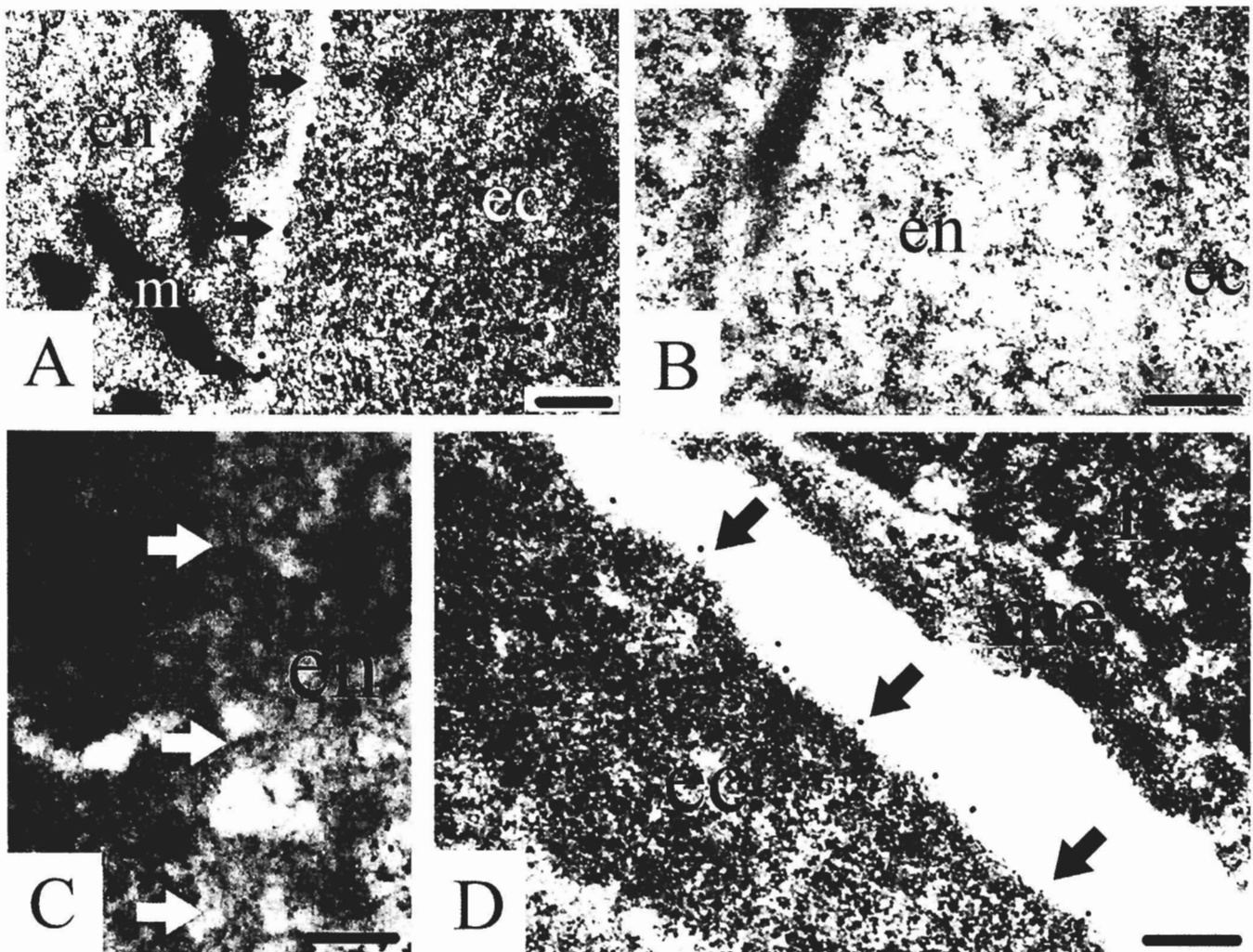

Fig. 3. A. Immunogold-histochemistry for collagen type IV (arrows) in a day 7 mouse embryo in the basement membrane between the ento- and ectoderm. m: mitochondrium. Bar: 0.16 μm. **B.** Staining for laminin-1 at the tip of the embryo between the ento- and ectoderm, where a basement membrane is still not visible. Bar: 0.25 μm. **C.** No staining for nidogen-1 (arrows) in the same area as shown in B. Bar: 0.16 μm. **D.** Nidogen-1 is present in an ectodermal basement membrane (arrows) within the embryo proper between the ectoderm and a mesodermal cell. Bar: 0.16 μm. en: entoderm; ec: ectoderm; me: mesoderm; n: nucleus. Reproduced with permission from Miosge et al., Nidogen-1, Expression and ultrastructural localization during the onset of mesoderm formation in the early mouse embryo. J. Histochem. Cytochem. 48, 229-237 (2000).

membrane, Reichert's membrane, is built with the help of the parietal endoderm cells (Salamat et al., 1995). In the mouse embryo on day 6 of development, the $\alpha 1$ chain of laminin and collagen type IV are present in the intercellular space at the border between ecto- and endoderm, where later a basement membrane is ultrastructurally visible. Laminin stimulates the neurite outgrowth (Edgar et al., 1984), the spreading of non-neuronal cells (Goodman et al., 1987) and has been connected to the migration of neural crest cells *in vivo* (Poelman et al., 1990). On day 7, the ultrastructurally detectable basement membrane between the ecto- and endoderm contains $\alpha 1$ chains of laminin in a random distribution. In those areas where the mesodermal cells leave the ectoderm they carry basement membrane remnants at their surface which are positive for laminin-1 especially at their outer border. Laminin-1 is oriented in such a way that cell binding domains are free to enable cell-cell contacts in the newly forming germ layer. The laminin molecule seems to be able to change its orientation in the basement membrane to fulfil new cell biological functions (Miosge et al., 1993).

On day 7 of mouse development, basement membrane research is particularly fruitful as there are three stages of basement membrane development to be found in one specimen. There are those that start to develop at the tip of the embryo between the ecto- and endoderm, there are fully developed basement membranes sealing the ectodermal cell layer in a different part of the embryo and those that have been degraded to allow the migration of the mesodermal cells to form the third germ layer. Laminin-1 and collagen type IV are found in all three stages of basement membrane development. In contrast, nidogen-1 is only present in fully developed basement membranes and not in those to be degraded or those at the beginning of their development (Miosge et al., 2000a). The investigation of early mouse embryos on day 7 supports the hypothesis of a stabilising action of nidogen-1 on basement membranes. Binding of nidogen-1 to laminin protects the complex from proteolytic degradation *in vitro* (Dziadek, 1995). As nidogen-1 is not found in degraded basement membranes *in vivo* this might indicate that nidogen-1 is one of the first proteins to be removed to destabilise and then disintegrate the basement membrane *in vivo* (Fig. 3).

It is well known that the $\alpha 1$, $\beta 1$ and $\gamma 1$ chains of laminin are found in the early mouse embryo. We have also found the laminin $\alpha 5$ and $\beta 2$ chains in the basement membranes of the day-7 mouse embryo (unpublished results) rendering it possible that there are more laminin isoforms present in the early mouse embryo than just laminin-1.

Orientation of laminin in adult kidney basement membranes

Immunogold histochemistry using antibodies against the C- and N-terminal domains of one molecule allows determination of the orientation of a molecule within a basement membrane (Miosge et al., 1995). Furthermore, co-localisations of two molecules can be achieved with antibodies coupled to colloidal gold particles of different sizes (Miosge et al., 1999a, 2000b). We were able not only to detect the entire laminin molecule with the help of postembedding immunogold histochemistry, but, furthermore, to localise specific domains of the molecule within the kidney basement membranes. We localised the E1 and E4 fragments of laminin mainly to the lamina lucida, while antibodies against the whole laminin molecule were found over the entire basement membrane (Herken and Miosge, 1991). Furthermore, we generated monoclonal antibodies against laminin-1 and characterised antibodies against the fragments E1 and E8 representing the N- and C-terminal pole of the laminin molecule, respectively. In the proximal tubule of the kidney the E1 fragment was always seen in the lamina lucida, while the E8 fragment was located in the lamina fibroreticularis. In the distal tubule both fragments were detected over the entire width of the basement membrane (Miosge et al., 1995). It is well known that basement membranes of different organs are composed of different isoforms of basement membrane components, e.g. laminin isoforms (Paulsson, 1993). We have as yet no indications that basement membranes of identical isoform composition might also exhibit a differing molecular arrangement. We showed, that the laminin-1 molecule is oriented in the proximal tubule of the mouse kidney, while it is randomly distributed throughout the distal tubule basement membrane. It remains to be determined why the cell binding E8 fragment of laminin-1 does not face the epithelial cell in the proximal tubule of the kidney to mediate the cell-basement membrane contact (Sorokin et al., 1990), but is found in the lamina fibroreticularis thereby pointing in the direction of the underlying extracellular matrix. It is known that also the E1 fragment of laminin-1 can bind cells *in vitro* (Sonnenberg et al., 1990). Our results lead to the speculation that the E1 fragment of laminin provides the contact to the epithelial cell in the proximal tubule of the kidney. The heterogeneity of basement membranes of different organs has been well described with regard to their isoform composition (Paulsson, 1993). We can now add the notion that also the molecular arrangement of the basement membrane components within a single basement membrane can be different (Miosge et al., 1995) and adapt to changing cell biological requirements (Korhonen et al., 1990; Virtanen et al., 1995, 1997).

Basement membrane architecture *in vivo*

With the help of single and double immunogold histochemistry, all three major basement membrane components, laminin-1, nidogen-1 and collagen type IV, were detectable over the entire width of tubular basement membranes in the kidney (Miosge et al., 1999a). In general, the molecular arrangement of

laminin-1, nidogen-1 and collagen type IV we found in kidney basement membranes *in vivo* is reflected by the recent basement membrane models summarised by Timpl and Brown (1996). In contrast, in the fetal basement membrane, collagen type IV is predominantly found in the lamina fibroreticularis. Furthermore, some segments of the adult proximal tubule basement membranes of the mouse kidney exhibited a molecular arrangement of nidogen-1 and laminin-1 in the outer layers (lamina lucida and lamina fibroreticularis) of the basement membrane, and not in the lamina densa. These results imply that the formation of basement membranes can also be more complex as suggested by the current models (Timpl and Brown, 1996).

From its binding repertoire, nidogen-1 is believed to be the link molecule for the connection of the laminin-1 and collagen type IV networks (Fox et al., 1991; Mayer et al., 1995) to stabilise the basement membrane (Timpl and Brown, 1996). Immunogold histochemistry with double labelling indeed reveals that nidogen-1 and laminin-1 are co-localised in basement membranes *in*

vivo (Miosge et al., 1999a). The co-localisation already seen in fetal basement membranes demonstrates its importance at early developmental stages. Nidogen-1 and nidogen-2 are both found in the same embryonic and fetal basement membranes (unpublished results). Nidogen-2 is also co-localised with laminin-1 in tubular basement membranes of the kidney (Fig. 4) and we have speculated that it can compensate as a link molecule for nidogen-1 in its absence (Miosge et al., 2000b).

Knock-out mice lacking the entire γ1 chain of laminin do not develop any basement membranes and die early (Smyth et al., 1999). Deletion of the nidogen-1 binding site on the γ1 chain of laminin results in mice dying at birth (Mayer et al., 1998). This model allows the investigation of basement membranes up to day 18 of development. We found some organs with ultrastructurally intact basement membranes, but also some with basement membrane defects (unpublished results). This implies that the nidogen-laminin interaction is only essential for the development of a few basement membranes. Deletion of nidogen-1 has no
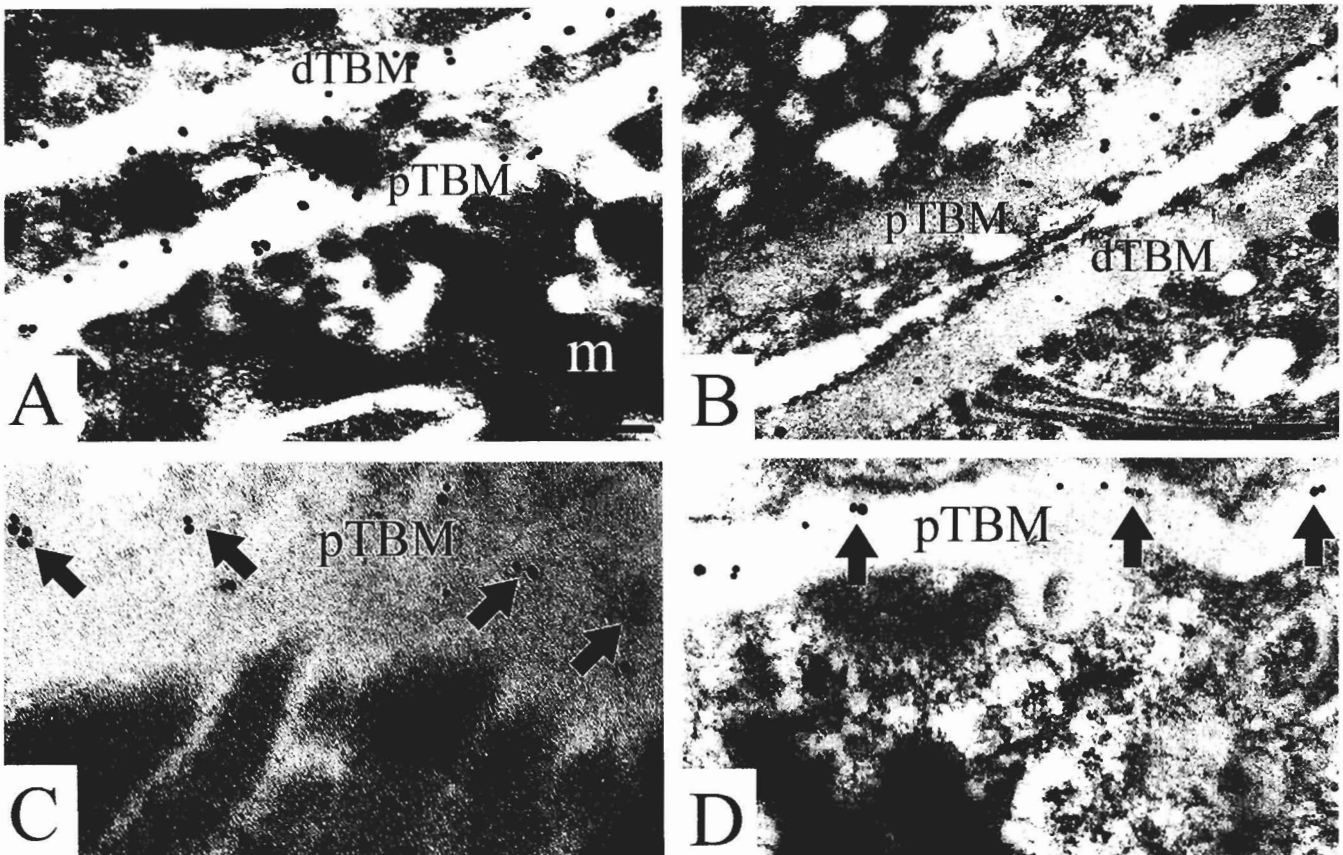

Fig. 4. Immunogold staining of nidogen-1 **(A, C)** and nidogen-2 **(B, D)** in adult mouse kidney. **A.** Labelling for nidogen-1 of adult proximal (pTBM) and distal (dTBM) tubular basement membranes, m = mitochondrion. Bar: 0.3 μm. **B.** Labeling for nidogen-2 of adult proximal (pTBM) and distal (dTBM) tubular basement membranes. Bar: 0.3 μm. **C.** Nidogen-1 is colocalized (arrows) with laminin-1 in the basement membrane of the proximal tubule (pTBM). Bar: 0.32 μm. **D.** Nidogen-2 is colocalized (arrows) with laminin-1 in the basement membrane of the proximal tubule. Bar: 0.3 μm. Nidogens are identified by large and laminin by small gold particles. Reproduced with permission from Miosge et al., Ultrastructural colocalization of nidogen-1 and nidogen-2 with laminin-1 in murine kidney basement membranes. Histochem. Cell Biol. 113, 115-124 (2000).

effect on basement membrane assembly (Murshed et al., 2000). Also loss of nidogen-2 does not affect the basement membrane ultrastructure, but there are preliminary results that the double knock-out mice exhibit altered basement membranes. This would support the hypothesis of a complementary biological function for the two nidogens.

Ultrastructural localisation of laminins and nidogens in the basement membranes of the myocyte and in the endothelial basement membrane

The main component of the myocyte basement membrane is laminin-2. We detected the $\alpha2$ chain of laminin in this basement membrane, but not the $\alpha1$ chain (Miosge et al., 1999b). The $\alpha4$ chain of laminin is not a component of the myocyte basement membrane (Talts et al., 2000), but collagen type IV, nidogen-1 and nidogen-2 are present (unpublished results). The basement membrane of the myotendinous junction is a specialised one which surrounds the interdigitated myocyte surface. Lack of $\alpha7$ integrin leads to a loss of these interdigitations; the myocyte has a rounded surface with a thickened basement membrane (Mayer et al., 1997). Furthermore, we demonstrated that $\alpha7$ integrin is the receptor for laminin-2/-4 at the myotendinous junction and that loss of $\alpha7$ integrin alters the composition of the

basement membrane at this site, with no laminin $\alpha2$ chain detectable. The basement membrane of the lateral site of the myocyte remains unaffected by $\alpha7$ integrin loss (Miosge et al., 1999b). Our ultrastructural investigation of $\alpha7$ integrin knockout mice demonstrated that $\alpha7$ integrin is the laminin-2/4 receptor at the myotendinous junction *in vivo*. Furthermore, we showed that lack of this receptor alters the molecular arrangement of the basement membrane at this tissue site. Therefore, the molecular composition of a basement membrane is not only influenced by the interaction of the molecules within the basement membrane but also by cell receptors.

Endostatin, the C-terminal domain of collagen type XVIII and a potent angiogenesis inhibitor, has been localised in basement membrane zones at the light microscopic level (Sasaki et al., 1998), but our ultrastructural investigation showed endostatin in the microfibrillar matrix adjacent to the endothelial basement membrane of capillaries. In contrast, endostatin is found in the tubular basement membranes of the kidney tubules (Miosge et al., 1999c). The endothelial basement membrane of capillaries in the mouse muscle is composed of $\alpha2$ and $\alpha1$ chains of laminin, the $\alpha4$ chain of laminin is found in the surrounding microfibrillar matrix (Fig. 5) and not in the basement membrane itself (Talts et al., 2000). A recent
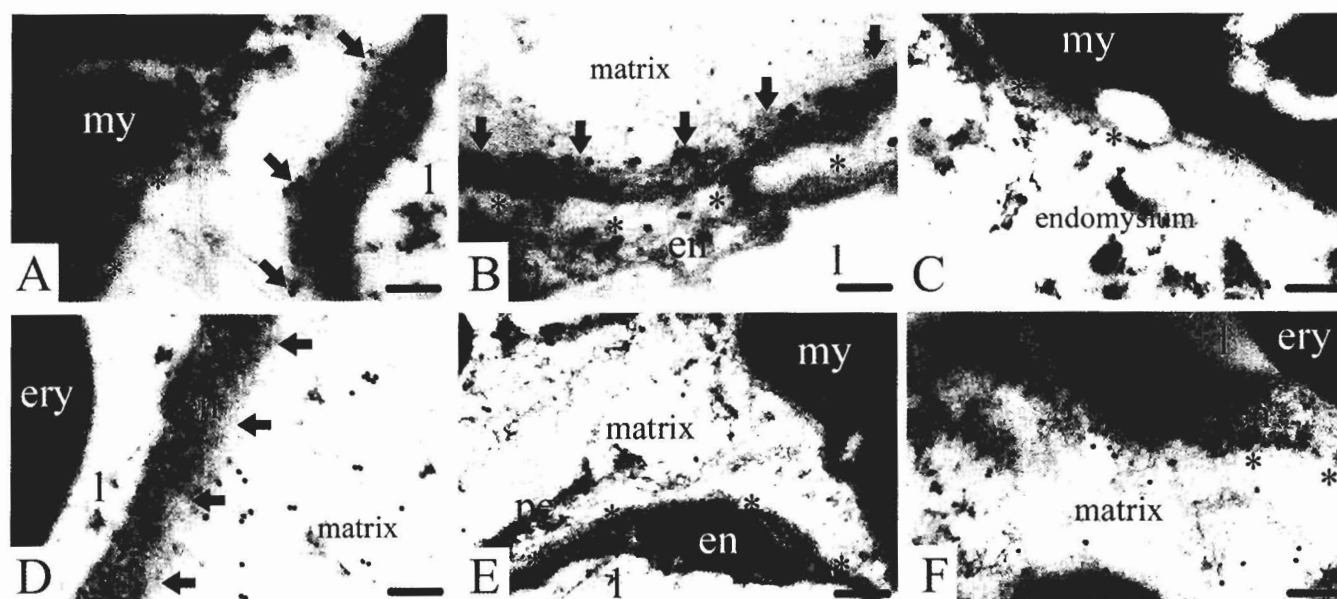

Fig. 5. **A.** Adult mouse soleus muscle, laminin $\alpha2$ chain is found in the basement membrane (asterisks) of a myocyte (my) and in the endothelial basement membrane (black arrows) adjacent to the endothelial cell (en) of a capillary. Bar: 0.32 μm. **B.** Adult mouse soleus muscle, laminin $\alpha2$ chain is found in the endothelial basement membrane (asterisks) next to the endothelial cell (en) and in the basement membrane of the pericyte (pe) of a small arteriole. l: lumen. Bar: 0.43 μm. **C.** Adult mouse heart, laminin $\alpha2$ chain is found in the matrix of the endomysium, but not in the basement membrane (asterisks) of the cardiomyocyte (my). Bar: 0.32 μm. **D.** Adult mouse soleus muscle, laminin $\alpha4$ chain is found in the matrix adjacent to a capillary, but not in the endothelial basement membrane (black arrows). ery: erythrocyte in the lumen (l) of the capillary; en: endothelial cell. Bar: 0.32 μm. **E.** The laminin $\alpha4$ chain is found in the endomysium and in the matrix adjacent to a capillary, not in the myocyte or endothelial basement membranes. Bar: 0.32 μm. **F.** The laminin $\alpha4$ chain is found in the matrix adjacent to a capillary, not in the endothelial basement membrane. Bar: 0.32 μm. Reproduced with permission from Talts et al., Structural and functional analysis of the recombinant G domain of laminin $\alpha4$ chain and its proteolytic processing in tissues. J. Biol. Chem. 275, 35192-35199 (2000).

investigation revealed the laminin ß1 and ß2 chains in the endothelial basement membrane, as well as the newly described γ3 chain of laminin. Also nidogen-1 and nidogen-2 are real basement membrane components of capillaries (unpublished results).

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