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Basement Membrane Proteins: Structure, Assembly, and Cellular Interactions

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ABSTRACT: Basement membranes are thin layers of a specialized extracellular matrix that form the supporting structure on which epithelial and endothelial cells grow, and that surround muscle and fat cells and the Schwann cells of peripheral nerves. One common denominator is that they are always in close apposition to cells, and it has been well demonstrated that basement membranes do not only provide a mechanical support and divide tissues into compartments, but also influence cellular behavior. The major molecular constituents of basement membranes are collagen IV, laminin-entactin/nidogen complexes, and proteoglycans. Collagen IV provides a scaffold for the other structural macromolecules by forming a network via interactions between specialized N- and C-terminal domains. Laminin-entactin/nidogen complexes self-associate into less-ordered aggregates. These two molecular assemblies appear to be interconnected, presumably via binding sites on the entactin/nidogen molecule. In addition, proteoglycans are anchored into the membrane by an unknown mechanism, providing clusters of negatively charged groups. Specialization of different basement membranes is achieved through the presence of tissue-specific isoforms of laminin and collagen IV and of particular proteoglycan populations, by differences in assembly between different membranes, and by the presence of accessory proteins in some specialized basement membranes. Many cellular responses to basement membrane proteins are mediated by members of the integrin class of transmembrane receptors. On the intracellular side some of these signals are transmitted to the cytoskeleton, and result in an influence on cellular behavior with respect to adhesion, shape, migration, proliferation, and differentiation. Phosphorylation of integrins plays a role in modulating their activity, and they may therefore be a part of a more complex signaling system.

KEY WORDS: basement membranes, collagen IV, laminin, entactin/nidogen, proteoglycans.

I. INTRODUCTION

Basement membranes are found in most tissues in the multicellular organism and exert a strong influence on adjacent cells. For this reason, the characterization of the biological properties of basement membranes has become an important theme in cell biology, developmental biology, and neurobiology. The purpose of the present review is to describe the structure and function of basement membranes with an emphasis on the molecular level.

From a biochemical standpoint, basement membranes are molecular composites of collagen, proteoglycans, and noncollagenous glycoproteins. The composite is formed by a process of self-assembly leading to a relatively regular structure. The constituents of the basement membrane contain binding sites for cells and the nature and number of such binding sites and the way they are presented are sensed by cell surface receptors.

A description is given of the macromolecules of basement membranes, how they assemble into a membrane structure with defined physical properties, and the mechanisms by which they interact with cells. As it is becoming increasingly clear that basement membranes in different locations

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differ in their structural and biological properties, the molecular heterogeneity of basement membranes is discussed in particular detail.

II. STRUCTURE OF BASEMENT MEMBRANE MACROMOLECULES

A. "Classical" Laminin

Laminin, the most abundant noncollagenous protein in basement membranes, was initially purified from the transplantable mouse Engelbreth-Holm-Swarm (EHS) tumor[1] and from cultures of the basement membrane-producing mouse embryonal carcinoma cell line M1536-B3.[2] Such tumor lines produce large amounts of a comparatively easily extracted basement membrane-like matrix, and their introduction led to a rapid development in basement membrane research. Most components isolated from the tumor sources have later been found in naturally occurring basement membranes, but in the meantime it is known that the tumor cells do not reflect the whole repertoire of gene expression found among normally differentiated basement membrane-producing cells. Laminin is one example of this, and, for lack of a better name, the molecule originally described from the tumor sources is referred to here as "classical" laminin.

This multidomain protein (M_r ca. 800,000) is composed of three genetically distinct polypeptide chains connected by disulfide bonds (see References 3 and 4). The polypeptides are referred to as the Ae (M_r ca. 400,000), B1e, and B2e (both M_r ca. 200,000) chains, respectively, in the modified nomenclature recently introduced[5] to provide systematic designations for laminin chain variants. By rotary shadowing electron microscopy, laminin was found to have the shape of an asymmetric cross with three short arms of 36 nm and a long arm of 77 nm.[6] More recent studies have shown that one of the short arms measures 48 nm, whereas the other two measure 34 nm,[7] which is consistent with the difference in the number of sequence repeats in domain III of the Ae and Be chains, respectively (see below).

The structure derived for "classical" laminin is depicted in Figure 1 and its fine details have been reviewed recently.[4] It is clear that in particular the B1e (1786 amino acid residues) and B2e (1607 amino acid residues) chains share a great deal of homology. In each case, domain I (M_r 40,000 to 43,000) located at the C-terminus contains numerous heptad repeats with hydrophobic residues in position 1 and 4 and charged residues in position 5 and 7, indicating the presence of a coiled-coil α-helix.[8-10] By circular dichroism (CD) spectroscopy of a proteolytic fragment (25K, the C-terminal segments of the B1e and B2e chains joined as a heterodimer by a single disulfide bond) it was possible to demonstrate that the C-terminal portions of the B1e and B2e chains are indeed in an α-helical conformation.[11] Domains II (M_r 22,000 to 24,000) also contain heptad repeats, and although these are less periodic than in domain I, an α-helical structure is predicted.[9,10] The length of the long arm as seen by electron microscopy,[6] as well as CD studies on a fragment covering nearly the entire long arm,[7] strongly support the assumption that this domain is also in a coiled-coil conformation. Domains III (M_r 37,000 to 44,000) are rich in cysteine and glycine and consist of periodic repeats with considerable homology to epidermal growth factor (EGF).[9,10] The EGF-like repeats in laminin do, however, contain eight cysteines, as opposed to six in EGF and in most other proteins with this sequence motif. Critical residues are conserved and the laminin type of EGF-like repeat could adopt the folding seen for EGF[12,13] if an extra disulfide bond is introduced, and the outer portions of the loop structures are allowed to vary.[14] Tandem repeats with an EGF-like folding would lead to an elongated compact structure, which is in good agreement with the rod-like structure seen for the inner parts of the short arms by electron microscopy.[6,7,15] Predictions based on the sequences of domains IV (M_r 22,000 to 26,000) indicate a mixture of α-helix, β-sheet and random coil, which corresponds well to the globular structure seen in this segment of the short arm by electron microscopy. Domains V (M_r 28,000 to 29,000) are similar to domains III in that they contain the same type of EGF-like repeats, and are likely to contribute the outer rod-like portion of the short arms. The N-terminal domains VI (M_r 28,000 to 29,000) show similarities to domains IV and make up the outer globules of the short arms.

The Ae chain (3084 amino acid residues) shows much homology to the Be chains, but does

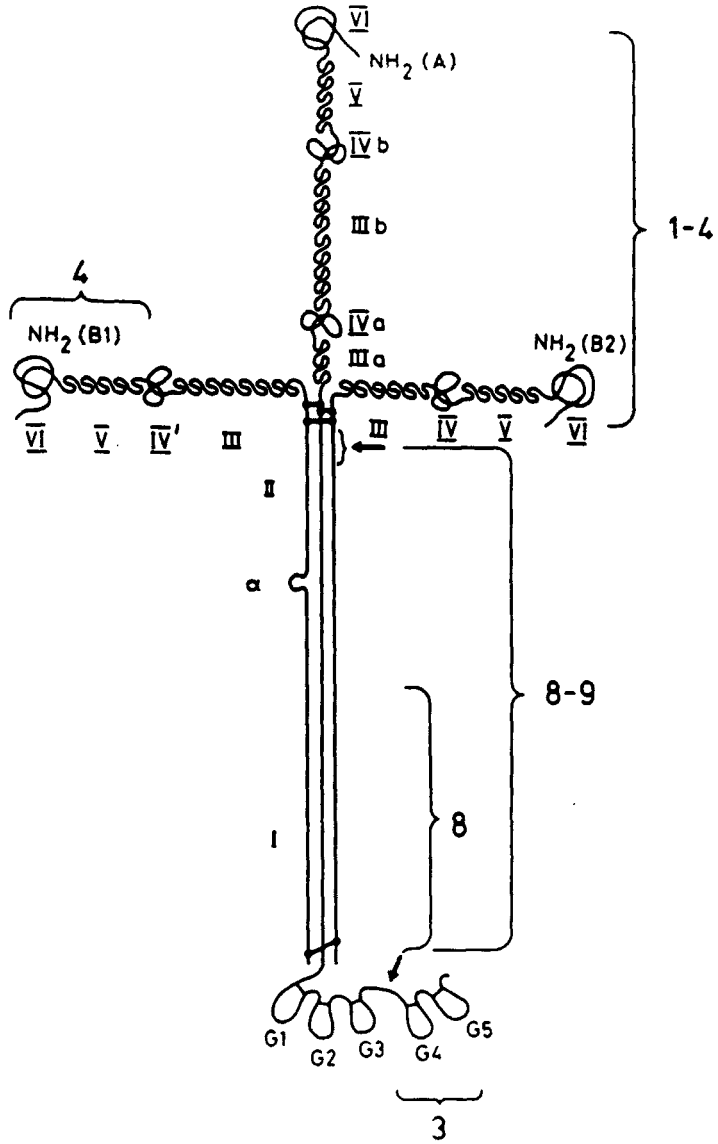

FIGURE 1. Schematic model of mouse EHS tumor laminin. Roman numerals designate structural domains and Arabic numerals refer to major proteolytic fragments. (From Beck, K. et al., *FASEB J.*, 4, 148, 1990. With permission.)

contain additional domains. Most clearly distinct is the C-terminal domain G (M_r 104,000)[16,17] for which no equivalent is found in the Be chains. Via electron microscopy[6] this domain is known to have a globular shape, and in proteolytic fragments subdomains within this globule were observed.[11] Sequence analysis[16,17] reveals the presence of five tandem repeats containing clusters of basic residues. Combining the data from sequencing and electron microscopy it can be assumed that the larger globule G is constructed from five smaller globules with a largely independent folding. All five subdomains can actually be seen by negative staining on some electron micrographs (cf. Figure 1B in Reference 4). The Ae chain contains domains I and II (combined M_r 64,000) similar to those found in the Be chains.[17] A coiled-coil α-helical structure can be predicted for these domains, but domains I and II in the Ae chain give a lower probability

value than the corresponding domains in the Be chains.[4] Although no absolute proof yet exists, all evidence points to domains I and II of the three chains forming a triple coiled-coil α-helix, which is likely to be the critical structure in the assembly of the molecule.[5,18] N-terminal from the α-helical portion of the Ae chain a domain IIIa (M_r 20,000) is found, which is similar to domain III in the Be chains in containing EGF-like repeats, but shorter. It is followed by domain IVa (M_r 23,000), similar to globular domain IV of the Be chains, by a second, longer EGF-repeat-containing domain IIIb (M_r 47,000), and by domain IVb (M_r 22,000), homologous to domains IV and IVa. Toward the N-terminus domains V (M_r 27,000) and VI (M_r 29,000) are found, which are highly homologous to their counterparts in the Be chains.[17,19] As a consequence, the short arm contributed by the Ae chain has three EGF repeat-containing, rod-like domains and three globular domains, as opposed to the two of each type found in the Be chains.

This structural description of laminin is based on the mouse sequence obtained by sequencing of cDNA clones from EHS tumor and from teratocarcinoma cell libraries[8-10,16,17,19,20] and by protein sequencing of mouse EHS tumor laminin.[11,16,19] For the Ae chain the human sequence is available[21,22] and shows 76 to 78% amino acid identity with the mouse Ae chain. The B1e and B2e chain sequences have been completed for human[23,24] and for *Drosophila* laminin.[25-28] The amino acid sequence of the human B1e chain showed 93% homology to that from mouse,[23] and the human B2e chain is also highly homologous to the mouse chain.[24] The domain structure is conserved between mouse and human in both of these chains. The *Drosophila* B1e chain also retains the domain structure found for mouse, but the extent of sequence homology is lower.[25] Interestingly, while the *Drosophila* sequence shows only 25% identity with the mouse sequence in domains I, II, and IV, the amino acid identity in domains III, V, and VI is about 55%. The fact that the EGF-like domains and the N-terminal globule are more highly conserved in evolution could indicate that these regions perform particularly important functions. The B2e chain is similarly conserved between mouse[10] and *Drosophila*[26-28] and shows the same domain structure. The *Drosophila* B2e chain is more highly homologous to the mouse B2e chain than to the *Drosophila* B1e chain, suggesting that these two chains result from a gene duplication prior to the divergence leading to chordates and arthropods.[28] Laminins from *Drosophila*,[29] sea urchin,[30] and leech[31] have been characterized by rotary shadowing electron microscopy, and a conservation of domain structure was seen, with the exception of the consistent observation of a variation in the length of the long arm in invertebrates, possibly due to extra domains.

The structure of laminin is stabilized by interchain disulfide bonds.[1] A disulfide bond between two cysteines close to the C-termini of the B1e and B2e chains, respectively, has been demonstrated.[11] At the center of the cross, domain II of each polypeptide contains two cysteines that could form a disulfide knot connecting all three chains.[17] At least one disulfide bond involving the Ae chain must be present in this region, as this chain cannot be released from the Be chains without reduction. The presence of disulfide bonds between all three chains would be in good agreement with much evidence from protein fragmentation, but formal proof is lacking. The presence of cysteines in the globular domains of laminin indicates that these domains are stabilized by intrachain disulfide bonds, but assignment of these bonds by means of protein chemistry has not been completed.

EHS tumor-derived laminin is rather highly glycosylated and the values for carbohydrate content obtained by compositional analysis range from 12 to 15[32,33] to 25 to 27%.[34] It must be remembered that glycosylation is a posttranslational modification, and it is not known how well glycosylation of tumor-derived laminin reflects glycosylation in normal tissues. The surprisingly large variation in values for carbohydrate content in EHS tumor laminin may even indicate that this tumor line has, over years of propagation in different laboratories, developed sublines that differ in posttranslational events. The sequences of the three chains of EHS tumor laminin contain a total of 73 potential attachment sites for N-linked oligosaccharides, 46 of which are on the Ae chain and the rest equally divided between the two Be chains.[9,10,17] The glycosylation sites are unevenly distributed over the molecule and are particularly enriched in the long arm.[4] As the carbohydrate composition shows an abundance of mannose and

N-acetylglucosamine, it appears that laminin contains only N-linked oligosaccharides.[34] These represent a very complex array of structures where it is clear that bi- and triantennary structures are present as well as the blood group I structure and polylactosaminyl chains.[33-35] The presence of high mannose and tetraantennary oligosaccharides that was reported by one group[33] has been challenged by other investigators.[34] The importance of this rich glycosylation is largely unknown, but it can be assumed that as a consequence the long arm of laminin is a highly hydrophilic structure. A characterization of the glycosylation of laminin from nontransformed sources would be of interest, in particular as the oligosaccharides have been implicated in cell-matrix interactions.[36-40]

B. Laminin Isoforms

A number of independent observations indicated that laminin present in different locations and expressed by different cell types is not always identical in structure. It is of some interest to summarize the evidence for heterogeneity among laminins that led to a more systematic search for laminin isoforms.

Pepsin-extracted laminin fragments from human placenta showed differences from those of EHS tumor laminin, indicating that they could be derived from a molecule related to but distinct from laminin.[41] Laminins isolated by high salt and detergent from human placenta did indeed show an additional polypeptide of M_r 240,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[42-45] This polypeptide, designated M, did, however, crossreact with antisera raised against tumor-derived laminin,[42] and it could not be excluded that it was a degradation fragment of the Ae chain.

Another line of evidence came from the study of the expression of the known laminin chains in different biological systems. It was noted that the Ae, B1e, and B2e chains are not synchronously expressed during early development of the mouse embryo, and, while the B1e and B2e chains could be identified at the 4- to 8-cell stage, the Ae chain was detected first at the 16-cell stage.[46] In other studies it was shown that monoclonal antibodies directed against a certain laminin chain stained only some but not all basement membranes, even though laminin was ubiquitous when polyclonal sera against the whole molecule were used.[47-49] When cDNA probes directed against laminin chains became available, it was noted that the mRNAs for the respective chains were expressed in a disproportionate manner in many organs.[50,51] Different amounts of mRNA do not, however, necessarily lead to unbalanced amounts of protein, as translation could proceed at varying rates. In embryonal kidney mesenchyme it was shown by immunochemistry[52] and Northern blot analysis[53] that while the Be chains are constitutively expressed the Ae chain is detected first after the embryonic induction, leading to the formation of a polarized epithelium, and that its expression is transient. In a survey of different organs of the embryonic mouse it was found that the Ae chain expression was much more restricted than that of the Be chains.[54] In studies of laminin-like proteins from rat schwannoma with neurite-outgrowth promoting activity, it was found that such molecules lacked the Ae chain and had an aberrant appearance in electron microscopy, showing only two short arms.[55,56] Indeed, in the mouse sciatic nerve very little (if any) Ae chain mRNA or polypeptide can be detected, even though the Be chains are expressed.[57]

The studies on expression indicate that a certain chain from "classical" laminin can be missing and despite this a laminin-like protein is present. Considering the structure of laminin, this would be more easily explained if another polypeptide, fulfilling the requirement of ability to participate in the formation a coiled-coil α-helix, replaced it. One such polypeptide, designated S-laminin, was detected by the use of monoclonal antibodies that selectively stained synaptic basement membranes.[58] By use of these antibodies cDNA clones could be isolated from a rat kidney library that coded for a protein of M_r 190,000, which showed extensive homology with the mouse laminin Be chains.[58] The homology was higher to the B1e than to the B2e chain, but the possibility that the S-laminin chain represents the rat B1e chain could be excluded, as the corresponding cDNA probes recognize distinct restriction fragments on Southern blots of genomic DNA. In the extended nomenclature[5] it is therefore referred to as B1s. The B1s chain has all domains contained in the B1e chain, and as observed also for the *Drosophila* B-chains, the ho-

mology is highest in domains III, V, and VI, corresponding to the regions with EGF-like repeats and to the N-terminal globular domain. Although antibodies against the B1s chain stain the basement membrane at the neuromuscular junction particularly intensely, B1s is not restricted to this location.[58,59] It is also detected in the perineural basement membranes of peripheral nerves, in the glomeruli of kidney, and in arterial basement membranes.[59] Its staining pattern is reciprocal to that observed with antibodies against the laminin B1e chain,[59,60] and indeed, when a mixture of heterotrimeric laminin-like molecules from placenta was studied by use of chain-specific monoclonal antibodies no molecular species containing both B1e and B1s could be identified.[60] It thus appears that B1s is an isoform of the B1e chain and that these are mutually exclusive in the formation of heterotrimers.

Merosin was first identified as a M_r 65,000 polypeptide in proteolytic digests and a M_r 80,000 polypeptide in tissue extracts of human placenta.[61] Immunofluorescence showed a distribution restricted to the basement membranes of Schwann cells, striated muscle, and trophoblasts.[61] When cDNA clones coding for merosin were sequenced, an open reading frame much longer than that corresponding to the M_r 80,000 polypeptide was found, and the so-far determined 1130 amino acid residues from the C-terminus of merosin showed a 39% identity with the sequence of the corresponding portion of the mouse laminin Ae chain.[62] The domain structure with five repeats in domain G is conserved. The M_r 80,000 fragment represents the C-terminal portion of this domain. An antiserum, raised against a peptide corresponding to a deduced sequence N-terminal of the M_r 80,000 polypeptide, recognized a large chain of M_r 300,000 in a placental extract, indicating that these two polypeptides are originally part of the same primary translation product, the M_r 80,000 polypeptide being the C-terminal part and the M_r 300,000 chain being the N-terminal portion.[62] This gene product is referred to as the Am chain in the following section.[5] The combined size of the two polypeptides is similar to that of the Ae chain. The cleavage is believed to be a physiological event, as it has so far not been possible to isolate an M_r 380,000 Am chain from tissue extracts.[62] The M_r 80,000 polypeptide remains associated with the rest of the molecule during purification.

In parallel, a laminin-like protein was purified from mouse heart and designated "heart laminin".[63] The critical step in the purification was a selective extraction with EDTA, presumably dissolving Ca^{2+}-dependent laminin aggregates in the tissue.[64,65] This laminin-like protein also contained a M_r 300,000 polypeptide and little or no Ae chain. Antibodies raised against the M_r 300,000 chain did not react with the Ae chain and vice versa, indicating that the two chains are distinct. Heart laminin contained Be chains and in rotary shadowing electron microscopy gave images similar to those of EHS-tumor laminin.[63] When the same purification procedure was applied to human placenta a laminin-like protein, containing the M_r 300,000 polypeptide of Am as its heavy chain, was obtained.[62] Comparison of the "heart laminin" M_r 300,000 chain and the merosin heavy chain by use of sequence-specific antibodies showed a high degree of similarity,[66] leading to the conclusion that the two preparations are species homologs of the same polypeptide, i.e., the Am chain. When laminin-like heterotrimers from human placenta were analyzed by chain-specific monoclonal antibodies, it appeared that the Am and the Ae chains were mutually exclusive.[60] Proteolytic fragmentation of heart laminin showed that the Am polypeptide spans the long arm in a way similar to the Ae chain in "classical" laminin,[66] and from the combination of these results it can be concluded that the Am chain is an Ae chain isoform and takes the place of the Ae chain in laminin-like proteins.

Evidence, although less direct, exists for the existence of still more laminin isoforms. By immunoprecipitation of keratinocyte culture media with antibodies against laminin, a family of oligomeric molecules with M_r values ranging from 370,000 to 950,000 were identified.[67] In addition to the Ae, B1e, and B2e chains, these immunoprecipitates contained polypeptides of M_r 195,000, 170,000 and 160,000.[67] One of these chains could be B1s, but even though proteolytic degradation cannot be excluded, the results indicate the presence of more B chain-sized laminin polypeptides. Unorthodox laminin-like proteins were also demonstrated in cultures of 3T3-L1

adipocytes[68] and of endothelial cells.[69] In those systems the B1e and B2e chains combine with a M_r 180,000 polypeptide, designated A′ by its discoverers, to form laminin-like complexes. From its size, A′ is unlikely to be similar to the Am chain. Interestingly, in the endothelial cells, which express both Ae and A′ chains, their expression is under hormonal regulation, and the angiostatic steroid medroxyprogesterone suppressed the A′ synthesis and stimulated the Ae production. The differences between Ae and A′ are in the polypeptide backbone and are not a result of differential glycosylation. Similarly, in the case of the laminin-like proteins from schwannoma cultures that have been shown to contain mainly B chain sized polypeptides,[55,56] biosynthesis experiments indicate that the lack of a normally sized Ae chain is not due to proteolytic processing.[56] Schwannomas express the Am chain, presumably as part of a laminin-like molecule,[70] and the heterogeneity of the laminin-like proteins expressed in these cells requires further examination.

C. Entactin/Nidogen

This molecule was first described as a M_r 158,000 sulfated glycoprotein present in a basement membrane-like matrix produced by the mouse endodermal cell line M1536-B3, and called entactin.[71] From the EHS tumor a M_r 80,000 polypeptide was purified and designated nidogen.[72] Immunoblotting with specific antibodies to nidogen showed that the M_r 80,000 polypeptide was derived from a protein of M_r 150,000,[73] which is highly sensitive to endogenous proteases present in the EHS tumor. The relationship between entactin and nidogen remained controversial until the sequences for the two preparations were completed and turned out to be identical.[74,75] In the following this protein is referred to as entactin/nidogen, but a better name would probably be the laminin C chain, as it is expressed together with laminin in normal tissues and is laid down in the basement membrane matrix as a noncovalent complex with laminin (see below). In an early paper entactin/nidogen was actually referred to as the laminin C chain, as it could be immunoprecipitated from culture media of parietal endoderm cells by an antiserum against laminin.[76]

Electron microscopy of the intact M_r 150,000 entactin/nidogen, purified from the EHS tumor, revealed a dumbbell-like structure containing one larger and one smaller globule connected by a 16-nm long rod-like domain[77] (Figure 2c). Analysis of the murine entactin/nidogen sequence[74,75] confirmed this model, showing that a 641 residues long N-terminal sequence forms the larger globular domain I (M_r 85,000). Domain II (M_r 27,000), i.e., the rod-like portion, is made up from five consecutive EGF-like repeats. These do, in contrast with those found in laminin, contain only six cysteine residues each, and therefore conform better to the classic EGF consensus motif. The C-terminal globular domain III has a M_r of 38,000 and corresponds to the smaller globule seen by electron microscopy.[77] The sequence of human entactin/nidogen is 85% homologous to that from mouse on the amino acid level and shows conservation of domain structure.[78,79] Posttranslational modifications of entactin/nidogen include glycosylation, with probably both N- and O-linked oligosaccharides making up a total of 5% of the molecular mass,[77] as well as tyrosine sulfation.[80] The entactin/nidogen sequence contains two consensus sequences for tyrosine sulfation and two potential acceptor sites for N-linked oligosaccharides.[74,75]

D. The Laminin-Entactin/Nidogen Complex

Soon after the discovery of entactin/nidogen, indications pointed to its association with laminin. As mentioned above, entactin/nidogen was coprecipitated by specific antibodies to laminin.[71,76] It has a broad distribution among basement membranes, similar but not always identical[81,82] to that of laminin.[71] Entactin/nidogen could be identified in guanidine HCl extracts of a variety of mouse tissues, and was found together with laminin in close to equimolar amounts in tissue extracts, while the stoichiometric expression was lost in some culture systems.[83] It was shown to interact with laminin in a variety of recombination assays.[73]

The isolation of laminin-entactin/nidogen

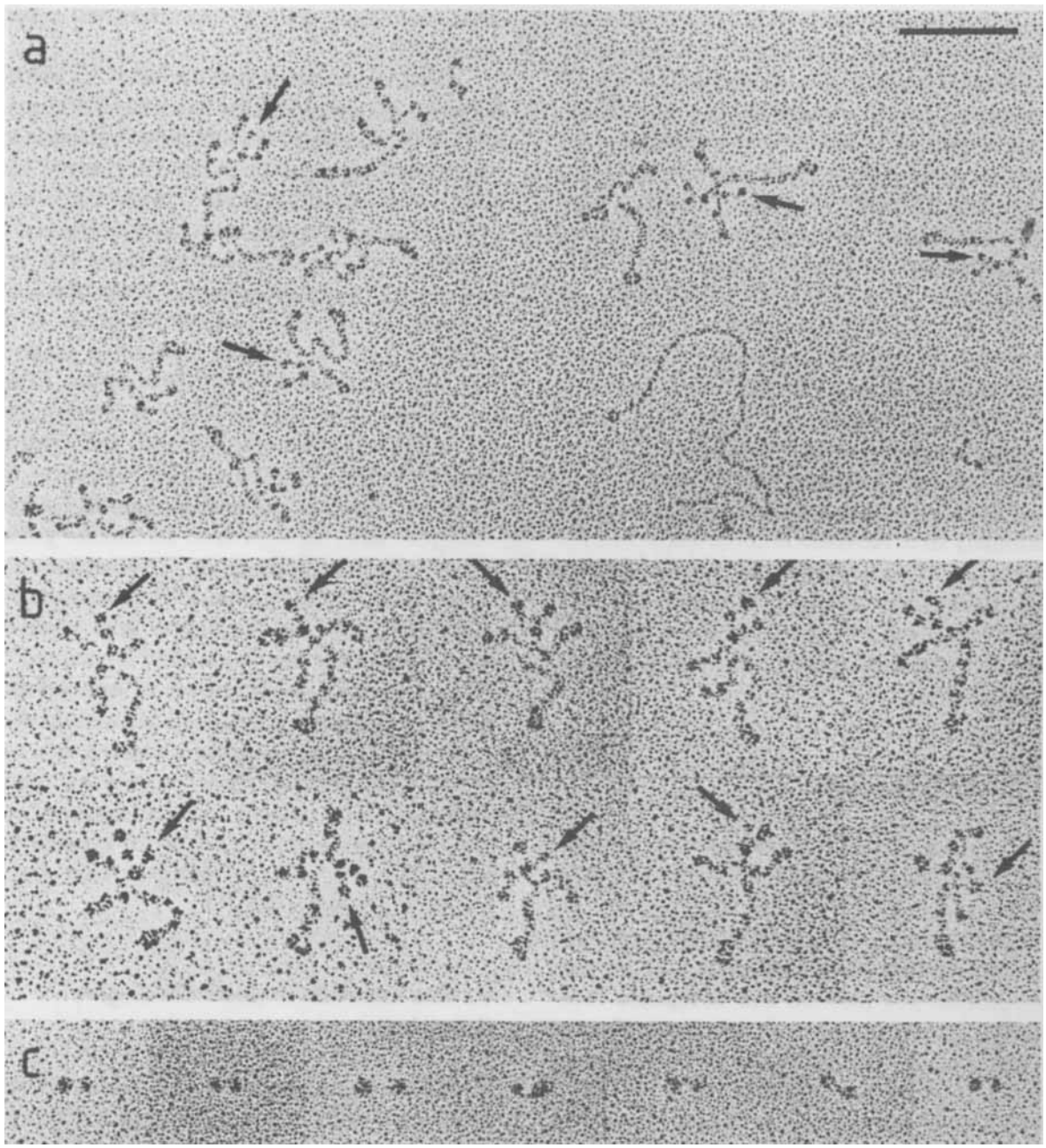

FIGURE 2. Rotary shadowing electron microscopy of (a,b) laminin-entactin/nidogen complexes and of (c) dissociated entactin/nidogen. (a) Shows an overview, while (b) and (c) display selected particles. Bar = 100 nm. (From Paulsson, M. et al., *Eur. J. Biochem.,* 166, 11, 1987. With permission.)

complexes remained difficult because of the susceptibility of entactin/nidogen to proteolytic degradation.[73] The conventionally used protocols for laminin extraction from EHS tumor tissue[1] yielded only fragments of entactin/nidogen. Purification of the intact complex became possible through the discovery that laminin-entactin/nidogen complexes are rapidly and selectively extracted from tissues by buffers containing chelating agents such as EDTA.[84] Analysis of such complexes demonstrated that laminin and entactin/nidogen do indeed occur in an equimolar ratio in the complex, and by rotary shadowing electron microscopy it could be shown that the entactin/nidogen molecule binds via one of its globular domains to the inner rod-like domain of one of the short

arms of laminin[84] (Figure 2). The laminin-entactin/nidogen complexes could be dissociated by treatment with 2 *M* guanidine HCl, and recombination experiments showed a high affinity of entactin/nidogen for fragment 1 of laminin, representing the inner portions of the laminin short arms[84] (Figure 1). More detailed studies employing proteolytic fragments of laminin and entactin/nidogen indicated that the interaction is mediated by the C-terminal globular domain III of entactin/nidogen, and that it has an apparent K_d of 10 to 20 n*M*.[85] Biosynthetic studies of the formation of the laminin-entactin/nidogen complex show that the complex is formed shortly after translation, and suggest that transport to the extracellular compartment requires complex formation,[86] in addition to a correct assembly of the laminin subunits.[87] Entactin/nidogen has also been found associated with laminin-like proteins extracted from normal tissues, such as heart muscle.[63] Although much evidence supports the notion that laminin and entactin/nidogen are always expressed together in naturally occurring basement membranes and that entactin/nidogen is an integral part of the laminin molecule, a lack of formal proof exists, and the question may be semantic.

E. Collagen IV

The collagen of basement membranes has been studied longer than the other molecular constituents. For a description of the pioneer work on this subject some earlier reviews are recommended.[88,89] Particularly important observations were that basement membranes contain a genetically distinct collagen, type IV,[88] which is, as opposed to the fibrillar collagens I to III, not proteolytically processed upon secretion.[90] As in the case of laminin, the introduction of the transplantable, basement membrane-producing, mouse EHS tumor[91] was of great importance in providing a plentiful source of material. Dissection of the EHS tumor collagen with proteolytic enzymes led to the isolation of the major triple helical fragment,[92,93] of a minor triple helical fragment designated 7S,[94,95] and a globular domain NC1.[96] Analysis of these fragments, as well as of more intact forms of collagen IV extracted by acetic acid or mild pepsin digestion, allowed the construction of a model for its domain structure and supramolecular organization.[96,97]

According to this model, collagen IV, consisting of three M_r 180,000 polypeptide chains, is divided into three major domains[96] (Figure 3a). At the N-terminus a 30-nm-long triple helical domain 7S is found (M_r 26,000 per polypeptide). After a short nontriple helical interruption the major, 360-nm-long, triple helix follows (M_r 120,000 per polypeptide). This triple helix contains frequent interruptions in the typical Gly-X-Y repeat structure of collagen helices, and is therefore more flexible and also more sensitive to proteolysis than the corresponding domains in the interstitial collagens I to III.[98] At the C-terminus the globular NC1 domain follows (M_r 25,000 per polypeptide), which does not have the characteristic collagenous amino acid composition, i.e., does not contain hydroyproline and only moderate amounts of glycine.[96] By use of rotary shadowing and electron microscopy it could be shown that four molecules of collagen IV are frequently connected by association of the 7S domains, forming a "spider-shaped" structure[97] (Figure 3b), and that two molecules can interact in a head-to-head fashion via the NC1 domains[96] (Figure 3b). The combination of these two types of interactions allows the formation of a network[96] that could serve as a scaffold for the basement membrane. This structural model, initially based on analysis of proteolytic fragments, could be confirmed with collagen IV isolated from the medium of cell cultures, where both kinds of associations were observed.[99,100]

Biosynthesis studies in several cell culture systems demonstrated two polypeptide chains, α1(IV) and α2(IV), which had different mobilities in SDS-PAGE, and which gave different cleavage patterns on protease digestion and CNBr fragmentation, indicating their distinct nature.[101-103] Characterization of avian,[104,105] bovine,[106] and human[107] collagen IV indicated the subunit composition $[\alpha 1]_2\alpha 2$; however, evidence exists for some homotrimers of α1 chains in rat.[108] The complete sequence has been determined for the α1 chain from man,[109,110] mouse,[111,112] and *Drosophila*,[113] and for the α2 chain from man[114] and mouse.[115] The sequences confirm the domain structure determined by electron microscopy,[96] showing at the N-terminus a 7S region with a triple-helical prediction and rich in cysteine and

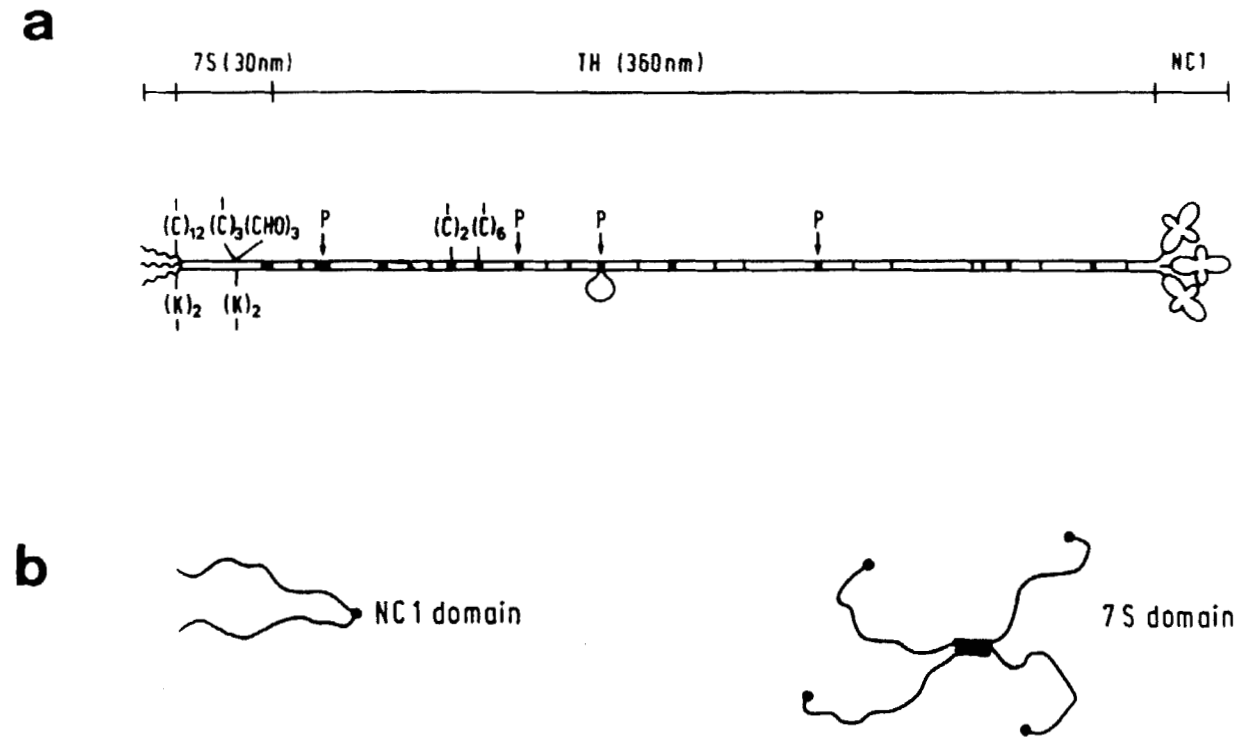

FIGURE 3. Schematic model of (a) a collagen IV molecule and (b) its mode of assembly into oligomers. Black bars along the triple helix (TH) indicate imperfections in the Gly-X-Y repeat. Crosslinking can occur via cysteine (C) or lysine/hydroxylysine (K) residues. CHO: N-linked carbohydrate. P: pepsin-sensitive peptide bonds. (From Timpl, R., *Eur. J. Biochem.*, 180, 487, 1989. With permission.)

lysine residues, and at the C-terminus a noncollagenous NC1 domain. The major triple helix in mouse does have 21 interruptions of the Gly-X-Y repeat in the α1 chain and 24 interruptions in the α2 chain.[112] Most of the interruptions are in the same position in the two chains, and on alignment a total of 26 imperfections are found. These do not show any sequence similarity between α1 and α2, but are well conserved for each chain between mouse and man.[112] The overall sequence identity between man and mouse is 90.6% for the α1 chain and 83.5% for the α2 chain, while there is only 43% identity between α1 and α2 in mouse. In *Drosophila* only one α chain has been detected, and presumably all of the collagen IV molecules are homotrimers.[116] The domain structure of the α chain in *Drosophila* is similar to that of α1 in man and mouse, and in particular the NC1 domain is highly conserved.[113] Of the 22 imperfections in the *Drosophila* sequence, 11 are found in the same site as in the human α1 chain, but the amino acid sequences in those interruptions differ.[113] It appears then that the positioning of the helix imperfections is on the whole more conserved, and perhaps more important, than the actual sequence at those sites.

Collagen IV contains numerous interchain disulfide bonds and lysine-derived crosslinks. The disulfide bonds are localized in the 7S domain in the major triple helix at a position about $^1/_3$ from the N-terminus, and in the NC1 domain.[117,118] The isolated NC1 domain, as obtained by collagenase digestion, is a hexamer consisting of three globular domains derived from each of the two associated molecules of collagen IV.[119] On dissociation in SDS both monomers and dimers are obtained, demonstrating the presence of crosslinks. As the dimers can be converted to monomers by reduction, and free sulfhydryls are present in the native NC1 domain, it appears that dimerization occurs through rearrangement of disulfide bonds during maturation of the assembled molecules.[120] The dimers formed by disulfide exchange always connect two α chains derived from the two different molecules of collagen IV that are assembled head-to-head via the NC1 do-

main.[118] The NC1 domain of collagen IV is the first basement membrane component that was crystallized.[121] A high-resolution structural analysis could provide much information about the association of collagen IV molecules. Collagen IV contains N-linked biantennary heteropolysaccharides bound to the 7S domain, in addition to the hydroxylysine-linked disaccharides common to most collagens.[122]

F. Isoforms of Collagen IV

The recent identification of isoforms of collagen IV is of particular interest, as the novel collagen IV chains α3 to α5 were found through investigation of different forms of kidney disease. The Goodpasture syndrome is a severe autoimmune disorder, consisting of the triad of glomerulonephritis, lung hemorrhage, and antiglomerular antibody formation (for a review, see Reference 123). The glomerular basement membrane antigen, against which the autoimmune antibodies are directed, was identified as a collagenase-resistant structure of a size and amino acid composition similar to the NC1 domain of type IV collagen.[124] A detailed analysis of NC1-derived monomer polypeptides from glomerular basement membranes did, however, show a more complex pattern than could easily be explained on the basis of only α1 and α2 chains.[125] N-terminal sequencing of two such novel monomers gave sequences distinct from each other and from those of the known α chains.[126] The chain carrying the Goodpasture epitope was designated α3[127] and the other novel chain α4.[128] By indirect immunofluorescence microscopy of glomeruli, it could be shown that antibodies against the α1 and α2 chains bind to mesangial matrix and along the subendothelial region of the glomerular capillary wall.[129] Antibodies against the novel chains bound to the phase dense aspect of glomerular basement membrane and to Bowman's capsule. These distinct patterns indicate a restricted topographic distribution of the different collagen IV isoforms.[129] More extended studies in a variety of tissues showed that α3 and α4 are much enriched in synaptic basement membranes in muscle, while they are absent from the extrasynaptic areas and from the basement membranes of nerves and arteries.[59] Although we lack conclusive evidence on the isoform composition of the triple helical collagen IV molecules, the tissue distribution indicates that expression of α3 and α4 is more closely coupled than that of these chains with α1 and α2.

Yet another novel collagen IV chain, α5, was identified through studies of the Alport type of familial nephritis. This is a hereditary disorder primarily characterized by progressive renal insufficiency and sensorineural hearing loss. Patients with Alport's syndrome have abnormalities in the ultrastructural appearance of the glomerular basement membrane, and after renal transplantation sometimes develop an autoimmune nephritis (see Reference 130 for a review). Antibodies from such a patient reacted with epidermal basement membranes of controls, but not with those of other Alport patients.[130] The antibodies recognized a distinct monomeric polypeptide in NC1 preparations, which is apparently altered in Alport's nephritis. Analysis of families suffering from Alport's syndrome by use of the antibodies, indicated an X-linked dominant inheritance.[130] Gel electrophoresis of the NC1 domain isolated from Alport's patients showed the absence of a M_r 28,000 monomer that occurs in controls, while the M_r 26,000 and 24,000 monomers derived from the α1 and α2 chains were present at normal levels.[131] cDNAs were isolated that coded for a polypeptide homologous to, but distinct from, the α1 to α4 chains of collagen IV, and the gene for this novel collagen IV chain, α5, was assigned to the Alport syndrome locus on the X chromosome.[132,133] On further analysis, mutations were found in the gene for the α5 chain in Alport kindreds,[134] clearly demonstrating that Alport's syndrome is due to a defect in this collagen IV polypeptide. In the meantime the C-terminal 95% of the amino acid sequence has been deduced.[135] The domain structure of the α5 is the same as in the α1 and α2 chains, and the positions but not the sequence of the interruptions in the Gly-X-Y repeats are conserved.

As in the case of laminin, the identification of novel collagen IV chains raises a number of biological questions that largely remain to be answered. It is important to determine which chains *in vivo* form collagen trimers with each other, and how the collagen IV isoforms contribute to the structural and functional diversity of basement membranes.

G. Basement Membrane Proteoglycans

The presence of a proteoglycan component in basement membranes was first recognized in morphological studies employing probes for anionic sites in kidney glomeruli, such as cationized ferritin and ruthenium red.[136] The anionic sites are considered important for the glomerular filtration properties, in particular with respect to retention of anionic plasma proteins in the circulation. Ruthenium red-positive particles, resembling connective tissue proteoglycans in morphology, were seen at regular 60-nm intervals along the glomerular basement membrane.[136] It was shown that the anionic particles could be removed by incubation with heparitinase or nitrous acid, treatments that result in a specific degradation of heparan sulfate, but not with enzymes degrading other types of glycosaminoglycans or sialoglycoproteins.[137] Shortly thereafter, glycosaminoglycans were isolated from glomerular basement membranes, and the major component directly identified as heparan sulfate.[138]

Isolation of the corresponding heparan sulfate proteoglycan was first achieved in the EHS tumor model. The proteoglycan was of high molecular mass, and antibodies raised against the tumor-derived proteoglycan stained basement membranes in skin, kidney, and cornea, showing that it is a widespread basement membrane component.[139] Synthesis of basement membrane-type heparan sulfate proteoglycans was demonstrated in cultures of parietal endoderm,[140] PYS-2 teratocarcinoma cells,[141] and aortic endothelial cells.[142] Fractionation of the heparan sulfate proteoglycans from the EHS tumor led to the isolation of two distinct forms.[143,144] One of these is a large proteoglycan displaying a low-buoyant density in CsCl gradient centrifugation, which indicates a low carbohydrate-to-protein ratio.[144] In addition, a smaller, high-density proteoglycan with a high proportion of heparan sulfate is present in the EHS tumor.[143,144] Similar populations of large and small proteoglycans could be identified in the culture medium of PYS-2 teratocarcinoma cells,[145] and in cultures of parietal yolk sac from mouse.[80]

A structural characterization of the low-density proteoglycan from the EHS tumor showed that the core protein has a M_r of about 500,000 in sedimentation equilibrium centrifugation and carries only three heparan sulfate chains.[146] The heparan sulfate chains are of variable length, dependent on the strain of EHS tumor studied. With a M_r range of 30,000 to 80,000 for each glycosaminoglycan, a M_r of 600,000 to 800,000 is obtained for the whole proteoglycan.[146] By electron microscopy the core protein was seen as a 80-nm-long tandem array of globular domains, with all three heparan sulfate chains attached to one terminal domain[146-148] (Figure 4). Partial sequences have been deduced for the proteoglycan core protein.[149] Interestingly, these demonstrate the presence of EGF-like cysteine repeats similar to those found in laminin,[9] as well as repeats containing two cysteines each, homologous to sequences forming loop structures in N-CAM and other members of the immunoglobulin family.[150] Similar or identical large heparan sulfate proteoglycans have been isolated from cultures of endothelial cells[151,152] and, more surprisingly, from the extracellular matrix produced by cultured fibroblasts.[153,154] The latter result indicates the possibility of basement membrane proteoglycans being derived from mesenchymal cells as well as from epithelium and endothelium.[154] Also, expression of collagen IV[155] and laminin[156] has been detected in mesenchymal cells, and it appears clear that such cells can contribute several components of basement membranes.

The nature of the small proteoglycans in basement membrane remains more uncertain, and much evidence points to the possibility that this is not a single molecule, but a mixture of several proteoglycan populations. The small high-density heparan sulfate proteoglycans isolated from the EHS tumor were described as having a M_r of 130,000 with a protein core of M_r 5000 to 12,000[143] (Figure 4) or as having a core protein of M_r 95,000 to 130,000.[144] Antibodies raised against the high- and low-density proteoglycan cross-reacted with both proteoglycans.[144,157] Pulse-chase studies with EHS tumor cells indicated that a large low-density proteoglycan is formed first, and with time proteolytically processed to a smaller high-density form.[158] Later studies on the metabolism of proteoglycans in minced EHS tumor tissue did in contrast show that even though large low-density proteoglycans were lost with time due to proteolysis these were not the precursors for the high-density proteo-

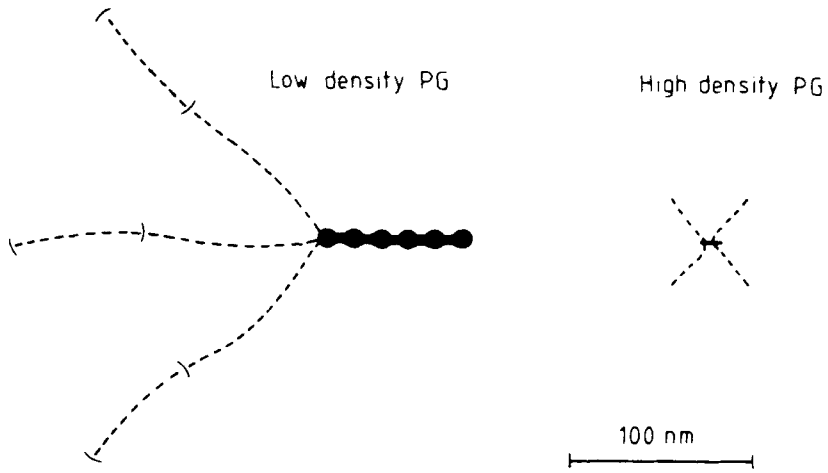

FIGURE 4. Structural models of basement membrane proteoglycans purified from the mouse EHS tumor. Solid structures are protein and dashed lines are heparan sulfate. Parentheses are used to indicate variability. (From Paulsson, M. et al., *J. Mol. Biol.*, 197, 297, 1987. With permission.)

glycans.[159] The high-density proteoglycans were described as a mixture of four different forms with core proteins in the range of M_r 21,000 to 34,000, which are glycosylated with both heparan sulfate and chondroitin sulfate.[159] The demonstration of chondroitin sulfate in the high-density proteoglycans is particularly important as this glycosaminoglycan has never been found in the low-density form, and this result would therefore not be compatible with a precursor-product relationship. A final answer will, however, come first if the high- and low-density proteoglycans from the EHS tumor system can be shown to have distinct protein sequences.

The proteoglycans from glomerular basement membrane also have been studied extensively. Early investigations described them as low molecular weight proteoglycans substituted with both heparan sulfate and chondroitin sulfate, but on separate core proteins.[160] In purified glomerular basement membranes the heparan sulfate proteoglycans are the major population, accounting for 70% of the total glycosaminoglycan.[161] Characterization of purified heparan sulfate proteoglycan from glomerular basement membranes indicated that it has a M_r of 130,000 to 150,000, with a core protein of M_r 18,000, and contains heparan sulfate chains of M_r 25,000.[162] Other studies of a glomerular basement membrane proteoglycan gave a somewhat larger size, M_r 200,000, and a much larger core protein of M_r 130,000, substituted with four heparan sulfate chains (M_r 14,000) clustered within a short peptide segment.[163-165] As the proteoglycans isolated from glomerular basement membrane were smaller than the large EHS tumor proteoglycan, it was long controversial whether they are related to this molecule or are distinct gene products. By use of antibodies against the large EHS tumor proteoglycan, it could be shown that the smaller glomerular core proteins are immunochemically similar to this proteoglycan, and are presumably derived by proteolysis from a precursor core protein of the same size as that in the EHS tumor.[166] This does not exclude the existence of additional proteoglycan populations in glomerular basement membrane. Recent studies of the proteoglycans synthesized by glomerular epithelial cells in culture show that although most of the glycosaminoglycan produced is heparan sulfate, some chondroitin sulfate/dermatan sulfate can also be identified, and among the heparan sulfate proteoglycans there is clear heterogeneity.[167-169] Similar complex patterns are also obtained after *in vivo* labeling.[170]

The presence of glycosaminoglycans other than heparan sulfate in basement membranes was highlighted in immunohistochemical studies with monoclonal antibodies specific for carbohydrate epitopes, showing a broad distribution of chon-

droitin-6 sulfate to basement membranes.[171] The parietal yolk sac of rodents is a much-studied basement membrane, and when yolk sacs were kept in culture the major proteoglycan synthesized was a chondroitin sulfate with a total M_r of 800,000 to 900,000 in rat[172] or 500,000 to 600,000 in mouse.[173] The size of the core proteins were M_r 130,000 in rat and M_r 160,000 in mouse, values sufficiently close so that it is quite possible that the studies concern the same proteoglycan from two species. For both proteoglycans it could be shown that they are intrinsic components of the yolk sac basement membrane,[173,174] and the mouse molecule was detected in a wide range of basement membranes, with the remarkable exception of the glomerular basement membrane.[175]

Not only the protein cores, but also the glycosaminoglycan portion may show biologically interesting variations. When heparan sulfate from the EHS tumor was compared with that from mouse yolk sac, very different structures were found. The EHS tumor heparan sulfate has an amount of N-sulfate groups within the range typical for heparan sulfates, while O-sulfates are unusually scarce.[176,177] Heparan sulfate from parietal yolk sac, on the other hand, carries N- and O-sulfate groups in about equal proportions, contains 3-O-sulfated glucosamine residues characteristic for the high-affinity antithrombin-binding structure of heparin, and does indeed bind to antithrombin with high affinity.[176] One possible explanation for the differences would be that the 3-O-sulfation of glucosamine is transformation sensitive. This hypothesis was supported in studies of heparan sulfate from basement membrane proteoglycan from cultured mammary epithelial cells, which showed a significant decrease in 3-O-sulfated glucosamine content and antithrombin affinity on transformation.[178] Also among heparan sulfates from normal tissues, a considerable variation of the proportion of heparan sulfate that binds with high affinity to antithombin can be found ranging from 4.7% of total heparan sulfate in kidney to 21.5% in brain.[179] The potential biological importance of the structural variations in basement membrane heparan sulfates is emphasized in recent studies that show that the major proportion of anticoagulantly active heparan sulfate in the vascular wall is present in the subendothelial basement membranes.[180]

Although basement membrane proteoglycans are not yet as well characterized as, for example, laminin or collagen IV, it is clear that this group of molecules has great potential for providing structural and functional diversity to basement membranes. Not only is it likely that different types of proteoglycan core protein can be expressed in a topographically and perhaps temporally restricted manner, but differences in post-translational modifications such as glycosylation might create variability among proteoglycans with the same core protein.

H. Basement Membrane-Associated Proteins and Novel Basement Membrane Components

SPARC/osteonectin/BM40 was identified in different systems and given several names before it became clear that it was one molecule. In the following it is referred to as SPARC (secreted protein acidic and rich in cysteine), as this is the most descriptive term, and appropriate to a protein where the function remains an enigma. SPARC was first purified from bone calcified matrix, where it is a major noncollagenous matrix component and more enriched than in any other tissue.[181] It was named osteonectin on the basis of the assumption that it was a bone-specific protein linking mineral to collagen.[182] Even though SPARC binds Ca^{2+} and may influence mineralization, it is not bone specific and the search for its biological role continues.

The reason that SPARC is often considered a basement membrane protein is that it is produced in large amounts by basement membrane producing cells in endothelium,[183] parietal endoderm,[184] and EHS tumor.[185] The similarity of SPARC from those sources and osteonectin from bone became obvious by sequence comparison, which showed such a high degree of homology that all differences could be due to species variation.[184,186,187] Interpretation of the SPARC sequence allowed the presentation of a tentative structural model[184,187,188] (Figure 5). At the N-terminus of the M_r 33,000 protein, domain I contains two segments of 14 to 15 amino acids in each of which 7 to 8 residues of glutamic acid are found, and for which an α-helical structure is predicted. Domain II is rich in cysteines and gives a prediction of β-structure alternating with

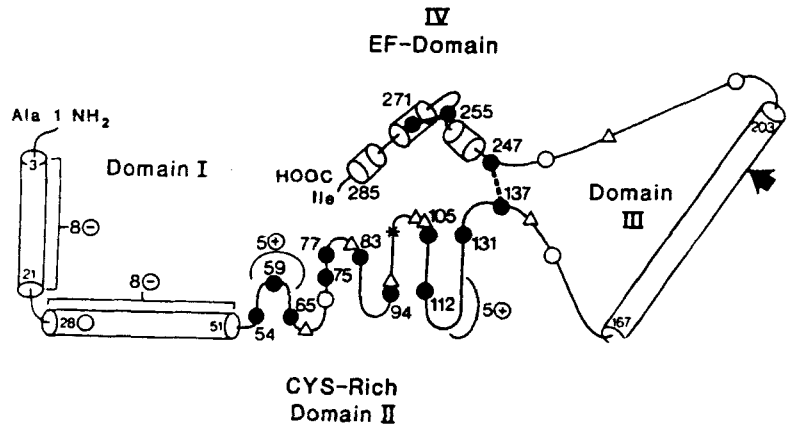

FIGURE 5. Schematic representation of SPARC. Cylinders indicate parts of the sequence that give an α-helical prediction. Cysteine (●), methionine (○), and serine (△) residues are indicated. The arrow indicates a preferred site for proteolytic cleavage. (From Engel, J. et al., *Biochemistry*, 26, 6958, 1987. With permission.)

β-bends. Within this domain there is a clear homology to the third domain of ovomucoid,[189] but so far it has not been possible to demonstrate that SPARC functions as a protease inhibitor. Domain III is predicted to be predominantly α-helical. Toward the N-terminus in domain IV, a distinctive feature is found in the form of a so-called EF-hand motif, i.e., a helix-loop-helix structure that functions as a Ca^{2+}-binding site in many intracellular proteins.[190] A Ca^{2+}-dependent increase in α-helix content could be demonstrated by CD spectroscopy, indicating that SPARC actually binds Ca^{2+}.[188]

Further studies have shown that SPARC has a broad tissue distribution. On the mRNA level particularly high expression was found in osteoblasts, odontoblasts, parietal endoderm, deciduum, hair follicles, peripheral nerves, skin, and submucosa of the stomach.[191] In immunohistochemistry a preferential location was epithelia in gut, skin, and glands that exhibit high rates of turnover.[192] In the fetus high levels of SPARC antigen were found in heart, thymus, lung, and gut, and in areas undergoing chondrogenesis, osteogenesis, and somatogenesis. SPARC is present in platelets,[193] is released upon stimulation with collagen or thrombin, but also expressed on the surface of the activated platelet;[194] it forms complexes with thrombospondin,[195] another protein present both in platelets and in extracellular matrix. It is difficult to see a clear pattern to this tissue distribution, and SPARC is certainly found in many other locations in addition to basement membranes. In recent studies a specific Ca^{2+}-dependent binding of SPARC to the triple helical portion of collagen IV has been demonstrated, both for the protein purified from the mouse EHS tumor[196] and for recombinant human SPARC.[197] This provides a mechanism for the anchorage of SPARC in basement membranes, even though it does not in itself prove a structural function. Other studies suggest a role for SPARC in modulating shape of endothelial cells, smooth muscle cells, and fibroblasts,[198,199] a function that would certainly be compatible with a localization to basement membranes.

A number of proteins associated with basement membranes are of great functional importance; although probably not structural components of the basement membrane in a narrow sense. Prominent among those is collagen VII,[200] the protein that forms the anchoring fibrils,[201,202] i.e., structures that connect basement membranes in skin to the underlying stroma (for a review, see Reference 203). Collagen VII is a trimer of α chains that are believed to be identical.[200] The pro-α-chain has a M_r of 350,000 and is made up from a C-terminal globular NC1 domain (M_r per chain: 150,000), a triple helical domain (M_r per chain: 170,000), and a N-terminal globular do-

main NC2 (M_r per chain: 30,000).[204,205] Two molecules of collagen VII form an antiparallel dimer of 780 nm length by an association of the N-terminal parts of the triple helix, which with time becomes covalently stabilized by disulfide bonds.[206,207] It appears that the N-terminal NC2 domain has been proteolytically removed in the tissue form of type VII collagen.[205] The C-terminal part of collagen VII shows a trident shape in rotary shadowing electron microscopy in which each part is contributed by the NC1 domain of one α chain,[207] and it is suggested that these arms interact with components of the basement membrane. The C-terminal globular domain of type VII collagen has been identified as the antigen against which patients with the blistering skin disease epidermolysis bullosa acquisita form autoantibodies.[208]

Agrin is a component of the specialized basement membrane in the synaptic cleft of the neuromuscular junction.[209] It was first purified from the electric organ of the marine ray *Torpedo californica* as a set of polypeptides of M_r 150,000 to 70,000.[210] The heterogeneity might be due to proteolysis of a large precursor. Agrin has the ability to cause clustering of acetylcholine receptors, acetylcholinesterase, and buturylcholinesterase on cultured myotubes, and is therefore considered a synaptic organizing protein.[210] Agrin also causes patching of laminin and heparan sulfate proteoglycan on cultured myotubes, indicating an interaction with those basement membrane components.[211]

Two novel proteins have been purified recently from the EHS tumor and suggested as potential basement membrane components. Bamin has a M_r of 72,000 to 80,000 and appears immunochemically distinct from known basement membrane components.[212] Antibodies raised against bamin stained glomerular basement membranes, indicating that it is present in at least some normal basement membranes. BM90 is a M_r 90,000 Ca^{2+}-binding glycoprotein found in basement membranes, but also in considerable amounts in serum.[213] The cellular source of BM90 is not yet known and it remains to be investigated if it originates in basement membranes, or if it becomes selectively adsorbed to these structures from serum.

Patients suffering from tubulointerstitial nephritis have autoantibodies directed against an antigen in the tubular basement membrane of the kidney. This antigen was recently purified and shown to be a protein of M_r 58,000 that comprises as much as 9% of the mass of the tubular basement membrane.[214] It is not found in the glomerular basement membrane, nor in the EHS tumor, and appears to be a novel, tissue-specific, noncollagenous basement membrane protein.

Several groups have demonstrated the presence of basic fibroblast growth factor (bFGF) in basement membranes.[215-217] The basement membranes appear to be a storage compartment for bFGF, which is bound to the heparan sulfate proteoglycans in the matrix.[218,219] Experiments showing blockade of endothelial cell proliferation on subendothelial extracellular matrix by antibodies against bFGF indicate that the basement membrane-bound growth factor is biologically active.[220] These findings demonstrate a new biological role for basement membranes as a potential growth factor presenting structure. As a side-product of these studies other novel, low-molecular-mass heparin-binding proteins of unknown function have been identified in basement membranes.[221] The presence of growth factors in basement membrane preparations should be kept in mind when proliferative responses are interpreted as being due to the action of basement membrane macromolecules.

Plasminogen activator is produced by trophoblasts and parietal endoderm during embryogenesis, and it has been suggested that this protease plays a role in the remodeling of the extraembryonal basement membranes.[222] Both plasminogen activator and plasminogen bind to immobilized laminin, and laminin is a substrate for plasmin.[223] EHS tumor matrix contains significant amounts of tissue type plasminogen activator and the protease remains associated with purified preparations of laminin.[224] As tissue type plasminogen activator also binds to and is activated by heparin,[225] a role for basement membrane proteoglycan in regulation of tissue proteolysis is possible. Further, endothelial cells, in particular microvascular cells, synthesize plasminogen activator inhibitor-type I together with basement membrane proteins.[226] Taken together, these findings indicate that basement membranes contain an intrinsic proteolytic system that might play a role in tissue remodeling and also in clearing debris to retain a filter function.

Other plasma proteins have been found associated with basement membranes. It has been suggested that complement factor C1q can bind to laminin, and that this interaction would provide a mechanism for deposition and retention of immune complexes in basement membranes.[227] Complement factor C3d has been detected in basement membranes of the glomerulus and trophoblast,[228] and C3d,g is present in epidermal basement membrane.[229] The biological relevance of these observations is still not clear, but C3 components in basement membranes could play a role in fixation of immune complexes and attraction of leukocytes. Also, serum amyloid P-component has been found associated with glomerular basement membranes.[230]

III. ASSEMBLY OF BASEMENT MEMBRANES

Basement membranes are formed by a process of self-assembly. The molecular components of the membrane do themselves contain the information needed for the assembly in the form of specific binding sites for other macromolecules. A large number of studies have described interactions among basement membrane proteins, but in many of these insufficient evidence for specificity and physiological relevance was provided. An uncritical reader could get the impression that each basement membrane protein can bind every other component present. In the following section an attempt is made to describe a small selection of such studies on interactions and assembly that have employed appropriate biochemical and biophysical methodology, and where the results appear to be well reproducible. This aspect of basement membrane structure has been the subject recently of a more specialized review.[231]

A. The Network of Collagen IV

The network formation of collagen IV has already been mentioned in the context of molecular structure. Dimerization via the NC1 domain and formation of tetramers via the 7S domain (see Figure 3) would give a network with a mesh size (length between two identical crosslinking sites) of about 800 nm if pictured in two dimensions.[96] It was demonstrated by gelation experiments and by electron microscopy of reformed collagen IV meshworks that lateral interactions between triple helices can also take place[232] in a manner related to fibril formation among fibrillar collagens. Incorporating such lateral interactions into the network model led to the suggestion of a layered hexagonal planar array with sides of 170 nm,[232] i.e., a considerably smaller mesh size than in the earlier model. The presence of lateral interactions was confirmed in ultrastructural studies on human amniotic basement membrane[233] and mouse EHS tumor.[234] The networks seen were, however, of rather irregular structure. An interesting novel feature was the observation of supramolecular helices, presumably representing twisting of laterally associated molecules of collagen IV.[233,234] A model for the collagen IV network based on this line of work is shown in Figure 6A. Lateral aggregation of collagen IV, as well as potential twisting into superhelices, would be compatible with the X-ray diffraction patterns obtained with the bovine lens capsule basement membrane.[235] Studies on water flow through the rat lens capsule point to the deformability and elasticity of the native basement membrane.[236] Although both the end-to-end and lateral association mechanisms can be considered as proven, we must be cautious in constructing more detailed models, as the specimens observed in the electron microscope have often been exposed to harsh or denaturing treatment, and we may be underestimating the dynamic nature of basement membrane structure.

B. Laminin Self-Aggregation

In gelation studies, similar to those classically employed for collagens, it was found that laminin forms aggregates in a time-, concentration-, and temperature-dependent manner.[64] The process was reversible upon lowering the temperature from the permissive range of 21 to 37°C to 4°C, and on addition of EDTA. The latter result indicated a dependence on divalent cations, and it could indeed be shown that laminin-entactin/nidogen complexes bind Ca^{2+}, and that

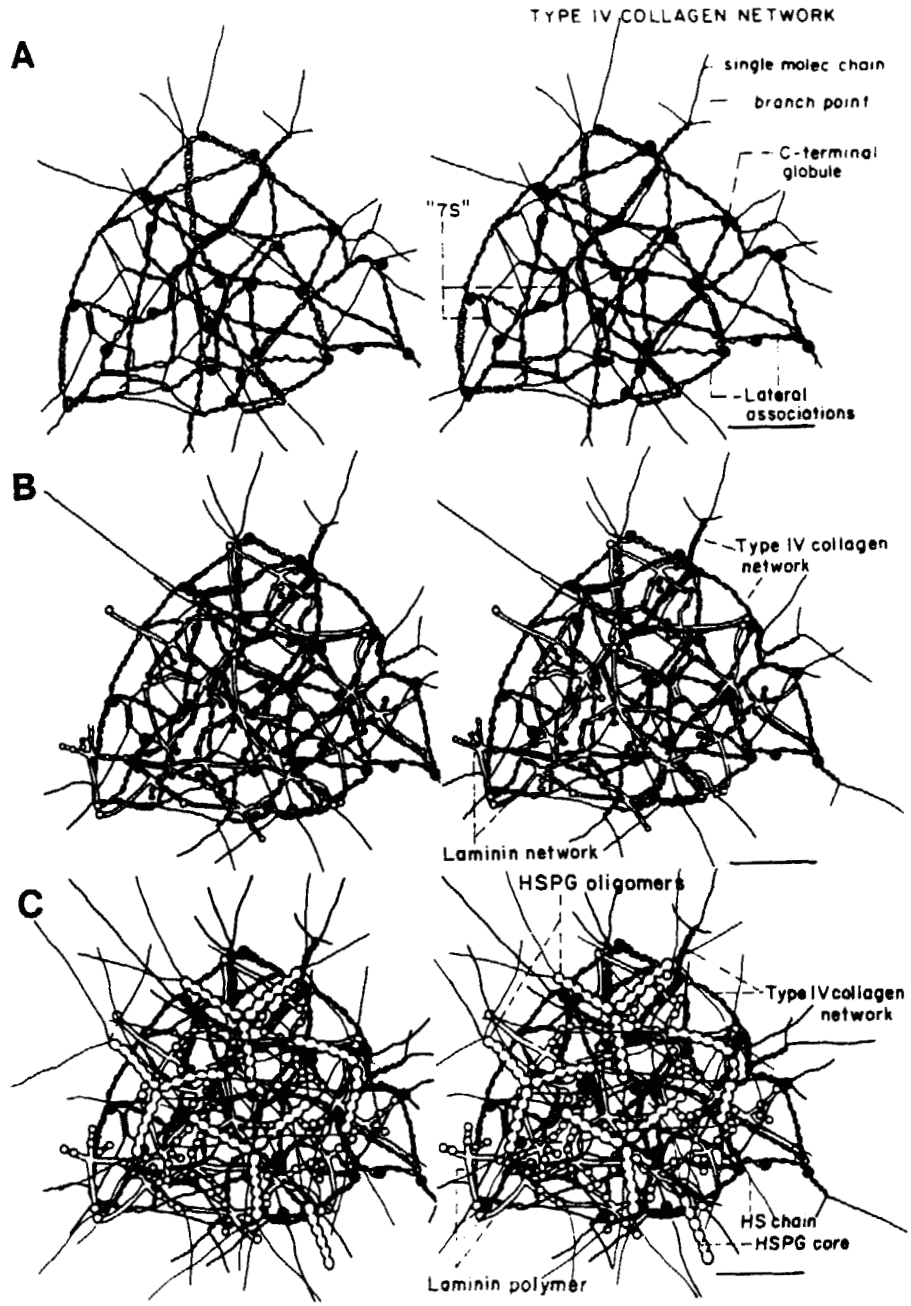

FIGURE 6. Tentative models for basement membrane assembly. In A the assembly of the type IV collagen network is shown, important features being both N- and C-terminal interactions of the monomers as well as lateral associations of the triple helical portions. In B a model is shown for the manner in which self-aggregating laminin molecules could be anchored in the collagen network. The laminin molecules aggregate primarily via binding sites on their short arms, and might be connected to collagen IV by the entactin/nidogen molecule. In C the low-density proteoglycans have also been introduced into the composite. The core proteins are believed to have capacity for self-association, but their mode of association with laminin and collagen IV is unknown. Bars = 100 nm. (From Yurchenco, P. D. and Schittny, J. C., *FASEB J.*, 4, 1577, 1990. With permission.)

Ca^{2+}-binding induces the aggregation.[65] Although initial studies indicated a two-step aggregation mechanism, involving binding sites on both the long and the short arms,[64] more recent work has pointed to sites on the short arms being of particular importance.[7,237,238] Evidence for the *in vivo* relevance of the divalent cation-dependent laminin self-aggregation comes from experiments showing that laminin or laminin-like proteins may be selectively solubilized from the mouse EHS tumor,[84] heart muscle,[63] and human placenta[62] by use of chelating agents. Laminin is apparently anchored in the tissue matrix by a divalent cation-dependent mechanism. The self-aggregation observed *in vitro* is likely to contribute to this anchorage, even though there might be additional mechanisms. Studies in embryonal sciatic nerve showed that laminin is largely soluble and becomes insoluble first postnatally.[57] Such results imply that not just expression and distribution, but also the interactions of laminin could be under developmental control. When laminin-like proteins are isolated from normal, mature tissues, nonreducible crosslinks within the laminin-entactin/nidogen complexes are found,[63] indicating that laminin anchorage in the basement membrane might with time become covalent. Similar crosslinking of laminin-entactin/nidogen complexes can be obtained *in vitro* by the action of liver transglutaminase,[239] and it is possible that transglutaminase-mediated isopeptide bond formation is also a mechanism for crosslinking in basement membranes *in vivo*.

C. Integrating the Assemblies of Collagen IV and Laminin

Each of the major basement membrane proteins appears to have a separate self-assembly mechanism. It is still not quite clear how dependent these assemblies are on each other. In early work a direct interaction of laminin with collagen IV was suggested on the basis of molecular electron microscopy of mixtures.[240,241] The binding was inhibited by antibodies against epitopes on the long arm of laminin,[242] and this domain was also often seen in contact with collagen IV in the electron micrographs. After the purification of entactin/nidogen and the laminin-entactin/nidogen complex, similar experiments were again performed and showed binding of entactin/nidogen and of the complex to collagen IV in both molecular electron microscopy and enzyme-linked immunosorbent assay (ELISA) -style interaction assays.[243] In this study, however, no affinity of laminin alone or of laminin fragments for collagen IV could be detected, and it is uncertain if the early results showing direct binding[240-242] were due to an at that time not recognized entactin/nidogen contamination in the laminin preparations. Under all circumstances it appears that the laminin-entactin/nidogen complex, which is presumably the *in vivo* form of laminin,[63,84] can bind to collagen IV and thereby create bridges between the polymers of collagen IV and laminin, forming a more stable composite (Figure 6B). It should be noted that both laminin[244] and collagen IV[245] can be detected separately and also outside actual basement membranes, and may be present in a soluble form or as independent molecular assemblies. Further, both collagen IV[155] and laminin[156] can be produced by mesenchymal cells. These basement membrane proteins may be more widely spread than originally thought and could be part of a variety of supramolecular structures.

D. Interactions of Basement Membrane Proteoglycan

Despite the wealth of studies on proteoglycan interactions, virtually nothing is known about the situation in basement membranes *in vivo*. It has been shown that purified large heparan sulfate proteoglycan from the EHS tumor forms dimers and oligomers via a protein-protein interaction.[147] As the proteoglycan was extracted by use of denaturing agents the results must be viewed with caution, but they are strengthened by the fact that proteolytic removal of a terminal portion of the core protein abolished the interaction. It was shown that laminin binds to heparin,[246] and that heparan sulfate proteoglycan from the EHS tumor binds weakly to laminin and collagen IV.[143] On the basis of this information, models for proteoglycan assembly, like those shown in Figure 6C, can be constructed, but these are at the moment only tentative. The simple fact that the EHS tumor proteoglycans require denaturing agents for their extraction would point to the importance of protein-protein interactions in their anchorage.

Laminin-glycosaminoglycan interactions appear to be much influenced by the degree of sulfation, and lowly sulfated heparan sulfates show only weak affinity.[247] The heparin-binding properties of collagen IV also have been subject to detailed study,[248,249] but again evidence for *in vivo* relevance is lacking. When laminin was purified from a variety of cell culture media, it was found to be present as a complex with a proteoglycan.[55,250-254] The stability of the complex against high salt concentrations[252] indicates that it is held together by protein-protein interactions rather than those that are heparan sulfate dependent. In one case a bound proteoglycan could even be shown to inhibit the biological activity of laminin.[251] Structural characterization of laminin-proteoglycan complexes from cell cultures is likely to provide information about the interactions relevant for basement membrane assembly.

E. Bridging the Gap between Molecular and Ultrastructural Levels

Purely ultrastructural studies of basement membrane fall outside the scope of the present article and readers are referred to a recent review.[255] On the whole it has been difficult to explain the morphological findings in molecular terms, but some more recent studies have had the specific aim of localizing particular proteins or protein domains within subcompartments of the basement membrane, and thereby providing information on molecular localization and orientation. The partial resolution of the collagen IV network by electron microscopy was mentioned above.[233,234] Other studies have used immunolabeling with antibodies against defined epitopes on laminin followed by electron microscopy to determine not only localization, but also orientation of molecules.[256,257] Results obtained with the epithelial basement membrane of the mouse cornea pointed to short arm structures of laminin being found mainly in the center of the basement membrane.[256] The long arm is variable in orientation but often points to one of the surfaces. Similar studies of the basement membranes in mouse kidney also showed localization of long and short arm structures to discrete microdomains.[257] The results did, however, indicate that orientation of laminin molecules varies with location, pointing to an organizational heterogeneity of basement membranes. When the rat kidney was investigated with antibodies against a broad range of basement membrane antigens, a marked compositional heterogeneity between the different basement membranes was seen.[258] It is likely that further work along those lines will reveal heterogeneity and also common features of basement membrane structure.

IV. CELLULAR INTERACTIONS OF BASEMENT MEMBRANE MACROMOLECULES

Numerous studies have been concerned with the effects of basement membrane macromolecules on a variety of cell types. It goes beyond the scope of the present article to summarize all the descriptive biology that has been performed with basement membrane-derived reagents. Even restricting the review to the more molecular and mechanistic work is difficult, as many controversial and in part contradictory results have been put forward. Gradually, though, a consensus is appearing that laminin and collagen IV are major mediators of contacts between basement membranes and cells, and that many but not necessarily all signals provided by these two proteins are transduced by the integrin class of cellular receptors. The author therefore focuses on describing integrin-mediated effects of laminin and collagen IV, but also briefly mentions alternative mechanisms for cell-basement interactions that have been suggested.

A. Binding Sites on Laminin for Cellular Receptors

Soon after laminin was first purified it was found to mediate the attachment of epithelial cells to a matrix of collagen IV.[259] Initially it was considered as a "bridging" protein, but was then shown to be able to promote cell attachment on its own.[260,261] In early studies it was often emphasized that laminin should preferentially promote the attachment of epithelial and carcinoma cells, while fibronectin should be specific for mesenchymal and sarcoma cells.[262,263] This dichotomy was with time found to be an oversim-

plification, and laminin can mediate the attachment of a broad variety of cells of both epithelial and mesenchymal lineage.[264] In an attempt to narrow down the location of cellular binding sites on laminin, proteolytic fragments were produced and tested for activity in assays for cell attachment.[265] Several active fragments were identified, but among these fragment 1, representing the center of the laminin cross (see Figure 1),[266] was found to be a particularly important tool in the characterization of the binding sites.

In parallel with the work on laminin in cell attachment, neurobiologists had noticed that laminin plays an important role in promoting neurite outgrowth.[267-269] Indeed, when so-called neurite outgrowth promoting factors were purified from culture media of different cells, these could be shown to be complexes between laminin and a proteoglycan.[55,252,253] The site on laminin mediating the neurite outgrowth effect could be localized to a domain at the end of the long arm, either by use of the proteolytic fragment 8 (see Figure 1), which retains the activity,[270] or by the use of blocking monoclonal antibodies.[271] Further characterization of the binding site on the long arm of laminin showed that this site could also promote attachment of a large range of non-neuronal cells.[264,272,273] A comparative analysis of the binding of fragment 1, fragment 8, and whole laminin to cells showed that binding of fragment 8 and laminin was competitive, while fragment 1 did not compete with binding of either fragment 8 or whole laminin.[274] This implies that there are distinct receptor mechanisms for fragment 1 and fragment 8, and that the binding site on fragment 8 is that which cells recognize in intact laminin. The binding site on fragment 1 appears to be latent, and becomes active first after proteolytic degradation of laminin.[274] The relevance of this site is dependent on whether this kind of proteolytic degradation of laminin occurs *in vivo*. The binding site on the long arm is highly sensitive to denaturation, indicating its conformation dependence, and appears to require the presence of all three chains of laminin.[275] It is therefore unlikely that it will be possible to narrow down the position of this site further by the use of synthetic peptides.

The characterization of cell binding sites on laminin have thus far been done mainly by use of "classical" laminin with the chain composition Ae,B1e,B2e. The B1s chain has been stated to promote attachment of neurons,[58,276] but as the native protein has not yet been purified these results must be considered preliminary. Am-containing laminin variants have been shown to mediate the attachment of a variety of cells and to promote neurite outgrowth, and in this respect cannot be distinguished from Ae-chain-containing laminin.[70]

B. Binding Sites on Collagen IV for Cellular Receptors

The role of collagen IV as a cell attachment protein has been largely neglected, in part because of early data that indicated that collagen IV was inert in this respect and needed laminin as a mediator of cell contact.[259] Even so, scattered reports on cell attachment to collagen IV occurred in the literature (see Reference 277). A screening of cell lines for attachment to collagen IV showed a variety of behaviors. Some cell types, including human embryo fibroblasts or human HT 1080 fibrosarcoma cells, showed a higher extent of attachment to collagen IV than to laminin or fibronectin.[277] One group of epithelial cells showed significant attachment only to mixtures of laminin and collagen IV,[277] i.e., the kind of observation that led to the original "bridging" theory.[259] Other cells did not show affinity for collagen IV under any of the conditions used. The binding of fibrosarcoma cells to collagen IV showed a requirement for a native triple helical structure. When fragments were tested the activity resided in the major triple helix, while the 7S and NC1 domains were inactive.[277] One binding site could be identified in a trimeric cyanogen bromide-derived fragment located 100 nm away from the N-terminus of the molecule.[278] In this fragment the triple helical conformation is stabilized by interchain disulfide bridges. Conformation-dependent attachment to the major triple helix was seen with primary chick embryo gizzard cells,[279] while murine melanoma cells attached to the NC1 domain as well.[280] The neuron-like rat pheochromocytoma cell line PC12 at-

taches equally well to collagen IV as to laminin, indicating that collagen IV might also influence neuronal systems.[281]

C. Integrins that Recognize Laminin and Collagen IV

Within a short period of time, the knowledge of the molecular mechanisms for cellular recognition of laminin and collagen has expanded rapidly. The critical factor was the application of techniques for integrin identification and isolation, which had been developed largely in the system of cell attachment to fibronectin (for recent reviews, see References 282, 283). From a rat neural cell line a laminin receptor was isolated that consisted of the integrin β_1-subunit in complex with an α-subunit of M_r 200,000.[284] By sequencing[285] this laminin-binding integrin was shown to correspond to the human VLA1 ($\alpha_1\beta_1$).[286] Laminin-binding integrins have also been isolated from glioma cells ($\alpha_3\beta_1$),[287-289] platelets ($\alpha_6\beta_1$),[290] and endothelial cells ($\alpha_2\beta_1$).[291] The relative importance of these and potential further laminin binding integrins[292-294] in different tissues and physiological events is being intensively investigated, but it is not yet possible to see a pattern. The issue is complicated by the recent observation that the same integrin, isolated from two different cellular sources, might differ in specificity.[295,296] It appears that $\alpha_6\beta_1$[294,297,298] and $\alpha_3\beta_1$[289] both bind to the site on the long arm of laminin. Integrin $\alpha_1\beta_1$ isolated from rat hepatocytes binds to both fragment 1 (center of cross) and fragment 8 (long arm),[299] while $\alpha_1\beta_1$-dependent cell attachment of human JAR choriocarcinoma cells is preferentially to fragment 1.[298]

Several studies have indicated $\alpha_1\beta_1$ and $\alpha_2\beta_1$ as the major integrins responsible for cell attachment to collagen IV.[279,300-302] The number of binding sites within the collagen IV molecule is still uncertain. The fact $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins appear to bind collagen IV as well as laminin leads to some conceptual difficulties, as these two ligand proteins do not appear to share any structural properties. The nature of the recognition mechanism is therefore unclear. The matter is complicated even more by the observation that while the mammalian $\alpha_1\beta_1$ binds to collagen I as well as to collagen IV and laminin,[301,302] the avian homolog does not show any affinity for collagen I.[279]

Integrins are connected to the actin cytoskeleton in a manner that allows mechanical information to be transmitted over the cell membrane. The fine details of integrin-cytoskeleton associations remain to be worked out, but the actin-associated proteins talin, vinculin, and α-actinin have been suggested as mediators of an interaction (see References 303 and 304). Further, phosphorylation of the intracellular portion of the integrin molecule can be induced by, for example, the phorbol-ester tumor promoter PMA and leads to an activation of the integrin-mediated cellular responses to extracellular ligands.[305,306] Integrins may be phosphorylated by the action of protein kinase C,[307] and inhibition of protein kinase C interferes with neurite outgrowth on laminin,[308] fibronectin, and collagen.[309]

Despite the many open questions with respect to specificity and relative importance of different integrins, the evidence for their role in mediating cell attachment to laminin and collagen IV is convincing. The binding can be demonstrated with purified receptor protein, as well as by inhibition with antibodies to receptor polypeptides in more intact systems. In laminin, the integrins bind to those sites that have earlier and independently been shown to be important for cell attachment and neurite outgrowth.

D. Other Potential Mechanisms for Cellular Recognition of Basement Membranes

The author has chosen to be selective, and to focus on such mechanisms for the cellular action of basement membranes where he is personally convinced of their validity. In the large literature in this area there are many more studies that might contain relevant information. In particular, several investigators have employed synthetic peptides as probes for biological activity in laminin, and so far seven different presumptive active sites have been postulated on this basis.[282] Entactin/nidogen has been shown to mediate attachment of some cell lines,[75,310] and might complement laminin in its cell binding activity.

In the early years of laminin research much

work was focused on a laminin-binding protein of apparent M_r 68,000 that was believed to function as a cell surface laminin receptor. Even though this line of work is not more widely pursued, it is often mentioned in the literature, and readers are referred to a comprehensive review on this subject.[311] Certain lectins have been shown to inhibit attachment and spreading of cells on laminin.[36] The effect cannot only be due to steric hindrance as an unglycosylated laminin obtained from tunicamycin-treated cells supports only attachment and not spreading.[37] In agreement, it has been reported that cell surface galactosyltransferase mediates cell spreading on laminin,[38] and is also important for neurite outgrowth by PC12 cells[39] and from chick dorsal root ganglia.[40] Cell membrane sulfatides may bind to laminin, at a site at the C-terminus of the Ae chain related to the heparin-binding site.[312] Even though most recent studies focus on integrin-mediated cell attachment to basement membranes, it should be considered that complementary and modulating mechanisms may also be needed.

E. Effects of Laminin on Cellular Proliferation and Differentiation

Most of the biological assays employed with purified basement membrane proteins have measured comparatively superficial parameters such as cell-substrate attachment, cellular shape, and motility, and this obviously limits the kinds of cellular responses that have been observed. A broader screening has been done with complex extracts containing basement membrane material, but where effects of co-isolated growth factors, plasma proteins, etc. cannot be excluded nor distinguished. The increased knowledge of the structure and receptor interactions of basement membrane macromolecules now provides tools for a more stringent analysis of the influence of these proteins on cellular function and phenotype. For example, purified laminin stimulated cell proliferation in established cell lines, and this activity appears to be contained in parts of the molecule rich in EGF-like repeats.[313] The hypothesis was put forward that EGF-like domains in matrix proteins might have more general importance as localized signals for growth and differentiation.[14] A number of recent studies have shown effects of laminin on proliferation, differentiation, and transdifferentiation events, particularly in the nervous system.[314-316] Such influences may also be studied by the use of biochemical markers. Laminin has been shown to increase both levels and activity of tyrosine hydroxylase, an enzyme of the catecholamine biosynthesis pathway, in calf adrenal chromaffin cells.[317] The emerging view that cytokines (growth factors) and biologically active extracellular matrix proteins form an integrated signalling system[318] provides further incitement to study the influence of basement membrane proteins on cellular proliferation and phenotype and is likely to promote our understanding of the interactions between cells and their extracellular environment.

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