

Localization of Type IV Collagen, Laminin, Heparan Sulfate Proteoglycan, and Fibronectin to the Basal Lamina of Basement Membranes

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ABSTRACT Electron microscopic immunostaining of rat duodenum and incisor tooth was used to examine the location of four known components of the basement-membrane region: type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin. Antibodies or antisera against these substances were localized by direct or indirect peroxidase methods on 60- μ m thick slices of formaldehyde-fixed tissues.

In the basement-membrane region of the duodenal epithelium, enamel-organ epithelium, and blood-vessel endothelium, immunostaining for all four components was observed in the basal lamina (also called lamina densa). The bulk of the lamina lucida (rara) was unstained, but it was traversed by narrow projections of the basal lamina that were immunostained for all four components. In the subbasement-membrane fibrous elements or reticular lamina, immunostaining was confined to occasional "bridges" extending from the epithelial basal-lamina to that of adjacent capillaries.

The joint presence of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin in the basal lamina indicates that these substances do not occur in separate layers but are integrated into a common structure.

Basement membrane is an extracellular matrix that separates connective tissue from epithelia, endothelia, muscle fibers, and the entire nervous system. The region of the basement membrane has been divided into layers that were given various names (1-3). Using the nomenclature of Kefalides et al. (1), the region includes three layers: (a) "lamina lucida" (also called lamina rara), a 10-50-nm thick layer in close apposition to the cells, which appears homogeneous but is occasionally crossed by fine filaments (2), (b) "basal lamina" (also called lamina densa), a 20-300-nm thick layer formed of fine filaments within an amorphous matrix and considered by some to be the "basement membrane proper" (1), and (c) subbasement membrane fibrous elements or "reticular lamina," a discontinuous layer of reticular and anchoring fibrils that is absent in some sites (1, 2). Several macromolecules, including type IV collagen, laminin, a heparan sulfate proteoglycan, and fibronectin, have been localized by immunostaining to the basement-membrane region in a variety of tissues (2, 4-14, Laurie, G. W., C. P. Leblond, G. R. Martin, and G. R. Grotendorst. Manuscript submitted for publication). The question arises as to the part of the basement-membrane region in which each substance is located. There is agreement that "type IV collagen" is present in the basal lamina (in kidney glomeruli [4], epidermis [5], lung alveoli [6], capillaries [7], and striated muscle

[8]). "Laminin" localization is the subject of controversy, as some investigators assign it to the basal lamina (in kidney glomeruli [9] and tubules [10], and in striated muscle [8]) and some to the lamina lucida (in glomeruli [2, 10] and in epidermis [11]). In the case of "heparan sulfate proteoglycan," indirect evidence provided by cationic markers or dyes has been used to assign it to the basal lamina (in embryonic salivary epithelium [15, 16]) or on each side of the basal lamina (in embryonic corneal epithelium [17] and embryonic lens [18]) or to the lamina lucida (in glomeruli [19, 20]). Finally, "fibronectin" has been assigned to the basal lamina and reticular lamina (in striated muscle [8] and embryonic neural tube [12]) or to the lamina lucida (in kidney glomeruli [13]) or only to interstitial connective tissue (in glomeruli and tubules [21]).

The present investigation reexamines the localization of the four substances within basement-membrane regions of duodenum and incisor tooth.

MATERIALS AND METHODS

Preparation of Antisera and Antibodies

The mouse EHS tumor was the source of type IV collagen (22), laminin (23), and heparan sulfate proteoglycan (14). Fibronectin was prepared from mouse serum (24). Antisera were raised in rabbits, and, in some cases, antibodies were extracted from them by affinity chromatography. The specificity of antisera and

antibodies was determined by enzyme-linked immunoassay (ELISA) (25). Antilaminin antibodies were further purified by sequential passage over Sepharose-bound type IV collagen, heparan sulfate proteoglycan and fibronectin. Similarly, the antiheparan sulfate proteoglycan antibodies were purified over laminin-Sepharose. The antifibronectin sera showed no reaction by ELISA with type IV collagen, laminin, heparan sulfate proteoglycan, and type V collagen. It was, therefore, used without further purification. The antitype IV collagen antibodies showed no reactivity by ELISA with laminin, heparan sulfate proteoglycan, type V collagen, and fibronectin, and were linked to peroxidase by the method of Avrameas and Ternynck (26).

Preparation of Tissues and Immunostaining

Sherman rats, aged 20 d, were perfused with cold 5% formaldehyde in 0.08 M sodium phosphate buffer (pH 6.0). Duodena and lower incisor teeth were dissected at 4°C and immersed in the same fixative for 1 h. After washing for 2 h in several changes of PBS (pH 7.3), the tissues were cut into 60- μ m slices on a Smith and Farquhar tissue chopper and immunostained with the dilutions of antibodies or antisera yielding optimal staining of basement membranes in tooth sections. The direct method was used in the case of the peroxidase-linked antitype IV collagen antibodies (0.3 mg IgG/ml), whereas the indirect PAP method was utilized for antilaminin antibodies (0.06 mg/ml), antiheparan sulfate proteoglycan antibodies (0.05 mg/ml), and antifibronectin antiserum (1:30,000), as previously described (7, 27). For control, nonimmunized rabbit IgG linked with peroxidase (0.01 mg IgG/ml; Cappel Laboratories Inc., Cochranville, PA) was used with or without PAP; as well, non-immune rabbit serum (1:1000; Cappel Laboratories Inc.) was employed with PAP. The antilaminin antibodies were also detected by an indirect method using ferritin as the antibody label. Formaldehyde-fixed slices were incubated overnight at 4°C with antilaminin antibodies (0.06 mg/ml), washed for 40 min with phosphate buffered saline, exposed for 60 min at room temperature to ferritin-labeled goat antirabbit IgG (1:50; Cappel Laboratories Inc.), osmicated for 15 min, then processed routinely for Epon infiltration. For control, nonimmunized rabbit IgG (0.01 mg/ml; Cappel Laboratories Inc.) was used.

With either the peroxidase or ferritin protocol, the antibodies in contact with the slices had access only to the surface and interstices exposed by the blade of the tissue chopper. For this reason, the slices were flat-embedded and serially sectioned in the following way. After infiltration with Epon, they were placed flat on a Teflon plate (200 mm \times 200 mm \times 0.7 mm) and covered with a drop of freshly prepared Epon. The flat end of a prehardened cylindrical Epon block was then applied firmly onto a slice and kept in this position through polymerization at 60°C. Each block with the slice embedded on its flat end was separated from the Teflon plate by bending the latter. After trimming without removal of superficial Epon, numerous thin (0.05 μ m) sections were serially cut from the surface of the slices and examined in the electron microscope.

RESULTS

Duodenum

The basement membrane of the epithelium examined at the base of duodenal villi was not stained with control nonimmune IgG or serum (Fig. 1). With antilaminin antibodies, strong immunostaining was observed in the basal lamina as shown at low (Fig. 2) and high magnification (Fig. 3). At high magnification, peroxidase immunostaining was distinguished as small dense dots. The bulk of the lamina lucida was unstained, but this layer was traversed by immunostained structures (Fig. 3) interpreted as fine filaments extending from the basal lamina to the adjacent epithelial cell. In the reticular lamina, immunostaining was not observed in collagen fibrils and fibroblasts, but in occasional strands of variable width, referred to as "bridges" (7), which joined the basal laminae of epithelium and adjacent blood vessels (7) and, in routine electron micrographs, appeared composed of material similar to that of basal laminae. The plasmalemma of epithelial cells showed some homogeneous rather than dotted staining considered to be artefactual (see Discussion). With antitype IV collagen antibodies, immunostaining again occurred in the basal lamina (Fig. 4). Immunostained projections extended across the lamina lucida. Bridges cut in longitudinal and cross-sections were moderately immunostained (Fig. 4). Immunostaining with an-

tiheparan sulfate proteoglycan antibodies (Fig. 5) yielded a similar, usually less intense pattern. Antifibronectin antiserum (Fig. 6) gave the same overall pattern, but was usually even less intense. Fibronectin immunostaining was also present in connective tissue, particularly at the surface of fibroblasts.

Incisor Tooth

The epithelial basement-membrane of the enamel organ examined 2 mm from the tooth's posterior extremity was not reactive in controls (Fig. 7). After immunostaining for laminin (Fig. 8), type IV collagen (Fig. 9), heparan sulfate proteoglycan (Fig. 10), and fibronectin (Fig. 11), the pattern was the same as in the duodenum, with dots of reaction product predominating in the basal lamina and its extensions across the lamina lucida and into connective tissue. When ferritin, instead of peroxidase, was used as label for the localization of antibody binding sites, no reaction was observed in controls (Fig. 13), whereas immunostaining for laminin was again observed in the basal lamina and in its extensions, but not along the plasmalemma (Fig. 14). As in duodenum, fibronectin immunostaining was present in connective tissue (Fig. 12).

When the capillary basement membrane was examined in duodenum and tooth, the lamina lucida was narrow and poorly distinguishable, but definitely unstained, whereas the distinct basal lamina was immunostained (Fig. 2).

DISCUSSION

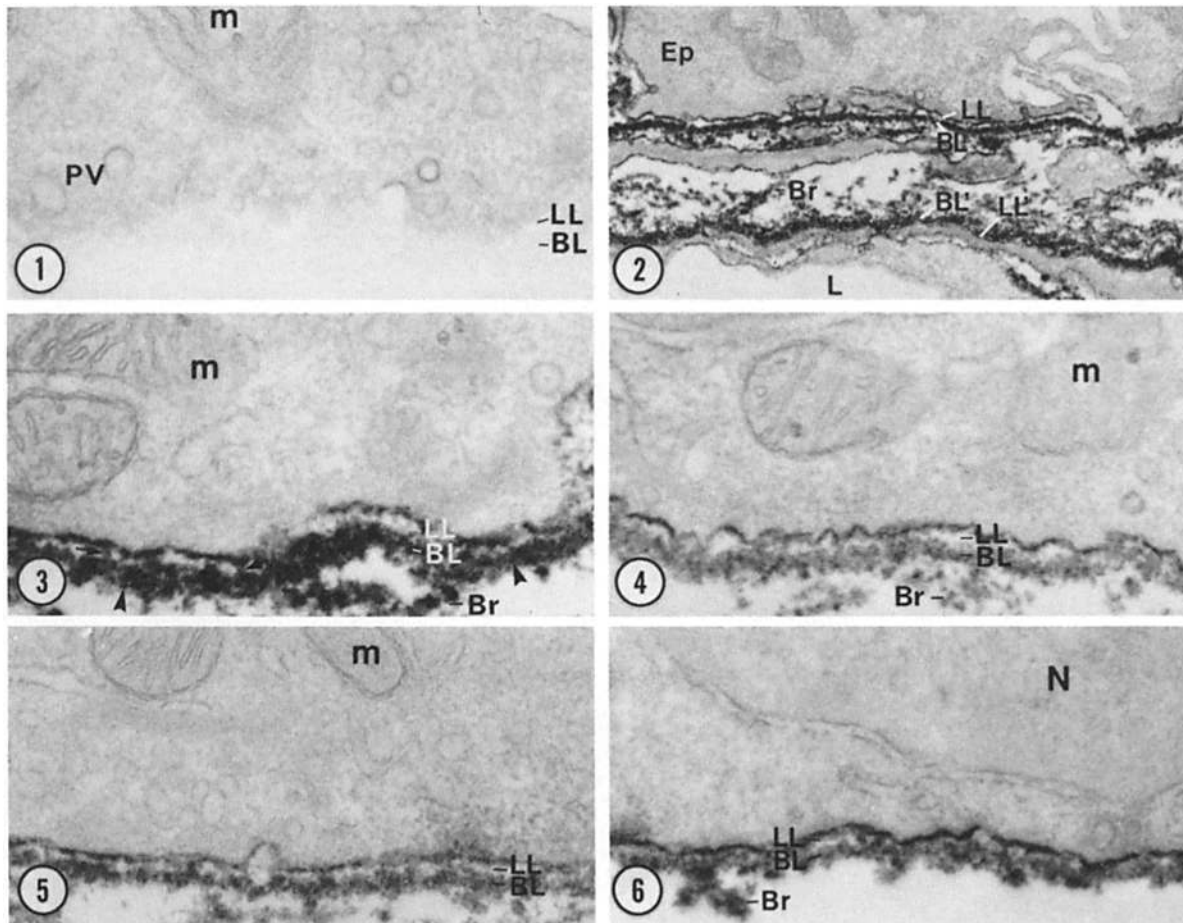
The ultrastructural localization of four components of the basement-membrane region: type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin, was reexamined. Immunostaining using peroxidase as antibody marker was characterized in the electron microscope by 18–40-nm dots (12, 28–30) believed to consist of insoluble diaminobenzidine polymers (31). Immunostaining for each substance was observed in the basal laminae of duodenal epithelium, outer-enamel epithelium, and blood-vessel endothelium. The lamina lucida was unstained except for a few reactive structures interpreted as fine filaments projecting from the basal lamina. The reticular lamina occasionally contained immunostained basal-lamina-like "bridges" (7) that extended from epithelial to endothelial basal laminae.

Although peroxidase immunostaining is distinguished by 18–40-nm dots, epithelial- and endothelial-cell membranes located near reactive basal laminae often showed homogeneous staining, as has been observed for cell membranes near sites immunostained by peroxidase-linked antithyroglobulin antibodies (32) and antitype I procollagen antibodies (27). Thus, cell-membrane staining was observed whatever antibody was used with the peroxidase label. When ferritin was the antibody label, the membrane was not stained (Fig. 14). It was concluded that the homogeneous membrane staining was artefactual (12), perhaps due to reactive sites releasing incompletely polymerized diaminobenzidine (31) that would react with membrane lipids.

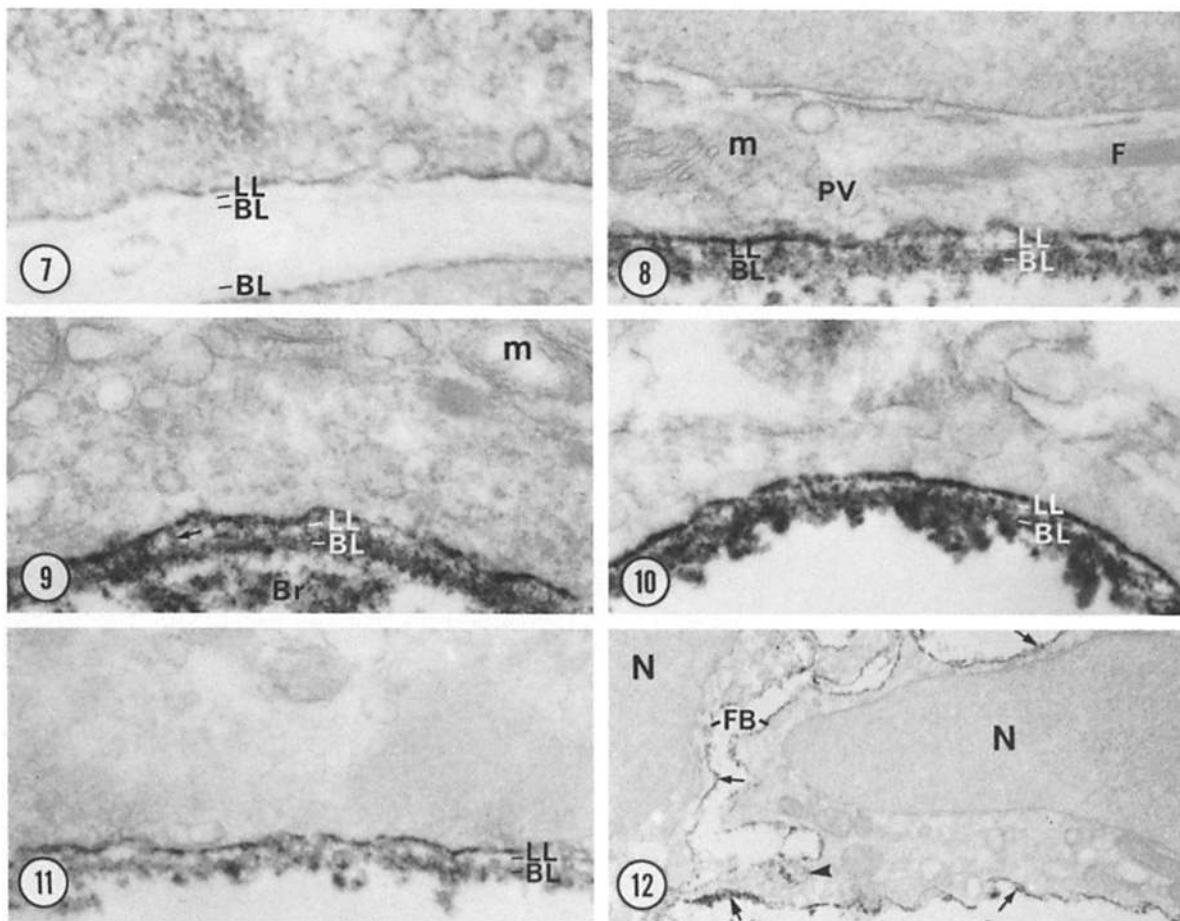
Type IV collagen antigenicity was localized to basal laminae in duodenum, enamel organ, and blood vessels in accordance with reports on other organs (4–8). The antigenicity was attributed to type IV collagen itself, because this substance had been extracted from glomerular basement membrane, lens capsule, Descemet's membrane (1), and Reichert's membrane (1, 33, 34). Laminin antigenicity was also localized to the basal-lamina layer of the basement membrane region. This was in accord-

ance with some reports (8, 9), but not with others that proposed a lamina-lucida location (2, 11). The use of ferritin instead of peroxidase as antibody label confirmed the localization of laminin antigenicity to the basal lamina. Because our procedure could localize extracellular as well as intracellular substances (7, 35, 36), the lack of immunostaining in the lamina lucida was not due to lack of penetration; indeed the projections from the basal lamina across the lamina lucida were immunostained. In any case, it was likely that the antigenicity of the basal lamina was due to the presence of laminin itself, because laminin had been extracted from Reichert's membrane (37-39). Heparan sulfate proteoglycan had not previously been immunolocalized at the level of the electron microscope. On

the basis of reactions with cationic markers and dyes, this substance had been assigned to the basal lamina (15, 16), to its surface (17, 18) or to the lamina lucida (19, 20). Cationic markers and dyes probably detected the exposed anionic portions of glycosaminoglycan side chains, which might be located at the edge of the basal lamina. In distinction, the antibodies used in this study detected the protein core of the heparan sulfate proteoglycan (14) and localized it throughout the thickness of the basal lamina. Finally, the localization of fibronectin antigenicity to the basal lamina was in accordance with conclusions reached by immunostaining of striated muscle (8) and embryonic neural tube (12), but not with observations on kidney (2, 10, 40), in which occasional immunostaining of the



FIGURES 1-6 Electron micrographs of the basal portion of columnar epithelial cells (*Ep*) and their basement membrane, taken from the base of rat duodenal villi. The background delineation of organelles is due to osmication; the peroxidase immunostaining appears as dense dotlike reactions (Figs. 3-6). Basal lamina (*BL*), lamina lucida (*LL*), bridges (*Br*), nucleus (*N*), mitochondria (*m*), plasmalemmal vesicle (*PV*). $\times 40,000$, except Fig. 2, which is $\times 12,500$. Fig. 1. Control preparation exposed to peroxidase-linked nonimmunized rabbit immunoglobulin and the PAP sequence. The cell is unstained, as is the basal lamina which is only barely distinguishable. The lamina lucida located between cell and basal lamina is unstained. Fig. 2. Low-power micrograph of immunostaining with antilaminin antibodies in the basal lamina of the epithelium (*BL*), in the basal lamina (*BL'*) of a subjacent venule (*L*; lumen) and in bridges located between the two. The venule's lamina lucida is thin, but unstained (*LL'*). The processes in the connective tissue space are portions of smooth-muscle cells whose basal laminae are partly visible. Fig. 3. Immunostaining with antilaminin antibodies is detected throughout the basal lamina in the form of fine dots (arrowheads). The lamina lucida is unstained, except for what are interpreted as a few projections of the basal lamina (arrows). Reactive "bridges" extend from the basal lamina into subjacent connective tissue. The cell and organelles are unstained. The homogeneous staining of the plasmalemma is an artifact of diaminobenzidine diffusion (see Discussion). Fig. 4. Immunostaining with antitype IV collagen antibodies again shows as reactive dots crowded in the basal lamina, and is absent in the lamina lucida except for occasional basal-lamina projections. A reactive bridge is present below the basal lamina. Fig. 5. Immunostaining with antiheparan sulfate proteoglycan antibodies is clearcut along the basal lamina, but scarce in the lamina lucida. In places, immunostained basal-lamina material extends through the lamina lucida to the cell. Fig. 6. Immunostaining with antifibronectin antiserum is present in the basal lamina. The lamina lucida is thin and contains discrete immunostained extensions of the basal lamina. A reactive bridge extends below the basal lamina.

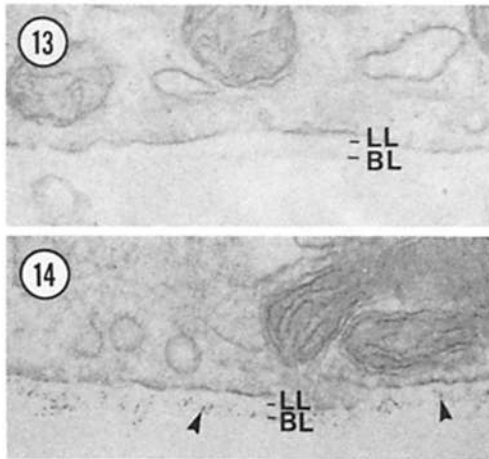


FIGURES 7-12 Electron micrographs taken from the rat incisor-tooth. Fig. 7-11 depict cells of the outer-enamel epithelium and adjacent basement-membrane. Basal lamina (*BL*), lamina lucida (*LL*), bridges (*Br*), nucleus (*N*), mitochondria (*m*), plasmalemmal vesicle (*PV*), filament bundle (*F*). Fig. 7, 8, and 10 $\times 50,000$; Figs. 9 and 11, $\times 40,000$; Fig. 12 $\times 10,000$. Fig. 7. Control preparation exposed to peroxidase-linked nonimmune rabbit IgG. At top is a portion of a cell from the outer-enamel epithelium and at bottom is a small part of an endothelial cell from a capillary. Both are lined by a faintly visible basal lamina. Neither cells nor laminae lucidae are stained. Only cells of the outer-enamel epithelium are shown in Figs. 8-11. Fig. 8. Immunostaining with antilaminin antibodies shows as dense dots over the basal lamina. In places, immunostained basal-lamina material extends through the lamina lucida and to the cell. The organelles of the outer-enamel epithelium are unstained. Fig. 9. Immunostaining with antitype IV collagen antibodies is present in the basal lamina and in its extensions (arrow), which traverse the lamina lucida to the cell. Associated with the other side of the basal lamina are portions of immunostained bridges (*Br*). Fig. 10. Immunostaining with antiheparan sulfate proteoglycan antibodies is found mainly in the basal lamina with some reactive dots in the lamina lucida as basal-lamina projections. Fig. 11. Immunostaining with antifibronectin antiserum is present in the basal lamina. In places, immunostained basal-lamina material extends through the lamina lucida to the cell. Fig. 12. Immunostaining of pulp of incisor tooth with antifibronectin antiserum is found along the plasmalemma of two fibroblast cells (*FB*, arrows). No intracellular staining is found in these except for a structure that appears to be multivesicular body (arrowhead).

glomerular basement-membrane (41) was attributed to the presence of soluble fibronectin in transit (21). The differences in the various reports may be in part explained by the low amount of fibronectin detectable in basement membranes (Laurie, G. W., C. P. Leblond, G. R. Martin, and G. R. Grotendorst. Manuscript submitted for publication). In our investigation, weak but definite fibronectin immunostaining was observed in the basal laminae of duodenal epithelium, outer-enamel epithelium, and blood vessels. Taken together, the results point to the presence of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin in the basal lamina and its projections.

In the past, it was generally held that the four substances were layered in the basement-membrane region, with laminin (2, 10, 11) and fibronectin (10, 13) in the lamina lucida, type IV collagen in the basal lamina (4-8), and heparan sulfate proteoglycan at the interface (17, 18). In contrast, the present

results indicate that these substances are not layered, but are integrated together in the basal lamina. The joint location is in keeping with their mutual interactions since laminin and fibronectin are both known to bind type IV collagen (42-44) and heparan sulfate (44, 45). Because the substances are probably of sufficiently large size to span the basal lamina, fibronectin, which is known to attach to fibroblasts and collagen fibrils (43, 44), could bind the basal lamina to connective tissue, whereas laminin, which is known to adhere to various epithelial and muscle cells (42, 43), could bind the basal lamina to these and other associated cells. The basal lamina may, therefore, be considered as the essential part of the basement-membrane region, in agreement with the concept of Kefalides et al. (1) and Sugrue and Hay (46). Indeed, Kefalides et al. (1) described the basal lamina as "basement membrane proper." In distinction the lamina lucida, although containing basal-lamina projections, may consist mostly of the cell coat or glycocalyx since



FIGURES 13 and 14 Ferritin as the antibody marker in electron micrographs of outer enamel epithelium and adjacent basement-membrane from incisor tooth. Basal lamina (BL); lamina lucida (LL). $\times 50,000$. Fig. 13. Control preparation exposed to nonimmune rabbit IgG. Ferritin particles are rare. Fig. 14. Preparation exposed to anti-laminin antibodies. Immunostaining is seen as a row of ferritin particles (arrowheads) in the basal lamina. No particles are along the plasmalemma.

the cell coat stains with colloidal iron as does the space interpreted to be the lamina lucida (47).

In conclusion, it is proposed that type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin form an integrated complex that constitutes the basal lamina and its extensions. The complex would provide support as well as adhesion to both the associated cells and the connective tissue stroma.

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