

# Ultrastructural Localization of Fibronectin and Laminin in the Basement Membranes of the Murine Kidney

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**ABSTRACT** Affinity-purified rabbit antibodies specific for two large noncollagenous glycoproteins—laminin and fibronectin—were used to study the distribution of these proteins in normal murine kidneys. Immunofluorescence staining of conventional frozen sections demonstrates fibronectin within mesangial areas of the glomerulus. Laminin is also found in mesangial areas. However, it also appears to be distributed in typical basement membranelike patterns on glomerular and tubular basement membranes and Bowman's capsule. At the ultrastructural level, by labeling 600–800-Å thick frozen sections with a three-stage procedure consisting of specific antibodies, biotinyl sheep anti-rabbit IgG, and avidin-ferritin conjugates, fibronectin is present only in the mesangial matrix and is specifically localized to areas immediately surrounding mesangial cell processes. Laminin, on the other hand, is found uniformly distributed throughout tubular basement membranes, the mesangial matrix, and Bowman's capsule. In glomerular basement membranes, laminin labeling is restricted to the lamina rara interna and adjacent regions of the lamina densa.

Basement membranes are complex structures containing collagenous and noncollagenous glycoproteins and proteoglycans. The collagenous components of basement membranes include several type-IV collagens (3, 6, 21, 22). In addition, the presence of type V (AB<sub>2</sub>) collagen has been demonstrated within basement membranes of lung, kidney, and selected vascular beds (18). Characterization of the noncollagenous basement membrane components has been hampered by many of the same problems observed in characterizing the collagenous moieties, namely low solubility and yield due to possible cross-linking, protease sensitivity, and difficulty in obtaining sufficient amounts of relatively homogenous basement membranes as starting material. Recently, Timpl et al. (20) described the isolation and purification of a large noncollagenous glycoprotein, laminin, from a transplantable murine sarcoma that produces an extracellular matrix of basement membranes. In immunofluorescence studies, antibodies to laminin were shown to react with basement membranes of normal tissues, suggesting that laminin or an immunologically related protein is a component of basement membranes in tissues (1, 20). Fibronectin, another large noncollagenous glycoprotein, has been found by some investigators to be associated with glomerular basement membranes (GBM) (12, 15). Although other investigators have found fibronectin associated with some basement membranes, they have not found it associated with the GBM (7, 8, 19). In this report, we demonstrate the differing ultrastructural localizations of these two noncollagenous glycoproteins in the kidney of the mouse, using ultrathin frozen-sectioning techniques coupled with immunoferritin reagents. Our results support a base-

ment membrane localization for laminin but not for fibronectin.

## MATERIALS AND METHODS

All chemicals used were of analytical grade. Bovine albumin (BSA) and 6 × recrystallized horse spleen ferritin (Pentex) were obtained from Miles Laboratories Inc., Elkhart, Ind. Glutaraldehyde was obtained from Fisher Scientific Co., Fair Lawn, N. J. Avidin (type III) was obtained from Sigma Chemical Co., St. Louis, Mo.

### Preparation of Antibodies

Cold insoluble globulin (CIG) was prepared from fresh human plasma as described by Murray et al. (11). Briefly, plasma was incubated overnight at 4°C with Sepharose 4B beads coupled with denatured calf type I acid-soluble collagen. The beads were washed extensively with phosphate-buffered saline (PBS). When the optical density of the washing buffer returned to base line, CIG was eluted with 1.0 M potassium bromide, 50 mM Tris-HCl, pH 5.3. The CIG was dialyzed against PBS and concentrated to 1 mg/ml using Aquacide (Calbiochem-Behring Corp., San Diego, Calif.). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) revealed a single band with an apparent molecular weight of 440,000 when unreduced and a doublet with apparent molecular weights of 200,000–220,000 after reduction with β-mercaptoethanol (data not shown).

Laminin was prepared as previously described (20). The Engelbreth-Holm-Swarm sarcoma grown in lathyritic mice was harvested and extracted with 0.5 M sodium chloride, 50 mM Tris-HCl, pH 7.6, containing protease inhibitors phenylmethanesulfonyl fluoride and *p*-chloromercuribenzoate. After salt precipitation, laminin was purified by chromatography on DEAE-cellulose, agarose, and carboxymethyl cellulose gels. The protein has an apparent molecular weight of 850,000 before reduction when run on polyacrylamide gel electrophoresis in the presence of SDS. After reduction, three bands were noted, one having an apparent molecular weight of 400,000 and a doublet with apparent molecular weights in the 220,000 range (data not shown).

Rabbit antiserum to each protein was raised by intradermal injection of 1 mg of the protein mixed with complete Freund's adjuvant. 4 wk after the first injection, two booster injections were given at 2-wk intervals. 2-wks after the last injection, the animals were exsanguinated, and the sera were harvested and stored at  $-20^{\circ}\text{C}$ . The fibronectin antisera were purified by absorption on and elution from an affinity column composed of CIG coupled to cyanogen bromide-activated Sepharose 4B beads (13). Laminin antisera were purified by cross-absorption on a type IV collagen-Sepharose affinity column and absorption and elution from a laminin affinity column as previously described (20).

The antibodies were tested for specificity using radioimmunoassays and enzyme-linked immunosorbent assay (ELISA) assays as previously described (17, 20). The purified antibodies were specifically inhibited by the antigen to which they were raised, and no cross-reactivity was detectable with type IV or V collagens (data not shown). The titer of the laminin antibodies was 1:15,000 by radioimmunoassay, and the titer of the fibronectin antibodies was 1:2,560 by ELISA assay.

Sheep anti-rabbit IgG was prepared as previously described (1). The antibodies were purified by immunoabsorption on rabbit IgG (Miles Laboratories Inc., Elkhart, Ind.) coupled to cyanogen bromide-activated Sepharose 4B (18).

Fluorescein-conjugated sheep anti-rabbit IgG was prepared using standard techniques (25). Biotinized sheep anti-rabbit IgG and avidin-ferritin were prepared as previously described (1). Immunofluorescence studies were performed as previously described (9).

Immunoferritin labeling of ultrathin frozen sections for electron microscopic analysis was done as previously described (18). Briefly, cubes of renal cortex were

fixed with glutaraldehyde, frozen in sucrose on copper chucks, and cut on a Sorvall-Christensen FTS/LCT-2 frozen thin sectioner (Dupont Instrument-Sorvall, Newtown, Conn.) at  $-80^{\circ}\text{C}$  with a dry glass knife. Sections (600–800-Å thick) were placed on Formar- and carbon-coated grids and labeled using the inverted drop method.

Alternately, cubes of renal cortex were fixed according to the method of McLean and Nakane (10) before ultrathin frozen sectioning.

## RESULTS

### Immunofluorescence

When 6- $\mu\text{m}$  thick frozen sections of kidney tissue were stained with antibodies to fibronectin and fluorescein-labeled anti-IgG, fibronectin apparently was present within mesangial areas of glomeruli in irregular granular distributions. Occasional small patches were also found in peritubular areas corresponding to interstitium. Labeling was not apparent on glomerular or tubular basement membranes or on Bowman's capsule (Fig. 1a).

In contrast, antibodies to laminin gave linear patterns of fluorescence suggesting the presence of this protein in association with glomerular and tubular basement membranes as well

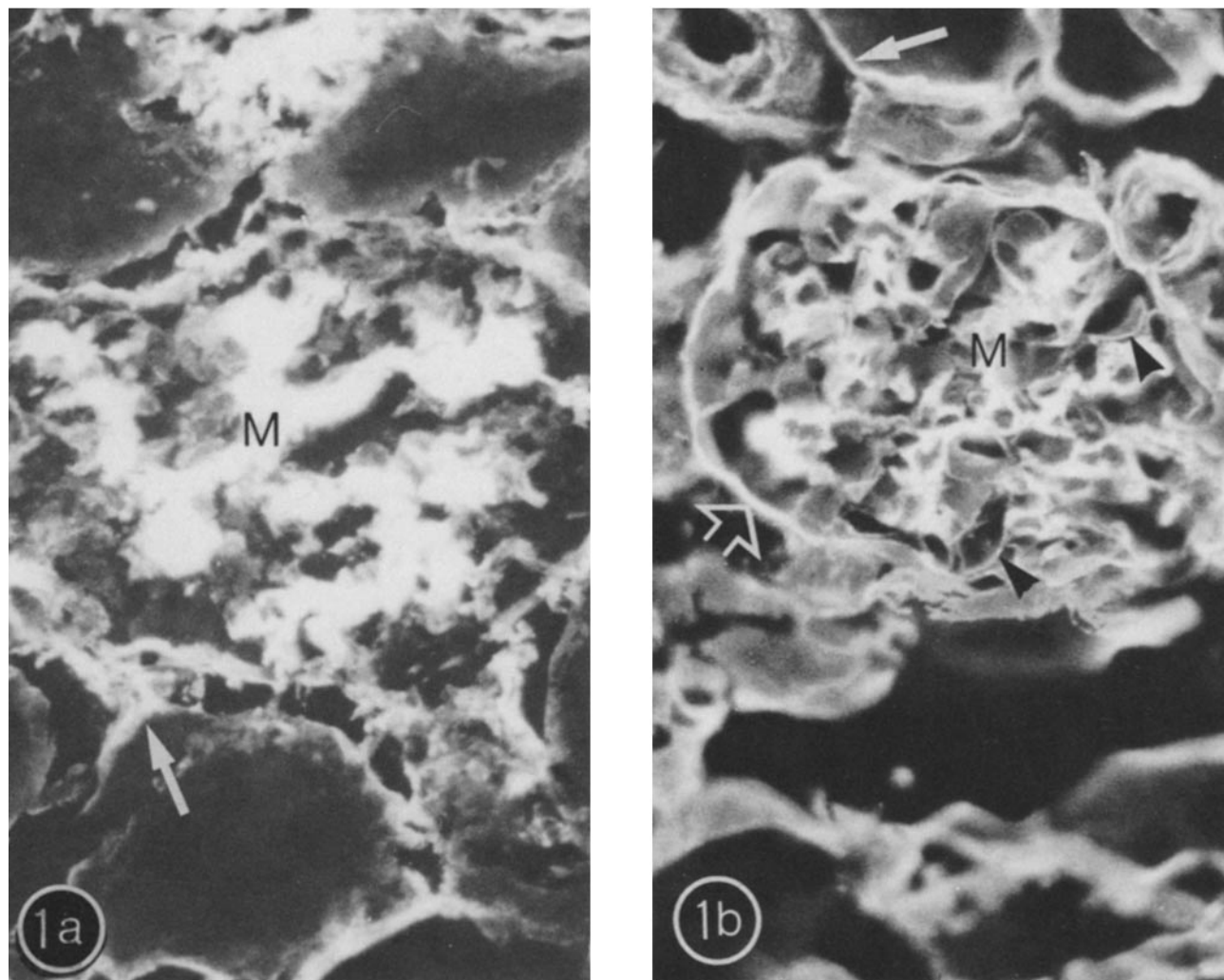


FIGURE 1 (a) Immunofluorescence microscopy of a 6- $\mu\text{m}$  frozen section through a glomerulus and adjacent tubules labeled with antibodies to plasma fibronectin and a fluorescein-labeled secondary antibody. Note the intense fluorescence in mesangial areas (M) with no label evident in glomerular basement membrane localizations. Fluorescence is also evident in peritubular interstitium (arrows).  $\times 500$ . (b) Immunofluorescence microscopy of a 6- $\mu\text{m}$  frozen section through a glomerulus and adjacent tubules labeled with antibodies to laminin and a fluorescein-labeled secondary antibody. Note the intense fluorescence in mesangial areas (M) as well as in a glomerular basement membrane localizations (arrowheads). Intense linear fluorescence patterns are also noted on Bowman's capsule (open arrows) and on tubule basement membranes (arrows).  $\times 500$ .

as with Bowman's capsule. In addition, mesangial areas appeared to be stained (Fig. 1 *b*).

Control sections incubated with preimmune rabbit serum or with antibodies preincubated with the specific antigen showed no fluorescence (data not shown).

Immunofluorescence technology does not allow one to ascertain which region(s) of the basement membrane are staining (lamina rara or densa), whether labeling occurs at basement membrane-cell contact sites, or where within the mesangium (matrix or cell processes) the antigens are located. We undertook this immunoelectron microscopic study to determine the ultrastructural localizations of these proteins with the hope of gaining some insight into possible functions for selected renal structures.

### Immunoelectron Microscopy

Ultrathin frozen sections of a portion of a glomerulus and of two adjacent tubular cells stained with uranyl acetate are seen in Fig. 2 *a* and *b*. Many of the ultrastructural components of the kidney are easily identified at low magnification.

### Glomerular Basement Membrane

Figs. 3 *a* and *b* are high magnifications of areas of GBM similar to those enclosed in the boxes labeled *A* on Fig. 2 *a*. Fig. 3 *a* is a representative section of a GBM area labeled with fibronectin antibodies: no ferritin grains are seen. Fig. 3 *b* is a representative section of GBM labeled with laminin antibodies. Ferritin grains are noted over the endothelial cell side of the

basement membrane corresponding to the lamina rara interna and possibly a portion of the lamina densa. When antibodies were preincubated with laminin or fibronectin or when normal rabbit IgG was used instead of specific antibodies, no labeling was noted (data not shown).

### Tubular Basement Membrane

Tubular basement membranes (similar to those seen in Fig. 2 *b* and labeled *C*) exhibited no ferritin labeling when incubated with fibronectin antibodies (Fig. 4 *a*). However, uniform labeling was seen over tubular basement membranes (TBM) when laminin antibodies were used (Fig. 4 *c*). Again, with normal rabbit IgG or antibodies preincubated with laminin, no labeling was observed (data not shown).

### Mesangium

Fibronectin antibodies were noted to be localized specifically in certain areas of the mesangial stalk (similar to that seen in Fig. 2 *a* and labeled *B*), in particular, to regions surrounding mesangial cell processes (that appear as dark areas; Fig. 5 *a*). In contrast, laminin antibodies were localized diffusely within areas of the light-grey amorphous mesangial matrix (Fig. 5 *b*). When preimmune rabbit IgG or inhibited antibodies were used as controls, no labeling was observed (data not shown).

### Bowman's Capsule

Bowman's capsule was not labeled by antibodies to fibro-

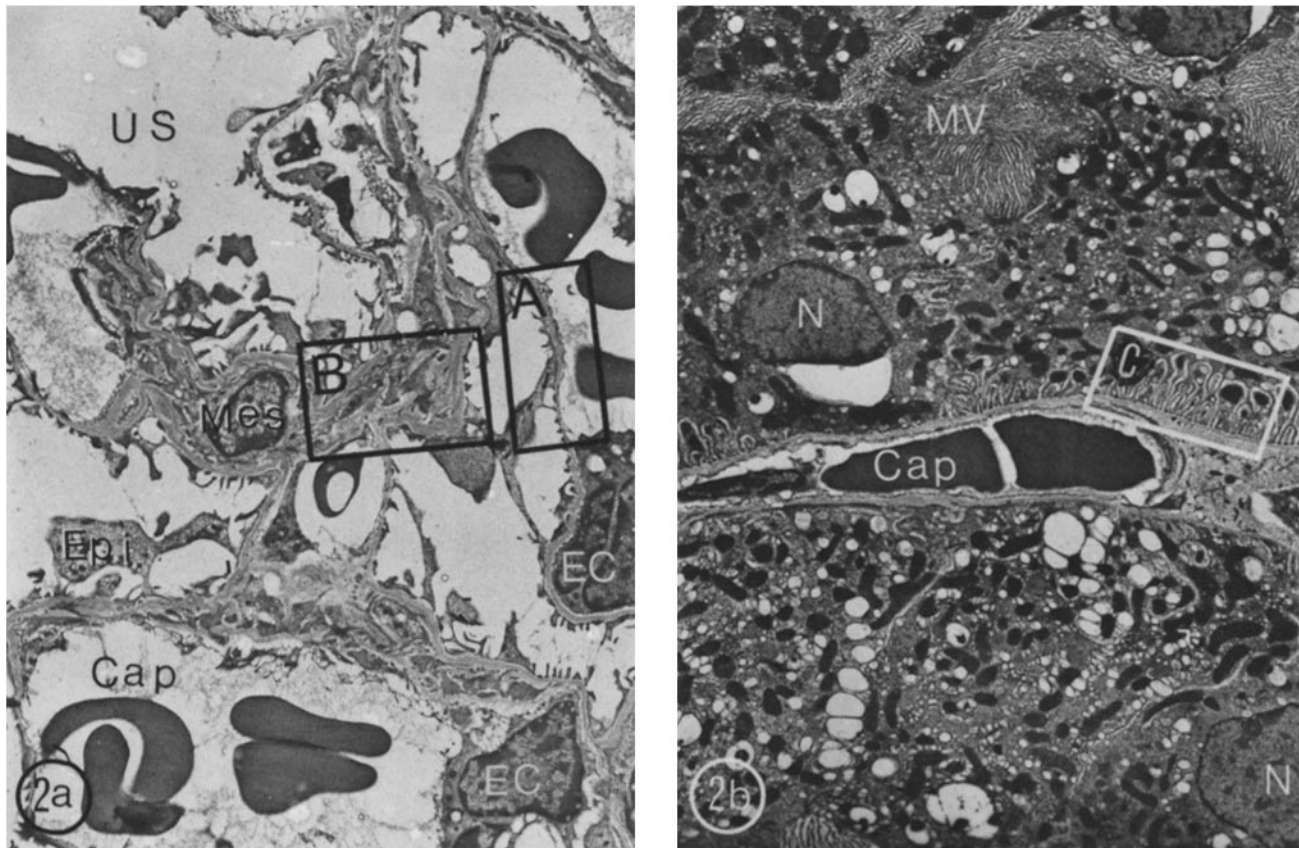


FIGURE 2 (a) Low-magnification electron micrograph of an ultrathin frozen section of a glomerulus showing characteristic glomerular structures including glomerular basement membranes bordered by endothelial (EC) and epithelial cell (Epi) processes (A) and mesangium (Mes) with mesangial cell processes interspersed within mesangial matrix (B). US, urinary space, Cap, capillary.  $\times 7,300$ . (b) Low-magnification electron micrograph of an ultrathin frozen section of two adjacent tubules showing tubular-epithelial-cell foot processes abutting the regular amorphous tubular basement membranes (C). A capillary (Cap) is noted, as are epithelial-cell microvilli (MV) and Nuclei (N).  $\times 7,800$ .

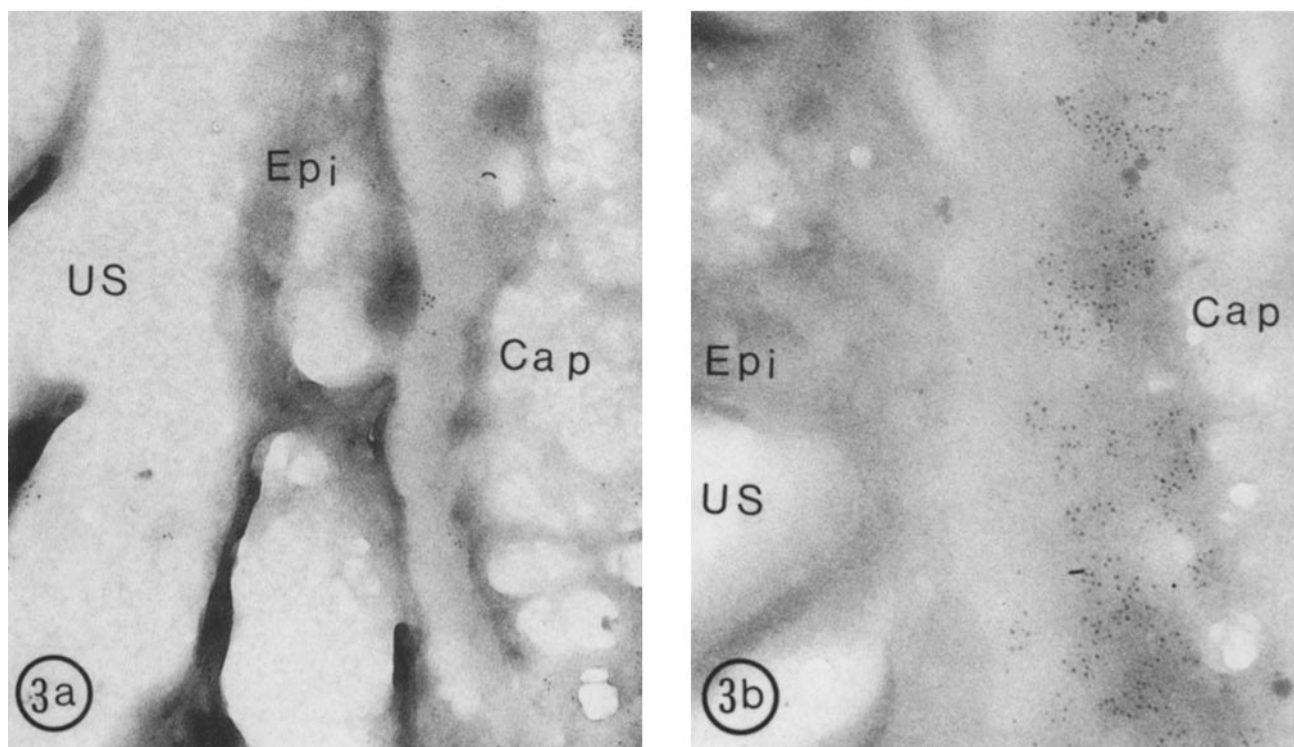


FIGURE 3 High-magnification electron micrographs of ultrathin frozen sections through areas of GBM similar to that observed in Fig. 2 a, box A labeled with (a) antibodies to fibronectin, biotinized sheep anti-rabbit IgG, and avidin-ferritin (note the complete absence of specific labeling) and (b) antibodies to laminin, biotinized sheep anti-rabbit IgG, and avidin-ferritin. Note the asymmetric labeling of the GBM. Labeling is restricted to the endothelial side of the GBM. Cap, capillary, US, urinary space, Epi, epithelial-cell foot processes.  $\times 60,000$ .

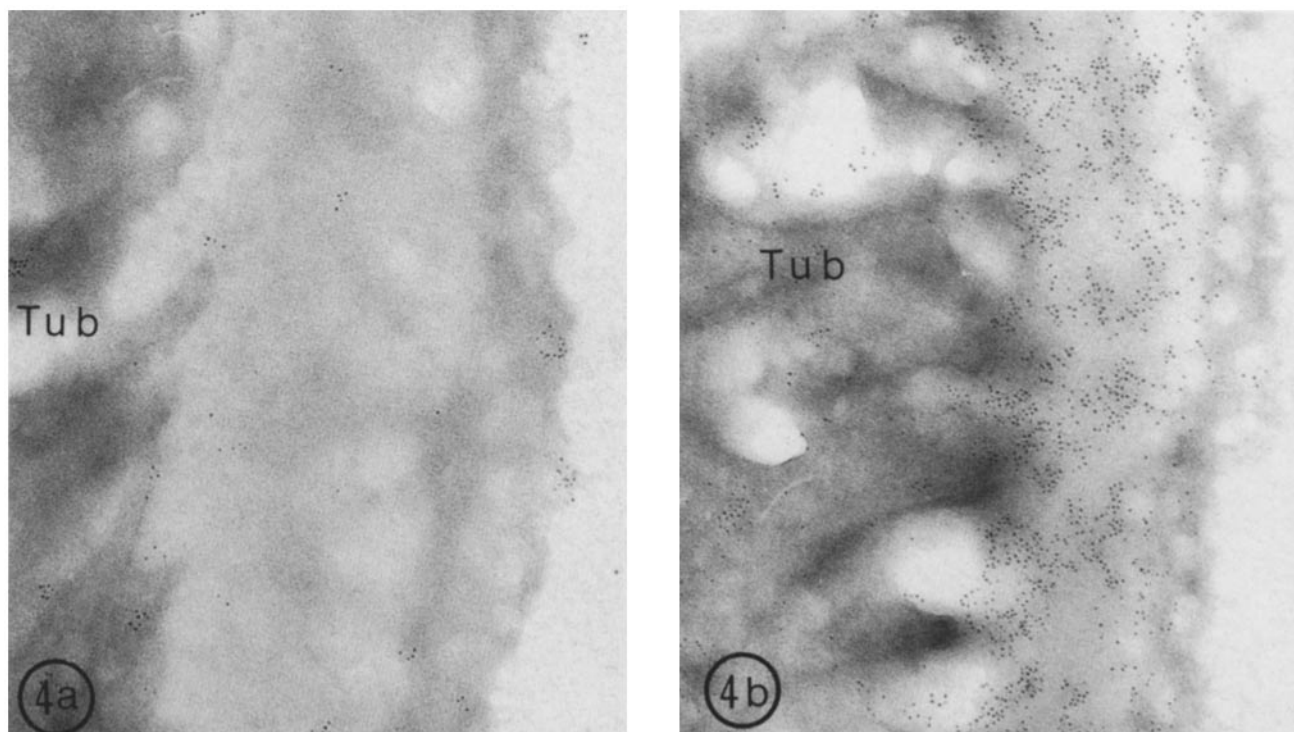


FIGURE 4 High-magnification electron micrographs of ultrathin frozen sections through areas of TBM similar to that observed in Fig. 2 b box C labeled with (a) antibodies to fibronectin, biotinized sheep anti-rabbit IgG, and avidin-ferritin (note the complete absence of specific labeling) and (b) antibodies to laminin, biotinized sheep anti-rabbit IgG, and avidin-ferritin. Note the uniform labeling spanning the complete width of the TBM. Tub, tubular epithelial cell foot processes.  $\times 60,000$ .

nectin, however laminin antibodies labeled Bowman's capsule in a uniform pattern (data not shown). Labeling of these structures was unchanged when tissues fixed according to the method of McLean and Nakane were used (data not shown).

Our findings regarding the localization of laminin and fibronectin as well as our previous localization of collagen types IV and V (AB<sub>2</sub>) within the glomerulus are schematically illustrated in Fig. 6 (18).

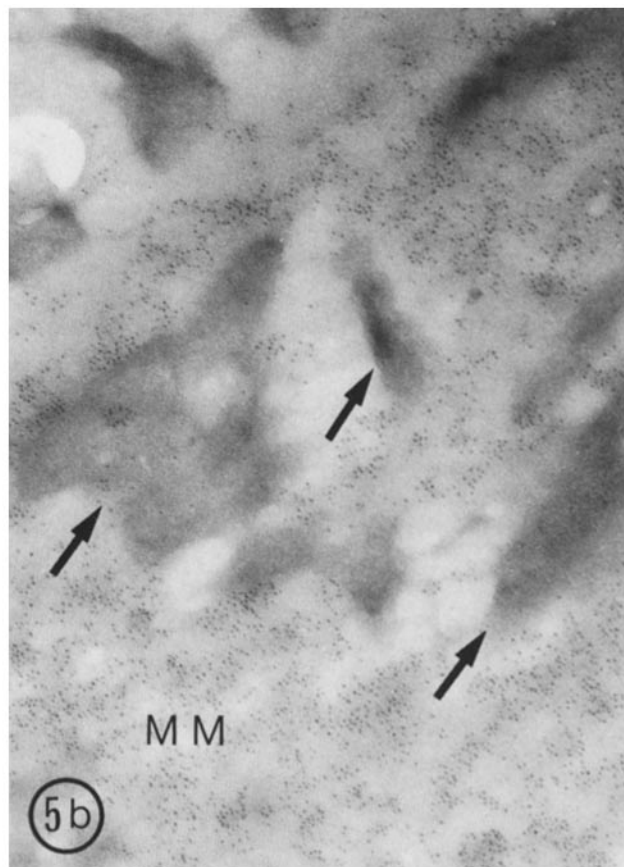
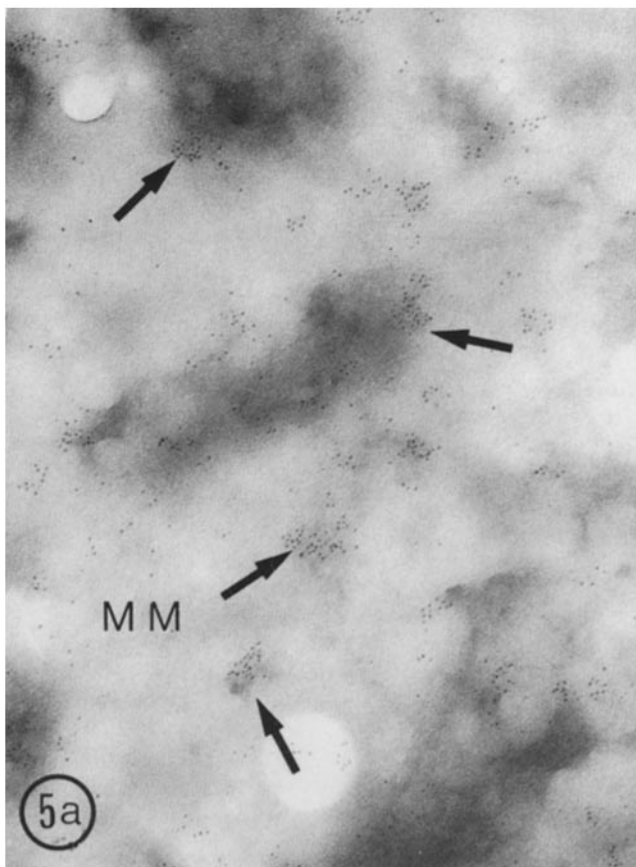


FIGURE 5 High-magnification electron micrographs of ultrathin frozen section through areas of mesangium similar to that observed in Fig. 2 a box B labeled with (a) antibodies to fibronectin, biotinized sheep anti-rabbit IgG, and avidin-ferritin. Note the specific labeling in areas of matrix-mesangial cell process contact. The mesangial cell processes appear as irregular dark areas interspersed throughout the lighter amorphous mesangial matrix; (b) antibodies to laminin, biotinized sheep anti-rabbit IgG, and avidin-ferritin. Note the uniform labeling over areas of amorphous mesangial matrix. Mesangial cell processes, arrows. MM, mesangial matrix.  $\times 60,000$ .

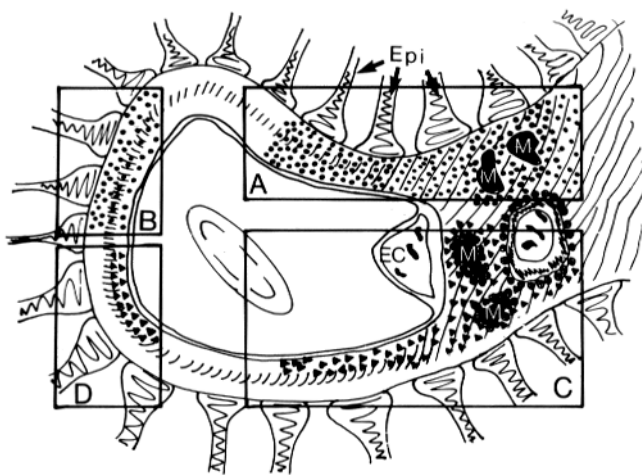


FIGURE 6 Schematic representation of the ultrastructural localization of types IV and V ( $AB_2$ ) collagen, laminin, and fibronectin in the murine glomerulus. Collagen types IV and V ( $AB_2$ ) (represented by black dots) are both present throughout the mesangial matrix (A) and GBM (B) in regular uniform patterns. Laminin (triangles) is uniformly present throughout the mesangial matrix (C) but has distinctive asymmetric localization on the GBM (D), being restricted to the endothelial cell (EC) side of the GBM. No laminin is noted on the epithelial cell (Epi) side of the GBM. Fibronectin (circles) is found exclusively in the mesangium (C) specifically in areas surrounding mesangial cell processes (M).

## DISCUSSION

The composition of basement membranes and the organization of collagenous and noncollagenous components within the membrane are topics of great interest inasmuch as functional properties depend upon structural integrity. The development of ultrathin frozen-section technology coupled with advances in the isolation, purification, and immunochemistry of noncollagenous and collagenous basement membrane components has made it possible to study the specific localization of some of these components (18). We have previously demonstrated the uniform distribution of basement membrane collagen types IV and V ( $AB_2$ ) in renal basement membranes over the entire width of glomerular, tubular, and Bowman's capsule basement membranes as well as over the mesangial matrix (Fig. 6, box A and B) (1). In this study, we show that laminin appears to share most localization sites with the basement membrane collagens studied previously, including tubular basement membranes, mesangial matrix, and Bowman's capsule. The one notable and perhaps functionally important exception apparently is the glomerular basement membrane of the capillary loops, in which laminin is localized specifically to the endothelial cell side of the basement membrane encompassing the lamina rara interna and a portion of the lamina densa (Fig. 6, box C and D). In contrast, fibronectin was found only in the mesangium around mesangial cell processes (Fig. 6, Box D).

The differences in distribution of these two large matrix glycoproteins may suggest specific roles for each component.

There is an abundance of data suggesting the notion that fibronectin mediates cell adhesion of mesenchymal cells to collagenous and noncollagenous substrata in vitro (5, 14) and that it is important for cell migration by processes that are poorly understood (4). In vivo fibronectin has been found in many tissues and its distribution as determined by immunofluorescence light microscopy is not inconsistent with this role as an adhesion molecule for mesenchymal cells.

There are reports that demonstrate the synthesis of fibronectin by some epithelial cells (16, 24), and immunofluorescence studies have localized fibronectin to certain epithelial basement membranes (19). Nevertheless, ultrastructural studies have not been done to determine whether fibronectin is located at the epithelial cell-basement membrane interface consistent with a role as an adhesion protein for epithelium. Furthermore, in vitro adhesion assays with epithelial cells do not as yet confirm such a function (11).

Fibronectin has been found in basement membranelike locations in selected vascular beds (19); however, with respect to the glomerular capillary bed, the findings are contradictory. Several light microscopic immunohistochemical studies of fibronectin in kidney tissue have found it present only in mesangium (7, 8, 19), whereas other such studies have reported its presence in a basement membrane distribution (12, 15). A recent ultrastructural immunohistochemical study of guinea pig kidney localized fibronectin to endothelial and epithelial cell processes abutting the GBM (12). This distribution was adduced as evidence that fibronectin may mediate adhesion of the cells to the GBM. Apart from a species difference, reasons for the discrepancy between that study and the data presented here are not clear. Because these investigators used the periodate-lysine-paraformaldehyde (PLP) fixative of McLean and Nakane (10), it is possible that the difference is due to loss of antigenic sites in kidney tissue fixed with glutaraldehyde. However, after PLP fixation, as previously described (see Methods), we still find labeling for fibronectin only about mesangial cell borders and not on epithelial or endothelial cell processes. We conclude that the discrepancy may be due to a species difference, to differing antibody specificities, or to problems with diffusibility of reagents through thick sections.

The fluorescent labeling of basement membranes with antibodies to laminin is in agreement with previously published reports (1, 20). The ultrastructural technique used here, however, allows one to recognize ultrastructural differences in distribution on different basement membranes. Whereas tubular basement membranes contain this macromolecule distributed throughout the entire thickness, a distribution also found in Bowman's capsule, the basement membrane of the glomerular loop is apparently organized in a different fashion. Laminin is restricted to the endothelial side of the basement membrane, possibly the lamina rara interna, and could serve two functions: (a) it may be synthesized by endothelial cells that may use it for adhesion to the underlying basement membrane and (b) as a glycoprotein containing a high proportion of acidic residues and sialic acid (R. Timpl, personal communication), it may also be one of the glomerular polyanions that are thought to form a charge barrier restricting passage of plasma proteins across the GBM (2, 23). This

asymmetry may reflect unique structural and/or functional roles for this glycoprotein, and furthermore this glycoprotein could be involved directly or indirectly in pathologic changes affecting cells or basement membranes.

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