

Ontogenesis of Glomerular Basement Membrane: Structural and Functional Properties

Michel Desjardins and Moïse Bendayan

Département d'anatomie, Université de Montréal, Montréal, Québec H3C 3J7, Canada

Abstract. Protein A-gold immunocytochemistry was applied in combination with morphometrical approaches to reveal the $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, and $\alpha 3(\text{IV})$ chains of type IV collagen as well as entactin on renal basement membranes, particularly on the glomerular one, during maturation. The results have indicated that a heterogeneity between renal basement membranes appears during the maturation process. In the glomerulus at the capillary loop stage, both the epithelial and endothelial cell basement membranes were labeled for the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen and entactin. After fusion, both proteins were present on the entire thickness of the typical glomerular basement membrane. At later stages, the labeling for $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen decreased and drifted towards the endothelial side, whereas the labeling for the $\alpha 3(\text{IV})$ chain increased

and remained centrally located. Entactin remained on the entire thickness of the basement membrane during maturation and in adult stage. The distribution of endogenous serum albumin in the glomerular wall was studied during maturation, as a reference for the functional properties of the glomerular basement membrane. This distribution, dispersed through the entire thickness of the basement membrane at early stages, shifted towards the endothelial side of the lamina densa with maturation, demonstrating a progressive acquisition of the permselectivity. These results demonstrate that modifications in the content and organization of the different constituents of basement membranes occur with maturation and are required for the establishment of the filtration properties of the glomerular basement membrane.

BASEMENT membranes are layers of specialized extracellular matrix associated with different cell types, among which are epithelial and endothelial cells. Along with their cell-supporting role, they are also involved in several cellular processes (Timpl, 1989) as well as in filtration of macromolecules. This is particularly significant in the kidney where the glomerular basement membrane acts as a selective filter, preventing the passage of serum proteins the size of albumin or larger to the urinary space (Bendayan et al., 1986; Kerjaschki et al., 1986). The understanding of the processes governing basement membrane function is still a matter of intense investigation (Kefalides et al., 1985). Insight into the possible mechanisms underlying the glomerular basement membrane permselectivity came with the characterization of the heparan sulfate proteoglycan, localized mainly in both laminae rarae and which seems to be responsible for the charge restriction selectivity of the basement membrane (Kanwar and Farquhar, 1979; Stow et al., 1985). Conversely, the lamina densa, in which type IV collagen is mainly present (Desjardins et al., 1990a), appears to be the structure responsible for size restriction selectivity (Bendayan et al., 1986; Batsford et al., 1987).

Studies have been performed to characterize the formation of the glomerular wall (Vernier and Birch-Anderson, 1962; Jokelainen, 1963; Kazimierzak, 1971; Reeves et al., 1978;

Saxén et al., 1986), as well as the origin of its basement membrane (Sariola et al., 1984; Abrahamson, 1985; Abrahamson and Perry, 1986a). These morphological studies were complemented by work on the glomerular permselectivity using electron dense tracers (Vernier and Birch-Anderson, 1963; Reeves et al., 1980; Bakala et al., 1985), which demonstrated that during maturation the glomerular basement membrane evolves from a highly permeable structure, allowing the passage of high molecular weight molecules, into an organized matrix restricting selectively the passage of plasma proteins. The nature of basement membrane modifications during this process are however poorly understood. Molecular changes of its components are likely to occur since immunohistochemical studies have revealed differences in patterns between fetal and adult renal basement membranes (Michael et al., 1983; Wan et al., 1984; Mounier et al., 1986; Wingen et al., 1987).

In the present study we have localized basement membrane components, the $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, and $\alpha 3(\text{IV})$ chains of type IV collagen as well as entactin, during the formation and maturation of rat renal tissue. Particular emphasis was directed towards the glomerular basement membrane and the acquisition of its permselectivity properties. Our results have indicated that maturation appears as a process characterized by the ontogenesis of basement membranes heteroge-

neity, through which they acquire their distinct structural and functional properties. This seems to be particularly true for the glomerular basement membrane, where modifications in the structural distribution of type IV collagen chains occur with the progressive acquisition of its ability to restrict the passage of endogenous albumin to the urinary space.

Materials and Methods

Sprague-Dawley rats 4, 14, 21, and 90 d old were used for the study (3 animals per group). The 4-, 14-, and 21-d-old animals were kept with their mother, whereas the 90-d-old animals were fed on a standard diet. The animals were killed by decapitation and the renal cortex was fixed by immersion for 2 h in paraformaldehyde 4%-lysine-periodate (McLean and Nakane, 1974) at 4°C. The tissue fragments were then processed for EM. They were dehydrated in graded methanol, embedded in Lowicryl K4M (Chemische Werke Lowi GmbH; Waldkraiburg, Germany) at -20°C and polymerized at -20°C under ultraviolet light as described previously (Bendayan, 1984). Semi-thin sections were cut, stained with toluidine blue and examined by light microscopy. Areas containing glomeruli profiles were selected and thin sections were cut, mounted on carbon and Parlodion-coated nickel grids, and processed for the protein A-gold immunocytochemical labeling of the $\alpha 1$ (IV) and $\alpha 2$ (IV) as well as the $\alpha 3$ (IV) chains of type IV collagen and entactin.

Antibodies against entactin and type IV collagen (α 1(IV) and α 2(IV) chains) were kindly provided by Drs. G. R. Martin and H. K. Kleinman (National Institutes of Health, Bethesda, MD). Entactin was revealed using a rabbit polyclonal antibody against entactin extracted from a mouse embryonal carcinoma-derived cell line from the parietal yolk sac (Carlin et al., 1981). The antibody was purified by affinity chromatography and further passed through a laminin-Sepharose column (Laurie et al., 1984). In addition, the absence of major reactivity of this antibody with laminin was assessed by immunocytochemical studies in which absorption of the entactin antibody with laminin before the labeling procedure did not affect the results (Desjardins and Bendayan, 1989a). The $\alpha 1$ (IV) and $\alpha 2$ (IV) chains of type IV collagen were revealed using a polyclonal antibody produced against type IV collagen extracted from the Engelbreth-Holm-Swarm (EHS) tumor (Laurie et al., 1980). The type IV collagen molecule was extracted by 0.5 M acetic acid and purified by salt precipitation and ion exchange chromatography. The anti-type IV collagen antibody was purified by immunoabsorption (Laurie et al., 1980). The specificity of this antibody for $\alpha 1$ (IV) and $\alpha 2$ (IV) chains was confirmed by Kleppel et al. (1986) who have demonstrated that type IV collagen extracted from the EHS tumor is composed of $\alpha 1$ (IV) and $\alpha 2$ (IV) chains. Previous studies have demonstrated the difference in labeling distributions obtained with this antibody and those against the $\alpha 3$ (IV) and $\alpha 4$ (IV) chains in various basement membranes (Desjardins and Bendayan, 1989a; Desjardins et al., 1990a). The $\alpha 3$ (IV) chain of type IV collagen was revealed using an IgG fraction of a polyclonal antibody kindly provided by Dr. J. Wieslander (Biocarb AB, Lund, Sweden). This antibody is directed against the NCI globular domain of the monomer M2* extracted from bovine glomerular basement membrane (Butkowski et al., 1985). Its specificity and major absence of cross-reactivity with the other monomers were well demonstrated by immunoblot experiments (Butkowski et al., 1987; Langeveld et al., 1988). Immunocytochemical studies have further demonstrated the specificity of the labelings obtained with this antibody when compared to those provided by antibodies against the $\alpha 1$ (IV) and the $\alpha 2$ (IV) chains (Desjardins et al., 1990a,b).

The ultrastructural localization of endogenous albumin was performed as described previously (Bendayan et al., 1986) to evaluate glomerular basement membrane permeability during maturation, using a specific anti-rat albumin antibody (IgG fraction; Cooper Biomedicals, Inc., Malvern, PA). The protein A-gold complex was prepared with 15-nm gold particles as described previously (Bendayan, 1984). The immunolabeling procedure described in previous studies was followed carefully (Bendayan, 1984; Desjardins and Bendayan, 1989a). In brief, the tissue sections were pre-incubated in 1% ovalbumin in 0.01 M PBS, pH 7.2 for 5 min and transferred to a drop of one of the antibody for 90 min at 20°C. This was followed by the incubation with the protein A-gold and staining in uranyl acetate and lead citrate. For revealing endogenous albumin, the preincubation with the 1% ovalbumin was omitted.

The specificity of the labelings was assessed through several control experiments. These included an absorption with the specific antigen before labeling and positive controls where non-specific antigens were added to the

antibody solution before labeling, as done previously (Desjardins and Bendayan, 1989a).

Morphometrical analysis of the labeling intensity obtained for the various type IV collagen chains and entactin was performed over the glomerular basement membrane and the Bowman's capsule basement membrane of each animal in the four different groups as follows: the tissues were first observed at low magnification, at which gold particles are undiscernible. Regions of glomerular loops and Bowman's capsule were then brought to a magnification of 16,900. For each animal, 10-12 micrographs of each basement membrane were recorded from at least four glomeruli and analyzed. The intensity of the labeling, evaluated as number of gold particles per squared micron of basement membrane, was determined using a Zeiss Videoplan 2 system (Carl Zeiss, Toronto, Ontario, Canada). The distribution of the labelings for the different type IV collagen chains, entactin and endogenous albumin was further determined over the thickness of the glomerular basement membrane for each group of animals, as performed previously (Bendayan et al., 1986; Desjardins and Bendayan, 1989b). For this, the distance, d , from the endothelial plasma membrane to the gold particles and the thickness, T , of the glomerular basement membrane at the same sites were measured. The ratio ($R = d/T$) of these values was calculated and the distribution ($0 < R < 1$) determined for each group of animals. An average of >1,000 gold particles was analyzed for each antigen in each group. The results are expressed as histograms of the percentage of labeling in function of its distribution over the glomerular basement membrane thickness.

Results

The ontogenesis of basement membrane heterogeneity was addressed in the present study using immunocytochemical localization of type IV collagen ($\alpha 1$, $\alpha 2$, and $\alpha 3$ chains) and entactin in renal tissue from 4-, 14-, 21- and 90-d-old rats. The study was focused on the glomerular and Bowman's capsule basement membranes since their continuity in areas of transition at the vascular pole of the glomerulus has proven to be useful for the demonstration of basement membrane heterogeneity (Fig. 1).

In a first step, the intensity of the labelings was evaluated over the Bowman's capsule basement membrane and that of the glomerulus of the various animals. At 4 d, both basement membranes were labeled for $\alpha 1$ (IV) and $\alpha 2$ (IV) chains of type IV collagen and entactin, whereas the labeling for the $\alpha 3$ (IV) chain appeared to be of low intensity. For any given labeling, only few gold particles were found over nuclei of the various cells or in extracellular regions such as the urinary space, demonstrating the low level of background staining. The 14-, 21-, and 90-d-old animals still displayed labelings of their basement membranes for these antigens, although changes in intensities did occur. The intensity of the labelings of the two basement membranes were compared during maturation and ratios of the glomerular basement membrane over the Bowman's capsule basement membrane values (GBM/BC)¹ were calculated. The results are presented in Table I, and reveal the appearance of a progressive heterogeneity between the two basement membranes. This is particularly striking in the area of transition between the two basement membranes at the vascular pole of the glomerulus. In this area, the 4-d animals display intense labeling for entactin in both basement membranes, whereas 90-d animals show labelings of much lower intensities in the Bowman's capsule basement membrane compare to the still highly labeled glomerular basement membrane (Fig. 1).

In a second step, the labeling for the $\alpha 1$ (IV) and $\alpha 2$ (IV)

1. *Abbreviations used in this paper:* BC, Bowman's capsule; GBM, glomerular basement membrane; RER, rough ER.

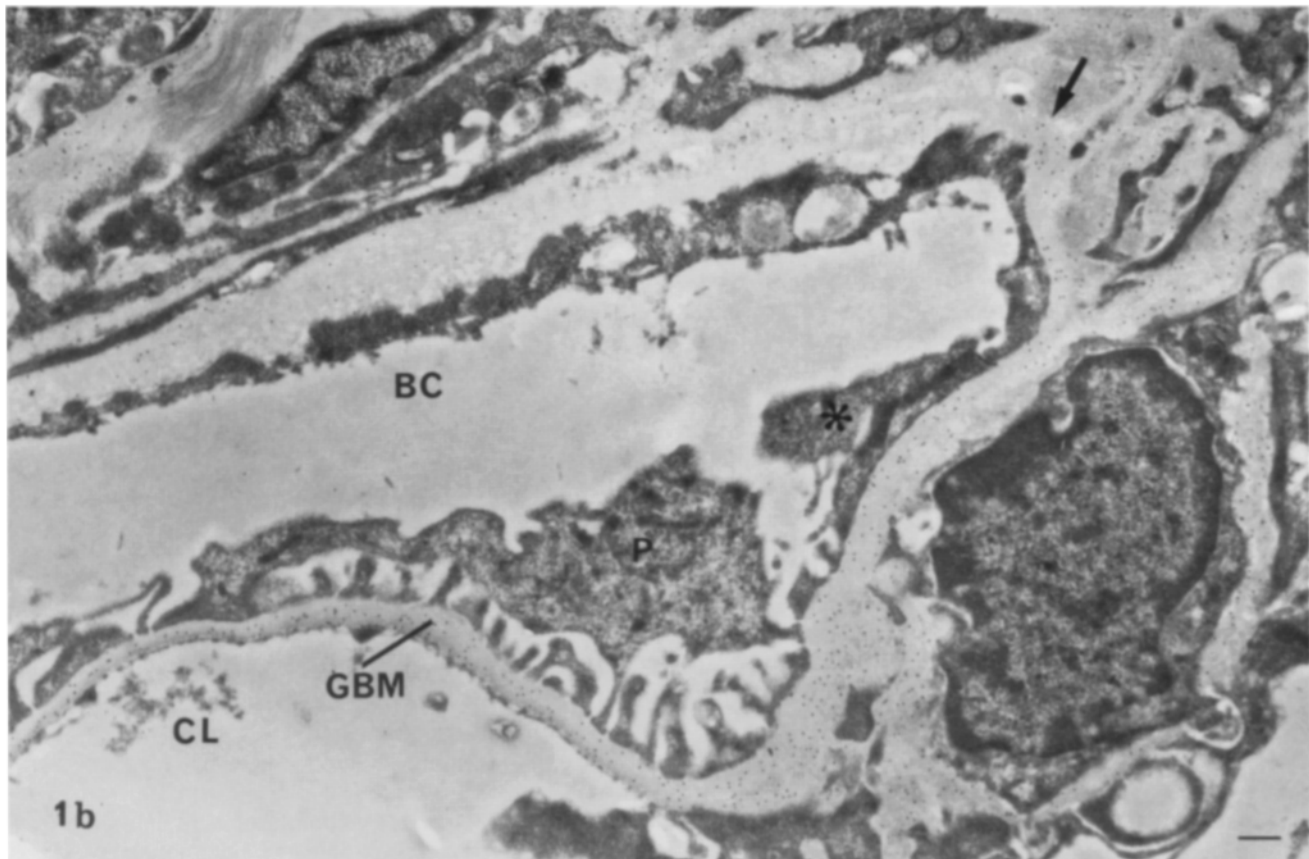
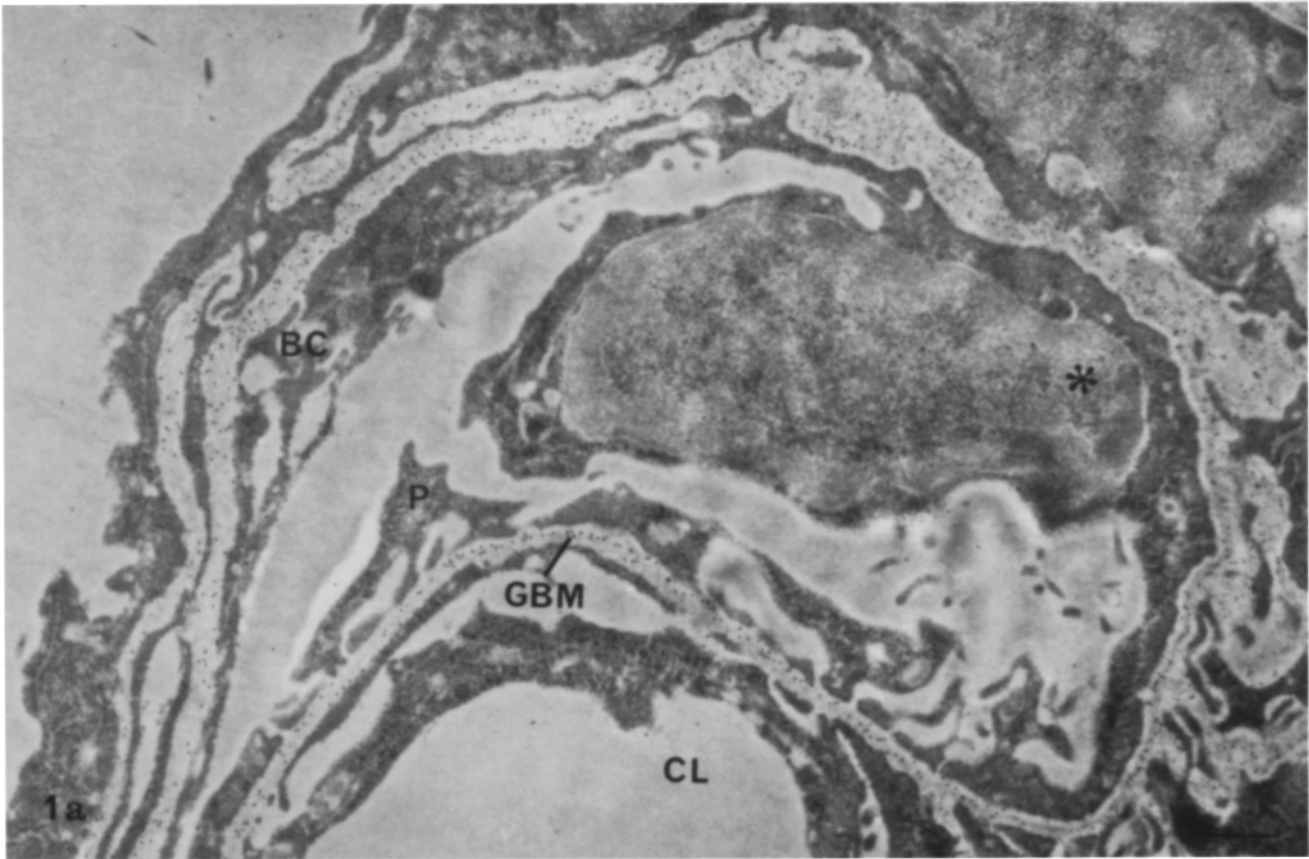


Figure 1. Localization of entactin over transitional areas at the vascular pole of glomeruli from 14- (a) and 90- (b) d-old rats. At 14 d, the labeling is present in the Bowman's capsule (BC) and the glomerular basement membranes (GBM). At 90 d, very few gold particles are present in the Bowman's capsule basement membrane, whereas the glomerular one remains highly labeled. The high labeling starts (arrow) at the level of the transitional area (*). CL, capillary lumen. Bars, 0.5 μ m.

Table I. Analysis of Labeling Intensities during Maturation (Gold Particles/ μm^2 Basement Membrane)

		4 d	14 d	21 d	90 d
$\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$ Chains of collagen IV	GBM*	61.79 \pm 3.22 [†]	62.61 \pm 2.42	31.82 \pm 1.89	15.30 \pm 0.79
	BC	87.99 \pm 5.43	108.57 \pm 3.57	91.68 \pm 6.25	58.94 \pm 1.86
	R	0.70	0.58	0.35	0.26
Entactin	GBM	91.39 \pm 5.53	118.89 \pm 4.44	77.31 \pm 2.25	54.51 \pm 4.88
	BC	62.62 \pm 3.45	64.67 \pm 4.37	34.41 \pm 3.22	7.33 \pm 0.83
	R	1.46	1.84	2.25	7.44

* GMB, glomerular basement membrane; BC, Bowman's capsule basement membrane; R, GBM/CB.

[†] Mean \pm SEM (three animals per group of age).

chains of type IV collagen over the glomerular basement membrane was compared to the one for the $\alpha 3(\text{IV})$ chain. The results presented in Fig. 2 show that the labeling for $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains progressively disappear during maturation, whereas that for the $\alpha 3(\text{IV})$ chain increases.

Further observations of the glomerular basement membrane labelings indicated changes in the spatial distribution of the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains during maturation. Before the fusion into a single glomerular basement membrane, the individual epithelial and endothelial cells basement membranes separated by a clear area at this capillary loop stage are labeled for these antigens as well as for entactin. Further, at the capillary loop stage, the epithelial cells display a continuous morphologically well organized basement membrane at their base, while in some cases the basement membrane of the endothelial cells appears discontinuous and less well defined (Fig. 3). Interestingly, the rough ER (RER) of the endothelial cells is labeled for $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen (Fig. 3 a) and entactin (Fig. 3 b), whereas fewer gold particles are observed in the RER of the epithelial cells at the capillary loop stage and later on through maturation. After fusion, the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen and entactin appear distributed to the entire thickness of the resulting typical glomerular basement membrane. At 4 d, 59.6 and 51.9% of the labeling are present within the endothelial first half of the glomerular basement membrane for $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen (Fig. 4 a) and entactin (Fig. 6 a), respectively, which reflects the central location of both antigens. During maturation, a shift of the labeling for the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains towards the endothelial side occurs (Fig. 4). At 14 d, 69.2% of that labeling is present on the endothelial side, whereas this value increases to 73.1 and 80.6%, respectively at 21 and 90 d (Fig. 4). No such changes are observed for the $\alpha 3(\text{IV})$ chain which remains centrally located throughout maturation. Indeed, 52.2, 51.5, 48.1, and 50.9% of the labeling for $\alpha 3(\text{IV})$ chain are present on the endothelial side of the glomerular basement membrane at days 4, 14, 21, and 90, respectively (Fig. 5). This is also the case for entactin which remains distributed rather centrally (Fig. 6), with 51.9, 50.4, 54.6, and 58.8% of its labeling on the endothelial side at 4, 14, 21, and

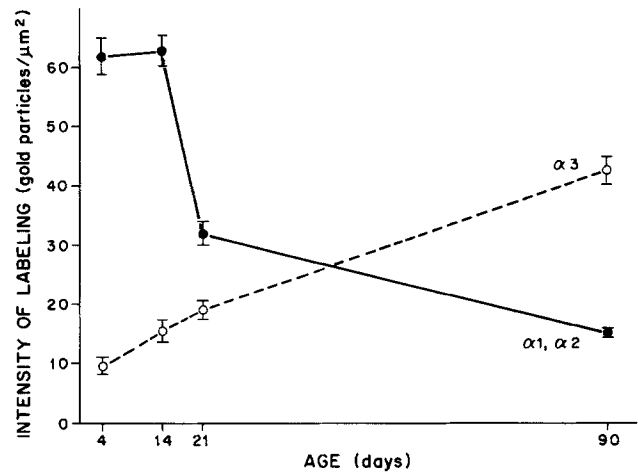
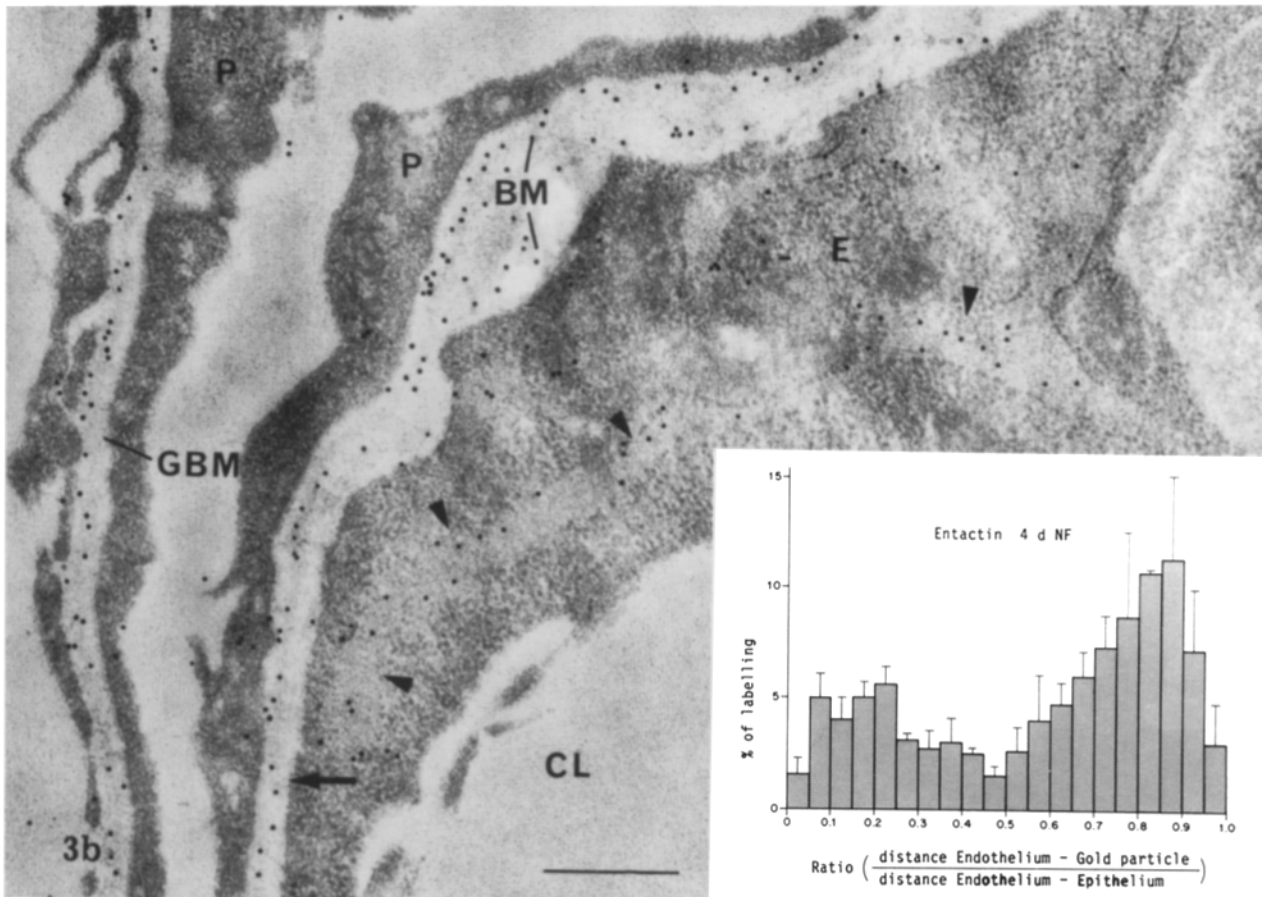
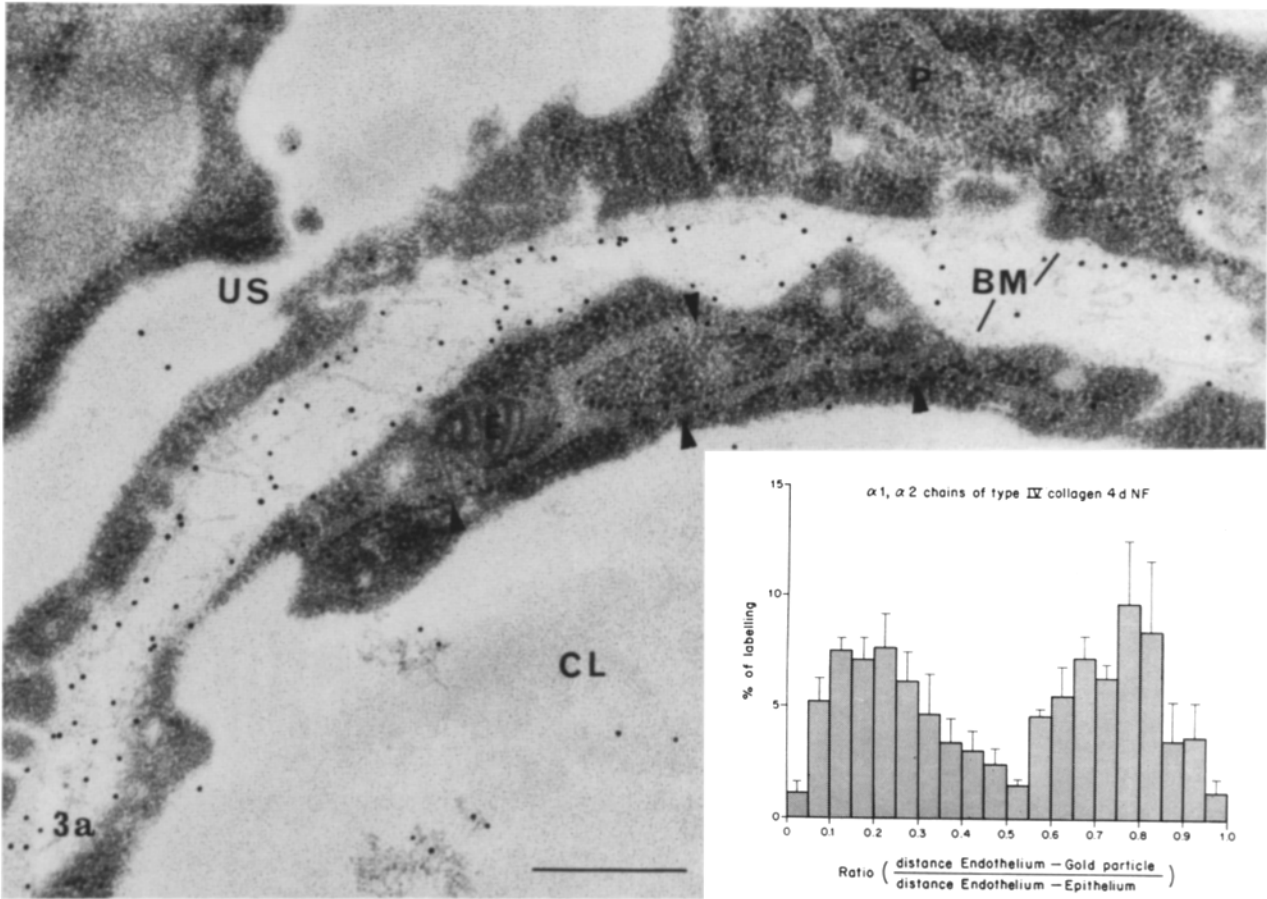


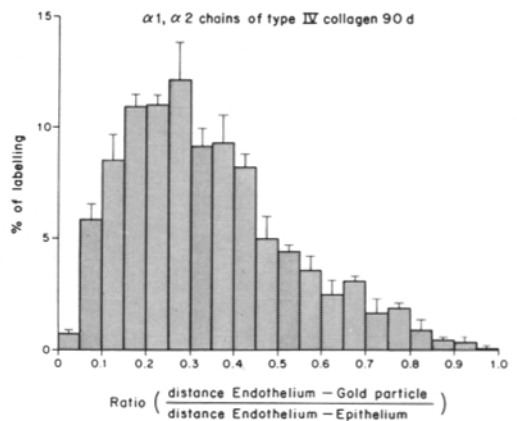
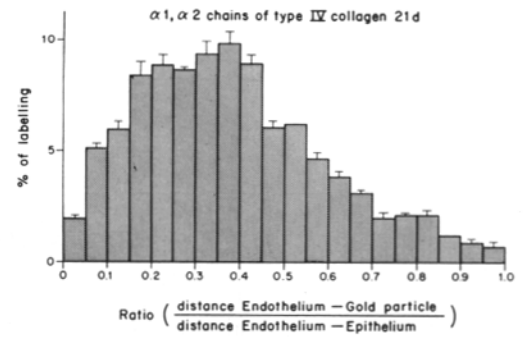
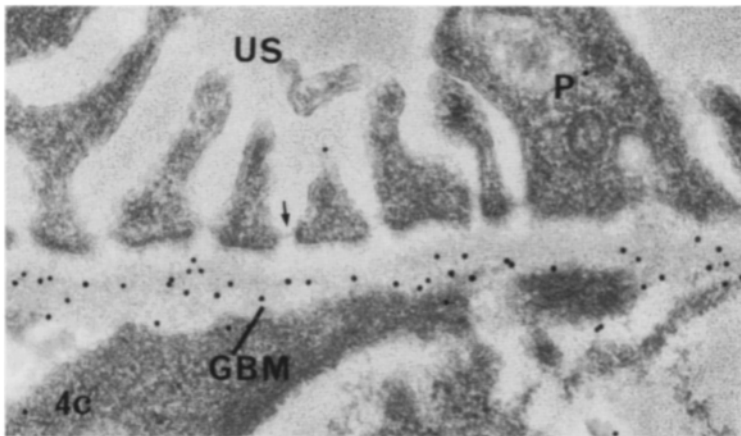
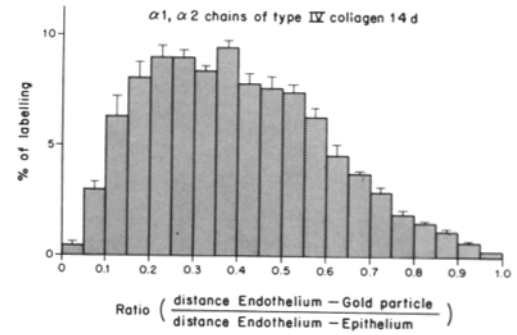
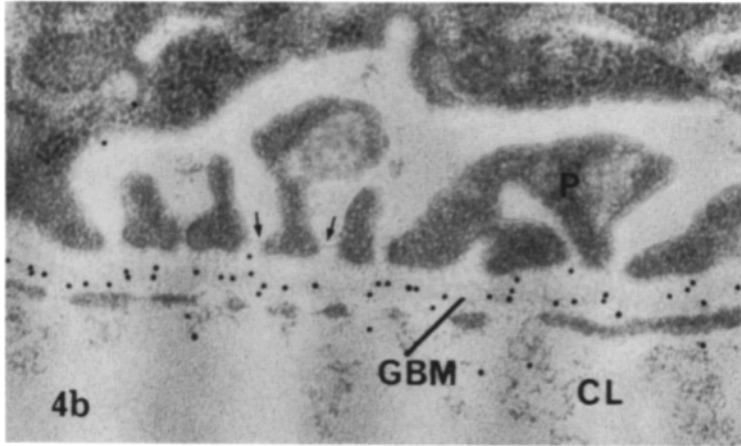
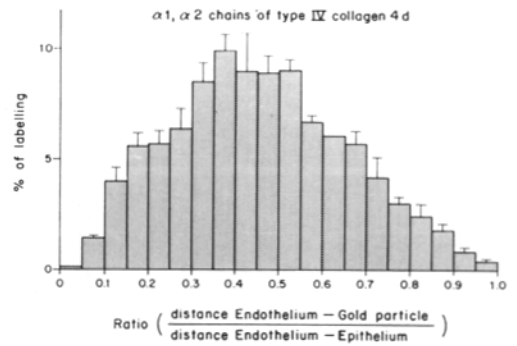
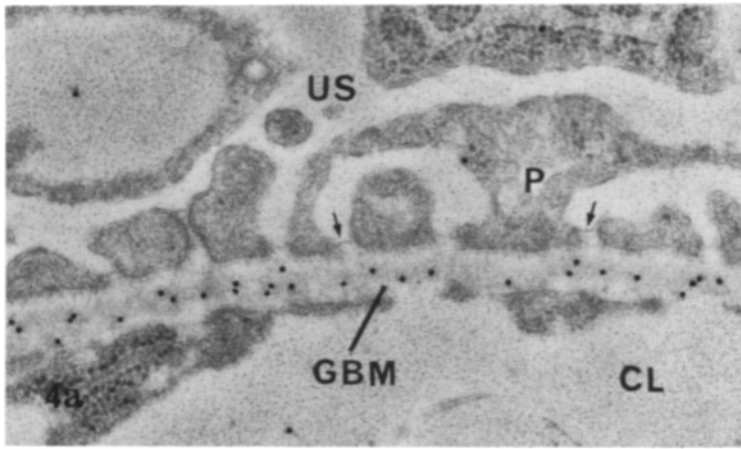
Figure 2. Labeling intensities of $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, and $\alpha 3(\text{IV})$ chains of type IV collagen in the glomerular basement membrane at different ages during maturation.

90 d, respectively. The analysis of the distribution of the labelings over the thickness of the glomerular basement membrane are presented in Figs. 3 to 6.

Finally, in the last part of our study, endogenous albumin was revealed on the renal tissue of the various animals. An intense labeling was observed in the capillary lumen demonstrating the retention of serum proteins by the fixation protocol. In the glomerular wall, the labeling was mainly restricted to the basement membrane (Fig. 7). At 4 d in fused glomerular basement membranes, 61.0% of the labeling for albumin is present on the endothelial side. This value increases during maturation, 71.5, 74.7, and 82.3% of the labeling being present on the endothelial side at 14, 21, and 90 d respectively. The distribution of endogenous albumin was used as a reference to evaluate the functional properties of the glomerular basement membrane during maturation and the shift found, demonstrates the restriction of the passage of this protein by the basement membrane from the capillary lumen to the urinary space.

Figure 3. Localization of type IV collagen $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains (a) and entactin (b) over basement membranes of epithelial (P) and endothelial (E) cells of the glomerulus. Both basement membranes are labeled before fusion into glomerular basement membrane (GBM). Active synthesis by the endothelial cell is demonstrated by the labeling present in the rough endoplasmic reticulum (arrowheads). Insets show the distribution of the labelings in both basement membranes (see Fig. 4). CL, capillary lumen; NF, nonfused basement membranes; P, podocyte; US, urinary space; arrow, region showing a fused basement membrane. Bars, 0.5 μm .





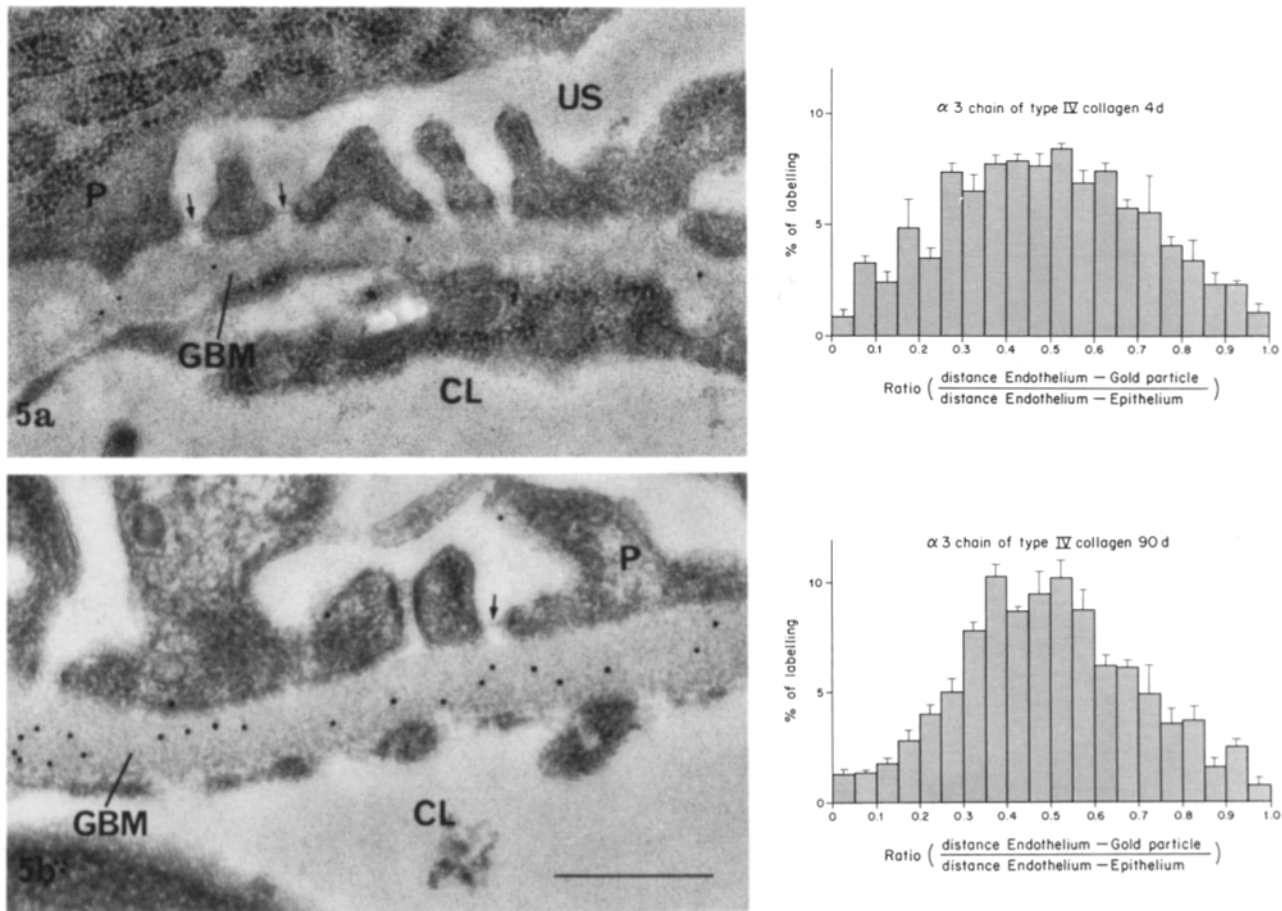


Figure 5. Localization of type IV collagen $\alpha 3(IV)$ chain in the glomerular basement membrane of 4- (a) and 90- (b) d rats. Histograms show the distribution of the labeling over the thickness of the glomerular basement membrane (GBM), expressed as ratio values. The labeling is present throughout the basement membrane. Bars, 0.5 μm .

Discussion

In the newborn rat, the kidney is not completely mature and the nephrogenesis continues until ~ 2 wk of age (for a review see Abrahamson, 1987). Therefore, the histological observation of a 4-d-old rat renal tissue shows all stages of glomerular formation from the nephrogenic vesicle to the maturing stage, at which the typical "tri-lamellar" glomerular basement membrane is present. This situation is thus particularly interesting and was taken advantage of to investigate the distribution of various basement membrane components during renal maturation. The protein A-gold immunocytochemical technique was chosen in the present study for the high resolution of the results provided and for the possibility of performing quantitative evaluations. A potential limitation of this approach consists in the possible masking of certain determinants and/or in some steric hindrance that would prevent access to the antigen with absence of labeling. The protein A gold is a postembedding technique which reveals all those antigenic sites exposed at the surface of the tissue sec-

tion by the cutting procedure (Bendayan, 1984). Since the tissue has been sectioned, masking of antigenic sites by neighboring proteins is unlikely to occur. Further to this, the use of polyclonal antibodies, which are directed against several epitopes of the same protein, reduces the impact of such a limitation. In a previous study (Desjardins and Bendayan, 1990a), we have further investigated this point and performed enzymatic treatments of the tissue sections before immunolabeling to "unmask" possible determinants. These were unsuccessful, the labeling patterns remaining unchanged after enzymatic treatments. Consequently, the masking of any antigen under our working conditions, appears quite unlikely to occur.

During maturation, basement membranes appear to be modified and to acquire their functional properties. In adult rats, we have previously demonstrated the existence of a heterogeneity in the distribution and concentration of the main components of basement membranes, namely type IV collagen, entactin, heparan sulfate proteoglycan and laminin, among renal structures (Desjardins and Bendayan, 1989a).

Figure 4. Localization of type IV collagen $\alpha 1(IV)$ and $\alpha 2(IV)$ chains in the glomerular basement membrane of 4- (a), 14- (b), 21- (c), and 90- (d) d-old rats. Histograms show the distribution of the labeling over the thickness of the glomerular basement membrane (GBM), expressed as ratio values. A progressive shift of the labeling towards the endothelial side of the basement membrane is observed with maturation. CL, capillary lumen; P, podocyte; US, urinary space; arrows, slit diaphragms. Bars, 0.5 μm .

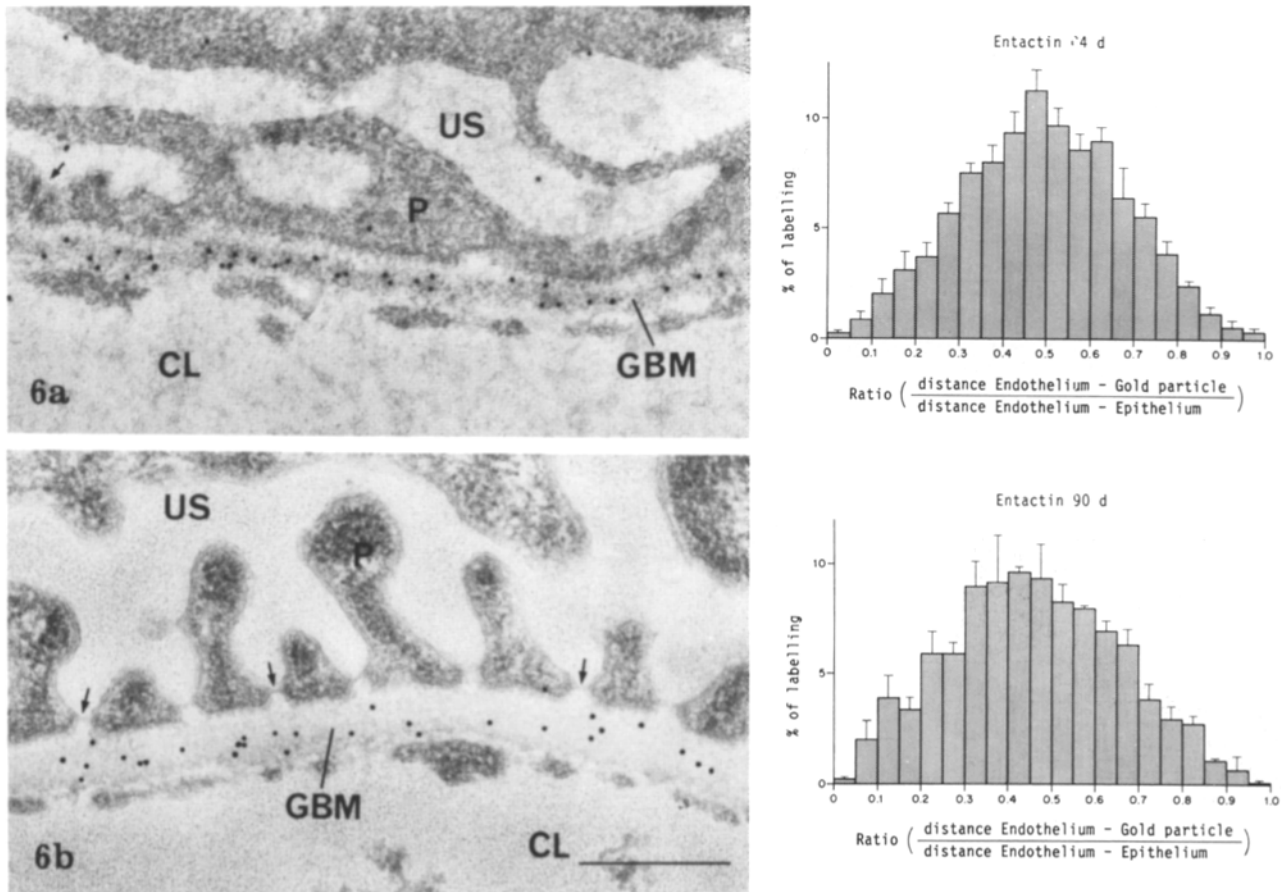


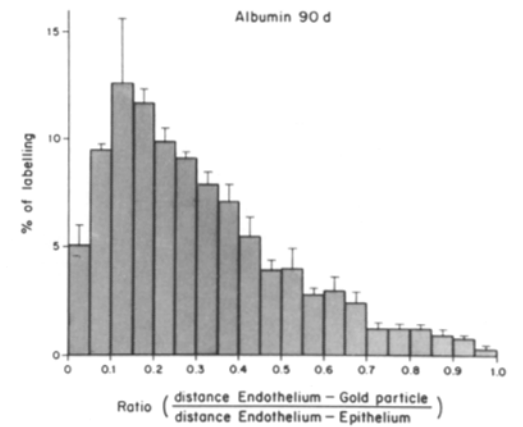
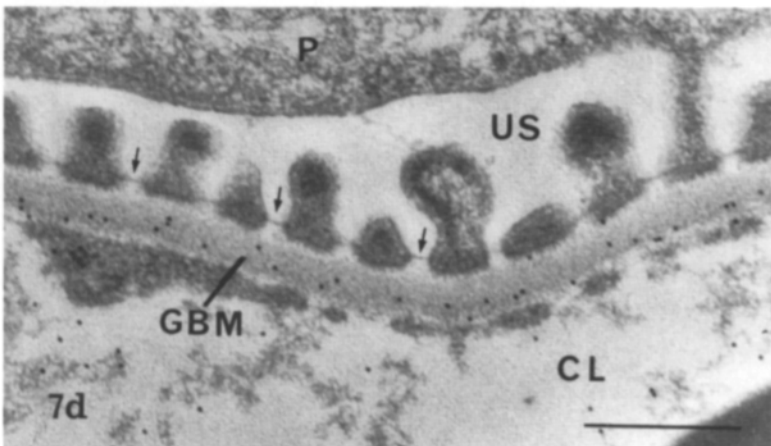
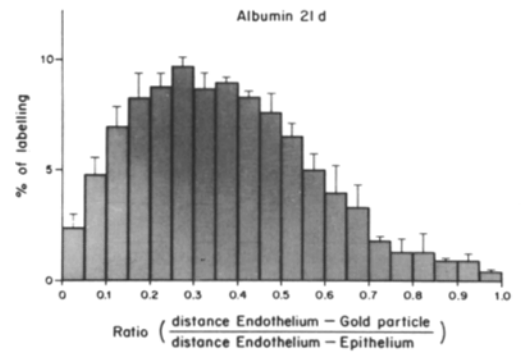
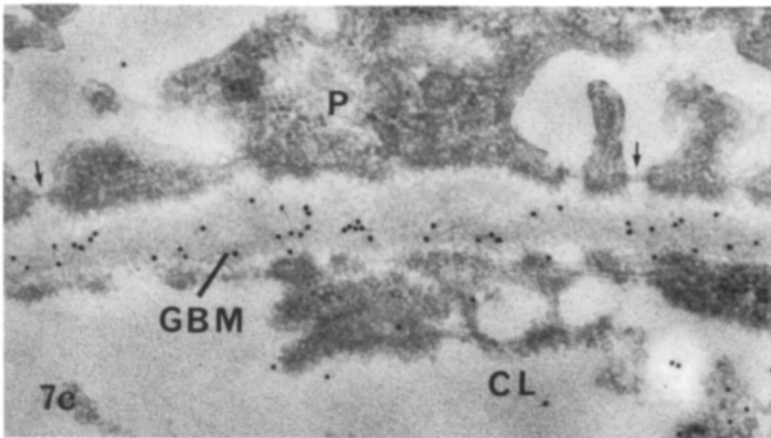
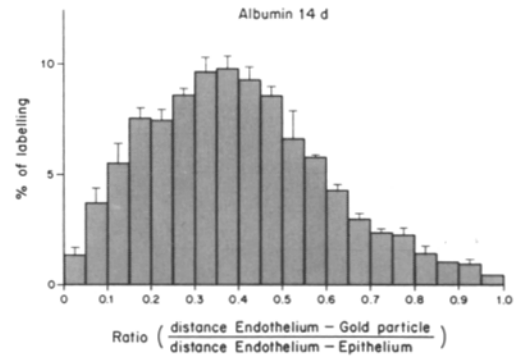
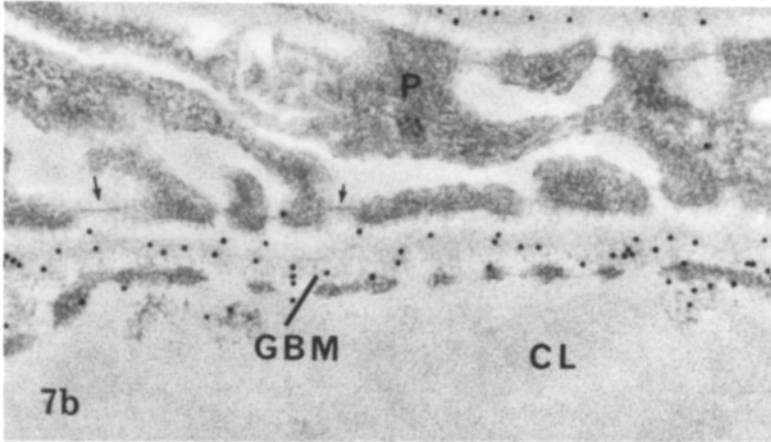
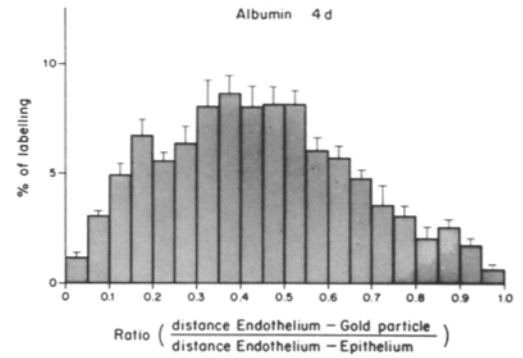
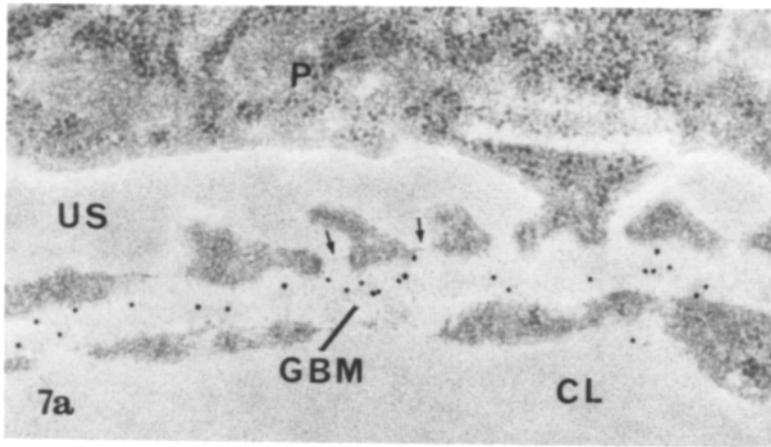
Figure 6. Localization of entactin in the glomerular basement membrane of 4- (a), and 90- (b) d rats. Histograms show the distribution of the labeling over the thickness of the glomerular basement membrane (GBM), expressed as ratio values. The labeling is present throughout the basement membrane. Bars, 0.5 μ m.

Since these proteins are known to interact and to form highly organized matrices (Yurchenco et al., 1986; Grant et al., 1989; Yurchenco and Schittny, 1990), our results were interpreted as reflecting variations in the tridimensional organization of the various renal basement membranes to allow for their distinct and respective roles. Further, specific chains of type IV collagen were also found to be heterogeneously distributed revealing variations in the nature of type IV collagen in basement membranes (Butkowski et al., 1989; Desjardins et al., 1990a). Developmental acquisition of basement membrane heterogeneity was shown to occur in the avian lens capsule (Fitch et al., 1983). The present study was thus performed to investigate changes in the distribution of basement membrane components during renal maturation leading to the heterogeneity observed in the adult tissue.

Basement membranes of parietal and visceral epithelial cells of the glomerulus, as well as the one of the proximal tubule originate from a condensate of undifferentiated neph-

rogenic mesenchyme at an early stage of renal development (Abrahamson, 1987). Our results indicate that the heterogeneity between the basement membranes associated to these different cell types establishes progressively during maturation. Indeed, dissimilar patterns of maturation were observed for two distinct, although closely related, basement membranes originating from the nephrogenic mesenchyme, that of the Bowman's capsule and the one of the glomerular wall. The labeling intensity for the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains of type IV collagen progressively decreases in the glomerular basement membrane, while it retains high intensities in the Bowman's capsule basement membrane throughout maturation. This is reflected by the decreasing ratios of GBM/BC labelings for type IV collagen (Table I). On the other hand, labeling for entactin remains high in the glomerular basement membrane through maturation, whereas it slowly decreases in the Bowman's capsule basement membrane. These results indicate that basement membranes are rather homog-

Figure 7. Localization of endogenous albumin in the glomerular basement membrane of 4- (a), 14- (b), 21- (c), and 90- (d) d rats. Albumin was used as a marker of permeability properties of the glomerular wall. Histograms show the distribution of the labeling over the thickness of the glomerular basement membrane (GBM), expressed as ratio values. While albumin is present on the entire thickness of the basement membrane at 4 d, it is restricted to the endothelial side in more mature animals as permeability properties are acquired. It is interesting to note that the distributions of albumin follow those of the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains of type IV collagen (see Fig. 4). Bars, 0.5 μ m.



enous at early stages of formation and get modified during maturation to reach the heterogeneity found in tissues of adult animals. Previous studies have documented changes in immunohistochemical detection of various antigens of basement membranes and extracellular matrix (Ekblom et al., 1980; Lelongt et al., 1988), among which type IV collagen (Ekblom, 1981; Ekblom et al., 1981; Michael et al., 1983; Mounier et al., 1986; Kleppel and Michael, 1990) and entactin (Avner et al., 1983) during glomerular development. Immunocytochemistry and *in situ* hybridization also revealed that coordinate synthesis of basement membrane components and of their respective receptors appear as key events in the early process of kidney formation (Laurie et al., 1989; Ekblom et al., 1990; Korhonen et al., 1990). However, the nature of the modifications leading to the elaboration of functional matrices are still mostly unknown.

In the present study, a particular emphasis was directed to the study of the formation and maturation of the glomerular basement membrane and to the acquisition of its functional properties. At 4 d, before their fusion into the typical glomerular basement membrane, both epithelial and endothelial cell basement membranes were labeled for entactin and the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen, whereas labeling for the $\alpha 3(\text{IV})$ chain was almost absent. Furthermore, both types of cells appear to synthesize these components, although at this stage the endothelial cells seem to be more active as suggested by the intense labeling of their RER. After fusion into the tri-lamellar structure, the labeling was then distributed through the entire thickness of the basement membrane. With maturation, the labeling for $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ decreased and was progressively restricted to the endothelial side of the basement membrane. On the other hand, the labeling for the $\alpha 3(\text{IV})$ chain, very low at 4 d, increased during maturation and remained distributed throughout the thickness of the glomerular basement membrane. Indeed in adult animals type IV collagen composed of the recently identified $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains (Hudson et al., 1989) was found to be present in the entire thickness of the glomerular basement membrane (Desjardins et al., 1990a).

Entactin, a sulfated protein of ~ 158 kD (Carlin et al., 1981), first isolated from the EHS tumor (Timpl et al., 1983), was recently found to be identical to nidogen (Timpl, 1989) and has been localized by immunocytochemistry in a variety of basement membranes (Hogan et al., 1982; Laurie et al., 1984; Desjardins and Bendayan, 1989a). Entactin and laminin form in equimolar ratio a stable complex (Carlin et al., 1983; Dziadek and Timpl, 1985; Paulsson et al., 1987), in which entactin appears to mediate the binding of laminin to type IV collagen (Aumailley et al., 1989; Timpl, 1989). Furthermore, immunocytochemical studies performed on various tissues have demonstrated the codistribution of these two components (Avner et al., 1983; Laurie et al., 1984; Schittny et al., 1988; Desjardins and Bendayan, 1989a). Laminin and entactin appear at early stages of embryogenesis, at the 2-cell and the 8- to 16-cell morula stages respectively (Dziadek and Timpl, 1985), indicating a sequential assembly of these proteins. During early nephrogenesis, laminin appears in basement membranes in a punctate pattern after induction by the nephrotic bud (Ekblom et al., 1980). Subsequently, immunostaining of entactin and laminin were found to be identical at all stages of nephrogenesis (Avner et al., 1983).

In our case, labeling for entactin was found to be intense during nephrogenesis but progressively disappeared during maturation in the Bowman's capsule basement membrane. Indeed, we have previously reported that entactin and laminin codistribute in renal basement membranes exhibiting low labelings in the Bowman's capsule (Desjardins and Bendayan, 1989a). In correlation to this, the ultrastructural aspect of this basement membrane is quite different from all others, displaying a multilayer or laminated pattern. Although type IV collagen undergoes modifications during maturation, the distribution of entactin in the glomerular basement membrane remained unchanged as it does in pathological conditions (Desjardins and Bendayan, 1990). This indicates that entactin, and possibly the entactin-laminin complex display affinities for type IV collagen molecules regardless of their α chain composition. The high sensitivity of entactin to proteases may influence the binding of laminin to type IV collagen and must play an important role regulating any remodeling occurring in basement membranes (Timpl, 1989) particularly during maturation.

Modifications of collagenous elements have been reported previously during renal development (Ekblom et al., 1981; Mounier et al., 1986), with the replacement of interstitial type I and III collagen by type IV collagen as renal structures differentiate. Furthermore, our results suggest that type IV collagen made mainly by $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains, present at early stages of renal formation, progressively disappears from the maturing glomerular basement membrane and gets restricted to the endothelial side, while type IV collagen made of novel chains emerges. Interestingly, Kleppel and Michael (1990), have shown that two different 28-kD peptides, possibly related to the novel chains of type IV collagen, are expressed in glomerular basement membrane at later stages of development compare to $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen, and increase in maturing glomerulus. The changes observed for the various chains of type IV collagen in the glomerular basement membrane may thus be related to a reorganization process leading to the acquisition of functional properties. In this line of thought, a possible involvement of the various chains of type IV collagen in the restrictive permeability of the glomerular wall was raised in our earlier studies (Bendayan et al., 1986; Desjardins and Bendayan, 1989b). The distributions of endogenous albumin and IgG's were shown to follow that of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen on the endothelial side of the glomerular basement membrane (Bendayan, 1985; Bendayan et al., 1986; Desjardins and Bendayan, 1989b; Desjardins et al., 1990a). Furthermore, the alterations in the filtration properties of the glomerular basement membrane occurring in diabetes, coincide with a complete redistribution of the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen (Desjardins et al., 1990b). The present study of endogenous albumin in the glomerular wall during maturation has also revealed a direct correlation between the distribution of albumin and that of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains of type IV collagen. These distributions are likely to reflect a progressive restriction of the passage of albumin through the glomerular basement membrane, and the gradual acquisition of its functional properties. Indeed, high molecular weight proteins are absent in urine samples of adult rats but present in those of 4-d-old animals (Schaeferbeke and Cheignon, 1980). Thus, the redistribution of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains and the appearance of

the $\alpha 3(IV)$ chain of collagen IV seem to be involved in the acquisition of the glomerular basement membrane permselectivity properties. However beside type IV collagen, other components could play important roles in the selective permeability properties of glomerular basement membranes. A reorganization of the anionic sites of the glomerular basement membrane, and a relocalization of the heparan sulfate proteoglycan from a central region of the forming basement membrane to both laminae rarae were reported (Reeves et al., 1980; Abrahamson and Perry, 1986b).

A working model regarding the process leading to the acquisition of size selectivity of the glomerular basement membrane would be that the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains of type IV collagen favor, through their self-assembly and their binding to other components, the organization of a loose matrix that allows passage of serum proteins. As $\alpha 1(IV)$ and $\alpha 2(IV)$ chains disappear, type IV collagen made of $\alpha 3(IV)$ and perhaps $\alpha 4(IV)$ chains appears (the $\alpha 3(IV)$ and $\alpha 4(IV)$ chains were shown to codistribute in adult normal renal basement membranes [Desjardins et al., 1990a]) with a reorganization into a tighter matrix leading to the establishment of efficient permselectivity. In support of this hypothesis, isolated acellular glomerular basement membrane was shown to display a looser structure on the endothelial side (enriched in $\alpha 1(IV)$ and $\alpha 2(IV)$ chains) compared with the more tightly arranged epithelial side (Carlson and Audette, 1989). Maturation thus appears as a process of coordinate synthesis and reorganization through which basement membranes acquire their structural and functional properties.

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