

Regional Distribution of *N*-Acetyl-D-galactosamine Residues in the Glycocalyx of Glomerular Podocytes

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ABSTRACT *Helix pomatia* lectin (HPL) bound to colloidal gold was used as a specific cytochemical probe for the localization of terminal nonreducing *N*-acetyl-D-galactosamine residues in thin sections of rat kidney. In the glomerulus, lectin-binding sites were associated only with the podocyte foot process bases and were not found on the free cell surface of podocytes or on any other glomerular components. Gold-particle label was often arranged in the form of clusters which extended from the foot process base to the lamina rara externa and lamina densa of the basement membrane. In contrast, wheat germ lectin (WGL)-binding sites (β -[1 \rightarrow 4] linked *N*-acetyl-D-glucosamine residues and *N*-acetylneuraminic acid residues) were found in all regions of the podocyte plasma membrane and on the cell surface of all other glomerular cell types. In addition, WGL-binding sites were present in all three layers of the glomerular basement membrane (GBM) as well as in the mesangial matrix. A quantitative evaluation of the pattern of labeling for HPL-binding sites together with the sugar specificity of this lectin suggest that a component of the glycocalyx is being detected rather than a basement membrane component. This was confirmed by the absence of *H. pomatia* lectin-binding sites in preparations of isolated GBM which retained, however, wheat germ lectin-binding sites. These data show that the glycocalyx of the foot process base is a highly specialized cell surface domain with respect to its carbohydrate composition.

Podocytes are highly specialized cells that form the visceral epithelium of Bowman's capsule in the kidney. Their cell body, which lies in the urinary space, sends out long foot processes which interdigitate with those of neighboring podocytes and which rest on the glomerular capillary basement membrane. The factors that are responsible for the maintenance of this characteristic architecture are unknown, but may include intracellular cytoskeletal elements (2) as well as specific properties of the plasma membrane.

In this respect, a number of studies have revealed a striking heterogeneity between two distinct regions of the podocyte plasma membrane, (a) the so-called "free surface," which includes the cell body as well as the foot process membrane adjacent to the urinary space above the slit membrane, and (b) the part of the foot process membrane below the slit membrane, the so-called foot process base which is embedded in the lamina rara externa of the basement membrane. These two plasma membrane regions label quite differently with cationic stains such as colloidal iron (10, 16) and ruthenium red (9, 13) or with phosphotungstic acid (11) and with various lectins (4, 7). Recently, different membrane compositions have also been

revealed between these two regions by both conventional freeze-fracture and freeze-fracture of tissue exposed to the cholesterol probe, filipin (16, 17).

To further define regional differences in membrane composition in the glomerulus, we used, as cytochemical probes, lectins that interact with specific sugar moieties on oligosaccharide chains of glycoconjugates (18). The lectins were bound to colloidal gold as an electron-dense marker (24) and were used in a postembedding labeling technique (20, 21) to reveal lectin-binding sites on thin sections of aldehyde-fixed, resin-embedded material. The advantage of this approach is that problems of uneven reagent penetration, common in pre-embedding techniques, are circumvented and that the highly electron-dense gold particles allow a precise localization of lectin-binding sites. In this report we describe the distribution of binding sites for *Helix pomatia* lectin (HPL), specific for terminal, nonreducing *N*-acetyl-D-galactosamine (GalNAc) residues (31) and wheat germ lectin (WGL) which recognizes β -(1 \rightarrow 4)-*N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid residues (1), in the glomeruli of intact kidney and in isolated glomerular basement membranes. We demonstrate the

existence of a discrete glycocalyx domain containing *N*-acetyl-D-galactosamine residues, which is associated only with the basal part of the podocyte foot process plasma membrane.

MATERIALS AND METHODS

Tissue Processing

Kidneys from adult (200–300 g) male Wistar rats were fixed by perfusion with 1% glutaraldehyde in isotonic phosphate-buffered saline (PBS, pH 7.4) at room temperature. The perfusion was carried out for 15 min, and small pieces of kidney were then fixed by immersion giving a total fixation time of 2 h. After several rinses with PBS, the kidney pieces were incubated with 0.1 M NH_4Cl in PBS for 45 min to block free aldehyde groups. Tissue was then dehydrated in ethanol and embedded in Epon 812 or in the hydrophilic resin Lowicryl K4M at low temperature, as previously described (25).

Preparation of Isolated Glomerular Basement Membranes

Glomerular basement membranes (GBM) were prepared by the method of Carlson et al. (5) but using a different protocol to obtain isolated glomeruli. Kidneys were perfused with isotonic phosphate buffered saline (PBS, pH 7.4) and cortical slices were finely minced with a razor blade. The cortical slush was dispersed in PBS in a 15-ml glass tube and the larger fragments were allowed to sediment. Glomeruli were enriched in the resulting supernatant and were isolated under dissecting microscopes by aspiration into micropipettes. The glomeruli were then subjected to hypotonic shock followed by treatment with Triton X-100, DNase and sodium deoxycholate as described by Carlson et al. (5). The resulting material was then fixed in 1% glutaraldehyde in PBS for 1 h, enclosed in Agar and embedded at low temperature in Lowicryl K4M (25).

Preparation of Colloidal Gold, Lectin-Gold, and Glycoprotein-Gold Complexes

Colloidal gold was prepared by reducing tetrachloroauric acid either with trisodium citrate (to give 15-nm gold particles) or with a saturated solution of white phosphorus in ether (to give 3-nm gold particles), as previously described (19). The preparation of lectin-gold complexes has been described in detail in previous publications (20, 21, 24). Briefly, the pH of the colloidal gold was adjusted to 7.4 by 0.2 M K_2CO_3 . The minimal amount of HPL (Pharmacia Fine Chemicals, Uppsala, Sweden) needed to stabilize the colloidal gold was 6.5 $\mu\text{g}/\text{ml}$ for 15-nm gold particles, and 25 $\mu\text{g}/\text{ml}$ for 3-nm gold particles. For localization of WGL-binding sites, the indirect technique of Geoghegan and Ackerman (8) using ovomucoid-gold complexes was applied. Colloidal gold (particle size ~15 nm) was adjusted to pH 4.8 with 0.1 N HCl. The minimal amount of ovomucoid needed to stabilize 10 ml of colloidal gold was 16 μg . The crude lectin-gold complexes and the ovomucoid-gold complexes were purified and concentrated by ultracentrifugation at 60,000 g for 45 min (15-nm gold particles) or 105,000 g for 1 h (3-nm gold particles). The supernatant was discarded and the sedimented complexes were resuspended in PBS to give a stock solution of ~100 μg HPL-gold ml^{-1} , and 100 μg ovomucoid-gold ml^{-1} , respectively, expressed in terms of protein concentration.

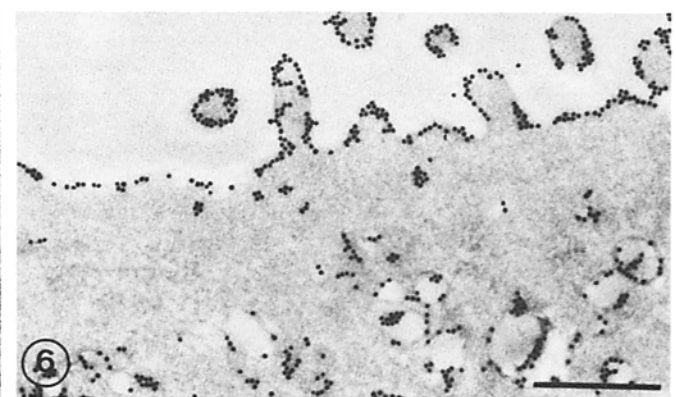
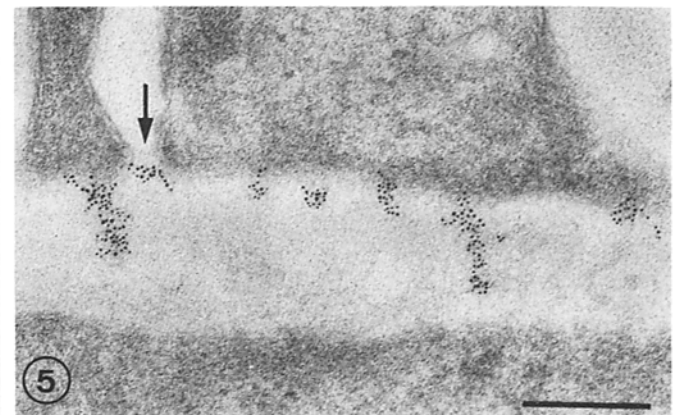
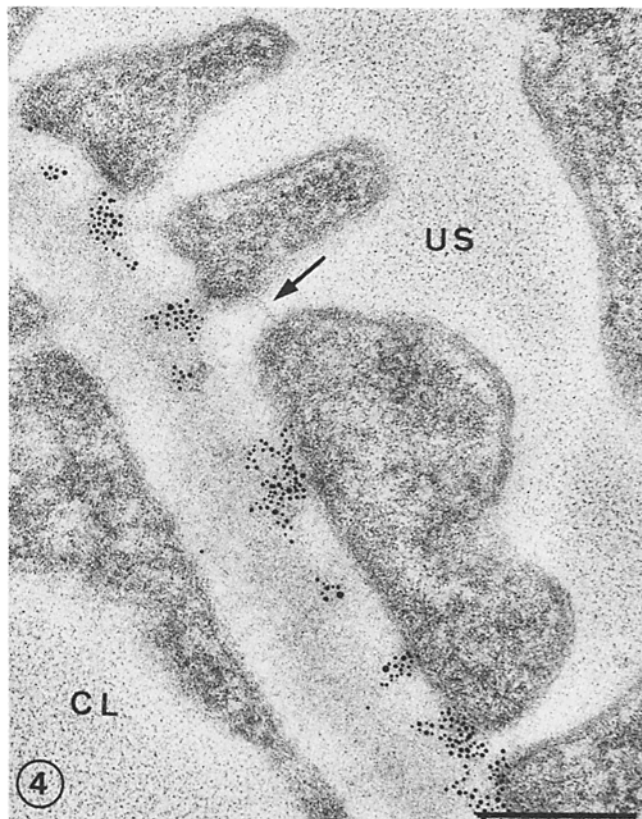
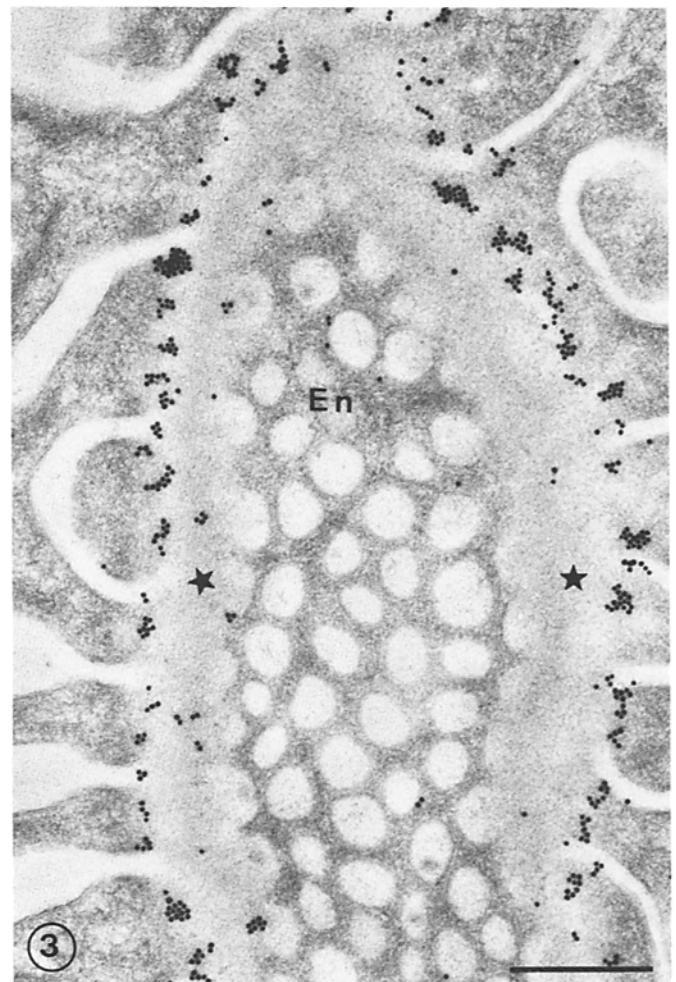
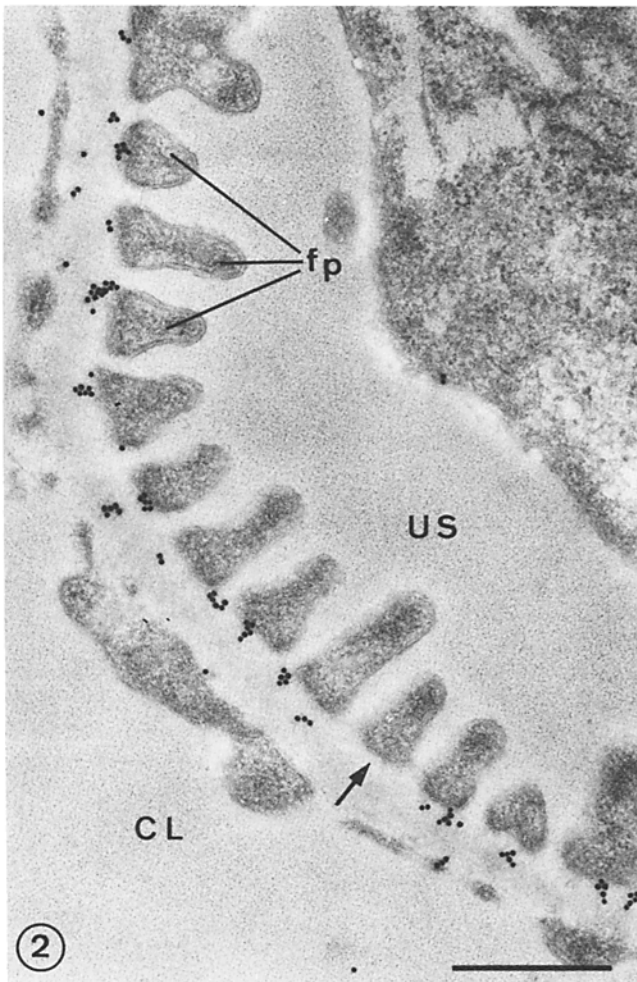
Cytochemical Procedures

LIGHT MICROSCOPY: Semi-thin sections from Epon-embedded material were used after removal of Epon. For removal of Epon, semi-thin sections on glass slides were treated for 3–5 min at room temperature with a freshly prepared solution consisting of 2 g KOH, 5 ml of propylene oxide and 10 ml of methanol (15). Sections were then rinsed with methanol/PBS (1:1) and finally PBS alone. The sections were incubated at room temperature for 30 min with 10 μg HPL-gold ml^{-1} in a moist chamber and were then washed in PBS, dehydrated, and re-



FIGURE 1 Rat kidney, Epon-embedding; semi-thin section stained with HPL-gold complexes. After Epon removal and rehydration, a discrete, often punctate labeling surrounds the capillary lumen (CL). Podocyte cell bodies (arrowheads), mesangium, and parietal Bowman's capsule (arrow) are virtually unlabeled. Proximal tubular cells (PT) are also stained. Bar, 20 μm . $\times 900$.

FIGURES 2–6 Figs. 2 and 3: Lowicryl K4M thin sections, postembedding staining with HPL-gold (15-nm) complexes. A peripheral region of a glomerular capillary is seen. The distribution of the lectin-binding sites as indicated by the electron-dense gold particles is restricted to the lamina rara externa and lamina densa. Label occurs as single particles and small clusters which extend from the foot process base (*fp*) to various depths into the basement membrane. Occasionally, foot process bases are unlabeled (arrow). Quantitative evaluation showed that 12.2% of the foot process bases were unlabeled when 15-nm gold particles were used for HPL-complex formation but only 2.5% were unlabeled when 3-nm gold particles were used (see Table II and Fig. 7). The plasma membrane of the free surface of the foot processes and of the cell body and the endothelium (*En*) shows no specific label. Only a very few gold particles lie over the lamina rara interna. Fig. 3 is a grazing section of a glomerular capillary which shows the preferential distribution of the gold particle label in both the lamina rara externa and lamina densa. Very few gold particles are related to endothelial fenestrae. *US*, urinary space. *CL*, capillary lumen. *Stars*, basement membrane. Fig. 2: bar, 0.5 μm ; $\times 42,000$. Fig. 3: bar, 0.5 μm ; $\times 38,500$. Figs. 4 and 5: Cross-sections of glomerular capillaries. The clustered appearance of the label is more evident by the use of HPL-gold complexes prepared with the 3-nm gold particles. The label is restricted to the GBM and extends from the foot process base to the lamina rara externa and lamina densa. Sometimes a labeling occurs over the space between the foot processes, beneath the slit membrane (arrows). Fig. 4: bar, 0.25 μm ; $\times 85,000$. Fig. 5: bar, 0.25 μm ; $\times 67,000$. Fig. 6: Thin section incubated with HPL-gold complexes showing part of the apical region of an epithelial cell in the distal convoluted tubule. Lectin binding sites are revealed along the plasma membrane as well as in cytoplasmic vesicles. Bar, 0.5 μm . $\times 34,000$.



embedded in Eukitt (Kindler, Freiburg, Federal Republic of Germany). Mounted sections were examined under bright-field illumination.

ELECTRON MICROSCOPY: Thin sections were prepared from Lowicryl K4M-embedded tissues and isolated GBM and were picked up on carbon/Parlodion coated nickel grids. They were first placed on a drop of PBS for 5 min and then transferred to a drop of HPL-gold complex (5 or 10 $\mu\text{g}/\text{ml}$) for a 30-min incubation at room temperature in a moist chamber. WGL-binding sites were revealed by incubating thin sections with native WGL (10–20 $\mu\text{g}/\text{ml}$) for 30 min followed by two rinses with PBS and a further incubation with ovomucoid-gold complex (5 $\mu\text{g}/\text{ml}$) for 30 min at room temperature in a moist chamber. Grids were then rinsed with PBS (2 \times 2 min) and finally with distilled water. The sections were counterstained with uranyl acetate and lead acetate, prior to examination in a Philips EM 300 electron microscope.

Cytochemical Controls

Different types of control incubations were performed to determine the specificity of labeling. Sections were exposed to the HPL-gold complex to which GalNAc, the specific inhibitory sugar, had been added at a concentration of 0.1 M 1 h prior to the incubation. Similarly, *N*-acetyl-D-glucosamine (0.5 M) was added to WGL prior to incubation. Second, sections were incubated in a first step with an excess (50 $\mu\text{g}/\text{ml}$) of native, nonlabeled HPL for 30 min, followed by a 30-min incubation with the gold-labeled HPL. Nonspecific binding of ovomucoid-gold complex to thin sections was checked by omission of the WGL incubation step.

Quantification of Labeling

Photographs were taken from regions of perpendicularly sectioned glomerular basement membrane with its associated endothelial cells and podocyte foot processes. For the quantification of gold particle labeling with HPL-gold, prints at a final magnification of 30,000 were used. A total of 90 photographs from three different staining experiments and control experiments (addition of the inhibitory sugar to HPL-gold) were evaluated and the relationship of the gold particles to the three layers of the basement membrane (i.e., lamina rara externa, lamina densa, and lamina rara interna) was assessed as the number of gold particles per 100 μm of the layer considered. On the same photographs, the percentage of HPL-gold-labeled foot process bases was estimated.

RESULTS

Light Microscopy

In semi-thin Epon sections stained by the HPL-gold complex, positive sites appeared red due to the inherent color of colloidal gold. In the glomerulus, a delicate staining was seen at the level of the capillary wall but its precise localization could not be determined by light microscopy (Fig. 1). However, podocyte cell bodies were clearly unlabeled. Other parts of the glomerulus, including Bowman's capsule, were not stained. In the cortex, the brush border of proximal tubules was heavily stained but basolateral regions showed only a faint staining. The apical pole of cells from the distal convoluted tubules was also heavily stained, as was the basal labyrinth in these cells. This staining was completely abolished under the cytochemical control conditions.

Electron Microscopy

When thin sections of Epon-embedded tissue were used,

only a very low degree of labeling was seen, whereas under the same staining conditions Lowicryl K4M-embedded material was intensely labeled (Figs. 2–9). This labeling was specific, as shown by the negative results under control conditions, and the background level of labeling was very low (Fig. 8, Table I). All the following data are, therefore, based on the examination of labeled thin sections of Lowicryl K4M-embedded kidney or isolated GBM.

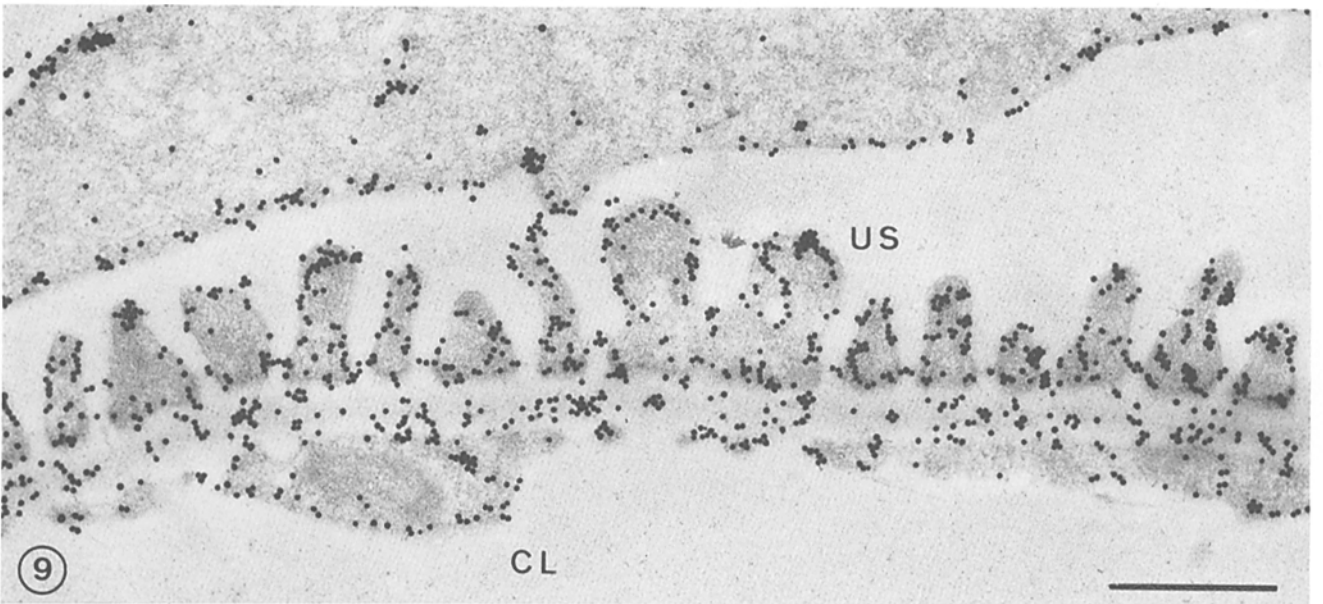
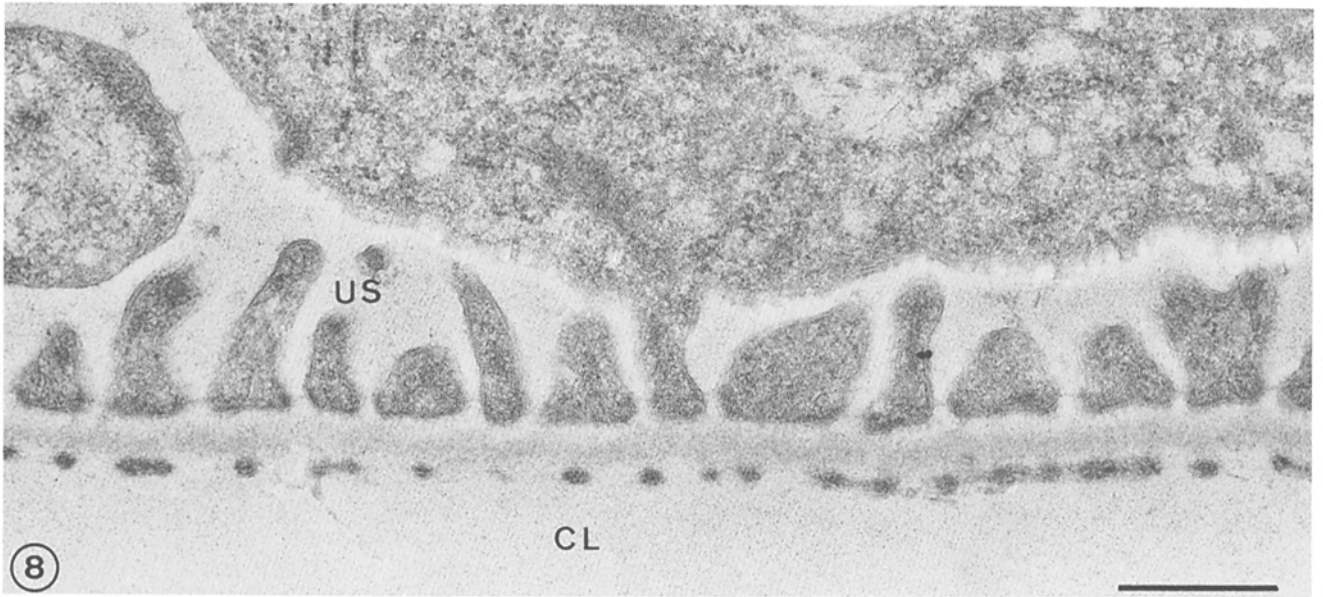
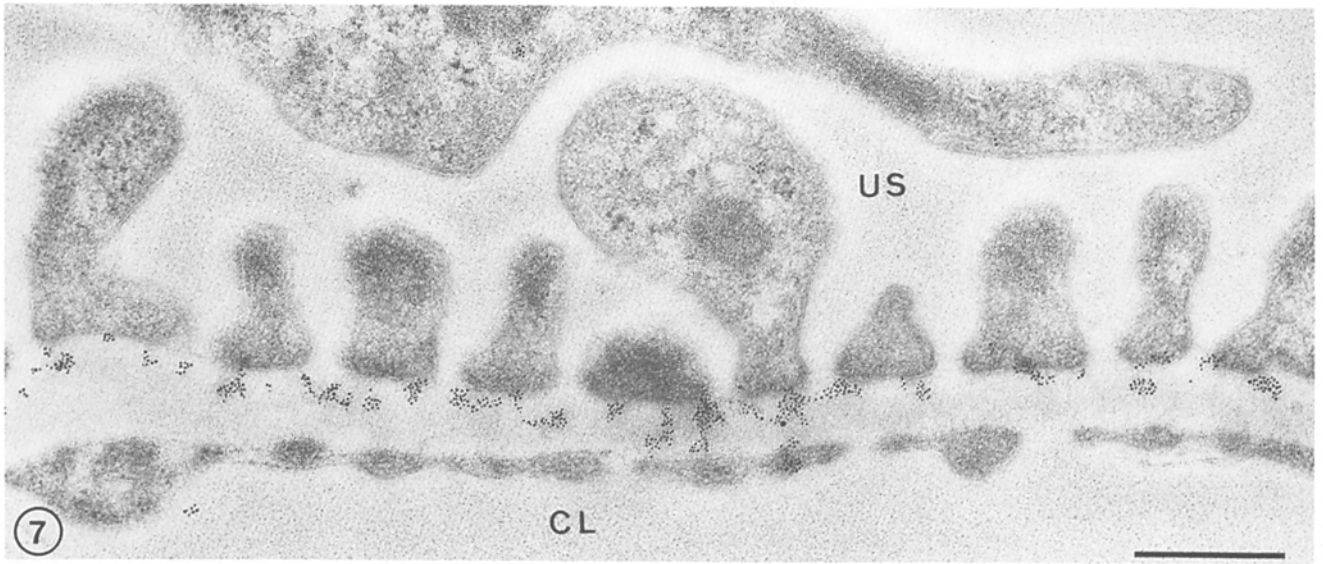
In the intact glomerulus, binding sites for HPL, revealed by the presence of electron-dense gold particles were found only over the glomerular capillary basement membrane (Figs. 2–5 and 7). Qualitatively, the gold particles appeared preferentially distributed over the lamina rara externa and the lamina densa, with very few particles over the lamina rara interna (Figs. 2, 4, 5, and 7). This distribution was even more pronounced in grazing sections of the basement membrane (Fig. 3). In addition to single gold particles, the labeling consisted of groups of particles which had various shapes and sizes, including linear arrays (Figs. 2, 4, and 7). In many instances, the particle clusters were closely associated with the plasma membrane of the foot process bases (which are embedded in the lamina rara externa), from which they extended to varying depths into the lamina rara externa and lamina densa. In addition, the space between foot processes, underlying the slit diaphragm, was sometimes labeled with gold particles (Figs. 4 and 5).

This labeling pattern was seen with lectin-gold complexes prepared using either 3- or 15-nm gold particles although, because of their smaller size, the organization of the label into groups was more pronounced with the 3-nm particles (compare Fig. 2 with Figs. 4, 5, and 7). In neither case, however, was there a clear indication that the particle clusters were arranged at regular intervals in the GBM as described for anionic sites in the lamina rara externa and interna by Kanwar and Farquhar (11).

No specific particle labeling was seen associated with any region of the plasma membrane of the foot processes above the slit diaphragm or of the cell body.

The qualitative observations of a preferential distribution of labeling over distinct regions of the GBM were confirmed by the quantitative evaluation of labeling intensity. The results in Table I show that the majority of gold particles extended over the lamina rara externa and lamina densa. The number of particles over the lamina rara interna was no higher than that of the background labeling found under control conditions. In many cases, the particles or groups of particles could be clearly seen to overlie either the lamina rara externa or the lamina densa and, in these cases, labeling appeared mainly as single particles or small groups of particles. However, due to the low electron-density of the GBM in nonosmified, Lowicryl K4M-embedded tissue, it was sometimes difficult to determine the exact structural limits underlying the groups of particles. Furthermore, many of the larger groups extended over both the

FIGURES 7–9 Figs. 7 and 8: Podocyte foot process bases are labeled with HPL-gold complexes (3-nm gold particles). In this region all the bases of the foot processes are positive and the labeling extends from the foot process base plasma membrane for various depths into the GBM (Fig. 7). This picture demonstrates the high degree of labeling obtained (97.1% of podocyte foot process bases labeled—Table II) using 3-nm gold particles for HPL-gold complex formation. The specificity of the labeling is demonstrated by the negative results when the inhibitory sugar (0.1 M *N*-acetyl-D-galactosamine) was added to the HPL-gold complex before incubation (Fig. 8 and Table I). *US*, urinary space. *CL*, capillary lumen. Fig. 7: bar, 0.5 μm ; \times 40,000. Fig. 8: bar, 0.5 μm ; \times 43,000. Fig. 9: This figure shows the distribution of WGL-binding sites. A similar region from a capillary loop is illustrated for comparison with Fig. 7. In contrast to HPL binding sites, WGL binding sites are present over the whole surface of podocytes and capillary endothelial cells as well as in all regions of the GBM. Note the very low degree of background over the tissue free regions of the section. Bar, 0.5 μm . \times 43,000.



lamina rara externa and lamina densa and, for these reasons, a compartment including both strata was included in the quantification. In addition, we evaluated the percentage of foot process bases labeled with HPL-gold complexes. The results in Table II show that the great majority of foot processes are labeled and that when 3-nm gold particles were used as markers this figure reached 97%. Examination of serial thin sections showed that the same foot process base which was negative in one section could become positive in the consecutive thin section.

Other glomerular structures including mesangial cells, mesangial matrix, Bowman's capsule parietal epithelial cells, and their basal lamina were not labeled. The basal lamina of the various tubule segments was similarly unlabeled, contrasting with the dense labeling of both plasma and intracellular membranes of the tubular cells (Fig. 6). In contrast, when sections were incubated to reveal WGL-binding sites, all three layers of the GBM and the glycocalyx of all regions of the podocyte plasma membrane were heavily labeled (Fig. 9). In addition, the glycocalyx of all other glomerular cell types as well as epithelial cells of proximal and distal convoluted tubules and their basement membranes were positive.

When thin sections from preparations of isolated GBM were incubated with HPL-gold complexes no specific labeling was seen (Fig. 10), whereas when WGL-ovomucoid-gold was applied a dense labeling appeared (Fig. 11).

To control whether the characteristic patchy labeling of

TABLE I
Distribution of the HPL-Gold Label over Regions of the
Glomerular Basement Membrane*

	Specific label		Control‡	
	Gold particles per 100 μm	%	Gold particles per 100 μm	%
Lamina rara externa	1,211	31.23	22	27.78
Lamina densa	779	20.09	43	54.16
Lamina rara interna	89	2.29	13	16.67
Lamina rara externa and lamina densa	1,634	42.14	—	—
Lamina rara interna and lamina densa	165	4.25	1	1.39

* For specific label counting a total length of 250 μm of basement membrane was evaluated and 9,795 gold particles were counted. Under control conditions 100 μm were evaluated. Background labelling found under control conditions accounted for 2.11% of the total labeling under specific staining conditions.

‡ 0.1 M *N*-acetyl-D-galactosamine was added to the HPL-gold complexes 60 min before use.

TABLE II
Percentage of Foot Process Bases Labeled with HPL-Gold
Complexes

	Positive			Negative %
	Direct*	Indirect		
HPL-gold 15-nm ($n = 1,221$)	60.2	27.6		12.2
HPL-gold 3-nm ($n = 807$)	89.6	7.5		2.9

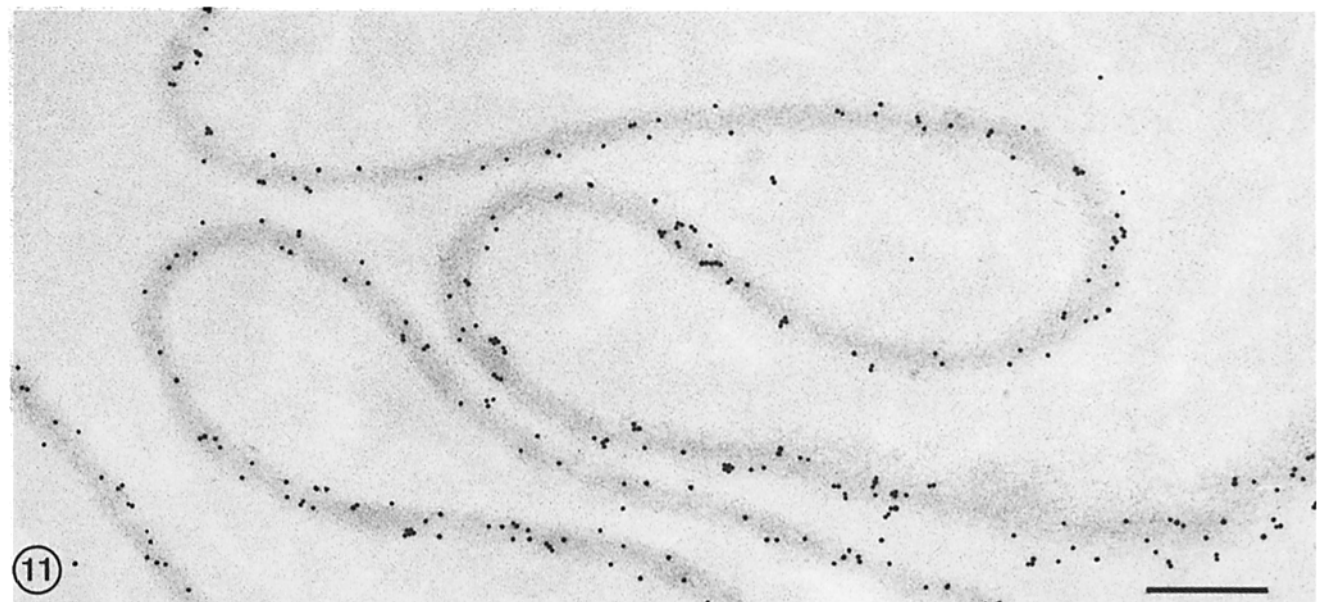
* Foot processes were scored as "directly" labeled if single gold particles or gold particles arranged in groups were in direct contact with the plasma membrane of the foot process base. When the gold particle label was present in the lamina rara externa and/or lamina densa underlying the foot process base, but was not in direct contact with plasma membrane, the labeling was scored as indirect.

podocyte foot processes was due to the conditions of tissue processing necessary for postembedding staining, we performed labeling experiments on ultrathin frozen sections prepared as described by Tokuyasu (28). To our surprise, label was very weak over podocyte foot process bases, although an intense labeling was still present over other regions which were also positive on K4M sections (e.g., proximal and distal tubules). Preliminary experiments indicate that this is an effect resulting from the presence of sucrose during the preparation of cryostat sections. Incubation of K4M sections with lectin solutions containing different concentrations of sucrose resulted in a differential inhibition of HPL-gold binding over different kidney regions. At concentrations >0.5 M sucrose, all binding (glomerular and tubular) was completely inhibited, whereas at lower concentrations, down to 50 mM, tubular labeling became progressively more intense while glomerular labeling was still poor, thus mimicking the effect seen on ultracryostat sections. The sucrose effect is currently being investigated in more detail, but it is pertinent to note that, in order to inhibit all labeling on K4M sections, >500 mM sucrose was required, whereas a complete inhibition was observed with only 1 mM GalNAc, the specific inhibitory sugar.

DISCUSSION

In this investigation, we used HPL-gold complex as a cytochemical probe for the localization of terminal nonreducing *N*-acetyl-D-galactosamine residues in thin sections of rat kidney. In the intact glomerulus, the lectin-binding sites were selectively associated with the basal foot process plasma membrane and the lamina rara externa and lamina densa of the GBM. The absence of such labeling in preparations of isolated GBM (which were labeled with WGL-ovomucoid-gold complexes) argues strongly that HPL-gold is detecting a glycoprotein associated with the glycocalyx of the podocyte foot process base rather than a glycocomponent of the basement membrane. The pattern of labeling revealed by HPL-gold in the intact glomerulus is quite different from the distribution of laminin, type IV collagen, and fibronectin recently described by Courtoy et al. (6). Apart from different staining patterns at the level of the GBM, all these three components were also detected in the mesangial matrix, which was not labeled with HPL-gold.

In addition to the negative results obtained with isolated GBM, there are other reasons for our interpretation that the HPL-gold is labeling part of the podocyte foot process glycocalyx. (a) In most cases, the gold-particle clusters extended from the outer leaflet of the foot process plasma membrane into the lamina rara externa and lamina densa. This pattern, together with the general labeling pattern described in Results, is consistent with the description of the foot process glycocalyx given by Latta et al. (13). These authors described ruthenium red-stained filaments which extended from the plasma membrane, across the outer layer and into the central layer of the GBM. (b) The HPL-gold complex specifically binds to terminal nonreducing GalNAc residues (31). Consequently, this lectin will recognize only *O*-glycosidically linked glycoproteins such as some mucin type glycoproteins with terminal GalNAc, and blood group A reactive glycoconjugates. Such glycoproteins have not been found as constituents of GBM. Two distinct types of carbohydrate units in glycoproteins account for almost all of the carbohydrate present in the (bovine) GBM (26, 27). One is a disaccharide unit containing glucose and galactose (found for example in type IV collagen of basement membranes) and the other is a heteropolysaccharide which does not



FIGURES 10 and 11 Lowicryl K4M embedded isolated GBM stained with HPL-gold complexes or with the WGL-ovomucoid-gold complex sequence. In loops of intact isolated GBM, no binding of HPL-gold complexes was observed using the same conditions of incubation as for sections from intact glomeruli (Fig. 10). In contrast, WGL binding sites as revealed by the presence of gold particles were found in isolated GBM (Fig. 11). Fig. 10: bar, 0.5 μm ; $\times 52,000$. Fig. 11: bar, 0.5 μm ; $\times 31,000$.

contain GalNAc. Indeed, basement membranes in other regions of the kidney (e.g. Bowman's capsule, proximal and distal tubules) were not labeled by HPL-gold, nor was the mesangial matrix labeled; furthermore, the positive labeling for WGL-binding sites of GBM both in situ and after isolation shows that glycocomponents are retained in the GBM during the isolation procedure. (c) In previous studies using HPL as a cytochemical probe, lectin-binding sites have been found in the glycocalyx of various cell types (22, 23, 29, 30) and in the present study we observed a strong labeling of the plasma membrane glycocalyx in epithelial cells of the urinary tubule.

Regional specializations in the glycocalyx composition of podocytes have been reported using colloidal iron, ruthenium red, phosphotungstic acid, cationic ferritin, and various lectins (3, 4, 7, 9-13). In general these reagents labeled the plasma

membrane of the "free" surface of podocytes (i.e., above the slit diaphragm and adjacent to the urinary space) while the membrane of foot process bases (embedded in the lamina rara externa) was either negative or only weakly stained. A different labeling pattern was observed with other lectins, however. Concanavalin A stained all regions of the podocyte plasma membrane uniformly as did WGL (reference 4 and this study) and *Ricinus communis* lectin labeled the foot process bases more heavily than the plasma membrane of the "free" surface of podocytes (4). All of these reagents also stained, to varying degrees, the GBM. In addition to these membrane surface characteristics, recent freeze-fracture data also show a differential content of intramembrane particles and filipin-cholesterol complexes between foot process bases and the rest of the podocyte plasma membrane (16, 17). The present results add,

therefore, a new parameter to the description of the podocyte plasma membrane which appears an exceptionally differentiated structure in keeping with the complex filtration process taking place at this level and we are currently investigating the labeling pattern in disease states in which flattening and spreading of the foot processes is found.

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