Expression of gp330 and gp330/α2-Macroglobulin Receptor-Associated Protein in Renal Tubular Differentiation

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ABSTRACT
The gp330/α2-macroglobulin receptor-associated protein (RAP) is a 39- to 45-kd protein that binds to the low-density lipoprotein receptor-related protein/α2-macroglobulin receptor and to gp330, a major glycoprotein of the brush border of proximal tubule cells. Despite evidence that gp330 functions as a receptor for several ligands and that soluble RAP inhibits ligand binding to gp330 in vitro, the physiologic function of RAP is unknown. Given the predominant location of RAP within the rough endoplasmic reticulum (RER), RAP might be involved in the intracellular processing and/or transport of gp330. The developing rat kidney was used as a dynamic model to study in detail the relationship between gp330 and RAP in vivo by immunohistochemical techniques. RAP was expressed in the renal vesicle and continued to be present, with a vesicular and perinuclear pattern of staining, in both proximal tubule cells and glomerular cells at subsequent stages. Immunoperoxidase electron microscopy demonstrated RAP in cisternae of the RER and in large subapical vesicles. gp330 was initially expressed in early proximal tubule cells in S-shaped bodies and was located in the perinuclear envelope and cytoplasmic vesicles as well as at the apical surface. Cytoplasmic gp330 staining was more evident at a stage subsequent to the S-shaped body, possibly related to more active biosynthesis. By comparative analysis of the patterns of immunofluorescence and immunoperoxidase staining, gp330 and RAP colocalized in the RER and in some large subapical vacuoles, but no definite RAP staining could be detected at the surface of proximal tubule cells at any stage, despite the presence of abundant gp330 in this location. The expression of gp330 at the apical surface of immature tubular cells was associated with the onset of fluid-phase endocytosis of fluorescein isothiocyanate-dextran and, therefore, of reabsorption of material from the tubular lumen, in the absence of concomitant changes in RAP expression in the same cells. These findings indicate that the role of endogenous RAP may not be directly related to ligand binding of gp330 at the surface of proximal tubule cells, although RAP may be involved in the processing and the intracellular trafficking of newly synthesized gp330, in particular in the delivery of gp330 to the plasma membrane.

Key Words: gp330, gp330/α2-macroglobulin receptor-associated protein, proximal tubule, exocytosis, nephron development

gp330 is a glycoprotein that is concentrated in the apical region of proximal tubule epithelial cells as well as cells in some other epithelia (1,2). After the original description of gp330 as the major target antigen of pathogenic antibodies in Heymann nephritis, an experimental model of membranous glomerulonephritis (3), numerous studies have addressed the structural and biochemical features of this molecule with the aim of establishing its specific function. Partial amino acid sequence analysis and cDNA cloning (4) have documented homology with the low-density lipoprotein (LDL) receptor and LDL receptor-related protein/α2-macroglobulin receptor (LRP/α2-MR), another member of the LDL receptor family, and recent reports have established that gp330 is able to bind several ligands in vitro, including apoprotein E–enriched β-VLDL (very low-density lipoprotein) (5), lipoprotein lipase (6), plasminogen (7,8), and plasminogen activator-plasminogen activator inhibitor type 1 complexes (uPA-PAI-1) (9). Binding of ligands was inhibited by a gp330-LRP/α2-MR-associated protein (RAP) (5,6,9). RAP is a 39- to 45-kd protein that copurifies both with gp330 and...
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LRP/α2-MR on affinity chromatography columns (10–13). RAP binds to gp330 (13,14) or LRP/α2-MR (13,15,16) immobilized on membranes and to the brush border of proximal tubule epithelial cells in the rat kidney, at sites where gp330 is detectable (14,17).

Because gp330 is found in clathrin-coated pits of the plasma membrane and possesses domains specialized for receptor-mediated endocytosis (1,4), these findings have been taken to suggest that, by analogy with LRP/α2-MR, gp330 is a multifunctional receptor and in the renal proximal tubule is involved in the endocytosis of filtered protein(s) from the tubule lumen. This possibility is supported by findings that RAP and uPA-PAI-1 microperfused into renal tubules are endocytosed by proximal tubule cells and that recombinant RAP inhibits the endocytosis of uPA-PAI-1 (9,14).

Despite progress in identifying specific ligands for gp330 and the purification of RAP, the physiologic role of the RAP-gp330 complex remains unknown. In proximal tubule cells of the adult rat kidney, RAP is confined to intracellular sites, predominantly the rough endoplasmic reticulum (RER) (17), and there is no clear evidence of targeting of endogenously synthesized RAP to the cell surface. On the basis of this location, as well as the structural and biochemical characteristics of the protein, a hypothetical function of RAP in this cell type is to act as a molecular chaperon, which would assist the processing and/or the intracellular transport of gp330. In addition, RAP might modulate ligand binding to gp330 in the intracellular transport pathways by an interaction similar to the binding of invariant chain to newly synthesized class II major histocompatibility complex (18,19).

We have recently obtained evidence for the existence of two forms of gp330, which differ in structure and, at least in part, subcellular location in proximal tubule cells (20). One form contains a cytoplasmic domain predicted from partial cDNA cloning (4) and is located in coated pits at the base of brush border microvilli, on the basis of staining with specific anticytoplasmic peptide antibodies. The other form, in which the same cytoplasmic domain is not detectable, is present on the surface of brush border microvilli and is possibly also in the coated pit region. The presence of gp330 in nonclathrin domains of the apical plasma membrane (2,20,21) may indicate that the function and trafficking of gp330 are not exclusively related to receptor-mediated endocytosis; the respective roles of the two forms of gp330 in endocytic events and their relationship with RAP are unknown.

A suitable model with which to study the expression of these molecules in relation to their initial synthesis and to the apical reabsorption of tubular fluid is the developing rat kidney, in which the patterns of distribution of several proteins are temporally regulated. Furthermore, by examination of fluid phase endocytosis, individual nephrons can be used to determine whether cells belonging to a particular tubule are exposed to tubular fluid and are capable of reabsorption (22). In addition, gp330 functions as a receptor for extracellular matrix components in primary cultures of rat proximal tubule epithelial cells (23), and RAP complexed with gp330 is able to bind to heparin (24), which is contained in the basement membranes of the kidney (25). Therefore, the gp330/RAP complex might also be involved in epithelial morphogenesis in light of the important roles of extracellular matrix components and receptors in renal development.

This study was designed to explore in detail by immunohistochemical methods the relationship between gp330 and RAP in different stages of maturation of the proximal tubule in vivo. We also compared the expression of these molecules with the onset of reabsorptive activity from the luminal fluid, as assessed by the ability of tubular cells to internalize fluorescein isothiocyanate (FITC)-dextran, a marker of fluid phase endocytosis. The results show that greater amounts of gp330 and RAP colocalize to the RER in a maturing stage of the proximal tubule in which the brush border is assembled, but only gp330 is delivered to the cell surface. The expression of gp330 at the apical surface of proximal tubule cells coincided with the onset of fluid-phase endocytosis, in the absence of concomitant changes in RAP staining. These findings may indicate that the role of RAP is not directly related to ligand binding of gp330 at the cell surface, and they are consistent with the involvement of RAP in the processing and the intracellular trafficking of newly synthesized gp330.

METHODS

Chemicals

Paraformaldehyde was obtained from Electron Microscopy Sciences (Fort Washington, PA). BSA, L-lysine HCl, and N-propyl gallate were from Sigma (St. Louis, MO), sodium pentobarbital was from H. Schein Inc. (Port Washington, NY), and sodium meta-periodate was from Fisher (Fair Lawn, NJ). Fixable lysine-FITC dextran (10 kd) was from Molecular Probes (Eugene, OR).

Antibodies

Affinity-purified rat gp330 was prepared from solubilized rat FXIA, a crude extract of rat kidney cortex, with an anti-gp330 monoclonal antibody (14c1) (26) as previously described (20,27,28), and displayed a broad band at 440-kd on reduced sodium dodecyl
sulfate–polyacrylamide gel electrophoresis. A monoclonal antibody (1H2) was obtained by immunizing mice with affinity-purified gp330. 1H2 reacts with purified gp330 by enzyme-linked immunosorbent assay and western blot analysis (M. Sy and T. Yahaya, unpublished observation) and with gp330 affinity purified on a column containing RAP (29). In addition, by western blot analysis of preparations of gp330, 1H2 has been shown to react with material that is also recognized by a monoclonal antibody against human gp330 (6). The production and specificity of the rabbit polyclonal anti-gp330 antiserum used in this study have also been described (21). These antibodies did not produce a band at 280-kd, indicating that they do not react with a protein designated gp280, which may be related to gp330 (30). Immunohistochemical studies have shown that both 1H2 and the rabbit anti-gp330 antiserum recognize extracellular epitopes of gp330 in the coated pit region of proximal tubule cells as well as on the microvillar surface in some tubules (20).

We have recently obtained a rabbit anti-gp330 cytoplasmic peptide antiserum using an 18-amino-acid peptide synthesized on the basis of cloned partial gp330 cDNA. The antiserum precipitated from Fx1A a protein that was specifically recognized both by 14c1 and 1H2. It stained material in Fx1A with a mass of approximately 440 kd by western blotting and, as revealed by immunohistochemistry, reacted with gp330 in the coated pit region of proximal tubule cells (20).

Rabbit antiserum against a rat RAP-glutathione transferase fusion protein and the corresponding preimmune serum were kindly provided by Dr. Joachim Herz (University of Texas, Southwestern Medical Center, Dallas, TX). The immune serum reacted with a protein of about 44 kd in preparations of gp330 by western blot analysis (12) and specifically stained RER cisternae of glomerular visceral epithelial cells and proximal tubule epithelial cells in the adult rat kidney (17).

Anti-rat dipeptidyl peptidase IV, which was a gift of Dr. Ann Hubbard (Johns Hopkins University School of Medicine, Baltimore, MD), is a rabbit polyclonal antiserum raised against rat liver dipeptidyl peptidase IV (31). It recognized a band at 100 to 110 kd by western blotting of rat renal brush border membrane vesicles.

FITC-conjugated goat anti-rabbit immunoglobulin G (IgG) (Kirkegaard and Perry, Gaithersburg, MD) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Jackson, West Grove, PA) absorbed with normal rat serum were used as secondary antibodies for immunofluorescence studies. Biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, VT) or goat anti-mouse IgG (Kirkegaard and Perry) was used for immunoperoxidase labeling.

### Tissue Preparation

One-, 4-, 6-, 11-, and 21-day-old Sprague-Dawley rats were used. The study was conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. Animals were anesthetized with sodium pentobarbital injected ip (0.1 mL/100 g body wt of a 65 mg/mL solution), and the kidneys were fixed by immersion overnight at 4°C with paraformaldehyde lysine periodate (PLP) fixative (32). Older animals were fixed by intracardiac perfusion with PLP after a brief perfusion with Hanks’ balanced salt solution. Kidneys were excised and fixed overnight in PLP. Subsequently, they were washed three times in phosphate-buffered saline (PBS) and kept at 4°C in PBS containing 0.02% azide until further processed.

### Immunofluorescence Microscopy

Immunofluorescence Microscopy. Both 5- and 1-μm-thick cryostat sections were used for immunofluorescence localization. Fragments of kidney were rinsed in PBS and immersed in 30% sucrose in PBS or, for 1 μm sections, 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.4, for at least 1 h (33). Sections of 5 μm were cut on a Reichert Frigocut 2800 (Leica Inc., Deerfield, IL) at −26°C. Sections of 1 μm were cut at −60°C on an FC 4D Reichert Ultracut cryomicrotome as previously described (33,34). Sections were placed on Superfrost Plus glass microscope slides (Fisher Scientific, Fair Lawn, NJ) and stained immediately or stored at −20°C. After being rinsed in PBS, they were bathed in PBS containing 1% BSA for 20 min to block nonspecific binding. The sections were incubated with the primary antibody for 1 h at room temperature or overnight at 4°C, and then washed 2 × 5 min in high-salt PBS (PBS containing 18 g NaCl/L) and 5 min in PBS. Fluorescein-conjugated goat anti-rabbit IgG at a concentration of 20 μg/mL was applied to sections for 45 min, followed by 2 × 5 min washes in high-salt PBS and 5 min in PBS. The sections were mounted with fluorescence mounting medium (100 mM Tris-HCl: glycerol 50:50, 2% n-propyl gallate; pH 8.0) (35) and viewed with a Nikon FXA fluorescence microscope (Nikon Inc., Melville, NY). Photographs were taken on 400ASA Kodak T-Max film push processed to 1600ASA (Eastman Kodak, Rochester, NY).

Double staining of 5-μm sections was performed by the following incubation sequence: anti-gp330 mouse monoclonal antibody (1H2, 5 μg/mL), followed by TRITC-conjugated goat anti-mouse IgG (20 μg/mL), and rabbit anticytoplasmic peptide antiserum (diluted 1:100) or rabbit anti-RAP antiserum (1:100), followed by FITC-conjugated goat anti-rabbit IgG (20 μg/mL). In control experiments, incubation sequences were inverted, and primary antibodies were omitted or substituted with preimmune serum.
Immunoperoxidase Labeling. Fixed pieces of kidney cortex from 1-day-old rats were cryoprotected for at least 1 h in 10% dimethylsulfoxide, frozen in liquid nitrogen, and sectioned at 30 μm at -26°C. Sections were incubated overnight by immersion in drops of the antiserum against RAP (diluted 1:100) or mouse anti-gp330 monoclonal antibodies (1H2, 5 μg/mL) in PBS containing 1% BSA to reduce nonspecific binding. In control experiments, sections were incubated in PBS containing 1% BSA alone. After extensive washes in PBS, they were incubated for 2 h in biotin-conjugated goat anti-rabbit IgG (Vector) or goat anti-mouse IgG-biotin (Kirkegaard and Perry) absorbed with normal rat serum, washed extensively for 1 h, incubated in avidin-biotin complex reagent (Vector), washed again, and finally incubated in 1 mg/ml diaminobenzidine and 0.01% H2O2 to reveal sites of peroxidase activity. The tissue sections were fixed briefly in 1% glutaraldehyde in PBS, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in LX-112 (Ladd Industries, Burlington, VT). Thin sections were examined with a Philips CM10 electron microscope (Lico Inc., Bedford, MA) either unstained or after heavy metal staining.

Analysis of Fluid Phase Endocytosis

One-day-old rats were injected ip with 100 μL of FITC-dextran (10 kd) diluted in PBS (1 mg/mL). Shortly before euthanasia, 15 min after the injection of the probe, the animals were anesthetized as described above. The kidneys were fixed in PLP, and frozen tissue sections were processed for fluorescence microscopy. To compare the location and intensity of the fluorescence probe with those of gp330 and RAP, serial 5-μm sections were cut and stained by indirect immunofluorescence with anti-gp330 antibodies and anti-RAP antibodies, respectively, followed by the appropriate secondary rhodamine-conjugated antibodies.

RESULTS

The heterogeneous pattern of nephron development in 1-day-old neonatal rats allows the analysis of different evolutive stages in an individual section. We also used kidneys from rats of various ages to identify possible differences in the temporal expression of proteins. However, the results from later times were similar to those obtained in 1-day-old rats. In immunofluorescence experiments, the stages of tubular differentiation were identified on the basis of morphology and patterns of aggregation of cells in renal vesicles, S-shaped bodies, and proximal tubules (Figure 1) and on the pattern of staining for dipeptidyl peptidase IV as previously described (36).

Developmental Expression of gp330

The S-shaped body was the earliest structure of the developing nephron in which gp330 could be detected by immunofluorescence on 5-μm sections, with either a polyclonal antiserum or a monoclonal antibody, both of which react with extracellular epitopes of the antigen. Staining was present in differentiating parietal epithelial cells and primitive podocytes at the periphery of the immature glomerulus and in early proximal tubule cells (Figure 1b). In general, the pattern was both cytoplasmic and apical, although many parietal cells showed more distinct or restricted apical staining. Apical and cytoplasmic gp330 staining was also observed transiently at the primitive stage of distal tubule formation in S-shaped bodies (Figure 1b). In glomeruli at an intermediate stage of maturation, gp330 was occasionally expressed in visceral epithelial cells lining the periphery of the early capillary tuft. Parietal epithelial cells were also variably stained. However, in more mature and fully differentiated glomeruli, no significant staining could be detected in either cell type.

In a stage of the proximal tubule subsequent to the S-shaped body, which was characterized by an increase in tubular size and more evident lumen in cross-sections, the cells displayed more cytoplasmic staining of gp330, both vesicular and perinuclear, associated with reactivity on the assembling brush border (Figure 1e). In some cells, marked cytoplasmic staining was present in the basolateral region of the cytoplasm. More differentiated proximal tubules were characterized by more distinct staining of the apical region associated with a decrease or absence of cytoplasmic staining. The staining of brush border microvilli was heterogeneous, with gp330 detectable over the microvilli of only some tubular profiles (Figure 1h) and more distinctly in animals at times later than 4 days. The presence of brush border microvilli that were not stained by anti-gp330 antibodies was confirmed by their reactivity with antidipeptidyl peptidase IV antibodies on the same or adjacent sections.

The antipeptide antiserum directed against the cytoplasmic domain of gp330 produced patterns of immunofluorescence staining that, on 5-μm sections of immature glomeruli and proximal tubules of S-shaped bodies, where microvilli were not developed, were similar to those obtained with antibodies to extracellular epitopes of the protein. Colocalization was confirmed by double immunofluorescence staining of individual sections (Figure 1c and f). However, in more differentiated proximal tubules, the brush border microvilli, which in some segments were stained with the other anti-gp330 antibodies, were never stained with the anticytoplasmic domain antiserum (Figure 1l). Moreover, the perinuclear and cytoplasmic vesicular staining obtained with this antiserum on semithin frozen sections of immature tu-
Figure 1. Nephron segments at different stages of development in a 5-μm section of newborn rat kidney. Panels a, d, and g are differential interference contrast micrographs of an S-shaped body (a), immature proximal tubules (d), and more differentiated proximal tubules (g). Double immunofluorescence staining was performed first with mouse monoclonal antibodies (1H2) against extracellular epitopes of gp330 revealed with rhodamine-conjugated goat anti-mouse IgG (b, e, and h) and then with a rabbit anti-gp330 cytoplasmic peptide antiserum followed by FITC-conjugated goat anti-rabbit IgG (c, f, and i). Note similar patterns of staining by these antibodies in the S-shaped body (b and c) and maturing proximal tubules (e and f), in which gp330 is visible in intracellular sites with a perinuclear and vesicular distribution and in the apical region of the cell. Subapical staining is produced by both reagents in more differentiated proximal tubules (h and i). In addition, however, the microvillar region of some of the brush borders (arrows) is recognized by antibodies against extracellular epitopes (h) but not by the anticytoplasmic peptide antiserum (i). Bar, 50 μm.

Developmental Expression of RAP: Relationship to gp330 Staining

RAP was already detectable by immunofluorescence in 5-μm sections of renal vesicles and comma-
Figure 2. Double immunofluorescence staining of extracellular epitopes of gp330 (a) and cytoplasmic domain of gp330 (b) in a 1-μm section of proximal tubules from a 1-day-old rat. Tubular cells contain large subapical vacuoles, which are stained for both external epitopes of gp330 and its cytoplasmic domain (arrows). Bar, 50 μm.

Figure 3. Comparison of the expression of gp330 and dipeptidyl peptidase IV by double immunofluorescence staining of 1-μm sections of proximal tubules at different stages of maturation. Proximal tubule cells that display cytoplasmic and apical staining for external epitopes of gp330 (a) also exhibit the presence of dipeptidyl peptidase IV on both the basolateral membrane and the apical region (b), a pattern characteristic of early and intermediate stages of maturation. In more differentiated tubules, gp330 is detectable only in the apical region, including the microvillar surface in some tubules (arrows) (c), and dipeptidyl peptidase IV is no longer visible on the basolateral membrane (d). Bar, 50 μm.

shaped bodies, in which the staining was weak (Figure 4a). It was also found in the S-shaped body, both in primitive glomerular epithelial cells committed either to the visceral phenotype or to the parietal phenotype and in early proximal tubule cells (Figure 4c). The location was intracellular, with a perinuclear and vesicular pattern of distribution. In addition, RAP and gp330 colocalized to intracellular compartments in immature proximal tubule cells by double immunofluorescence staining (Figure 4c and d). However, no distinctive reactivity for RAP was present on the cell surface at these as well as at any later maturation stages of the proximal tubule. The RAP staining was similar in distribution but more intense in proximal tubules at a subsequent stage (Figure 4e). Cytoplasmic gp330 was also more abundant in these tubules and could be colocalized with RAP to the same intracellular sites (Figure 4e and f). Proximal tubules in which gp330 was exclusively apical were characterized by a variable intensity of RAP cytoplasmic staining (Figure 4g and h). The perinuclear staining appeared less abundant compared with that observed in tubule cells at an intermediate stage and was similar to that of the adult kidney (17).

Ultrastructural Localization of gp330 and RAP

Immunoperoxidase electron microscopy was performed to identify intracellular compartments containing gp330 and RAP in maturing proximal tubule cells. Material reactive with anti-gp330 antibodies was present in cisternae of the RER of cells in which the brush border microvilli were not developed. In
some cells, many RER cisternae were stained in the basolateral region of the cell. Reaction product could also be detected in the perinuclear envelope, on the membrane of some large subapical vacuoles and apical vesicles, and on the cell surface (Figure 5a). No staining was visible in cisternae of the Golgi apparatus or on the basolateral membrane. At later stages, in which the brush border was almost completely formed, cisternae of RER and the perinuclear envelope were less consistently stained or were even unstained (Figure 6a). However, reaction product was still observed in the membrane of large subapical vacuoles, which were most prominent at these stages of microvillar formation and were occasionally found in continuity with the apical membrane (Figure 6a, inset). Staining was also associated with subapical vesicles, dense apical tubules, and the luminal surface of coated pits. The microvilli membrane was stained in some tubules. Thus, the staining pattern at a late developmental stage was similar to that of the adult rat kidney.

RAP staining, in contrast, was almost exclusively confined to the perinuclear envelope and cisternae of the RER, both in proximal tubule cells (Figure 5b) and in glomerular podocytes and parietal epithelial cells. However, membranes of some large subapical vacuoles in cells of tubules that exhibited various degrees of brush border microvillar differentiation were also reactive (Figure 6b). Reaction product was rarely found in apical vesicles and coated pits of proximal tubule cells, and no staining was observed in dense apical tubules, lysosomes, and other intracellular compartments.

The Expression of gp330 at the Cell Surface but Not That of RAP Parallels the Development of Fluid-Phase Endocytosis

To evaluate the possible relationship of the expression of gp330 and RAP to the initial reabsorption of material contained in the tubular fluid at the apical pole of proximal tubule cells, neonatal rats were injected with FITC-dextran, a marker of fluid-phase endocytosis. The locations of the internalized probe, gp330, and RAP were compared by immunofluorescence with TRITC-conjugated secondary antibodies on serial sections. The uptake of FITC-dextran by proximal tubule epithelial cells occurred only in cells that expressed gp330 and only at those sites in the apical region of the cell where gp330 was detectable (Figure 7). Conversely, no relationship could be found between FITC-dextran uptake and the intensity and the pattern of tubular staining for RAP.

DISCUSSION

The purpose of this study was to investigate the distribution of RAP and different epitopes of gp330 in tubular epithelial cells that are establishing epithelial polarity and reabsorptive activity in vivo. On the basis of the available biochemical and immunocytochemical data, the prediction was that, if RAP has a role in the synthesis of gp330, the appearance of these proteins in intracellular biosynthetic compartments of these cells should follow similar patterns. We first analyzed the expression of gp330 using antibodies that recognize either extracellular or intracellular epitopes of the molecule and bind to the apical region of the proximal tubule cell (20). In the adult rat, the form that contains a cytoplasmic domain predicted on the basis of partial cDNA cloning (4) is localized to the apical region of the proximal tubule cell (20). No notable differences were found between the patterns of staining obtained with these anti-gp330 antibodies and RAP in developing nephron than on the microvilli of proximal tubules, in which only extracellular epitopes were detectable. This situation is identical to that previously described in the adult kidney (17).

The initial appearance of gp330 occurred in the S-shaped body, in which it was detected within cells committed to glomerular differentiation and cells of maturing proximal and distal tubules. gp330 labeling of glomerular cells was less evident at later stages of differentiation as well as in the adult kidney (17). In addition, the presence of gp330 in distal tubules was also limited to an early stage of development and was restricted to the apical region of these cells. The detection of relatively abundant amounts of gp330 transiently in early glomerular cells and in immature but not fully differentiated distal tubular cells is in agreement with data from a recent report (30).

gp330 labeling was intense in the perinuclear envelope and RER cisternae of immature proximal tubule cells at a developmental stage subsequent to the S-shaped body. Although cytoplasmic gp330 staining was not described by Biemesderfer et al. in a study on the assembly of the tubular brush border in the developing rat kidney (36), its detection in this study confirms a pattern of distribution that has been reported more recently (30). This distribution pattern extends to the form of gp330 that contains the cytoplasmic domain recognized by our antipeptide antibodies. Differences in epitopes of gp330 recognized by the antibodies used in these different studies cannot be ruled out to explain discrepancies in localization, but our finding of much weaker signals in semithin frozen sections indicates that different section thickness may be important in the ability to detect intracellular gp330. Nonetheless, we were still able to detect cytoplasmic staining for gp330 even on
Figure 4. Double immunofluorescence staining of RAP (a, c, e, and g) and gp330 (b, d, f, and h) in a 5-μm section of the kidney of a 4-day-old rat. The section was incubated both with 1H2 anti-gp330 monoclonal antibodies and anti-RAP antiserum by the same procedure described in the legend to Figure 1. Panels a and b show a comma-shaped body that is recognized only by anti-RAP antibodies. In Panels c and d, cells of an S-shaped body destined to become proximal tubule epithelia are stained by both antibodies (arrows). Glomerular cells in these S-shaped bodies (asterisks) show no or little gp330, but abundant RAP. Maturing proximal tubules with distinct periluclear and vesicular cytoplasmic staining and colocalization of
semithin sections, and this allowed us to compare the distribution of gp330 in biosynthetic pathways relative to the establishment of cell polarity, as reflected by the distribution of dipeptidyl peptidase IV. In the proximal tubule, dipeptidyl peptidase IV is located initially both on the basolateral and on the apical membrane of immature cells and then becomes restricted to brush border microvilli (36). Cytoplasmic staining of gp330 was detectable in tubules in which dipeptidyl peptidase IV was expressed on the basolateral membrane but not in those tubules in which dipeptidyl peptidase IV was restricted to brush border microvilli. This probably indicates that high rates of initial biosynthesis of gp330 are temporally related to the assembly of the brush border. The loss of gp330 from intracellular biosynthetic compartments suggests that a down-regulation of its synthesis occurs after brush border assembly.

The characteristic pattern of sequential changes described above, and in particular the identification of a developmental stage characterized by a high level of gp330 in biosynthetic pathways in parallel with brush border assembly, allowed us to explore in detail the relationship between the developmental expression of gp330 and RAP. Small amounts of RAP staining were already found in the renal vesicle, before gp330 could be detected. Subsequently, beginning from the portion of the S-shaped body destined to form the proximal tubule, the RAP staining pattern paralleled that of cytoplasmic gp330. Notably, in immature tubules, RAP and gp330 colocated to the perinuclear region and to cytoplasmic structures, which by immunoperoxidase electron microscopy, were identified as cisternae of the RER; the intensity of staining was also similar. These findings are consistent with the possibility that RAP is involved in the synthetic processing of gp330. Thus, although the presence of RAP but not gp330 in cells of the renal vesicle might reflect the potential association of RAP with other proteins, gp330 is the only protein, among those known to bind RAP, to be expressed in the proximal tubule, at least in the adult rat. However, gp330 and a 280-kd protein related to gp330 have been reported to appear initially in the renal vesicle (30), and we cannot rule out the possibility that small amounts of these proteins not detectable by the antibodies used in this study may also be synthesized at this early stage. By pulse chase and enzymatic digestion experiments on tissue fragments from neonatal rat kidney, it has recently been docu-
Figure 6. Immunoperoxidase electron microscopy of gp330 and RAP in proximal tubule cells from the kidney of 1-day-old rat. (a) gp330 is clearly detectable in a large subapical vacuole (asterisk) and in coated pits at the base of the brush border but, unlike the less mature cells in Figure 5a, not in the perinuclear envelope. The inset in Panel a shows continuity of the membrane of a large subapical vacuole with the apical plasma membrane. (b) In contrast to gp330, RAP is located in the perinuclear membrane (arrows) and RER cisternae (inset) and not on the cell surface. Note RAP staining in a large subapical vacuole (asterisk). Bar, 0.5 μm.

It is noted that RAP associates with gp330 in the RER to form a heterodimer within 30 min after the synthesis of gp330 (37). A large hetero-oligomer then forms, suggesting that RAP may have a role in the folding of gp330 and its transport to the Golgi apparatus. The results of our localization experiments, coupled to the biochemical features of the RAP/gp330 complex, support the hypothesis that endogenously synthesized RAP may serve as a molecular chaperon that assists proper folding and assembly of gp330 within the endoplasmic reticulum and possibly facilitates its movement to other cellular compartments. Moreover, the fact that, in this study, the uptake of a marker of fluid-phase endocytosis at the apical pole of proximal tubule cells occurred only at sites where gp330 was present, and was not paralleled by changes in RAP expression, also suggests that RAP is not directly related to the function of gp330 on the cell surface. In agreement with this interpretation, RAP is almost entirely restricted to intracellular sites in several other types of epithelia, such as in the epididymis, efferent ducts, endometrium, and thyroid, which express gp330 at the cell surface (38). The presence of RAP in intracellular sites of epithelial cells that do not contain gp330 but express LRP/a2-MR (38) is also consistent with a role of RAP in the assembly and oligomerization of different proteins, by analogy to molecules with established chaperon function (39).

Although RAP contains a putative signal sequence (HNEL) for retention in the endoplasmic reticulum (40), its detection in the membrane of some large subapical vacuoles and, very rarely, in apical vesicles and coated pits on the apical membrane, in which gp330 is present, demonstrates that these molecules may interact even in compartments distal to the Golgi apparatus. Large subapical vacuoles containing gp330 were prominent in tubular cells at late stages of microvillar formation, and in some instances, their membrane appeared to fuse with the apical plasma.
membrane. The abundance of such organelles may be related to the generation of a completely differentiated microvillar domain. In this context, a potential function of similar large vesicles during development is the delivery of newly synthesized membrane to the apical domain (41,42). Indeed, recent studies on Madin-Darby canine kidney cells with incomplete intercellular contacts have documented that structures remarkably similar to large subapical vacuoles are externalized on exposure to stimuli that are required for establishing complete surface polarity (43,44). Because the membrane of these organelles (vacuolar apical compartment VAC) displays microvilli and contains apical, but not basolateral, markers, they have been proposed to contribute to the biogenesis of epithelial surface polarity (43) and perhaps are involved in its maintenance, as well as its derangement under pathologic conditions (45). It is not known whether the large subapical vesicles of developing proximal tubule cells represent a completely homogeneous population of organelles, nor if or how they interact with other compartments involved in intracellular vesicular trafficking. The observation that most large subapical vesicles were stained for gp330, whereas RAP was found less constantly, raises the possibility that the gp330/RAP complex might dissociate at this level, after which gp330 alone is targeted to the cell surface.

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The text is too long to be fully transcribed here. However, it appears to be a bibliography or reference list related to research on rat hepatocyte plasma membrane proteins and other related topics. The references are cited with various authors and years, and the topics range from fixation methods to the interaction of proteins in various cellular processes. The text is formatted in a standard reference list style, with authors' names, journal names, and page numbers listed.