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Comparative Immunochemistry and Ontogeny of Two Closely Related Coated Pit Proteins

The 280-kd Target of Teratogenic Antibodies and the 330-kd Target of Nephritogenic Antibodies

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We have previously shown that monoclonal antibodies specific for a 280-kd protein (gp280) concentrated within the coated pits of renal and yolk sac brush border–induced fetal malformations, whereas antibodies specific for gp330, another coated pit protein with a similar distribution, had no deleterious effect on embryonic development. In this study, we show that gp280 and gp330 are closely related proteins, as indicated by 1) similarities in peptide maps obtained after cyanogen bromide cleavage, 2) immunological cross-reactivity related to a minor contingent of antibodies that do not have teratogenic activity, and 3) asynchronous but related expressions during ontogenesis. During the early stages of development, the expression of the two glycoproteins was limited to (gp330) or predominant in (gp280) the clathrin-coated pits and intermicrovillar areas. In the pre-implantation embryo, gp330 was expressed by trophectodermal cells, which became negative in day-6 embryos trapped in endometrial infoldings. At this stage, gp280 and gp330 were both simultaneously detectable at the apical pole of the first entoblastic cells and remained expressed by the brush border of visceral yolk sac epithelial cells until the end of pregnancy. In addition, gp330 was expressed by amniotic cells and neurectodermal structures. During nephrogenesis, in contrast, the expression of gp280 and gp330 by the intermicrovillar areas of the proximal tubule cell was the result of a complex maturation process. gp280 and gp330 were diffusely distributed in S-shaped bodies in the presumptive areas of the glomerulus, proximal tubule, and distal tubule (gp330). During development of the nephron, the pattern of expression became progressively restricted to the proximal tubule and glomerulus (gp330), and selective localization in the intermicrovillar areas was only achieved in filtrating nephrons. (Am J Pathol 1993, 142: 1654–1667)

The teratogenic and fetotoxic action of heterologous antibodies raised against kidney and yolk sac was first demonstrated 30 years ago in the pioneering studies of Brent et al[1–3] and David et al.[4] The first biochemical information concerning the specificity of the teratogenic antibodies was brought by Leung[5] who isolated from the renal brush border (BB) a 340-kd glycoprotein described as diffusely expressed on the entire area of the microvilli. The antisera raised against this protein reacted with the visceral yolk sac endodermal cells and embryonic endoderm but identified in these structures a protein of lower molecular mass.[6]

In the wake of studies performed on Heymann's nephritis,[7–10] we identified a protein of 280-kd that is selectively concentrated in clathrin-coated areas of the proximal tubule and yolk sac brush borders.[11–12] We further showed that monoclonal antibodies specific for the 280-kd protein were teratogenic when in-

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jected in pregnant rats. Our experiments therefore associated the target antigen of the teratogenic antibodies with structures involved in endocytosis. They thus provided some support to the pathogenetic process proposed in this model, which suggests that antisera cause developmental abnormalities by interfering with the uptake and degradation of nutrients by epithelial cells of the visceral yolk sac.[13–16] They further showed that this process was highly specific, because monoclonal or polyclonal antibodies reactive with the Heymann antigen, a 330-kd glycoprotein[17–20] co-expressed with gp280, had no effect on the outcome of pregnancy. Subsequent work by Leung et al[21] using pre- and postembedding immunogold studies indicated that the 340-kd antigen identified by their antisera was, in fact, restricted to the coated pits. Because this molecular mass is very similar to that of the Heymann antigen, the object of this study was to investigate the relationship between gp330 and gp280. To this effect, we first purified to homogeneity the two proteins and used the material for peptide mapping and preparation of polyclonal antisera. Homologies in cyanogen bromide (CNBr) peptide maps and immunological cross-reactivity indicate that the two proteins are closely related. However, the bulk of the antibodies induced by gp330 and gp280 are specific for the immunizing protein and cross-reactive antibodies are not teratogenic. We have also carried out a detailed immunohistological study of the ontogenesis of gp280 and gp330 showing asynchronous expression and distinct distribution of the two proteins, gp280 being delayed and restricted to the extraembryonic endoderm until appearance in the metanephros. However, ultrastructural localization of the two proteins at the level of membrane domains was very similar: both were directly restricted to—or predominant in—the coated pits of primitive endoderm and yolk sac epithelium; in contrast, within the developing nephron, gp280 and gp330 were first expressed diffusely on the membrane of epithelial cells within S-shaped bodies and only subsequently became concentrated within the intermicrovillar areas of proximal tubule, when glomerular filtration was initiated.

Materials and Methods

Monoclonal Antibodies

The production, purification, and characterization of the monoclonal antibodies (MAbs) used in this work have been previously reported.[9, 10] MAb75 is specific for gp280, MAb12 is specific for gp330, and MAb180 is specific for maltase (gp300).

BB Membrane Preparations

Rat renal tubular membrane preparations were obtained by gently forcing cortical fragments through a 106-μ sieve as previously described.[9] Membranes were dissolved at a final concentration of 10 to 15 mg/ml in 1% sodium desoxycholate and dialyzed against 0.1% sodium desoxycholate. Yolk sacs were sampled at 20 days of gestation, extensively washed in phosphate-buffered saline (PBS), containing 1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L N-ethyl-maleimide, 2 mmol/L benzamidine, 5 mmol/L ethylenediaminetetraacetic acid, and dissolved in sodium desoxycholate as described for renal membranes.

Purification of gp280, gp330, and gp300

Immunoaffinity chromatography was carried out as previously described.[10] MAb 12, MAb 75, or MAb 180 were individually coupled to CNBr-activated Sepharose 4B (Pharmacia, Saint Quentin en Yvelines, France) at a concentration of 10 mg purified IgG/ml of gel. Approximately 60 mg of BB membrane proteins were applied to 3 ml of antibody-coupled Sepharose 4B. Extensive washing of the unbound material was carried out using 0.5% NP-40 in phosphate-buffered saline supplemented with protease inhibitors. Specifically bound proteins were eluted with 50 mmol/L diethylamine pH 11, dialyzed against water, and lyophilized.

Production of Polyclonal Antisera

The material obtained by immunoaffinity chromatography was either directly incorporated in complete Freund's adjuvant (one rabbit) or further purified by 4% polyacrylamide gel electrophoresis–sodium dodecyl sulfate PAGE-SDS[22] (two rabbits): individual bands were located by negative staining by 1 mol/L KCl, cut out of the gel, dialyzed against distilled water, ground, and incorporated in complete Freund's adjuvant. Rabbits received 4 to 5 subcutaneous injections at 4-week intervals, containing approximately 15 μg protein.

Peptide Mapping

For this purpose, proteins trapped in the polyacrylamide bands after PAGE-SDS were electroeluted, concentrated, digested with CNBr, and processed for two-dimensional (2D) electrophoresis as previously described in detail.[23]

Immunoblotting

For immunoblotting, BB membranes from renal tubule or yolk sac were boiled in sample buffer (125 mmol/L Tris, pH 6.8, containing 20% glycerol, 5% SDS) and separated by PAGE-SDS as described above under reducing (5% 2-mercaptoethanol) or nonreducing conditions. Proteins were transferred to nitrocellulose and immunodetected as previously described.[23] The end point titers of the various antisera varied from 1/20000 to 1/50000. They were routinely used at a final dilution of 1/5000. In some experiments, immunoblotting was used to analyze the specificity of immunoglobulins eluted from nitrocellulose bands. For this purpose, gp330 and gp280, purified by immunoaffinity chromatography, were submitted to PAGE-SDS and transferred onto nitrocellulose. Approximately 10 nitrocellulose bands containing one of the purified glycoproteins were cut in small pieces and incubated under continuous agitation for 90 minutes with 5 ml of a 1/50 dilution of the serum under study. After careful washing, the bound antibodies were eluted with glycine buffer pH 2.2 containing 0.1% bovine serum albumin and immediately neutralized by addition of phosphate buffer, according to Smith and Fisher.[24] Reactivity of the eluate was analyzed by immunoblotting as described above, except that bound Ig were detected using alkaline phosphatase–labeled anti-rabbit IgG.

Immunohistology

Pre-implantation eggs (the day when spermatozoa were detected on vaginal smears was designated day 0) were obtained 4 and 5 days after conception by careful washing of the uterine cavity with periodate-lysine-paraformaldehyde[25] containing 2 to 4% paraformaldehyde. Postimplantation embryos were obtained from day 6 to day 8 by serial sectioning of uterine horns and from day 10 to day 12 by individual dissection up to the membrane of Reichert. From day 17 to day 20, visceral yolk sacs were sampled *in toto*. Kidney, lung, and brain were sampled at days 19, 20, and 21 of gestation and 3 days after birth after intracardiac perfusion with Hanks' solution followed in some instances by fixative. Uterine horns and oviducts were sampled from virgin and pregnant rats. Samples to be studied at the light microscopic level were not fixed or lightly fixed using periodate-lysine-paraformaldehyde containing 2 to 4% paraformaldehyde. Immunoultrastructural analysis was carried out on samples fixed with periodate-lysine-paraformaldehyde, using preembedding techniques.[7]

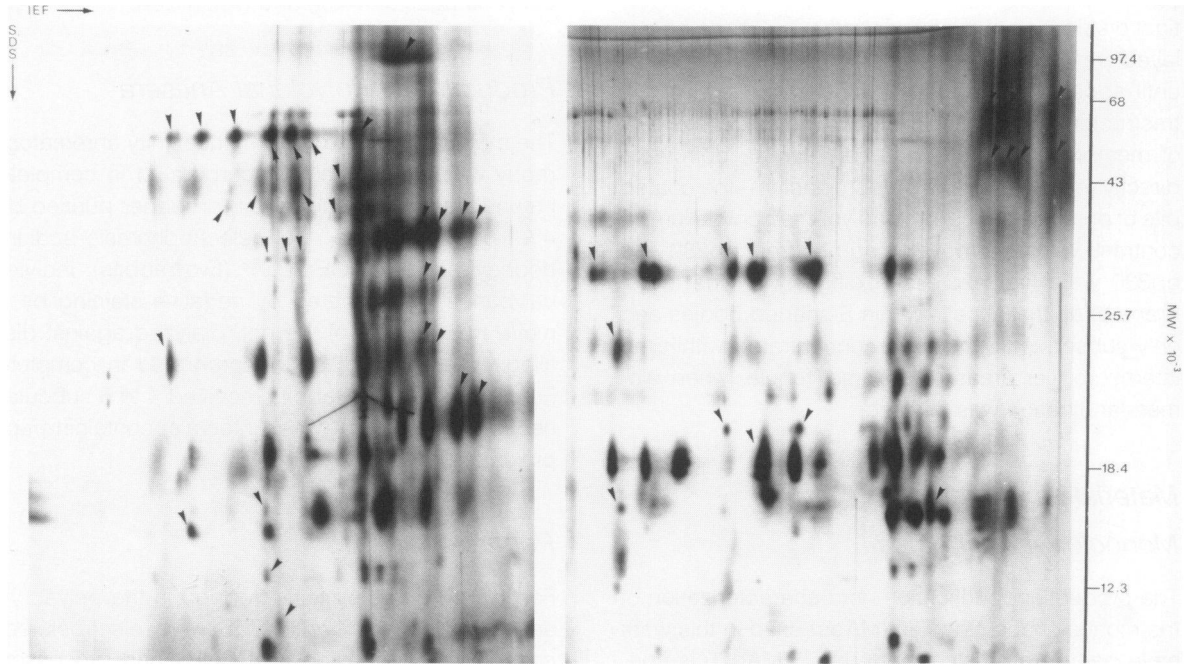


Figure 1. *2D PAGE of CNBr digests of gp280* (left) *and gp330* (right). *Arrows indicate spots (or group of spots) specific of each protein. The streaking observed in the acidic region of the gels is probably related to the complex chemical treatments involved in CNBr digestion. MW standards are given on the right of the figure.*

Teratogenicity

The potential role of anti-gp330 antibodies on embryonic development was tested under two distinct experimental conditions. First, gp330 specific antibodies (3 ml rabbit antiserum/rat) were administrated intravenously within 72 hours of the beginning of pregnancy. Second, antibodies raised against gp330 but also reactive with gp280 were injected intravenously at a dose of 3 ml/rat 9 days after the beginning of pregnancy.

Results

Peptide Mapping

gp330 and gp280 prepared from renal BB by affinity chromatography and preparative electrophoresis were digested with CNBr and analyzed by 2D electrophoresis. Three independent cleavage experiments were performed, and each sample was analyzed at least twice. Comparison of the 2D maps was performed by superimposition of the gels; differences were considered significant when found reproducibly in the three experiments. Figure 1 shows a large region of the 2D gels stained by silver nitrate; some of the spots too close to the borders have been excluded. Ninety-one and 78 spots were respectively found on gp280 and gp330 maps; 57 were considered as common. The 18-kd region in which direct superimposition of major peptides can be achieved is a good illustration of the homology between gp280 and gp330. Copies on tracing paper of each of the gels were made (Figure 2) and a composite map was drawn to summarize the results.

Immunochemistry

Despite the similarities found by peptide mapping, analysis by Western blotting of the immunoreactivity of polyclonal anti-gp330 and anti-gp280, raised against the native antigens purified only by affinity chromatography, showed that the proteins exhibited only limited cross-reactivity, thus suggesting that the immunologically dominant segments were distinct. Indeed, most of the antibodies produced were strictly specific for the immunogenic protein, gp280 or gp330, as illustrated in Figure 3. The left side of this figure is an immunoblot prepared from yolk sac plasma membranes in which individual lanes were cut in half and incubated with antibodies raised against purified gp280 (lane 1A) or purified gp330 (lanes 1B and 2A) or a mixture of the two antisera

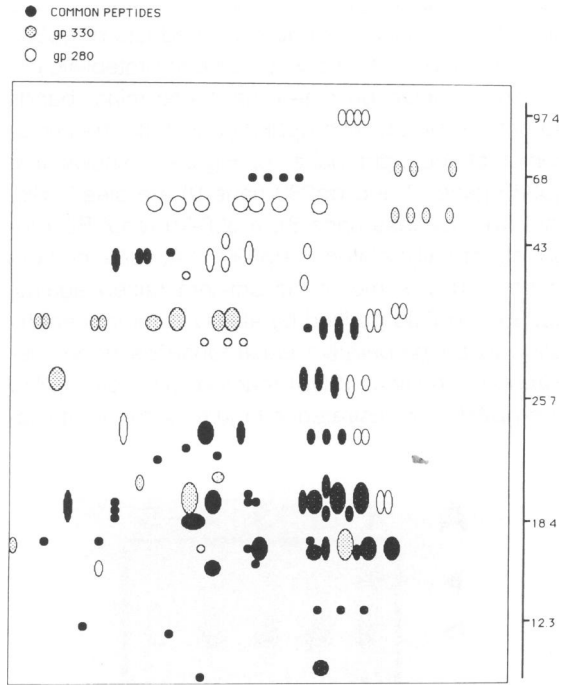


Figure 2. *Composite map of gp280 and gp330 derived from three independent experiments. Dark-shaded symbols: peptides shared by gp280 and gp330; light-shaded symbols: peptide specific of gp330; open symbols: peptides specific of gp280.*

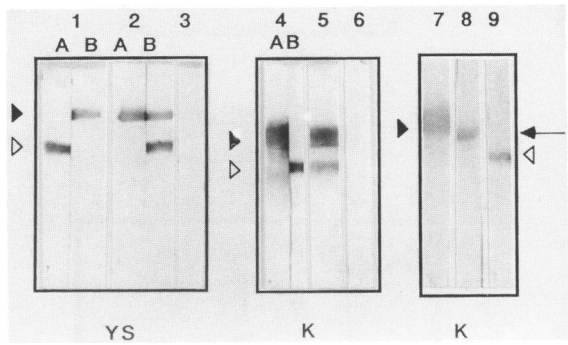


Figure 3. *Immunoreactivity of yolk sac and renal BB preparations with monospecific anti-gp280 and anti-gp330 antibodies. BB preparations from yolk sac (YS,* left panel*) and kidney cortex (K,* center *and* right panels*) were separated under reducing conditions in 4% polyacrylamide SDS gels and blotted onto nitrocellulose. Individual lanes, or lanes cut in half and denoted A and B, were stained with the following polyclonal antibodies:* Left panel: *1A: anti-gp280; 1B: anti-gp330; 2A: anti-gp330; 2B: mixture of anti-gp330 and anti-gp280; 3: normal rabbit serum.* Center panel: *4A: anti-gp330; 4B: anti-gp280; 5: mixture of anti-gp330 and anti-gp280; 6: normal rabbit serum.* Right panel: *7: anti-gp330; 8: anti-maltase (gp300); 9: anti-gp280. (▶), (▷), and arrow indicate respectively the migration of MAb affinity-purified gp330, gp280, and gp300.*

(lane 2B). It can be seen that each antiserum identified a single protein band although the two antigens were easily demonstrable on each strip. The central part of Figure 3 presents a similar experiment performed with renal BB membranes: the anti-gp330 antibodies (lane 4A) stained massively the gp330 band and very weakly two bands of lower

molecular weight (MW). The latter are diffuse and probably represent degradation products of gp330 that are known to be very sensitive to proteolytic enzymes. It should be noted that these minor bands do not co-migrate with gp280 (lane 4B). The immunoblot of the right panel of Figure 3 shows that gp330 (lane 7) and gp280 (lane 9) are clearly distinct from maltase (lane 8), a 300-kd renal BB protein structurally related to gp330.[26] At variance from these results, some of the antisera raised against gp280 or gp330 purified by affinity chromatography followed by preparative electrophoresis under denaturing conditions (SDS) reacted with both gp330 and gp280. As illustrated in Figure 4A for an antiserum raised against gp280, the titer of the cross-reactive antibodies was low (1/2000) compared with the titer observed against the immunogen (1/32000). Because of the procedure used to prepare the immunogen, it was unlikely that the reactivity observed could be explained by contamination of the immunogen. To provide direct proof for the presence of cross-reactive antibodies, antisera raised

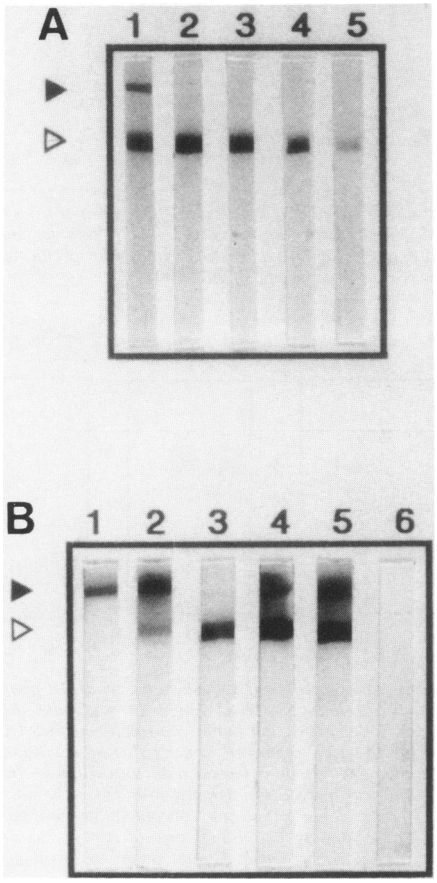


Figure 4. *Identification of antibodies reactive with gp330 and gp280. BB preparations from yolk sac were separated under reducing conditions in 4% polyacrylamide SDS gels, blotted onto nitrocellulose and incubated with the antibodies.* A: *serial twofold dilutions (1/2000 to 1/32000) of an antiserum raised against gp280 (purified by affinity chromatography followed by preparative electrophoresis). Note reactivity in the low dilution with both gp330 (▶) and gp280 (▷).* B: *1: polyclonal antibody specific for gp330; 2: antiserum reactive with gp330 and gp280; 3: polyclonal antibody specific for gp280; 4: eluate from a gp280 nitrocellulose band incubated with an antibody raised against gp330 but also reactive with gp280; 5: eluate from a gp330 nitrocellulose band incubated with an antibody raised against gp280 but also reactive with gp330; 6: control eluate from a gp330 nitrocellulose band incubated with an antiserum specific for gp280.*

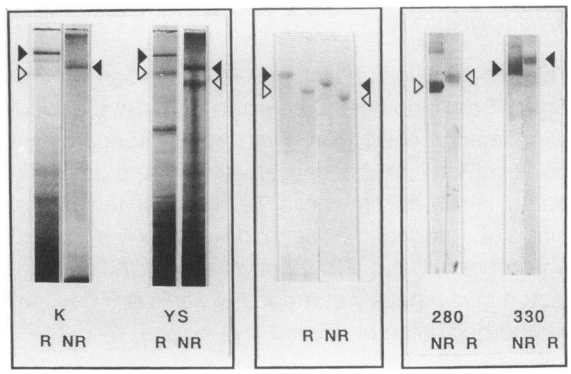


Figure 5. *Migration of gp280 and gp330 under reducing and nonreducing conditions.* Left panel: *brush border preparations from kidney cortex (K) and yolk sac (YS) were separated in 4% polyacrylamide SDS gels under reducing conditions (R) and in the absence of reducing agent (NR). Gels were stained with coomassie blue. Note that gp330 (solid arrowhead) and gp280 (open arrowhead) migrate faster under nonreducing conditions.* Center panel: *individual bands of gp280 (open arrowhead) and gp330 (solid arrowhead) were cut out from 4% PAGE-SDS gels of YS proteins run under nonreducing conditions and rerun on a new PAGE-SDS gel in the presence (R) or the absence (NR) of reducing agent. Note that reduction does not release any detectable polypeptide chain from gp330 or gp280; the apparent MW of the two proteins is lower under nonreducing conditions.* Right panel: *immunoblot analysis of gp280 and gp330 separated in the absence of reducing agent (NR) and in the presence of dithiothreitol (R). Note faster migration of immunoreactive gp330 and gp280 under nonreducing conditions. A small amount of high MW material is not dissociated.*

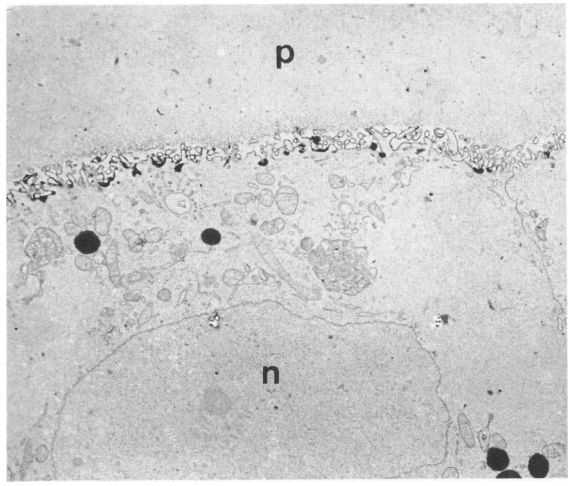


Figure 6. *Immunoultrastructural localization of gp330 on day-4 pre-implantation blastocyst. Note positivity of intermicrovillar areas. p: zona pellucida; n: nucleus. Original magnification ×3000.*

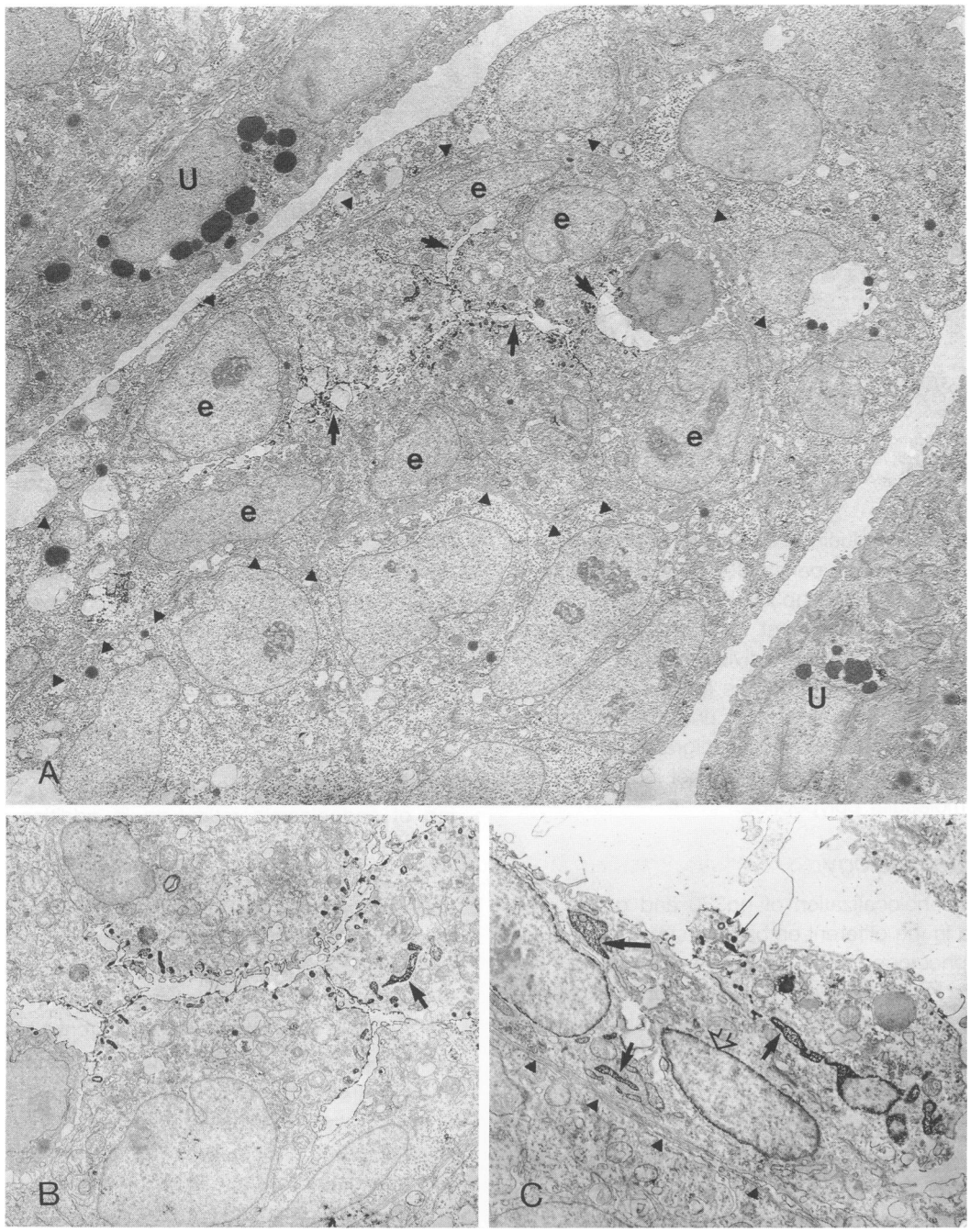


Figure 7. *Ultrastructural immunolocalization of gp280* (**A, B**) *and gp330* (**C**) *on pre-implantation blastocysts.* **A:** *Low-power electronmicrograph of a blastocyst trapped in endometrial infoldings. Note staining of the luminal aspect of primitive entoblastic cells (e) that line the blastocelic cavity (arrows) and are separated from the inner cell mass and the trophectodermic cells by a thin basement membrane (▲). Uterine epithelium (U) and trophectodermic cells are unstained. Original magnification: ×1100.* **B:** *High-power view of entoblastic cells. Staining for gp280 is essentially located on the coated pits facing the blastocelic cavity and to a lesser extent on noncoated areas of the plasmalemma. Note (arrow) staining of endoplasmic reticulum. Original magnification: ×3000.* **C:** *Expression of gp330 by entoblastic cells. Note staining of coated pits and vesicles (thin arrows), endoplasmic reticulum (solid arrows), and perinuclear cisternae (open arrow). Basement membrane is outlined by (▲). Original magnification: ×3000.*

against gp330 but reactive with gp280 were incubated with nitrocellulose transfers of gp280. As shown in Figure 4B (lane 4) the immunoglobulin eluted from these bands were equally reactive with gp330 and gp280. Similar results were obtained when antibodies raised against gp280 but reactive with gp330 were eluted from gp330 bands (lane 5).

In the course of the present experiments, we noted (Figure 5) that proteins in the 300-kd MW range migrated with an apparent lower MW when

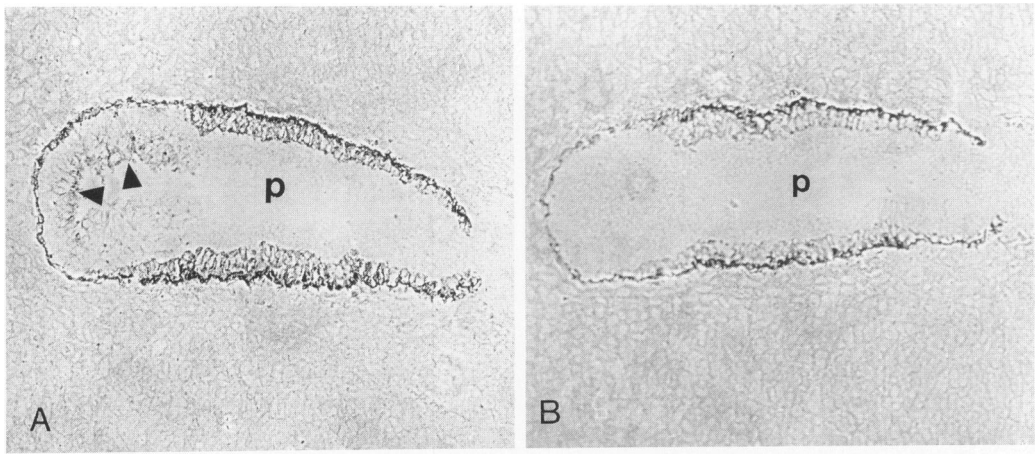


Figure 8. *Immunolocalization of gp330* (A) *and gp280* (B), *on postimplantation embryos obtained by cryosectioning of uterine horns: immunoperoxidase, day 8. Note apical staining of endodermic cells by anti-gp330 and anti-gp280. In addition, gp330 is detectable (arrowheads) on neuroectodermic cells facing the proamniotic cavity (p). Original magnification:* ×200.

run under nonreducing conditions. Analysis by immunoblotting (right panel of Figure 5) confirmed that both gp280 and gp330 migrated faster in the absence of reducing agent. As shown in the central panel of Figure 5, when individual bands of gp330 and gp280 were cut out of the gels run under nonreducing conditions and rerun under reducing and nonreducing conditions, they migrated to the expected positions. Reduction did not induce release of low molecular polypeptide.

Immunohistology

The immunolocalization of gp330 and gp280 was studied in the different embryonic structures including trophectoderm and its derivatives, yolk sac entoblastic cells, intraembryonic and amniotic structures, and maternal derivatives of mullerian structures.

Early Stages of Development and Extraembryonic Structures

At day 4, trophectodermal cells were clearly positive for gp330. As shown in Figure 6, the protein was expressed only within the intermicrovillar areas on the outer surface of the cells lining the blastocelic cavity and overlaying the inner cell mass; under the same conditions, staining for gp280 remained consistently negative. Subsequently, primitive endodermal cells first appear at the blastocelic pole of the inner cell mass and migrate around the blastocelic cavity. As shown in Figure 7, blastocysts trapped at day 6 in endometrial mucosal infoldings were positive for gp330 and gp280 and could be examined at the ultrastructural level. Whereas the inner cell mass and the trophectoderm were negative, the primitive endodermal cells (which line the blastocelic cavity and are separated from the inner cell mass and the trophectoderm by a thin basement membrane) were positive for gp280 (Figure 7, A and B) and gp330 (Figure 7C). The two proteins were concentrated within the coated pits facing the cavity. In the case of gp280, mild to moderate staining of the plasma membrane could also be detected. Evidence of biosynthesis was found for gp280 and gp330 as shown by staining of endoplasmic reticulum; in addition, gp330 was detected in perinuclear cisternae.

In postimplantation embryos (days 7 and 8), all yolk sac endodermal cells (Figure 8, A and B) were positive for gp280 and gp330 and remained so until parturition. By indirect immunofluorescence on day 18 of gestation, the apical aspect of the visceral cells of the yolk sac, which differentiate a well-developed BB, was brightly stained (not shown). At the ultrastructural level, gp330 and gp280 (Figure 9, A and B) were essentially confined to the intermicrovillar areas and to the coated vesicles. gp280 was also detectable to a limited extent along the microvilli; when detectable in noncoated areas, staining for gp330 was mild. For both gp330 and gp280 (Figure 9C), staining of the endoplasmic reticulum was also observed, thus providing clear-cut morphological evidence of persistent biosynthesis. In addition, large apical vesicles located between lysosomes and the tubular vesicular system were consistently stained. As expected, cuboidal cells of the Duval's sinuses that derive from the yolk sac were positive for gp330 and gp280. Primitive ectoderm lining the proamniotic cavity at the embryonic pole of the ovum (day 7) and epithelial cells lining the amniotic cavity (Figure 8A) after day 8 were

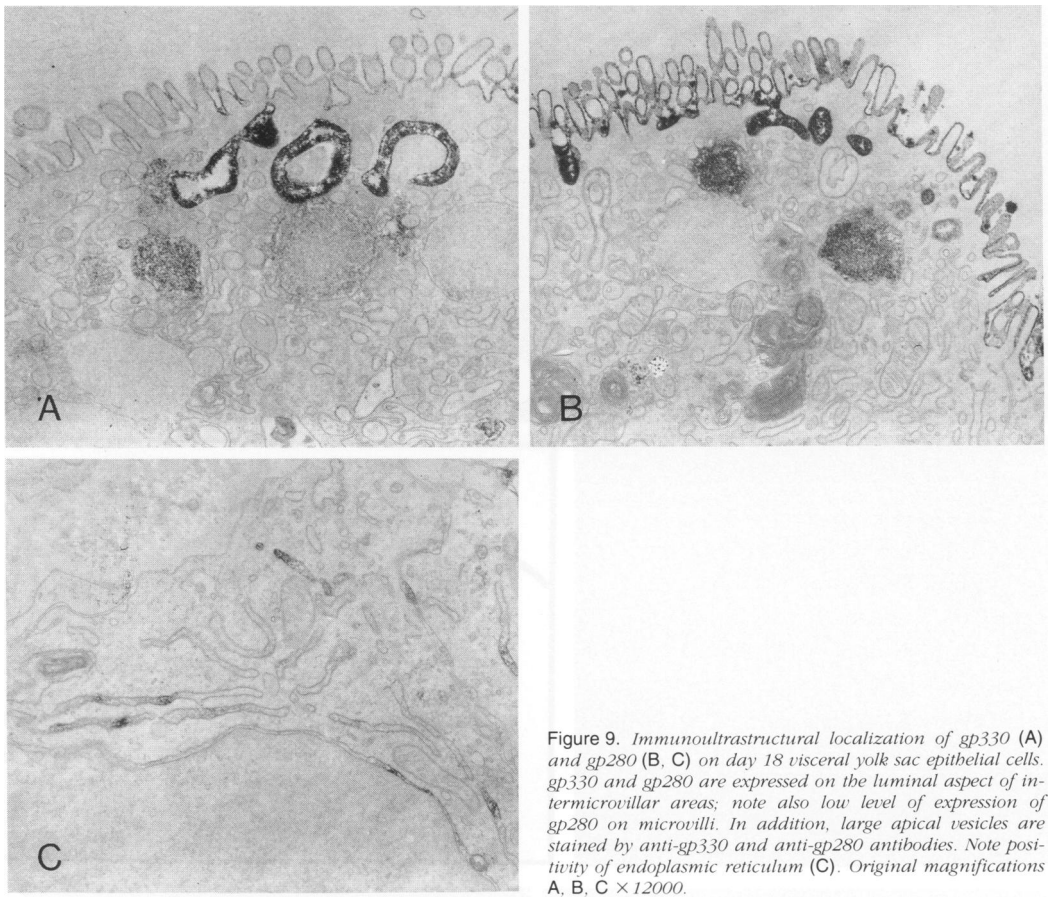


Figure 9. *Immunoultrastructural localization of gp330* (A) *and gp280* (B, C) *on day 18 visceral yolk sac epithelial cells. gp330 and gp280 are expressed on the luminal aspect of intermicrovillar areas; note also low level of expression of gp280 on microvilli. In addition, large apical vesicles are stained by anti-gp330 and anti-gp280 antibodies. Note positivity of endoplasmic reticulum* (C). *Original magnifications* A, B, C ×*12000.*

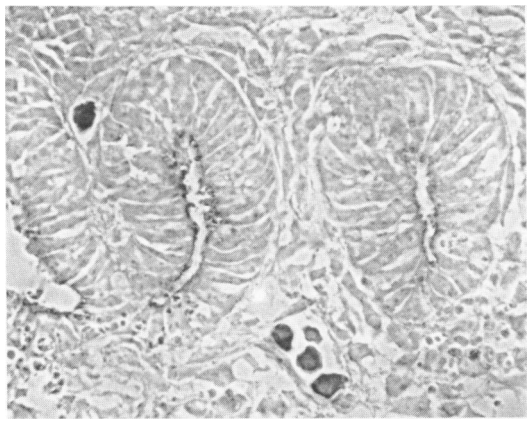
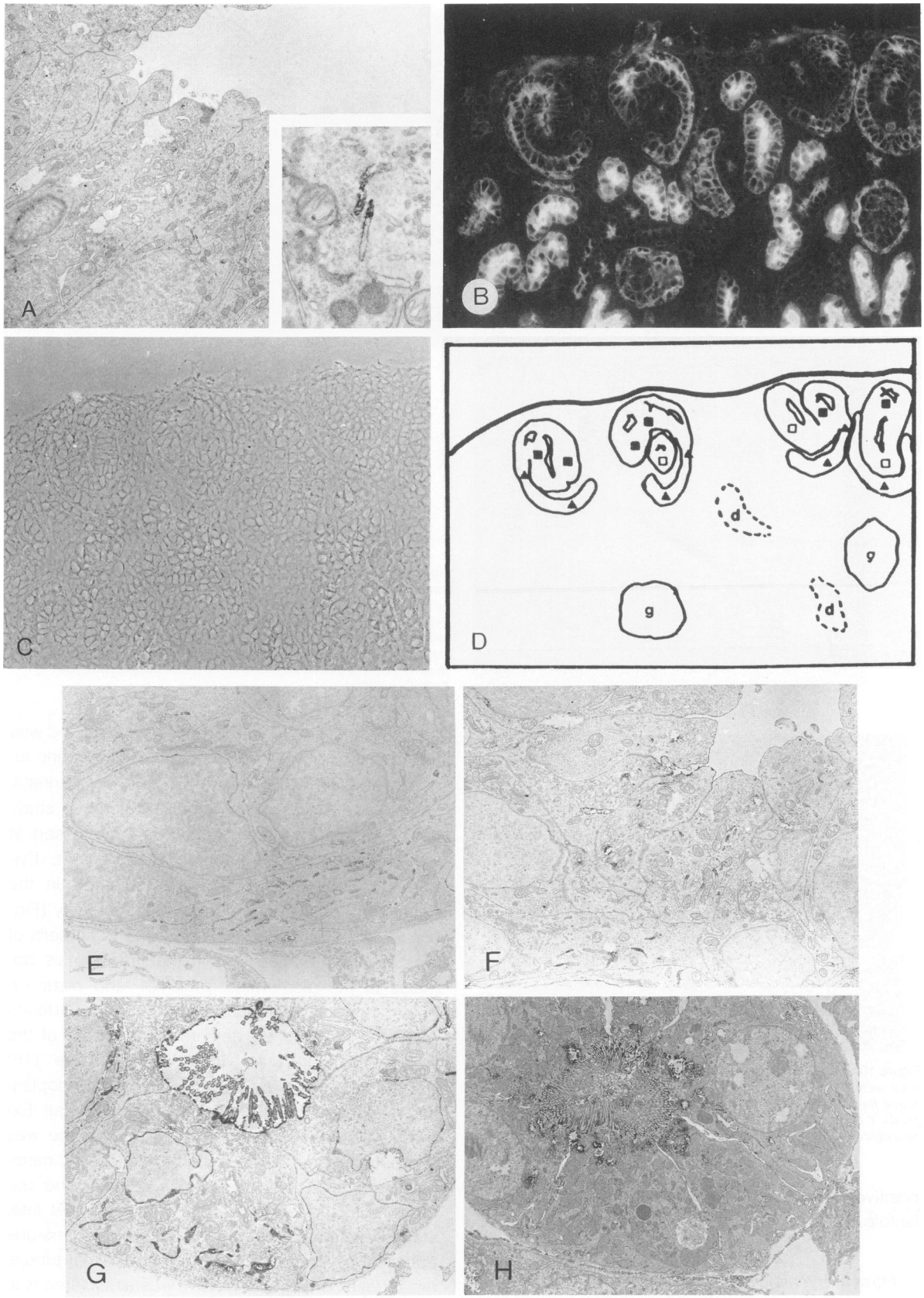


Figure 10. *Immunolocalization of gp330 in the mesonephros. Cryostat section on day-12 embryo stained by immunoperoxidase technique for gp330 and examined under phase-contrast; note reaction product at the luminal pole of mesonephric tubule cells. Original magnification:* ×*200.*

positive for gp330. Trophoblastic cells and the allantoic placenta were consistently negative.

Intraembryonic Structures

gp330 and gp280 had contrasting distributions within intraembryonic structures. As expected, gp330 was detected within renal structures and was first identified at the luminal pole of mesonephric tubules at day 12 (Figure 10). In the perinatal kidney—the most easily available material to study the developing nephron, gp330 was expressed at the earliest steps of nephron development. Evidence of biosynthesis was first detected in the nephronic vesicle (Figure 11A). Subsequently (Figure 11, B to F), gp330 was localized in all cells of the S-shaped bodies, i.e., in the presumptive domains of the glomerulus, proximal, and distal tubules. At the ultrastructural level, clear-cut evidence of synthesis could be detected within cells of the lower (Figure 11E) and intermediate (Figure 11F) limbs as indicated by staining of rough endoplasmic reticulum profiles and perinuclear cisternae. Expression of gp330 at the plasma membrane was fine but rather diffuse: it was not restricted to membrane invaginations and extended beyond the apical pole to the lateral aspect of the cells. At later stages, expression became restricted to glomerular epithelial cells and cells lining the proximal tubule. Initial development of microvilli was associated with morphological evidence of intense synthesis and predominant expression by the apical pole (Figure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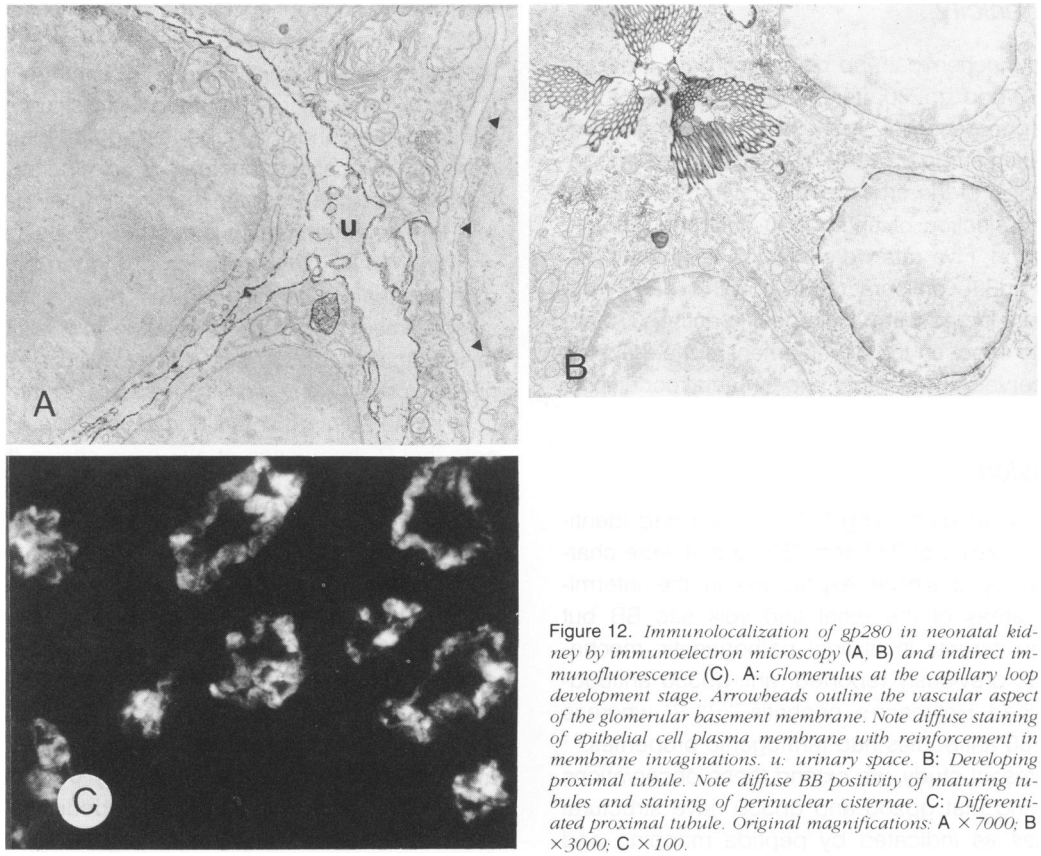

Figure 12. *Immunolocalization of gp280 in neonatal kidney by immunoelectron microscopy* (A, B) *and indirect immunofluorescence* (C). A: *Glomerulus at the capillary loop development stage. Arrowheads outline the vascular aspect of the glomerular basement membrane. Note diffuse staining of epithelial cell plasma membrane with reinforcement in membrane invaginations. u: urinary space.* B: *Developing proximal tubule. Note diffuse BB positivity of maturing tubules and staining of perinuclear cisternae.* C: *Differentiated proximal tubule. Original magnifications:* A ×*7000;* B ×*3000;* C ×*100.*

11G). When microvilli were fully developed (Figure 11H), gp330 was localized in the intermicrovillar areas and morphological evidence of biosynthesis was scarce, a pattern closely resembling that observed in the adult kidney. gp330 was initially detected in the canalar portion of the ureteric bud but was no longer expressed in the collecting duct. In addition, gp330 was detected in neurectodermic cells from day 8 until birth. Ultrastructural immunoelectron microscopy confirmed that staining was located on the coated pits of the apical membrane facing the amniotic and neural cavities (not shown). In the perinatal stage, gp330 was also expressed by choroid plexuses and type II pneumocytes.

Before birth and in the neonatal kidney, gp280 was only detectable in metanephric structures. Within a given nephron, it was first detectable in the S-shaped bodies in the presumptive domains of the glomerulus and the proximal tubule. As previously described for gp330, staining for gp280 at the ultrastructural level was initially diffuse as shown for an immature glomerulus (Figure 12A) and a developing, but not fully differentiated, proximal tubule (Figure 12B). Subsequently, expression of gp280 became confined to the differentiated proximal tubule (Figure 12C).

Mullerian Structures

In the virgin rat, the epithelial cells lining all segments of the oviduct were brightly stained by anti-gp330 antibodies. In addition, after mating and during pregnancy, epithelial cells lining the endometrial cavity became positive (data not shown). No staining was observed with anti-gp280.

Figure 11. *Immunolocalization of gp330 within neonatal kidney by immunoelectron microscopy* (A, E *to* H) *and indirect immunofluorescence* (B). A: *Nephronic vesicle. Note positivity of endoplasmic reticulum profiles (insert).* B: *Indirect immunofluorescence of cryostat section of neonatal kidney stained for gp330. Note staining of S-shaped bodies, proximal tubules at various degrees of maturation, and immature glomerulus.* C: *Phase contrast of B.* D: *Schematic of* (B). *Four S-shaped bodies have been outlined and divided into lower limb (▲), intermediate limb (■) and upper limb (□); g: maturing glomerulus; d: distal tubule.* E: *Lower limb of S-shaped body in the presumptive area of the glomerulus.* F: *Intermediate limb of S-shaped body in the presumptive area of the proximal tubule.* G: *Maturating proximal tubule at the time of formation of microvilli.* H: *Fully differentiated proximal tubule. Note at the early stages diffuse membrane positivity and evidence of biosynthesis indicated by staining of perinuclear cisternae and endoplasmic reticulum. Staining becomes restricted to the intermicrovillar domain in differentiated proximal tubule epithelium. Original magnifications:* A ×*3000; insert* ×*12000;* B ×*50;* E, F ×*4400;* G ×*3000;* H ×*2500.*

Teratogenicity

The immunochemical and ontogenic data provided for gp330 and gp280, i.e., shared antigenicity and earlier appearance of gp330 detected on the preimplantation embryo, led us to analyze the teratogenic potential of the cross-reactive paratopes and of the early injection of anti-gp330 antibodies before implantation. Five rats were injected with two different anti-gp330 antisera reactive with gp280; four rats were injected with monospecific anti-gp330 antisera. No effect on the development of the embryos was observed under either experimental conditions.

Discussion

In previous studies using MAb,[9–11] we had identified two proteins of 280 and 330-kd that were characterized by a similar expression in the intermicrovillar areas of the renal and yolk sac BB but served as targets in distinct pathogenetic mechanisms: as previously reported, anti-gp330 antibodies induced Heymann's nephritis,[10,18–20] whereas anti-gp280 antibodies had teratogenic properties.[11] In the present study, we extend these observations by showing that gp280 and gp330 have structural similarities as indicated by peptide mapping and limited shared antigenicity. We also show that, although they are coexpressed in the extraembryonic endoderm, their overall sequential expression during ontogenesis is asynchronous.

The peptide maps presented in this work clearly show the generation of common peptides by CNBr cleavage of gp330 and gp280. The degree of homology is not as high as would be expected if gp280 were derived from gp330 by cleavage of a 50-kd peptide but sufficient to indicate that the two glycoproteins are related structurally. The use of silver staining and the potential presence of repeat sequences preclude any further quantitative estimations. The detection of antibodies reactive with gp330 and gp280 provides further evidence of their structural relationship. It is likely that they belong to a group of proteins that also includes maltase, a 300-kd glycoprotein, because homologies in glycopeptide mapping of gp330 and gp300 have been reported by Kerjaschki et al.[26] The same group also showed that antibodies raised against gp330 reacted with gp300 and immunoprecipitated a protein with a MW of 280 kd. Despite these observations relating gp280 and gp330, it is striking to note that 1) most of the antibodies produced in this study are specific for either 280 or 330 and 2) the specificities of the pathogenic antibodies are distinct. The structural bases accounting for the pattern of antibody production observed are unknown, but one may note that the apparent MW on SDS gels of gp280 and gp330 increase when the proteins are run under reducing conditions, suggesting that the conformation of the native monomers is constrained by intrachain disulfide bridges. This could play a critical role in the immunogenic properties of the proteins and account for the presence of hidden but common antigenic determinants. This possibility is also in agreement with our observation that antibodies raised against gp330 but reactive with gp280 do not have teratogenic activity. Concerning the specificity of the pathogenic antibodies, it is interesting to note that Pietromonaco et al[27] have shown that the antibodies bound in the glomerulus during the induction of passive Heymann's nephritis by polyclonal anti-gp330 antibodies have a limited spectrum of reactivity, because eluates prepared within 72 hours identified only two CNBr peptides. The reactivity of the eluates widened as the disease evolved but remained restricted when compared to that of the injected antibodies. Detailed information on the targets of the teratogenic antibodies is not available but the pathogenic activity of some (but not all) monoclonal anti-gp280 antibodies and the lack of effect of antibodies raised against gp330 but reactive with gp280 suggest that the number of critical binding sites is limited. It would thus seem that in the two systems under consideration involving two related proteins, the pathogenic antibodies react with a limited number of antigenic determinants as previously reported in other models of immunologically mediated disease.[28,29]

The exact functions of gp280 and gp330 are unknown, but their expression by the clathrin-coated areas of a limited number of epithelia suggests that they are both involved in some aspects of endocytosis specific for these organs. Because the mode of action of teratogenic antibodies is classically related to an inhibitory effect on the endocytic process by yolk sac visceral epithelial cells, it was surprising to note that fetal malformations were only induced by antibodies to gp280. Although we had shown that gp330 was expressed by yolk sac epithelial cells on day 11 of pregnancy, one of the possibilities that had to be considered to explain the lack of effect of antibodies to gp330 on embryonic development was a delayed expression of gp330 by endodermal cells. This led us to analyze chronologically the expression of gp330 and gp280 throughout pregnancy.

During the initial phase of gestation, as previously shown in the mouse by Buc-Caron et al,[30]

gp330 was first detectable on trophectodermal cells. Expression was transient, however, and persisted only during the pre-implantation phase. Subsequently, gp280 and gp330 were detected on primitive endodermic cells and remained expressed throughout pregnancy on the BB of the visceral epithelial cells of the yolk sac and to a lesser extent by the parietal cells that do not develop an apical BB. As soon as they were detectable, the two glycoproteins assumed a coated pit restricted expression, suggesting that they were already involved in the endocytic process. This was in particular the case for the expression of gp330 by trophectodermic cells and for that of gp330 and gp280 on endodermal cells, even at a time when microvilli were not yet formed. It is thus likely that the different effects of anti-gp280 and anti-gp330 antibodies are not related to a delayed expression of the latter but rather reflect different properties (or functions) of the target proteins. It is in this context interesting to note that whereas the expression of gp330 is virtually restricted to the clathrin-coated intermicrovillar domain, the ultrastructural distribution of gp280 at the subcellular level includes mainly coated pits but also to some extent noncoated areas. This pattern may be of significance as we[31] and others before us[13,15,16,32] have assessed the effect of teratogenic antibodies on endocytosis using mainly fluid phase markers that might be internalized via nonclathrin-coated vesicles. The existence of such a nonclathrin-dependent pathway has been the object of debate,[33] but recent studies by Hansen et al[34] suggest that half of the vesicles formed at a given time may be uncoated. It is thus conceivable that gp280 and gp330 may be involved in different, perhaps overlapping, internalization pathways: because of its more widespread distribution, gp280 might partake in more general aspects of endocytosis in such a way that the corresponding antibodies might have a notably greater impact on the internalization process.

During the development of renal structures, the expression of the two glycoproteins is also asynchronous: gp330 is first detectable (in the absence of gp280) in the mesonephros and in the nephronic vesicle as well as in the ureteric bud; subsequently, gp330 and gp280 are detected in metanephric structures. At variance from observations made on endodermal cells, morphological evidence suggests that their expression in the adult proximal tubule is the result of a maturation process. Indeed within S-shaped bodies, gp330 and gp280 are diffusely distributed on the apical and lateral aspects of the plasma membrane and expressed in areas that will give rise to proximal and distal tubules and to the glomerulus. During development of the nephron, their pattern of expression narrows to the proximal tubule and to the glomerulus (for gp330), and they become progressively concentrated in clathrin-coated intermicrovillar areas when microvilli develop. This stage is also characterized by the establishment of the vascularization of the glomerulus initiating glomerular filtration: it is thus tempting to suggest that the concentration of gp330 and gp280 in the intermicrovillar domains is related to their functional role in proximal tubule reabsorption. This is in agreement with studies of Biemesderfer et al[35] who reported that at this stage there is a close relationship between expression of gp330 and the ability of proximal tubule cells to reabsorb marker proteins such as peroxydase. At variance from our results, this group, however, reported that the first expression of gp330 was in clathrin-coated intermicrovillar areas during formation of microvilli. The reason why these authors did not detect gp330 before this stage and in particular in S-shaped bodies is unknown. It should however be mentioned that immunofluorescence detection on semi-thin cryosections of prefixed material is more precise but predictably less sensitive than the conventional immunofluorescence on unfixed tissue used in our experiments. In support of this possibility, one may note that in this study, which uses the same fixative as Biemesderfer et al,[35] the positivity at the electron microscope level is not as important as anticipated from the immunofluorescence data.

The observations presented in this paper indicate that the 280-kd coated pit protein target of teratogenic antibodies is related structurally and immunologically to another 330-kd coated pit protein that serves as the target of nephritogenic antibodies. During ontogenesis, the synthesis of gp330 and gp280 is asynchronous although the two glycoproteins are co-expressed by yolk sac and proximal tubule cells. In the former, they are localized from the time of their initial expression in clathrin-coated areas of the membrane, whereas in the latter they undergo a complex maturation process to achieve their adult expression in coated pits.

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