Origin and Deposition of Basement Membrane Heparan Sulfate Proteoglycan in the Developing Intestine

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Abstract. The deposition of intestinal heparan sulfate proteoglycan (HSPG) at the epithelial–mesenchymal interface and its cellular source have been studied by immunocytochemistry at various developmental stages and in rat/chick interspecies hybrid intestines. Polyclonal heparan sulfate antibodies were produced by immunizing rabbits with HSPG purified from the Engelbreth-Holm-Swarm mouse tumor; these antibodies stained rat intestinal basement membranes. A monoclonal antibody (mAb $4C_1$) produced against lens capsule of 11-d-old chick embryo reacted with embryonic or adult chick basement membranes, but did not stain that of rat tissues. Immunoprecipitation experiments indicated that mAb $4C_1$ recognized the chicken basement membrane HSPG.

Immunofluorescent staining with these antibodies allowed us to demonstrate that distribution of HSPG at the epithelial–mesenchymal interface varied with the stages of intestinal development, suggesting that remodeling of this proteoglycan is essential for regulating cell behavior during morphogenesis. The immunofluorescence pattern obtained with the two species-specific HSPG antibodies in rat/chick epithelial/mesenchymal hybrid intestines developed as grafts (into the coelomic cavity of chick embryos or under the kidney capsule of adult mice) led to the conclusion that HSPG molecules located in the basement membrane of the developing intestine were produced exclusively by the epithelial cells.

These data emphasize the notion already gained from previous studies, in which type IV collagen has been shown to be produced by mesenchymal cells (Simon-Assmann, P., F. Bouziges, C. Arnold, K. Haffen, and M. Kedinger. 1988. *Development (Camb.).* 102:339–347), that epithelial–mesenchymal interactions play an important role in the formation of a complete basement membrane.

EPITHELIAL–STROMAL tissue interactions are a prerequisite for cytodifferentiation of intestinal epithelial cells during ontogenesis (21) and in the adult organ (12). Substantial evidence supports the view that extracellular matrix components and, in particular, basement membrane molecules are involved in such cell interactions in various organs (for review see 32). Basement membrane is a thin sheet of extracellular matrix that forms a boundary between connective tissue and epithelial, endothelial, muscle, and fat cells. Identification of the macromolecular components of basement membranes have been severely hampered by the extreme insolubility of these structures. To date heparan sulfate proteoglycan (HSPG)[1] (16), laminin (40), nidogen-entactin (7, 9, 42), type IV collagen (41), and, more recently, BM 40 (10) have been identified as integral components of basement membranes. In the intestine, a descriptive study has shown that compositional changes of some extracellular matrix molecules are temporarily related to intestinal morphogenesis and differentiation (35); indeed, it was reported that during development of the intestine, the interstitial collagens and fibronectin are distributed in a heterogeneous manner, related to morphogenetic events, while basement membrane constituents (laminin, nidogen, and type IV collagen) are always evenly distributed along the crypt–villus axis at the epithelial–mesenchymal junction like in the adult (14).

In vitro studies have shown that single matrix molecules do not allow survival or elicit terminal differentiation of intestinal epithelial cells, processes which are only triggered by viable mesenchymal or fibroblastic cells (23). Related to this, the formation of a true basement membrane required the presence of both epithelial and fibroblastic cells (13, 24, 36). One could demonstrate that mesenchymal components are involved in the elaboration of an adequate extracellular matrix. In particular, basement membrane type IV collagen has been shown to be produced by the mesenchymal cells. This cell population also plays a predominant role in the modifications of the glycosaminoglycan synthesis pattern, which occur in epithelial–fibroblastic cocultures in parallel to epithelial cell differentiation (4).

1. *Abbreviations used in this paper*: Cm/Re, chick mesenchyme/rat endoderm; EHS, Engelbreth-Holm-Swarm; HSPG, heparan sulfate proteoglycan; Rm/Ce, rat mesenchyme/chick endoderm.

In the present study, we have been investigating HSPG molecules located in the basement membrane that separates epithelial cells from the closely associated mesenchyme; these molecules appear to be implicated in the development and differentiation processes as well as with alterations found in cancer (for reviews see 2, 11, 18). Possible remodeling processes in the basement membrane HSPG molecules were examined during intestinal development. Furthermore, interspecies combinations of rat and chick tissue anlagen were used as a model to trace the cellular source of HSPG molecules in the intestinal basement membrane with species-specific antibodies.

Materials and Methods

Animals

Fetuses from pregnant Wistar rats bred in our laboratory, whose gestation had been accurately timed, were removed by cesarean section at various stages between the 14th d of gestation and birth. The day on which a vaginal plug was found was designated as day 0, and the developmental stages of the fetal rats were determined according to the number of days of gestation.

White Leghorn chick embryos were used. The chicken eggs were incubated at 38 ± 1°C, and the developmental stages were referred to as days of incubation.

Production of Antibodies

Polyclonal Antibodies. HSPG was purified from Engelbreth-Holm-Swarm (EHS) mouse sarcoma as described previously (16). This procedure led to the purification of three types of HSPG: a high density proteoglycan that is extractable in saline buffer and high and low density proteoglycans that are extractable in urea. The low density proteoglycan probably corresponds to the native proteoglycan in the basal lamina (16). The results of SDS-PAGE before and after treatment of the low density proteoglycan with heparitinase were identical to those reported by Hassell et al. (16). The protein core has been identified as a 400-kD polypeptide, and no laminin or other contaminating components were detected (data not shown).

Antibodies against the low density form of HSPG and against laminin (purified from EHS tumor as previously described [40]) were prepared in rabbits. The specificities of the antibodies were tested either by ELISA or Western blot. Antibodies against HSPG were purified from the serum by cross-immunoabsorption on a laminin affinity column to remove laminin-reacting antibodies.

Monoclonal Antibodies. Mice were immunized with lens capsule from 11-d-old chick embryos. In brief, the lens capsules were washed three times in distilled water containing 1% Triton X-100 and then crushed in liquid nitrogen. For the first immunization, the lens capsule powder (100–200 μg) was emulsified in a 50:50 mixture of water and Freund's adjuvant and injected subcutaneously in the foot pad. The animals were similarly boosted 3 wk later and rested for an additional 3 wk. 3 d before being used for a hybridoma fusion, they received an intraperitoneal injection of antigen without Freund's adjuvant.

The immune spleen cells were fused with NS1 myeloma cells following methods previously described (25). The resulting hybridoma cultures were screened for production of antibodies using indirect immunofluorescent microscopy on frozen sections of eyes taken from 6- or 11-d chick embryos (see below). Hybridomas were cloned by limiting dilution.

The selected hybridomas were propagated as ascite tumors in BALB/c mice. Monoclonal antibodies were purified from pooled ascitic fluids by chromatography on protein A–Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden).

Purified mAb 33, identified as anti-chicken HSPG (1) was a generous gift of Dr. D. Fambrough (Carnegie Institute of Washington, Baltimore, MD).

Characterization of the Monoclonal Antibodies

Culture of Chicken Myotubes and Labeling Conditions. Chicken myotubes were obtained from 11-d-old chick embryos as described by Vallette et al. (43). They differentiate in a medium composed of 3:1 MEM/medium 199 (Eurobio, Paris, France) with 10% horse serum (Gibco Laboratories, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Differentiated myotubes (after 7–8 d in culture) were labeled with 100 μCi/ml [^{35}S]methionine (1,475 Ci/mmole; Amersham Corp., Arlington Heights, IL) in methionine-free MEM supplemented with 2% FCS for 40 h. After exposure, the labeled medium was removed and centrifuged for 20 min at 20,000 *g* to remove cellular debris. The myotubes were scraped with a rubber policeman and homogenized in a potter glass teflon homogenizer in the following extraction buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.02% SDS, and 0.5% Trasylol. The homogenate was then centrifuged at 20,000 *g* for 30 min at 4°C. The supernatant fraction containing the soluble, labeled proteins constituted the cell extract.

Heparitinase Treatment. Heparitinase was obtained from Miles Laboratories Inc. (Elkhart, IN). The labeled medium was incubated with 10 μg/ml of heparitinase (910 U/mg) in PBS containing 3 mM of Ca_2CO_3 for 1 h at 37°C. In the same conditions, heparitinase treatment of the low density proteoglycan purified from the EHS tumor released a protein of ~400 kD, which corresponds to the protein core of the proteoglycan as previously described by Hassell et al. (16) (data not shown).

Immunoprecipitation. The procedure used for immunoprecipitation of cell lysates and media was adapted from that of Bumol and Reisfeld (5) and Rotundo (31). Protein A–Sepharose was allowed to swell in saline buffered with 10 mM Tris-HCl, pH 7.4, containing 0.5% Triton X-100 (buffer A). To reduce background, the cell extracts or media were preabsorbed as follows: 50 μl of protein A–Sepharose was incubated with 20 μl of nonimmune serum with constant agitation at room temperature for 1 h. The IgG–protein A complex was washed three times with 1 ml of buffer A. The cell lysate or the medium was then incubated with the IgG–protein A complex with constant agitation for 1 h at 25°C. The absorbed lysates or media were recovered after removal of the IgG–protein A–Sepharose complex by centrifugation. The complex was discarded. This preabsorption procedure was repeated using another batch of nonimmune serum.

The immunoprecipitation per se was then performed. 50 μl of protein A–Sepharose was incubated with 20 μl of immune serum, affinity-purified antibodies against laminin (40 μg), or purified monoclonal antibodies against HSPG (40 μg) for 1 h with agitation at 4°C. Nonimmune serum was used as control. The IgG–protein A complex was washed as described above and then incubated with either absorbed lysate or medium at 4°C for 1 h. The resulting antigen–antibody protein A–Sepharose complex was recovered by centrifugation (30 s at 200 *g*). The complex was washed twice with 1 ml of extraction buffer; twice with 1 ml of buffer A plus 1 M NaCl; twice with 1 ml of buffer A plus 0.02% SDS, 0.3 M NaCl; and twice with buffer A. The resulting complex was denatured at 100°C for 3 min in 62 mM Tris, pH 6.8, 2.5% SDS, 10% glycerol, 65 mM DTT and was then electrophoresed on a 7% polyacrylamide gel according to the method of Laemmli (27). After electrophoresis the gels were fluorographed with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and exposed to RP/2 Royal X-omat film (Eastman Kodak Co., Rochester, NY) at −70°C for varying periods of time.

Immunocytochemistry. Antibodies were tested (as described below) on the following tissues: whole eye or intact anterior eye segments from chick embryos or from post hatched chicks (up to 3 wk), skeletal anterior latissimus dorsi from 3-wk-old chickens, and soleus or gastrocnemus from 2-mo-old rats.

Interspecies Intestinal Recombinants

Associations between rat and chick intestinal tissue components have been performed using an experimental procedure described previously (20). Briefly, 5-d chick embryonic and 14–15-d fetal rat intestinal anlagen were removed. The mesenchyme was separated from the endoderm after incubation of the intestinal segments in an 0.03% solution of collagenase (1 h at 37°C). Two types of interspecies recombinations of the isolated endodermal and mesenchymal components were performed: chick mesenchyme/rat endoderm (Cm/Re) and rat mesenchyme/chick endoderm (Rm/Ce). After overnight culture on agar-solidified medium to ensure their cohesion, the associations were grafted either into the coelomic cavity of 3-d chick embryos or under the kidney capsule of adult nude mice (nu/nu Swiss mice). The developed intestinal segments were harvested 10–14 d later.

Immunofluorescent Staining of HSPG on Intestinal Tissue

Intestinal segments at different developmental stages and interspecies recombinants were processed similarly. They were embedded in Tissue-Tek

compound (Miles Laboratories Inc.), frozen in Freon cooled in liquid nitrogen, and stored at −70°C until use. Transverse sections (5–6 μm thick) realized at −25°C were placed on gelatin-coated slides.

Cryostat sections were then incubated for 30 min at room temperature with the antibodies diluted in PBS in a moist chamber. The anti-mouse HSPG antibody (from the mouse EHS tumor) was used at 1:500 dilution, and the anti-chick HSPG antibody (mAb $4C_1$) was used at 1:20 dilution. Slides were rinsed with PBS and washed in two changes of PBS for 5 min each. Sections were then incubated either with FITC-conjugated goat anti-rabbit γ globulin (1:20 in PBS; Nordic Immunological Laboratories, Tilburg, The Netherlands) or sheep anti-mouse IgG antibodies (1:200; Institut Pasteur, Paris, France). Slides were washed, mounted in glycerol/PBS/phenylenediamine under a coverslip, observed under a microscope (Ortoplan; E. Leitz, Inc., Wetzlar, FRG), and photographed using HP5 film (ASA 400; Ilford Ltd., Basidon, Essex, England).

Control sections were processed as above, but first affinity-purified antibodies were omitted; these controls did not show any fluorescence.

Results

Production and Characterization of Antibodies

Antibodies against the low density form of HSPG purified from the EHS tumor were prepared in rabbits. These antibodies did not react with laminin, type IV collagen, or nidogen-entactin purified from the same tumor. They intensively stained mouse eye basement membranes (15, 19, 44).

Monoclonal antibodies against chick lens capsule have been produced. In three independent fusions, ~400 growing hybridoma cultures were obtained. 24 hybridomas were selected on the basis of their production of antibodies reacting with the lens capsule of 11-d embryos. 14 monoclonal antibodies stained all the basement membranes tested, whereas the others revealed a heterogeneity of staining for the different basement membranes (data not shown). Our studies here are directed only to the monoclonal named mAb $4C_1$. This antibody reacted with all the embryonic or adult chick basement membranes tested; in contrast, it did not stain that of rat tissues (Fig. 1).

Metabolic labeling of chicken myotubes with [^{35}S]methionine and isolation of the neosynthesized antigen(s) by immunoprecipitation followed by SDS-PAGE have been performed. In the myotube culture medium there was a protein of 250–300 kD that firmly bound to any protein A–IgG complex (Fig. 2 *a*, lane *a*). This component is a major secreted molecule, and two preabsorption steps did not completely deplete the labeled medium from this unidentified material (see additional comments in the legend of Fig. 2). mAb $4C_1$ immunoprecipitated a material that did not migrate into the gel (Fig. 2 *a*, lane *e*) like purified mAb 33, identified as anti-chicken HSPG, did (Fig. 2 *a*, lane *d*). After heparitinase treatment, the immunoprecipitated material that did not migrate into the gel (Fig. 2 *b*, lane *b*) was converted into a protein of ~400 kD (Fig. 2 *b*, lane *a*)—i.e., the molecular mass of the protein core expected for the basement membrane HSPG.

In immunoprecipitation experiments performed with the labeled cell lysate, mAb $4C_1$ precipitated the 400-kD protein core, which corresponds to the cellular form of the HSPG molecules in their process of biosynthesis (data not shown). However, one should notice that, in this case, several additional proteins were nonspecifically immunoprecipitated and could not be completely removed from the labeled cell lysate by the two preabsorption steps.

Immunoprecipitation with polyclonal antibodies against the low density form of HSPG purified from the EHS tumor gave rise to a similar electrophoretic pattern (Fig. 2 *a*, lane *c*). It is noteworthy that the intensity—i.e., the quantity of the immunoprecipitated material—was lower using the polyclonal antibodies than the two monoclonal antibodies. This fact probably reflects a weak cross-reactivity of the polyclonal antibodies vs. the chicken HSPG. The material immunoprecipitated by the HSPG antibodies is clearly different from that precipitated by the anti-laminin antibodies. Immunoprecipitation performed with affinity-purified anti-lami-

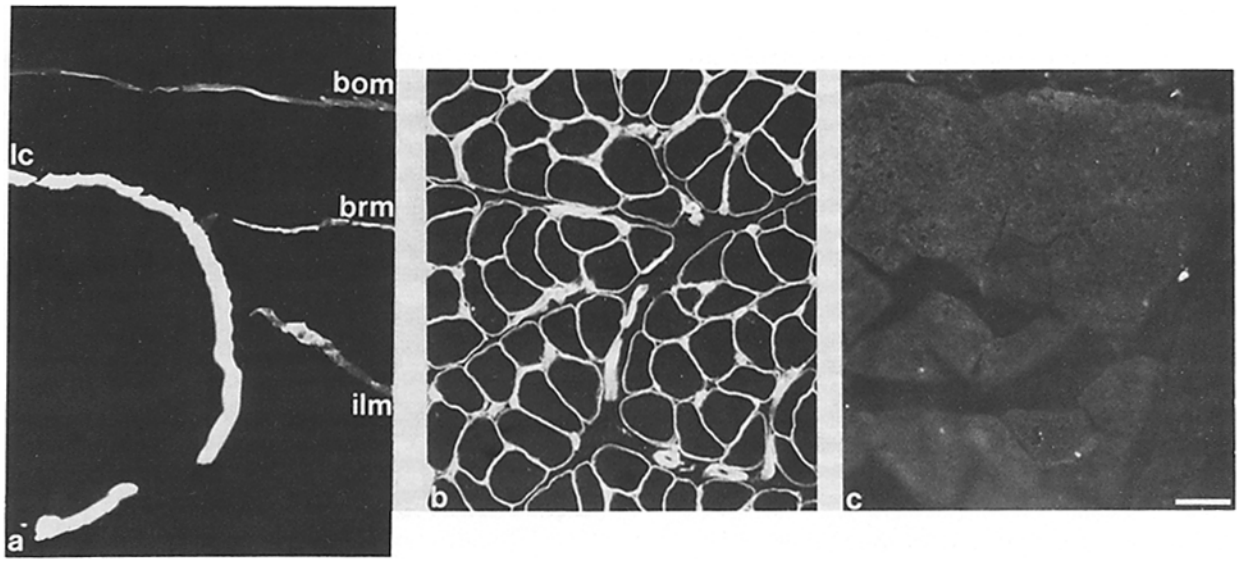

Figure 1. Immunofluorescence staining using mAb $4C_1$. (*a*) Indirect immunofluorescence micrograph illustrating the staining pattern in sections of the eye basement membranes of 6-d-old chick embryo: *brm*, Brusch's membrane; *bom*, Bowman's membrane; *ilm*, inner limiting membrane; and *lc*, lens capsule. (*b*) Immunofluorescence staining of sections of adult chicken anterior latissimus dorsi. (*c*) Immunofluorescence staining of sections of adult rat soleus. Rat muscle basement membrane was not stained. Bar, 50 μm.

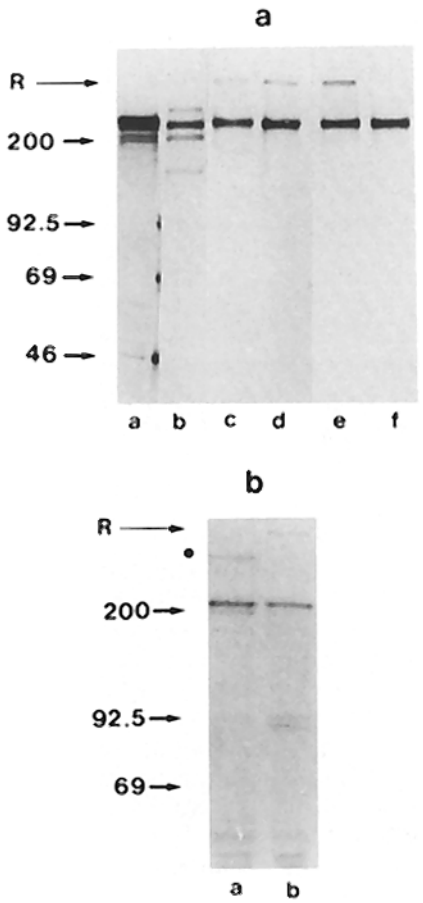

Figure 2. (*a*) Electrophoretic analysis of [^{35}S]methionine-labeled proteins secreted by 11-d-old chicken myotubes. Medium was preabsorbed twice with protein A–Sepharose previously incubated with two different nonimmune sera. Then the proteins were immunoprecipitated with the different antibodies. Immunoprecipitates were electrophoresed on a 7% SDS–polyacrylamide gel. (Lane *a*) Material precipitated by nonimmune serum (second preabsorption step); (lane *b*) material immunoprecipitated by polyclonal antibodies against laminin; (lanes *c–e*) material immunoprecipitated, respectively, by polyclonal antibodies against EHS tumor HSPG, mAb 33, and mAb 4C$_1$; and (lane *f*) no specific protein was immunoprecipitated using another monoclonal antibody (mAb 4G$_3$) produced against lens capsule. (*b*) Heparitinase treatment: (lane *a*) heparitinase-treated medium immunoprecipitated by mAb 4C$_1$ and (lane *b*) medium incubated in the same conditions but without heparitinase and immunoprecipitated by mAb 4C$_1$. (•) The heparitinase treatment released a 400-kD protein. ^{14}C-myosin (200 kD), -phosphorylase b (92.5 kD), -albumin (69 kD), and -ovalbumin (46 kD) (New England Nuclear) were used as globular molecular mass standards (*arrows*). (*R* →) Top of the running gel. Note the presence in all the immunoprecipitates of a major 250–300-kD secreted protein. Because of its strong affinity for the protein A–IgG complex, this protein could correspond to fibronectin. However, under nonreducing conditions this material was resolved in two bands, one with the same molecular mass (250 kD) and another with a higher molecular mass (~450–500 kD) corresponding presumably to nonreduced fibronectin. The intensity of the two bands was similar (data not shown). Furthermore, chromatography of the labeled medium on a gelatin-Sepharose column only partially depleted the medium from the unidentified material. These results suggest that the contaminating band can partially be attributed to fibronectin.

nin antibodies precipitated three polypeptides of 400, 200, and 150 kD corresponding, respectively, to the A and B chains of laminin and to the nidogen 150-kD entactin known to be precipitated with laminin antibodies (28) (Fig. 2 *a*, lane *b*). Anti–entactin antibodies immunoprecipitated the same polypeptides (data not shown).

Immunolocalization of HSPG in Rat Intestine

The polyclonal anti–HSPG antibodies were first used to examine the distribution of HSPG in the mature rat intestine and during its morphogenesis.

Adult Organ. In the adult rat intestine, immunostaining with the anti–HSPG antibodies was found in the basement membrane lining the epithelium (Fig. 3 *a*); HSPG was present all over the crypt–villus axis, although the staining was more uniform and linear at the base of the villi and around the crypts (Fig. 3, *b* and *c*) than at the upper part of the villi (Fig. 3 *d*). The epithelial cells were completely negative. In the lamina propria, the basement membrane of blood vessels, lymph vessels, and smooth muscle cells were decorated (Fig. 3, *a* and *d*). The submucosa region was almost devoid of labeling in contrast to the muscularis mucosae, which exhibited a bright staining; in the longitudinal and circular muscular layers, the antigen delineated well-defined rings around each cell (Fig. 3, *a* and *e*).

Developing Organ. At 14 d of gestation, before the onset of villus morphogenesis, anti–HSPG antibody revealed a strong labeling at the basement membrane zone. In addition, some immunostaining was seen around cells scattered over the whole thickness of the mesenchyme (Fig. 4, *a* and *b*).

At 18 d of gestation (Fig. 4 *c*), the stage at which villus primordia had developed, label was present and continuous at the basement membrane zone along the entire villi. HSPG was associated with some cellular and fibrillar structures within the lamina propria. In addition, the peripheral zone of the mesenchyme, which could be at this stage clearly identified as being the muscular layers, reacted with anti–HSPG antibody.

As villi elongated until adult stage (Fig. 4, *d–f*), the labeling still present along the whole crypt–villus axis became less regular and less continuous mainly in the upper part of the villi, while staining intensity remained unchanged. This phenomenon was particularly visible during the perinatal period (Fig. 4, *e* and *f*). It should be noted that the epithelial cells of postnatal intestines often revealed a greenish background.

Cellular Origin of the Intestinal Basement Membrane HSPG

The cellular origin of the HSPG located at the basement membrane has been analyzed by means of interspecies tissue recombinations.

Screening of the HSPG Antibodies on Chick and Rat Intestinal Tissue. The monoclonal antibody raised against the HSPG of the chick species (mAb 4C$_1$) has been checked for its species specificity on intestinal tissue cryosections. This antibody (1:20 dilution) applied to a 13-d embryonic chick intestine clearly delineated the basement membrane underneath the epithelium; it also labeled—but to a lesser extent—some cellular elements within the lamina propria as well as

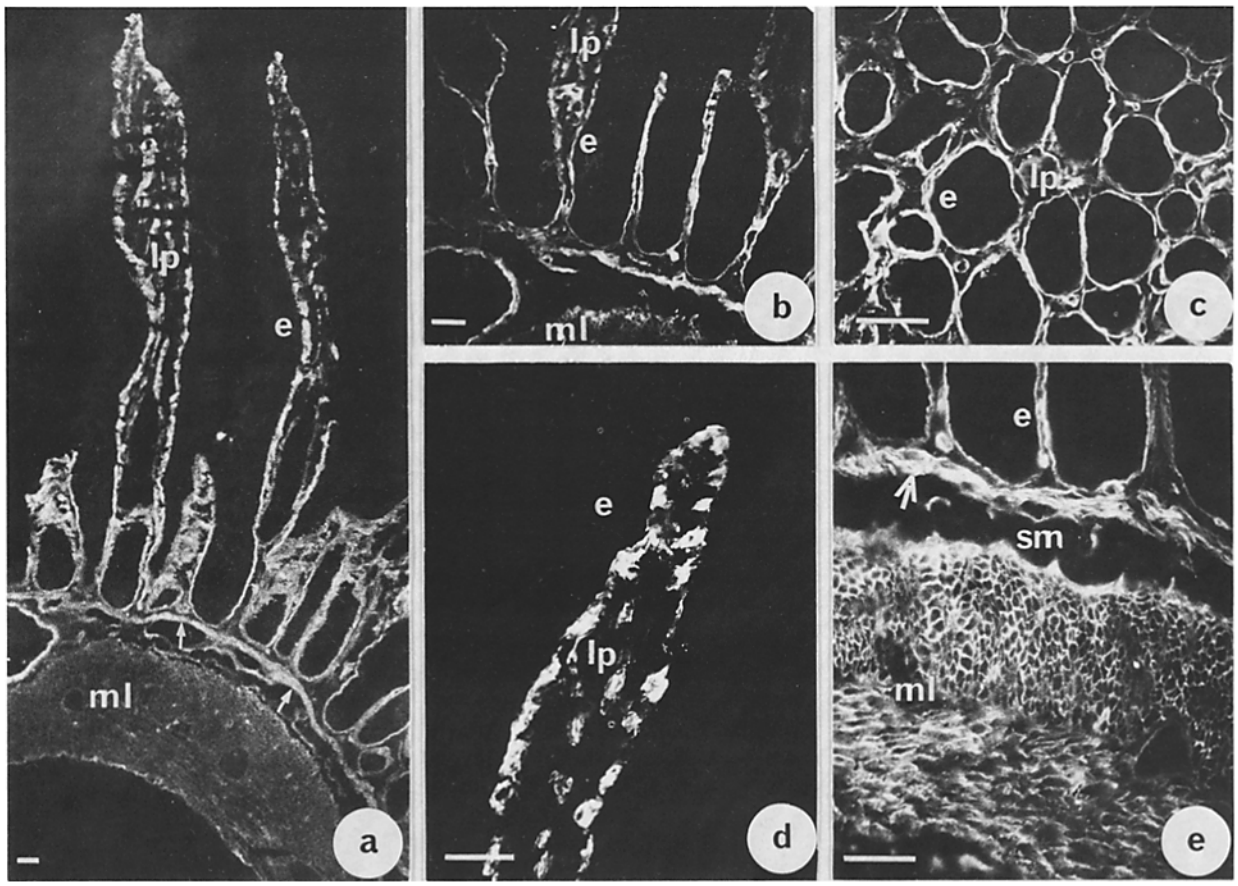

Figure 3. Representative indirect immunofluorescence micrographs of HSPG molecules using polyclonal antibodies in sections of the whole rat intestinal wall (*a*); base of the villi and crypt zone (*b* and *c*); upper part of the villus (*d*); and muscular layers (*e*). (*c*) Transverse sections across *b*. *e*, epithelium; *ml*, muscular layers; *lp*, lamina propria; and *sm*, submucosa. The arrows point to the muscularis mucosae. Bar, 30 μm.

the muscular layers (Fig. 5 *a*). This antibody used at the same dilution did not stain sections of rat tissue (Fig. 5 *b*). The polyclonal antibody raised against the HSPG of the mouse species used efficiently at the 1:500 dilution on rat intestine (Fig. 3) did not cross react with chick intestine (Fig. 5 *c*). It has to be noted that, when applied at 1:50 dilution, a very faint staining occurred on chick intestinal segments accompanied by a greenish background (not illustrated).

Interspecies Hybrid Intestines. Previous data have shown that interspecies tissue recombinants, grown as grafts in the chick embryo or as intrarenal grafts, develop into vascularized intestinal structures (20, 22); the endoderm gives rise to the epithelium, while the mesenchyme forms the lamina propria, the muscularis mucosae, as well as the muscular layers.

The results of the immunolocalization of HSPG in such hybrid intestines using the species-specific antibodies are summarized in Table I. In Cm/Re recombinants, developed inside the coelomic cavity of chick embryos, the polyclonal anti-mouse HSPG antibodies strongly underlined the subepithelial basement membrane; some scattered punctuated fluorescence was found in the near underlying lamina propria (Fig. 6 *a*). The same type of associations grafted under the kidney capsule of nude mice revealed a closely similar staining with the anti-mouse HSPG antibodies at the basal surface of the epithelial cells (Fig. 6, *c* and *e*). The basement membranes of the blood vessels, which have invaded the hybrid intestines developed in the mouse host, were also clearly delineated with the anti-mouse HSPG antibodies. The muscular layers were always devoid of staining.

Monoclonal anti-chick HSPG antibodies (mAb $4C_1$) applied to Cm/Re recombinants revealed no basement membrane staining whatever the grafting conditions, while the muscular layers as well as the lamina propria were evenly stained (Fig. 6, *b*, *d*, and *f*); blood vessels were labeled only in the associations grafted in chick hosts.

In Rm/Ce hybrid intestines, developed in the chick embryo or in the adult mouse host, anti-mouse HSPG antibodies stained obviously the lamina propria and the muscular layers (Fig. 7, *a* and *d*). However, basement membrane of Rm/Ce recombinants was stained only with anti-chick antibodies (Fig. 7, *b* and *e*). HSPG immunoreactivity was also observed around the invading blood vessels of the associations grafted into the chick embryo with anti-chick antibodies (Fig. 7, *b* and *c*) and vice versa of associations grafted in the mouse host with anti-mouse antibodies (Fig. 7 *d*).

Discussion

In this paper, we first describe the production of two antibod-

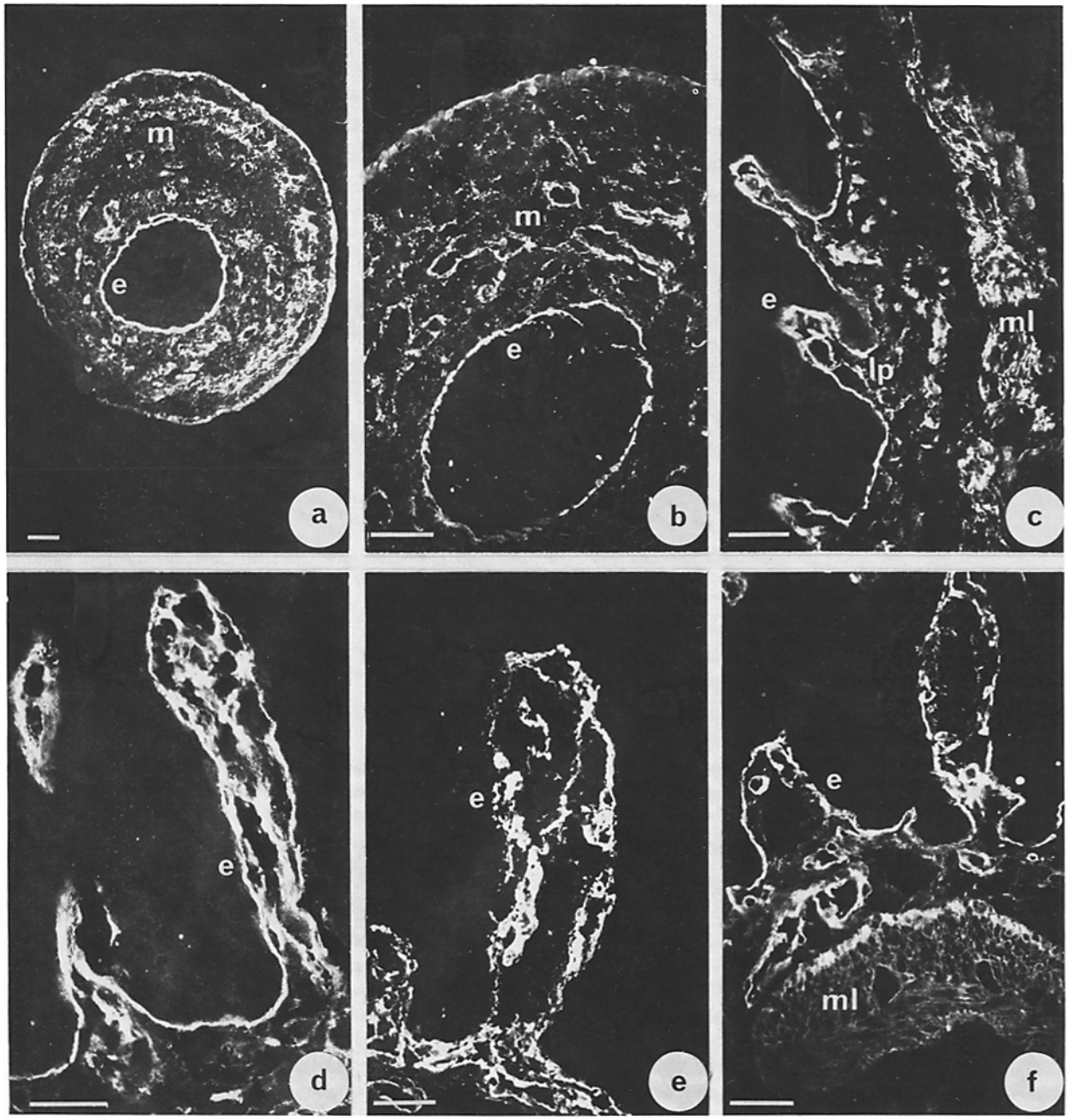

Figure 4. Representative indirect immunofluorescence micrographs of HSPG molecules using polyclonal antibodies in transverse sections of rat intestine at various developmental stages: 15- (*a* and *b*), 17- (*c*), and 18-d (*d*) fetal intestines or of intestines at birth (*e*) and 4 d after birth (*f*). *e*, endoderm or epithelium; *m*, mesenchyme; and *ml*, muscular layers. Bar, 30 μm.

ies specific for rat and chick HSPGs, respectively. In the second part of the work, we have examined the immunolocalization of this extracellular matrix component as a function of rat intestinal morphogenesis as well as in experimental conditions allowing to explore the cellular source of HSPG of the basement membrane. These latter experiments involve the use of hybrid intestinal segments made up by enzymatically dissociated endoderm and mesenchyme and allow us to conclude that the HSPG at the intestinal epithelial–mesenchymal interface has its origin in the epithelial cells.

Concerning the specificity of our antibodies, the polyclonal antibody against mouse EHS tumor HSPG was found to react specifically with HSPG (15, 19, 44), but did not react with laminin, nidogen-entactin, or type IV collagen purified from the same tumor. When applied at a 1:500 or 1:1,000 dilution it clearly delineated the basement membranes of the intestine from the rat species, but did not stain chick basement membranes. In contrast, at higher dilutions (1:50), this antibody revealed a very faint staining of chick basement membranes. Although our antibody exhibits a weak cross-reaction with the chicken HSPG, confirmed by the immunoprecipitation data, it can be considered as species specific

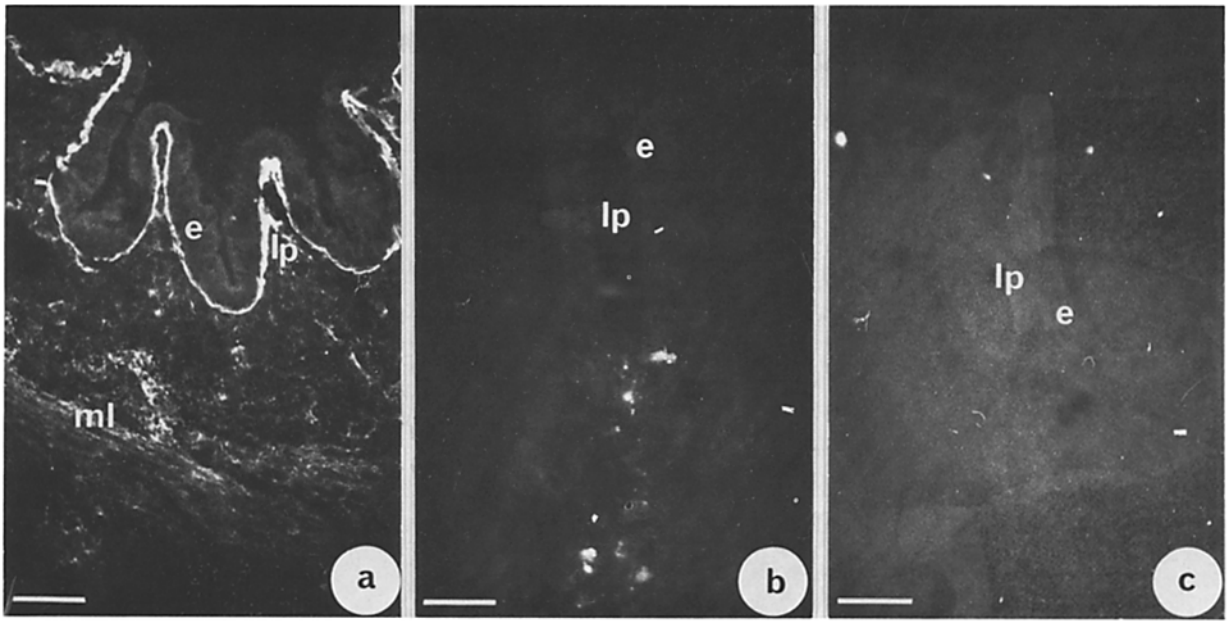

Figure 5. Immunofluorescence pattern of HSPG molecules in (*a*) 13-d chick embryonic intestine stained with anti-chick $4C_1$ antibodies; (*b*) adult rat intestine incubated with anti-chick $4C_1$ antibodies; and (*c*) 13-d chick embryonic intestine incubated with polyclonal anti-mouse antibodies against HSPG purified from the EHS tumor. *e*, epithelium; *lp*, lamina propria; and *ml*, muscular layers. Some unspecific yellowish fluorescence is found within the lamina propria of the rat intestine in *b*. Bar, 30 μm.

when used at a 1:500 dilution. mAb $4C_1$ did not stain any rat or human (data not shown) basement membranes, indicating that the corresponding epitope can be only detected in chicken basement membranes. This antibody has been characterized as anti-HSPG and gives identical immunoprecipitation data as the mAb 33 (anti-chicken HSPG, a gift of Dr. D. Fambrough; reference 1).

Examination of the immunolocalization of the HSPG molecules during intestinal development in the rat by using the polyclonal antibodies revealed that the antigens were found throughout life in the basement membrane lining the epithelium: at early stages, when the epithelium is still stratified and undifferentiated, as well as when the epithelium is restricted to a single layer of cells. Moreover, immunostaining was obvious around cellular elements present within the embryonic mesenchyme and later on within the lamina propria, in the basement membrane of blood and lymph vessels and of smooth muscle cells. When muscular layers are well differentiated, the antigen also delineated well-defined rings around each cell.

Table I. Comparative Localization of HSPG Immunostaining in Interspecies Recombinants by Species-specific Antibodies

Hybrid intestines	Host	Antibodies	Basement membrane	Lamina propria (cellular elements)	Muscular layers	Blood vessels
Cm/Re	Chick	Anti-HSPG*	+	−§	−	−
		mAb $4C_1$‡	−	+	+	+
	Mouse	Anti-HSPG	+	−	−	+
		mAb $4C_1$	−	+	+	−
Rm/Ce	Chick	Anti-HSPG	−	+	+	−
		mAb $4C_1$	+	−	−	+
	Mouse	Anti-HSPG	−	+	+	+
		mAb $4C_1$	+	−	−	−

* Polyclonal antibodies against HSPG purified from the mouse EHS tumor.
‡ Monoclonal antibodies against chicken HSPG.
§ Punctuated fluorescent deposition within the lamina propria can, however, be observed.

The overall distribution of HSPG is similar to that described previously for other basement membrane components, such as laminin, nidogen, and type IV collagen (35). However, contrasting with the regular deposition of the latter components at all stages of development, changes in the staining pattern of HSPG are observed during intestinal morphogenesis. Indeed, in the developing intestine around birth, the labeling of the basement membrane became discontinuous and irregular, a phenomenon particularly obvious from the middle towards the tip of the villi. These data can be interpreted as focal disruptions in the basement membrane that could be correlated to the histological observation of gaps or fenestrations in the basal lamina during the perinatal period, allowing epithelial–mesenchymal cell contacts at strategic phases of intestinal development (6, 29) and towards the apex of the villi in the adult intestine (26). Similar transient microheterogeneities in the deposition of extracellular matrix molecules have been described in other organs undergoing morphogenetic movements (for review see 3, 39). They suggest that basal lamina remodeling is involved in the regulation of cell behavior during morphogenesis.

The discontinuous and irregular deposition of HSPG molecules at the basement membrane level could also result from variations in the turnover of these molecules, affecting their biosynthesis and/or degradation. Indeed, in various organs, expression of HSPG was lost as the epithelial cells ap-

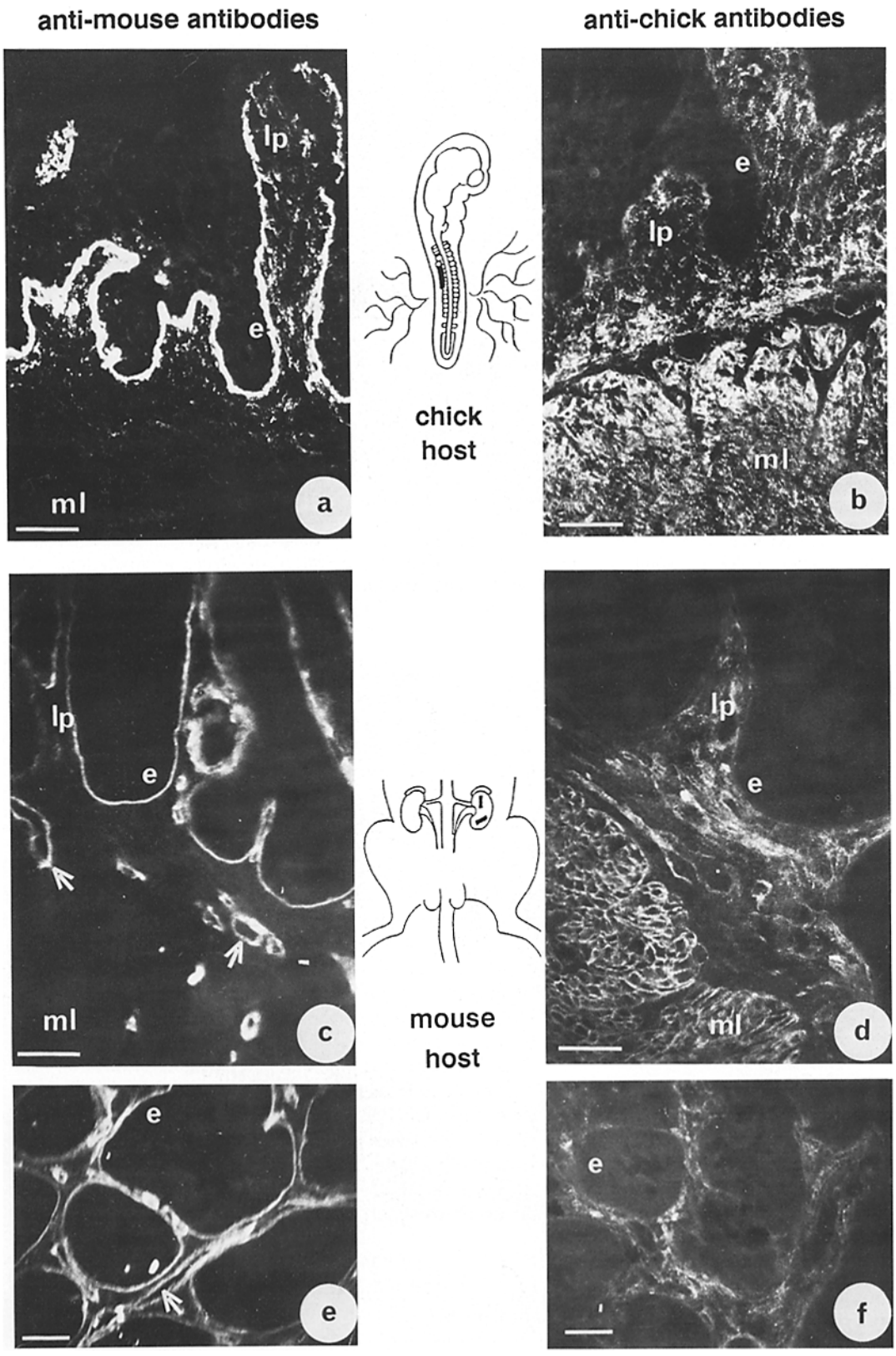

Figure 6. Immunodetection of HSPG molecules with anti-mouse (*a*, *c* and *e*) and anti-chick (*b*, *d*, and *f*) antibodies on Cm/Re hybrid intestines developed in the coelomic cavity of chick embryos (*a* and *b*) or under the kidney capsule of nude mice (*c–f*). *e* and *f* are sections across the base of the villi of hybrid intestines depicted, respectively, in *c* and *d*. *e*, epithelium; *lp*, lamina propria; and *ml*, muscular layers. The arrows show invading vessels of the mouse host revealed by the anti-mouse antibodies. Bar, 30 μm.

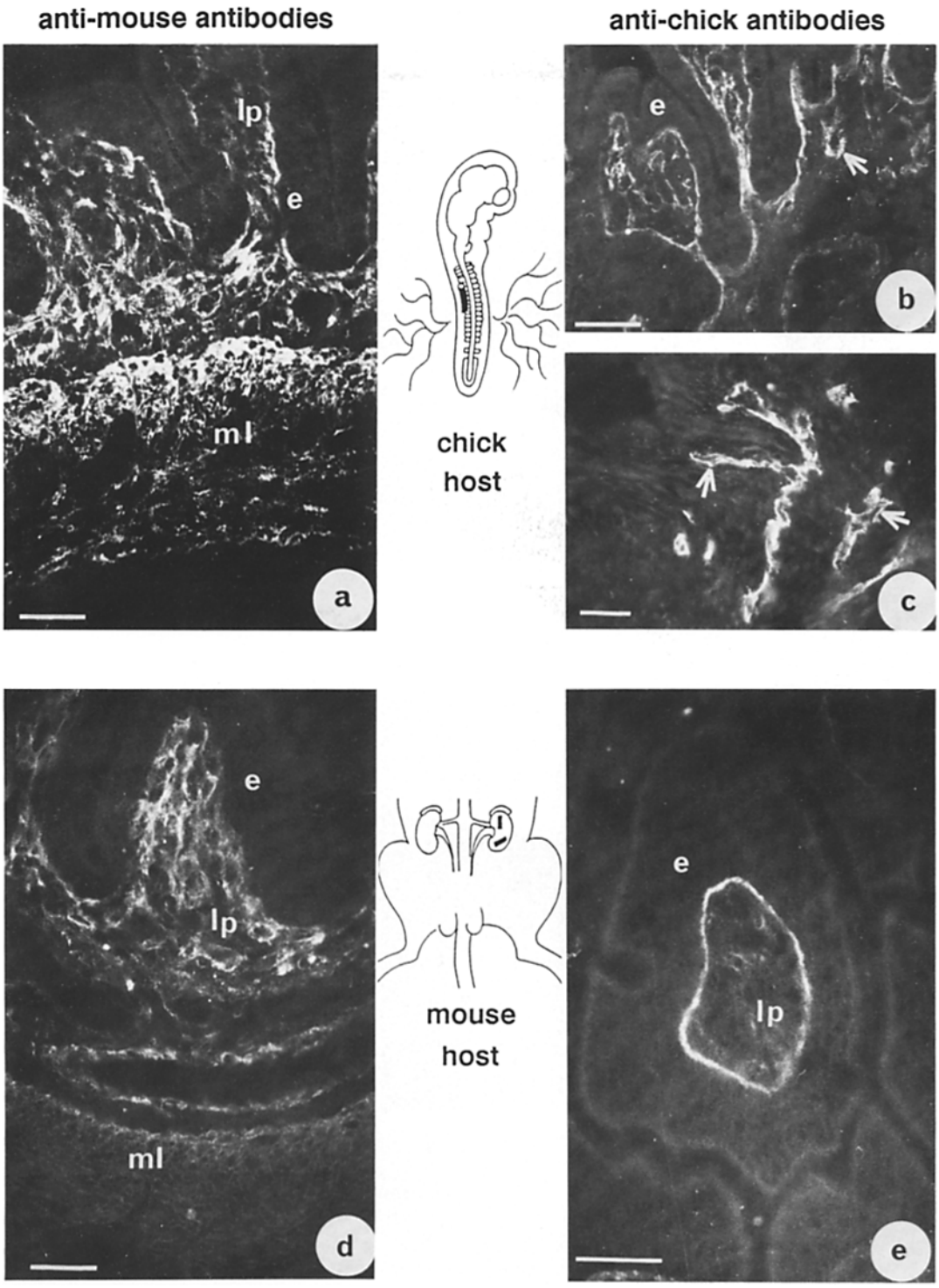

Figure 7. Immunodetection of HSPG molecules with anti-mouse (*a* and *d*) and anti-chick (*b*, *c*, and *e*) antibodies on Rm/Ce hybrid intestines developed in the coelomic cavity of chick embryos (*a–c*) or under the kidney capsule of nude mice (*d* and *e*). (*c*) Detail of the blood vessels of the chick embryo host invading the muscular layers of the hybrid intestine. (*e*) Transverse sections across a villus. *e*, epithelium; *lp*, lamina propria; and *ml*, muscular layers. Arrows show invading vessels. Bar, 30 μm.

proached terminal differentiation (17, 38). In the mature intestine, the undifferentiated dividing crypt cells migrate and differentiate towards the top of the villi. One could postulate that HSPG is preferentially synthesized by the undifferentiated crypt cells, the discontinuous labeling of the upper part of the villi from birth onwards being in this case linked to temporal changes in the degradation rate of these molecules. Furthermore, the difference between the transient fragmented deposition of HSPG and the continued and regular one of the other basement membrane components strengthen the concept of variations in turnover among the basement membrane components; such differences in the accumulation and deg-

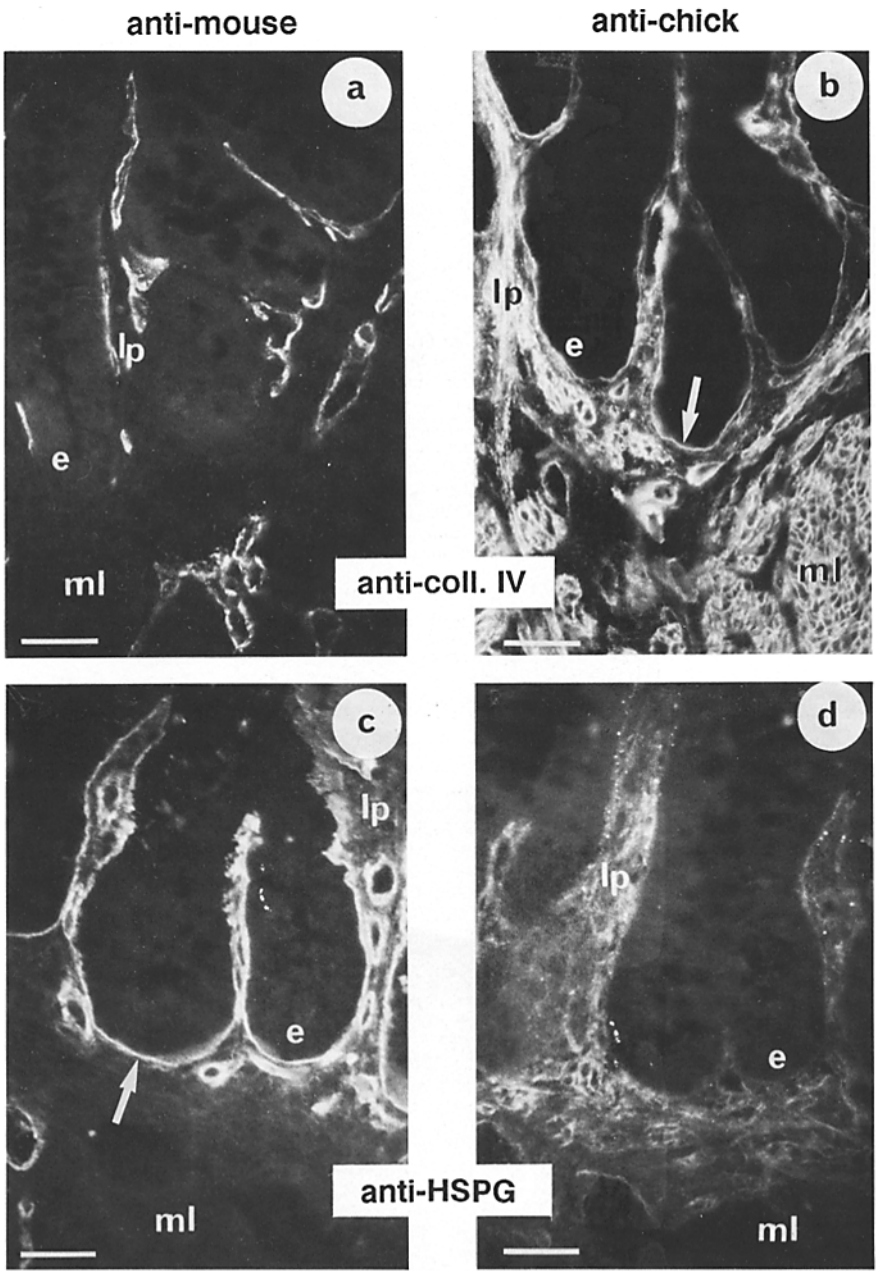

Figure 8. Experiments showing the dual epithelial–mesenchymal origin of the intestinal basement membrane. Consecutive cryosections of a Cm/Re hybrid intestine developed under the kidney capsule of a nude mouse were stained in parallel with species-specific antibodies recognizing rodent (*a*) and chick (*b*) type IV collagen as described previously (36) or rodent (*c*) and chick (*d*) HSPG. *e*, epithelium; *lp*, lamina propria; and *ml*, muscular layers. Arrows show basement membrane labeled with anti-chick antibodies in the case of type IV collagen (*b*) and with anti-rodent antibodies in the case of HSPG (*c*), indicating, respectively, their mesenchymal and epithelial origin. Bar, 30 μm.

radation steady state between extracellular matrix molecules have been already postulated (30).

In a consecutive step, we have analyzed the cellular origin of HSPG in the basement membrane zone of developing intestine. The combined use of interspecies associations of rat and chick embryonic tissue anlagen and of species-specific antibodies enabled us to definitely conclude that, in the intestine, the epithelial cells are the cellular source of basement membrane HSPG molecules. Moreover, the fact that, in the hybrid intestines (Cm/Re), scattered fluorescent granules were revealed with the anti-mouse antibody in the mesenchyme underlying the epithelium clearly emphasizes the essential role of the mesenchyme for degradation of this basement membrane component, a phenomenon already demonstrated during morphogenesis of submandibular salivary (2, 37) and mammary glands (34).

It has been shown that a variety of cell types synthesize HSPG molecules in culture (for review see 18); yet to our knowledge there was no clear-cut demonstration of the epithelial origin of these molecules located at the epithelial–mesenchymal interface. However from the comparison of the present data with preceding experiments, it appears that the epithelial origin of the basement membrane components cannot be considered as a general phenomenon. Indeed, using a similar technology, we could show previously that basement membrane type IV collagen was produced by the intestinal mesenchyme (reference 36 and Fig. 8); this last result is further strengthened by current data showing, by in situ hybridization, that mRNA for type IV collagen accumulate in the mesenchyme (Simon-Assmann, R., F. Bouziges, J. N. Freund, F. Perrire-Schmitt, and M. Kedinger, manuscript submitted for publication). In relation to this conclu-

sion, laminin mRNA expression has been shown to be confined to the muscularis externa and the lamina propria in the gut during embryogenesis (33).

Taken together, these data demonstrate the dual epithelial–fibroblastic origin of the intestinal basement membrane. This notion is reinforced by coculture experiments in which epithelial and fibroblastic cell contacts have been shown to be a prerequisite for the structural organization of the basement membrane (24, 36). Although the precise mechanism involved in the assembly of the basement membrane is not yet known, its strategic position at the epithelial–fibroblastic interface is believed to constitute the recognition system that delivers, via receptors, much of the information needed for cell differentiation. It is of interest to note that, among these receptors, HSPG appears to be closely involved in a transmembrane cytoskeletal–matrix interaction (8, 45).

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