Assembly of the Intestinal Brush Border: Appearance and Redistribution of Microvillar Core Proteins in Developing Chick Enterocytes

Toshiko Shibayama, Joan M. Carboni, and Mark S. Mooseker

Department of Biology, Yale University, New Haven, Connecticut 06511-8112

Abstract. The assembly of the intestinal microvillus cytoskeleton during embryogenesis in the chick was examined by immunochemical and light microscopic immunolocalization techniques. For these studies, affinity-purified antibodies reactive with three major cytoskeletal proteins of the adult intestinal microvillus, fimbrin, villin, and the 110-kD subunit of the 110K-calmodulin protein complex were prepared. Immunocytochemical staining of frozen sections of embryonic duodena revealed that all three proteins were present at detectable levels at the earliest stages examined, day 7–8 of incubation (Hamilton/Hamburger stages 25–30). Although initially all three proteins were diffusely distributed throughout the cytoplasm, there was a marked asynchrony in the accumulation of these core proteins within the apical domain of the enterocyte. Villin displayed concentrated apical staining by embryonic day 8 (stage 28), while the apical concentration of fimbrin was first observed at embryonic day 10 (stage 37). Diffuse staining of the enterocyte cytoplasm with the anti-110K was observed throughout development until a few days before hatch. By embryonic day 19–21 110K staining was concentrated at the cell periphery (apical and basolateral). The restricted apical localization characteristic of 110K in the adult brush border was not observed until the day of hatching. Immunoblot analysis of whole, solubilized embryonic duodena confirmed the presence of 110K, villin, and fimbrin throughout development and indicated substantial increases in all three proteins, particularly late in development. Immunoblot staining with anti-110K also revealed the presence of a high molecular mass (200 kD) immunoreactive species in embryonic intestine. This 200-kD form was absent from isolated embryonic enterocytes and may be a component of intestinal smooth muscle.

THE cytoskeletal apparatus which underlies and supports the apical brush border (BB)[1] surface of the intestinal epithelial cell consists of a highly ordered array of actin filaments and associated binding proteins. The organization and major proteins of the BB cytoskeleton have been well characterized (for review see Mooseker, 1985). For this reason, the BB is an excellent system for analysis of various aspects of cytoskeletal assembly. The BB is assembled both during embryonic development and during the continuous differentiation of enterocytes in the mature intestine. The study presented here considers the developmental assembly of the BB during embryogenesis of the chick.

Ultrastructural studies on the development of the intestinal epithelium of the chick (Overton and Shoup, 1964; Chambers and Grey, 1979) have revealed that the assembly of the BB cytoskeleton is a complex and gradual process. This ultrastructural differentiation is superimposed on the concurrent macroscopic changes in tissue organization of intestinal organogenesis (Grey, 1972). Between ~5 and 8 d of incubation (embryo stages 25–34; Hamburger and Hamilton, 1951) the lumen of the embryonic intestine changes from slightly oval to an elongated ellipse in cross-sectional profile (Burgess, 1975). During this time the apical surface of the enterocyte is dome-shaped and protrudes into the lumen. The base of the dome is delineated by the circumferential junctional complex. The apical surface contains a sparse population of microvilli of variable lengths. Chambers and Grey (1979) have reported that these microvilli contain filamentous meshworks rather than the well-ordered filament bundles typical of later developmental stages and of the mature BB. However, our own ultrastructural studies have revealed the presence of filament bundles within the microvilli on cells with domed apices (Mooseker, M., unpublished observations). With time, the density of microvilli gradually increases, and the apex of the cell flattens until the apical surface parallels the height of the junctional complex. By the onset of the formation of the first previllous ridges, at ~9–10 d of incubation, the apical surface contains a relatively dense population of short microvilli of fairly uniform length (0.2–0.5 μm), each containing a supporting filament bundle. Elongation of the basal "rootlet" ends of the microvillar bundles occurs next (11–15 d of incubation). Microvilli remain quite short until approximately the day before hatching (day 20–21). During the subsequent week there is a dramatic increase (to 2–3 μm) in microvillar length (Chambers and Grey, 1979; Stidwill and

1. *Abbreviations used in this paper*: BB, brush border; 110K-CM, 110K-calmodulin complex; NC, nitrocellulose; TBS, Tris-buffered saline.

Burgess, 1986). This increase in microvillus length is accompanied by an increase in the total cellular actin, and, more importantly, by a transient increase in the relative amounts of G:F-actin during the time when microvillar core filaments are elongating (Stidwill and Burgess, 1986).

Except for the junctional complex, which is present very early in the developing embryo (e.g., Overton and Shoup, 1964) the terminal web cytoskeleton does not assemble until relatively late in development (at ~16 d of incubation to day 3 posthatch; Chambers and Grey, 1979). Consistent with these ultrastructural observations, Glenney and Glenney (1983b) observed that the terminal web protein, TW 260/240, a BB-specific isoform of spectrin that forms cross-links between the rootlet ends of microvillar cores (Pearl et al., 1984; Glenney and Glenney, 1983*a*), is not detectable immunocytochemically until embryonic day 16.

In the present study, we have used a combination of immunochemical and light-microscopic level immunolocalization techniques to examine the temporal expression and cellular distribution of three major proteins of the microvillar actin bundle in day 7 to day 21 embryos as well as in the posthatch chick. The three proteins examined were villin, fimbrin, and the 110-kD subunit (110K) of the 110K-calmodulin (110K-CM) complex. Two of these proteins, villin and the 110K, are thought to be BB-specific, while fimbrin has been identified immunohistochemically in a variety of non-BB-containing cell types (Bretscher and Weber, 1980). Based on the interaction of these three microvillar proteins with actin in vitro, each has a potential to play important roles in the assembly of the microvillus. Villin is a Ca^{++}-binding protein that has a number of Ca^{++}-dependent effects on actin assembly and filament structure (Mooseker et al., 1980; Glenney et al., 1980). For example, at Ca^{++} <10 nm, villin cross-links filaments into bundles. At Ca^{++} >1 μm, villin binds to and caps the barbed, fast assembly end of the filament. Above 10 μm Ca^{++}, villin severs filaments. Fimbrin is thought to be the primary filament cross-linker of the microvillus core. This is based on the observation that when added to actin filaments in vitro, it forms bundles of uniformly polarized filaments that are structurally similar to those observed within microvilli in vivo (Glenney et al., 1981; Matsudaira et al., 1983). The 110K-CM complex is thought to comprise, at least in part, the spirally arranged bridges which tether the microvillar core laterally to the membrane. Recent studies describing its ATP-dependent interaction and ATPase activities (Howe and Mooseker, 1983; Collins and Borysenko, 1984; Swanljung-Collins and Collins 1985; Mooseker et al., 1986; Conzelman and Mooseker, 1987) have suggested that the 110K-CM complex is functionally analogous to myosin.

The results presented here indicate that all three of these microvillar core proteins are present at detectable levels quite early in development (from day 7–8 of incubation). However, initially all three proteins are diffusely distributed throughout the cytoplasm of the enterocyte and each exhibits a different timetable for assuming a concentrated distribution at the apical surface of the cell; villin arrives at ~day 7, and fimbrin becomes concentrated at the apical surface at day 9–10. The 110K subunit does not display the discrete BB-specific pattern of localization characteristic of the mature enterocyte until the day of hatching.

Materials and Methods

Production and Characterization of Antisera

Antisera reactive with the three microvillar core proteins, villin, fimbrin and the 110-kD subunit of 110K-CM were prepared by subcutaneous injection of the purified protein into rabbits using standard procedures (e.g., Fujiwara and Pollard, 1976). All three antigens were purified from BBs isolated by the method of Keller and Mooseker (1982) from chicken intestinal epithelial cells. Villin was purified from high salt extracts of BBs as described in Coleman and Mooseker (1985). Fimbrin was purified by the method of Glenney et al. (1981) except that final purification from fimbrin-enriched fractions was achieved by SDS-PAGE and subsequent electroelution of the fimbrin was achieved with cut gel bands and an electroelution apparatus (Isco, Inc., Lincoln, NE). Similarly, fractions enriched for the 110K subunit were prepared by the method of Glenney and Glenney (1984), and the final purification was achieved by electroelution from gel slices.

Affinity-purified antibodies were prepared by passage of whole antisera over agarose columns with covalently linked antigen (villin, fimbrin, or 110K) using cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The villin preparations used for affinity chromatography were isolated as described above. Native fimbrin prepared by the method of Glenney et al. (1981) was used rather than SDS-denatured protein. Affinity purification of 110K-specific antibodies was achieved by using 110K purified as described above, and also by using native 110K-CM complex purified by the method of Howe and Mooseker (1983) as modified by Conzelman and Mooseker (1986). The antibodies retained by the affinity matrix were eluted by addition of 0.1 M glycine-HCl, pH 2.8. The purified antibodies were dialyzed against Tris-buffered saline (TBS; 0.15 M NaCl, 20 mM Tris, pH 7.6) containing 0.02% NaN_3.

The specificity of the three antisera for their respective antigens was assessed by immunoblot analysis. Samples of purified villin, fimbrin, and the 110K subunit as well as SDS-solubilized isolated BBs were subjected to electrophoresis with 5–15% acrylamide mini-gels (Matsudaira and Burgess, 1978) in the presence of SDS. The gels were then electrophoretically transferred (Trans-blot Cell; Bio-Rad Laboratories, Inc., Richmond, CA) onto 0.22-μm pore nitrocellulose (NC) paper (Schleicher & Schuell, Inc., Keene, NH) for 24 h (45 V, 4°C) with a transfer buffer consisting of 192 mM glycine, 20 mM Tris, pH 8.2, 16% methanol, and 0.05% Tween-20. The NC transfers were stained for total protein with 0.2% ponceau S in 3% trichloroacetic acid, and destained with H_2O. Before incubation with antibodies, nonspecific protein binding to the NC was blocked in a solution of 5.0% nonfat dry milk in TBS (TBS-BLOTTO; Johnson et al., 1984) for 30 min at 37°C. The blocked strips were then reacted overnight at room temperature with the affinity-purified antibodies (3 μg/ml in TBS-BLOTTO), washed in TBS containing 0.05% Tween-20, and then incubated for 1 h with peroxidase-conjugated goat anti-rabbit Ig (1:500 dilution in TBS-BLOTTO; Cappel Laboratories, Malvern, PA). They were first washed in TBS containing 0.05% Tween-20, then in 100 mM Tris pH 7.6. Immune complexes were visualized by incubation in 0.05% 3,3′ diaminobenzidine, 0.01% H_2O_2 in 100 mM Tris, pH 7.6.

Immunolocalization and Immunoblot Analysis of Embryonic Intestine

Immunolocalization Studies. Fertilized eggs (White Leghorn; Hall Brothers Hatchery, Wallingford, CT) were maintained at 37.5°C in a forced-draft incubator. Under these conditions, hatch generally occurred during days 21 or 22 of incubation. Duodena were dissected from embryos beginning with days 6.5–7 of incubation; all successive days were examined up to the day-5 posthatch chick. Since incubation time is not a reliable method for uniformly staging embryos, particularly during the first 8–9 d of incubation (Hamburger and Hamilton, 1951), the Hamburger-Hamilton developmental stage of the 6–9 d embryos used for immunocytochemical studies was estimated by relating the cross-sectional profile of the intestines in cryostat sections to those reported by Burgess (1975) (generally the embryos used were at least a day behind in developmental stage compared with those analyzed by Burgess). For the early stages of development, when changes in the distribution of both fimbrin and villin were observed (day 6.5 to day 10; stages 25 to 35) at least five different sets of embryos were examined immunohistochemically for each day of incubation. At least two sets of 13–18 day embryos were examined for each day (no major changes in distribution were observed during this time). Four sets of embryos were examined between

day 19 and day 1 posthatch, the time at which marked changes in the distribution of 110K were observed.

Dissected intestines were fixed for 1 h on ice in 4% paraformaldehyde, containing 0.1 mM EGTA in 0.1 M Na phosphate buffer, pH 7.4. After fixation, the tissues were washed with 0.1 M Tris-Cl, pH 7.4, 0.1 mM EGTA, and then incubated for 10 min in the same buffer containing 0.05% Na borohydride (4°C) to quench free aldehydes. The intestines were transferred to 1 M sucrose, in 0.1 M Tris-Cl, pH 7.4, 0.1 mM EGTA for 2 h and then embedded and frozen (in a liquid N_2 bath) in O.C.T. compound (Miles, Naperville, IL) before cryostat sectioning. The tissue sections (4–6 μm) were mounted on microscope slides (coated with 0.5% gelatin, 0.05% chromium potassium sulfate) and either used immediately or stored at −80°. Before antibody staining the slides with mounted sections were immersed for 1–2 min in a Coplin jar containing −20°C acetone and washed in water. Each tissue section was then covered with 30 μl of blocking solution (3% normal goat serum, 1% BSA in PBS), incubated for 30 min in a humidified chamber, and subsequently rinsed with PBS (Carboni and Condeelis, 1985). The sections were then incubated (1 h) in affinity-purified antibodies to one of the three microvillar proteins (at 20 μg/ml) or with the same concentration of rabbit IgG fraction (Sigma Chemical Co., St. Louis, MO), rinsed three times (15 min each) in Coplin jars containing 0.1% BSA in PBS, and each section incubated (1 h) in 30 μl of fluorescein-labeled goat anti-rabbit Ig (Cappel Laboratories) at a 1:500 dilution in PBS containing 1% BSA. Slides were rinsed as above, then 10 μl of 0.1 M *n*-propyl gallate in a 1:1 mixture of PBS and glycerol was added to each section before application of a coverslip. The stained sections were examined with a Zeiss light microscope equipped with epi-illumination and filter inserts appropriate for fluorescein, with either a 40× neofluar or 63× planapo objective. Photomicrographs of fluorescently stained sections were made using Tri-X film (85-s exposures) developed with Diafine developer (Acufine Inc., Chicago, IL). To obtain a rough estimate of the contribution of the intestinal epithelium to the total intestinal tissue mass during the course of development, the same sections were photographed at low power with a 10× phase-contrast objective, and the cross-sectional area of the intestinal epithelium versus the total tissue area was determined morphometrically with a planimeter.

Immunoblot Analysis. Dissected intestines (10–20 for early embryos; 1–2 for later stages) were placed in a 1-ml Dounce homogenizer, 9 vol of SDS-PAGE sample buffer (2% SDS, 3 mM Tris-Cl, pH 6.8, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 3 μg/ml aprotinin, 10 μg/ml chymostatin, 2% sucrose) was added, followed by rapid immersion in a boiling water bath for 3 min. Solubilization of the tissue was aided by homogenization during this step. The samples were made 2% betamercaptoethanol, 30 mM dithiothreitol and reheated after removal of an aliquot for protein determination (Markwell et al., 1978) with BSA as standard. The samples were then subjected to electrophoresis (using equivalent total protein loadings) and electrotransferred to NC paper by the methods outlined above. In addition to analyzing whole intestinal tissue, we isolated epithelial cells from late-stage embryos (day 19–21). This was done by placing the intestine over a 20-gauge canula needle and then massaging the intestinal wall against the needle surface to dissociate the epithelial cells, which were flushed from the intestine with cell dissociation buffer (76 mM Na_2HPO_4, 19 mM KH_2PO_4, 12 mM EDTA, 200 mM sucrose, 0.2 mM PMSF). The dissociated epithelial cells were collected by centrifugation (200 *g* for 5 min), washed by resuspension and sedimentation in cell dissociation buffer, solubilized in 9 vol of SDS-PAGE sample buffer, and then subjected to electrophoresis and electrotransferred to NC paper as described above.

Results

Specificity of Antisera

For the immunocytochemical and immunoblot analyses described below, antisera reactive with the microvillar core proteins villin, fimbrin, and the 110-kD subunit of 110K-CM were used. The specificity of the affinity-purified antisera used in these studies was ascertained by their selective reactivity with the appropriate antigen on immunoblots of both purified antigen (results not shown) and solubilized BBs isolated from adult chickens (Fig. 1). All three antisera react with a single band of the appropriate molecular mass on immunoblots of electrophoretically transferred BB proteins. Depending on the particular BB sample used for such analysis, these three antisera also reacted at times with bands with faster migration rates than the intact antigen (results not shown). This is most probably due to proteolysis of the intact microvillar core proteins. The most frequently observed putative proteolytic fragment was on immunoblots of BB samples with the 110K antibodies (e.g., Fig. 6*f*), which often reacted with a 90–95-kD band (in addition to the 110K band), which is a common proteolytic fragment of this protein (Mooseker and Stephens, 1980).

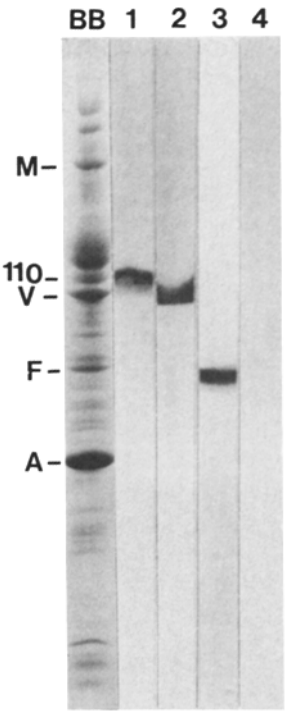


Figure 1. Immunoblot characterization of affinity-purified antibodies generated against the microvillus core proteins 110K, villin, and fimbrin. Affinity-purified antibodies against either 110K (lane *1*), villin (lane *2*), fimbrin (lane *3*), or nonimmune rabbit IgG (lane *4*) were reacted with NC strips containing electrotransferred adult BB proteins separated by SDS-PAGE. Lane *BB* is a Coomassie Blue–stained lane of the gel used for electrotransfer. The positions of BB myosin (*M*), the 110K subunit (*110*) villin (*V*), fimbrin (*F*), and actin (*A*) on both the gel and the NC strips are indicated.

Localization of Microvillar Core Proteins during Embryogenesis

The distribution of the microvillar core proteins villin, fimbrin, and the 110-kD subunit of 110K-CM complex was determined by immunocytochemical staining of frozen sections of intestine obtained from embryos throughout the course of development, beginning with the day 6.5–7 of incubation (Figs. 2–5). Between 5–9 d of incubation, the intestine undergoes three distinct stages of morphogenesis termed circle, ellipse, and triangle based on the morphology of the intestinal lumen in cross section (Burgess, 1975). At ~9–10 d of incubation the first three previllous ridges form (Burgess, 1975). It is at this time that flattening of the cell apex and the increase in microvillus density occurs (Overton and Shoup, 1964; Chambers and Grey, 1979). In our study, embryos examined at 6.5–7.5 d of incubation (Fig. 2) were generally at either the circle stage or at the initial phase of the ellipse stage (termed "small ellipse" by Burgess [1975]), corresponding to stages 25–30 of Hamburger and Hamilton [1951]). In the earliest developmental stages examined (obtained from eggs between 6 and 7 d of incubation) variable levels of diffuse cytoplasmic staining, generally slightly above those ob-

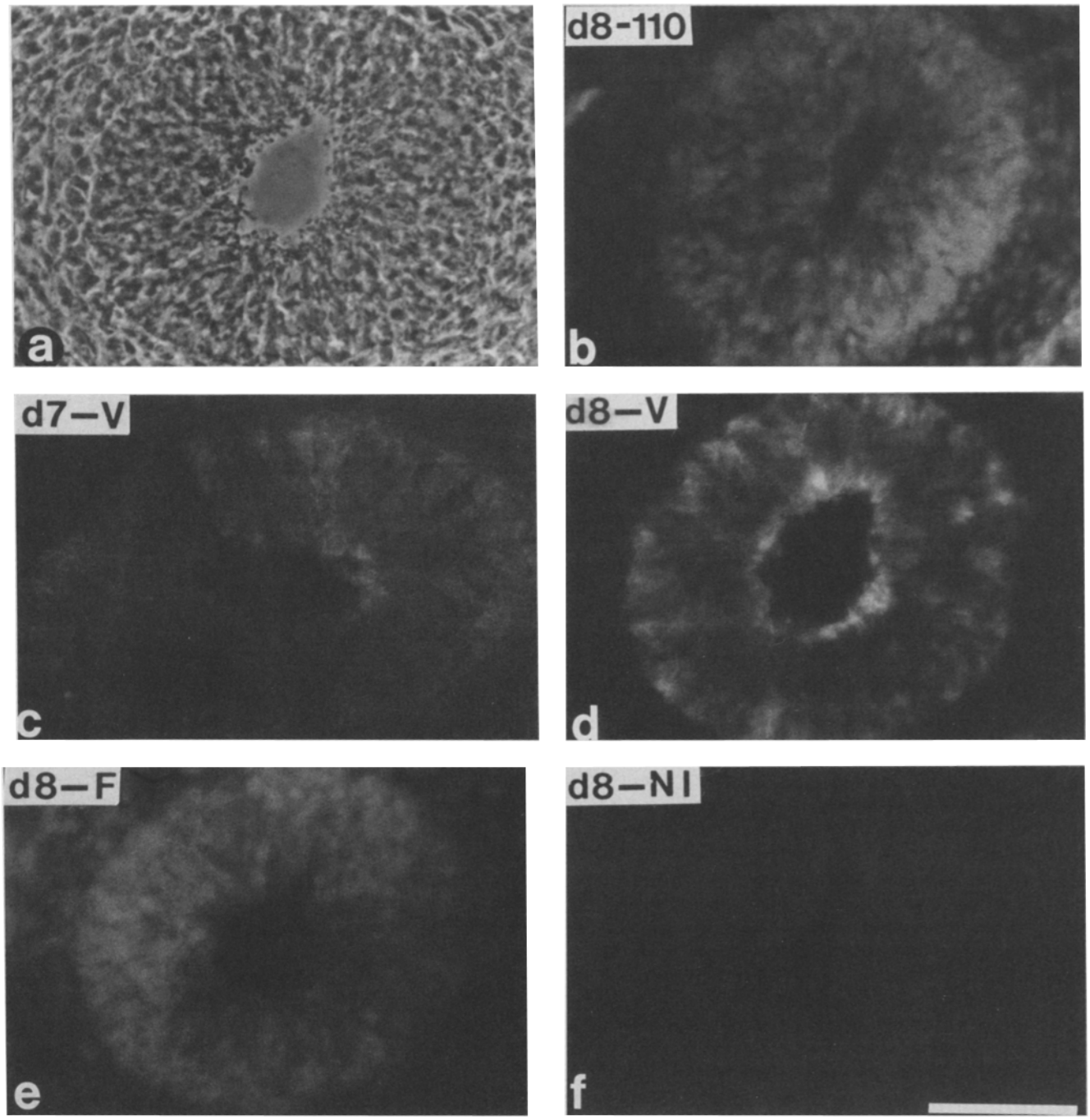
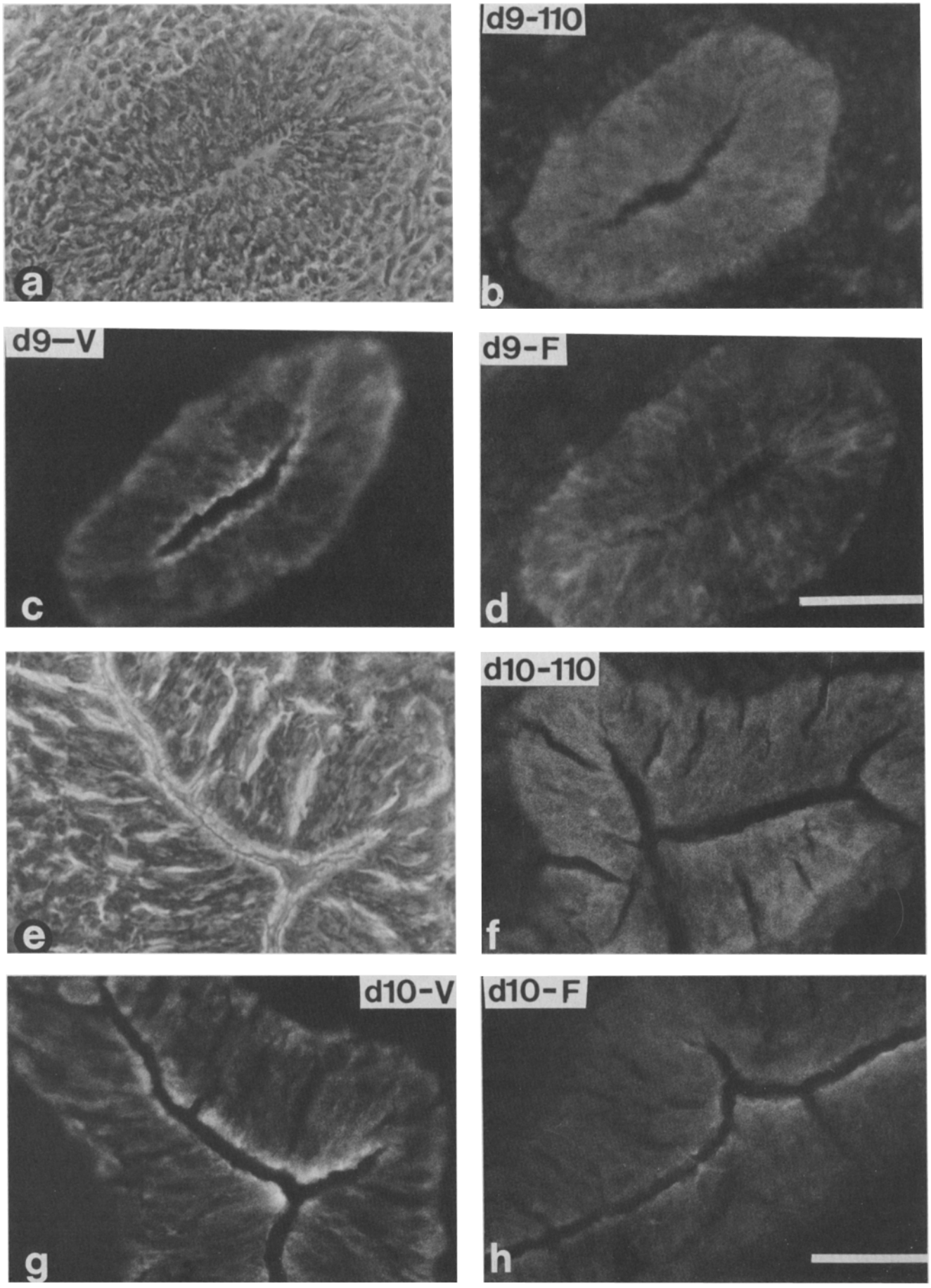


Figure 2. Immunolocalization of 110K, villin, and fimbrin in embryonic duodena from stage 25–30 embryos (6.5–8 d of incubation). Cryostat sections, with the lumen cut in cross section and stained with affinity-purified antibodies are shown. (*c*) A circle stage intestine (stage 25–26, 6.5 d) stained with anti-villin. (*b*, *d*, and *e*) Sections of duodena from the small ellipse stage (stage 28–29, 7.5–8 d) stained with anti-110K (*b*), anti-villin (*d*), or anti-fimbrin (*e*). (*f*) Stage 30 (8 d) intestine stained with nonimmune rabbit IgG. (*a*) Phase-contrast micrograph of section in *d*. (*b–f*) Immunofluorescence light micrographs. Bar, 50 μm.

served in sections stained with nonimmune antibodies (see Fig. 2 *f*), were observed (see Fig. 2 *c* for staining with anti-villin; results for fimbrin and 110K not shown).

By the small ellipse stage (approximately stage 26–27) significant staining of the epithelium with each of the antisera was observed (Fig. 2, *b*, *d*, and *e*). All three antisera gave diffuse staining throughout the epithelial cell cytoplasm. However, the staining pattern with anti-villin (Fig. 2 *d*) also indicated that appreciable concentration of villin within the apical surface of the epithelial cells had occurred by this

Figure 3. Immunolocalization of 110K (*b* and *f*), villin (*c* and *g*), and fimbrin (*d* and *h*) in day 9 (stage 33–35) and day 10 (stage 36–37) embryonic intestines. (*a–d*) Day 9; (*e–h*) day 10. (*a* and *e*) Phase-contrast micrographs of sections in *d* and *g*, respectively. Bars: (*a–d*) 50 μm; (*e–h*) 30 μm.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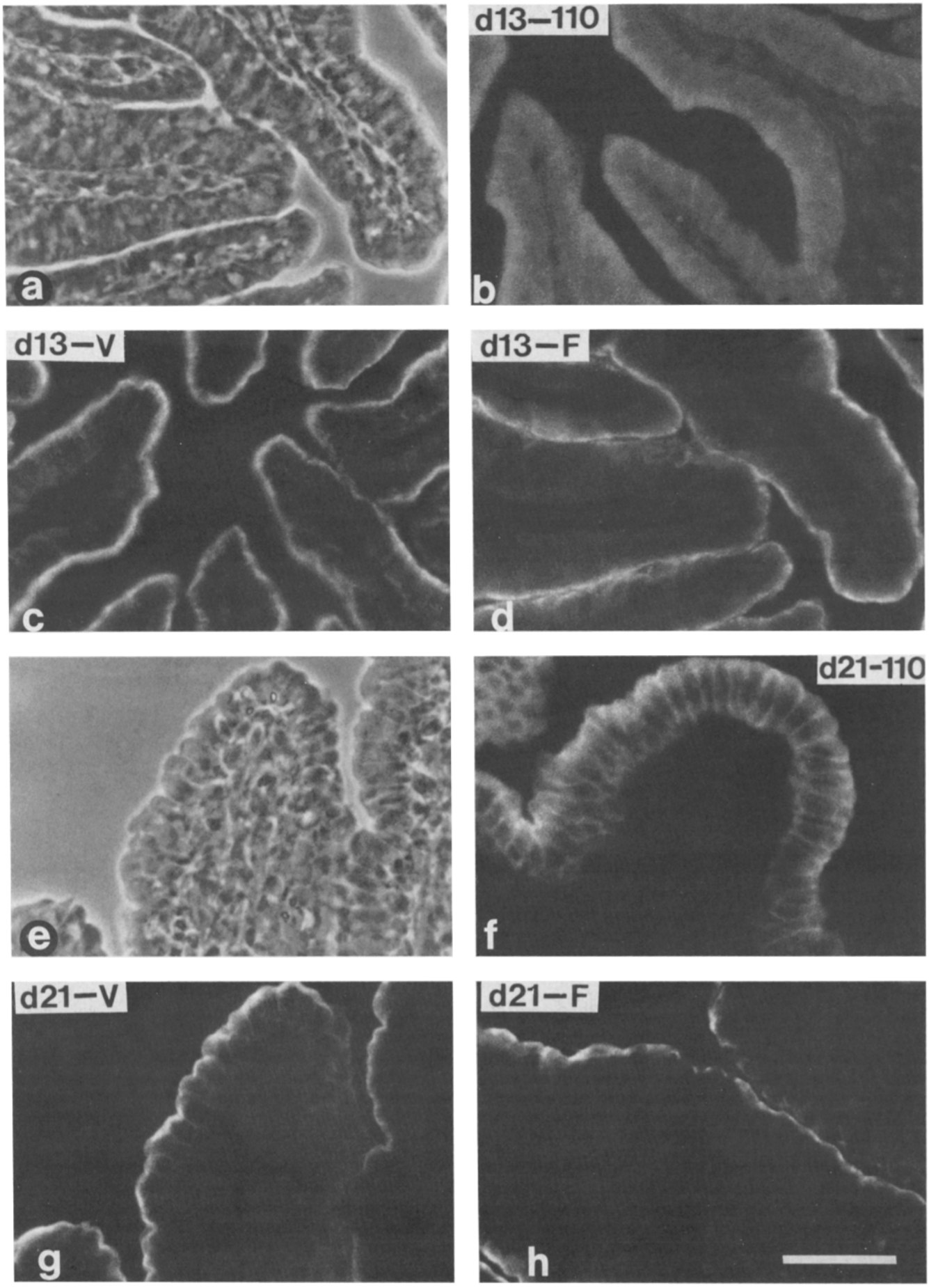
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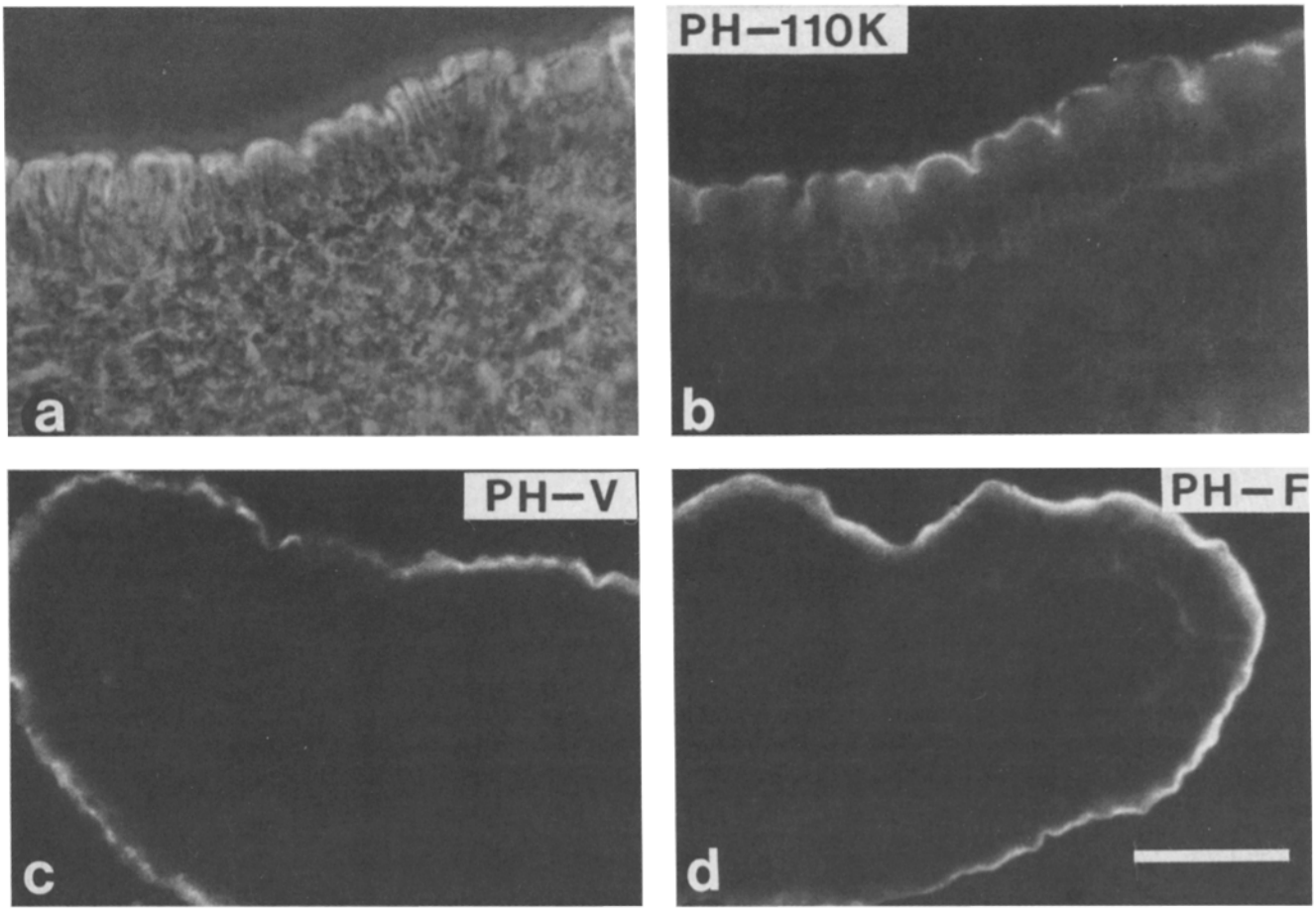

Figure 5. Immunolocalization of 110K (*b*), villin (*c*), and fimbrin (*d*) in intestines from just-hatched chicks. (*a*) A phase-contrast micrograph of the section in *b*. Bar, 30 μm.

stage. At this and later stages staining with anti-villin was restricted to the epithelium. However, the 110K and fimbrin antibodies also stained cells below the epithelial layer (Fig. 2, *b* and *e*). Intestines obtained from incubation day 8–9 embryos were generally in the "elongated ellipse" stage corresponding to stages 30–34 (Fig. 3, *a–d*). By this time villin displayed prominent localization to the apical surface of the epithelium, which continued throughout later stages of development (Figs. 3 *g*; 4, *c* and *g*; and 5 *c*). The diffuse cytoplasmic staining with anti-fimbrin and 110K persisted, although the staining with anti-fimbrin was more mottled than with anti-110K (cf. Fig. 3, *b* and *d*).

The apical concentration of fimbrin (Fig. 3 *h*) was first observed in intestines that had begun initial stages of previllous ridge formation (9–10 d of incubation), was quite pronounced by day 13 (Fig. 4 *d*), and remained so in later developmental stages (Figs. 4 *h* and 5 *d*). However, the diffuse cytoplasmic distribution of the 110K persisted throughout successive developmental stages (e.g., Fig. 4 *b*) until a few days before hatch. In day 19–21 embryos (e.g., Fig. 4 *f*) an apparent movement of the 110K to the cell periphery, as indicated by a greater intensity of fluorescence associated with the apical and basolateral surfaces of the enterocyte, was observed. The restricted apical localization of the 110K that is characteristic of its distribution in the adult enterocyte was first observed in hatchlings (Fig. 5 *b*).

Immunochemical Analysis of Microvillar Core Proteins during Embryogenesis

Immunoblot analysis of SDS-solubilized intestinal tissue beginning with day 6.5–7 embryos, revealed that immunoreactive polypeptides similar in molecular mass to fimbrin, villin, and 110K were present at detectable levels throughout development (Fig. 6, *a–d*). However, photographic documentation of the low levels of the three antigens present in the day 7–8 embryo samples (and detectable by eye) was difficult. This analysis also revealed that there was a marked increase in the relative amounts of each protein during later stages of development. While this may reflect an increase in the amount of microvillar core proteins per enterocyte, there is also a dramatic increase in the relative contribution of the epithelium to the total tissue mass of the intestine during embryogenesis. Based on morphometry of tissue cross sections, there is roughly a fourfold increase in the epithelial composition of the intestine, from ~5–10% of total tissue in the day

Figure 4. Immunolocalization of 110K (*b* and *f*), villin (*c* and *g*), and fimbrin (*d* and *h*) in day 13 (*a–d*) and day 21 (*e–h*) embryonic intestines. (*a* and *e*) Phase-contrast micrographs of sections in *d* and *g*, respectively. Bar, 30 μm.

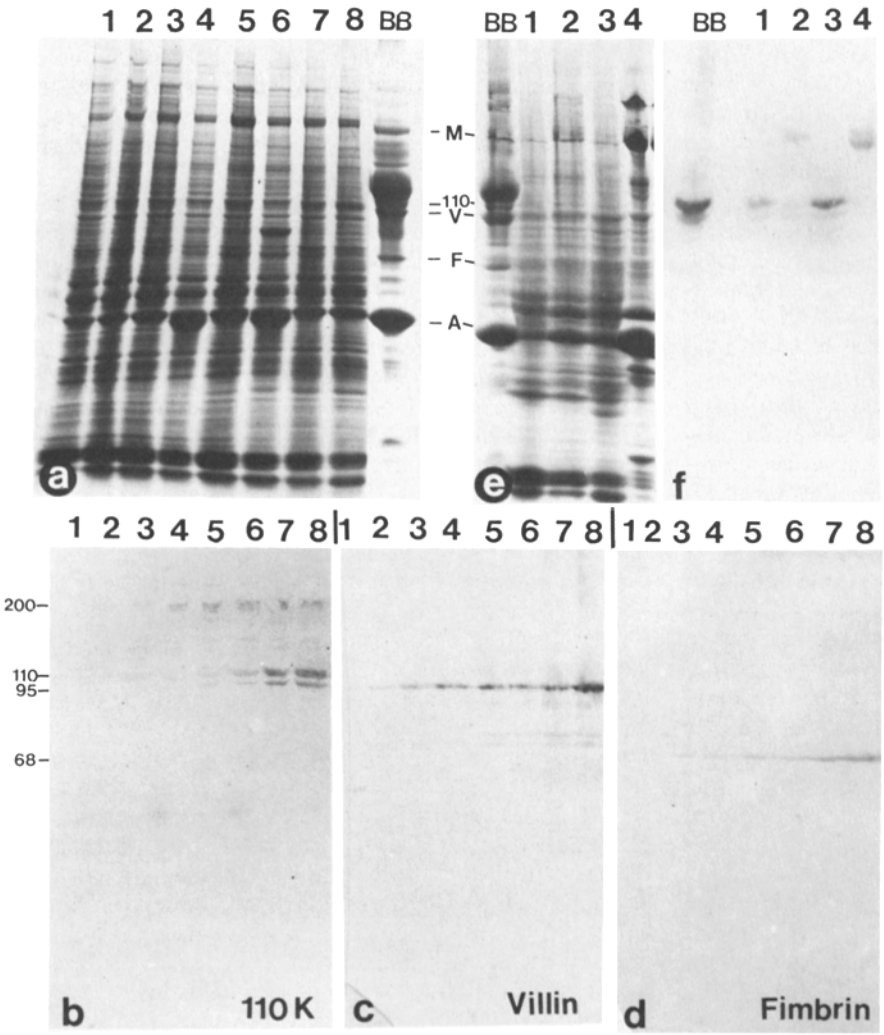

Figure 6. (*a–d*) Immunoblot analysis of 110K, villin, and fimbrin during intestinal development. (*a*) A Coomassie-stained gel of the samples used for immunoblot analysis in *b–d*. (*b–d*) Immunoblots of the above samples reacted with affinity-purified antibodies to 110K (*b*), villin (*c*), and fimbrin (*d*). Whole dissected duodena were solubilized in sample buffer, separated by SDS-PAGE, and transferred to NC paper. Lane *1*, day 7 embryo; lane *2*, day 9 embryo; lane *3*, day 12 embryo; lane *4*, day 14 embryo; lane *5*, day 17 embryo; lane *6*, day 19 embryo; lane *7*, day 1 posthatch; lane *8*, day 5 posthatch; lane *BB* is isolated adult BB samples. Migration positions of key BB proteins are indicated (for abbreviations see Fig. 1) (*e* and *f*) SDS-PAGE (*e*) and immunoblot analysis (*f*) of the distribution of 110K-immunoreactive proteins in intestinal tissues. Lanes *BB*, isolated adult BBs; lanes *1*, isolated day 19 embryonic intestinal epithelial cells; lanes *2*, whole homogenates from day 19 embryonic intestine; lanes *3*, isolated adult intestinal epithelial cells; lanes *4*, adult intestinal smooth muscle. The 200-kD immunoreactive form observed on anti-110K immunoblots of whole, embryonic intestines (*b* and lane *2* in *f*) is absent in isolated intestinal epithelia (lanes *1* and *3* in *f*), and is enriched in the smooth muscle sample (lane *4* in *f*).

7 embryo to 25–30% in the day 1 posthatch chick. It should be noted that because of significant differences in antibody titer, these data cannot be used to determine the relative ratios of 110K/villin/fimbrin.

The immunoblot analysis (using the villin and 110K antibodies) also revealed the presence of immunoreactive bands in some of the intestinal tissue samples, which did not correspond to the molecular mass of the corresponding protein in the mature BB. Most of these bands correspond in molecular mass to common proteolytic fragments of villin and 110K, (based on the de novo appearance of similar-sized fragments in preparations of the respective purified protein). This includes the 90–95-kD band seen on the 110K blots (Fig. 6 *b*), and the doublet of ~60–70 kD seen on the villin blots (Fig. 6 *c*). The 110K immunoblots also revealed the presence of high molecular mass immunoreactive bands. The band that was most prominant and that appeared most often was ~200 kD (Fig. 6 *b*). The 200-kD protein(s) was not present in immunoblots of epithelial cells isolated from day 19 embryonic and adult intestine (Fig. 6, *e* and *f*), and thus is probably a protein present in nonepithelial cells of the intestine (e.g., smooth muscle). Conversely, the 110K immunoreactive band is enriched in the isolated epithelial cell preparation (cf. Fig. 6 *f*, lanes *1* and *2*). The 200-kD immunoreactive species comigrates with the heavy chain of myosin, and in fact may be smooth muscle myosin, since the 110K shares antigenic determinants with both smooth and skeletal muscle (but not BB) myosin (Carboni, J. M., T. Shibayama, K. A. Conzelman, and M. S. Mooseker, unpublished observations). In this regard note that there is a 200-kD immunoreactive band in the adult intestinal smooth muscle sample (Fig. 6 *f*, lane *4*).

Discussion

The results presented here demonstrate that immunoreactive forms of the microvillar core proteins villin, fimbrin, and 110K are all expressed quite early in embryonic development, but that there is marked asynchrony in their concentration at the apical surface of the enterocyte. Villin is the first microvillar core protein to display a restricted apical localization (Fig. 2 *d*). This occurs at a stage of development (at 7.5–8 d of incubation, approximately stage 29) when the density of microvilli on the apical surface is quite low (Chambers and Grey, 1979). This suggests that villin may somehow play a critical role in microvillus assembly, perhaps involving its remarkable Ca^{++}-dependent effects on actin assembly (for review see Mooseker, 1985). The more pressing

question concerns the molecular basis for the localization of villin within the apical cytoplasm at a time when there is little ultrastructurally recognizable actin-based cytoskeleton in the presumptive BB region with which this actin-binding protein might be expected to associate. Perhaps villin interacts with the cytoplasmic surface of the apical membrane, where it might function to nucleate the assembly of microvillar core filaments. Alternatively, it might be primarily associated with the few microvilli that are present at this time. There is hope that such questions can be approached through immunolocalization studies at the ultrastructural level, as well as through cell fractionation studies designed to examine the association state of villin in the embryonic enterocyte.

Fimbrin is the next core protein to display a concentrated apical localization (Fig. 3 *h*). The timing (at ~10 d of incubation) of its apparent movement into the apical surface correlates with the first ultrastructurally recognizable stages of BB assembly. This process is manifested by the flattening of the previously dome-shaped cell apex and the appearance of a dense lawn of short microvilli of fairly uniform length (Chambers and Grey, 1979). Fimbrin is an actin-filament–bundling protein, and could thus play an important role in the formation of microvillar cores, depending upon the actual mechanism of microvillus assembly. One model for microvillus assembly, initially proposed by Tilney and Cardell (1970), is that assembly is mediated by nucleated assembly (perhaps regulated by villin) of core filaments from sites on the membrane. Fimbrin could then "zip" filaments together into bundles, forming a well-ordered, uniformly polarized microvillus core. In this case, the nucleated assembly of filaments, not fimbrin, would be responsible for generating the uniform polarity of the filament bundle. Alternatively, Chambers and Grey (1979) have suggested that initial stages of microvillus formation involve a transition from microvilli containing random filamentous arrays to more ordered bundles. Given the ability of fimbrin to form uniformly polarized microvillus-core–like bundles in vitro, this core protein might play an important role in such a transition. Future studies will be required to carefully examine, ultrastructurally, the transitional stages of microvillus assembly that occur in the interval between the "triangle" stage and the initial stages of previllous ridge formation (stages 35–39; see Chambers and Grey, 1979; Burgess, 1975). Such a study, coupled with correlative localization of fimbrin, might help resolve the potential involvement of this core protein in the assembly of the microvillus.

The most striking observation in the present study is the very late stage at which concentration of the 110K protein into the apical surface of the cell occurs. The apparent movement of the 110K from a diffuse cytoplasmic distribution to the restricted localization within the apical surface, which is characteristic of its distribution in the adult, appears to occur in two stages. In the last few days of embryonic development, immunofluorescent staining becomes more concentrated at the cell periphery, suggesting an increased association of the 110K subunit with the plasma membrane (Fig. 4 *f*). At or around the time of hatching, the 110K assumes its final, restricted distribution within the BB (Fig. 5 *b*). Initially we were concerned that the diffuse cytoplasmic distribution revealed by immunostaining might actually reflect the distribution of the 200-kD immunoreactive protein (Fig. 6 *b*) rather than that of the 110K. However, the absence of the 200-kD protein in immunoblots of isolated embryonic epithelial cells suggests that this is not the case (Fig. 6, *e* and *f*) and may explain the anti-110K staining of cells below the epithelial layer (e.g., Figs. 2 *b*, 3 *b*, and 4 *b*). This also rules out the possibility that the 110K is a proteolytically processed product of the 200-kD immunoreactive form.

One plausible explanation for the late concentration of the 110K within the BB is that its localization is linked to either the differentiation of the apical membrane and/or the elongation of microvillus that accompanies this differentiation (Stidwell and Burgess, 1986). With respect to the few membrane-associated enzymes examined thus far (e.g., Shehata et al., 1984), expression of the BB membrane hydrolases occurs quite late in development and continues in the posthatch chick. Since the 110K-CM complex may be a myosin-like mechanoenzyme (Collins and Borysenko, 1984; Swanljung-Collins and Collins, 1985; Mooseker et al., 1987; Conzelman and Mooseker, 1986) it is tempting to speculate that this complex is actively involved in the movement of newly synthesized membrane to the apical surface. On the other hand, Courdrier et al. (1983) have presented indirect evidence for the involvement of a 200-kD microvillar integral membrane protein in the binding of 110K-CM to the membrane. Thus, the synthesis and microvillus membrane insertion of such a tethering protein might be required for the movement (either active or passive) of the 110K to the microvillus. Future studies will hopefully clarify the involvement of such a protein in the association of the 110K-CM with the membrane. Another important question raised by these observations concerns the association state of the 110K subunit with calmodulin. Could the movement of 110K be keyed to the formation of a mature 110K-CM complex either because of a sudden increase in cellular levels of calmodulin, or because of posttranslational regulation of the interaction of the 110K with calmodulin?

In conclusion, these simple, descriptive studies on the expression and distribution of major proteins of the intestinal microvillus cytoskeleton during embryogenesis have set the stage for more detailed examination of possible mechanisms of cytoskeletal assembly in this system. Although these results do not reveal such mechanisms, they do at least help to eliminate some possibilities. For example, the fact that villin, fimbrin, and the 110K protein are all expressed early in development tends to argue against a self-assembly mechanism for microvillus assembly governed simply by the sequential expression of these three major microvillar core proteins. However, one cannot rule out the possibility that self-assembly is regulated by posttranslational modification of these microvillar components or by the sequential temporal expression of a minor protein of the microvillus not assessed in this study. This is in marked contrast to the expression of at least one protein of the terminal web cytoskeleton, TW 260/240, whose first appearance (as detected by immunocytochemical techniques) apparently coincides temporally with initial stages of terminal web assembly. It will also be important to examine if similar patterns of microvillus core protein expression are observed in other modes of BB assembly such as that seen during fetal development of mammals or in the differentiation of crypt cells in the adult intestine. In this regard, Dr. C. Rochette-Egly and Dr. K. Haffen (manuscript submitted for publication; personal communication) have used our 110K antibodies to examine the distribution of

110K during fetal development in the rat. Like the chick intestine, the 110K fails to display apical localization until late (day 18 fetus) in development.

The authors thank the following members of our laboratory for their help and advice during the course of this work: Jim Anderson, Tom Coleman, Karen Conzelman, Michelle Peterson and Bruce Stevenson. Special thanks to Karen Conzelman for the considerable help she provided in reading and revising the manuscript, and in teaching us how to dissect embryos. We thank Dr. D. Kankel and Dr. M. Constantine-Paton for the use of cryostat equipment in their laboratories, and Dr. C. Rochette-Egly and Dr. K. Haffen for sending us a preprint of their manuscript on the localization of 110K in fetal rat intestine. We also thank Dr. Hiroshi Maruta and Dr. Tomoko Hashimoto for their helpful advice, and we especially thank Professor Y. Nakamura, Showa University, Tokyo, Japan, for allowing Dr. Shibayama to conduct this study. Finally, we acknowledge the excellent technical assistance of Michael Boyd and Anna Goglia, and Kristine Hall Mooseker's help in preparing this manuscript.

This work was supported by March of Dimes basic research grant 1-924, National Institutes of Health grant AM 25387 to M. S. Mooseker, and by a National Institutes of Health postdoctoral fellowship to J. M. Carboni.

Received for publication 16 December 1986, and in revised form 19 March 1987.

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