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

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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) 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 (Moosker, 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
throughout the cytoplasm of the enterocyte and each exhibits 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 (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. Filamin 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 microvillar core filaments of G:F-actin during the time when microvillar core filaments are elongating (Stidwill and Burgess, 1986).

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 antibodies were purified 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 electrophoretography 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 antibodies retained by the 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% NaN3.

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, 1976) 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 H2O. 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 then 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% H2O2 in 100 mM Tris, pH 7.6.

Materials and Methods

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 immunocytochemically 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

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day 19 and day 1 posthatch, the time at which marked changes in the distri-

bution 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 fixa-
tion, 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 boro-

hydride (Nikon) 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 N2 bath) in OCT. (Miles, Naperville, IL) before cryostat sectioning. The tissue sections (4–6 μm) were

mounted on microscope slides (coated with 0.5% gelatin, 0.05% chro-

mium potassium sulfate) and either used immediately or stored at −80°. Be-

fore 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 con-

centration 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

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 propyl gallate

in a 1:1 mixture of PBS and glycerol was added to each section before appli-
cation 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. Pho-
tomicrographs of fluorescently stained sections were made using Tri-X film

(35-s exposures) developed with Diafine developer (Acufine Inc,. Chicago,

IL). To obtain a rough estimate of the contribution of the intestinal epithe-
lum to the total intestinal tissue mass during the course of development,

the same sections were photographed at low power with a 10X phase

counter 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 1:10 Dounce homogenizer, 9 vol of

SDS-PAGE sample buffer (2% SDS, 3 mM Tris-Cl, pH 6.8, 1 mM EDTA,

0.2 mM phenylmethylsulfon 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% betamercap-
toethanol, 30 mM diethiothreitol and heated 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 pro-

tein loadings) and electrotransferred to NC paper as described 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 in-
etestine over a 20-gauge canula needle and then massaging the intestinal wall
deriving from the needle surface to dissociate the epithelial cells, which were

flushed from the intestine with cell dissociation buffer (76 mM Na2HPO4,

19 mM KH2PO4, 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 elec-

trophoresis and electrotransferred to NC paper as described above.

### Results

**Specificity of Antisera**

For the immunocytochemical and immunoblot analyses de-
scribed 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 react-

ivity with the appropriate antigen on immunoblots of both

purified antigen (results not shown) and solubilized BBs iso-

lated from adult chickens (Fig. 1). All three antisera react

with a single band of the appropriate molecular mass on im-

munoblots of electrophoretically transferred BB proteins.

Depending on the particular BB sample used for such analy-
sis, 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. 6f), 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).

**Localization of Microvillar Core Proteins during Embryogenesis**

The distribution of the microvillar core proteins villin, fim-

brin, and the 110-kD subunit of 110K-CM complex was deter-

mined by immunocytochemical staining of frozen sections of

intestine obtained from embryos throughout the course of de-

velopment, 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 previllus 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 1. Immunoblot characterization of affinity-purified antibodies 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 electrophoresis. 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.
Figure 2. Immunolocalization of II0K, 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-II0K (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.

In sections stained with nonimmune antibodies (see Fig. 2f), 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 stage.

Figure 3. Immunolocalization of II0K (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.
At this and later stages staining with anti-villin was restricted to the epithelium. However, the II0K 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 II0K persisted, although the staining with anti-fimbrin was more mottled than with anti-II0K (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 pre-villous 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 II0K 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 II0K 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 II0K 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 II0K 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

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Figure 4. Immunolocalization of II0K (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.
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 prominent 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 Ca²⁺-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-kDa immunoreactive protein (Fig. 6 b) rather than that of the 110K. However, the absence of the 200-kDa 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 h, 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 microvilli 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; Swanjung-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 key 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.

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References

Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surfaces. J. Cell Biol. 86:335-340.
Burgess, D. R. 1975. Morphogenesis of intestinal villi. II. Mechanism of formation of previllous ridges. J. Embryol. Exp. Morphol. 34:723-740.
Carboni, J. M., and J. Condeelis. 1985. Ligand-induced changes in the location of actin, myosin, 95K (alpha-actinin), and 120K protein in amebae of Dictyostelium discoideum. J. Cell Biol. 100:1884-1893.
Chambers, C., and R. D. Grey. 1979. Development of the structural components of the brush border in absorptive cells of the chick intestine. Cell Tissue Res. 204:387-405.
Coleman, T. R., and M. S. Mooseker. 1985. Effects of actin filament cross-linking and filament length on actin-myosin interaction. J. Cell Biol. 101:1850-1857.
Collins, J. H., and C. W. Borysenko. 1984. The 110,000-dalton actin- and calmodulin-binding protein from intestinal brush border is a myosin-like ATPase. J. Biol. Chem. 259:14128-14135.
Conzelman, K. A., and M. S. Mooseker. 1987. The 110-kD protein-calmodulin complex of the intestinal microvillus is an actin-activated MgATPase. J. Cell Biol. 105:313-324.
Couedrier, E., H. Reggio, and D. Louvard. 1983. Characterization of an integral membrane glycoprotein associated with microfilaments of pig intestinal microvilli. EMBO (Eur. Mol. Biol. Organ.) J. 2:469-475.
Fujiiwa, K., and T. D. Pollard. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and the mitotic spindle of human cells. J. Cell Biol. 71:848-857.
Glenney, J. R., and P. Glenney. 1983a. Spectrin, fodrin, and TW 260/240: a family of related proteins lining the plasma membrane. Cell Motil. 3:671-682.
Glenney, J. R., and P. Glenney. 1983b. Fodrin is the general spectrin-like protein found in most cells whereas spectrin and the TW protein have a restricted distribution. Cell. 34:503-512.
Glenney, J. R., and P. Glenney. 1984. The microvillus 110K cytoskeletal protein is an integral membrane protein. Cell. 37:743-751.
Glenney, J. R., P. Kaufus, P. Matsudaïra, and K. Weber. 1981. F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of the microvillus core filaments. J. Biol. Chem. 256:9283-9288.
Grey, R. D. 1972. Morphogenesis of intestinal villi. I. Scanning electron microscopy of the duodenal epithelium of the developing chick embryo. J. Morph. 137:193-214.
Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49-92.
Howe, C. L., and M. S. Mooseker. 1983. Characterization of the 110-kdalton actin-, calmodulin-, and membrane-binding protein from microvilli of intestinal epithelial cells. J. Cell Biol. 97:974-985.
Johnson, D. A., J. W. Gauths, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing non fat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. 1:3-8.
Keller, T. C. S., and M. S. Mooseker. 1982. Ca2+-calmodulin-dependent phosphorylation of myosin, and its role in brush border contraction in vitro. J. Cell Biol. 95:943-959.
Markwell, M. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87:206-210.
Matsudaïra, P. T., and D. R. Burgess. 1978. SDS microslab linear gradient polycrylamide gel electrophoresis. Anal. Biochem. 87:386-396.
Matsudaïra, P., E. Mandelkow, W. Renner, L. K. Hesterberg, and K. Weber. 1983. Role of fimbrin and villin in determining the interfilament distances of actin bundles. Nature (Lond.). 301:209-214.
Mooseker, M. S. 1985. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. Annu. Rev. Cell Biol. 1:209-241.
Mooseker, M. S., T. A. Graves, K. A. Wharton, N. Falco, and C. L. Howe. 1980. Regulation of microvillus structure: calcium-dependent solation and cross-linking of actin filaments in the microvilli of intestinal epithelial cells. J. Cell Biol. 87:809-822.
Mooseker, M. S., T. R. Coleman, and K. A. Conzelman. 1986. Calcium and the regulation of cytoskeletal assembly, structure, and contractility. Ciba Found. Symp. 122:232-249.
Mooseker, M. S., and R. E. Stephens. 1980. Brush border alpha-actinin? Comparison of two proteins of the microvillus core with alpha-actinin by two-dimensional peptide mapping. J. Cell Biol. 85:466-474.
Overton, J., and J. Shoup. 1964. Fine structure of cell surface specializations in the maturing duodenal mucosa of the chick. J. Cell Biol. 21:75-85.
Pearl, M., D. Fishkind, M. S. Mooseker, D. Keene, and T. C. S. Keller. 1984. Studies on the spectrin-like protein from the intestinal brush border, TW 260/240, and characterization of its interaction with the cytoskeleton and actin. J. Cell Biol. 98:66-78.
Shehata, A. T., J. Lerner, and D. S. Miller. 1984. Development of nutrient transport systems in chick jejunum. Am. J. Physiol. 246:G101-107.
Stidwill, R. P., and D. R. Burgess. 1986. Regulation of intestinal brush border microvillus length during development by the G- to F-actin ratio. Dev. Biol. 114:381-388.
Swanljung-Collins, H., and J. H. Collins. 1985. Calcium and actin stimulation of the ATPase activity of brush border 110,000 Dalton protein-calmodulin complex. J. Cell Biol. 101(No. 5, Pt. 2):163a (Abstr.).
Tilney, L. G., and R. M. Cardell, Jr. 1970. Factors controlling the reassembly of the microvillus border of the small intestine of the salmon. J. Cell Biol. 47:408-422.