Characterization of rat epimorphin/syntaxin 2 expression suggests a role in crypt-villus morphogenesis

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Goyal, Alka, Renu Singh, Elzbieta A. Swietlicki, Marc S. Levin, and Deborah C. Rubin. Characterization of rat epimorphin/syntaxin 2 expression suggests a role in crypt-villus morphogenesis. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G114–G124, 1998.—The rodent intestinal mucosa undergoes a remarkable morphogenesis as the crypt-villus axis is formed. Endoderm-mesenchymal interactions play a critical role in this process. Epimorphin is a mesenchymal protein postulated to play a role in lung and skin morphogenesis. The rat homologue, syntaxin 2, belongs to a family of integral membrane proteins that function in vesicle docking and fusion. To clarify its role in fetal gut morphogenesis, epimorphin expression was examined during ontogeny, in an isograft model of ischemic injury and mucosal repair, and during intestinal adaptation after small bowel resection. Epimorphin/syntaxin 2 mRNA levels were increased in fetal gut during lumen formation and villus morphogenesis. mRNA levels remained elevated in the first 2 wk after birth and then declined at weaning. In situ hybridization showed epimorphin/syntaxin 2 mRNA in gestational day 14 (G14) and G15 intestinal mesenchymal cells and in the mucosal lamina propria during villus formation. Epimorphin/syntaxin 2 mRNA expression increased during villus repair in the isograft. In contrast, in the early stages of intestinal adaptation after small bowel resection, epimorphin/syntaxin 2 mRNA expression was suppressed in the adapting gut. We conclude the cell-specific and temporal patterns of epimorphin expression in the models used in this study suggest a role in the morphogenesis of the crypt-villus axis.

THE MAMMALIAN small intestine contains a continuously proliferating and differentiating epithelium. The primary anatomic and functional component of this epithelium is the crypt-villus unit. Anchored stem cells are located in the crypts of Lieberkühn and give rise to proliferating daughter cells that differentiate into four major intestinal epithelial cell types, including enterocytes, enteroendocrine and goblet cells, and Paneth cells (8). Cellular differentiation occurs coincident with migration from the stem cell/proliferative region onto the villus or to the base of the crypts.

Crypt-villus axis morphogenesis begins in the rodent small intestine during late gestation. Before that time, the intestinal tube is composed of a single layer of undifferentiated endoderm surrounded by mesenchymal cells. From gestational day 13 (G13) to G14, the endoderm and mesenchyme proliferate and a stratified epithelium is produced. In the next several days, a central lumen forms, accompanied by the emergence of small villi covered by a differentiating epithelium (26, 46). Proliferating cells, initially scattered randomly throughout the epithelium, become confined to the intervillus epithelial region at birth (G22), as nascent crypts are formed (19). From G18 through G22, the villus-associated epithelium continues to differentiate, preparing to digest and absorb nutrients immediately after birth.

The molecular basis of gut morphogenesis and crypt-villus axis formation has been the subject of intensive investigation (41). In the intestine and in other epithelial tissues such as skin and lung, direct cell-cell contact between mesenchyme and endoderm is required for epithelial morphogenesis and cellular differentiation (4, 22, 25, 27). The presence of mesenchyme is essential for generation of the normal crypt-villus axis containing all four differentiated cell types, as demonstrated previously in elegant endodermal-mesenchymal coculture and implantation experiments (24, 25). Investigators have begun to identify the specific mesenchymal molecules required for appropriate morphogenesis. For example, hepatocyte growth factor is produced and secreted by mesenchyme and interacts with the c-met receptor on endodermal cells. Experiments in cell culture suggest the importance of hepatocyte growth factor in lumen formation (47), and studies on human embryos have revealed that MET immunostaining was positive in the basal domain of the fetal intestinal epithelia between 5 and 13 wk of gestation, which coincides with the time of organ differentiation (28). Another important mesenchymal gene is a member of the forkhead family of winged transcription factors. Deletion of the fkh 6 gene, normally expressed in embryonic mesenchyme adjacent to endoderm, results in enhanced proliferation in the crypts with distortion, villus hyperplasia, and the formation of mucinous cysts (23). Further identification of these critical factors is essential for understanding the molecular basis of crypt-villus axis formation.

Another potential candidate mesenchymal morphogenic factor is epimorphin. Epimorphin is a membrane-associated protein that has been postulated to regulate morphogenesis of the lungs and skin (22). Transfection of the epimorphin cDNA into fibroblasts followed by coculture with lung endoderm induced the formation of a large lumen and well-developed air sacs. In addition, incubation of skin or lung organ cultures with epimorphin antibody led to disorganization of hair follicle and air sac structure, respectively (22). cDNA sequence analysis revealed homology to the syntaxin family of proteins, and cloning experiments designed to isolate homologues of neuronal syntaxin led to the identification of a rat cDNA that shared 96% amino acid identity with epimorphin and between 20% and 65% amino acid identity with neuronal syntaxins.
identity with other members of the syntaxin family (3). These integral membrane proteins act as target membrane receptors for vesicle docking and fusion in a variety of tissues, including neurons and nonneuronal secretory tissues such as pancreas, where they play a role in calcium-regulated exocytosis (13). Yet, in several instances, syntaxins appear to play a different role in development, distinct from their function in mature tissues (6, 10, 15, 32).

The role of epimorphin/syntaxin 2 in organ morphogenesis has not been clarified. To address the hypothesis that epimorphin/syntaxin 2 is important in lumen formation and villus morphogenesis in the developing fetal gut, the cell-specific and temporal regulation of epimorphin expression was examined during ontogeny. Also, epimorphin regulation was studied in an isograft model system of fetal villus ischemic injury and regeneration. To begin to understand the role of epimorphin in the adult intestine, expression patterns were analyzed in the mature intestine and during intestinal adaptation, when epithelial cell proliferation, migration, and differentiation are altered in the remnant gut. The results suggest a role for epimorphin/syntaxin 2 in gut morphogenesis, particularly in lumen and crypt-villus axis formation.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were housed under a strict light-dark cycle and fed standard laboratory chow. Pregnant female Sprague-Dawley rats (Sasco) were precisely timed for gestation based on the presence of a vaginal plug (defined as day 0 of gestation) and killed on G14–G22 (birth). Pups were killed at postnatal week 1 (P1), P2, P3, P4, P6, and P8. Suckling pups remained with their dams until they were killed. Male Sprague-Dawley rats (220–260 g) were used for small intestinal resection experiments. Fetuses from pregnant female Fisher rats killed on G19 were used as donors for fetal intestinal isografts, and 4- to 6-wk-old male Fisher rats were used as hosts.

Tissues and RNA isolation. Total RNA was isolated from intestinal tissues using the single-step method of Chomczynski and Sacchi (9). On G14–G17, RNA was isolated from the whole intestine, without further subdivision due to its small size. From G18 through P8, proximal intestine (jejunum) or ileum was used for analysis. For fetal intestinal RNA isolation, three to six pregnant females were killed for each time point, each containing 6–10 fetuses. For RNA isolation on P1, P2, and P3, two to three litters of 8–10 pups were killed for each date. For P4, P6, and P8, at least four rats were killed for each time point.

Cloning of the rat intestinal epimorphin/syntaxin 2 cDNA. The reverse transcriptase-polymerase chain reaction (RT-PCR) method was used to isolate a partial cDNA encoding rat intestinal epimorphin/syntaxin 2. Nucleotide primers were designed based on the mouse epimorphin sequence, from nucleotides 600 to 620 (sense primer) and nucleotides 924 to 946 (antisense primer). The first strand was synthesized from E17 rat intestinal RNA using reverse transcriptase, and PCR (40 cycles) was performed using Taq polymerase. As predicted, a 350-bp fragment was isolated. Complete sequence analysis of this partial cDNA revealed 96% nucleic acid identity with mouse epimorphin and 100% identity with rat syntaxin 2 (3).

RNase protection assays. We determined by Northern blot analysis that epimorphin/syntaxin 2 mRNA levels were detectable but rare in the adult gut. Therefore RNase protection assays were utilized to examine the ontogenic and spatial expression of epimorphin/syntaxin 2 and its regulation during gut adaptation. The 350-bp PCR fragment was subcloned into the plasmid pGEM-2, and a [32P]labeled epimorphin/syntaxin 2 antisense RNA probe was prepared with high specific activity [32P]CTP and T7 polymerase. The probe was purified by 6% polyacrylamide gel electrophoresis and hybridized overnight at 42–45°C to 10 µg of total RNA from each intestinal segment and developmental time point. After RNase digestion, samples were run on a 6% polyacrylamide gel, and autoradiography was performed. A single band was noted and quantitated using NIH Image 1.55 analysis of digitized images (W. Rasband, National Institute of Mental Health) obtained with a UMAX PS-2400X scanner, using UMAX Magiscan v. 2.1 (UMA Technologies, Fremont, CA). To verify the results, these experiments were repeated three to six times. A low specific activity 18S ribosomal probe (Ambion, Austin, TX) was used to normalize for differences in RNA concentration or loading. Hybridization of this probe to ~0.5 µg total RNA produces two closely migrating bands, as indicated by the manufacturer (Ambion) and shown in Fig. 1. The band at the top was used to normalize the RNase protection experiments.

Northern blot hybridization. Northern blot hybridization was performed to quantitate epimorphin mRNA expression in
G116 EPIMORPHIN/SYNTAXIN 2 EXPRESSION IN GUT MORPHOGENESIS

isograft epithelium, as previously described (36). Total RNA was prepared from individual isografts, and 20 µg RNA were electrophoresed on 1.2% agarose and 2.2 M formaldehyde gels. Gels were stained with ethidium bromide and photographed to verify mRNA integrity. RNA was transferred onto nitrocellulose membranes, prehybridized at 42°C for 2–3 h in 50% formamide, 6× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M Na2HPO4, and 0.001 M EDTA, pH 7.4), 5× Denhardt’s solution, 0.5% SDS, and 50 mg/l denatured salmon sperm DNA, and probed with an [α-32P]dCTP radiolabeled epimorphin cDNA probe. Probe was labeled using the random oligo priming method (Amersham Life Sciences, Arlington Heights, IL), hybridized at 42°C overnight, and washed at 55°C in 0.1× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). Blots were placed on film at −70°C and developed after 1 wk. Quantitation of hybridization bands was performed by scanning laser densitometry. Blots were normalized to the ethidium bromide signal of the 18S ribosomal band, which was quantified with the Eagle Eye image analysis system (Stratagene, La Jolla, CA) to control for differences in gel loading. Relative mRNA levels were reported as the ratio of specific transcripts to 18S RNA.

In situ hybridization analyses. In situ hybridization analysis to detect the cell-specific expression of epimorphin/syntaxin 2 in fetal and postnatal intestine was performed as previously described (16, 35). Briefly, tissues were fixed in 4% paraformaldehyde in PBS overnight, incubated in 30% sucrose in PBS until saturated, and then frozen in optimal cutting temperature solution. Cryostat sections (8 µm) were prehybridized by proteinase K digestion (1 µg/ml for 10 min at 37°C) and acetylated. A radiolabeled antisense epimorphin/syntaxin 2 RNA probe or sense (control) probe was synthesized using high specific activity [35S]-labeled UTP. Sections were hybridized with 5 × 10⁶ disintegrations per minute (dpm) of probe per milliliter of hybridization solution at 55°C overnight. Standard washing conditions were performed as described previously (16, 35) and included a stringent wash in 0.1× SSC at 55°C. Slides were dipped in Kodak NTB-2 emulsion, exposed for 10–16 days at 4°C, developed, and counterstained with hematoxylin and eosin. Photographs were taken using a Nikon Microphot camera under dark-field and light microscopy. For each time point, tissues were used from at least four to six animals, and at least four sections for each animal were analyzed.

Intestinal isograft implantation analyses. Rat intestines were harvested from fetuses of pregnant female Fisher rats on G18–G19. Jejunal segments were implanted into the dorsal subcutaneous space of young adult male Fisher rat hosts as previously described (16, 37, 39). Briefly, intestinal segments were isolated from fetal pups and placed into RPMI medium on ice. Hosts were anesthetized, and a midline incision along the dorsal paravertebral surface was made. The subcutaneous fascia was dissected by forceps, and isografts were placed into this space and sutured using 7-0 silk. Isografts were harvested 24–96 h after implantation and analyzed for cellular epimorphin/syntaxin 2 expression by Northern blot analysis. The developmental age of the 96-h isograft was considered equal to G22, calculated as previously described (16, 37, 38). Briefly, 24 h were allowed for vascularization of the isograft. The age of the graft was then expressed as the fetal age plus the total number of days of implantation minus 1 (for vascularization).

70% Small bowel resection surgical protocol. Male Sprague-Dawley rats (220–260 g) were randomly assigned to receive either 70% small intestinal resection or control, sham intestinal resection surgery, as previously described (11, 39). All animals were fasted overnight after being acclimated to our animal facility. For 70% small bowel resection surgery, the intestine was cut 5 cm distal to the ligament of Treitz and 15 cm proximal to the ileocecal valve. The resected part of the intestine was removed, with construction of an end-to-end anastomosis. In sham-resected animals, bowel bisection and reanastomosis were performed 5 cm distal to the ligament of Treitz. After surgery, animals received buprenorphine (0.4 mg/kg sc) for analgesia and were caged individually with access to 5% dextrose in water containing oxytetracycline (4.5 g/l) for up to 2 days. Animals were killed at 2, 4, 8, 16, 48, and 168 h after surgery (n = 3 per group). Portions of the middle part of the remnant ileum and the equivalent fragment in the control animals were rapidly frozen and saved for total RNA isolation.

RESULTS

Isolation of a partial cDNA encoding rat intestinal epimorphin/syntaxin 2. To isolate the rat cDNA encoding intestinal epimorphin/syntaxin 2, we used RT-PCR. PCR primers were based on the mouse epimorphin

Fig. 2. Postnatal intestinal expression of epimorphin. RNase protection assay was performed as described in Fig. 1 legend. Lanes 1–4, jejunum from postnatal weeks 1 through 4 (P1–P4), respectively. Lane 5, P6 jejunum. Top: 350-bp protected fragment. Bottom: 18S bands as per Fig. 1.
sequence isolated from a C3H10T1/2 expression library (22). A 350-bp fragment of the epimorphin/syntaxin 2 cDNA was cloned. Alignment of this partial cDNA sequence with the mouse epimorphin cDNA revealed 96% nucleotide identity. Alignment with the rat syntaxin 2 cDNA, the rat homologue of mouse epimorphin cloned from several cDNA libraries, including liver, brain/spinal cord, macrophages, pancreas, and pituitary gland (3), indicated 100% identity in this region. Thus this part of the coding sequence of rat intestinal epimorphin/syntaxin 2 is identical to syntaxin 2 from other rat tissues.

Epimorphin/syntaxin 2 mRNA is maximally expressed in the developing fetal intestine. Crypt-villus axis morphogenesis begins on approximately G13–G14 and continues until several days after birth. To test the hypothesis that epimorphin/syntaxin 2 might play a role in regulating part of this morphogenetic process, we examined the developmental regulation of its expression in the intestine using RNase protection assays (Figs. 1–4). Fetal and postnatal rat intestine were harvested, and total RNA was prepared from each segment. An antisense epimorphin/syntaxin 2 cRNA probe was hybridized to 10 µg of total RNA from each sample. Typical RNase protection assays are shown in Figs. 1 and 2. A single protected band of ~350 bp represents epimorphin/syntaxin 2 mRNA. The results of several independent RNase protection assays are quantitated in Fig. 3A (fetal ontogeny) and Fig. 3B (postnatal ontogeny). Epimorphin/syntaxin 2 mRNA was most abundantly expressed in intestine on fetal days 14–17, coincident with the commencement of crypt-villus morphogenesis (Fig. 3A). By G18, mRNA levels decreased in proximal gut but remained elevated in distal gut (Fig. 4). Subsequently, on G19 through birth (G22), mRNA levels declined in all segments but were still readily detectable. A second peak of epimorphin/syntaxin 2 mRNA expression was found during the suckling period (P1 and P2; Figs. 2 and 3B), but mRNA levels decreased to basal levels by P3, the onset of weaning. mRNA levels remained low but were still detectable in adult jejunum (P6 and P8).

Epimorphin/syntaxin 2 mRNA is expressed in fetal mesenchymal cells and in the lamina propria of the nascent villus, coincident with villus formation. To begin to determine the function of epimorphin/syntaxin 2 in gut morphogenesis, we examined its cell-specific expression, using in situ hybridization techniques (Figs. 5 and 6). On fetal day 15, the endoderm is a stratified, multicellular layer of undifferentiated cells surrounded by mesenchyme. There is a central lumen, and villus structures have not yet formed. Expression of epimorphin/syntaxin 2 mRNA was most prominent in the mesenchyme that surrounds endoderm (Fig. 5, A and B). Small thumblike villi appeared by G16, and mesenchymal expression persisted (Fig. 5, C and D). At this stage, epimorphin/syntaxin 2 mRNA was not detectable in endoderm (Fig. 5, C and D; thin arrows). A sense epimorphin/syntaxin 2 control RNA probe hybridized to fetal day 15 intestine demonstrated the specificity of the probe; no specific signal is seen (Fig. 5, E and F).

Fig. 3. Quantitative analysis of ontogeny of epimorphin mRNA expression. Data are expressed as mean relative mRNA level ± SE. Fetal intestinal values (A) were derived from n = 3–6 independent RNase protection assays, and postnatal values (B) were from n = 3 independent RNase protection assays. Fetal RNA samples were prepared from whole intestine from fetal days 14, 15, 16, and 17, and from jejunum only for fetal days 18, 19, 20, and 22. All postnatal RNA was prepared from jejunum.

Fig. 4. Ontogeny of epimorphin expression along the horizontal axis of the intestine. RNase protection assay was performed as described in legend to Fig. 1. RNA samples were prepared from whole intestine from G14 through G17 or were divided into proximal (p) and distal (d) segments for G18, G19, and G20. Intestine harvested at birth (G22) (B) was further subdivided into proximal, mid- (m), and distal segments because of its larger size. 1wp, 1 wk proximal segment; 6wp, 6 wk proximal segment.
By P1, well-defined villi were present and epimorphin expression was found in the villus mucosal lamina propria and muscularis layers (Fig. 6, A and B, black arrows). There was no expression in the villus-associated epithelium (white arrows) or in the crypt epithelium. Probe specificity was again demonstrated by using a sense RNA probe hybridized to P1 intestine (Figs. 6, C and D). White grains indicating the presence of epimorphin mRNA were not found in the lamina propria (thick black arrows) or on the villus (thin black arrows). The large white cells noted at the base of the villi had no specific grains overlying them. As we have shown previously, these intestinal cells are intensely refractile under dark-field microscopy, leading to the white color (11), but inspection of multiple sections revealed no specific grains on these cells.

Epimorphin/syntaxin 2 mRNA levels increase after isograft implantation. Intestinal isograft implantation techniques have been used by our laboratory and
others to examine the influence of luminal contents on spatial differentiation in the intestine, from duodenum to colon and from crypt to villus tip (16, 37, 38). When fetal intestine is removed from its normal environment and isografted into the subcutaneous tissue of a host rat, it develops into a well-differentiated intestine that, despite the absence of luminal substances, expresses enterocytic and enteroendocrine gene products in an appropriate region-specific manner.

We have used this isograft model to begin to determine whether or not epimorphin/syntaxin 2 may play a role in villus repopulation after ischemic injury to neonatal bowel after nascent villi have formed. G19 jejunum was implanted in the subcutaneous space of the host rat and examined for expression of epimorphin/syntaxin 2 mRNA in the lamina propria of postnatal intestine. Cell-specific expression of epimorphin was assessed in P1 A-D and P6 jejunum E-H. Antisense probe was hybridized to P1 A, B, and P6 sections E, F. Sense control probe was hybridized to P1 jejenum C, D, or P6 jejenum G, H. In situ hybridization analyses were performed as described for Fig. 5. A: P1 jejunum, viewed by dark-field microscopy. Black arrows show signal in villus core and mucosal lamina propria. Thin white arrows indicate epithelium that does not express epimorphin. B: same section as in A, viewed by light microscopy. C: P1 jejenum viewed under dark-field microscopy, hybridized to sense control probe. No specific lamina propria signal (thick black arrows) or epithelial signal (thin black arrows) is seen. Cells at base of villi, which appear white under dark-field microscopy, do not exhibit white grains specific for epimorphin mRNA. D: same section as in C, viewed under light microscopy. E: section of P6 jejenum, hybridized to antisense epimorphin cRNA probe, viewed under dark-field microscopy. Thick white arrows show large mesenchymal lamina propria cells that express epimorphin. Thin white arrows indicate negative epithelium. F: high-power view of P6 jejenum hybridized to antisense epimorphin cRNA probe, viewed under dark-field microscopy. Thick white arrows point to large cells positive for epimorphin mRNA; thin white arrows point to negative epithelium. G: P6 jejenum hybridized to sense control probe (dark-field). No specific signal is seen in epithelium (thin white arrow) or in lamina propria (thick white arrows). H: same section as in G, under light microscopy. Magnification: ×320 (A and B); ×250 (C and D); ×100 (E); ×200 (F-H).
young adult male rats. At 24 h after implantation, we confirmed that isografts demonstrate intraluminal hemorrhage with villus loss and denuding of the intestinal epithelium. However, surviving cells proliferate and nascent villi reappeared by 48–72 h after implantation (data not shown).

The expression of epimorphin/syntaxin 2 was examined in the repairing bowel 48–96 h after implantation. Epimorphin/syntaxin 2 mRNA was readily detectable even by Northern blot hybridization as early as 48 h after implantation (Fig. 7A). Furthermore, mRNA levels increased by 96 h after implantation, coincident with the formation of many new villi (Fig. 7B). To compare epimorphin/syntaxin 2 expression in isografts with the equivalently aged normal intestine, total RNA was isolated from a G22 pup (birth) and from a fetal day 19 isograft harvested 96 h after implantation (thus equivalent to G22 intact intestine; see MATERIALS AND METHODS), and a Northern blot was prepared. Epimorphin/syntaxin 2 mRNA levels were sevenfold higher in the isografted intestine than in the equivalent segment of intact intestine harvested from a pup at birth (Fig. 7C).

Due to the relatively small size of the grafts, direct histological correlation of crypt-villus formation in the same grafts from which RNA was prepared was not possible. However, hematoxylin and eosin staining was performed on other grafts harvested at the same time points, and a marked increase in the size and number of villi was noted by 72–96 h (data not shown).

Epimorphin/syntaxin 2 mRNA is expressed in the lamina propria of the adult gut, and mRNA levels decrease in the early stages of intestinal adaptation after small bowel resection. Epimorphin mRNA levels declined to low but detectable levels in the adult intestine, as shown in Fig. 2. To begin to determine the function of epimorphin in mature intestine, we determined cell-specific expression in adult gut. As shown in Fig. 6, E and F, a subpopulation of large lamina propria cells expresses epimorphin in the adult gut. The diffuse expression in the mucosal and submucosal lamina propria noted in fetal and early postnatal life is no longer apparent. When stained with hematoxylin and eosin, these cells resemble macrophages. Expression in this cell type is consistent with previous data indicating that macrophages produce syntaxin 2, which functions in phagocytic pathways (17). Comparison of these sections with those in Fig. 6, G and H, hybridized to the sense control probe, shows the specificity of the signal. Although large white cells are seen, there are no specific white grains overlying them.

After loss of functional small bowel surface area, the gut epithelium undergoes marked morphological and functional changes in a remarkable process of adaptation. In the early stage of the adaptive response (i.e., the first 48 h after resection), there is a rapid increase in the crypt cell production rate (12, 18, 48). In addition, we have shown that the enterocytic expression of several genes involved in lipid uptake and processing increases rapidly after resection (39). In the second stage of adaptation, which peaks 1–2 wk after resection, enhanced crypt cell production rates persist, result-

Fig. 7. Intestinal isografts express epimorphin/syntaxin 2 during villus regeneration after implantation. G19 intestine was implanted as an isograft in normal Fisher rat hosts as described in MATERIALS AND METHODS. Isografts were harvested at 48, 72, and 96 h after implantation, and total RNA was prepared from each graft separately. Loss of nascent G19 villi at 24–48 h after implantation, followed by regeneration after implantation (at 72–96 h), was verified by routine hematoxylin and eosin staining of grafts (data not shown). A: Northern blot hybridization of total RNA prepared from individual isografts harvested at various times after implantation. Total RNA (20 μg) was electrophoresed on an agarose formaldehyde gel and transferred to nylon membranes. Blots were probed with [32P]dCTP-radiolabeled 350-bp epimorphin cDNA, and autoradiography was performed. Each lane represents total RNA isolated from an individual isograft. B: quantitation of relative epimorphin mRNA levels in isografts. Autoradiographic bands were quantitated by scanning laser densitometry and were normalized to the ethidium bromide signal of the 18S ribosomal band. Data are expressed as mean relative densitometric units for each time point (n = 3–4 grafts per time point). C: Northern blot hybridization of total RNA prepared from intact jejunum harvested at birth or from a fetal day 19 isograft harvested at 96 h after implantation. A sevenfold increase in epimorphin mRNA expression was seen in the isograft compared with normal intestine.
AND METHODS. Epimorphin/syntaxin 2 mRNA levels were detected in adaptation early after small bowel resection, epimorphin/syntaxin 2 mRNA expression was unchanged in resected compared with sham-resected control ileum. Rats were subjected to 70% small intestinal resection as described in MATERIALS AND METHODS. Epimorphin/syntaxin 2 mRNA levels were detected in adaptive ileum or control sham-resected ileum by RNase protection assay and normalized by simultaneous hybridization to an 18S probe, as described in Figs. 1 and 2. Digitized images were quantitated as described in MATERIALS AND METHODS. Data are expressed as the ratio of resected to sham-resected intestine, in arbitrary units (resected units/sham-resected units).

DISCUSSION

We have shown that epimorphin/syntaxin 2 mRNA expression is developmentally regulated in the rodent small intestine. mRNA levels increase in the fetal gut coincident with lumen and villus formation. A second peak of expression occurs in the suckling period during P1 and P2, followed by a decline at weaning. Abundant expression can be detected in fetal mesenchymal cells that underlie the nascent villus epithelium. Gut endoderm and newly differentiating enterocytes do not appear to express epimorphin/syntaxin 2 mRNA. We also found that epimorphin/syntaxin 2 mRNA expression is increased in the isografted intestine during villus repopulation after ischemic injury to its nascent epithelium. Yet in the adult intestine, during intestinal adaptation early after small bowel resection, epimorphin/syntaxin 2 mRNA levels are suppressed in adaptive ileum compared with sham-resected controls.

This study is the first to examine in detail the cell-specific and temporal patterns of expression of epimorphin during ontogeny. Our in situ hybridization analyses are particularly important in establishing the cell-specific expression of epimorphin in fetal intestine, since there are conflicting data from studies of other tissues. For example, epimorphin mRNA has been shown to be present in ameloblasts, which are epithelium-derived cells (31). Also, immunohistochemical analyses have also yielded variable results, since the epimorphin antibody cross-reacts with syntaxin 1 (7). Our data agree with those reported originally by Hirai et al. (22), describing epimorphin as a mesenchymal skin and lung protein in fetal tissues.

The patterns of epimorphin/syntaxin 2 expression during gut development and in the isograft model of villus repair suggest that its critical function in the intestine is duringontogeny, specifically in regulating lumen formation and villus morphogenesis. In particular, the persistently increased epimorphin mRNA levels in fetal day 18 distal gut compared with fetal day 18 proximal gut also support a role in morphogenesis, since the formation of the villus proceeds in a wave-like fashion from proximal to distal gut during fetal life. This presumed function is consistent with previous studies demonstrating that epimorphin expression can be upregulated by retinoic acid, a potent morphogen (34), and with experiments in other epithelia suggesting that epimorphin may be an important mesenchymal regulator of morphogenesis. For example, transfection of epimorphin into NIH/3T3 fibroblasts followed by coculture with fragments of lung endoderm induces the formation of air sacs that appear normal, with a large lumen (22). In comparison, native fibroblast-endoderm cocultures resulted in small tubular structures without a dominant lumen. Also, in fetal organ cultures, air sac formation from fetal lung and hair follicle formation from fetal lip skin were blocked by incubation with epimorphin antibody. The formation of a central lumen is important in gut as well as lung morphogenesis, and the morphology of the hair follicle (in which there is a proliferative compartment that gives rise to well-differentiated cells) mimics the organization of the crypt-villus axis. Thus the existence of general morphogenetic processes in most epithelial organs suggests that common molecules may play similar roles in different tissues. It is likely that epimorphin/syntaxin 2 functions in the same manner in the intestine. Studies in which intestinal endoderm or cultured intestinal cells are grown with epimorphin-transfected fibroblasts could provide further support for this hypothesis.

The mechanism by which epimorphin/syntaxin 2 influences tissue morphogenesis is not well understood. Sequence analysis of the encoded epimorphin protein revealed homology to the syntaxin family (3, 44). These are integral membrane proteins that are thought to act as target membrane receptors during secretory vesicle docking and fusion. There are multiple isoforms ex-
pressed in different tissues. This family was defined by low-stringency screening of rat cDNA libraries, using syntaxins 1A and 1B (neuronal syntaxins) as probes (3). Syntaxin 2 is the rat homologue of epimorphin, sharing ~60% amino acid homology with syntaxins 1A and 1B. Most studies have shown that syntaxin 2 is located on the plasma membrane, although there are multiple isoforms that differ in their carboxy terminals, and one in particular has a truncated domain of insufficient length to span the membrane (3). When transfected into Madin-Darby canine kidney cells, syntaxin 2 targets both apical and basolateral membranes (30), although in pancreatic acinar cells it was found in the apical plasma membrane and in the cytoplasm (14). It is postulated to be important in targeting zymogen granules to the apical membrane during regulated secretion (14) and appears to play a role as a calcium-sensitive regulator of exocytosis in pancreatic acinar cells (13). These proteins have generally been found to have an intracellular orientation (3). However, when syntaxin 1 was expressed in Xenopus oocytes, it exhibited glutamate receptor properties, with an extracellular orientation (5, 42). Also, Hirai (20) obtained data supporting an extracellular domain for epimorphin. Oka and Hirai (33) have also suggested a different role for epimorphin, indirectly regulating cell proliferation and mesenchymal cell migration. A specific NH2-terminal modified fragment of the epimorphin molecule inhibited proliferation and induced morphogenetic changes in human umbilical vein endothelial cells (HUVECs) cultured in collagen, resulting in their rearrangement into circular venule-like formations. In addition, HUVECs adhered to dishes coated with this same modified fragment. The effects of this polypeptide on the morphology of cultured intestinal epithelial cells have not yet been examined.

Thus the precise localization and function of epimorphin, particularly during ontogeny, have not been clarified. How can these disparate data, suggesting very different roles for epimorphin/syntaxin 2 (i.e., regulation of vesicle docking and fusion vs. epithelial morphogenesis) be reconciled? One unifying hypothesis is that epimorphin may play a role in morphogenesis as a vesicle docking protein, by regulating the secretion of diffusible morphogens such as hepatocyte growth factor (44). Recent studies by Hirai et al. (21) indicate that growth factors such as hepatocyte growth factor and epidermal growth factor require the presence of epimorphin to induce growth and branching morphogenesis of mouse mammary epithelial cells. A second possibility is that the role of this gene product is different in developing compared with adult intestine. The function of syntaxins in other in vivo systems provides support for this premise. For example, the Drosophila homologue of syntaxin 1, syx, is expressed at the earliest stages of Drosophila development and is found on newly forming membranes (40). Mutations of syx prevented normal cellularization of the Drosophila embryo, which is dependent on massive membrane addition and the migration and segregation of cytoskeletal proteins (6). In addition, syntaxin 1 is developmentally regulated in neuronal tissues. Its localization changes during rat neuronal development (2, 15, 32). In fact, it is expressed before synapse formation, again suggesting a role for this protein in ontogeny that is independent of regulating synaptic transmission, its presumptive function in the adult brain (15, 32). The very different cell-specific patterns of expression found in developing vs. mature intestine suggest that epimorphin/syntaxin 2 plays distinct roles depending on the ontogenic stage.

Epimorphin/syntaxin 2 mRNA levels declined in adapting compared with sham-resected control intestine 4–16 h after resection and then returned to basal adult levels. Decreased expression does not appear to be due to a general suppressive effect of surgery, since mRNA levels decreased in comparison with sham-operated controls that underwent identical anesthesia as well as transection and reanastomosis. We have also shown that expression of a variety of other intestinal gene products increases early in adaptation (11, 38). Little is known about the molecules that may regulate epimorphin expression, so it is difficult to postulate a specific humoral, neuroendocrine, or other intestinal product that may mediate the decrease in expression. It has been suggested that epimorphin has an antiproliferative effect, since proliferation of HUVECs, an endothelial cell line, is inhibited when these cells are cultured on epimorphin-coated wells (33). If this is indeed true, suppression of epimorphin expression might be required to permit enhanced cell proliferation during adaptation, particularly in the crypt and submucosal compartments. However, these data are difficult to interpret in light of other findings regarding the role of epimorphin/syntaxin 2 as a vesicle docking and/or fusion protein in mature cells and our discovery that its expression in adult gut is confined to lamina propria cells, although some positive cells do lie in close proximity to the crypts.

A major gap in the understanding of mesenchymal-epithelial interactions during intestinal epithelial morphogenesis is that critical mesenchymal regulatory genes are still largely unknown. Although its precise function in intestinal ontogeny is unclear, the unique cellular and ontogenic patterns of expression of epimorphin make it a valuable model gene for identifying mesenchymal trans-acting factors that regulate its expression. Once identified, the role of these factors in the gut and in general epithelial tissue morphogenesis can be explored, similar to experiments in which the function of critical intestinal endodermal-epithelial trans-acting factors (43, 45) or mesenchymal factors in other epithelial tissues [e.g., isl 1 in pancreas (1)] has been deciphered. In addition, future experiments that elucidate the role of epimorphin/syntaxin 2 in developing and mature mammalian tissues may provide novel insights into how a specific ontogenic stage influences the function of individual proteins.

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