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Peptide YY expression is an early event in colonic endocrine cell differentiation: evidence from normal and transgenic mice

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SUMMARY

The hormone peptide YY is produced by endocrine cells in the pancreas, ileum and colon. We have previously shown that peptide YY is coexpressed in all four islet cell types in the murine pancreas when they first appear, suggesting a common peptide YY-producing progenitor. In the colon, peptide YY has been frequently identified in glucagon-expressing L-type endocrine cells. Characterization of colonic endocrine tumors in transgenic mice expressing simian virus 40 large T antigen under the control of the peptide YY gene 5′ flanking region revealed tumor cells producing not only peptide YY and glucagon, but also neurotensin, cholecystokinin, substance P, serotonin, secretin, and gastrin. This suggested that multiple enteroendocrine lineages were related to peptide YY-producing cells. Subsequent examination of the ontogeny of colonic endocrine differentiation in nontransgenic mice revealed that peptide YY was the first hormone to appear during development, at embryonic day 15.5. Between embryonic days 16.5 and 18.5, cells expressing glucagon, cholecystokinin, substance P, serotonin, secretin, neurotensin, gastrin and somatostatin first appeared and peptide YY was coexpressed in each cell type at this time. Peptide YY coexpression continued in a significant fraction of most enteroendocrine cell types throughout fetal and postnatal development and into adulthood, with the exception of serotonin-producing cells. This latter population of cells expanded dramatically after birth with rare coexpression of peptide YY. These studies indicate that expression of peptide YY is an early event in colonic endocrine differentiation and support the existence of a common progenitor for all endocrine cells in the colon.

Key words: peptide YY, peptide tyrosine tyrosine, transgenic mice, enteroendocrine differentiation, large T antigen, mouse

INTRODUCTION

The hormone peptide YY (PYY) is synthesized by endocrine cells in the colon, ileum, islets of Langerhans, antrum of the stomach, and in small numbers of neurons in the brainstem. PYY was isolated and purified on the basis of its N-terminal tyrosine and C-terminal tyrosine amide residues (Tatemoto, 1982a). The hormone has widespread inhibitory effects on gastrointestinal and pancreatic function including secretion of gastric acid, exocrine pancreatic secretion, as well as gastric and intestinal motility (Lundberg et al., 1982; Pappas et al., 1986, 1985). Physiologic release of PYY is not well understood although intraluminal fat appears to be a major enteral secretagogue (Aponte et al., 1985, 1988; Greeley et al., 1989a). In addition, neuroendocrine signals arising from the foregut also appear to stimulate release of this hormone (Greeley et al., 1989b). PYY is closely related to two other regulatory peptides, pancreatic polypeptide and neuropeptide Y (Tatemoto, 1982b). Examination of the precursor structure of PYY deduced from its cloned cDNA, as well as the structural organization of the PYY gene, indicate that this peptide belongs to a multigene family that arose from the duplication of a common ancestral gene (Krasinski et al., 1991; Leiter et al., 1987).

In the pancreas, PYY has been localized to glucagon-producing α cells of several species (Ali-Rachedi et al., 1984; Bottcher et al., 1993, 1984; Lozano et al., 1991; Nilsson et al., 1991; Rombout et al., 1986). We have previously examined the ontogeny of PYY expression in the developing murine pancreas of both normal mice and transgenic mice expressing simian virus 40 large T antigen (Tag) under control of the rat PYY gene promoter (Upchurch et al., 1994). Transgenic mice developed insulinomas, raising the possibility that PYY cells may be related to additional islet lineages besides α cells. In nontransgenic mice, we identified PYY immunoreactivity in the earliest islet progenitors in the fetal pancreas at embryonic day 9.5, and in each of the four established islet cell types when they first appeared and throughout development. The coexpression of PYY in all islet cell types as they first emerge suggested that they arise from a common PYY-producing progenitor cell (Upchurch et al., 1994).

PYY-like immunoreactivity in the intestine has been identified in most, if not all, glucagon-producing endocrine cells in the colon and ileum (Ali-Rachedi et al., 1984; Bottcher et al., 1986,

1984; El-Salhy et al., 1983). Ultrastructural studies have identified PYY and glucagon in the same secretory granules. Expression of the PYY gene occurs before birth in the ileum and colon in the rat, antedating the presence of its presumed enteral secretagogues (Krasinski et al., 1991). The abundance of PYY and glucagon gene transcripts parallel one another during development in the rat. Both mRNAs were detectable by day 17 of gestation, rising to adult levels 3 days later (2 days before birth). The presence of PYY before birth suggests its expression may be an early event in enteroendocrine differentiation.

More recent work using multiple label immunohistochemistry revealed that PYY is frequently coexpressed in colonic enteroendocrine cells producing neurotensin, cholecystokinin, and glucagon (Roth et al., 1992). In contrast, PYY immunoreactivity could not be identified in enteroendocrine cells expressing either substance P or serotonin, although serotonin was frequently coexpressed in substance P-producing endocrine cells. On the basis of coexpression studies, two distinct lineage branches were proposed for endocrine cells in the colon (Roth et al., 1992). The first consisted of cells producing PYY, glucagon, neurotensin, and cholecystokinin whereas cells producing serotonin and substance P, constituted a second differentiation pathway. Studies in transgenic mice expressing human growth hormone under control of the 500 bp liver fatty acid binding protein gene 5′ flanking region were also interpreted to support the existence of two distinct endocrine differentiation pathways in the colon. In these animals, the reporter gene was frequently coexpressed in enteroendocrine cells producing PYY, glucagon, cholecystokinin, and neurotensin but not in serotonin or substance P-producing cells (Roth et al., 1992).

We have examined the major colonic endocrine cell types to determine if they arise from a PYY-producing progenitor. Two approaches were utilized in this study. First, transgenic mice were generated which expressed a viral oncoprotein under control of the PYY gene 5′ flanking region, resulting in the development of well-differentiated colonic endocrine tumors. In the second approach, we examined the ontogeny of enteroendocrine cells in the colon of normal nontransgenic mice. Three new findings arose from these studies. First, all of the hormones commonly seen in colonic endocrine cells were expressed in tumors of PYY-Tag transgenic mice. Second, PYY was the first hormone to appear in normal colonic endocrine differentiation. Finally, PYY was coexpressed in each of the major enteroendocrine cell types when it first appeared in the developing colon.

MATERIALS AND METHODS

Transgene construction and production of transgenic mice

A 2.8 kb *Bgl*II/*Xho*I fragment (−2800 to +32 bp of exon 1) of the rat PYY gene was cloned 5′ to a *Stu*I/*Bam*HI fragment containing the early region of simian virus 40 which includes sequences encoding large T antigen (Upchurch et al., 1994). The transgene was excised and separated from vector sequences and used to generate 16 founder transgenic mice by standard pronuclear microinjection of $B_6D_2F_1 \times B_6D_2F_1$ mouse embryos (Hogan et al., 1986). Transgenic founders were identified by dot blot hybridization of a Tag probe to tail DNA. By 3 to 4 months of age, 12 founder mice became wasted and died shortly thereafter. At autopsy, colonic enteroendocrine tumors were identified in seven of these. Five founders could not be analyzed due to extensive autolysis postmortem, although they presumably succumbed from tumors. The remaining four founders did not appear to express the transgene. Pedigrees were established from male founders and maintained on an outbred CD-1 background.

Immunohistochemistry

Fetal tissues were obtained from pregnant mothers using noon on the day of the vaginal plug as day 0.5 of gestation. For histological examination, fresh tissues were fixed in Bouin's fixative, embedded in paraffin, and sectioned at 4 μm. Sections were incubated with primary antisera against Tag, glucagon and serotonin as described previously (Lopez et al., 1995). Additional primary antisera included: guinea pig anti-PYY (#4120, G. Aponte, U. C. Berkeley, 1:5000 immunoperoxidase (IP), 1:400 immunofluorescence (IF)), rabbit anti-neurotensin (IncStar, 1:4000 IP, 1:300 IF), rabbit anti-secretin (#13/4, D. Gossen, Brussels, 1:1500 IP, 1:500 IF), rabbit anti-somatostatin (R. Lechan, Boston, 1:300 IF), rabbit anti-cholecystokinin (#RPZ-7.1 W. Chey, Rochester, N.Y., 1:2500 IP), rabbit anti-gastrin (#2604, J. Rehfeld, Copenhagen, 1:1000 IP, 1:200 IF), rabbit anti-substance P (R. Kream, Boston, 1:1000 IP, 1:100 IF). Controls included non-immune primary sera, mismatched primary and secondary antisera, known positive sections, and absorption with specific and heterologous antigens. In all cases, absorption of primary antisera with homologous peptides abolished all immunostaining, and absorption with heterologous antigens did not affect specific immunostaining. The antiserum to cholecystokinin (anti-CCK33) was preabsorbed with 10 μM gastrin to avoid crossreactivity with intracellular gastrin-immunoreactive material. Antisera 2604 (J. Rehfeld, Copenhagen), directed against the N terminus of gastrin, has minimal cross-reactivity with cholecystokinin and was used to detect gastrin immunoreactivity (Lüttichau et al., 1993; Rehfeld et al., 1972).

Immunoperoxidase labeling was performed with a Vectastain ABC kit (Vector Labs) using DAB (diaminobenzidine) precipitation or AEC (aminoethylcarbazole) for detection. Tag detection was enhanced with subtilisin pre-digestion and DAB-nickel chloride precipitation (Rindi et al., 1990). Tissues were counterstained with dilute hematoxylin or methyl green. Hormone colocalization was determined by double immunofluorescent labeling with FITC-, Texas red, or Cy3-conjugated donkey anti-guinea pig and anti-rabbit IgG secondary antibodies which were immunoadsorbed for multiple labeling (Jackson ImmunoResearch). Single-labeled sections incubated with mis-matched secondary antibodies showed no immunostaining, confirming the specificity of the secondary antisera. Immunofluorescence was observed on an Olympus BH-2 microscope fitted with appropriate barrier filters to achieve complete color separation for the different fluorophores. Images were recorded either by standard photomicroscopy or with an Optronics color, low light videocamera using Optimas Bioscan software.

RESULTS

Pedigrees were established from five of the seven transgenic founder mice which developed colonic tumors. Each of the pedigrees exhibited a similar phenotype, with multiple enteroendocrine tumors arising throughout the large intestine. The results presented here are from one representative pedigree. Individual enteroendocrine cells with nuclear Tag staining were seen as early as embryonic day (E)16.5 and later in gestation appeared occasionally in pairs or small clusters of 3-4 cells. Multiple label immunohistochemistry revealed that

almost all of these cells had cytoplasmic staining for both PYY and glucagon similar to normal intestinal L-type endocrine cells. (Fig. 1A,B). By 3 to 6 weeks of age, multiple small mucosal neoplasms with nuclear staining for Tag were evident throughout the large intestine (Fig. 1C,D). Analysis of these small tumors by immunohistochemistry revealed that these were well-differentiated endocrine tumors. Most cells in the tumors showed intense immunostaining for PYY, glucagon, cholecystokinin, or neurotensin (Fig. 1C-F).

When examined at two to three months of age, small tumors had coalesced into bulky obstructing tumors visible without magnification (Fig. 2A). Invasion into the muscularis layer and surrounding lymphatics was regularly seen at this stage of tumorigenesis and most cells in these large tumors revealed intense nuclear staining for Tag (Fig. 2B). Cells in large tumors of older transgenic mice continued to stain for PYY and glucagon (Fig. 2C,D). In addition, smaller foci of tumor cells

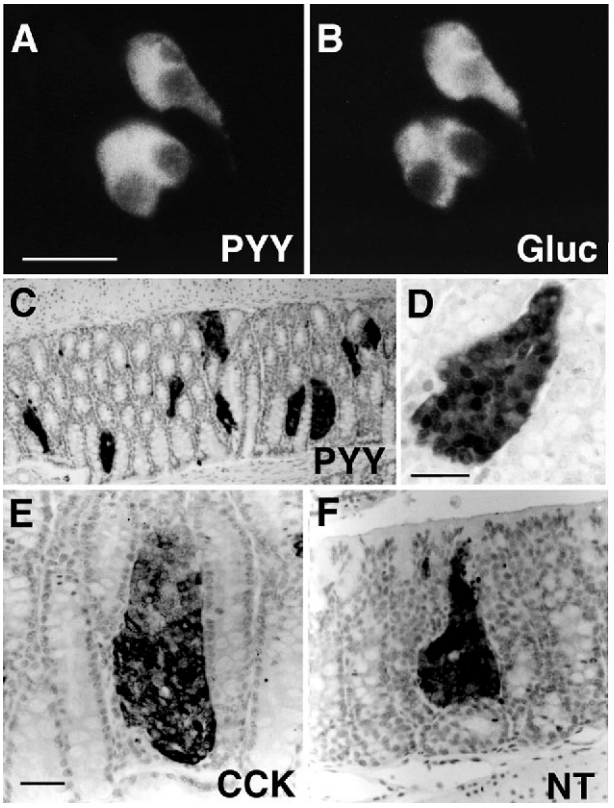

Fig. 1. Development of multiple well-differentiated colonic enteroendocrine tumors in transgenic mice. (A,B) Double immunofluorescent staining of three adjacent cells in a 1 day old transgenic mouse, presumably the earliest stage of abnormal proliferation of colonic PYY cells. (A) PYY (FITC) and (B) glucagon (Texas red) are coexpressed in all three cells. Bar, 10 μm. (C) Photomicrograph showing several small intraepithelial tumors with cytoplasmic staining for PYY from a 6 week old transgenic mouse. (D) Double immunoperoxidase staining of a small enteroendocrine tumor showing black, nuclear Tag staining in most cells that colocalizes with lighter cytoplasmic immunostaining for PYY, in a 6 week old transgenic mouse. Bar, 25 μm. (E,F) Small intraepithelial tumors in 6 week old transgenic mice with cytoplasmic staining for either cholecystokinin (E) or neurotensin (F). Bar, 25 μm. PYY, peptideYY; Gluc, glucagon; CCK, cholecystokinin; NT, neurotensin.

revealed immunostaining for secretin, gastrin, substance P, and serotonin (Fig. 2E-H), as well as for neurotensin and cholecystokinin. At all stages of tumor development, colonic tumor cells failed to stain for several other neuroendocrine peptides including gastric inhibitory polypeptide (GIP), somatostatin, motilin, vasoactive intestinal peptide (VIP), pancreatic polypeptide, and insulin.

Nontransgenic mice were examined to determine whether PYY-producing cells in the colon also coexpress other hormones during normal murine enteroendocrine development. Colonic sections from mice between E14.5 and adult ages were examined using multiple label immunohistochemistry and the results are summarized in Fig. 3. Endocrine cells were first detected about E15.5 of gestation in the fetal colon. At this stage of development these few cells produced PYY but none of the other major gut hormones (Figs 3, 4A). One day later many more PYY cells were identified, of which approximately half showed glucagon immunoreactivity (Fig. 4B,C). For the remainder of gestation and throughout development most PYY cells coexpressed glucagon. Other endocrine cell types first appeared at either E17.5 (cholecystokinin, substance P, serotonin, secretin) or E18.5 (neurotensin, gastrin, somatostatin). While examining tissues from developing mice, we noted that gastrin and secretin, two hormones not generally appreciated as products of colonic endocrine cells, could be seen around the time of birth (Fig. 5). Enteroendocrine cells expressing gastrin were observed between E18.5 and postnatal day 1, but were not detectable thereafter. Most, if not all, gastrin-containing cells coexpressed PYY (Fig. 5). Cells staining for secretin immunoreactivity were first seen at E17.5 and were numerous by birth. Most coexpressed PYY (Fig. 5). By postnatal day 10, very few secretin containing cells could be identified and by adulthood were rarely seen. Most neurotensin immunoreactive cells also contained PYY (Fig. 5). About half of the small number of somatostatin cells (Fig. 5) and cholecystokinin cells (Roth et al., 1992) in the colon coexpressed PYY. Endocrine cells expressing GIP, motilin, pancreatic polypeptide, and insulin were not seen at any stage of development in the colon, consistent with others' observations (Roth et al., 1990).

Of particular interest was the unanticipated presence of substance P- and serotonin-expressing cells in tumors originating from expression of Tag under control of the PYY gene. The coexpression of these latter two hormones with PYY was examined by multiple label immunohistochemistry in both small, well-differentiated tumors and in normal tissue (Fig. 6). Tumor cells expressing substance P frequently also showed PYY immunoreactivity (Fig. 6A-C). A smaller percentage of serotonin-staining tumor cells also stained for PYY (Fig. 6D). In light of the observed expression of substance P and serotonin in transgenic mouse tumors, we reexamined substance P and serotonin-expressing cells in the developing colon of normal nontransgenic mice for colocalization of PYY, since it had been previously reported that PYY was not coexpressed in these cell types. Small numbers of enteroendocrine cells expressing serotonin and substance P first appeared in the colon at E17.5 (Fig. 3). PYY was coexpressed in the majority of substance P-producing cells (Fig. 6E,F) and in smaller numbers (approx. 15-20%) of serotonin-expressing cells at this time (Fig. 6G,H). A significant proportion (approx. 40%) of

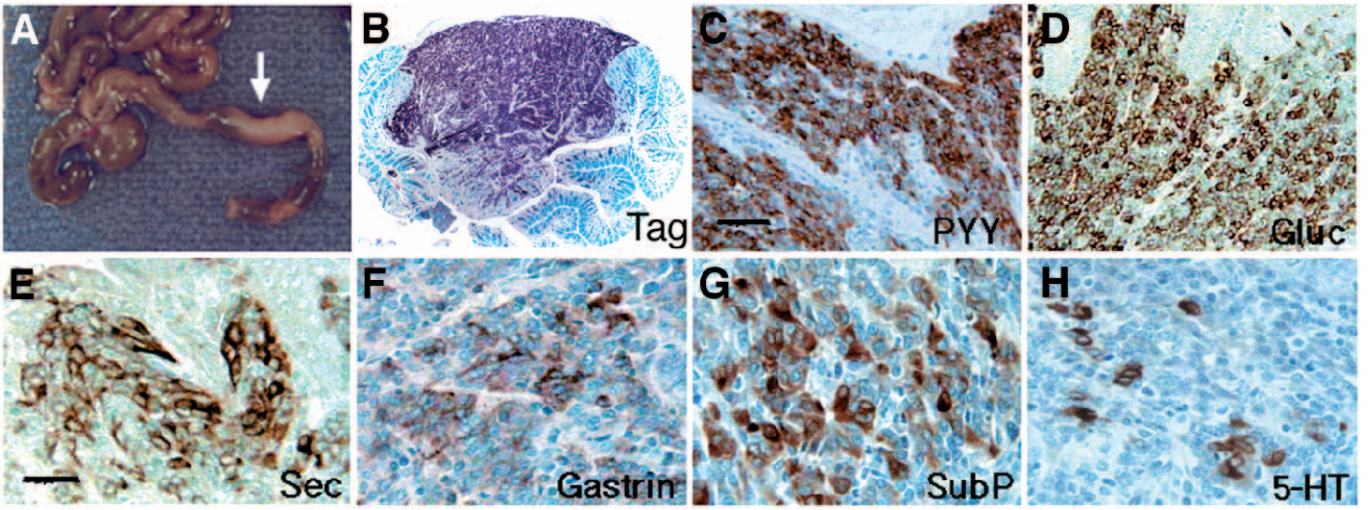

Fig. 2. Presence of multiple hormones in large enteroendocrine tumors of transgenic mice. (A) Colon, cecum, terminal ileum from a 10 week old transgenic mouse revealing thickening of the proximal colon (arrow) from endocrine carcinoma. (B) Low power photomicrograph showing black nuclear immunostaining for Tag in a large tumor found in the colon shown in A. (C-H) Cytoplasmic immunoperoxidase staining of many but not all cells for (C) PYY and (D) glucagon (Gluc). Bar, 50 μm. Smaller clusters of cells showed cytoplasmic staining for (E) secretin (Sec); (F) gastrin; (G) substance P (Sub P); and (H) serotonin (5-HT). Bar, 25 μm.

substance P-containing enteroendocrine cells continued to coexpress PYY in the adult mouse colon. In contrast, PYY was rarely seen in serotonin-expressing cells after birth, despite the rapid expansion of this cell population to eventually become the most abundant enteroendocrine cell type.

DISCUSSION

The work described here presents several novel observations with respect to the development and differentiation of endocrine cells in the large intestine. First, we have identified PYY as the first major gut hormone to be produced in the colon during development. Second, we have shown that all enteroendocrine cell types in the large intestine appear to coexpress PYY at the time they first differentiate. Finally, the development of tumors expressing multiple hormones, in transgenic mice expressing a viral oncoprotein under control of the PYY gene, further suggests that PYY cells are multipotential. It is well established that all epithelial cells of the intestine, including enteroendocrine cells, arise from a common totipotent stem cell. However, it is not known whether all enteroendocrine cells arise from a common multipotential progenitor following commitment to the endocrine differentiation pathway. Our developmental studies suggest that most if not all of the major endocrine cell types in the mouse colon arise from a common endocrine progenitor which produces PYY. Later in development, the serotonin-expressing cell population expands to become the most frequent endocrine cell type, with cells rarely coexpressing PYY. The loss of PYY expression may indicate that serotonin cells are directed to a differentiation pathway distinct from the remaining endocrine cell types which continue to coexpress PYY. These new observations suggest that some models for enteroendocrine differentiation need to be revised (Fig. 7).

In this study we have used 2.8 kilobases of 5′ flanking

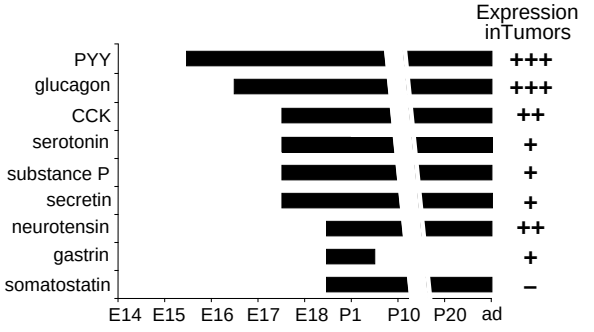

Fig. 3. Emergence of colonic endocrine cell types. Presence of cells expressing different hormones was determined at the indicated stages of development in normal CD1 mice. Column at the right indicates relative frequency of hormone-expressing cells in tumors of adult transgenic mice. E, embryonic day; P, postnatal day; ad, adult.

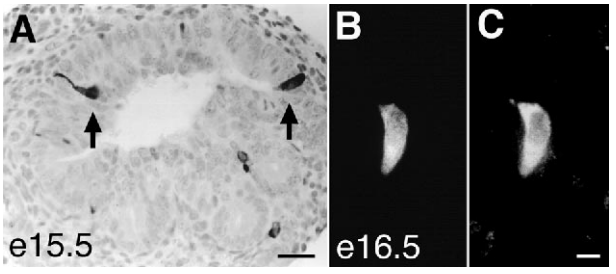

Fig. 4. PYY and glucagon-producing cells appear earliest in the colon. (A) Transverse section of CD1 mouse colon at E15.5 with immunoperoxidase staining of two cells for PYY (arrows). Bar, 20 μm. (B,C) Double immunofluorescent staining of a single cell at E16.5 showing colocalization of PYY (B) and glucagon (C) using FITC- and Cy3-conjugated secondary antibodies respectively. Bar, 5 μm.

sequence of the rat PYY gene to direct the expression of a viral oncoprotein, SV40 large T antigen, to endocrine cells of the large intestine of transgenic mice. Expression of the transgene was directed to the proper endocrine cell types in the colon at the appropriate stage of development. Analysis of endocrine tumors that developed in transgenic mice indicated that tumor cells shared many characteristics of normal colonic endocrine cells, producing PYY, glucagon, neurotensin and cholecystokinin. In addition, tumors in PYY-Tag transgenic mice revealed that tumor cells were capable of producing several additional hormones not generally associated with L-type enteroendocrine cells including substance P, serotonin, gastrin, and secretin. The tumors in these transgenic mice share a number of similarities with colonic enteroendocrine tumors arising in transgenic mice expressing large T antigen under the control of 2 kb of glucagon gene 5′ flanking sequence (Lee et al., 1992). The glucagon-Tag mice developed multiple colonic neoplasms expressing glucagon and PYY in most cells as well as small foci of cells expressing cholecystokinin. However, these tumors did not appear to produce the other gut hormones described in the PYY-Tag tumors. The expression of a wider spectrum of neuroendocrine genes in the PYY-Tag tumors could be related to the earlier onset of PYY gene expression relative to enteroglucagon in the developing colon. Thus in PYY-Tag transgenic mice, the viral oncoprotein may be expressed at an earlier stage of endocrine cell differentiation than in glucagon-Tag mice, prior to segregation of different enteroendocrine cell types.

A relatively limited number of studies have explored lineage relationships among the different enteroendocrine cell types populating the large intestine. In adult mice, pairwise examination of coexpression of different neuroendocrine gene products in the colon revealed that PYY, glucagon, cholecystokinin and neurotensin were frequently coexpressed in single cells, leading to the proposal that these cell types make up one lineage branch for endocrine differentiation (Roth et al., 1992). Substance P and serotonin were frequently identified together in single cells but never with the preceding four hormones. These results led to the hypothesis that substance P and serotonin producing cells constituted a second pathway for enteroendocrine differentiation. Further evidence to support this model was obtained from the expression of human growth hormone under control of 596 bp of the liver fatty acid binding protein gene in transgenic mice. Although L-FABP is not normally expressed in the colon, this particular transgene was ectopically expressed in most cells expressing glucagon, PYY, neurotensin and cholecystokinin but not in cells expressing substance P or serotonin. The reasons why coexpression of PYY in substance P and serotonin cells was not observed

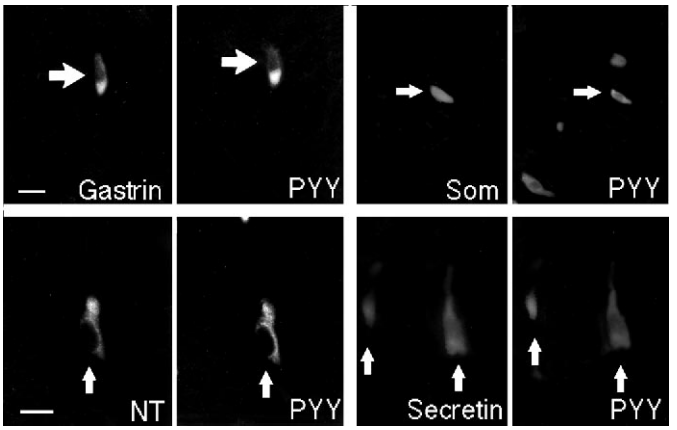

Fig. 5. Coexpression of PYY in enteroendocrine cells producing gastrin, somatostatin, neurotensin, and secretin in the developing mouse colon. Double immunofluorescent staining showing colocalization of PYY and other peptide hormones in endocrine cells (arrows) in the colon of non-transgenic mice at E18.5. Peptides were localized with FITC- (PYY) or Texas red-conjugated (gastrin, somatostatin, secretin, and neurotensin) second antibodies. Som, somatostatin. Bar, 20 μm (top row); 10 μm (bottom row).

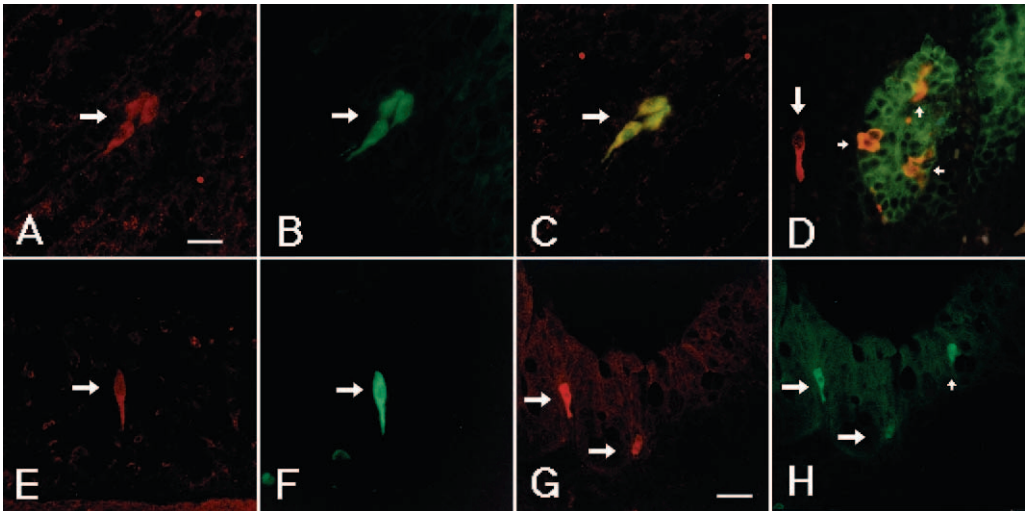

Fig. 6. Coexpression of PYY with substance P and serotonin in tumors and in normal tissues. Double immunofluorescent staining for PYY and either substance P or serotonin, localized with FITC- and Cy3-conjugated secondary antibodies, respectively, in colonic tissues of transgenic mice (A-D) or fetal nontransgenic CD1 mice (E-H). (A-C) Cluster of three cells (arrows) in a 6 week old transgenic mouse shows colocalization of substance P (A; cy3), and PYY (B; FITC). (C) Multiple exposure photomicrograph revealing yellow doubly labeled $SubP^+/PYY^+$ cells. (D) Multiple exposure photomicrograph showing labeling for serotonin (red) and PYY (green) in a small tumor from a 3 week old transgenic mouse. A small subpopulation of tumor cells are $serotonin^+/PYY^+$ (yellow; small arrows). A normal red serotonin-producing cell is seen adjacent to the tumor (large arrow). (E,F) Double immunofluorescent staining of a single cell (arrows) from an E18.5 normal fetus for substance P (E) and PYY (F). (G,H) Serotonin coexpression (G, arrows) in two PYY cells (H, large arrows) in an E17.5 mouse. A third PYY cell (small arrow) does not coexpress serotonin. Bar, 20 μm (A-F); 40 μm (G,H).

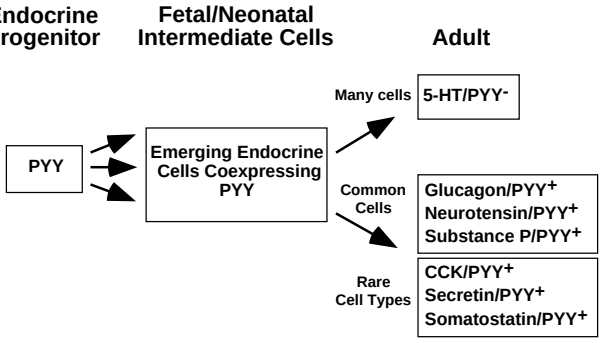

Fig. 7. Proposed model for the differentiation of colonic endocrine cells from a PYY-producing progenitor. The first enteroendocrine cell to appear expresses PYY. When each of the other hormone-producing cells appear during development, most cells coexpress PYY. This coexpression continues into adulthood for all cell types except serotonin (5-HT) cells.

earlier are not clear. The ability to observe coexpression of peptide YY in these two cell types may be highly dependent on the sensitivity of the antisera used to immunostain each peptide.

Secretin and gastrin have not been generally recognized as hormones produced by endocrine cells of the large intestine. We have noted previously that cells staining for secretin can frequently be identified in the mouse colon about the time of birth and that human growth hormone expressed under control of the secretin gene in transgenic mice is similarly expressed in colonic endocrine cells (Lopez et al., 1995). A potential relationship to L-type enteroendocrine cells was further suggested by the appearance of glucagon-producing colonic endocrine tumors in transgenic mice expressing Tag under control of the secretin gene (Lopez et al., 1995). By adulthood, secretin transcripts are present in very low abundance in the colon and only rare secretin-immunoreactive cells are seen by immunohistochemistry (Lopez et al., 1995). Expression of progastrin mRNA has been previously described in colorectal tumors, although it does not appear to be efficiently processed to gastrin (Van Solinge et al., 1993). Analysis of RNA from the developing rat colon suggests that in rats, gastrin transcripts are transiently expressed just prior to birth but not thereafter (Lüttichau et al., 1993). However, gastrin peptide had not been identified in enteroendocrine cells prior to the present study showing it in PYY cells.

We have previously suggested that the four principal endocrine cell types in the murine pancreas arise from a common PYY-producing progenitor (Upchurch et al., 1994). The present work is consistent with the existence of a PYY-producing endocrine progenitor cell in the colon as well, from which cells expressing glucagon, neurotensin, cholecystokinin, secretin, gastrin, somatostatin, substance P and serotonin differentiate. Expression of peptide YY may therefore represent an important early event in endocrine differentiation in these two tissues which are derived from primitive gut endoderm.

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