Enteroendocrine Localization of GLP-2 Receptor Expression in Humans and Rodents

BERNARDO YUSTA,* LILLY HUANG,* DONALD MUNROE,† GABRIELLE WOLFF,§ ROBERT FANTASKE,† SADHANA SHARMA,† LIDIA DEMCHYSHYN,† SYLVIA L. ASA,§ and DANIEL J. DRUCKER*

*Department of Medicine, Toronto General Hospital, Banting and Best Diabetes Centre, University of Toronto, Toronto, Ontario; §Department of Pathology, Mount Sinai Hospital, University of Toronto, Toronto, Ontario; and †NPS Allelix Inc., Mississauga, Ontario, Canada

Background & Aims: **Glucagon-like peptide (GLP)-2, a product of the proglucagon gene, is expressed in enteroendocrine cells of the small and large intestine and is trophic to the gastrointestinal mucosa. GLP-2 also inhibits gastric acid secretion and emptying and upregulates intestinal hexose transport. GLP-2 acts via binding to a single G protein–coupled GLP-2 receptor (GLP-2R), but the cellular targets for the diverse actions of GLP-2 remain unknown.** ***Methods:*** **GLP-2R expression in rodent and human tissues was examined using a combination of Northern blotting, reverse-transcription polymerase chain reaction (RT-PCR), and immunocytochemistry.** ***Results:*** **A single major GLP-2R messenger RNA transcript was detected by Northern blot analysis in rodent stomach, duodenum, jejunum, ileum, and colon, but not in rodent esophagus. GLP-2R expression was also detected by RT-PCR in RNA from the hypothalamus, brain stem, and lung. Immunocytochemical localization of human GLP-2R expression using specific antisera detected GLP-2R immunopositivity in subsets of endocrine cell populations in the epithelium of the stomach and both the small and large bowel.** ***Conclusions:*** **These findings suggest that enteroendocrine-derived GLP-2 acts directly on endocrine cells to induce one or more downstream mediators of GLP-2 action in the gastrointestinal tract.**

The proglucagon gene encodes multiple peptides related in amino acid sequence that are liberated in a tissue-specific manner in the brain, pancreas, and the enteroendocrine cells of the gastrointestinal tract. Glucagon, a 29–amino acid peptide, is secreted from islet A cells and plays a key role in glucose homeostasis. In contrast, several peptides with distinct biological activities are secreted from enteroendocrine cells of the small and large bowel, including glicentin, oxyntomodulin, and 2 glucagon-like peptides (GLPs) designated GLP-1 and GLP-2.[1]

Although glicentin and oxyntomodulin are cosecreted with GLP-1 and GLP-2 from the gut, much less is known about their biological activities in vivo. Glicentin stimulates intestinal growth in rodents,[2] and oxyntomodulin regulates gastric acid secretion and intestinal hexose transport,[3] but receptors for these peptides have not yet been defined and the physiologic relevance of glicentin and oxyntomodulin remains uncertain. In contrast, the biological activities of GLP-1 have been extensively characterized and include regulation of gastric emptying, stimulation of glucose-dependent insulin secretion, and inhibition of glucagon secretion, appetite, and food intake.[1] The multiple actions of GLP-1 result in lowering of blood glucose levels in rodents, normal human subjects, and patients with diabetes, raising the possibility that GLP-1 may be a useful adjunct for the treatment of patients with non–insulin-dependent diabetes.

More recent experiments have elucidated a role for GLP-2 as a regulator of intestinal mucosal growth. GLP-2 administered to rodents promotes increased crypt cell proliferation and decreases apoptosis, leading to increased villus height in the intestinal small bowel epithelium.[2,4] Coinfusion of GLP-2 and parenteral intravenous nutrition in rats prevents the mucosal hypoplasia that occurs in the absence of enteral nutrition.[5] Furthermore, GLP-2 reduces the severity of experimental intestinal injury in rodents with ischemic intestinal injury or colitis,[6,7] and enhances intestinal adaptation after major small bowel resection in the rat.[8] Together, these findings have stimulated considerable interest into the mechanism of GLP-2 action in vivo.

Abbreviations used in this paper: FABP, fatty acid–binding protein; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP, glucagon-like peptide; GLP-2R, glucagon-like peptide 2 receptor; RT-PCR, reverse-transcription polymerase chain reaction; SDS, sodium dodecyl sulfate.

0016-5085/00/$10.00
doi:10.1053/gast.2000.16489

Both glucagon and GLP-1 are known to exert their actions through binding to distinct 7-transmembrane domain, G protein–linked receptors that are encoded by different genes.[9,10] A separate GLP-2R has recently been isolated by expression cloning and shows significant amino acid homology to both the glucagon and GLP-1 receptors.[11] The GLP-2R specifically recognizes GLP-2, but not related members of the glucagon peptide superfamily. After GLP-2 binding to the transfected receptor, adenosine 3′,5′-cyclic monophosphate levels are increased,[11,12] consistent with findings from studies of related members of the glucagon/GLP-1R family.

Although RNase protection assays showed GLP-2R expression in the rat gastrointestinal tract,[11] little is known about intestinal GLP-2R expression. Whether the GLP-2R gene gives rise to a single RNA transcript, as is the case for the glucagon receptor, or multiple RNAs, as shown for the GLP-1R,[10] remains unknown. Furthermore, the multiple actions of GLP-2 on proliferation, apoptosis, gastric emptying, and hexose transport[13] raise important questions as to whether these diverse effects are direct or indirect; however, the cell types that express the GLP-2R in the gastrointestinal tract have not yet been identified. To gain further insight into the mechanism of GLP-2 action, we have studied GLP-2R expression in rodent and human tissues using a combination of reverse-transcription polymerase chain reaction (RT-PCR), Northern blotting, and immunocytochemistry.

Materials and Methods

Animals

Mice transgenic for the rat GLP-2R complementary DNA (cDNA) under the control of the intestinal fatty acid–binding protein (FABP) promoter[14] were generated and propagated in accordance with the guidelines of the Toronto General Hospital Animal Care Committee. These mice were generated to assess the effects on enterocyte biology of targeted expression of the GLP-2R to a localized intestinal epithelial compartment.[14] Because FABP–GLP-2R mice express high levels of the translated rat GLP-2R in the murine small bowel epithelium, FABP–GLP-2R mice were used as a positive control for GLP-2R immunohistochemistry experiments. Sprague–Dawley rats and CD1 mice were obtained from Charles River Canada (Toronto, Ontario).

Tissues and Cell Lines

Histologic sections of human stomach, small intestine, and large intestine were obtained from autopsy material from the Department of Pathology, Mount Sinai Hospital, University of Toronto, Canada. Carcinoid tumors (locally invasive endocrine tumors from the gastrointestinal tract) were also obtained from the Department of Surgical Pathology, Mount Sinai Hospital. Cell lines were obtained from American Type Culture Collection or from the endocrine or cancer research laboratories of the Toronto General Hospital/Ontario Cancer Institute. Intestinal cell lines IEC6, IEC14, IEC17, IEC18, IEC19, and IEC20, as well as CaCo2, Colo201, Colo205, Colo320, SW620, SW1116, and HT29 cells were a generous gift from Dr. D. Gauci (Ontario Cancer Institute, Toronto, Ontario). Rat pancreatic lines RIN14B, RINSF, and RINM and cell lines IMR32, SK-N-MC, PC12, and T84 were purchased from American Type Culture Collection. The GLUTag and STC-1 murine intestinal endocrine cell lines were from the Drucker Laboratory (Toronto, Canada).[15,16]

Cell Culture

IEC cell lines were maintained in α–minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% glucose, and 1% L-glutamine and harvested at 60%–70% confluency, 80%–90% confluency, and 100% confluency. Adenocarcinoma cell lines CaCo2, Colo201, Colo205, Colo320, HT29, SW620, SW1116, and HT29 were grown in α-MEM supplemented with 10% FBS and 1% glutamine. T84 cells were grown in 50% Ham's F12 media, 50% Dulbecco's modified Eagle medium supplemented with 10% FBS, and 1% glutamine. Rat pancreatic cells were maintained in RPMI 1640 media supplemented with 10% FBS and neuroblastoma cells in Dulbecco's modified Eagle medium/F12 media supplemented with 10% FBS. Adenocarcinoma cell lines CaCo2, HT29, SW620, and Colo205 were differentiated either spontaneously over several days (CaCo2), in the presence of 2 mmol/L sodium butyrate (Colo205 and SW620) or 5 mmol/L sodium butyrate (HT29). GLUTag and STC-1 cells were propagated as previously described.[15,16]

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from rat and mouse tissues by the guanidinium thiocyanate method.[17] PolyA$^+$ RNA was isolated using the polyATract System (Promega, Madison, WI) and quantified by fluorometry. For Northern blotting, 2 μg of polyA$^+$ RNA was electrophoresed in a 1% agarose-formaldehyde gel, transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH), and UV cross-linked using a Bio-Rad UV chamber (Bio-Rad Laboratories, Richmond, CA). After prehybridization, membranes were hybridized with a ^{32}P-labeled cDNA probe overnight at 42°C in 40% formamide-containing buffer. ^{32}P-labeled cDNA probes were generated by random priming from rat GLP-2R cDNA,[11] rat GLP-1R,[18] rat GIP receptor,[19] mouse glucagon receptor,[18] and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[20] generated by RT-PCR (as indicated later). Blots were washed at high stringency (0.1× standard saline citrate [SSC] plus 0.1% sodium dodecyl sulfate [SDS], 65°C) and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at −70°C with intensifying screens for the indicated time periods. Before rehybridization with subsequent probes, membranes were stripped at 80°C in 0.01× SSC plus 0.5% SDS.

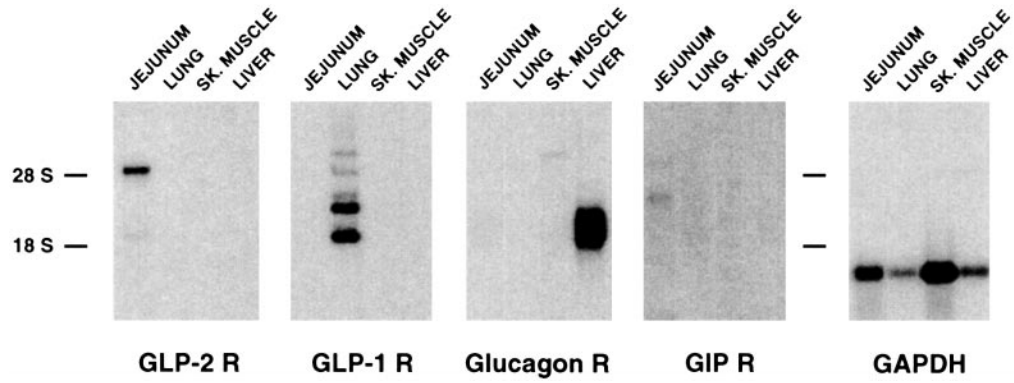


Figure 1. Northern blot analysis of rat RNA for members of the glucagon receptor superfamily. PolyA$^+$ RNA prepared from the jejunum, lung, skeletal muscle, and liver was hybridized with cDNA probes labeled with comparable specific activities for GLP-2R, GLP-1R, glucagon receptor (Glucagon R), gastric inhibitory polypeptide receptor (GIP R), or GAPDH. The relative migration positions of the 28 and 18s ribosomal RNA subunits are shown. The film exposure times for the different blots are as follows: GLP-2R, 4 days; GLP-1R, 6 hours; glucagon receptor, 14 hours; GIP receptor, 10 days; and GAPDH, 2 hours.

Semiquantitative RT-PCR

For first-strand cDNA synthesis, 5 μg of DNase I–treated total RNA from rat tissues underwent reverse transcription at 42°C for 50 minutes using random hexamers and SuperScript II (Life Technologies, Gaithersburg, MD). Aliquots of the first-strand reactions (1/20 vol) were used as templates for PCR using *Taq* polymerase (Life Technologies). PCR was performed over a range of cycles spanning the exponential phase of amplification kinetics, and linearity was shown for relative cDNA input across 2 orders of magnitude. Primer pairs selected for PCR were: rat GLP-2 R 5′-TTGTGAACGGGCGCCAGGAGA-3′ and 5′-GATCTCACTCTCTTCCAGAATCTC-3′; rat GAPDH 5′-TCCACCACCCTGTTGCTGTAG-3′ and 5′-GACCACAGTCCATGACATCACT-3′. Amplification of rat GLP-2R cDNA was performed at an annealing temperature of 65°C for 26 cycles, resulting in the generation of a 1672–base pair (bp) product spanning the entire GLP-2R open reading frame. For human GLP-2R RT-PCR, 0.15 μmol/L primers HWBR-F1 (5′-CTTATTCCTTTCCTGGC-3′) and HWBR-R4 (5′-GACAGGTAGGACATCCACC-3′) were used. Samples were denatured at 94°C for 2 minutes and submitted to 30 cycles of PCR (94°C for 40 seconds, 58°C for 40 seconds, and 72°C for 30 seconds) with an additional extension at 72°C for 8 minutes. Positive controls for human GLP-2R RT-PCR consisted of COS-7 or baby hamster kidney (BHK) cells transiently transfected with the human GLP-2R. GAPDH cDNA amplification was performed at an annealing temperature of 60°C for 24 cycles, resulting in the generation of a 452-bp product. GAPDH cDNA amplification was used as an internal control for the efficiency of first-strand cDNA synthesis. The specificity of each amplification reaction was monitored by control reactions in which amplification was performed on samples in which the RT was omitted from the RT reaction mixture (RT−).

PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and quantification by computer imaging (GAPDH cDNA) or by Southern blotting (GLP-2R cDNA). RT-PCR Southern blots were hybridized with a ^{32}P-labeled GLP-2R probe using nucleotide sequences internal to the PCR primers to verify the authenticity of the PCR product. Blots were washed at 65°C in 0.1× SSC, 0.1%

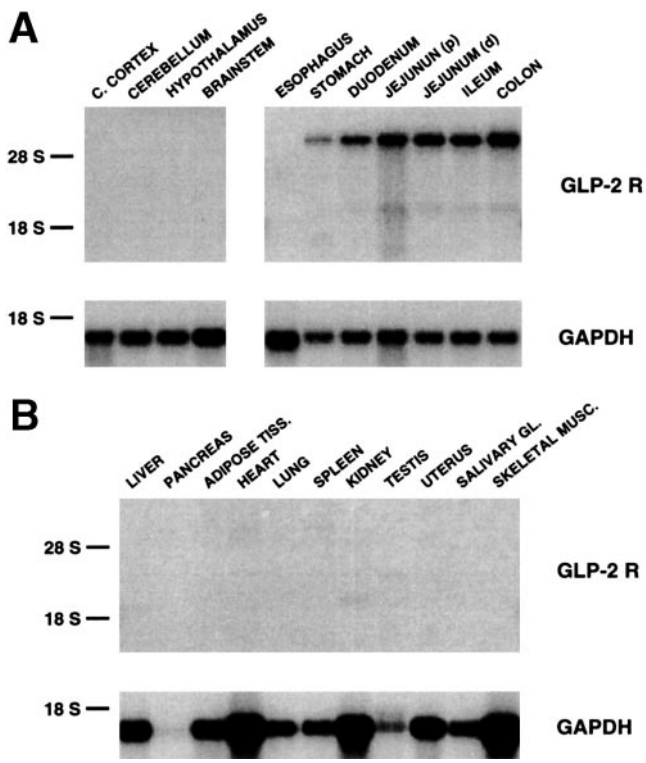


Figure 2. (*A*) Northern blot analysis of polyA$^+$ RNA from mouse brain or different regions of mouse intestine hybridized with cDNA probes for GLP-2R (blot exposed for 8 days) or GAPDH (blot exposed for 2.5 hours). The relative migration positions of the 28 and 18s ribosomal RNA subunits are shown. (*B*) Northern blot analysis of polyA$^+$ RNA from different mouse tissues hybridized with cDNA probes for GLP-2R (blot exposed for 8 days) or GAPDH (blot exposed for 5 hours). The relative migration positions of the 28 and 18s ribosomal RNA subunits are shown.

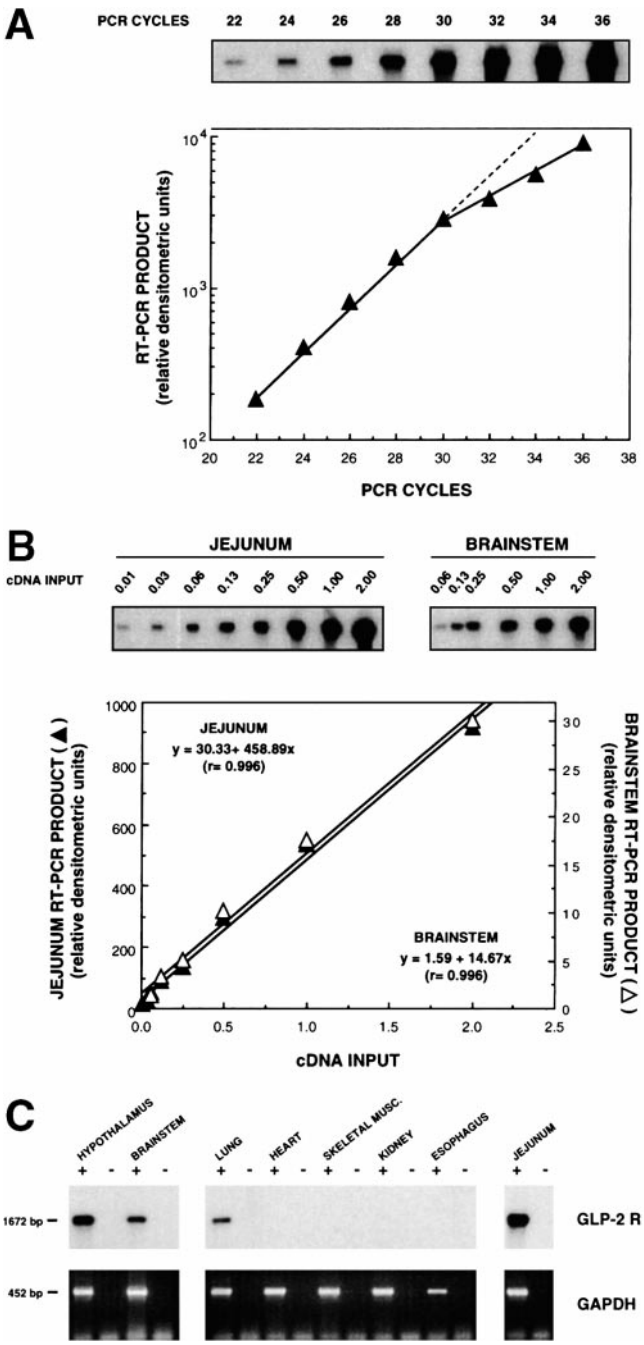


Figure 3. (*A*) RT-PCR analysis of GLP-2R expression. RNA isolated from rat jejunum underwent reverse transcription and was analyzed over a range of cycle lengths by RT-PCR. PCR products were subsequently analyzed by Southern blotting with an internal GLP-2R–specific cDNA probe. The relative intensity of the signals obtained was quantitated using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and plotted against the number of PCR cycles. (*B*) Semiquantitative linear relationship between cDNA input and GLP-2R RT-PCR products obtained from rat jejunum and brain stem first-strand cDNA, as assessed by RT-PCR and Southern blotting for GLP-2R. (*C*) RT-PCR analysis of GLP-2R expression in rat tissues. Identical cDNA aliquots were analyzed for GAPDH expression. −, PCR performed in the absence of first-strand cDNA synthesis; +, PCR performed after first-strand cDNA synthesis. The exposure time for the blot containing rat jejunum RT-PCR products was 5 hours; the exposure time for the blot containing the remaining RT-PCR products was 2 days.

SDS, and exposed against a phosphorimaging screen for quantification on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Antisera and immunocytochemistry. GLP-2R antisera were raised in rabbits against a GST–GLP-2R fusion protein generated in *Escherichia coli.* The fusion protein contained amino acids 457–550 of the GLP-2R, and was purified from bacterial extracts by GST column chromatography before immunization. Antisera were affinity-column purified against the original immunogen before use in immunocytochemistry. Tissues obtained from rodents or from human patients at autopsy were fixed overnight in buffered formalin, embedded in paraffin, and used for immunocytochemical analysis, as described previously.[21] GLP-2R antisera were used at a dilution of 1:800 to 1:1600. Nonspecific positivity was assessed by analysis of staining with preimmune sera, by omission of primary antisera, and by analysis of wild-type BHK cells and BHK cells transfected with the rat or human GLP-2R. Histologic sections from the small intestine of an FABP-promoter rat GLP-2R transgenic mouse[14] were also used as positive controls to verify the presence of antisera that recognized the rat GLP-2R in tissue sections in vivo.

Antisera recognizing endocrine cell secretory products were used as previously described.[22] For analysis of the relative numbers of GLP-2R–immunopositive endocrine cells in different regions of the gut, several hundred serotonin- and chromogranin-immunopositive cells were examined and compared with the number of GLP-2R–immunopositive cells in adjacent serial sections. Histologic sections from different regions of the human gastrointestinal tract were analyzed using the avidin-biotin-peroxidase technique and primary antisera directed against cholecystokinin (CCK) (1:2000; Serotec, Oxford, England), chromogranin (1:1000; Biomeda, Foster City, CA), gastrin (1:100; Zymed, San Francisco, CA), GIP (1:400; Peninsula, Belmont, CA), GLP-1 (1:2500; Drucker Laboratory), PYY (1:2000; Peninsula), secretin (1:100; Biogenex, San Ramon, CA), serotonin (1:50; Dako, Carpinteria, CA), and somatostatin (1:1000; Dako).

Western Blot Analysis

Whole cell–membrane extracts from STC-1 cells, GLUTag cells, BHK cells transfected with the rat GLP-2R, and 293 EBNA cells expressing a transfected human GLP-2R[11] were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions, transferred to nitrocellulose membranes, and analyzed by Western blotting, as described previously.[23] Loading and transfer conditions were assessed by staining of the gel with Coomassie or the membrane with Ponceau S. Membranes were incubated with preimmune or GLP-2R–immune antisera overnight at a dilution of 1:1000, and proteins were visualized by using a secondary antibody conjugated to horseradish peroxidase, detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Toronto, Ontario).

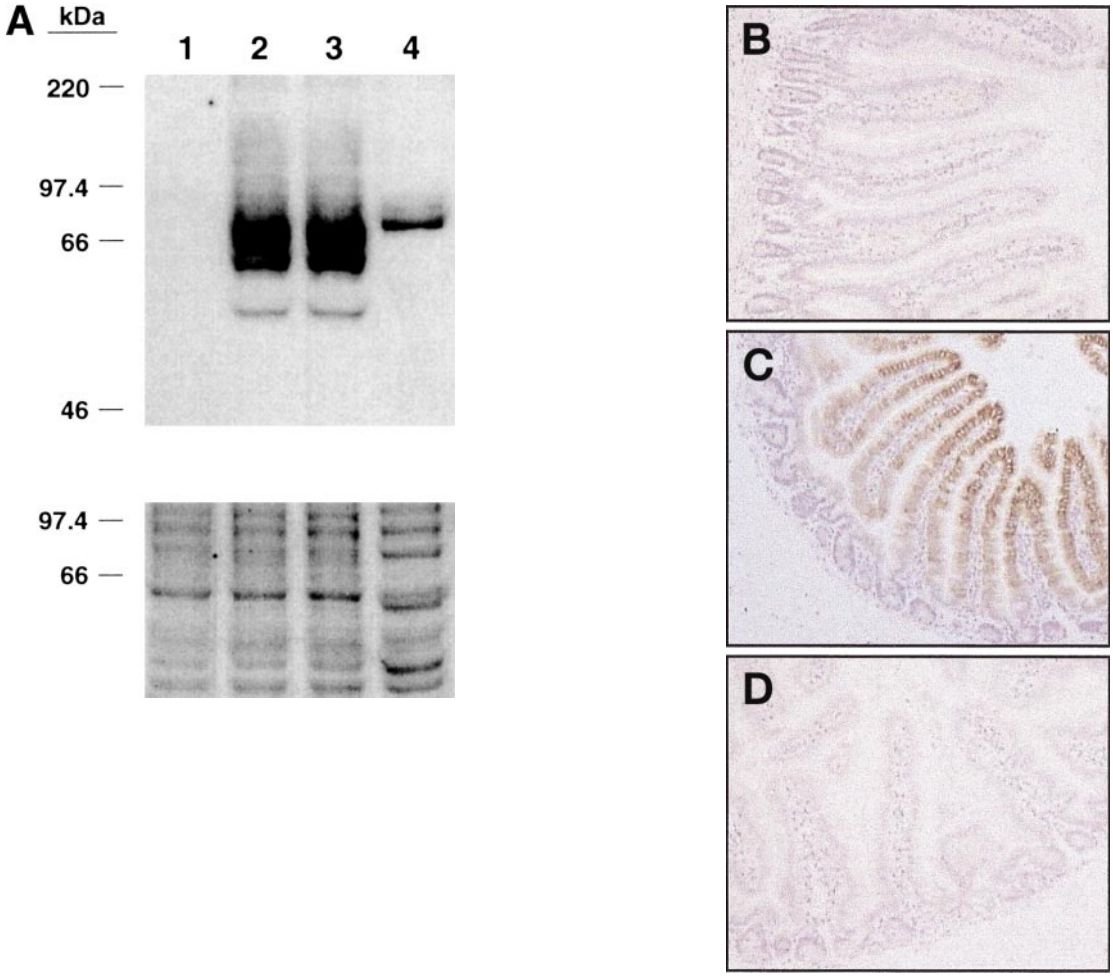
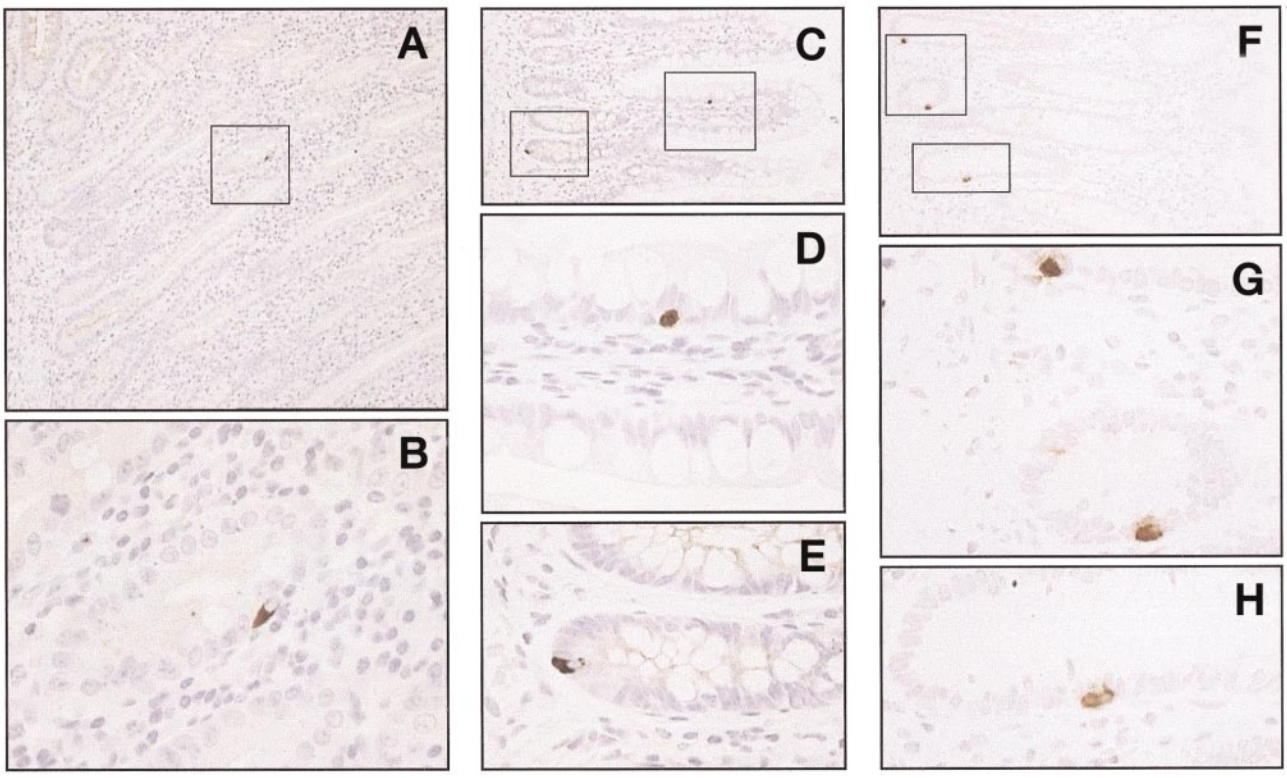


Figure 4. (*A*) Detection of GLP-2R by Western blotting in transfected cells. (*Top*) Whole-cell membrane extracts from BHK cells transfected with the expression vector alone (*lane 1*), 2 different BHK stable cell lines expressing the transfected rat GLP-2R (*lanes 2* and *3*), or EBNA cells stably transfected with the human GLP-2R (*lane 4*) were analyzed by Western blotting after SDS-PAGE under reducing conditions. The relative migration positions of coelectrophoresed molecular-weight markers are shown. (*Bottom*) Ponceau S staining of the Western blot membrane shown on *top*. Immunohistochemical detection of GLP-2R immunopositivity in (*B*) wild-type mouse small intestine, (*C*) FABP–GLP-2R transgenic mouse small intestine, and (*D*) FABP–GLP-2R transgenic mouse small intestine after absorption of the GLP-2R antisera with recombinant immunogen. (Original magnification 100× for *B, C,* and *D*.)



Results

Multiple GLP-1R RNA transcripts have been detected using Northern blotting and GLP-1R–specific cDNA probes[10,24]; however, whether the GLP-2R gene also gives rise to multiple transcripts is not currently known. Because messenger RNA (mRNA) transcripts for related members of the glucagon receptor superfamily potentially cross-hybridize with GLP-2R probes, we first ascertained the specificity of Northern blotting for detection of GLP-2R mRNA transcripts. RNA isolated from rat jejunum, lung, skeletal muscle, and the liver was hybridized in separate experiments with cDNA probes for the GLP-2R, GLP-1R, glucagon receptor, and GIP receptor (Figure 1). A single major GLP-2R–mRNA transcript of ~5.8 kb was detected in polyA$^+$ RNA from rat jejunum, but no GLP-2R–RNA transcripts were detected in polyA$^+$ RNA from the rat lung, muscle, or liver. In contrast, 2 major GLP-1R–mRNA transcripts were detected in the rat lung that migrated more rapidly than the larger jejunal GLP-2R mRNA (Figure 1). However, no GLP-1R–mRNA transcripts were detected in the rat jejunum, muscle, or liver, consistent with results from previous Northern blotting experiments.[10] Similarly, a single glucagon receptor–mRNA transcript was detected in the rat liver, but not in RNA from jejunum, lung, or muscle (Figure 1). A single GIP receptor–mRNA transcript was detected in polyA$^+$ RNA from jejunum, ~4.0 kb in size, clearly smaller in size than the jejunal GLP-2R–mRNA transcript. Together, these findings establish the specificity of Northern blotting for the detection of GLP-2R–mRNA transcripts and show that transcription of the GLP-2R gene gives rise to a single mRNA transcript.

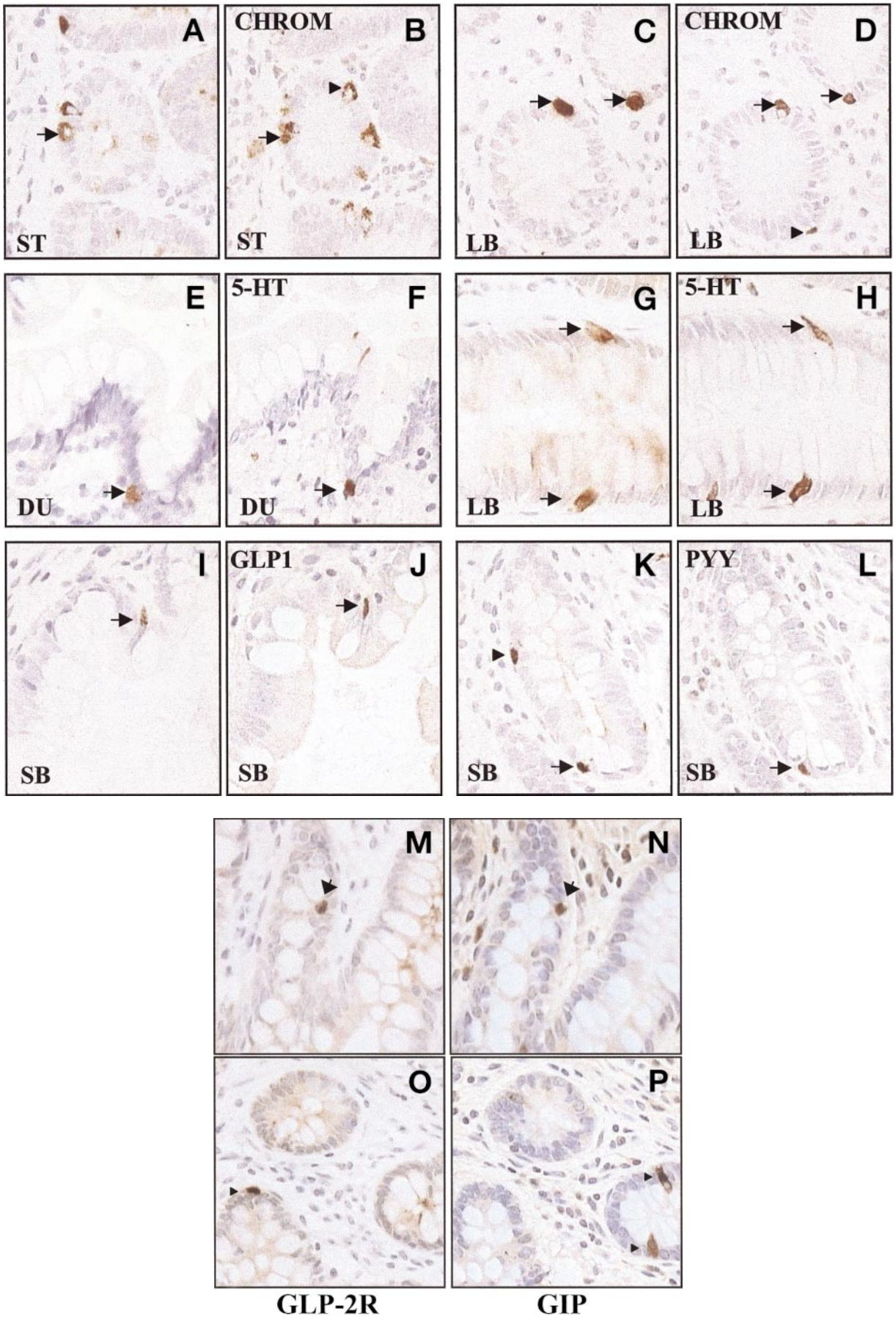
The results of several studies have established that GLP-2 promotes mucosal growth in both the small and large bowel, and inhibits gastric emptying and meal-stimulated gastric acid secretion.[25–27] We initially used Northern blotting to analyze the distribution and relative abundance of GLP-2R–RNA transcripts along the gastrointestinal tract. GLP-2R–mRNA transcripts were not detected in the esophagus, but were observed in more distal regions of the gastrointestinal tract, with relatively higher levels of expression observed in the small and large bowel than in the stomach (Figure 2*A*). In contrast, although a GLP-2R cDNA was cloned from a hypothalamic cDNA library,[11] we did not detect GLP-2R–mRNA transcripts by Northern blotting analysis in polyA$^+$ RNA from the hypothalamus, cortex, cerebellum, or brain stem (Figure 2*A*). No GLP-2R–mRNA transcripts were detected by Northern blotting in a range of peripheral tissues, including the liver, pancreas, kidney, gonads, and skeletal muscle tissue (Figure 2*B*).

The inability to detect GLP-2R–mRNA transcripts by Northern blotting in tissues such as the hypothalamus prompted us to use more sensitive techniques such as a RT-PCR assay for detection of low-abundance mRNA transcripts. We first analyzed GLP-2R–mRNA transcripts in total RNA from rat jejunum over a range of PCR cycles to establish conditions for semiquantitative detection of gene expression. A linear relationship was obtained between PCR product and cycle number between 22–30 cycles (Figure 3*A*), hence, 26 cycles were chosen for further RT-PCR experiments. To ascertain the use of this assay for detection of GLP-2R–mRNA transcripts in the gastrointestinal tract and brain, we analyzed GLP-2R expression over a range of cDNA inputs by using cDNA from rat jejunum and brain stem. GLP-2R–mRNA transcripts were detected, in a linear semiquantitative manner, over a broad range of input (cDNAs) in both tissues, establishing the sensitivity of the RT-PCR assay (Figure 3*B*). Furthermore, the relative abundance of GLP-2R expression by RT-PCR was at least 20–30-fold greater in the jejunum than brain stem, consistent with the results from Northern blotting experiments.

We next examined GLP-2R expression by RT-PCR using RNA from multiple tissues. GLP-2R–mRNA transcripts were detected by RT-PCR (Figure 3*C*) in RNA from the hypothalamus, brain stem, and lung. In contrast, GLP-2R–mRNA transcripts were not detected by RT-PCR in most of the tissues examined, including the heart, muscle, and kidney.

To localize the specific cell types that express the GLP-2R, we generated antisera directed against amino acids 457–550 in the carboxy-terminal region of the rat GLP-2R. Several antisera gave positive staining of GLP-2R–transfected cell lines, and 1 immunopurified antiserum was subsequently used for detection of GLP-2R immunopositivity in histologic sections from rodent and human tissues. To verify that immunopositivity in immunocytochemical analyses was consistent with the detection of a GLP-2R protein, we performed Western blot analysis of GLP-2R–transfected cell lines (Figure 4*A*). The predicted molecular weight of the human GLP-2R

Figure 5. Localization of GLP-2R–immunopositive cells in the human gastrointestinal tract. A few isolated GLP-2R–immunopositive cells are detected in the (*A* and *B*) stomach, (*C*, *D*, and *E*) small bowel, and (*F*, *G*, and *H*) large bowel. (Original magnification 100× [*A*, *C*, and *F*] and 400× [*B*, *D*, *E*, *G*, and *H*].)


A
ST
CHROM
B
ST
C
LB
CHROM
D
LB
E
DU
5-HT
F
DU
G
LB
5-HT
H
LB
I
SB
GLP1
J
SB
K
SB
PYY
L
SB
M
N
O
P
GLP-2R
GIP

in the absence of posttranslational modifications, after cleavage of a putative signal peptide, is ~56.2 kilodaltons. A single major band of ~72 kilodaltons was recognized in EBNA cells transfected with the human GLP-2R.[11] A similarly migrating 72-kilodalton band and a smaller ~60-kilodalton band were detected in extracts from BHK cells transfected with the rat GLP-2R. A minor band of ~50 kilodaltons, consistent with a possible degradation product, was also observed in BHK cell extracts. In contrast, no GLP-2R–immunoreactive proteins were detected in control BHK cell extracts (Figure 4*A*, lane 1). Similarly, GLP-2R–immunoreactive proteins were not detected in transfected cells when using preimmune sera (data not shown).

To ascertain whether the GLP-2R antisera identified GLP-2R–immunopositive cells in histologic tissue sections, we first analyzed histologic sections from FABP–GLP-2R transgenic mice as a positive control. These mice overexpress the rat GLP-2R under the control of the intestinal FABP.[14] No GLP-2R immunopositivity was detected in enterocytes from wild-type nontransgenic mouse intestine (Figure 4*B*). In contrast, FABP–GLP-2R transgenic murine enterocytes were strongly immunopositive with the GLP-2R antisera (Figure 4*C*) and the GLP-2R immunopositivity was eliminated after preincubation of the GLP-2R antisera with recombinant GLP-2R immunogen (Figure 4*D*).

Because the GLP-2R is a member of the glucagon/secretin receptor superfamily that exhibits regions of amino acid homology in conserved subdomains, we next assessed whether the GLP-2R antisera cross-reacts with either of the structurally related GLP-1R or glucagon receptor. No immunopositive pancreatic islet or exocrine cells or hepatocytes were detected in sections of rat or human pancreas and liver (data not shown), consistent with the lack of detectable GLP-2R–mRNA transcripts in these tissues (Figure 2). Furthermore, the GLP-2R antisera did not recognize the GLP-1R in extracts from cells transfected with the GLP-1R in vitro (not shown). Because islets and liver express the GLP-1 and glucagon receptors, these findings show that the GLP-2R antisera specifically detect the GLP-2R, but not the glucagon or GLP-1R in histologic tissue sections.

To localize the specific intestinal cell types that express the GLP-2R, we studied tissue sections from different regions of the human gut. Analysis of multiple histologic sections showed occasional GLP-2R–immunopositive cells in the epithelium of the stomach and both the small and large intestine (Figure 5*A–H*). The GLP-2R–immunopositive cells were rare in all regions of the gastrointestinal tract, ranging from 0 to 2 detectable cells in most low-power histologic sections of the stomach, and both the small and large intestine. In the stomach, the majority of GLP-2R–positive cells were localized to the lower portion of the pyloric antrum. Occasional, rare low-power fields from the human colon had up to 5–10 GLP-2R–positive cells. In the small bowel, GLP-2R–positive cells were seen both at the base of the villous epithelium, and immunopositive cells could also be detected at various locations along the length of the entire crypt-to-villus axis.

The abundance, appearance, and location of the GLP-2R–positive cells within the epithelium suggested that GLP-2R was expressed in intestinal endocrine cells. Consistent with this hypothesis, analysis of serial sections stained with antisera against either the GLP-2R or gut endocrine cell markers showed that GLP-2R–immunopositive cells in the stomach and both the small and large intestine also contained chromogranin immunopositivity (Figure 6*A–D*). Similarly, endocrine cells in the duodenum and large intestine were identified that contained both GLP-2R and serotonin immunopositivity (Figure 6*E–H*).

Although the majority of GLP-2R–positive cells contained chromogranin immunoreactivity, the majority (>80%) of the chromogranin-positive cells in the stomach and duodenum did not contain GLP-2R immunopositivity. Similarly, >70% of chromogranin-positive cells in the small bowel were GLP-2R immunonegative. In contrast, ~50% of all chromogranin-positive cells in the colon contained GLP-2R immunopositivity. In the colon, there were twice as many serotonin-positive cells as GLP-2R–positive cells, but most GLP-2R–positive cells also contained serotonin immunoreactivity.

In contrast to the localization of GLP-2R immunopositivity in endocrine cells, GLP-2R–immunopositive cells were not detected in nonendocrine gastric cell lineages or in enterocytes of the small bowel villous epithelium (Figure 5). Similarly, serosa and muscle layers in the stomach and both the small and large bowel did not contain GLP-2R–immunopositive cells (Figure 5 and data not shown).

Figure 6. Colocalization of immunopositivity for GLP-2R and enteroendocrine cell products in serial sections from the human gastrointestinal tract. *A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O* were stained with antisera against the GLP-2R. Adjacent sections were stained with antisera to chromogranin (CHROM: *B* and *D*), serotonin (5-HT: *F* and *H*), GLP-1 (*J*), peptide YY (PYY: *L*), and glucose-dependent inhibitory polypeptide (GIP: *N* and *P*), respectively. ST, stomach; DU, duodenum; SB, small bowel; LB, large bowel. *Arrows* show cells that exhibit immunopositivity for both GLP-2R and an endocrine cell product. *Arrowheads* show cells that stain positive for either the GLP-2R or an endocrine product. (Original magnification 400×.)

To determine the hormonal phenotype of the subset of enteroendocrine cells that expressed GLP-2R, we analyzed adjacent serial sections from the human stomach, and both the small and large intestine, for GLP-2R and hormone immunopositivity. The majority of all endocrine cell populations identified with different antisera in the stomach and both the small and large intestine did not contain GLP-2R immunopositivity. The majority of gastrin-positive cells in the stomach, and secretin- and somatostatin-positive cells in the small bowel, were GLP-2R immunonegative. An occasional duodenal endocrine cell exhibited both GIP and GLP-2R positivity, but most GIP-positive K cells did not contain GLP-2R immunoreactivity (Figure 6*M–P*). Several cells were identified in the ileum that contained both PYY and GLP-2R immunoreactivity (Figure 6*K* and *L*). Intriguingly, occasional endocrine cells that produce GLP-1 and GLP-2 were also GLP-2R immunopositive in the small bowel (Figure 6*I* and *J*). In contrast to the findings in the small bowel, and despite the relative abundance of GLP-1–immunopositive cells in the large bowel, we did not observe enteroendocrine cells in the large bowel that contained both GLP-1 and GLP-2R immunopositivity. Similarly, no somatostatin- or CCK-immunopositive cells were detected that also contained GLP-2R immunopositivity in either the small or large bowel.

After the localization of GLP-2R immunopositivity to normal gut endocrine cells, we hypothesized that human carcinoid tumors arising from intestinal endocrine cells might also exhibit GLP-2R immunopositivity. Although the majority of cells in the 4 human intestinal carcinoid tumors studied did not contain GLP-2R immunopositivity, small focal areas of GLP-2R positivity were observed in 3 of 4 tumors examined (Figure 7*A* and *B*). Consistent with the restriction of GLP-2R expression to gut endocrine lineages, we did not detect GLP-2R mRNA transcripts by RT-PCR in IEC6, IEC14, IEC17, IEC18, IEC19, IEC20, CaCo-2, Colo201, Colo205, Colo320, SW480, SW48, DLD-1, SW620, SW1116, T84, HCT-81, and HT-29 cell lines from the small and large intestine. Similarly, no GLP-2R–mRNA transcripts were detected in central nervous system–derived neuroblastoma cells (IMR32, SK-N-MC, CHP-126, NMB/N7), pheochromocytoma cells (PC12), or in pancreatic islet cell lines (RIN14B, RIN5F, and RINM cells). Furthermore, no GLP-2R–mRNA transcripts or GLP-2R–immunoreactive protein were detected in RNA or cell extracts from the STC-1 or GLUTag gut murine endocrine cell lines (Figure 7*C* and *D*).

Discussion

Our studies show that GLP-2R expression, as assessed by a combination of Northern blotting, RT-PCR, and immunocytochemistry, is predominantly restricted to the gastrointestinal tract and brain. The observation that GLP-2R–mRNA transcripts are expressed at low levels in the gastrointestinal tract, and detectable only by sensitive techniques such as Northern blotting with polyA$^+$ RNA, RNAse protection assays, or RT-PCR, is in keeping with previous efforts that failed to detect GLP-2 binding in the gastrointestinal epithelium when various experimental approaches were used.[28] Indeed, our studies clearly show that GLP-2R–immunopositive cells are not abundant in representative histologic sections of the small and large bowel, consistent with the low levels of GLP-2R–mRNA transcripts detected by Northern and RT-PCR analyses. In contrast to the more widely expressed GLP-1 and glucagon receptors,[18,29] the comparatively restricted pattern of GLP-2R expression suggests that the direct actions of GLP-2 are likely to be considerably more localized, predominantly to the gastrointestinal tract.

The localization of GLP-2R immunopositivity to endocrine cells of the stomach and both the small and large intestine was somewhat unexpected. These findings suggest that at least some of the effects of GLP-2 on crypt cell proliferation, apoptosis, intestinal hexose transport, and both gastric motility and acid secretion are indirect, and likely mediated by as yet unidentified factors released from GLP-2R–positive enteroendocrine cells in vivo. The localization of the majority of GLP-2–producing endocrine cells to the distal small bowel and colon suggests that GLP-2 actions on endocrine cells in the stomach and proximal jejunum may be mediated via circulating GLP-2 in an endocrine manner. Nevertheless, it remains possible that locally derived GLP-2 also acts on adjacent endocrine cells in a paracrine manner (Figure 8). The lack of GLP-2R immunopositivity in small bowel enterocytes or in colonic mucosal epithelial cells is in keeping with our inability to detect GLP-2R expression in immortalized intestinal cell lines derived from the small bowel or from large bowel tumors. In contrast, consistent with the findings of GLP-2R expression in gastrointestinal endocrine cells, rare scattered groups of GLP-2R–positive cells were detected in several human gastrointestinal carcinoid tumors.

What are the implications of GLP-2R expression in enteroendocrine cells of the gastrointestinal tract for understanding GLP-2 action? The gut contains a complex population of endocrine cells that exhibits region-specific differences in abundance and hormonal pheno-

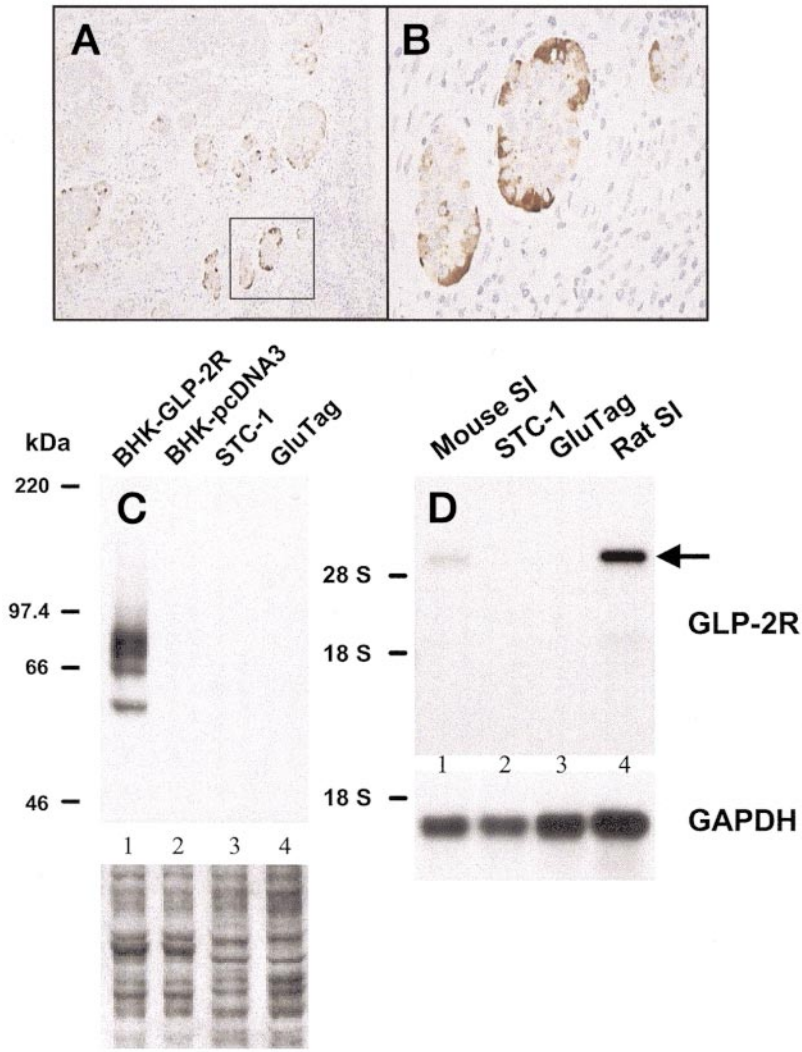


Figure 7. (*A* and *B*) Identification of GLP-2R immunopositivity in a human intestinal carcinoid tumor. Focal areas of tumor cells contain GLP-2R immunopositivity (original magnification 100× and 400× for *A* and *B*, respectively). (*C*) Western blot analysis of GLP-2R–immunoreactive protein from BHK–GLP-2R cells (*lane 1*), BHK cells transfected with control plasmid alone (*lane 2*), STC-1 cells (*lane 3*), and GLUTag cells (*lane 4*). The Coomassie stain of the gel is shown below the Western blot. (*D*) Northern blot analysis of GLP-2R–mRNA transcripts in enteroendocrine cell lines. PolyA$^+$ RNA (2 μg) from mouse jejunum (*lane 1*), STC-1 cells (*lane 2*), GLUTag cells (*lane 3*), and rat jejunum (*lane 4*) was analyzed by Northern blotting using rat GLP-2R cDNA probe as described in Materials and Methods. The blot was rehybridized with a cDNA for GAPDH (*bottom panel*) as a loading control.

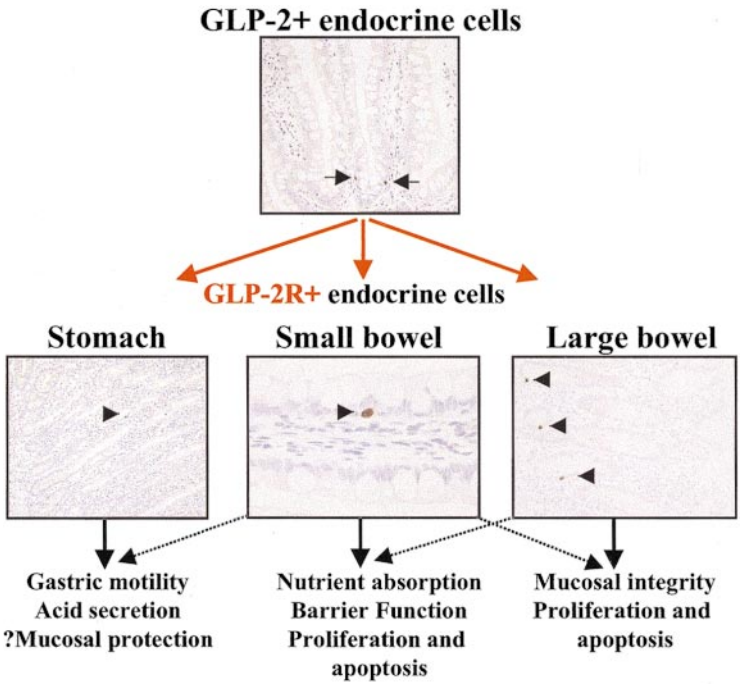


Figure 8. Model for understanding biological actions of GLP-2 in the gastrointestinal tract. GLP-2 produced in gut endocrine cells (*small arrows*) acts in a paracrine or endocrine manner via receptors (GLP-2R) on subpopulations of gastrointestinal endocrine cells (*arrowheads*) in the stomach and both the small and large intestine. The downstream effectors of GLP-2 action liberated after stimulation of GLP-2R–positive endocrine cells remain unknown.

type. For most of the endocrine cell populations examined in our experiments, the majority of gut endocrine cells studied did not contain GLP-2R immunopositivity. The finding that the GLP-2R is not directly expressed on proliferating crypt cells or villous enterocytes, integrated together with the identification of GLP-2R–positive endocrine cells, points toward the existence of 1 or more downstream mediators of GLP-2 action (Figure 8).

Many neuroendocrine peptides exhibit trophic activity in the gastrointestinal tract, including gastrin, neurotensin, gastrin-releasing peptide, and peptide YY.[30,31] Furthermore, molecules with potent intestinotrophic activity such as TGF-α and fibroblast growth factors have been localized to endocrine cells in the human gastrointestinal tract.[32,33] Hence, it seems likely that enteroendocrine-derived GLP-2 promotes gut growth and repair by stimulating the liberation of 1 or more trophic molecules from specialized endocrine cells that express the GLP-2R. The identity of the enteroendocrine-derived downstream mediators of GLP-2 action (Figure 8) remains unknown and is clearly an important issue for future investigation.

Intriguingly, enteroendocrine cell populations have been noted to be increased in several human intestinal diseases including celiac disease, Hirschprung's disease, and inflammatory bowel disease.[34–38] Furthermore, in the setting of intestinal inflammation, intestinal endocrine cells may express molecules such as the trefoil factor pS2 that potentially exerts protective effects on adjacent intestinal epithelium.[38] Hence, some of the effects of exogenous GLP-2 in experimental models of murine inflammatory bowel disease[7,39] and in enhancing barrier function in normal mice[40] may be caused by the liberation of enteroendocrine-derived molecules that exert diverse actions, including stimulation of mucosal repair mechanisms and protection from further epithelial injury in vivo.

Although the biology of intestinal endocrine cells remains poorly understood, increasing evidence suggests that some enteroendocrine cells are regulated by circulating or locally derived hormones through expression of G protein–linked peptide-hormone receptors. For example, GLP-1 release from enteroendocrine cells is stimulated by gastrin-releasing peptide and GIP in both primary intestinal cell cultures and in animals in vivo.[41–43] Furthermore, the parathyroid hormone 2 receptor, NPY/PYY Y–1 receptor, and somatostatin-2A receptor have been localized to gastrointestinal endocrine cells in vivo.[44–46] These findings, together with our results of GLP-2R localization on gut endocrine cells, imply the existence of a complex peptidergic communication system for transduction of regulatory signals to and from specialized endocrine cells of the gastrointestinal tract.

What are the implications of the molecular and cellular biology of GLP-2R expression for understanding GLP-2 action in vivo? The single major GLP-2R–mRNA transcript detected by Northern analysis, taken together with the single RT-PCR product obtained with primers spanning the entire GLP-2R coding sequence, implies that alternative exon splicing to generate multiple receptor isoforms with differing biological activities is unlikely to be a feature of GLP-2R biology. Given the highly limited cellular expression of the GLP-2R in enteroendocrine cells of the intestinal epithelium, it is perhaps not surprising that gastrointestinal cell lines that express the endogenous GLP-2R have not yet been reported. Further characterization of the endocrine cell populations that express the GLP-2R, and identification of the growth factors and peptides secreted by the GLP-2–stimulated GLP-2R–immunopositive enteroendocrine cells, is clearly an important next step to further advance our understanding of the biology of GLP-2 action.

References

1. Drucker DJ. The glucagon-like peptides. Diabetes 1998;47:159–169.
2. Drucker DJ, Ehrlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proc Natl Acad Sci U S A 1996;93:7911–7916.
3. Schjoldager BTG, Baldissera FGA, Mortensen PE, Holst JJ, Christiansen J. Oxyntomodulin: a potential hormone from the distal gut. Pharmacokinetics and effects on gastric acid and insulin secretion in man. Eur J Clin Invest 1988;18:499–503.
4. Drucker DJ, Shi Q, Crivici A, Sumner-Smith M, Tavares W, Hill M, DeForest L, Cooper S, Brubaker PL. Regulation of the biological activity of glucagon-like peptide 2 by dipeptidyl peptidase IV. Nat Biotechnol 1997;15:673–677.
5. Chance WT, Foley-Nelson T, Thomas I, Balasubramaniam A. Prevention of parenteral nutrition-induced gut hypoplasia by coinfusion of glucagon-like peptide-2. Am J Physiol 1997;273:G559–G563.
6. Prasad R, Alavi K, Schwartz MZ. Glucagonlike peptide-2 analogue enhances intestinal mucosal mass after ischemia and reperfusion. J Pediatr Surg 2000;35:357–359.
7. Drucker DJ, Yusta B, Boushey RP, Deforest L, Brubaker PL. Human [Gly2]-GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. Am J Physiol 1999;276:G79–G91.
8. Scott RB, Kirk D, MacNaughton WK, Meddings JB. GLP-2 augments the adaptive response to massive intestinal resection in rat. Am J Physiol 1998;275:G911–G921.
9. Jelinek LJ, Lok S, Rosenberg GB, Smith RA, Grant FJ, Biggs S, Bensch PA, Kuijper JL, Sheppard PO, Sprecher CA, O'Hara PJ, Foster D, Walker KM, Chen LHJ, McKernan PA, Kindsvogel W. Expression cloning and signaling properties of the rat glucagon receptor. Science 1993;259:1614–1616.
10. Thorens B. Expression cloning of the pancreatic β cell receptor for the gluco-incretin hormone glucagon-like peptide 1. Proc Natl Acad Sci U S A 1992;89:8641–8645.
11. Munroe DG, Gupta AK, Kooshesh P, Vyas TB, Rizkalla G, Wang H, Demchyshyn L, Yang ZJ, Kamboj RK, Chen H, McCallum K, Sumner-Smith M, Drucker DJ, Crivici A. Prototypic G protein–coupled receptor for the intestinotrophic factor glucagon-like peptide 2. Proc Natl Acad Sci U S A 1999;96:1569–1573.

12. Yusta B, Somwar R, Wang F, Munroe D, Grinstein S, Klip A, Drucker DJ. Identification of glucagon-like peptide-2 (GLP-2)-activated signaling pathways in baby hamster kidney fibroblasts expressing the rat GLP-2 receptor. J Biol Chem 1999;274: 30459–30467.
13. Drucker DJ. Glucagon-like peptide 2. Trends Endocrinol Metab 1999;10:153–156.
14. Yusta B, Tavares W, Boushey RP, Munroe DG, Drucker DJ. Restricted cellular specificity of GLP-2 action in FABP-GLP-2 receptor transgenic mice. Gastroenterology 1999;116:A948,G4127.
15. Gajic D, Drucker DJ. Multiple cis-acting domains mediate basal and adenosine 3',5'-monophosphate-dependent glucagon gene transcription in a mouse neuroendocrine cell line. Endocrinology 1993;132:1055–1062.
16. Drucker DJ, Jin T, Asa SL, Young TA, Brubaker PL. Activation of proglucagon gene transcription by protein kinase A in a novel mouse enteroendocrine cell line. Mol Endocrinol 1994;8:1646–1655.
17. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–159.
18. Campos RV, Lee YC, Drucker DJ. Divergent tissue-specific and developmental expression of receptors for glucagon and glucagon-like peptide-1 in the mouse. Endocrinology 1994;134:2156–2164.
19. Wheeler MB, Gelling RW, McIntosh CHS, Georgiou J, Brown JC, Pederson RA. Functional expression of the rat pancreatic islet glucose-dependent insulinotropic polypeptide receptor: ligand binding and intracellular signaling properties. Endocrinology 1995;136:4629–4639.
20. Piechaczyk M, Blanchard JM, Marty L, Dani C, Panabieres F, El Sabouty S, Fort P, Jeanteur P. Post-transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase gene expression in rat tissues. Nucleic Acids Res 1984;12:6951–6963.
21. Asa SL, Singer W, Kovacs K, Horvath W, Murray D, Colapinto N, Thorner MO. Pancreatic endocrine tumour producing growth hormone-releasing hormone associated with multiple endocrine neoplasia type I syndrome. Acta Endocrinol 1987;115:331–337.
22. Asa SL, Lee YC, Drucker DJ. Development of colonic and pancreatic endocrine tumors in mice expressing a glucagon-SV40 T antigen transgene. Virchows Arch 1996;427:595–605.
23. Trinh KY, Jin T, Drucker DJ. Identification of domains mediating transcriptional activation and cytoplasmic export in the caudal homeobox protein Cdx-3. J Biol Chem 1999;274:6011–6019.
24. Lankat-Buttgereit B, Goke R, Fehmann H-C, Richter G, Goke B. Molecular cloning of a cDNA encoding for the GLP-1 receptor expressed in rat lung. Exp Clin Endocrinol Diabetes 1994;102: 341–347.
25. Drucker DJ. Intestinal growth factors. Am J Physiol 1997;273: G3–G6.
26. Wojdemann M, Wettergren A, Hartmann B, Holst JJ. Glucagon-like peptide-2 inhibits centrally induced antral motility in pigs. Scand J Gastroenterol 1998;33:828–832.
27. Wojdemann M, Wettergren A, Hartmann B, Hilsted L, Holst JJ. Inhibition of sham feeding-stimulated human gastric acid secretion by glucagon-like peptide-2. J Clin Endocrinol Metab 1999;84: 2513–2517.
28. McGregor GP, Goke R, Goke B. Biological actions of glucagon-like peptide(GLP)-2 revealed—how pluripotential is the glucagon gene? Exp Clin Endocrinol Diabetes 1998;106:25–28.
29. Bullock BP, Heller RS, Habener JF. Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide 1 receptor. Endocrinology 1996;137:2968–2978.
30. Podolsky DK. Regulation of intestinal epithelial proliferation: a few answers, many questions. Am J Physiol 1993;264:G179–G186.
31. Taylor RG, Fuller PJ. Humoral regulation of intestinal adaptation. Baillieres Clin Endocrinol Metab 1994;8:165–183.
32. La Rosa S, Chiaravalli AM, Capella C, Uccella S, Sessa F. Immunohistochemical localization of acidic fibroblast growth factor in normal human enterochromaffin cells and related gastrointestinal tumours. Virchows Arch 1997;430:117–124.
33. Yamamoto M, Sasaki H, Iseki S. The occurrence of acidic fibroblast growth factor-like immunoreactivity in subpopulations of endocrine cells in the pancreas and intestine of the rat. Arch Histol Cytol 1995;58:475–484.
34. Sjolund K, Alumets J, Berg NO, Hakanson R, Sundler F. Duodenal endocrine cells in adult coeliac disease. Gut 1979;20:547–552.
35. Sjolund K, Alumets J, Berg NO, Hakanson R, Sundler F. Enteropathy of coeliac disease in adults: increased number of enterochromaffin cells in the duodenal mucosa. Gut 1982;23:42–48.
36. Bishop AE, Pietroletti R, Taat CW, Brummelkamp WH, Polak JM. Increased populations of endocrine cells in Crohn's ileitis. Virchows Arch 1987;410:391–396.
37. Soeda J, O'Briain DS, Puri P. Mucosal neuroendocrine cell abnormalities in the colon of patients with Hirschsprung's disease. J Pediatr Surg 1992;27:823–827.
38. Poulsom R, Chinery R, Sarraf C, Lalani EN, Stamp G, Elia G, Wright N. Trefoil peptide expression in intestinal adaptation and renewal. Scand J Gastroenterol Suppl 1992;192:17–28.
39. Boushey RP, Yusta B, Drucker DJ. Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. Am J Physiol 1999;277:E937–E947.
40. Benjamin MA, McKay DM, Yang PC, Cameron H, Perdue MH. Glucagon-like peptide-2 enhances intestinal epithelial barrier function of both transcellular and paracellular pathways in the mouse. Gut 2000;47: 112–119.
41. Roberge JN, Brubaker PL. Regulation of intestinal proglucagon-derived peptide secretion by glucose-dependent insulinotropic peptide in a novel enteroendocrine loop. Endocrinology 1993; 133:233–240.
42. Roberge JN, Gronau KA, Brubaker PL. Gastrin-releasing peptide is a novel mediator of proximal nutrient-induced proglucagon-derived peptide secretion from the distal gut. Endocrinology 1996; 137:2383–2388.
43. Damholt AB, Buchan AM, Kofod H. Glucagon-like-peptide-1 secretion from canine L-cells is increased by glucose-dependent-insulinotropic peptide but unaffected by glucose. Endocrinology 1998;139:2085–2091.
44. Usdin TB, Hilton J, Vertesi T, Harta G, Segre G, Mezey E. Distribution of the parathyroid hormone 2 receptor in rat: immunolocalization reveals expression by several endocrine cells. Endocrinology 1999;140:3363–3371.
45. Jackerott M, Larsson LI. Immunocytochemical localization of the NPY/PYY Y1 receptor in enteric neurons, endothelial cells, and endocrine-like cells of the rat intestinal tract. J Histochem Cytochem 1997;45:1643–1650.
46. Sternini C, Wong H, Wu SV, de Giorgio R, Yang M, Reeve J Jr, Brecha NC, Walsh JH. Somatostatin 2A receptor is expressed by enteric neurons, and by interstitial cells of Cajal and enterochromaffin-like cells of the gastrointestinal tract. J Comp Neurol 1997;386:396–408.

Received August 26, 1999. Accepted April 19, 2000.

Address correspondence to: Daniel Drucker, M.D., Toronto General Hospital, 200 Elizabeth Street, CCRW3-845, Toronto, M5G 2C4, Canada. e-mail: d.drucker@utoronto.ca; fax: (416) 978-4108.

Supported by a Senior Scientist Award (to D.D.) from the Medical Research Council (MRC) of Canada and in part by operating grants from the MRC of Canada and by an Ontario Research and Development Challenge Fund Award. Dr. Drucker is a consultant to NPS Allelix Inc., and GLP-2 is the subject of a licensing agreement between the Toronto General Hospital, University of Toronto, and NPS Allelix Inc.

The authors thank Dr. D. Irwin for technical assistance.