Syncollin is differentially expressed in rat proximal small intestine and regulated by feeding behavior

SANDRA TAN AND SHING C. HOOI
Department of Physiology, Faculty of Medicine, National University of Singapore, Singapore 119260

Tan, Sandra, and Shing C. Hooi. Syncollin is differentially expressed in rat proximal small intestine and regulated by feeding behavior. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G308–G320, 2000.—Gradients of gene expression are maintained along the proximal-distal axis of the mammalian small intestine despite a continuously regenerating epithelium. To study the molecular mechanisms responsible for this phenomenon, we utilized a subtractive hybridization strategy to isolate genes differentially expressed in the duodenum but not ileum. We isolated and sequenced 15 clones. The clones were fragments of genes encoding lipases, proteases, and an esterase. A novel clone was characterized and subsequently shown to encode syncollin, a secretory granule protein that binds to syntaxin in a calcium-sensitive manner. RT-PCR and S1 nuclease protection assay were used to clarify the 5′-end of syncollin. Syncollin was expressed in the rat pancreas, spleen, duodenum, and colon. In situ hybridization localized syncollin expression in the pancreas to acinar cells and in the duodenum to villus epithelial cells.

THE SMALL INTESTINE MUCOSA comprises a heterogeneous population of cells that provide the opportunity to study processes involved in cellular differentiation and regulation of gene expression. The crypts of Lieberkühn located at the base of the intestinal villi contain ~150 proliferating cells each, providing a steady source of new cells (19). These cells differentiate into enterocytes, goblet cells, enteroendocrine cells, and Paneth cells as they migrate along the length of the villi (28). Cells at the tip of the villi are extruded into the intestinal lumen every day. On average, the intestinal epithelial cells are present in the villi for ~3 days before they are shed. The mechanisms regulating the rapid turnover and differentiation of intestinal epithelial cells are unclear.

In addition to cellular differentiation along the length of the small intestinal villi, there are also phenotypic differences among intestinal cells along the proximal-distal axis of the gut. Many genes are differentially expressed along the proximal-distal axis of the gut. The pattern of expression appears to be established late in fetal life and is maintained throughout the lifetime of the animal (22, 23). Genes that exhibit a proximal-distal gradient of expression include those encoding alkaline phosphatase (29), disaccharidases (e.g., maltase and lactase-phlorizin hydrolase), proteases (e.g., dipeptidyl peptidase IV and cathepsin B), and fatty acid binding protein (22).

The mechanisms regulating the differential gene expression in the proximal-distal axis of the small intestine are unclear. However, several trans-acting factors like hormones, pancreato-biliary secretions, and even microorganisms (15) are thought to be involved in establishing the proximal-distal gradient of gene expression. Recently, in a series of elegant experiments, investigators identified a 20 bp regulatory element in the 5′-flanking region of the gene encoding intestinal fatty acid binding protein that appears to be responsible for suppressing gene expression in the distal intestine, crypt progenitor, and Paneth cells, giving rise to the observed proximal-distal and enterocyte/goblet cell-specific gene expression pattern observed for this gene (21, 22, 30).

In the present study, we sought to identify other genes that may also be expressed in a similar proximodistal gradient along the small intestine. The study of the regulation of these genes will provide further insights into the regulation and molecular mechanisms responsible for the establishment and maintenance of specific patterns of gene expression in the small intestine. We used the technique of subtractive hybridization (8) to identify genes that are either specific to or expressed in higher concentrations in the proximal compared with the distal small intestine.

MATERIALS AND METHODS

Subtractive Hybridization and Cloning

The proximal and distal 12 cm of the small intestine were obtained from a male Sprague-Dawley rat (220 g). Pancreatic tissue adhering to the proximal gut was completely removed. Total RNA was isolated using the guanidinium thiocyanate method (5). The RNA was enriched for mRNA using oligo(dT) columns (Pharmacia, Uppsala, Sweden). Two micrograms of mRNA from each sample were used for subtractive hybridization (PCR-select cDNA subtraction kit; Clontech, Palo Alto, CA) following the manufacturer’s instructions. Briefly, mRNA from the proximal and distal small intestine was reverse transcribed at 42°C for 1 h using Moloney murine leukemia virus reverse transcriptase. Second-strand synthesis was carried out with an enzyme cocktail containing DNA polymer-
ase I, RNAse H, and E. coli DNA ligase. The cDNA obtained was blunt-ended with the restriction enzyme Rsal. cDNA derived from the proximal intestine was divided into two portions, each of which was ligated to different adaptors provided in the kit, using T4 DNA ligase. Both the adaptors were supplied by the manufacturer. Subtractive hybridization was performed with both portions of proximal small intestinal cDNA, using an excess of distal small intestinal cDNA as the driver. Subsequent to the subtractive hybridization, a second round of hybridization was performed with the two primary hybridization samples mixed together (without denaturation) and with an excess of driver cDNA. The mixture was then subjected to PCR, using the appropriate PCR and nested-PCR primers, supplied by the manufacturer, to amplify the differentially expressed genes.

The amplification products were extracted using chloroform-isoamyl alcohol (24:1) and ligated into pMOSBlue T vector (Amersham, Amersham, UK). The transformants were selected in bacterial agar plates containing ampicillin (50 µg/ml), tetracycline (15 µg/ml), and isopropylthiogalactoside-X-gal (100 mM, 50 mg/ml). Fifteen white colonies were selected for further characterization.

5′- And 3′-Rapid Amplification of cDNA ends

The 5′- and 3′-ends of a novel clone were obtained by 5′- and 3′-rapid amplification of cDNA ends (5′- and 3′-RACE), respectively, using the AmpliFINDER RACE kit (Clontech). Briefly, gene-specific forward primers prsip3 and prsip4 and reverse primers prsip1 and prsip2 were synthesized using a Beckman 1000M oligonucleotide synthesis machine (Beckman, Palo Alto, CA). The sequences of the primers (Fig. 1) were based on the sequence of the cDNA fragment obtained from the subtraction (Fig. 1). To obtain the 3′-end of the gene, mRNA was purified from rat proximal small intestine and reverse transcribed using a modified oligo(dT) primer with an anchor sequence tagged to its 5′-end [5′-CCTCTGAGGTTCCAGAATCAGAGGTTAGAATCGATAGGAATTCT(18)(G/C/A/N-3′)] and avian myeloblastosis virus (AMV) reverse transcriptase (Clontech). The mRNA template was removed by RNAse H treatment. This was followed by a primary PCR using primer prsip3 and the anchor primer (5′-CTGCTGCCCACCTCTGAGGTTCCAGAATCAGAGGTTAGAATCGATAGGAATTCT(18)(G/C/A/N-3′)) and KlenTaq polymerase (Clontech). Subsequently, a secondary PCR using prsip4 and the anchor primer was performed.

![Fig. 1](http://ajpgi.physiology.org/)

**Fig. 1.** Comparison of sip9 and syncollin cDNA sequence. ATG start codons of the sip9 and syncollin open reading frames (ORF) are underlined. Sequence of sip9 cDNA fragment is italicized. Primers used for 5′ (prsip1 and 2)- and 3′ (prsip3 and 4)-rapid amplification of cDNA ends (RACE) and RT-PCR are indicated. Prsip22 and 24 correspond to ORF reported by Edwardson et al. (9); prsip23 and 24 correspond to sip9 ORF.
To amplify the 5'-end of the gene, 2 µg of mRNA obtained from rat proximal small intestine were first reverse transcribed with the prsip1 primer and AMV reverse transcriptase (Clontech). The RNA template was hydrolyzed with 0.375 M NaOH. The cDNA was purified using Geno-Bind included in the Amplifinder RACE kit (Clontech). A single-stranded oligomer (5'-P-CACGAATTCACTATCGATTCTGGAACCTTCAGGG-NH2-3') was ligated to the 5'-end of the cDNA using T4 RNA ligase (Clontech). Subsequently, 1 µl of a 1:10 dilution of the ligation mixture was amplified with the anchor primer and primer prsip2. The 5'- and 3'-end cDNA fragments were cloned into pMOSBlue T-vector and sequenced as described in DNA Sequencing.

Amplification of 5'-Nontranscribed Genomic Fragment

To obtain the 5'-nontranscribed region of the novel clone (sip9), gene-specific primers prsip1 and 2 (Fig. 1), together with AP1 (5'-GTAATACGACTCACTATAGGGC-3') and AP2 (5'-ACTATAGGGCACGGTGTGT-3'), were used to amplify rat genomic DNA that had been restricted with Dra I and ligated to appropriate adaptors (Rat PromoterFinder DNA Walking Kit; Clontech). The genomic fragment was gel purified, cloned, and sequenced.

RT-PCR

RT-PCR was performed to amplify the open reading frames (ORF) of sip9 and syncollin (9). Total RNA from rat pancreas and duodenum was reverse transcribed using an oligo(dT) primer and AMV reverse transcriptase (Clontech) at 42°C. PCR was performed with 100 ng of template using primer pairs prsip22 and 24 (corresponding to the ORF reported by Edwardson et al. [9]) and prsip23 and 24 (corresponding to the sip9 ORF), respectively (Fig. 1). The results were analyzed by agarose gel electrophoresis.

S1 Nuclease Protection Assay

Two oligonucleotides were synthesized: the oligonucleotide prsip29 was complementary to nucleotides –50 to –20 of the sip9 gene, whereas the oligonucleotide prsip30 corresponded to nucleotides +70 to +101 of the syncollin cDNA (10) (Fig. 2). The oligonucleotides were end-labeled as described below. The probes were then hybridized to 5 µg of duodenal and pancreatic total RNA in separate reactions. Hybridization was carried out at 30°C for 16 h in S1 hybridization buffer (50% formamide, 40 mM Pipes, 400 mM NaCl, 1 mM EDTA). After hybridization, S1 nuclease digestion was carried out in 1× S1 nuclease buffer (0.28 M sodium chloride, 0.05 M sodium acetate, pH 4.5, 4.5 mM zinc sulfate, and 3 µg single-stranded salmon sperm DNA) using 300 U of S1 nuclease at 30°C for 1 h. The reaction mixture was subsequently analyzed on a sequencing gel.

DNA Sequencing

DNA minipreps were prepared using the Hybaid Recovery Plasmid Miniprep kit (Hybaid). DNA sequencing of the minipreps was performed using the Sequitherm Cycle Sequencing Kit (Epicentre Technologies, Madison, WI). Sequencing reactions were separated on a denaturing 6% acrylamide gel (Stratagene, La Jolla, CA). The gel was vacuum dried and exposed to Kodak X-Omat autoradiography film (Kodak, Rochester, NY).

Animals and Tissues in Gene Expression Studies

Sprague-Dawley rats (Sembawang Animal Centre, Singapore) were used for all experiments.

Tissue-Specific Expression of sip9 in Rat

A 220-g male rat was killed by decapitation. Tissues were dissected from the animal, immediately frozen on dry ice, and stored at –80°C until use. To determine the expression of sip9 along the rat gastrointestinal tract, the entire small intestine was dissected and divided into 2-cm samples from the gastro-duodenal junction to the ileocecal junction. The first 10 cm of the small intestine was designated as duodenum, the distal 6 cm as ileum, and the remaining small intestine as jejunum.

Developmental Expression of sip9 in Rat

The entire small intestine was obtained from fetuses at gestational day 21, 1-day-old pups (suckling and nonsuckling), and 2-day- and 7-day-old suckling pups. Nonsuckling (1 day) pups were removed from their mothers at birth. There were six animals in each group. The intestines were snap frozen in liquid nitrogen and stored at –80°C until use.

Regulation by Fasting and Refeeding

Male Sprague-Dawley rats weighing between 220 and 240 g were randomly divided into three groups. Controls (CTR, n = 4) were given free access to food and water. Food was removed from the 1D Fast (n = 4) group from 0900 on day 0 to 0900 on day 1. Food was removed from the 3D Fast (n = 5) group from 0900 on day 0 to 0900 on day 3. At the end of the experiment, the animals were killed by decapitation. Six centimeters of the duodenum, beginning one centimeter distal to the pyloric junction, were dissected from each animal after death. The duodenum was carefully dissected free from adhering pancreatic tissue. In a second experiment, 21 male Sprague-Dawley rats (220–240 g) were used. The rats were

-20 +1 +50

Fig. 2. Sequence and location of primers used for S1 nuclease protection assays. Primer prsip29 was complementary to nucleotides –20 to +50 of the sip9 gene, and prsip30 was complementary to first 70 nucleotides of syncollin cDNA (10).
randomly divided into three groups (n = 7 in each group). Controls (CTR) were given free access to food throughout the experiment. The 1D Fast group was fasted from 0900 on day 0 to 0900 on day 1. The Refeed group was also fasted from 0900 on day 0 to 0900 on day 1, but food was replaced from 0900 on day 1 and the rats were killed at 0900 on day 2. The CTR group was also killed on day 2 at 0900 h. After decapsulation, 0.5 cm of the duodenum beginning 1 cm distal to the pyloric junction was removed, immediately frozen on dry ice, and stored at −80°C until use.

Preparation of Probes

cDNA fragments were amplified by PCR using Taq polymerase (Promega, Madison, WI) and gel purified using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The cDNA was labeled with [-32P]dCTP (NEN Life Science Products, Boston, MA) by random prime labeling (Rediprime, Amersham). A synthetic rat 18S rRNA oligonucleotide probe (5′-GACAAGCATATGCTACTGGC-3′) (31) was synthesized using a Beckman 1000M oligonucleotide synthesis machine (Beckman). The oligonucleotide probe was end-labeled using polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-32P]ATP (NEN Life Science Products).

Northern Blot Analysis

Total RNA was extracted using the guanidinium thiocyanate method as previously described (5). Fifteen micrograms of total RNA were denatured in Northern blot buffer [50% formamide, 16% formaldehyde, and 1× MOPS (0.02 M MOPS, 5 mM sodium acetate, and 0.1 mM EDTA, pH 7.0)] and separated on a 1.5% agarose gel containing 1.8% formamide and 1× MOPS. The RNA was transferred to nylon membrane filters (Qiabrand, Qiagen) by capillary blotting. The RNA was cross-linked to the membranes using a Stratalinker (Stratagene).

For cDNA probes, blots were prehybridized in a solution (Hyb) containing 6× SSC (0.9 M sodium chloride, 90 mM sodium citrate), 0.6% SDS, 100 μg/ml denatured salmon sperm, and 50% formamide at 50°C for 6 h. Hybridization was carried out in fresh Hyb solution containing 0.5 x 10^6 cpm/ml cDNA probe at 50°C overnight. After hybridization, the blots were washed twice in 2× SSC-0.1% SDS at room temperature for 15 min each, followed by another two washes in 0.2× SSC-0.5% SDS at 50°C for 15 min each. The membranes were exposed to autoradiographic film (Biomax, Kodak). The films were developed in a Kodak RP X-Omat developer. The blots were stripped by shaking in boiling hot 0.5% SDS solution for 10 min followed by a 5-min wash in 2× SSC at room temperature.

For the 18S oligonucleotide probe, blots were prehybridized in 6× SSC, 5× Denhardt’s solution, 100 μg/ml denatured salmon sperm DNA, and 0.1% SDS at 42°C for 1 h. Hybridization was carried out at a concentration of 0.5 x 10^6 cpm/ml. Blots were washed twice in 6× SSC-0.1% SDS at room temperature for 15 min followed by another wash at 55°C in 6× SSC-0.1% SDS for 15 min.

The intensity of the bands on the autoradiographic film was quantified by densitometric analysis using a digital imaging system (BIO-1D, Vilber Lourmat) and the accompanying software (Bio-Profil).

Statistical Analysis

mRNA concentrations were expressed as relative densitometric units (RU) against 18S rRNA (or actin) for the control group was normalized to 100 RDU for easy reference. The means of the various treatment groups were compared using one-way ANOVA followed by the Newman-Keuls multiple-comparisons test (SPSS for Windows). Differences were considered significant at P < 0.05.

In Situ Hybridization
cDNA probe production. A cDNA fragment corresponding to nucleotides 77−543 of the rat syncollin cDNA was obtained by PCR from a sip9 template using forward primer prsip42 (5′-CGGGGATTCCTGCTTCAGGTCGCCCGAGA-3′) and reverse primer prsip42 (5′-CGGGTCAGACCTGTAGCAGGTAAT-3′), respectively. The PCR product was then digested with EcoRI and Xho I and cloned into a pBluescript SK plasmid (Stratagene) that had been appropriately digested. The purified plasmid was linearized with Xho I to create a template for sense probe production or with EcoRI to create an antisense template (enzymes from Promega, Madison, WI). Sense and antisense probes were synthesized using T3 and T7 RNA polymerases, respectively (digoxigenin RNA labeling kit, Boehringer Mannheim).

Tissue preparation. A 220-g male Sprague-Dawley rat was killed by decapitation. The duodenum and pancreas were excised and snap frozen in liquid nitrogen. The tissues were mounted in tissue-freezing medium (Leica Instruments); 6-μm cryostat sections were cut, placed on poly-L-lysine-coated slides, and fixed for 20 min in cold 4% paraformaldehyde. After fixation, they were acetylated for 2 × 15 min in 1× PBS containing 0.1% diethyl pyrocarbonate (Sigma, St. Louis, MO). The slides were then heated at 65°C for 2 h in 2× SSC to destroy endogenous alkaline phosphatase activity. After a 15-min equilibration in 5× SSC, the sections were prehybridized in DAKO hybridization mixture, consisting of 60% formamide, 5× SSC, blocking agent, RNase inhibitor, and hybridization accelerator (DAKO). Each tissue section was exposed to 30 μl of this solution for 2 h at 50°C in a moist chamber. The solution was subsequently removed, and 15 μl of heat-denatured probe (400 ng/ml) in DAKO hybridization solution were added. The slides were hybridized overnight in a moist chamber at 50°C with the sections covered by coverslips.

After the overnight hybridization, the coverslips were floated in 2× SSC at room temperature. Excess RNA probe was removed by incubating with RNaseA (20 μg/ml) in NTE (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) for 30 min at 37°C. The slides were then washed in 2× SSC for 1 h, followed by a second wash in 0.1× SSC, both at 50°C. Subsequently, the slides were incubated in buffer 1 (100 mM Tris and 150 mM NaCl, pH 7.5) and blocked with buffer 2 (buffer 1 containing 5% blocking agent and 2% normal sheep serum) for 30 min. Alkaline phosphatase coupled antidigoxigenin antibody, diluted 1:1,000 in buffer 2, was then added and incubated at room temperature for 2 h. Excess antibody was removed by two washes in buffer 1 (15 min each), after which the slides were equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl2, pH 9.5). Color was developed by incubating in detection buffer containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (20 μl/ml) (Boehringer Mannheim) at room temperature for 5 h. Staining was terminated by incubating in 1× TE (10 mM Tris and 1 mM EDTA, pH 8.0) for 15 min. Nonspecific staining was removed by gentle agitation in 95% ethanol for 1 h, followed by a 15-min rinse in water. The slides were counterstained with orexin, dehydrated in xylene, and mounted.
RESULTS

Proximal Small Intestinal-Specific Genes

The candidate sip-specific cDNAs were cloned into pMOSBlue T vector. Fifteen clones were isolated. All 15 clones were confirmed to be differentially expressed in the proximal but not distal 12 cm of the rat small intestine by Northern blot analysis (Fig. 3). The clones were sequenced, and the sequence obtained was compared with known sequences in GenBank using the NCBI BLAST search program. The results of the BLAST search are shown in Fig. 3. Fourteen of the clones were either identical to or showed a high degree of homology to known genes. However, one of the clones, sip9, did not show a significant match with any of the sequences in GenBank. The sequence of the sip9 cDNA fragment is italicized in Fig. 1. We proceeded to isolate the 5' - and 3' -ends of this novel gene.

Characterization of sip9

5' - and 3' -RACE were used to obtain the 5' - and 3' -ends of the sip9 cDNA, respectively. The DNA sequences of the 5' - , 3' - , and original sip9 fragments were aligned using the Contig Manager program (DNA-SIS 2.1 for Windows, Hitachi). The contiguous sequence of the entire sip9 clone is shown in Fig. 4. Both strands of each fragment were sequenced in their entirety at least twice to obtain the definitive sequence. In addition, a separate clone was obtained by reverse transcription-amplification (RT-PCR). Proximal small intestinal mRNA was reverse transcribed using an oligo(dT) primer, and an aliquot of the reaction was amplified with KlenTaq polymerase (Clontech). Primers used for the primary and secondary amplifications were prsip5 and 7 and prsip6 and 8, respectively (Fig. 4). The fragment obtained was cloned into pMOSBlue T vector. Both strands of the cDNA fragment were sequenced twice. The sequence obtained from the RT-PCR fragment was identical to the original sequence.

The full-length sip9 clone was analyzed for ORF using an ORF search program (Molecular Biology Tools Pack, Adelaide University, Australia). The predicted ORF is shown in Fig. 4. sip9 encodes a putative peptide of 134 amino acids with a molecular mass of ~15 kDa. The peptide possesses a strong hydrophobic NH₂-terminal region.

<table>
<thead>
<tr>
<th>sip1/sip14</th>
<th>sip3/sip7/sip11</th>
<th>sip4/sip13/sip17</th>
<th>sip9</th>
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<tr>
<td>R. norvegicus sterol esterase mRNA</td>
<td>Rat pancreatic triglyceride lipase mRNA</td>
<td>Rat pancreatic phospholipase A2 mRNA</td>
<td>Novel</td>
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<td>P D</td>
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Fig. 3. Representative Northern blots showing specific expression of sip clones in proximal (P) and distal (D) 12 cm of small intestine. Locations of 28S and 18S rRNA bands are indicated. Hybridization of respective blots to an 18S oligonucleotide probe is shown in respective bottom panels.

<table>
<thead>
<tr>
<th>sip5/sip18</th>
<th>sip6/sip19</th>
<th>sip8</th>
<th>sip15</th>
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<tr>
<td>Rat carboxypeptidase B gene, exons 10 and 11</td>
<td>H. Sapiens mRNA for chymotrypsin-like protease</td>
<td>Rat vitamin dependent calcium binding protein mRNA</td>
<td>Rat mRNA encoding pancreatic trypsinogen I</td>
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Determining 5'-End of sip9 cDNA: Resolving a Discrepancy

Soon after sip9 was deposited in GenBank (accession no. AF012887), a similar cDNA sequence encoding the secretory granule protein syncollin was reported by Edwardson et al. (9). Syncollin was cloned from a rat pancreatic cDNA library. Comparison between the sip9 and syncollin sequence is shown in Fig. 1. The sequence reported in this study is 20 nucleotides shorter at the 5'-end of the cDNA, resulting in a truncation of 11 amino acids at the NH2 terminus of the putative peptide. We performed several experiments to clarify the 5'-end of the syncollin sequence.

5'-RACE. Repeated 5'-RACE was performed using different enzyme mixes, primers, and mRNA templates from both the pancreas and duodenum of different rats. All the fragments obtained consistently yielded the 5'-end of the sip9 cDNA.

RT-PCR. RT-PCR was performed on both rat pancreatic and duodenal mRNA using the primer pairs indicated in Fig. 1. Using primer pairs prsip23 and 24, we amplified a 400-bp fragment, corresponding to the expected size of the sip9 ORF, from both rat duodenal and pancreatic mRNA. However, amplification using prsip22 and 24 [corresponding to the ORF given by Edwardson et al. (9)] was unsuccessful (Fig. 5).

Genomic sequence. We have also cloned 2.5 kb of the 5'-flanking region of rat syncollin gene. No sequence corresponding to the extra 20 bp reported by Edwardson et al. (9) was found anywhere within the 2.5 kb of sequence upstream of the transcriptional start site.

S1 nuclease protection assay. S1 nuclease protection was also performed to clarify the 5'-end of the sip9/syncollin cDNA. Two 70-mer oligonucleotides, prsip29 and 30, were synthesized. Primer prsip30 was comple-
mentary to nucleotides +1 to +70 of the syncollin cDNA (9). prsip29 was complementary to nucleotides −20 to +50 of the sip9 genomic sequence. As shown in Fig. 6, S1 nuclease digestion of the prsip30 reaction resulted in a protected fragment corresponding to the 5’-end of sip9, not syncollin (lane 4). Similarly, S1 nuclease digestion of the prsip29 reaction resulted in a protected fragment corresponding to the 5’-end of sip9 (Fig. 6, lane 2).

Tissue-Specific Regulation of sip9 Expression in Rat

The sip9 cDNA probe hybridized to specific bands in the rat multitissue Northern blot (Fig. 7A). The gene is expressed in the duodenum, pancreas, spleen, and colon. The probe also hybridized to several bands of lower molecular mass (especially in the pancreas and spleen). The bands persisted even with more stringent washes, indicating the likelihood of multiple related mRNAs to syncollin in these tissues. Syncollin is not expressed in the jejunum and ileum (Fig. 7B). Within the duodenum, it is expressed in a graded manner with maximal expression in the proximal duodenum (Fig. 7B).
In situ hybridization was performed to identify the cells expressing sip9 in the duodenum and pancreas. As shown in Fig. 8, A and C, strong hybridization signals were observed in the exocrine acinar cells of the pancreas, whereas the endocrine islets of Langerhans, the interlobular septa, blood vessels, and connective tissues showed no hybridization. In the duodenum, expression was localized to the epithelial cells along the intestinal villus. The lamina propria, muscle layers, submucosa, and Brunner’s glands were negative (Fig. 8, E and G). No hybridization signals were observed in adjacent sections when the sense probe was used (Fig. 8, B, D, F, and H).

To determine the regulation of sip9 expression in the proximal small intestine, rats were fasted for 1 and 3 days. Northern blot analysis showed that fasting significantly decreased sip9 expression in the proximal small intestine by ~60 and 80% after 1 and 3 days, respectively (Fig. 9A). When rats that had been fasted for 1 day were given free access to food, gene expression increased to threefold higher than control levels after 1 day of feeding (Fig. 9B).

Developmental regulation of syncollin in the rat gut during embryonic and postnatal development was analyzed by Northern blots. As shown in Fig. 10, sip9 mRNA expression was not detected in the late gestational fetal gut. In neonates that were allowed to suckle, sip9 expression was detected in the small intestine within the first 24 h after birth (PD-1 group). Its expression continued to increase in 2- and 7-day-old...
pups that were allowed to suckle. Sip9 mRNA levels exhibited a fourfold increase by postnatal day 7, compared with the PD-1 group. However, when pups were denied suckling, expression of sip9 was delayed and was nondetectable 24 h after birth.

**DISCUSSION**

All 15 clones isolated were differentially expressed in the proximal small intestine compared with the ileum. Sequence analysis of the 15 clones revealed that 14 of them were known genes encoding proteins involved in digestion. One of the fifteen, sip9, was novel at the time of characterization.

Clones sip1 and sip14 were fragments of the gene encoding rat sterol esterase mRNA (4). Sterol esterase has a broad substrate range and hydrolyzes phospholipids, vitamin esters, cholesterols, and triglycerides. Hence, it is also known as cholesterol esterase, carboxyl ester lipase, carboxyl ester hydrolase, and lysophospholipase (24). Sterol esterase mRNA was originally cloned from the pancreas. Proteins with sterol esterase activity have been detected in extrapancreatic tissues such as the intestine and aorta and in macrophages and milk (2). However, Reue et al. (19a) reported that carboxyl ester lipase (sterol esterase) mRNA was absent in rat intestine and it was suggested that the esterase activity in the intestine was derived from the pancreas. The data in this paper show that rat sterol esterase is expressed only in the more proximal part of the small intestine.

Clones sip3, sip7, and sip11 were cDNA fragments of the gene encoding rat pancreatic triglyceride lipase mRNA. Pancreatic lipase is known to be expressed in mouse liver (3) and rodent pancreas (1). This study demonstrates that it is expressed in the rat proximal small intestine as well.

Clones sip4, sip13, and sip17 were different cDNA fragments of the gene encoding rat pancreatic phospholipase A2 (PLA2) mRNA (18). Pancreatic PLA2 mRNA expression has been detected in rat gastric mucosa, lung (26), spleen (32), and pancreas (26). PLA2 activity has also been associated with the intestinal brush border in the rat (16). This study confirms the expression of this gene in the rat intestine and shows that this gene is differentially expressed in the proximal small intestine.

Clones sip5 and sip18 were cDNA fragments of the gene encoding rat carboxypeptidase B gene. This enzyme belongs to a larger family of carboxypeptidases that aid in the digestion of proteins. In addition, some members of this family are involved in the proteolytic processing of neuropeptides in various tissues. The carboxypeptidase B mRNA was cloned from the rat pancreas (6). This study shows that this gene is also expressed in the rat proximal small intestine.

Clones sip6 and sip19 were highly homologous to the human gene encoding chymotrypsin-like protease (14). In addition to the pancreas, chymotrypsin-like activity has been detected in other tissues in the body, including the duodenum. Duodenase is an enzyme purified from bovine duodenal mucosa that has been shown to have this activity (35). The identification of a gene encoding chymotrypsin-like protease in the rat duodenum is consistent with the presence of this activity in this tissue.
Clone sip8 was a cDNA fragment of the gene encoding rat vitamin-dependent calcium binding protein (calbindin) mRNA. There are two isoforms of this protein. Three distinct mRNA species (1.9, 2.8, and 3.2 kb) encode the larger 28-kDa calbindin. The larger isoform is expressed in rat kidney and brain (33). The sip8 fragment was a cDNA fragment of the gene encoding the smaller 9-kDa isoform of calbindin, Dk9 (7). In the placenta, calbindin Dk9 mRNA is expressed in the trophoblast epithelium. In the intestine, its expression is limited to the duodenum, where it exhibits a crypt-to-villus and a cephalocaudal gradient of expression (34). The data here are consistent with the known pattern of expression of this gene in the small intestine.

Clone sip15 corresponds to rat pancreatic trypsinogen I. Trypsinogen I is the major mRNA in pancreas accounting for 2–5% of total mRNA there. Trypsin is responsible for the activation of several digestive enzymes including chymotrypsinogens, proelastase, and procarboxypeptidase in the duodenal lumen. Most of the differentially expressed sip genes that were identified encoded digestive enzymes, mainly lipases and proteases. The small intestine functions to digest and absorb food and nutrients. The presence of these enzymes early in the digestive process as food transits along the small intestine ensures efficient digestion.

It is noted that a number of our proximal small intestine-specific genes are also expressed in the pancreas. This is not surprising, because the pancreas arises from the foregut endoderm during development. Also, it is likely that the pancreas and duodenum share common transcriptional regulatory mechanisms. PDX-1, a homeodomain transcription factor that is necessary for pancreatic-specific gene expression and development, also plays an important role in the normal differentiation and development of the duodenum (17).

The clone sip9 showed no homology to other known genes when it was isolated. We proceeded to isolate the complete cDNA of the gene. Figure 5 shows the complete cDNA nucleotide sequence and the amino acid sequence of the putative peptide encoded. The sequence was deposited in GenBank (accession no. AF012887). The cDNA is 580 nucleotides, excluding the polyAs. The predicted ORF begins at position 14 and ends at position 416, giving rise to a putative peptide of 134 amino acids. The ACC sequence preceding the ATG start codon is consistent with the consensus ACCATG/GCCATGG translation initiation sequence determined by Kozak (13).

Subsequently, it was shown by others (9) that the sip9 sequence encoded a secretory granule protein, syncollin, that binds to syntaxin in a calcium-sensitive manner. A comparison between the sip9 sequence and syncollin shows that sip9 is short by 20 nucleotides at the 5'-end of the mRNA, resulting in a truncation of 11 amino acids at the NH2 terminus of the putative peptide. Repeated 5'-RACE with both pancreatic and duodenal mRNA using different primers and different en-
zyme preparations consistently yielded fragments with the 5′-end reported in this study. In addition, RT-PCR using both rat pancreatic and duodenal mRNA showed that specific amplification products were obtained only when primers corresponding to the sip9 ORF was used. No amplification product was obtained when the primer pair corresponding to the syncollin ORF was used (Fig. 5).

Using a rat promoter-finder kit, we obtained a 2.5-kb fragment of the 5′-flanking region of syncollin. No sequence corresponding to the additional 20 bp reported by Edwardson et al. (9) was present in this fragment. More convincingly, when S1 nuclease protection assay was performed using a primer synthesized according to the proposed 5′-end of the syncollin cDNA given by Edwardson et al. (9), the extra 20-bp sequence at the 5′-end was not protected. The 5′-end of the protected fragment corresponds to the sip9 cDNA. We also subjected the extra 20 bp at the 5′-end of the syncollin cDNA sequence to a BLAST search. It was shown to be identical to nucleotides +1,999 to +2,018 of the rat multifunctional protein-2 cDNA (GenBank accession no. X94978).

The results from experiments to clarify the 5′-end of syncollin provide strong evidence that the 5′-end reported in this paper is the authentic one. It is likely that the syncollin cDNA isolated by Edwardson et al. (9) included a portion of the rat multifunctional protein cDNA that was ligated to the 5′-end of the cDNA.

Syncollin was detected in the membrane fractions of the pancreas and parotid gland (9). This study shows the expression of syncollin mRNA in the proximal small intestine, pancreas, spleen, and colon. It is likely that syncollin may be involved in secretory activities in these tissues. In the small intestine, syncollin expression was confined to the duodenum, where secretory activity is high. The rat duodenum is ~10 cm in length and is defined as the length of the small intestine immediately after the pyloric junction to the ligament of Treitz. Syncollin expression in the duodenum was not uniform. Highest expression was detected in the proximal two-fifths of the duodenum (Fig. 7B), probably reflecting higher secretory activity in this part of the duodenum.

In situ hybridization showed that syncollin was expressed in the exocrine acinar cells of the pancreas and the mucosal epithelium of the duodenum. This pattern of syncollin expression in the pancreas and intestine is similar to that of other proteins that may be involved in Ca2+-regulated exocytosis. For example, Prot17 has been localized to the basolateral and microvillous zymogen granule and subluminal vesicle membranes of intestinal epithelium and pancreatic acinar cells (27). Similarly, a novel 28-kDa Ca2+-regulated protein, CRHSP28, was localized to zymogen-granule-rich areas in the exocrine acinar cells of the pancreas, parotid, submandibular gland, and lacrimal gland. It was also highly expressed in mucosal epithelial cells (11).

Various soluble N-ethylmaleimide-sensitive fusion protein attachment receptor (SNARE) isoforms, such as syntaxin-3, SNAP-23, TI-VAMP, cellubrevin, and munc18–2 are expressed in intestinal and colonic epithelial cells, where they are proposed to play a role in exocytotic events in these cells (20). Syncollin, a secretary granule protein, may interact with SNAREs in the duodenal enterocyte to regulate secretory activities associated with digestion. The regulation of syncollin expression by feeding is consistent with this proposal. Fasting, which removes tactile and nervous stimuli on the duodenum, results in a decrease in enterocyte secretion and syncollin expression. Feeding after a 1-day fast increases expression of syncollin to levels about threefold higher than controls, consistent with a greater stimulation of the duodenum caused by an increase in food intake occurring at this time.

Unlike the human small intestine, in which development is largely completed well before birth in the first trimester, organogenesis in the rodent gut progresses much more slowly, being completed only at postnatal week 3. After birth, the major developmental milestones in the rat gut occur on introduction of milk and at weaning, during which significant cellular and functional maturation take place.

Syncollin expression was detected in the rat gut 1 day after birth and was increased fourfold by postnatal day 7. The upregulation of the syncollin gene after birth was dependent on stimuli-associated feeding. In the absence of feeding, gene expression was still undetectable 24 h after birth. It is likely that the expression of syncollin reflects the cellular and functional maturation of the enterocyte occurring after birth, with feeding. The expression of syncollin is required to allow secretory activities associated with digestion to take place.

Although it was reported that syncollin was absent in the membrane fraction of the spleen (9), our Northern blot analyses have consistently shown that syncollin mRNA is expressed in this tissue. In addition, Soares et al. also reported the cloning of the 3′-expressed sequence tag of rat syncollin (GenBank accession no. AA926002) from an adult rat spleen library, thus confirming the validity of our Northern blot data. This discrepancy may be caused by two possibilities. First, syncollin may not be membrane bound in the spleen. This raises the possibility of another role for syncollin in the spleen. Recently, it has been shown that syntxin, another membrane-associated protein involved in calcium-mediated exocytosis, may have multiple functions in cells. Rat syntxin-2 and its mouse homolog, epimorphin, may be involved in fetal gut, lung, and skin morphogenesis during ontogeny (10). Second, it is possible that translational regulation may account for the presence of syncollin mRNA but not the protein in the spleen. An example is rat acidic calcium-independent PLA2 (aiPLA2), which was recently cloned from rat granular pneumocytes. Although aiPLA2 mRNA was detected in other tissues such as brain, heart, liver, kidney, spleen and intestine, the aiPLA2 protein was only detected in the alveolar macrophages and cells of the bronchial epithelium. A role for translational...
control of aiPLA2 was suggested for the above observations (12).

In conclusion, we have demonstrated that subtractive hybridization is a systematic way to isolate genes that are differentially expressed in the proximal small intestine. In this study, we have also identified and characterized a novel gene, sip9, which was shown to encode syncollin (9). Several experiments were performed to clarify the 5′-end of syncollin, and the results confirm the 5′-end reported in this paper. Other than the pancreas and duodenum, syncollin mRNA was also detected in the spleen and colon. In situ hybridization localized syncollin to the duodenal villus epithelial cells and the exocrine acinar glands of the pancreas. In addition, this study also shows physiological and developmental regulation of syncollin.

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Address for reprint requests and other correspondence: S. C. Hooi, Dept of Physiology, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (E-mail: phshsc@nus.edu.sg).

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