Peptide YY and Neuropeptide Y Induce Villin Expression, Reduce Adhesion, and Enhance Migration in Small Intestinal Cells through the Regulation of CD63, Matrix Metalloproteinase-3, and Cdc42 Activity*

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Peptide YY (PYY) and neuropeptide Y (NPY) are regulatory peptides synthesized in the intestine and brain, respectively, that modify physiological functions affecting nutrient assimilation and feeding behavior. Because PYY and NPY also alter the expression of intestine-specific differentiation marker proteins and the tetraspanin CD63, which is involved in cell adhesion, we investigated whether intestinal cell differentiation could be linked to mucosal cell adhesion and migration through these peptides. PYY and NPY significantly decreased cell adhesion and increased cell migration in a dose-dependent manner prior to cell confluency in our model system, non-tumorigenic small intestinal hBRIE 3801 cells. Both peptides reduced CD63 expression and CD63-dependent cell adhesion. CD63 overexpression increased and antisense CD63 CDNA decreased intestinal cell adhesion. In parallel, both PYY and NPY increased expression of matrix metalloproteinase-3 (MMP-3) to a level sufficient to induce cell migration by activating the Rho GTPase Cdc42. The effects of both peptides on cell migration were blocked in cells constitutively overexpressing dominant-negative Cdc42. PYY and NPY also significantly induced the expression of the differentiation marker villin, which could be eliminated by an MMP inhibitor at a concentration that inhibits cell migration. Increased MMP-3 activity, which enhanced cell migration, also induced villin mRNA levels. Therefore, these data indicate that the alteration of adhesion and migration by PYY and NPY occurs in part by synchronous modulation of three proteins that are involved in extracellular matrix-basolateral membrane interactions, CD63, MMP-3 and Cdc42, and that PYY/NPY regulation of expression of mucosal proteins such as villin is linked to the process of cell migration and adhesion.

The maintenance of mucosal epithelial cells in a structure of well regulated spatial organization of specific cell phenotypes likely requires the coordination of events that are sensitive to a feedback regulation involving luminal signals (at the apical cell surface), signals derived from the mesenchyme, the extracellular matrix (ECM); at the basolateral surface), and receptosecretory cells. Receptosecretory cells of the gastrointestinal mucosa are endocrine cells in close contact with the luminal surface that synthesize and secrete bioactive peptides, many of which are found both in neuronal cells of the central and peripheral nervous systems and in endocrine cells in the mucosa of the anterior and posterior intestine. These peptides are responsive, either directly or indirectly, to the nutritional status of the organism and act to modify a number of responses ranging from behavior (neuropeptide Y (NPY), peptide YY (PYY), ghrelin, and cholecystokinin (1–3), digestion and absorption (gastrin, secretin, cholecystokinin, PYY, and motilin) (4), to metabolism (somatostatin, glucagon-like peptides, and glucose-dependent insulinotropic polypeptide) (4–7). Like many growth factor receptors, the receptors for these peptides are found in both neuronal and epithelial cells, where these peptides act as paracrine, neurocrine, and/or endocrine agents. Thus, these bioactive peptides are uniquely positioned to be part of a mechanism of feedback regulation coordinating mucosal homeostasis, such as cell proliferation, migration, and differentiation, in response to environmental (luminal) signals as well as neuronal (central and peripheral nervous systems) signals. How signals from these peptides are coordinated with those originating from exogenous sources, such as the diet, is still unclear. Two peptides that are responsive to nutrient cues are PYY and NPY.

PYY and NPY are 36-amino acid peptides that share 50% amino acid identity as well as a hairpin-like three-dimensional structure (PP-fold) with an amidated C terminus (8). PYY is synthesized in enteroendocrine cells of the mucosa of the distal small intestine and colon. It is released from the gastrointestinal tract in response to a meal and is directly released in response to free fatty acids (9). Its classical effects include the inhibition of intestinal motility, gall bladder and pancreatic exocrine secretion, peripheral inhibition of fluid and electrolyte secretion in the intestinal tract (10), and vasoconstriction (11). PYY is also localized to neurons (12), where it may have not only peripheral action, but also action affecting the central nervous system and behavior such as food intake (1). NPY is widely distributed throughout both the central nervous system and sympathetic peripheral nerves and is particularly abundant in the hypothalamus and cerebral cortex (13, 14). NPY

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1 The abbreviations used are: ECM, extracellular matrix; NPY, neuropeptide Y; PYY, peptide YY; I-FABP, intestinal fatty acid-binding protein; MMP, matrix metalloproteinase; IMDM, Iscove’s Modified Dulbecco’s medium; RT, reverse transcription; PBS, phosphate-buffered saline; PBD, p21-binding domain; NNGH, N-isobutyl-N-(4-methoxyphenyl)glycylhydroxamic acid; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MT, matrix type; LPH, lactase phlorizin hydrolase.
induces food intake in several species when injected intracerebroventricularly and decreases feeding when its hypothalamic expression is reduced (15, 16). Centrally, NPY is also implicated in decreased thermogenesis (17) and inhibition of sedation and memory (18–20) and plays a role in neuronal development (21, 22). Both PYY and NPY bind to PP-fold receptors called the neuropeptide Y receptors, which are members of the rhodopsin-like receptor family belonging to the heterogeneous superfamily of G protein-coupled receptors. Five subtypes of Y receptors have been cloned from mammals (Y1, Y2, Y4, Y5, and Y6) (23). PYY and NPY have similar binding affinity compared to the Y1, Y2, and Y5 receptors (23–26), which likely contributes to the sharing of several biological functions, including inhibition of gastrointestinal motility, chloride ion secretion, and pancreatic enzyme secretion.

There have been extensive studies as to the physiological responses of these peptides, but fewer studies characterize their effects on gene regulation (27–30). We previously reported that PYY and NPY induce the expression of a late marker of differentiation-dependent marker I-FABP (hBRIE 380i cells) (31, 33, 34). We investigated whether PYY and NPY regulation of CD63 affects the expression of differentiation-dependent proteins such as villin before the cells develop a fully differentiated phenotype and whether this process involves modulation of cell adhesion and/or migration.

We present data demonstrating for the first time that, in non-tumorigenic intestinal epithelial cells, both PYY and NPY decrease cell adhesion and induce cell migration and that these occur through the interaction of three protein families: tetraspanins, Rho GTPases, and matrix metalloproteinases. We show that, in pre-confluent cells, PYY and NPY decrease the expression of CD63 and cell adhesiveness, the opposite of what occurs when cells have been confluent for several days. Thus, the regulation of gene expression by these neuroregulatory peptides in enterocytes can be bimodal, dependent on cell state or age. In parallel, PYY and NPY induce intestinal cell migration mediated by Rho GTPase Cdc42 activation and induction of the matrix metalloproteinase (MMP)-3 (stromelysin) transcript. PYY/NPY-induced villin mRNA is blocked upon inhibition of MMP activities, and the villin transcript increases with constitutively elevated expression of MMP-3. These data suggest that expression of proteins specific to a mucosal cell phenotype in situ might be regulated differentially by a peptide hormone depending on the cell’s position along the crypt-to-villus axis and that this expression can be modulated through the process linked to cell migration.

MATERIALS AND METHODS

Cell Culture Conditions—The cell line utilized in this study was a well characterized subclone of the Berkeley rat intestinal epithelial hybrid cell line expressing PYY/NPY-binding sites and the enterocyte differentiation-dependent marker I-FABP (hBRIE 380i cells) (31, 33, 35). The cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 10% bovine calf serum (Hyclone Laboratories),(100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen) in T75 flasks (Corning) and kept in an atmosphere of 5% CO2 and 95% air at 37 °C. For the experiments, cells were trypsinized and plated in T25 flasks coated with collagen type I. Cells were pretreated at rat tails as described previously (31, 35). Cells were at pre-confluency 24 h after being laid down at a density of 5 × 104 cells/flask, at near confluency 48 and 72 h after seeding, and at post-confluency 3 days after reaching confluency.

Cells grown in T25 flasks coated with collagen type I were treated with or without 100 nM PYY(1–36) or NPY(1–36) (human sequences; American Peptide Co. Inc.) for 6 h in limiting medium (IMDM containing 0.1% bovine serum and 4 µg/ml transferrin) before total RNA was isolated for semiquantitative reverse transcription (RT)-PCR analysis. Preceding affinity precipitation of Cdc42, cells in collagen type I-coated T75 flasks were pre-starved for 15 h in limiting medium and starved in serum-free IMDM for 2 h before treatment with or without PYY or NPY at 100 nM. The peptides were dissolved in phosphate-buffered saline (PBS); therefore, in the experiments involving peptide treatment, the same amount of PBS was added for the control.

RT-PCR Analysis and PCR Cloning—Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocol. One µg of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) in a 20-µl reaction under the conditions described previously (32) using 0.5 µg of random hexamer (Invitrogen). One µl of the RT mixture was used for semiquantitative PCR analysis using a 25-µl reaction using Taq DNA polymerase (Promega) with the primers (Qiagen, Inc.) listed in Table I. The cycling parameters for PCR were: denaturation at 94 °C for 20 s, annealing at 58 °C for 15 s, and extension at 72 °C for 30 s. The numbers of cycles listed in Table I were chosen for when the amplification was still at the exponential phase. Control RT-PCR was done to detect possible interference from genomic DNA contamination. The level of 18 S RNA expression in each sample was also analyzed as an RNA content control between samples. The specificity of the PCR products was confirmed by restriction enzyme mapping and/or DNA sequencing (DNA Sequencing Facility, University of California, Berkeley, CA).

For semiquantitative measurement of CD63, villin, and MMP-3 levels, a PCR standard curve was prepared using the amplified 741-bp CD63, 516-bp villin, and 587-bp MMP-3 cDNAs, respectively. The fragments were purified and quantitated on agarose gel containing 100-bp DNA mass ladder (New England Biolabs Inc.) as a standard. The purified fragments were serially diluted and PCR-amplified with the amplified number of cycles as listed in Table I to determine the appropriate concentration range for a standard curve for a given RT-PCR result. The band intensity on agarose gel was scanned densitometrically and quantitated using Molecular Analyst software (Bio-Rad).

Peptide Amplification to isolate rat CD63, Cdc42, PAK3 Cdc42-binding domain (PBD), and MMP-3 cDNAs was carried out using Pfu DNA polymerase (Strategene) with the primers listed in Table I. DNA sequencing was performed to verify sequences of the amplified fragments and the orientation of the isolated cDNA for expression relative to the driving promoter of the vector.

Plasmid DNA Construction for CD63 and Antisense CD63 Overexpression, MMP-3 Overexpression, and Dominant-negative and Constitutively Active Cdc42—The open reading frame of rat CD63 cDNA was PCR-amplified (38 cycles of denaturation at 94 °C for 40 s, annealing at 59 °C for 40 s, and extension at 72 °C for 1.5 min) and cloned into the Smal site of the pCI-neo mammalian expression vector (Promega) for CD63 overexpression. Antisense CD63 experiments utilized the plasmid construct described previously (32), which contained the full CD63 open reading frame inserted into the expression vector in the antisense orientation.

The expressed fragment of rat MMP-3 cDNA was isolated by two-step PCR amplification. The first step was five cycles of denaturation at 94 °C for 40 s, annealing at 68 °C for 40 s, and extension at 72 °C for 2 min. After 100-fold dilution, 1 µl of the PCR mixture was used as the template of the second amplification, comprising 35 cycles of denaturation at 94 °C for 40 s, annealing at 58 °C for 40 s, and extension at 72 °C for 2 min. The amplified MMP-3 cDNA was then cloned into the EcoRV site of pCNA6/V5-HisA (Invitrogen).

The dominant-negative and constitutively active Cdc42 mutants constructed had mutations T17N and G12V, respectively. The open reading frame of rat Cdc42 cDNA was PCR-amplified and cloned into the EcoRV site of pBlueScript II SK(+) (Strategene). The mutation was performed using the QuikChange site-directed mutagenesis method (Strategene). Ten ng of pBlueScript II SK(+) containing Cdc42 cDNA was PCR-amplified using the QuikChange site-directed mutagenesis method containing Cdc42 cDNA was PCR-amplified using the QuikChange site-directed mutagenesis method. The primers used to synthesize dominant-negative Cdc42 were 5'-GTTGGTAAATACT-GTCCTCTGATATCCTACACAAC-3' and 5'-ATCAGGAGACCTTATTACCAAGCACCATCAC-3' (with the nucleotides mutated shown in boldface, conferring mutation T17N). The primers for constitutively active Cdc42 were 5'-TTCGAGATGTTCAGTGGTTTAAAACATGCTC-3' and 5'-ACCAGCAACAATCACCAGCACAACACACTTA-A.
The primer sequences are listed in the 5′ to 3′ direction and were derived from GenBank®/EBI Data Banks with the indicated accession numbers. The villin sequence is of a cDNA fragment corresponding to exons 16–19 of rat villin. The other expected amplified fragments from the RT-PCR analysis are diagrammed in Fig. 7. The amplified CD63 cDNA for overexpression spans from 45 nucleotides before the start codon to 37 nucleotides after the stop codon, the MMP-3 fragment from 6 nucleotides before the start codon to 25 nucleotides after the stop codon, and the Cdc42 fragment from the start codon to 7 nucleotides after the stop codon. The forward primer for MMP-3 includes an additional 6 nucleotides before and 3 nucleotides after the start codon to ensure maximal expression. Similarly, the forward primer for Cdc42 includes an additional 6 nucleotides before the start codon to 7 nucleotides after the stop codon to ensure maximal expression. The forward primer of PBD includes one of the BamHI sites (in boldface) for subcloning into pGEX-2T; the reverse primer has a stop codon (in boldface).

**RT-PCR**

| Gene          | Accession No. | Forward primer | Reverse primer | PCR cycles |
|---------------|---------------|----------------|----------------|------------|
| CD63          | NM_017125     | CAGAGATACAGATGAGAGGACG | CATAGGAAAAGACAGACGAGC | 35 |
| MMP-3         | NM_135232     | TGTCTATTGTACCTGACCACT | CCACTGAGAGGACGACGCTG | 35 |
| Cdc42         | NM_171994     | TCTACCATGCCTCTCTCTTC | TCTAGTTCCTCTCTCTCT | 41 |
| PAK3 (PBD)    | U33314        | GCGCTGCTTGTGTCTCTTG | CTCACTGAGATCTCTCAG | 28 |

**Cloning**

CD63  
N=017125  
GAGGATACACACCATCCTCTCT | CATAGGAAAAGACAGACGAGC |
MMP-3  
NM_135232  
TGTCTATTGTACCTGACCACT | CCACTGAGAGGACGACGCTG |
Cdc42  
NM_171994  
TCTACCATGCCTCTCTCTTC | TCTAGTTCCTCTCTCTCT |
PAK3 (PBD)  
U33314  
GCGCTGCTTGTGTCTCTTG | CTCACTGAGATCTCTCAG |

**Oligonucleotide primers used for RT-PCR analyses and cDNA cloning**

- A-3′ (with the nucleotides mutated shown in boldface, conferring mutation G12V). The native (methylated) plasmid template was eliminated by DpnI digestion. After transformation into Escherichia coli and purification, the mutated Cdc42 cDNA was excised by HindIII and XbaI digestion and subcloned into the same restriction enzyme sites of pcDNA3/V5-HisA.

**Cell Adhesion Assay**—Proliferating cells were plated at a density of 4 × 10⁵ cells/T25 flask and allowed to grow for 24 h. Three μg of plasmid DNA was transfected using 15 μl of Superfect (Qiagen, Inc.) following the manufacturer’s protocol. After 36 h, the cells were trypsinized and plated according to requirements of the subsequent experiments. Total RNA was also isolated from a cell aliquot to measure the levels of expression of the transfected gene.

To determine the efficiency of the transfection method, hBRIIE 380i cells were transfected with enhanced green fluorescent protein (Clontech) subcloned into pCI-neo. Twenty-four h after transfection, the cells were plated on 6-well glass microscope slides. After allowing the cells to grow for an additional 24 h, the expression of enhanced green fluorescent protein was examined using a UV light source (using a filter with excitation at 460–500 nm and emission at 505–560 nm). Percent transfection efficiency was calculated as the percentage of enhanced green fluorescent protein-expressing cells over the total number of cells. Cells transfected with an efficiency of at least 50% were used for experiments.

**Cell Migration/Motility Assay**—Cell Migration/Motility Assay—

**Construction and Purification of Glutathione S-Transferase (GST)-PBD Fusion Protein**—The cDNA fragment of rat PAK3 expressing amino acids 65–136 (PBD, an activated Cdc42-binding domain) (37) was PCR-amplified (31 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s) cloned into pBluescript II SK(+) at the EcoRV site, excised with BamHI, and subcloned into pGEX-2T (Amersham Biosciences) at the BamHI site. The plasmid construct was transformed into XL1-Blue, which was then grown in LB medium containing 100 μg/ml ampicillin. A single colony overnight culture was diluted 10 times with fresh medium supplemented with 100 μg/ml carbenicillin and grown to A₂₅₀ ~ 1. Subsequently, the expression of GST-PBD fusion protein was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside and allowed to proceed for 3 h. The bacterial cells were collected by centrifugation and washed once with ice-cold PBS. The fusion protein was purified as described (38) with modifications. The pellet from 100 ml of isopropyl β-D-thiogalactopyranoside-induced culture was resuspended in 1.5 ml of lysis buffer containing 50 mM Tris-Cl (pH 7.5), 200 mM NaCl, 5 mM MgCl₂, 5 mM dithiothreitol, 1 mM EDTA, 0.5 mg/ml lysozyme, 1 mg/ml DNase I, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After a 15-min incubation at 37 °C with gentle agitation, the
suspension was supplemented with 1.5% Sarkosyl and subjected to three cycles of freezing and thawing, followed by 5 s of vigorous vortexing. Bacterial cell debris was spun down at full speed in a microcentrifuge at room temperature for 5 min. The supernatant was then made into 2% Triton X-100 and incubated with end-to-end rotating at 4 °C with 50 μl of 50% slurry of glutathione-Sepharose beads (Amersham Biosciences) that had been washed with PBS and lysis buffer. Following incubation, the beads were washed five times with buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 1% (v/v) Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride and once with the same buffer containing 10% glycerol. Approximately the same volume of the glycerol-containing buffer was added to the washed beads before storage at −80 °C. The amount of purified GST-PBD was estimated by SDS-PAGE using known quantities of bovine serum albumin as standards.

Cdc42 Activity Assay—Cdc42 activity was assayed by GST-PBD affinity precipitation basically as described (39). Cells grown in T75 flasks coated with collagen type I at pre-confluency were preconditioned before treatment with PYY or NPY as follows. Cells were laid down in T75 flasks coated with collagen type I at a density of −1 × 106 cells/flask and incubated in normal medium. After 24 h, the cells were pre-starved for 15 h in limiting medium, followed by a 2-h incubation in serum-free medium. Following treatment with PYY or NPY at 100 nM for a period ranging from 0 to 5 min, the cells were washed with ice-cold Tris-buffered saline before being scraped off the flasks in buffer containing 50 mM Tris-Cl (pH 7.5), 500 mM NaCl, 10 mM MgCl2, 1% (v/v) Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation at 10,000 g for 10 min at 4 °C. Total protein content of the lysate was measured using Coomassie Blue reagent according to the Bradford method (Bio-Rad). Lysate with 300 μg of total protein was incubated with 20 μg of GST-PBD (Sepharose beads) for 1 h at 4 °C with end-to-end rotating. The beads were washed four times with ice-cold buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 1% (v/v) Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The beads were then boiled in the same volume of Laemmli sample buffer (40). One-third of each sample was subjected to electrophoresis on a 12% SDS gel and semidry Western blot transfer onto a polyvinylidene difluoride membrane (Millipore Corp.). The presence of activated Cdc42 was detected with rabbit anti-Cdc42 polyclonal antibody and horseradish peroxidase-coupled goat anti-rabbit antibody (both from Santa Cruz Biotechnology) using Western Lightning™ chemiluminescence reagent plus (PerkinElmer Life Sciences). As a control, total Cdc42 in each lysate (20 μg of total protein) was also analyzed.

RESULTS

At a stage of late confluency, hBRIE 380i cells develop clusters of differentiated cells, in which intestine-specific differentiation-dependent proteins such as I-FABP are expressed maximally (35). At this stage, PYY and NPY induce the expression of I-FABP (31, 35) and also of CD63. Under conditions of reduced CD63 mRNA, there is a delay of cluster formation and a reduction of I-FABP expression (31). CD63 is associated with cell adhesion (34), and increased expression of molecules associated with mucosal cell adhesion, such as β-catenin and E-cadherin, decreases cell migration (41). This brings forth the possibility that PYY and NPY effects on I-FABP expression, as an indicator of differentiation, could be a function of the state of cell adhesion and/or migration. To test this hypothesis, it was first necessary to determine whether PYY or NPY alters cell adhesion and migration. We chose to examine the effects of PYY and NPY in hBRIE 380i cells before reaching a confluent monolayer, a point at which cells would be likely more responsive to signals modulating adhesion/migration. To determine whether these alterations were linked to the timing of expression of markers of intestinal cell differentiation, we had to select an early marker of differentiation that is expressed initially when cells are in a state of transition from migration to contact inhibition (near confluency). Therefore, we first characterized hBRIE 380i cells for the timing of expression of differentiation protein markers known to exhibit specific expression patterns along the crypt-to-villus axis in the intestine.

The hBRIE 380i cells plated on type I collagen were examined at the state of pre-confluency (in the log phase of replication), near/early confluency (when cells start forming a confluent monolayer), and post-confluency (3 days from initial confluency, when cells develop clusters of differentiated cells). The characteristics of hBRIE 380i cells under these non-synchronous conditions are as follows: at near/early confluency, the cells are still replicating, but at a highly reduced rate compared with cells at the pre-confluence state, whereas cells at post-confluency are primarily non-replicative as determined by bromodeoxyuridine uptake (33). Endogenous expression of villin, I-FABP, and LPH was measured by RT-PCR (agarose gel image of amplified fragments not shown). These three protein markers displayed different patterns of expression depending on the state of cell confluency. Villin expression was the most abundant and could be seen starting at near/early confluency, continuously increasing to post-confluency. Consistent with
our previous observation (35), I-FABP message was quite abundant, but could be detected only after cells reached confluency. The expression level of LPH was the lowest even at 3 days post-confluency.

The timing of expression of villin, LPH, and I-FABP in the hBRIE 380i cells could be viewed as analogous to the expression pattern observed in the mucosa. In the intestine, villin expression is initiated early along the crypt-to-villus axis when cells have migrated out of the crypt and are non-replicative and still undifferentiated. I-FABP expression is highest toward the upper third of the villus, and LPH is expressed more toward the villus tips. We selected villin as a marker in our study because it is expressed in the intestine before cells enter the differentiated state, and its expression could be detected in hBRIE 380i cells before forming a confluent monolayer.

**PYY and NPY Reduce CD63 mRNA Expression in Pre-confluent Cells and Reduce Cell Adherence in a Time- and Dose-dependent Fashion**—PYY/NPY induce I-FABP and CD63 in post-confluent fully differentiated hBRIE 380i cells (31, 32). The expression levels of CD63 affect those of I-FABP. Because CD63 is necessary for cell adhesion in melanoma KM3 and MelJuso cell lines (42, 43), we tested whether CD63 also mediates adhesion of non-tumorigenic small intestinal hBRIE 380i cells and whether CD63 effects on the expression of differentiation markers such as I-FABP are a consequence of the role of CD63 in cell adhesion.

Unlike the increase in CD63 mRNA observed in cells at late confluency treated with PYY or NPY at 100 nM (Fig. 1A, P and N lanes versus C lane), identical treatment of cells at near confluency resulted in decreased CD63 expression (Fig. 1B, P and N lanes versus C lane). Cells treated with PYY or NPY at 100 nM or with a combination of 50 nM PYY and 50 nM NPY displayed a similar reduction of cell adhesion (Table II), suggesting that the activation of the Y receptor(s) by these two peptides has a similar net effect on cell adhesion. This decrease in adhesion occurred in a dose- and time-dependent fashion (Fig. 2, A and B).

**Reduced Expression of CD63 Increases PYY- and NPY-induced Cell Migration and Reduces Adherence of hBRIE 380i Cells**—To determine whether CD63 affects intestinal cell adhesion, hBRIE 380i cells were transiently transfected with either sense CD63 cDNA (for constitutive overexpression) or antisense CD63 cDNA and the empty vector as a control. Overexpression of CD63 resulted in significant increased cell adhesion (Fig. 3, black bars, CD63, C compared to Vector, C). This greater adherence to the substratum in cells overexpressing CD63 is expected if, in hBRIE 380i cells, CD63 has its reported role in cell adhesion (42, 43). Reduction of the CD63 transcript level in cells transfected with antisense CD63 cDNA conversely resulted in a significant decrease in cell adhesion (Fig. 3, black bars, CD63 AS, C compared to Vector, C). To establish that decreased CD63 expression directly caused the decreased adhesion observed upon PYY/NPY treatment, cell adhesion of the transfectants expressing elevated or reduced CD63 levels was assayed in the presence of 50 nM PYY and 50 nM NPY. Cells overexpressing sense or antisense CD63 cDNA were no longer affected by PYY and NPY. This does not preclude involvement of other proteins in cell adhesion. Consistent with the profile of PYY and NPY effects on cell adhesion, either PYY or NPY at 100 nM no longer significantly affected CD63 levels in cells transfected with the sense or antisense CD63 construct, although the peptides reduced CD63 expression from 60 to 40% in the control cells (Fig. 4).

Treatment with PYY or NPY induced hBRIE 380i cell migration in a dose-dependent manner (Fig. 5). As seen with cell adhesion, PYY or NPY at 100 nM or a mixture of 50 nM PYY and 50 nM NPY had similar effects on cell migration (Table II). To
test whether there was an inverse correlation between PYY/ NPY effects on cell migration and cell adhesion mediated by CD63, we examined the effects of altered CD63 levels on cell migration. The hBRIE 380i cells overexpressing CD63 had ~40% reduced migration (Fig. 3, white bars, CD63, C compared to Vector, C), whereas those transfected with antisense CD63 cDNA had significantly induced migration compared with control cells (Fig. 3, white bars, CD63 AS, C compared to Vector, C). PYY and NPY increased migration of cells transfected with vector alone to ~150%, but the extent of reduction in response to the peptides was less in cells transfected with sense and antisense CD63 cDNAs (Fig. 3, white bars, P/N versus C).

In summary, PYY and NPY down-regulated CD63 expression in hBRIE 380i cells at near confluency, causing a decrease in adhesion that correlated with induced migration. However, changes in CD63 expression alone could not account for the still significant increase in PYY/NPY-induced migration of cells overexpressing sense and antisense CD63 cDNAs.

Induced MMP-3 Levels/Activities Increase PYY/NPY-enhanced Cell Migration—Given the reciprocal relationship between cell adhesion and migration, we investigated whether PYY/NPY-enhanced cell migration and PYY/NPY-decreased adhesion are linked by substratum-plasmalemma interactions occurring through MMPs. We first tested whether decreased
tant contributor to this cellular response. MMP-3 appears to be an important mediator of PYY/NPY-sensitive migration, as observed by the significant reduction in cell migration when the MMP-3-specific inhibitor NNGH was added to the cells treated with PYY or NPY (Fig. 6). Therefore, although a number of MMPs may be involved in the PYY- and NPY-induced cell migration, the addition of NNGH significantly reduced cell migration in comparison to the control (Fig. 8). This suggests that MMP-3 might contribute to the reduced adhesion associated with PYY or NPY. However, cells treated with GM6001 no longer displayed a significant increase in cell migration in the presence of PYY or NPY (Fig. 6).

Given that a number of MMPs could be involved in the enhanced cell migration observed upon PYY and NPY treatment, we tested their expression in hBRIE 380i cells to identify candidate PYY/NPY-responsive MMPs, including those known to be inhibited by GM6001. Table I and Fig. 7 show the MMPs that were significantly attenuated in the presence of 25 μM GM6001, indicating that those MMPs whose activities were inhibited by GM6001 do not significantly contribute to reduced adhesion associated with PYY or NPY. However, cells treated with GM6001 no longer displayed a significant increase in cell migration in the presence of PYY or NPY (Fig. 6).

We decided to focus on MMP-3 because it was reported to be inhibited by GM6001. Overexpression of MMP-3 significantly attenuated the migration of hBRIE 380i cells treated with or without 100 nM PYY or NPY (Fig. 9a). When we quantitated MMP-3 transcript levels in hBRIE 380i cells treated with or without 100 nM NPY or PYY, we observed a 3–4-fold increase (Fig. 8b), suggesting that MMP-3 might contribute to PYY- and NPY-induced cell migration. The addition of the MMP-3-specific inhibitor NNGH significantly reduced cell migration almost to the same extent as GM6001 (Fig. 9a, bars b and c compared to bar a). Enhanced cell migration in the presence of 100 nM PYY or NPY was blocked by NNGH (Fig. 9b). Therefore, although a number of MMPs may be involved in PYY/NPY-sensitive migration, MMP-3 appears to be an important contributor to this cellular response.

**FIG. 5.** PYY- and NPY-enhanced migration of hBRIE 380i cells is concentration-dependent. Migration of near confluent cells was determined as described in the legend to Fig. 3 and under “Experimental Procedures” with or without PYY or NPY at concentrations from 1 to 100 nM. Percent change is the ratio of cell migration with PYY or NPY over the control (0), which was arbitrarily assigned a value of 100%. Bars are means ± S.D. a, p < 0.05, relative to the control.

**FIG. 6.** PYY and NPY effects on hBRIE 380i cell adhesion and migration are attenuated by GM6001. Cells at near confluence were assayed for their adhesion (black bars) and migration (white bars) capabilities as described in the legends to Figs. 2 and 3 in the presence of 25 μM GM6001 (an inhibitor of MMP-1, -2, -3, -8, and -9) or the same volume of Me2SO (DMSO) in limiting medium alone (C) or with PYY (P) or NPY (N) at 100 nM. The adhesion and migration of cells treated with Me2SO without the peptides (black and white bars, +DMSO, C) were arbitrarily designated 100%. Values are means ± S.D. Significant difference (p < 0.05) is indicated as follows: a, relative to +DMSO, C; b, relative to +GM6001, C.

**FIG. 7.** Expression of several MMPs tested in hBRIE 380i cells. Cells were grown to near confluency. RT-PCR was performed to test for the presence and to measure the levels of several MMPs as described under “Experimental Procedures.” The primers used and the number of PCR cycles as well as the GenBankTM/EBI accession numbers of the sequences are listed in Table I. Shown are the sizes and positions of the amplified fragments relative to the translated regions (gray boxes) of the cDNA. Numbers under the lines indicate nucleotides, with +1 assigned to the A of the start codon, except for 18S RNA, with 1 assigned to the start of the 18S RNA. Horizontal brackets indicate the amplified PCR fragments, with the sizes of the fragments given above. The expression of the enteroocyte differentiation-dependent markers villin and I-FABP and of 18S RNA was also measured for comparison.

**PYY and NPY Activation of Cdc42 Results in Increased hBRIE 380i Cell Migration Due to Induced Expression of MMP-3 and Correlates with Increased Villin Expression**—The hBRIE 380i cells treated with PYY or NPY exhibited actin cytoskeleton rearrangements (32), suggesting an involvement of small
Fig. 8. MMP-3 increases cell migration, and its expression is enhanced by PYY and NPY in hBRIE 380i cells at near confluency. Cells were transiently transfected with the empty vector or the MMP-3 cDNA construct as described under “Experimental Procedures.” The migration capability of the transfectants was determined as described in the legend to Fig. 3 (A). Percent change is the ratio of migration of MMP-3-overexpressing cells to vector-transfected cells, with the control (Vector lane) as described in the legend to Fig. 1 (B). RT-PCR was performed as described under “Experimental Procedures.” A typical result after agarose gel electrophoresis is shown. MMP-3 levels were estimated by densitometry and calculated as described under “Experimental Procedures.” The results are presented as -fold change, with the control arbitrarily assigned a value of 1.

Fig. 9. MMP-3 plays a significant role in PYY- and NPY-induced hBRIE 380i cell migration. A, migration of cells grown to near confluence was assayed in the presence of 25 μM GM6001 (an inhibitor of MMP-1, -2, -3, -8, and -9) (bar b), 80 μM NNGH (an MMP-3-specific inhibitor) (bar c), or MeSO (the solvent of both inhibitors) (bar a). Cell migration was also measured in the absence (C) or presence of PYY (P) or NPY (N) at 100 nM with MeSO (DMSO) or 80 μM NNGH. The migration of cells in MeSO without peptide treatment (bar a) and +DMSO, C) was arbitrarily designated 100%. Values are means ± S.D. Significant difference (p < 0.05) is indicated as follows: a, relative to bar a; b, relative to +DMSO, C.

Constitutively active Cdc42 led to a significant increase in cell migration, and conversely, dominant-negative Cdc42 resulted in a significant reduction in cell migration (Fig. 11, T17N and G12V, C compared to Vector, C). To determine whether Cdc42 activation is an initial step leading to PYY- and NPY-induced cell migration, cells expressing dominant-negative Cdc42 were treated with 50 nM PYY and 50 nM NPY. PYY and NPY induction of migration of these cells became negligible (Fig. 11, T17N, PIN versus C). Similarly, the effects of PYY and NPY on migration of cells expressing constitutively active Cdc42 were <50% of those on migration of cells transfected with the empty vector (Fig. 11, G12V, PIN versus C compared to Vector, PIN versus C), suggesting that the increase in migration due to activation of endogenous Cdc42 was less pronounced because the exogenous constitutively active Cdc42 was dominant. Because Cdc42 can induce AP-1 transcriptional activity (47, 48) and MMP-3 expression can be regulated by AP-1 (45), we tested whether activation of Cdc42 leads to PYY and NPY induction of MMP-3. MMP-3 transcript levels in hBRIE 380i cells expressing constitutively active Cdc42 were significantly higher than those on cells transfected with the empty vector (Fig. 12A, G12V and T17N, lanes compared to Vector lane). The regulation of MMP-3 by Cdc42 activities allows us to conclude that Cdc42 effects on hBRIE 380i cell migration are not only through direct changes in actin reorganization as established in other cell types, but also through changes in the expression of genes whose protein products are involved in ECM remodeling.

PYY and NPY Induce Villin Expression through a Pathway Involving MMP-3 Activity—Previously, we observed that modulation of cell-substratum interaction mediated by CD63 could be a pathway of PYY/NPY-induced expression of I-FABP (a late marker of cell differentiation) (32). To explore the possibility that PYY/NPY-induced cell migration also has an effect on the expression of villin (an early marker of cell differentiation) in hBRIE 380i cells, we first determined whether alteration of MMP-3 levels affects villin expression. We measured villin transcript levels in hBRIE 380i cells transfected with MMP-3 cDNA. Overexpression of MMP-3 correlated with an increase in...
FIG. 10. PYY and NPY increase the level of activated Cdc42 in hBRIE 380i cells. Cells at near confluency were laid down in T75 flasks coated with collagen type I at a density of ~1 × 10^5 cells/flask and allowed to grow in normal medium (10% bovine calf serum). After 24 h, the cells were pre-starved for 15 h in limiting medium (0.1% bovine calf serum), followed by a 2-h incubation in serum-free medium. The cells were then treated with PYY (A) or NPY (B) at 100 nM for the indicated times. Cdc42 activities were assayed by GST-PBD affinity precipitation, followed by Western blotting as described under “Experimental Procedures.” The intensity of the bands was quantified densitometrically. -Fold change is the ratio of active Cdc42 to total Cdc42 with peptide treatment for 0 min, which was assigned a value of 1.

![Cell images](Image)

FIG. 11. Activated Cdc42 modulates hBRIE 380i cell migration and partially mediates PYY/NPY effects. Cells were transiently transfected with a plasmid construct expressing constitutively active Cdc42 (G12V) or dominant-negative Cdc42 (T17N) or with the empty vector. Cdc42 activities in the cells transfected with these two mutants were assayed as described in the legend to Fig. 10 (inset). Changes in migration of the transfectants were assayed in limiting medium alone (C) or in the presence of a mixture of 50 nM PYY and 50 nM NPY (P/N) as described in the legend to Fig. 3. Values are means ± S.D. Significant difference (p < 0.05) is indicated as follows: a, relative to Vector; b, relative to G12V, C.

villin expression (Fig. 13). We also determined that modulation of MMP-3 levels by Cdc42 activities (Fig. 12A) was enough to alter villin transcript levels (Fig. 12B). Collectively, these data indicate that Cdc42 activities regulate MMP-3 levels and that, subsequently, either MMP-3 itself or migration mediated by MMP-3 modulates villin expression (or cellular processes that regulate villin expression).

We then attempted to establish whether the inhibition of MMP-3 activity and MMP-3-associated migration by NNGH at a concentration sufficient to inhibit cell migration could also inhibit the expression of villin. After repeated attempts, no significant difference in villin expression was observed (data not shown). We then treated hBRIE 380i cells with the broad-spectrum MMP inhibitor GM6001 in the presence or absence of 100 nM PYY or NPY. The relative expression of villin was measured by RT-PCR. As with I-FABP expression (31), villin transcript levels were also induced by a 6-h treatment with 100 nM PYY by an average of 3-fold (Fig. 14, +DMSO, P lane compared to C lane). A similar induction was observed when cells were treated with 100 nM NPY (Fig. 14, +DMSO, N lane compared to C lane). Treatment with 25 μM GM6001 eliminated that induction (Fig. 14, +GM6001, P and N lanes compared to C lane). These data suggest that inhibition of MMP-3 alone is sufficient to inhibit cell migration and that overexpression of MMP-3 induces expression of villin, but that elimination of NPY- and NPY-induced villin expression involves inhibition of the activity of more than one MMP. The PYY and NPY induction of MMP-3 expression leading to increased villin expression and the abrogation of that induction by GM6001 further establish the potential importance of cell-substratum interaction for the observed PYY and NPY effects.

The pathway of PYY/NPY induction of villin expression in hBRIE 380i cells involving MMP-3 can be summed up as follows. PYY/NPY activates Cdc42, leading to induction of MMP-3 expression, and, as a consequence, increases cell migration. Increased cell migration (possibly together with other MMP activities) in turn induces villin message levels or initiates cellular events indicated by increased villin expression.

**DISCUSSION**

The functional integrity of the small intestine is very sensitive to the withdrawal of food and is more adversely affected by the absence of food than any other organ in the body. The presence of diet in the lumen of the intestine is vital to the maintenance of gastrointestinal structure and function as well as systemic functions such as immunoresponsiveness. Changes in the intestinal mucosa resulting from luminal dietary stimulus, such as those seen from parenteral to enteral nutrition and from starvation to fed state, result in adaptive responses characterized by increased villus height and crypt depth, brush-border enzyme changes, and increased crypt cell proliferation and enterocyte differentiation as well as changes in enterocyte migration and programmed cell death (49–55). Conversely, the loss of dietary stimulus in the lumen or systemic fasting causes intestinal atrophy and functional adaptive changes such as reduction in villus height, crypt depth, epithelial turnover, and migration (50, 56, 57). Nutritional and non-nutritional regulators of intestinal adaptation include glutamine, epidermal growth factor, insulin-like growth factor I, glucagon-like peptide-2, and leptin (49, 51, 55, 58), and enteric nervous stimulation and free fatty acids (55, 59–61). The mechanism(s) by which the diet stimulates changes in the mucosa, either through contact in the lumen or humorally, remains unresolved.

The luminal presence of oleic acid directly induces the release of PYY from I-cells in the mucosa (9). Concomitantly, PYY induces I-FABP in hBRIE 380i cells at a concentration and time course similar to those of PYY release in vivo in response to a meal (62). This suggests that nutrients such as long chain free fatty acids cause the endocrine release of PYY or the vagal release of NPY, leading to the activation of Y...
FIG. 12. Activated Cdc42 modulates MMP-3 and villin expression. Cells were transfected with constitutively active Cdc42 (G12V) and dominant-negative Cdc42 (T17N) as well as with the empty vector. The transfectants were allowed to grow for 36 h, and RT-PCR was performed to measure MMP-3 (A) and villin (B) transcript levels as described under “Experimental Procedures.” The density of the bands was used to estimate expression levels using a PCR standard curve as described under “Experimental Procedures.” After normalization to 18 S RNA levels, the results are expressed as -fold change; the control (Vector) was arbitrarily designated as 1.

FIG. 13. Overexpression of MMP-3, which enhances migration, also increases villin expression in hBRIE 380i cells. Cells were transiently transfected with an MMP-3 overexpression vector (lane 2) or with the empty vector (lane 1). The transfectants were allowed to grow for 36 h, RT-PCR was performed as described under “Experimental Procedures.” MMP-3 and villin levels were measured using a PCR standard curve as described under “Experimental Procedures” and normalized to the level of 18 S RNA, with the vector control (lane 1) designated as 1.

The effects of PYY and NPY on migration of non-tumorigenic cells in culture would be expected to differ for cells at higher densities of a tight confluent monolayer compared with cells that have space for growth. We observed that a 30-min exposure to PYY/NPY induced stress fiber formation and the presence of filopodia and lamellipodia only in cells at the periphery of a monolayer (32). Therefore, to increase the number of responsive cells, we examined hBRIE 380i cells that were close to confluency, a transition state from maximal cell migration (and proliferation) to entering the state of non-replication and early differentiation. At this state of cell growth, PYY and NPY decreased the expression of the tetraspanin CD63, a member of the transmembrane 4 superfamily, and decreased cell adhesion. Correspondingly, PYY and NPY increased the expression of MMP-3, which correlated with increased cell migration. PYY and NPY also activated Cdc42, and MMP-3 levels were Cdc42-sensitive. However, this study does not preclude the likelihood that other MMPs are involved in the migration process or that CD63 and MMP activities are directly coupled.

Cell migration in a two- or three-dimensional matrix environment necessitates the loss of adhesion to the ECM and requires that there be ECM remodeling by matrix-degrading enzymes (63). Tetraspanins are structural components of dynamic adhesion complexes involving α3β1 integrins at the distal ends of lamellipodial and filopodial extensions (34). CD63 is also involved in the internalization of MT1-MMP from the cell surface to the CD63-positive lysosomes, where there is subsequent recycling or proteolytic degradation of MT1-MMP (64), suggesting that CD63 may be involved in the regulation of cell migration or invasion through the up- or down-regulation of MT1-MMP or other MMPs. Thus, CD63 could be in the position of affecting both adhesion and de-adhesion processes in cell migration.

The relationship between CD63 and actin reorganization is not clear. It is possible that CD63 might be associated with the signal transduction pathway through protein kinase C to activate Cdc42 as reported for the tetraspanin CD151 (65, 66). Cdc42 is required for direction sensing during chemotaxis in neuronal cells and induces formation of filopodia possibly containing receptors that might act as sensors of extracellular signals that could be processed into the cells (67). The localization of Cdc42 at the plasma membrane as well as the Golgi complex suggests that Cdc42 may also have an overlapping function in regulating vesicle trafficking (68, 69). Cdc42 appears to regulate polarized secretion in epithelial cells. Dominant-negative Cdc42 mutants selectively inhibit basolateral transport of membrane proteins, without affecting apical secretion (69, 70). This raises the possibility that tetraspanins such as CD63, which recycles from the plasma membrane to endosomes, if localized on the basolateral plasmalemma, could act as part of a sensing mechanism for the intestinal mucosal cells to respond to cues arising from the ECM. hBRIE 380i cells exhibit a high degree of invagination along the basolateral plasma membrane, which increases in cells treated with PYY. Thus, PYY and NPY activation of Cdc42 and inhibition of the CD63 transcript may not only modulate cell migration, but also...

2 G. W. Aponte, unpublished data.
induce vesicular endocytic activity at the basolateral surface.

Rho family GTPases mediate the formation of actin-containing structures that are associated with cell migration (71–73) and also regulate other processes important for cell migration, such as cell-substrate adhesion, cell-cell adhesion, protein secretion, vesicular trafficking, and transcription. Cdc42 does not appear to be required for cell motility in fibroblasts, but is necessary for vectorial migration in chemotaxis or wound healing (74). However, in undifferentiated unpolarized small intestinal crypt IEC-6 cells, Cdc42 activation induces cell migration (75). Rho GTPases have been shown to alter the secretion of proteases, although their effects on the regulation or activation of secreted proteases have not been studied extensively. Cdc42 activates JNK (76–78) and p38 MAPK (79, 80) in vitro. Studies using ectopically expressed wild-type or activated Cdc42 in various fibroblast cell lines provide evidence that Cdc42 induces c-Jun AP-1 transcriptional activity through a JNK-dependent phosphorylation event (47, 48). The Y1 receptor has been shown to couple to an MAPK pathway (81), which could lead to AP-1 and Ets transcription factor activation (82, 83). MMP-3 and several other MMPs have conserved regulatory binding elements for AP-1 and PEA3 (an Ets family member) in their promoter regions and can be induced by growth factors, cytokines, and ECM contacts (84–87). Given that both PYY and NPY are ligands of the Y1 receptor, there is a possibility that the increase in MMP-3 activity by transcriptional activation or even by post-translational modification is through an MAPK pathway.

The regulatory events in the process of intestinal epithelial cell migration along the crypt-to-villus axis have been studied primarily in models of disease states, such as those of inflammatory disorders (Crohn’s disease, ulcerative colitis, celiac disease, and related ulcers), cancer growth, and wound healing (restoration). The regulation mechanism of epithelial cell migration in non-pathological states has received far less attention even though it raises a fundamental question as to how cells coordinate movement with the timing of protein expression. Although epithelial cells in the intestinal mucosa undergo continual cell migration from the crypt to villus, the four mechanistic characteristics described for cell migration (lamellipodial extension, formation of new adhesions, cell body contraction, and tail detachment) (88) have not been observed in vivo. In the various mechanisms proposed for intestinal cell migration, such as through basement membrane flow and smooth muscle contraction (89), mitotic pressure (90), and active movement (91), the interaction of the basement membrane with the ECM is a common factor for each of the models. The PYY/NPY activation of Cdc42 and regulation of CD63 and MMP-3 expression bring forth the possibility that cell migration may be linked to positional expression of differentiation-dependent proteins by a mechanism that involves membrane recycling at the ECM-basolateral membrane interface of the mucosal cells with the lamina propria. The observed increase in villin expression by PYY and NPY appears to be MMP-dependent and is linked to migration. This suggests that, in the mucosa, pattern or timing of specific protein expression along the crypt-to-villus axis could be altered by factors that alter ECM-cell membrane interactions, such as PYY/NPY induction of MMP-3. PYY release in response to dietary stimuli raises the possibility that cell adhesion/migration and/or enterocyte protein expression is modulated by luminal cues and that PYY and NPY may be part of a homeostatic system that allows cells of the mucosa to adapt to altered states of nutriature.

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Peptide YY and Neuropeptide Y Induce Villin Expression, Reduce Adhesion, and Enhance Migration in Small Intestinal Cells through the Regulation of CD63, Matrix Metalloproteinase-3, and Cdc42 Activity

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