Peptide YY (PYY) and neuropeptide Y (NPY) are peptides that coordinate intestinal activities in response to luminal and neuronal signals. In this study, using the rat hybrid small intestinal epithelial cell line, hBRIE 380i cells, we demonstrated that PYY- and NPY-induced rearrangement of actin filaments may be in part through a Y1α and/or a nonneuronal Y2 receptor, which were cloned from both the intestinal mucosa and the hBRIE 380i cells. A number of PYY/NPY-responsive genes were also identified by subtractive hybridization of the hBRIE 380i cells in the presence or absence of a 6-h treatment with PYY. Several of these genes coded for proteins associated with the cell cytoskeleton or extracellular matrix. One of these proteins was the transmembrane-4 superfamily protein CD63, previously shown to associate with the extracellular domain, was highest in the differentiated populations of hBRIE 380i cells (9). In the current study, using these differentiated clusters. These studies suggest a new role for NPY and PYY in modulating differentiation through cytoskeletal associated proteins.

Regulatory peptides common to the intestine and nerves (neuro-gut regulatory peptides) are unique chemical mediators released from receptsecreatory and/or neurosecretory cells having multiple biological actions. These peptides can act as neurotransmitters, nociceptive, endocrine, or paracrine agents and share a common function in the coordination of intestinal activities in response to luminal signals or signals originating from peripheral tissues. Despite the range of diversity of their biological effects, there is one recurring biological observation that seems to link many of these peptides, their ability to act as factors affecting growth or differentiation of the intestinal mucosa. Differentiation and regulation of growth of complex tissues, such as the intestine, are not only determined by growth factors but also by intracellular signals generated by the interactions between cells and their extracellular matrix (ECM)¹ (1).

In intestinal epithelial cells, part of the ECM occurs in the form of a basement membrane that provides these cells with positional information and signals that initiate the organization of intracellular structure and cellular behavior. A factor probably important for the positional cues for differentiation in the intestinal mucosa is the potential link between the ECM of the lamina propria and the process of cellular migration from crypt to villus. Although over the past years there have been studies on the growth effects for specific peptides such as cholecystokinin (2), bombesin (3), vasopressin, gastrin (4, 5), and peptide YY (PYY) (6), to name a few, the contribution of these peptides to intestinal differentiation remains relatively unexplored. Such investigations were limited in the past primarily due to the lack of an appropriate cell culture model that mimicked some of the critical complex cell to cell interactions of the intestine.

To investigate the actions of regulatory peptides on mucosal cell differentiation, we have utilized intestinal cell lines developed by somatic cell hybridization (7). We have characterized one of these cell lines extensively, the Berkeley rat small intestinal epithelial cell hybrid (hBRIE 380i cells). These cells retain many characteristics of the enterocyte in situ including the formation of a nonreplicating polarized differentiated subpopulation of cells derived from a replicating progenitor-like cell population and expression of intestine specific structures, proteins, and receptors (7–10). To date, these are the only small intestinal epithelial cell lines that are not of embryonic origin that differentiate. A great advantage of these cells is that they develop a differentiated phenotype only under suitable hormonal and substratum conditions. The hBRIE 380i cells can therefore be used to dissect the mechanism(s) by which regulatory peptides and the basement membrane interact to regulate cell growth and/or differentiation.

We previously reported that a 6-h treatment of the hBRIE 380i cells with PYY results in an increase in intestinal fatty acid binding protein (I-FABP) transcripts only in the differentiated nonproliferating cell population (9). Because I-FABP has been well established as a differentiation-dependent protein expressed only in the mature intestinal mucosa (11, 12) and has often been used by others as a marker of intestinal cell differentiation (13), we suspected that PYY or NPY could have more general effects on differentiation. We presently report that both PYY and NPY stimulated an increase in actin stress fiber assembly in the hBRIE 380i cells, but only NPY appeared to initiate the formation of membrane ruffling. These data indicate that more than one receptor and effector system could be activated in response to NPY/PYY in these cells. Previous examination of PYY/NPY binding sites on hBRIE 380i cells has revealed receptors on both the nondifferentiated and differentiated populations of hBRIE 380i cells (9). In the current stud-

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¹ The abbreviations used are: ECM, extracellular matrix; PYY, peptide YY; NPY, neuropeptide Y; I-FABP, intestinal fatty acid binding protein; TM4SF, transmembrane-4 superfamily protein; IMDM, Iscove’s modified Dulbecco’s medium; BCS, bovine calf serum; RT, reverse transcription; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); TM, transmembrane region; RACE, rapid amplification of cDNA ends.

This paper is available on line at http://www.jbc.org
ies, we have cloned two receptors from the hBRIE 380i cells and rat intestinal epithelial cells that bind NPY and PYY. One is the Y1 receptor, and the other is a peripheral Y2 receptor found outside of nerves.

Regulation of differentiation of intestinal epithelia by regulatory peptides such as PYY and NPY may not only be by multiple receptor pathways but also dependent on interaction of the cell to the surrounding stroma or ECM. To determine gene-specific responses in hBRIE 380i cells to NPY and PYY that were also dependent on the cell basement membrane or cytoskeletal organization, subtractive hybridization was performed on hBRIE 380i cells grown on collagen gel matrix and exposed to PYY for 6 h. A number of genes were isolated that code for cytoskeletal related proteins in other tissues and species, such as the transmembrane-4 superfamily protein (TM4SF or tetraspan) CD63. We have verified that PYY induces the induction of CD63 mRNA levels in the hBRIE 380i cells and the presence of CD63 transcripts in the intestinal mucosa. Among its putative actions in other cell types, CD63 is thought to be part of a protein complex that binds integrin and participates in the processes of cell motility, adhesion, and differentiation (14, 15). We present data demonstrating that hBRIE 380i cells transfected with CD63 antisense cDNA fail to undergo differentiation. In the present study, we propose a mechanism whereby neuro-gut regulatory peptides can affect mucosal cell differentiation through the regulation of cytoskeletal-extracellular matrix interactions by modulation of the tetraspan CD63.

**Experimental Procedures**

**Cell Culture Conditions**—A subclone of the Berkeley rat intestinal epithelial hybrid cell line (hBRIE 380 cells) expressing PYY/NPY binding sites and the I-FABP, the hBRIE 380i cells, was used in the present study (7–9). Culture conditions were as described previously (8). Briefly, the hBRIE 380i cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM) (Life Technologies, Inc.) supplemented with 10% bovine calf serum (BCS) (Hyclone Labs, Logan, UT), 100 units/ml penicillin, and 100 units/ml streptomycin (Life Technologies) in T75 flasks (Corning Glass) coated with soft collagen type I gels, prepared from rat tails as described previously (8). The cells were exposed to PYY or NPY for 30 min at 4°C. The cellulose column was then washed with 50 mM Tris-HCl, pH 8.0, permeabilized, and/or association with the cytoskeleton were selected for further screening. We have previously described methods (8, 16). Poly(A)+ RNA was isolated from total RNA as described above for subtractive hybridization using an oligo(dT) cellulose column. Total RNA (30 μg/lane) and poly(A)+ RNA (6–10 μg/lane) were separated on denaturing agarose gels, transferred to nylon membranes (Micron Separations Inc., Westborough, MA), and hybridized to [32P]dCTP-labeled probes according to our standard methods (8). Control probes were synthesized from human cDNA fragments for β-actin (1.8 kb) (CLONTECH) and glyceraldehyde-3-phosphate dehydrogenase (1.1 kb) (CLONTECH) and used in each analysis. To identify the CD63 mRNA, a probe corresponding to the cytoplasmic domain of rat CD63 (18) was used in the hybridization reaction. This probe was synthesized from a template derived by PCR using primers specific for codons 104–109 and 197–202 of the rat CD63 cDNA (18). To verify the identified CD63 transcripts, another CD63 cDNA probe was synthesized from 878 bp of the mouse CD63 cDNA including the 714-bp coding region (19) obtained from an expressed sequence tag (EST) clone (GenBank accession number AA097085). A clone was sequenced and then synthesized using the published rat cDNA clone (accession number AA038369). The cDNA fragment was amplified by RT-PCR from the hBRIE 380i cells using primers specific for codons 1–26 and 688–714 of the CD63 coding sequence (see above). Two subtractive libraries were constructed using PCR products. Subtracted cDNAs from the induced and repressed hybridizations were labeled with [32P]dCTP by the random primed method (Promega, Madison, WI) and used as probes to identify the subtraction products. The subtractive hybridization reactions were labeled with [32P]dCTP in the random primed method (Promega, Madison, WI) and used as probes to identify the subtraction products. The subtractive hybridization reactions were labeled with [32P]dCTP by the random primed method (Promega, Madison, WI) and used as probes to identify the subtraction products. The subtractive hybridization reactions were labeled with [32P]dCTP by the random primed method (Promega, Madison, WI) and used as probes to identify the subtraction products.
region with the addition of restriction enzyme sites for ApII and BamHI for directional insertion into a pcDNA6/V5-His A vector (Invitrogen, Carlsbad, CA) that confers resistance to blasticidin S (Invitrogen). The CD63 cDNA inserts were constructed so that either the sense or antisense sequence would be transcribed. Plasmids containing either CD63 cDNA (CD63-expressing plasmid) or the intact pcDNA6/V5-His A plasmid were stably transfected into the hBRIE 380i cells using the calcium phosphate method previously described (7). One day prior to transfection, the hBRIE 380i cells were seeded at 0.5 × 10^6 cells/60-mm tissue culture plate in normal culture medium (IMDM supplemented with 10% FCS, penicillin, and streptomycin, as described above). Twenty-four h after transfection, selection of positive hBRIE clones containing the plasmid constructs was by the addition of selective medium; 10% BCS in IMDM otherwise. RNA was denatured at 70 °C for 10 min, followed by incubation for further studies were selected by determining the amount of decrease in CD63 mRNA levels respectively, when compared with amplification of a 594-bp cDNA fragment. Antisense and sense clones identified by RT-PCR, using primers directed to codons 5–11 (CP1) and 9–19 (CP2), were designed for the Y receptors.

For identification of Y1 receptor in hBRIE 380i cells, RT was performed on the RNA from hBRIE 380i cells, using the extracellular domain of recombinant CD63 (CD63ex) corresponding to the seventh transmembrane region (TM) (25, 26). The PCR primers were Y1P1 (5′-AACARAAAGAGAGTACATGGT-3′) and Y1P2 (5′-CGCTTGTGCTGCAGTCGGACAG-3′), corresponding to codons 66–73 (between TM1 and TM2), and Y1P2, which were designed to be specific for the Y1 receptor based on the rat cDNA sequence with the human Y1 sequence taken into consideration (25, 26). PCR was performed for 35 cycles under the following conditions: denaturation at 94 °C for 0.5 min; annealing at 62 °C for 1 min (five cycles), at 60 °C (15 cycles), and at 58 °C (15 cycles); and extension at 68 °C for 3 min. The resulting 754-bp amplified fragment was cloned into pGEM-T vector (Promega) and sequenced. To clone the entire coding sequence of Y1 receptor cDNA, RT was performed on the RNA from hBRIE 380i cells, with an antisense primer (Y1P3) (5′-ATGACCTACTCAGTCAGCAG-3′), corresponding to codons 1–8, and Y1P4 (26). The PCR conditions were as follows: denaturation at 94 °C for 0.5 min; annealing at 62 °C for 15 min (five cycles), at 60 °C (15 cycles), and at 58 °C (15 cycles); and extension at 72 °C for 1.5 min. The resulting 1149-bp cDNA was cloned into pGEM-T vector and sequenced.

To identify Y2 receptor in hBRIE 380i cells, 5 μg of poly(A)+ RNA was reverse transcribed using primer Y2P2 (5′-TARTTTISRTTCTACATC-AICCC-3′) designed for codons 326–332, followed by PCR using primers Y2P1 (5′-ATGGGGGARTGGAAATGCGG-3′), spanning codons 81–87 in TM3, and Y2P2. The primers were designed based on the human neuroblastoma (27) and mouse brain Y2 cDNA sequences (28). PCR was performed for 35 cycles under the following conditions: denaturation at 94 °C for 0.5 min; annealing at 62 °C for 1 min (five cycles), at 60 °C (15 cycles), and at 58 °C (15 cycles); and extension at 68 °C for 3 min. The resulting 659-bp amplified fragment was cloned into pGEM-T vector and sequenced. To clone the full coding sequence of Y2 receptor cDNA, RT was performed on the RNA from hBRIE 380i cells and the intestine of the rat (29). Y2P2 (5′-ATGACCTACTCAGTCAGCAG-3′) was designed for the last nine codons including the stop codon, followed by PCR using primers Y2P3 (5′-ATGGGCGCCRTAGTGGCCAGAAG-3′), corresponding to codons 1–7, and Y2P4 (27, 28). The PCR conditions were as follows: denaturation at 94 °C for 0.5 min; annealing at 62 °C for 5 min (five cycles), at 60 °C (15 cycles), and at 58 °C (15 cycles); and extension at 72 °C for 1.5 min. The resulting 1149-bp cDNA was cloned into pGEM-T vector and sequenced.

To determine the presence of the Y2 and Y1 receptors, total RNA from the hBRIE 380i, BRIE 291, PC12 cells, rat small intestinal epithelial cells, and rat cerebral cortex was isolated as described previously (16) and was reverse transcribed using oligo(DT)12. Aliquots of 1 μg of the reaction mixture were then subjected to PCR to amplify an 811-bp cDNA fragment of rat β-actin (29) and a 947-bp cDNA of the Y2 receptor spanning the start codon and TM7 (27, 28) or a 407-bp cDNA of the Y1 receptor spanning codon 248 to the stop codon (25, 26). The primers used for rat β-actin corresponded to regions between 54 and 35 nucleotides before the start codon (5′-TACACACCTCCTCGAGAGGGAACAG-3′) and between the 197th and 203rd codons (5′-TAGTTTTGCTGTTGTTGCTG-3′) of rat β-actin (23). The PCR was for 30 cycles under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 58 °C for 0.5 min, and extension at 72 °C for 1.5 min. For verification, the PCR mixture was then subjected to agarose gel electrophoresis and staining with ethidium bromide. For identification of β-integrin in the hBRIE 380i cells, RT was performed using oligo(dT)17. The PCR primers corresponded to regions between 4 nucleotides before the start codon and the sixth codon (5′-AACAGTATGTCGAGACAGG-3′) and between the 197th and 203rd codons (5′-TAGTTTTGCTGTTGTTGCTG-3′) of rat β-integrin (23). The PCR was for 30 cycles under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 58 °C for 0.5 min, and extension at 72 °C for 1.5 min. For verification, the PCR mixture was then subjected to agarose gel electrophoresis and staining with ethidium bromide.
codons 5–11 (18), and a tailed oligo(dT) primer (5’-GTCGACGCGT(GGI)5G-3’) under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 58 °C for 0.5 min, and extension at 72 °C for 1 min for a total of 35 cycles. The reaction mixture for 5’-RACE was incubated at 70 °C for 15 min followed by incubation at 37 °C for 30 min in the presence of 1 unit of RNase H and 1 μg of RNase A. The buffer was subsequently exchanged with water by three consecutive dilutions and concentrations in a Microcon-100 microconcentrator (Amicon, Beverly, MA). The cDNA was tailed with poly(dC) in a 20-μl reaction mixture containing 10 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, 10 μM dCTP, and 18 units of terminal deoxynucleotidyl transferase (Amersham Pharmacia Biotech) at 37 °C for 15 min. The reaction was stopped by incubation at 70 °C for 10 min. Five μl of the reaction mixture was subjected to PCR for 35 cycles using a tailed oligo(dG) primer (5’-CATATGTCGACGCGT(GGI)5G-3’) and primer CP2 under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 58 °C for 0.5 min, and extension at 72 °C for 1 min. To verify the specificity of the amplified fragments from the RACE reactions, the bands detected on the agarose gel were excised and subjected to PCR using primers CP1 and CP2 under the conditions of the RACE PCR, resulting in the expected 300-bp fragments.

**RESULTS**

**NPY and PYY Induced Reorganization of Actin Stress Fibers**—To investigate if cellular adhesion and growth in the intestine could be modulated by activation of members of the Y receptor family, we tested the effects of PYY and NPY on actin filament reorganization in the hBRIE 380i cells. Nonconfluent hBRIE 380i cells were treated with PYY or NPY for 30 min. Under these nonsynchronous conditions, both PYY and NPY caused a small increase in cell proliferation as determined by bromodeoxyuridine uptake (data not shown, but also reported by others (6)) and had a pronounced effect on actin rearrangement of stress filaments (Fig. 1). The PYY response seemed distinct from that of NPY. PYY elicited an immediate increase in stress fiber assembly (Fig. 1, A and B), whereas NPY induced the formation of membrane ruffling followed by stress fiber formation (Fig. 1, C and D). The termination points of stress fibers at the plasmalemma are most likely where focal adhesions form and proteins such as the integrins are clustered to form a link between the actin cytoskeleton and the ECM. We verified that at least one member of the integrin family was expressed in the hBRIE 380i cells, the β1-integrin. The presence of β1-integrin was first determined by RT-PCR, resulting in the identification of a fragment corresponding to nucleotides 41–653 of the published rat cDNA sequence (23) (data not shown). Then the expression of the entire mRNA for β1-integrin was confirmed by Northern analysis (see below and Fig. 2A).

**PYY Regulation of Genes Coding for Proteins Involved in Cell Growth, Adhesion, and the Cytoskeleton**—We previously established that I-FABP mRNA, used as a marker for differentiation...
in the hBRIE 380i cells, was induced in response to 100 nM PYY after 6 h of incubation (9). A slight increase in I-FABP mRNA levels was also observed when the cells were treated with 100 nM NPY for 6 h. These data demonstrated that PYY/NPY induced the expression of a differentiation-dependent protein marker, I-FABP, and indicated that the peptides might regulate other processes related to cellular growth/differentiation in the intestinal epithelium. To further explore genes that could be involved in cellular differentiation and would rapidly respond to the activation of Y receptors, subtractive hybridization was performed using a recently developed PCR-select cDNA subtraction methodology that enables the detection of low abundant transcripts. Two subtractive libraries from the hBRIE 380i cells were constructed corresponding to PYY-induced and -repressed mRNAs. Two subsequent screenings of each library resulted in over 200 clones that could be PYY-regulated genes (not yet verified). Selected cDNA clones were sequenced and compared with nucleotide sequences in the GenBank™ data base. Several of these clones shared identities with genes whose products are involved in cell growth, motility, and the cytoskeleton. One of these was a 965-bp fragment containing a 296-bp region with 94% identity to human and mouse EST clones with regions of homology to the actin-binding protein 8, anillin, suggested to play a role in the stabilization of contractile domains of the actin cytoskeleton during cytokinesis (22). The regions of similarity include the third proposed nuclear translocation domain of anillin. Another cDNA clone, a 1.5-kb fragment, had 87% identity with TMP from mouse brain tumors, which is also highly expressed in embryonic stem cells (21). The transcripts corresponding to the TMP and anillin-like genes were of low abundance. Only the embryonic stem cells (21). The transcripts corresponding to the cDNA clone, a 1.5-kb fragment, had 87% identity with TMP during cytokinesis (22). The regions of similarity include the actin-binding protein 8, anillin, suggested to play a role in the mouse EST clones with regions of homology to the containing a 296-bp region with 94% identity to human and BankTM data base. Several of these clones shared identities with regulated genes (not yet verified). Selected cDNA clones were each library resulted in over 200 clones that could be PYY-produced and -repressed mRNAs. Two subsequent screenings of low abundant transcripts. Two subtractive libraries from the cDNA subtraction methodology that enables the detection of induced and -repressed mRNAs. Two subsequent screenings of each library resulted in over 200 clones that could be PYY-regulated genes (not yet verified). Selected cDNA clones were sequenced and compared with nucleotide sequences in the GenBank™ data base. Several of these clones shared identities with genes whose products are involved in cell growth, motility, and the cytoskeleton. One of these was a 965-bp fragment containing a 296-bp region with 94% identity to human and mouse EST clones with regions of homology to the actin-binding protein 8, anillin, suggested to play a role in the stabilization of contractile domains of the actin cytoskeleton during cytokinesis (22). The regions of similarity include the third proposed nuclear translocation domain of anillin. Another cDNA clone, a 1.5-kb fragment, had 87% identity with TMP from mouse brain tumors, which is also highly expressed in embryonic stem cells (21). The transcripts corresponding to the TMP and anillin-like genes were of low abundance. Only the message for the anillin-like protein could be detected in the hBRIE cells by Northern analysis and was determined to be about 4 kb (data not shown). This message size is similar to that for anillin in Drosophila reported to be 4.0 and 4.3 kb (22). Due to the low levels of expression of both the anillin-like protein and TMP, it has not yet been verified that PYY induced these messages in the hBRIE 380i cells.

Another cDNA clone contained a 123-bp cDNA fragment with identical sequence to a region of the cDNA for rat clusterin, a protein found to inhibit cell aggregation (30–32). Full-length transcripts for clusterin (2 kb) have been reported for several tissues in different species (30). Northern analysis revealed a 1.8–2.0-kb mRNA in the hBRIE 380i cells (Fig. 2B). This transcript was abundantly expressed in the hBRIE cells and appeared to be slightly induced in response to both PYY and NPY (Fig. 2J). After normalizing the signals to glyceraldehyde-3-phosphate dehydrogenase used as a control (lower panel), the response to PYY (lane P) was greater than for NPY (lane N) when compared with cells incubated with medium alone (lane C). The apparent small changes in mRNA levels are probably due to localization of transcripts to specific cell populations of the hBRIE 380i cells. We have previously demonstrated that these cells form both a differentiated cluster cell population and less mature monolayer cells and that the differentiation-dependent marker I-FABP was only expressed and regulated in the more differentiated cluster cells (9).

Another cDNA fragment of 601 bp had sequence identity to the rat transmembrane-4 glycoprotein termed CD63, a protein previously shown to associate with β2-integrin in various cell lines and presumed to mediate cellular adhesion properties (14, 19). To verify that the CD63 transcripts were regulated by PYY (as indicated by the partial cDNA sequence obtained from the subtractive library), the hBRIE 380i cells were incubated with PYY for 6 h, under similar culture conditions used for construction of the subtractive library, and subjected to Northern analysis. Typical results are shown in Fig. 2C, demonstrating a strong signal for CD63 mRNA in the hBRIE 380i cells at approximately 1.1–1.2 kb. Previous studies indicated similar size transcripts for CD63 in rat mast cells, rabbit aorta, and murine kidney, lung, intestine, and macrophages (18, 19, 33). Lanes P and C represent cells incubated with PYY and control media, respectively. Only PYY appeared to induce CD63 mRNA levels when normalized to the signal for β-actin (lane P and lower panel). No increase in CD63 message levels was observed with the addition of NPY to the culture medium (lane N). The difference in response to PYY and NPY might be explained by the expression of multiple subtypes of the Y receptor family and/or coupling of these receptors to different intracellular signaling systems. Although the increase in the message level was higher for CD63 than for clusterin in response to PYY, both observations may be an underestimation of the responsiveness if these transcripts, like I-FABP mRNA, were localized to a specific cell population of the hBRIE 380i cells. Small changes in CD63 mRNA levels as determined in the heterogeneous cell population could represent large differences in a specific cell population, as previously demonstrated for I-FABP (9). It remains to be determined whether CD63 expression also is regulated in this manner.

In these initial studies, we chose to focus on the CD63 gene because of the proposed relationship with cell adhesion/migration through interactions with integrins. The CD63 cDNA fragment obtained from our subtractive hybridization corresponding to nucleotides 36–646 of the CD63 cDNA from rat basophilic leukemia cells (RBL-2H3) (18) is diagrammed in Fig.2A. A diagram of rat CD63 cDNA and RT-PCR analysis of CD63 mRNA in hBRIE 380i cells. A schematic representation of rat CD63 cDNA (18) is illustrated in A. The box indicates the open reading frame, with the regions for transmembrane domains shaded. The 611-bp fragment represents a partial cDNA obtained through subtractive hybridization. The 800- and 500-bp fragments are the results of 3’- and 5’-RACE. Typical results from RT-PCR and RACE analysis of CD63 transcripts in the hBRIE 380i cells are represented by the agarose gel. B, fragments of approximately 800 and 500 bp (and 600 bp) were obtained from the 3’- and 5’-RACE reactions, respectively (lanes 1 and 2). Amplification of the 800-bp cDNA (lane 1) using primers CP1 and CP2 resulted in an expected 308-bp fragment (lane 3). Further PCR analysis of the 500- and 600-bp fragments (in lane 2) using the CP1 and CP2 primers also resulted in the expected 308-bp cDNA (lanes 4 and 5). The molecular weight markers (100-bp ladder) are shown (lane M).

2 G. Halldeén, M. Hadi, H. T. Hong, and G. W. Aponte, unpublished data.
3A. To verify the size of the CD63 transcript in the hBRIE 380i cells, RT-PCR was performed. Amplification of the 3' cDNA end resulted in an approximately 800-bp fragment (Fig. 3B, lane 1), while amplification of the 5' cDNA end resulted in two fragments of approximately 500 and 600 bp (Fig. 3B, lane 2) that might represent alternatively spliced messages. All three fragments were confirmed to be CD63 cDNAs by amplifying a 308-bp region (Fig. 3B, lanes 3–5) using two gene-specific primers (Fig. 3A, CP1 and CP2). The results suggest the presence of full-length CD63 transcripts of about 1.0 and 1.1 kb in the hBRIE 380i cells, in agreement with results from the Northern analysis (Fig. 2C). The isolated cDNA corresponded to the previously reported full-length CD63 message containing the 714-bp coding region (18).

To determine whether the PYY-inducible CD63 transcript identified in the hBRIE 380i cells was also present in the native intestinal epithelium, Northern analysis was performed on total RNA isolated from an epithelial cell fraction of rat small intestine. The presence of CD63 mRNA in intestinal epithelial cells is shown in Fig. 4. The message was of identical size to that observed in the hBRIE 380i cells and is in agreement with the RT-PCR data (see above and Fig. 2C). It was also confirmed that the mRNA species identified by Northern blot analysis was identical to the cloned rat CD63 cDNA by using a labeled cDNA probe specific for codons 104–202 (18), corresponding to the extracellular domain of the protein. Using this probe, a strong mRNA signal of identical size was observed in both the hBRIE 380i cells and the intestinal epithelium (data not shown). The CD63 mRNA appeared to be highly abundant in both native intestinal epithelial cells and the hBRIE 380i cells (compared with β-actin).

To further investigate a potential link between PYY receptor activation, rearrangements of the actin cytoskeleton, and induction of CD63, we tested for the regulation of β1-integrin expression whose presence in the hBRIE 380i cells we had already established by RT-PCR (not shown). The hBRIE 380i cells were treated with 100 nM of either NPY or PYY for 6 h; total RNA was isolated; and mRNA levels were determined using Northern analysis. Typical results are shown in Fig. 2A, demonstrating that the β1-integrin message was not regulated by either PYY or NPY (lanes P and N, respectively) when compared with the control (lane C, medium alone) and normalized to β-actin shown in the lower panel. The β1-integrin mRNA was determined to be about 3 kb (Fig. 2A) as expected from previous cloning data reporting the β1-integrin coding region to be 2.4 kb in rat oligodendrocytes (23).

**CD63 Expression in Cells Forming Differentiated Clusters in the hBRIE 380i Cells**—CD63 was found to be most abundantly expressed in the clusters of hBRIE 380i cells, which are composed primarily of differentiated cells. These differentiated clusters of cells have been previously shown to be localized in areas where the cells have become morphologically columnar, polarized, with apical microvilli, and to express proteins such as I-FABP (Fig. 5, A and B, and Fig. 7B, arrow). Cells dispersed from these clusters revealed CD63 immunoreactivity in vesicular structures that appeared similar to lysosomal compartments (Fig. 5C, arrow). This pattern of cellular distribution was similar to that reported in a number of cell lines (18). No immunoreactivity was observed when the antisera had been preabsorbed with recombinant CD63. Bar, 30 μm (C and D).

To determine whether CD63 could be involved in the formation of differentiated clusters in the hBRIE 380i cells, the cells were transfected with CD63 antisense cDNA. Cells expressing decreased levels of CD63 mRNA were identified using RT-PCR. The data presented in this study are from one clone of these cells (hBRIE 380iCD63-as cells) whose CD63 mRNA levels were nondetectable (Fig. 6, lane 3). For each cell clone, a 594-bp CD63 cDNA was amplified, and the results were normalized to rat β-actin cDNA levels (Fig. 6, lower panel). The controls (Fig. 6, lanes 1 and 2), expressing normal levels of CD63 mRNA, were hBRIE 380i cells (lane 1) and cells transfected with CD63 sense cDNA (hBRIE 380iCD63+ cells) (lane 2).

Unlike untransfected hBRIE 380i cells (Fig. 7, A and B, and Fig. 8, A and B) and cells transfected with the CD63 sense cDNA (Fig. 8, C and D), the hBRIE 380iCD63-as cells failed to produce differentiated clusters of cells (Fig. 7, C and D). In untransfected cells, cluster formation was dependent on the presence of a collagen substratum. In the absence of this matrix, cells formed ridges (Fig. 7A, arrow) rather than clusters (Fig. 7B, arrow) and lacked the phenotypic characteristics of differentiated cells, as described previously (8, 9). The hBRIE 380iCD63-as cells displayed a strong reduction of cell cluster formation as well as cell ridges (Fig. 7, C and D). This occurred independently of the presence of the collagen matrix. Cells transfected with CD63 antisense cDNA, which had moderately...
repressed CD63 mRNA levels when compared with normal cells or hBRIE 380iCD63-as cells, demonstrated more collagen-dependent cluster formation than the hBRIE 380iCD63-as cells but less than untransfected cells (data not shown). These data suggest that CD63 is necessary for the cells to maintain interactions with the ECM, such as cell adhesion, that are required for differentiation.

Two Y Receptors Are Expressed in the hBRIE 380i Cells and the Intestine—Numerous Y receptor subtypes have previously been described in both the intestinal epithelium and enteric nervous system and reported to activate biological activities such as motility and secretion in the gut. Our data demonstrate that both NPY and PYY induce actin rearrangement in the hBRIE 380i cells and that the response was different for each peptide. In addition, the induction of mRNA levels for CD63 and clusterin by PYY was more pronounced than by NPY. Together these observations indicate the activation of multiple Y receptors and/or intracellular signaling systems in the hBRIE 380i cells. The expression of the Y1 and Y2 receptor subtypes in the intestine has previously been determined (34, 35); however, the Y2 receptor had not yet been identified in intestinal epithelial cells. Therefore, we tested for the presence of a number of Y receptors previously identified in various tissues and species. In the present study, we established the presence of both the Y1 and Y2 receptors in the hBRIE 380i cells and intact intestinal epithelial cells by RT-PCR.

The Y1 receptor was cloned using primers (Fig. 9A) synthesized based on the rat forebrain Y1 cDNA sequence (25). The Y1 receptor is alternatively spliced in murine tissues, resulting in two transcripts coding for an a and b subtype (36), with the b subtype lacking the C terminus and part of the seventh transmembrane domain. The a subtype was demonstrated to be the predominant form of the receptor in adult murine tissue (36). Using primers Y1P1 and Y1P2, designed to be specific for the Y1a receptor, we obtained an expected 745-bp fragment from the hBRIE 380i cells, confirmed by sequencing to be identical to the Y1 receptor cDNA (Fig. 9A) (25). The RT-PCR was repeated on RNA isolated from both the hBRIE 380i cells and native intestinal epithelial cells to clone the entire coding region of the Y1 receptor cDNA using primers Y1P3 and Y1P4. We obtained a 1149-bp fragment from each of the RT-PCR reactions with the same sequence as the published rat Y1 sequence (Fig. 9A). These results demonstrated that Y1 receptor transcripts were present in the hBRIE 380i and intestinal epithelial cells, indicating that the Y1 receptor is expressed in the intestinal epithelium.

To test if the hBRIE 380i cells also expressed the Y2 receptor, RT-PCR analysis was performed using degenerate primers, Y2P1 and Y2P2, designed based on the rat Y2 receptor amino acid sequence (37). A 659-bp cDNA fragment spanning trans-
Membrane regions 3–7 was obtained as expected and confirmed by sequencing to be the correct Y2 receptor cDNA fragment (Fig. 10A). To verify the presence of Y2 receptor transcripts in the hBRIE 380i cells and intestinal epithelial cells, we repeated the RT-PCR assay using a pair of degenerate primers, Y2P3 and Y2P4, synthesized based on the published rat Y2 amino acid sequence (37) amplifying the entire coding region of the receptor (Fig. 10A). We obtained a 1146-bp fragment that, after sequencing, was confirmed to be Y2 cDNA based on its homology with the published human neuroblastoma (27) and mouse brain Y2 cDNA sequence (28). This sequence was also identical to that of the Y2 receptor cDNA from rat hypothalamus that was reported during the course of our study (38), and the deduced amino acid sequence was identical to the previously published rat Y2 amino acid sequence (37). These results clearly demonstrate the presence of Y2 receptor transcripts in the epithelial cell population of intestinal cells in addition to the previously reported expression only in the enteric nervous system (34, 39).

To further verify the specificity of the RT-PCR for both the Y1 and Y2 receptors, RT-PCR was performed on tissues and cells previously established to be positive and negative for the receptors. Primers Y1P5 and Y1P4 (Fig. 9A) were used to amplify a 407-bp fragment specific for the Y1a cDNA in these cells and tissue preparations (Fig. 9B). Similarly, a 447-bp fragment of the Y2 cDNA was amplified in the same cells and tissue preparations using primers Y2P3 and Y2P5 (Fig. 10, A and B). As expected, rat cerebral cortex, a tissue previously reported to have a high density of both Y1 and Y2 receptors (40), expressed both the Y1 and Y2 subtypes (Figs. 9B and 10B). The pheochromocytoma cell line, PC12, also expressed both the Y1 and Y2 receptors (Figs. 9B and 10B) as previously determined (41). The rat intestinal epithelial cell line, BRIE 291, was positive only for the Y1 receptor (Figs. 9B and 10B), and the hBRIE 380i cells like the intestinal epithelium expressed both the Y1 and Y2 subtypes (Figs. 9B and 10B), in agreement with our previous cloning data (see above). The hBRIE 380i cells were created by somatic cell fusion of the rat small intestinal epithelial cell line, BRIE 291 cells, with terminally differentiated intestinal epithelial cells (7). Therefore, these results confirm that both the Y1 and Y2 receptor expression in the hBRIE 380i cells were of epithelial origin.

**DISCUSSION**

The high rate of proliferation of the intestinal epithelium and its balance with cell migration, adhesion, and terminal differentiation in a temporal and spatially specific manner is a process that must be closely regulated to preserve functional cell steady state. Critical questions lie in the potential contribution of peptide growth factors to these processes. Several peptide growth factors have been described for the intestine such as the epidermal growth factors, transforming growth factors α and β, insulin-like growth factors, and the fibroblast growth factors (for a comprehensive review see Ref. 42). However, the role of neuro-gut regulatory peptides such as NPY and PYY on differentiation of the gut remains relatively unexplored. Because these peptides can both be secreted and act locally, they are likely candidates as regulators of intestinal protein expression. Other diverse elements may be equally important in the regulation of differentiation. Elements that probably modulate the effects of peptide growth factors are the constituents of the ECM that are produced by both the epithelial cells and other cellular populations within the lamina propria, such as pericyelial fibroblasts (43, 44).

PYY and NPY are members of a 36-amino acid regulatory peptide family that includes pancreatic polypeptide. PYY shares a common structural motif with NPY as well as more than 70% sequence identity (45). While PYY-secreting cells
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occur mainly in the distal small intestine and the large intestine; NPY is abundantly expressed in all noradrenergic nerves innervating blood vessels and heart, as well as brain (46), adrenal medulla, intrinsic cardiac neurons (47), and the myenteric plexus of the gut (48–50). In addition to being localized to sympathetic neurons, NPY is also colocalized with catecholamines, acetylcholine, and enkephalins. PYY and NPY share several biological functions including inhibition of GI motility and chloride ion secretion.

In the present study, we demonstrated that NPY and PYY induced rearrangement of actin filaments in the hBRIE 380i cells. The PYY response seemed to be distinct from that of NPY. PYY elicited an immediate increase in stress fiber assembly, whereas NPY induced the formation of membrane ruffling followed by stress fiber formation. The termination points of stress fibers at the plasmalemma are thought to be where focal adhesions form and where proteins such as integrins are clustered and form a link between the actin cytoskeleton and the ECM. This process has been suggested to be dependent on Rho p21, while membrane ruffling, which was observed in the presence of NPY, has been reported to be a process requiring Rac p21 in Swiss 3T3 fibroblasts (51, 52). In epithelial cells small Rho-like GTPases have also been shown to be required for both cell migration and cell-to-cell adhesion. Recent data suggest that the activity of Rho-like GTPases is also required to maintain the cytoskeletal architecture of polarized Madin-Darby canine kidney cells (53) and that Rac is necessary for cell motility (54). Activation of GTP-binding proteins and focal adhesion kinase may be central in the signaling cascade initiated by integrins (55–57). These pathways may overlap with those initiated by regulatory peptides such as PYY and NPY to function synergistically.

Data from subtractive hybridization demonstrated a number of genes to be either induced or repressed by NPY and PYY. Several of the sequenced and identified genes were similar to those coding for proteins previously reported to participate in cell growth, adhesion, and migration in other tissues and cell lines. Examples of those gene products were a TMP (21), an anillin-like protein (22), clusterin (30–32), and CD63 (14, 19). We chose to investigate the association between Y receptor activation and CD63 mRNA induction because of data suggesting that integrin-CD63 interactions play a role in cell adhesion (14), a process regulating reorganization of actin filaments.

CD63 belongs to a family of newly identified TM4SF membrane proteins (58) that are characterized by four transmembrane domains. Members of this family such as CD9, CD37, CD63, CD81, and CD82 (59, 60) have been found to be expressed in leukocytes and a variety of other mammalian tissues. CD63 appears on the surface of most cultured cell lines at a moderate level (18). It is also found incorporated into membranes of different types of intracellular granules, including lysosomes (18, 61), endothelial Weibel-Palade bodies (62), platelet-dense granules (63), and the major histocompatibility complex class II compartment (64).

CD63 has been reported to suppress random tumor cell motility and enhance migration toward the β1-integrin substrates: fibronectin, laminin, and collagen. Specific associations between membrane proteins in the TM4SF and certain β1 integrins including α5β1, αvβ3, and αvβ1 have been previously described (65–67). A role for these TM4SF proteins in signaling is suggested by the demonstration of their modulation of intracellular calcium, tyrosine phosphorylation, and cell proliferation (57, 68). However, the mechanism whereby TM4SF proteins are involved in this signaling is not understood. It has been reported that phosphatidylinositol 4-kinase is associated with α5β1 integrin and TM4SF proteins (15). Therefore, the integrins-TM4SF complexes could be a point of merger with integrin and TM4SF protein signaling (15). Cell attachment mediated by transmembrane receptors in the integrin family has been shown to trigger signal transduction cascades that regulate cell proliferation, apoptosis, motility, and morphology.

We demonstrated that CD63 antisense cell clones (hBRIE 380iCD63-as cells) lost the expression of CD63, determined by immunocytochemistry, and did not display the differentiated cluster cells typical of the hBRIE380i cells. The hBRIE 380i cells grown on a collagen matrix form clusters of cells that appear 3–4 days after confluency comprising primarily nondividing differentiated cell populations (8, 9). Unlike the hBRIE 380i cells the hBRIE 380iyCD63-as cells had a similar appearance of newly confluent hBRIE 380i cells grown on tissue culture plastic without the collagen substrate. Additionally, the hBRIE 380iyCD63-as cells lost the expression of the differentiation marker I-FABP (data not shown). The loss of the characteristic properties of differentiated hBRIE 380i cells with decreased levels of CD63 implicates an important role for this tetraspan in intestinal cell differentiation possibly through its effects on cellular adhesion (cell to cell or cell to matrix). These studies utilizing the hBRIE 380iyCD63-as suggest that neuro-gut regulatory peptides can regulate cytoskeletal protein expression by facilitating protein signals from the basement membrane to induce cell differentiation.

CD63 immunoreactivity, determined utilizing GpAb4120ex made against the extracellular domain of recombinant rat CD63, was localized in vesicular structures distributed throughout the cell cytoplasm as well as in a punctate pattern of distribution on the cell surface. Our observations are consistent with reports of CD63 as a lysosomal membrane glycoprotein expressed on the surface of other cells, such as T-lymphocytes (69), neutrophils (70), and basophils (71). CD63 immunoreactivity was also localized primarily to the clusters of hBRIE 380i cells. This morphologically distinct region has been determined to be the site of differentiation-dependent protein expression such as I-FABP (9). CD63 localization to these clusters indicates that determination of mRNA levels by Northern analysis or ribonuclease protection assay could be underestimating the changes in expression in response to stimuli. For example, I-FABP transcripts were demonstrated to change 2-fold in response to PYY as determined by ribonuclease protection assay (9). By quantitative in situ hybridization, it was found that I-FABP transcripts were confined only to the differentiated cluster population of cells. In these cell populations, there was over a 5-fold induction of the message level (9). Although further studies need to be performed to determine the responsiveness of the heterogeneous hBRIE 380i cells to PYY and NPY, it is clear that these cells as a total population display different sensitivities to these peptides. This raises the possibility that the actions of PYY and NPY on cytoskeletal elements in the epithelial cells may occur through more than one pathway and by more than one Y receptor subtype.

A number of Y receptor subtypes have been previously identified in various species and tissues by characterization of ligand binding and receptor activation using binding and functional assays (34, 72–75). At least six different subtypes have been determined based on the order of agonist potency and antagonist specificity in various tissues, with each cell type displaying a specific receptor profile (40, 75–79). All characterized Y receptors appear to be G-protein-coupled (76). The cDNAs for Y1, Y2, Y4, Y5, and Y6 have been cloned from a number of species and tissues (26, 80–83). However, the presence of transcripts for each specific Y receptor subtype in native intestinal epithelial cells has not been clearly determined. Recently, partial cDNAs for the Y2, Y4, and Y5 receptors were
isolated from epithelial cells in the rat small and large intestine by RT-PCR, while the Y1 cDNA was found predominantly in nonepithelial colonic tissue (84). To determine what Y receptor subtype could be involved in the response to PYY and NPY in the hBRIE 380i cells, we designed degenerate PCR primers based on previously published sequence data for the Y1–Y5 receptors from various species (25, 26, 79–82). In this study, we establish the presence of two of these receptors, the Y1α and Y2 subtypes, in both the hBRIE 380i cells and the intact intestinal epithelium. It has previously been reported that the Y1 subtype is present in the intestinal mucosa, in epithelial cells as well as enteric neurons (34, 35, 73, 85). The presence of a Y2 receptor in the intestine has also been demonstrated, although this receptor subtype has been suggested to be localized to the neuronal cells rather than the epithelial cells (34, 39). With the exception of the identification and sequencing of a small nucleotide fragment from rat intestinal mucosa (84), the present study is the first to identify the complete coding region for the Y2 receptors outside of nervous tissues.

It has been reported that the Y3, Y4, and Y5 subtypes are also present in the small and large intestine using specific agonists and antagonists (77). However, it has not been conclusively demonstrated which Y receptor subtype is responsible for specific biological effects such as motility, anti-secretory activities, or modulation of cell growth and differentiation in the intestine. Reasons for this include (i) difficulties involved in the isolation of a pure population of intestinal cells from a mixture of cells including nerve plexa, intraepithelial lymphocytes, enteroendocrine, and smooth muscle cells; (ii) low abundance of receptors; (iii) overlap in specificity of ligands; and (iv) small differences in binding affinities of ligands used in functional assays that often cross-react with several of the Y receptors. Both NPY and PYY bind with similar affinities to all subtypes except to the Y3 receptor, which has been demonstrated to bind NPY but not PYY (76, 86). A peripheral PYY-prefering receptor subtype has been proposed but not yet identified (87, 88). Consequently, neither the differences in actin reorganization that we observed with the two peptides nor the higher level of induction of CD63 and clusterin transcripts in response to PYY can be fully explained by the presence of the Y1 and Y2 receptors in the hBRIE cells. While each Y receptor may account for specific biological responses, multiple receptors may have overlapping actions on cell maturation as indicated by the induction of proteins associated with the cytoskeleton and cell differentiation (i.e. CD63, clusterin, and I-FABP) and the induction of actin rearrangement by NPY and PYY. Both the Y1 and Y2 subtypes can activate at least two separate intracellular messenger cascades (inhibition of Ca2+ accumulation and stimulation of inositol 1,4,5-trisphosphate followed by elevated intracellular Ca2+ in a tissue- and cell type-specific manner (77, 78). Either the Y1 or Y2 receptor could act through one of these pathways in the intestine and also act synergistically with other receptors through intracellular cross-talk to potentiate effects on cell maturation and CD63 expression. Therefore, the observed differences in actin rearrangement and induction of CD63 and clusterin transcripts, in response to PYY and NPY, could be due to the activation of a combination of the Y1α, the Y2, or another yet-to-be-identified Y receptor subtype in the hBRIE 380i cells.

The present study provides a mechanism whereby intestinal neuroregulatory peptides could regulate mucosal cell migration and differentiation through the modulation of tetraspanins. This mechanism helps build a model to explain how extracellular cues could establish the temporal and spacial expression of proteins in the intestine. The regulation of CD63 expression might be a point of convergence where the effects of ECM factors necessary for growth and differentiation are modulated by neuro-gut peptides. It is possible that Y1/Y2 activation induces TM4SF complex formation with β-integrins such as CD81-P14K-CD63-integrin (15) through CD63, which in turn helps initiate the process of actin polymerization necessary for cell adhesion and the basolateral “sampling” of ECM components laid down by the surrounding cells along the crypt to villus axis. The presence of specific proteins in these areas, detected by receptors or through basolateral endocytic processing (an event also associated with CD63 (89)), could give rise to protein expression in both a temporal and positional fashion along the villus. This model may explain how mucosal cell differentiation and growth could be closely linked to cell migration and adhesion and regulated by neuro-gut regulatory peptides.

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