Enterophilins, a New Family of Leucine Zipper Proteins Bearing a B30.2 Domain and Associated with Enterocyte Differentiation*

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Enterocyte terminal differentiation occurs at the crypt-villus junction through the transcriptional activation of cell-specific genes, many of which code for proteins of the brush border membrane such as intestinal alkaline phosphatase, sucrase-isomaltase, or the microvillar structural protein villin. Several studies have shown that this sharp increase in specific mRNA levels is intimately associated with arrest of cell proliferation. We isolated several clones from a guinea pig intestine cDNA library. They encode new proteins characterized by an original structure associating a carboxyl-terminal B30.2/RFP-like domain and a long leucine zipper at the amino terminus. The first member of this novel gene family codes for a 65-kDa protein termed enterophilin-1, which is specifically expressed in enterocytes before their final differentiation. Enterophilin-1 is the most abundant in the small intestine but is still present in significant amounts in colonic enterocytes. In Caco-2 cells, a similar 65-kDa protein was recognized by a specific anti-enterophilin-1 antibody, and its expression was positively correlated with cell differentiation status. In addition, transfection of HT-29 cells with enterophilin-1 full-length cDNA slightly inhibited cell growth and promoted an increase in alkaline phosphatase activity. Taken together, these data identify enterophilins as a new family of proteins associated with enterocyte differentiation.

The self-renewing small intestinal epithelium represents an attractive and valuable system to study various processes occurring during cell life such as proliferation, differentiation, or apoptosis. Proliferation is limited to the crypts of Lieberkühn, where multipotent stem cells achieve continuous renewal of four main epithelial lineages. At the top of the crypt, cells lose their proliferative ability and complete differentiation during a highly organized migration along the crypt-villus axis. Enterocytes, mucus-producing goblet cells, and enteroendocrine cells evolve during a vertical migration to the villus apex (1–3). This process requires 4 to 5 days and results in well-differentiated cells that are finally released into the intestinal lumen upon programmed cell death. In contrast, Paneth cells undergo terminal differentiation while moving down to the base of the crypt (4–8). The differentiation process and the function of the epithelium also vary along the horizontal axis (from proximal to distal intestine), and in both cases, functional differences reflect various patterns of gene expression as well as the nature of the epithelial cell type (7).

How these various events are regulated at the genomic level and the intracellular signaling pathways involved in this differentiation process are still poorly understood. Recently identified genes were found to be necessary either to maintain the proliferative status of stem cells (Tcf-4) (9) or to direct the differentiation process (homeobox transcription factors cdx1 and cdx2) (10–12). Most of the previous studies were performed with enterocytes (95% of differentiated cells of the villus) and have essentially described the specific expression of genes contributing to the morphological and functional phenotype of mature cells. For instance, villin participates in the formation of the typical cytoskeleton underlying the microvilloisities of absorptive cells, whereas various brush border membrane hydrolases are involved in the digestive function of enterocytes. These include alkaline phosphatase, aminopeptidase N, sucrase-isomaltase (13–15), or phospholipase B, also called Ad-RabB (16–19). The promoter of the sucrase-isomaltase gene has been studied extensively to delineate the molecular mechanisms regulating the expression of genes involved in enterocyte differentiation (20). Interestingly, a common structure for enterocyte-specific promoters has been suggested for the sucrase-isomaltase and phospholipase B genes (21).

During our first attempts to clone phospholipase B cDNA, we isolated various clones encoding new proteins containing a B30.2 domain (22, 23). The B30.2 domain is a 160–170-amino acid globular domain present at the carboxyl-terminal half of a rapidly growing number of proteins classified according to the nature of their amino-terminal part (24–26). The main category consists of nuclear proteins with a B box and a RING motif, such as RFP (27–29), Sjögren syndrome type-A or Ro/SS-A antigen (30), and Xenopus laevis nuclear factor Xnf7 (31). The importance of these proteins was illustrated by the discovery of two genes, MEFV and MID1, whose mutations in the region encoding the B30.2 domain appear to be responsible for two genetic deficiencies, familial Mediterranean fever (32, 33) and Opitz G/BBB syndrome (34), respectively. Unlike other RING finger proteins, the MID1 product is not found in the nucleus but is associated with microtubules (35), and a conserved fibronectin type III
Enterophilins and Intestinal Differentiation

18353

domain was identified in the protein (36).

Butyrophilin, a membrane protein expressed in milk fat globule membrane, is the prototype of a second group of proteins whose intracellular B30.2 domain is linked to two extranuclear immunoglobulin-like motifs (IgV-IgC1) by a single transmembrane segment (37–39). Recently, a gene sharing high homology with butyrophilin and encoding a new protein called erythroid membrane-associated protein (ERMAP) was described (40). A third group consists of extracellular proteins such as the α and β subunits of strontiospin, two hypotensive and lethal toxins isolated from stonefish (41).

We now describe a new group of intestinal proteins bearing a carboxy-terminal B30.2 domain and an extended leucine zipper containing up to 45 regular heptad repeats in their amino terminus. One member of this novel gene family codes for a 65-kDa protein termed enterophilin-1. Both the endogenous expression pattern and transfection experiments reported herein indicate a close relationship between enterophilin-1 expression and enterocyte differentiation.

EXPERIMENTAL PROCEDURES

Tissue and Cells—The entire small intestine was removed and excised into fragments corresponding to duodenum, jejunum, ileum, and colon for regional dissection. The whole ileum was cut into two fragments (proximal and distal), and the jejunum and colon were divided into three equal segments (proximal, middle, and distal). Mucosa was scraped and frozen in liquid nitrogen for protein analysis. Intestinal epithelial cells were isolated and separated as a villus to crypt gradient according to Weiser (42). COS-7, HT-29, and Caco-2 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium with glucose as a carbon source, supplemented with 2 mM glutamine, 10% fetal bovine serum, and 100 μg/ml penicillin/streptomycin in a humidified atmosphere containing 5% CO2. Fetal bovine serum was increased up to 20%, and nonessential amino acids were added.

RNA Isolation—Total RNA was extracted from different tissues or from isolated intestinal cells by the cesium chloride/guanidinium thiocyanate method (43). Polyadenylated RNA was prepared using oligo(dT) cellulose columns (Amersham Pharmacia Biotech). cDNA Library Construction and Screening—Guinea pig intestinal cDNA library was constructed in λZAP II vector using 5 μg of polyadenylated RNA from differentiated villus enterocytes according to the instructions of the manufacturer (ZAP cDNA synthesis kit, Stratagene, La Jolla, CA). The library was packaged into phage particles using the Gigapack II system (Stratagene) and amplified once in XL1-Blue MRF' (Stratagene).

For immunological screening, the nitrocellulose filters were probed with anti-phospholipase B antiserum (18) and then probed with an anti-rabbit immunoglobulin-G antibody conjugated with alkaline phosphatase. Positive plaques were purified by three runs and recovered as BlueScript plasmids by in vivo excision using ExAssist helper phage (Stratagene).

For DNA screening, the library was plated on XL1-Blue MRF’ E. coli, and replicas on nylon membranes (Hybond N; Amersham Pharmacia Biotech) were prepared. Phage DNA was denatured (1.5 μ NaCl, 0.5 μ NaOH) and cross-linked to the membrane by exposure to UV. The 9D1 F1 probe was used as a probe for hybridization as described previously (19). Positive clones were purified through three runs and recovered as BlueScript plasmids by in vivo excision using ExAssist helper phage and SOLR E. coli (Stratagene).

Radioactive Probes and Northern Blots—Enterophilin-1 probe (850 bp) was obtained by digestion of the 3.9D1 clone with EcoRI/XhoI. Phospholipase B probe is a 386-bp fragment amplified by polymerase chain reaction (19). The glyceraldehyde-3-phosphate dehydrogenase probe (350 bp) was prepared by polymerase chain reaction using human lung cDNA as a template (44). The probes were labeled by random primer extension using the Nonaprimer Kit (AppiGene Oncor) and α-32P-CTP from Amersham. RNA electrophoresis and filter hybridization were performed as described previously (19).

Construction of Eukaryotic Expression Vectors—The BlueScript-3.9D1 clone containing the full-length cDNA of enterophilin-1 was used as a template for these constructions. pCI-Ent1 was obtained by digestion of pBluescript-3.9D1 with XhoI and KpnI, and the insert was ligated in pCI (Promega) previously digested with NheI and KpnI. To generate pGFP-Ent1 and pCDNA3-Ent1, double digestions were made with NotI/NheI and EcoRI (pCDNA3) or BglII (pGFP-C1). The enterophilin-1 cDNA ligated respectively in pGFP-C1 (CLONTech) and pCDNA3.1/Myc-His (Invitrogen) at the corresponding sites. We generated enterophilin-1 in frame with the carboxy terminus of green fluorescent protein (GFP) and carboxy-terminal c-myc and polyhistidine tag.

Transfection and Fluorescence Microscopy—COS-7 or HT-29 cells were plated in 60-mm dishes or 6-well plates and transiently transfected with the different plasmids using a calcium phosphate method (AMINE, Life Technologies, Inc.) according to the manufacturer's protocol. For transfection experiments with the GFP fusion constructs, COS-7 cells were grown on sterile glass coverslips placed in 35-mm culture plates and fixed as described previously (45). Fluorescence was visualized at 65 h with a Zeiss Axioskop microscope at a magnification of 40.

Preparation of B30.2 Fusion Protein—The pBluescript-3.9D1 plasmid was digested with EcoRI and XhoI, and the obtained fragment (residues 336–529) corresponding to the 3′ region of enterophilin-1 cDNA (B30.2 domain) was inserted into pGEX-KG vector (a gift of Dr. J. E. Dixon, University of Michigan Medical School, Ann Arbor, MI). This construction was introduced into E. coli strain XL1-Blue, and production of the fusion protein was induced with isopropyl-1-thio-β-D-galactoside (IPTG). The glutathione-S-transferase domain (GST) fusion protein was purified by affinity chromatography on glutathione-agarose beads (Sigma).

Preparation of Anti-B30.2 and Anti-Peptide Polyclonal Antibodies—After SDS-polyacrylamide gel electrophoresis under reducing conditions according to Laemml (46) and Coomassie Blue staining, the 55-kDa band corresponding to the GST-B30.2 domain fusion protein was cut out and used to immunize a New Zealand white rabbit. The antisera was purified by a two-step procedure, including preadsorption on GST bound to glutathione-agarose beads, followed by adsorption of the subsequent supernatant on GST-B30.2 fusion protein bound to glutathione-agarose beads. The specific anti-B30.2 antibody was eluted by acidification with 0.1 M citric acid, pH 2.7, and immediately neutralized with 1 M Tris-HCl, pH 11.0. The purified antibody was desalted and used for immunoblotting.

A rabbit polyclonal anti-peptide antibody was produced by Eurogentec (Seraing, Belgium) using a peptide of 16 residues (CQTERDKLRRQIEIDDKR) corresponding to amino acids 313–328 of enterophilin-1 (Fig. 1), in which the amino-terminal leucine was replaced by a cysteine to allow conjugation to the keyhole limpet hemocyanin carrier.

Preparation of Cell Extracts and Immunoblotting—All the procedures described were performed at 4 °C. Nine fractions of guinea pig enterocyte extracts were isolated (47). The whole ileum or PPS containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM leupeptin, and then disrupted by 10 strokes with a Potter homogenizer. Caco-2 cells were harvested with trypsin/EDTA either at confluence (mainly undifferentiated cells) or at various times (up to 23 days) after reaching confluence (differentiated cells). They were washed twice in PBS and resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 2 mM leupeptin).

Nuclei were isolated as described previously (47). Briefly, washed cells (about 10⁶ cells) were gently suspended in hypotonic buffer containing 0.5% Nonidet P-40. They were incubated on ice for 10 min, and nuclei were pelleted by centrifugation for 5 min at 1,000 × g. They were then washed twice with hypotonic buffer without detergent and then suspended in the same buffer containing 0.5 mM MgCl2.

Protein concentration was determined according to the method of Bradford (48). Protein samples were submitted to SDS-polyacrylamide gel electrophoresis (46) and transferred onto nitrocellulose membranes according to standard protocol (49).

Monoclonal antibody against β-actin was from Sigma. Anti-villin antibody was from Affiniti, and anti-lamin B was from Nova Castra Laboratories.

3H/Thymidine Incorporation—HT-29 cells were transfected as described previously and maintained in the presence of serum. 3H/Thymidine (0.5 μCi/ml) was added to the cells at 24, 48, or 72 h after transfection, and incubation was prolonged for 4 h. Cells were washed twice in PBS, and 5% trichloroacetic acid was added for 10 min at room temperature. The solution was withdrawn, and solubilization was performed at room temperature with 0.5 M NaOH for at least 30 min.
Radioactivity in cell lysates was measured by liquid scintillation counting (1900 TR; Packard).

Alkaline Phosphatase and Sucrase Activities—Caco-2 or transfected HT-29 cells were scraped in PBS and sonicated, and the homogenates were incubated in the presence of saccharose or \( p \)-nitrophenyl phosphate as substrates according to the instructions of the manufacturer (Roche Molecular Biochemical). Data are expressed in IU (micromole of substrate hydrolyzed per minute) per gram of protein. Cell proteins were determined according to the method of Bradford (48).

**RESULTS**

Isolation of cDNA Coding for Enterophilins—A ZAP II guinea pig intestinal cDNA library was constructed and screened for enterophilin- or intestinal differentiation-related sequences. Two clones, Ent-1 and Ent-2S, were isolated that code for enterophilins.

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**Fig. 1.** Sequence alignments of guinea pig Ent-1, the short form of enterophilin-2 (Ent-2S), human butyrophilin (BT), and marenostrin/pyrin (M/P). Leucine residues of the heptad repeats are in bold. Positions with full identity between enterophilins are indicated by an asterisk above the sequence, whereas + denotes a partial conservation of the following groups: D, E, N, Q; S, T; Y, F, V, L, I; and R, K, H. The same symbols below the sequences compare the B30.2 domains of the three proteins. The sequence comprised between residues 170 and 195 of Ent-2S (discontinuously underlined), which corresponds to a change in the open reading frame, is not considered for sequence comparison. Dashes correspond to gaps allowing maximal alignment. Typical motifs of B30.2 domain are in bold (LDW, WEF, LDY). Double-underlined and bold amino acid (1114) in Ent-1 corresponds to an amino acid insertion introducing an irregularity in the heptad repeats. Mutations observed in M/P of familial Mediterranean fever patients are given below the sequence comprised between amino acids 667 and 727 (32, 33).

**Fig. 2.** Comparison of enterophilin heptad repeats. End view of the Ent-2S (A) and enterophilin-1 (B) leucine zipper, looking from the amino terminus. The letters inside each circle represent standard nomenclature for the seven amino acids found in unique positions in a coiled coil. The sequence of the residues at each position of the helix is to be read outward of the helix. For enterophilin-1, the asterisk showed the irregularity in repeats due to an alanine insertion at position 114.
screened using a polyclonal antibody raised against phospholipase B (18, 19). Three identical positive clones (7D1, 8D1, and 9D1) carrying 1.5-kb insertions were isolated and sequenced. Because none of them apparently contained an ATG initiation codon, isolation of the full-length cDNA was attempted using the PstI 1,100-bp fragment of 9D1 as a probe. Seven positive clones were selected and sequenced. Among them, 3.9D1 corresponded to a complete nucleotide sequence of 1999 bp. The open reading frame designed according to Kozak (50) included 1,587 nucleotides (positions 142–1728) and encoded a 64.5-kDa protein consisting of 529 amino acids. The lack of signal sequence or putative transmembrane domain in the polypeptide suggested an intracellular localization. As shown in Fig. 1, the carboxyl-terminal region of the sequence (residues 352–508) was highly identical to the B30.2 domain of butyrophilin (41% identity) (37, 38), and marenosin/pyrin (40% identity) (32, 33). Because of its preferential intestinal expression (see below) and according to its homology with the B30.2 domain of butyrophilin, the product of the 3.9D1 clone was named enterophilin-1.

As also indicated in Fig. 1, the amino-terminal part of enterophilin-1 (residues 11–326) contained 45 heptad repeats preceded by a leucine residue (32 repeats), an aromatic amino acid (3 repeats), or a basic residue (7 histidines, 2 lysines, and 1 arginine). This sequence did not reveal significant homology with any known protein.

Besides five other clones related to enterophilin-1 but differing by their amino-terminal end, the seventh clone (7.9D1) coded for a putative protein containing 18 heptad repeats. However, despite the presence of the whole B30.2 domain, a

![Fig. 3. Subcellular localization of enterophilin-1. A, wild type and recombinant plasmids (pCI and pCI-Ent-1, respectively) were used for in vitro coupled transcription/translation in the presence of [35S]methionine and a reticulocyte lysate (Promega). B, COS-7 cells were grown in 60-mm dishes, transfected with wild type (pCI) or recombinant vector (pCI-Ent-1), and analyzed 48 h after transfection by Western blotting using the anti-peptide antibody. C, Weiser fraction 6 homogenate (H) was fractionated into nuclei (N) and postnuclear supernatant (S) as described under “Experimental Procedures.” 100 μg of protein from each fraction were analyzed by Western blotting using the antibody directed against the carboxyl-terminal peptide of leucine zipper (top panel). Villin and lamin B were detected using specific monoclonal antibodies (middle and bottom panels, respectively). Note that besides the typical 67-kDa lamin B form, an additional 70-kDa band might represent the lamin B2 isotype expressed at lower levels in mammals. D, COS-7 cells were transfected with pGFP-Ent-1 (D, 1 and 2) or with wild type pGFP-C1 (D, 3), cultured for 65 h, and analyzed by fluorescence microscopy (D, 1 and 3) or observed by light microscopy (D, 2).](image)

![Fig. 4. Northern blot analysis of enterophilin-1 mRNA in various guinea pig tissues. Total RNA isolated from various guinea pig tissues (30 μg) was run on 1% agarose, and ethidium bromide staining of rRNA is presented (bottom panel). After migration, RNA was transferred onto a nylon membrane and hybridized with 32P-labeled 850-bp enterophilin-1 probe (top panel). After autoradiography, the nylon membrane was stripped and rehybridized with the glyceraldehyde-3-phosphate dehydrogenase probe as a loading control (middle panel).](image)

![Fig. 5. Expression of enterophilin-1 along the small intestine horizontal and vertical differentiation axes. A, 100 μg of protein from homogenates corresponding to different guinea pig intestinal segments from duodenum to colon were analyzed by Western blotting with anti-peptide antibody. P, proximal; M, middle; D, distal. For the colon, the distal segment was divided into D1 and D2 (more distal than D1). B, enterocytes were separated into nine different fractions along the villus-crypt axis according to Weiser (42) and homogenized. 100 μg of protein from homogenates were analyzed by Western blotting with anti-peptide antibody.](image)
frameshift introduced a premature stop codon limiting the protein sequence to the leucine zipper domain. As shown in Fig. 1, the corresponding protein was referred to as enterophilin-2-short (Ent-2-S). Finally, the sequence of the 9D1 clone perfectly matched the sequence of Ent-2-S but still contained the intact B.30.2 domain. This was referred to as enterophilin-2-long (Ent-2-L), but its sequence is not presented herein because it did not contain the methionine initiation codon.

These sequence data indicated that enterophilins could be the prototypes of a new subgroup among the proteins bearing a B30.2 domain. Their most remarkable feature is a very long leucine zipper. As illustrated in Fig. 2, this allowed the prediction of an almost perfect geometrical alignment of amino acid residues at each position of the α helix: whereas position α displays only leucine, subsequent positions by rotating around the helix were successively basic (KHKKHK . . . at position e), acidic (EDEEDE . . . at position f), neutral (KTEKTE . . . at position c), and again predominantly acidic (DKEDEEDEE . . . at position d). In contrast, Ent-1 did not display the same geometrical regularity, and some of the heptad repeats contained a basic amino acid instead of a leucine or aromatic residue, as mentioned above (Fig. 2B).

Subcellular Localization of Enterophilin-1—Using the anti-peptide antibody described under “Experimental Procedures,” enterophilin-1 was detected as a 65-kDa protein in isolated guinea pig enterocytes (Fig. 3C). This corresponded to its predicted molecular mass, indicating that enterophilin-1 probably did not undergo any posttranslational modification. These data were confirmed by in vitro coupled transcription translation with the pCI-Ent-1 construct (Fig. 3A), and they fitted with the detection of an identical polypeptide in pCI-Ent-1-transfected COS-7 cells (Fig. 3B). Moreover, following an efficient fractionation of enterocytes, as shown by the segregation of villin from lamin B, enterophilin-1 was found to be concentrated in the nuclear fraction, although some amounts could still be detected in the cytosol.

Further attempts to localize enterophilin-1 by immunohistochemical techniques remained unsuccessful, probably because the epitopes recognized by both the anti-peptide and the anti-B30.2 domain antibodies remained buried either in the protein itself or by associated proteins. Thus, indirect evidence for a nuclear localization of enterophilin-1 was obtained using COS-7 cells transfected with pGFP-Ent-1, allowing the expression of enterophilin-1 in fusion with GFP. As shown in Fig. 3D, fluorescence microscopy analysis showed that cells transfected with pGFP-Ent-1 displayed a diffuse cytosolic staining, whereas a strong nuclear signal could be observed. In contrast, GFP alone was distributed throughout the cell (Fig. 3D).

Northern Blot Analysis of Enterophilin-1 mRNA Tissue Distribution—A number of guinea pig tissues were analyzed by
Northern blotting to detect the presence of enterophilin-1 mRNA (Fig. 4). A large 2-kb transcript was highly expressed in intestine and slightly detected in heart. Moreover, a weak band corresponding to a larger mRNA was observed in the lung. The smeared signal detected in the intestine suggested a possible RNA degradation. However, rRNA staining (Fig. 4, bottom panel) and glyceraldehyde-3-phosphate dehydrogenase probe hybridization signal (Fig. 4, middle panel), which were used to compare RNA loading, suggested that this was not the case. The smeared signal probably results from the diversity of enterophilin mRNA, as already suggested by data from cDNA cloning. In support of this, Northern blotting performed with 9D1 as a probe (which codes for Ent-2-L; see above) resulted in a similar smeared signal (data not shown).

Expression of Enterophilin-1 in Guinea Pig Intestine—The intestine can be structurally and functionally divided into several different regions, namely the duodenum, jejenum, ileum, and proximal and distal colon. Previous studies have shown differences in the expression of several genes along the intestine, reflecting this functional diversity (7). As shown by Western blotting experiments, the highest expression level of enterophilin-1 was observed in the proximal part of the intestine (duodenum and jejenum) and then declined in the ileum to remain at a significantly lower level in the different segments of colon (Fig. 5A).

Experiments were also performed to define the expression of enterophilin-1 along the crypt-villus axis. Guinea pig enterocytes were isolated at different stages of differentiation according to Weiser (42). As shown in Fig. 5B, enterophilin-1 was expressed in all fractions, with an increased level in fractions 5 and 6, whereas it declined in the most differentiated enterocytes (fractions 1–3). These data suggest that enterophilin-1 is expressed at the early stages of the differentiation process and that its expression level is increased when cells undergo terminal differentiation.

Enterophilin-1 Expression Is Related to Enterocyte Terminal Differentiation—Using the anti-peptide and the anti-B30.2 antibodies, we could identify in Caco-2 cells an immunoreactive 65-kDa protein displaying the same subcellular localization as guinea pig intestinal enterophilin-1 (data not shown). Although we have no definitive proof that this protein is the human orthologue of enterophilin-1, we decided to study its expression pattern during Caco-2 cell differentiation (51). Fig. 6A shows a typical growth curve of Caco-2 cells. Proliferation began after a lag time of 2 days, cells reached confluence after 7 days in culture, and then growth leveled off between 9 and 12 days. At confluence, domes characteristic of functional differentiation appeared all over the monolayer as described previously (52, 53). We confirmed this observation by following the expression of sucrase-isomaltase and alkaline phosphatase, two well-known intestinal brush border enzymes used as differentiation markers. As shown in Fig. 6C, both hydrolase activities were low during the proliferative phase and increased when growth reached a plateau (12 days). In parallel, Western blot analysis showed that enterophilin-1-related protein displayed an increased expression beginning slightly before (9 days) that of sucrase-isomaltase and alkaline phosphatase, with a high level of expression being maintained during the stationary phase (Fig. 6, B and C).

Upon adding butyrate to the culture, Caco-2 cells started
Enterophilins and Intestinal Differentiation

DISCUSSION

Enterophilins, whose cDNA was actually isolated by serendipity in a phage screen using anti-phospholipase B serum, appear to be a new subfamily of proteins with a B30.2 domain. Their structure is particularly interesting because of the presence of an unusually long and regular leucine zipper. Long leucine zippers are detected in proteins of the bZIP superfamily such as murine LZIP-1 and LZIP-2 (54), their human homologue Luman (55), hMAF (human MAF) (56), the light-regulated transcription factor ATB2 from Arabidopsis thaliana (57), or the zipper protein isolated from the brush border membrane of chicken intestine, which regulates actin/myosin 1 interactions (58, 59). However, the latter one contains only 27 heptad repeats that do not display the same regularity as enterophilins.

The large number of heptad repeats in enterophilins suggests that these proteins could be able to homo- or heterodimerize. Indeed, sequence analysis of enterophilin-1 using “Prosite” allows us to predict two coiled coil regions corresponding to residues 10–15 and 126–334. However, only enterophilin-1 displays neutral or hydrophobic residues at position d of the α helix, which contributes to the interface between dimerized helices (60, 61). It thus remains to be determined whether all enterophilins display any ability to dimerize by discriminating between zipper and non-zipper structures (62). Further investigations will be undertaken to characterize the other members of the enterophilin family. Accordingly, it is interesting to note that multiple transcripts have already been reported for other members of the B30.2 family such as Ro/SS-A, RoRet, or MID1 (30, 63–65). For the latter gene, other mutations responsible for Opitz syndrome were recently described, and authors reported the presence of at least six isoforms of mRNA (66).

The recently described signaling domain SPRY, which is a subdomain of the B30.2 region (23, 67), is also present in enterophilin-1 between position 403 and 523 of the amino acid sequence. The SPRY domain was identified in several products of genes involved in severe diseases such as pyrin/marenosin and midin, suggesting that enterophilin-1 could be involved in some pathologies.

We have focused our investigations on enterophilin-1, which appeared to display a nuclear localization. This may be linked to the large number of basic residues, concentrated at specific positions of the α helix, as discussed above. No classical nuclear localization signal was apparent in the protein (68). Alternative mechanisms such as transport by a cofactor as described for human MxB protein (69) may account for its nuclear localization. Furthermore, the presence of some putative phosphorylation sites in the sequence of enterophilin-1 suggests a role of phosphorylation events in its intracellular localization.

Enterophilin-1 has tissue-restricted expression and appears to be synthesized mainly in the small intestine. Analysis of Weiser fractions demonstrated that it is abundant in the early differentiating cells of the mid-villus region (fractions 5 and 6), where enterocytes progressing along the crypt-villus unit enter their terminal differentiation program.

Although we have no definitive evidence that Caco-2 cells express the putative human orthologue of enterophilin-1, the detection of an enterophilin-1-related protein displaying the same subcellular localization is strongly suggestive of the presence of a very similar if not identical protein in human bowel. Moreover, upon in vitro differentiation of Caco-2 cells, enterophilin-1-related protein displayed an expression pattern that was very reminiscent of our observations on Weiser fractions. Altogether, these data strongly suggest that enterophilin-1 is expressed rather early during the differentiation program, at least slightly before brush border hydrolases.

differentiation in a more coordinated way than by confluence-induced differentiation. Western blot analysis of enterophilin-1-related protein expression showed a sharp increase 1 day after butyrate addition (Fig. 7). An increase in alkaline phosphatase activity was also observed, but the peak was delayed (4 days after induction). Sucrase activity was also induced, but to a lower extent.

Finally, to explore the possible functional role of enterophilin-1 in enterocyte differentiation, undifferentiated HT-29 cells were transiently transfected with pcDNA3-Ent1 and analyzed for cell proliferation and differentiation. DNA synthesis was estimated by [3H]thymidine incorporation, and data in Fig. 8A indicate that enterophilin-1-transfected cells exhibited a slower growth rate than cells transfected with the vector alone. This resulted in a 30% decrease in [3H]thymidine incorporation 48 h after cell transfection. A slight reduction of cell number was also observed at 72 h in enterophilin-1-transfected cells compared with control cells (Fig. 8B). Finally, 72 h after transfection, a 2.5-fold increase in alkaline phosphatase activity was observed in cells transfected with enterophilin-1 cDNA compared with those transfected with vector alone (Fig. 8C). No alkaline phosphatase activity could be detected after 24 or 48 h under our experimental conditions.

Fig. 8. Effect of enterophilin-1 overexpression on HT-29 cell growth and differentiation. HT-29 cells were transiently transfected as described under “Experimental Procedures” with empty vector pcDNA3 (white box) or with pcDNA3-Ent-1 (shaded box). At the indicated times, they were analyzed for [3H]thymidine incorporation (A) or harvested with trypsin/EDTA and counted (B). In parallel, cells were scraped in PBS, and alkaline phosphatase specific activity was measured. Data are expressed as increases in enterophilin-1-transfected cells compared with cells treated with empty vector (C). All the values are the means ± S.E. from three independent experiments.
In addition, enterophilin-1 expression in HT-29 promoted the cell growth of alkaline phosphatase and slightly inhibited cell growth. However, we cannot conclude from our present data that enterophilin-1 plays a direct causal role in the late enterocyte differentiation program. The diversity of enterophilins, as suggested by cDNA cloning as well as by Northern analysis, might explain why overexpression of enterophilin-1 alone is not sufficient to induce a dramatic effect on cell proliferation. Additional studies will be required to better depict and understand the observed changes.

The involvement of a protein with a B30 domain in the differentiation process was illustrated by the work of Harada et al. (70), demonstrating that mouse hematopoiesis-specific ring finger protein (HERP-1) is required for terminal differentiation of erythroid cells. Furthermore, the expression of the estrogen-responsive finger protein (eFP) is regulated during osteoblast differentiation (71). The ability of these proteins to induce differentiation could be related to their DNA binding properties, suggesting a role in transcriptional regulation, as described previously for eFP (72). However, the sequences of the promoters targeted by these factors remain to be determined.

The familial Mediterranean fever protein (pyrin) is expressed preferentially in granulocytes and myeloid bone marrow precursors (32, 33), and recent data indicated that its level was enhanced in HL-60 cells undergoing stimulated granulocytic differentiation (73). A very recent study suggested that at some stage of its functional pathway, pyrin resides in the cytoplasm and might be related to protein sorting by the Golgi apparatus (74).

In conclusion, identification of enterophilins adds a new subgroup to the rapidly expanding family of proteins with a B30 domain. Very few of these proteins have been characterized on a functional point of view. Additional studies of enterophilins might provide data allowing us to progress in our understanding of the molecular mechanisms involved in enterocyte differentiation and colonic neoplasia. Because of its leucine zipper structure, enterophilin-1 could be part of a multiprotein complex. Therefore, identification of its cellular partners as well as the use of loss or gain of function in transgenic mice might open some promising fields.

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