Changes in Villin Synthesis and Subcellular Distribution during Intestinal Differentiation of HT29-18 Clones

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Abstract. Brush border in enterocytes is a cell surface specialization intimately associated with terminal differentiation of these cells. HT29-18, a clone derived from the HT-29 human colonic adenocarcinoma cell line, and HT29-18-C1, a subclone from HT29-18 described in the companion paper (Huet, C., C. Sahuquillo-Merino, E. Coudrier, and D. Louvard, 1987, J. Cell Biol., 105:345-357), undergo terminal differentiation with brush borders in the absence of glucose or upon replacement of glucose by galactose in the medium. Taking advantage of this clone and its subclone which can be manipulated in vitro, we have studied the synthesis and subcellular distribution of villin, one major protein in the microvillus core of the brush border. For this study, a monoclonal antibody against villin (BDID2C3) has been isolated and characterized in detail. In addition an ELISA has been set up to measure villin accurately in total cell extracts. Villin content in differentiated HT29-18 cells is close to that seen in normal human colonic cells but 10 times lower in undifferentiated HT29-18 cells. The rate of villin synthesis is dramatically increased in the course of enterocytic differentiation, while villin is remarkably stable after synthesis. We have recently shown, using a cDNA probe for villin, that this change is controlled either at the transcription level or by RNA stabilization (Pringault, E., M. Arpin, A. Garcia, J. Finidori, and D. Louvard, 1986, EMBO (Eur. Mol. Biol. Organ.) J., 5:3119-3124). As shown by immunofluorescence and immunogold labelings, villin is targeted to the brush border area of differentiated HT29-18 cells but remains diffusely distributed in undifferentiated ones.

We have recently isolated a clone, HT29-18, from a human colonic adenocarcinoma cell line HT-29 (Godefroy, O., C. Huet, L. Blair, and D. Louvard, manuscript in preparation). As reported by Pinto et al. (1982) for the uncloned HT-29 cell line, HT29-18 also undergoes a typical enterocytic differentiation in media lacking glucose. In addition Huet et al. (1987 companion paper) have shown that this clone is pluripotent. Subcloning a mixture of HT29-18 differentiated cells leads to the isolation of stable differentiated subclones. One of these subclones, designated HT29-18-C1, displays the phenotype of absorptive intestinal cells. By morphological criteria HT29-18-C1 grown in galactose medium is well differentiated and forms a brush border. In contrast to HT29-18, this subclone grown in glucose medium keeps some of its differentiated morphological character.

Villin, one major protein in the microvillus core of the brush border in intestinal epithelial cells, was originally localized in the microvilli of the brush border in both intestinal and renal epithelial cells (Bretscher et al., 1981; Reggio et al., 1982). Although the presence of villin can be correlated with the existence of well-organized brush borders in enterocytes or kidney proximal tubule cells, we have recently reported the presence of this protein in undifferentiated dividing cells of adult intestinal mucosa and embryonic intestine (Robine et al., 1985). We have reported preliminary data showing that morphologically undifferentiated HT29-18 cells produce a significant amount of villin, even before HT29-18 cells undergo terminal differentiation and construct a typical brush border (Robine et al., 1985). These observations led us to use this actin-binding protein as a marker to follow the assembly of the intestinal brush border in HT-29 clones.

Chicken villin was first isolated and characterized by Bretscher and Weber (1979). It is a 95-kD acidic polypeptide (Bretscher and Weber, 1980) with three calcium-binding sites of high affinity (Hesterberg and Weber, 1983a, b). Villin interacts with actin in vitro (Mooseker et al., 1980; Craig and Powell, 1980; Glenney et al., 1981a, 1981b; Glenney and Weber, 1981a; Matsudaira and Burgess, 1982a, b) and exerts control over the polymerization of actin in a Ca++-dependent manner (for review see Mooseker, 1985). Villin recently was purified from two different mammalian species, pig (Gerke and Weber, 1983) and rat (Alicea and Mooseker, 1987), and purified mammalian villin exhibits the same actin-binding properties.

Functional domains of villin have been isolated and characterized after controlled proteolysis. Glenney and co-workers (Glenney and Weber, 1981; Glenney et al., 1981b) used V8 protease to cleave chicken villin into two fragments, a core fragment of 90 kD that retains Ca++-dependent nucleation and severing activities but does not contain bundling fila-
ments activity, and a head piece fragment of 8.5 kD that binds actin in a Ca**+ independent manner. The amino-acid sequence of the head piece fragment consists of 76 amino acids derived from the carboxy-terminal end of the villin molecule (Glenney et al., 1981b). Matsudaira et al. (1985) reported that cleavage by trypsin leads to a 45-kD fragment derived from the amino terminus of the molecule, which retains severing activity, and a 51-kD fragment containing the head piece.

In the present work we have analyzed the synthesis, stability, and steady-state expression of villin and its subcellular distribution in HT29-18 clones using biochemical methods and immunocytochemical approaches at the light and electron microscope levels. We have also compared the quantitative expression of villin as well as its immunolocalization in HT29-18 cells with those observed in the human small and large intestine. In addition to a polyclonal antibody already available (Robine et al., 1985), we have prepared a monoclonal antibody (BDID2C3) specific for villin. The isolation and properties of this antibody as well as a direct ELISA, which permits quantitation of villin in total cell extracts, are reported.

Materials and Methods

Cells and Tissues

The human adenocarcinoma-derived cell line HT-29 originally established by Fogh and Trempe (1975) was obtained from Dr. Zweibaum (Institut National de la Santé et de la Recherche Médicale U-178, Villejuif, France) and maintained as described by Pinto et al. (1982). The characterization and properties of the HT29-18 clone derived from this cell line are described in detail elsewhere (Hu et al., 1987; companion paper). The human cervix epithelial carcinoma-derived HeLa and the human larynx epidermoid carcinoma-derived Hep-2 cell lines were grown using standard conditions. Small intestine and colon were taken from freshly killed rats. Human small intestine and colon samples were directly obtained from surgery.

Preparation of Cultured and Intestinal Cell Lysates

**Single Cell Suspensions.** Cultured cells were washed twice in PBS (10 mM potassium phosphate [KPi] pH 7.4, 0.15 M NaCl) and incubated for 5 min at 37°C with 0.05% trypsin, 0.02% EDTA. Dissociated cells were counted in a hemocytometer. Cell pellets were obtained after low speed centrifugation (1,000 g, 5 min).

To isolate normal adult enterocytes from rat colon and small intestine we used the method described by Gustin and Goodman (1981). Briefly, everted organs were filled with ice-cold 30 mM NaCl, 5 mM Na2 EDTA, and 8 mM Hepes/Tris, pH 7.6, incubated in 200 ml of the same cold buffer for 45 min under vigorous stirring, and then cell suspensions were collected and counted in a hemocytometer. After centrifugation at 500 g for 10 min at 4°C, the resulting pellets containing epithelial cells were used in the following experiments.

**Preparation of Cell Lysates.** Cell pellets from cultured cells or isolated enterocytes from rat intestine were homogenized in a buffer consisting of 20 mM Tris, pH 8.8, 7.5 mM CaCl2, and 1 mM phenylmethylsulfonyl fluoride supplemented with the protease inhibitor mixture proposed by Craig and Lancashire (1980). Protein content was determined using the Bradford protein assay (Bio-Rad Laboratories, Munich, FRG). These homogenates were treated as described by Garrels and Gibson (1976) and stored at -80°C before analysis using the Western blot technique. For quantitative studies using the ELISA a form of villin that does not sediment at high speed was required. For this purpose, Triton X-100 was added (to 2%) to the cell homogenates, left for 5 min at room temperature, and centrifuged for 3 min in microfuge (Beckman Instruments, Inc., Palo Alto, CA). The supernatant (S0) was collected, and the pellet (P0) was washed once by resuspension in the same buffer and recentrifugation. The supernatant (S0) of this wash was collected. Preliminary experiments with Western blots revealed that villin was quantitatively recovered in S0. No villin was detected in P0 or S1 (data not shown). Therefore S0 was used in the ELISA as a nonsedimentable fraction representative of the cellular villin content.

Metabolic Labelling of HT-29 Cells. Optimum conditions for [35S]methionine incorporation in HT29-18 cells were defined. Two labeling procedures were used. Preconfluent monolayers of HT29-18 clones were grown in 35-mm diam petri dishes in media containing either 5 mM galactose (HT29-18/gal) or 25 mM glucose (HT29-18/gl). After incubation of monolayers for 30 min at 37°C in culture medium lacking methionine, the monolayers were washed with ice-cold PBS and incubated 60 min at 37°C with 0.6 M of culture medium containing 200 µCi of [35S]methionine. Incorporation of [35S]methionine into TCA-precipitable material was found to be linear for 1 h and equivalent in preconfluent cultures of differentiated HT29-18/gal and undifferentiated HT29-18/gl cells. In pulse-chase experiments, after a 60-min incorporation of [35S]methionine (200 µCi), the cells were washed with PBS and incubated from 1 to 24 h with cell culture medium containing a 10-fold higher concentration of methionine than normal.

Production of Monoclonal Antibodies against Pig Villin

**Immunization and Cell Fusion.** Highly purified porcine villin, kindly provided by Drs. V. Gerke and K. Weber (Max-Planck Institute for Biophysical Chemistry, Göttingen, FRG) was used to elicit antibodies in 4-6 wk old female BALB/c mice. 50 µg villin in 0.1 ml PBS was emulsified in 0.1 ml Freund's complete adjuvant before intraperitoneal injection (day 0). Intraperitoneal booster injections were given at day 15 and at day 21 using incomplete adjuvant. After 4 wk (day 28) mice were boosted again by an intramuscular injection of 20 µg villin in 50 µl PBS. At day 31, animals were bled and the presence of antibodies against villin was assayed by immunoblotting. At day 32, an intramuscular injection of 10 µg villin in 20 µl PBS was given; at day 36 spleens were removed and cell fusion carried out according to the Köhler and Milstein (1975) procedure using the Sp20/Agi4 myeloma cell line.

**Screening Techniques.** Hybridoma culture fluids were first screened for secreted antibodies against villin using purified porcine villin as antigen either absorbed on nitrocellulose filters (Dot Blot) or coated on polystyrene wells (ELISA, see below). Hybridomas with antibodies against porcine villin were tested further for cross-reactivity with avian and human species by immunoblotting on purified chicken villin (generous gift from Dr. P. Matsudaira, Whitehead Institute, Cambridge, MA) and on total cell extracts from the human intestinal cell line HT29-18. Finally, hybridoma culture fluids selected by the above procedures were checked by immunocytochemistry on formaldehyde-fixed frozen sections of rat and human intestinal mucosa. One hybridoma successively cloned three times by the limiting dilution technique using nonirradiated macrophages as a feeder layer was designated BDID2C3, and selected because it fulfilled all screening procedures listed above.

**Characterization of the BDID2C3-secreted Immunoglobulin.** The type of immunoglobulin secreted by the BDID2C3 hybridoma was determined by ELISA using specific anti-μ (IgG), IgG2a, IgG2b, and IgG3 antibodies. The light chain content was also determined by ELISA using anti-κappa and anti-lambda light chain antibodies (a gift from D. L. Leclercq, Pasteur Institute, Paris). The BDID2C3 hybridoma produced IgG1 and kappa light chains.

**Tissue Production and Purification of Monoclonal Antibody (BDID2C3) Against Villin.** The cloned cells were injected into pristane-primed Balb/c mice to form ascites fluid rich in monoclonal antibody (Koprowski et al., 1977). Ascites fluids were collected, dialyzed overnight at 4°C against KPi buffer (10 mM, pH 8.0), and loaded on a DEAE-Trisacryl resin (LKLB Instruments, Inc., Bromma, Sweden) equilibrated in the same buffer. The monoclonal antibody was recovered in the column flow through. The fraction was dialyzed for 24 h against KPi (15 mM, pH 6.8) and loaded on a hydroxypatite agarose chromatography column (LKLB Instruments, Inc.) equilibrated in the same buffer. IgGs were eluted by a linear ionic gradient from 0 to 100 mM KPi, pH 6.8. A single symmetric peak was collected and dialyzed overnight against PBS containing 50% glycerol. The homogeneity of the preparation was checked on a 10% polyacrylamide gel in the presence of SDS. A typical peak of homogenous IgG was observed, slightly contaminated by a 70-kD polypeptide.

ELISA

Screening of Monoclonal Antibodies. Polystyrene flat-bottom microtiter plates (Nunc, Roskilde, Denmark) were coated with pure porcine villin (5 µg/ml in KPi buffer (0.05 M, pH 8.0)) and incubated 2 h at 37°C, followed by overnight incubation at 4°C. After saturation of the plastic surface with
PBS supplemented with 0.1% Tween 20 (Merck, Darmstadt, FRG) and fraction V BSA (4 mg/ml; Sigma Chemical Co., St. Louis, MO) (PBS-Tween-BSA). Hybridoma supernatants were incubated 1 h at room temperature or overnight at 4°C. Plates were washed and incubated under the same conditions with beta-galactosidase-labeled goat anti-mouse IgG diluted 1:1000 in PBS-Tween-BSA (a gift from Dr. T. Ternynck, Pasteur Institute, Paris).

After extensive washings with PBS-Tween, the enzymatic reaction was allowed to develop at 37°C in KPi (0.1 M, pH 7.0) containing 1 mM MgSO4, 0.2 mM MnSO4, 2 mM magnesium titriplex (Merck), 0.1 M 2-mercaptoethanol, and 2.5 mM p-nitrophenyl-beta-D-galactopyranoside (Sigma Chemical Co.), according to Guesdon et al. (1978). After the yellow color of the reaction product was developed (overnight at 37°C), the absorbance was measured at 444 nm in a Titertek Multiskan Photometer (Flow Laboratories Inc., McClean, VA).

Properties of Villin in Tissue or Cell Extracts. Polystyrene flat-bottom microtiter plates were coated with purified monoclonal anti-villin IgG. Coating was carried out for 2 h at 37°C followed by overnight at 4°C in KPi buffer (0.05 M, pH 8.0, 5 μg/ml purified IgG, 50 μl/well). The wells were then washed five times with PBS supplemented with 0.1% Tween 20 and free-binding sites were saturated in PBS-Tween-BSA for 30 min at 4°C. Cell lysates (protein content 1-5 mg/ml) were serially diluted in PBS-Tween-BSA (dilutions, 1:2 to 1:1024) and incubated on the coated plates overnight at 4°C (60 μl per well). The microtiter plates were then washed six times with PBS-Tween-BSA and filled with beta-galactosidase-linked rabbit anti-pig villin IgG that had been diluted in PBS-Tween-BSA. The dilution used was optimized for each batch of the enzyme-linked IgG. After 3 h at 4°C, five additional washings with PBS-Tween were made and the enzymatic reaction was performed as described above. A standard curve with purified porcine villin (a gift from Dr. V. Gerke and Dr. K. Weber) diluted in PBS-Tween-BSA was carried out simultaneously for each experiment. To measure nonspecific binding, we performed control experiments by replacing the antigen with PBS-Tween-BSA.

Immunochromesul Experiments

Immunoreplica Procedure. Proteins from cultures or tissues were extracted as described above. SDS-PAGE was performed as described by Laemmli (1970) in 7% acrylamide slab gels. Electrophoresis was performed under reducing conditions, using 0.1% SDS in water and 1% mercaptoethanol. The gel was fixed overnight in 25% ethanol and 10% acetic acid and then dried. The gel was used as a template for the replica of the membrane. After fixing, the membrane was rinsed in water and incubated in distilled water. The membrane was then dried for 10 min at 80°C. After drying, the membrane was incubated in a solution of 50% polyethylene glycol and 50% glycerol. The membrane was then placed on the gel in a sealed chamber and allowed to equilibrate for 1 h. After equilibration, the membrane was removed from the gel and the gel was allowed to dry. The membrane was then stained with Coomassie Blue R-250 and destained in water.

Immunoprecipitation Procedure. After washing with PBS, the labeled cells were scraped with a rubber policeman and centrifuged. The pellet was resuspended in 0.250 ml of 0.5% SDS in water and boiled 3 min. The immunoprecipitation was performed essentially as described by Mostov and Blobel (1983). After boiling, the sample was centrifuged at 13,000 g for 10 min. The supernatant was then added to 2 mg/ml of protein A-Sepharose and incubated overnight at 4°C. The immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography on Kodak X-OMAT AR film at ~80°C.

Immunofluorescence Microscopy. Modified procedures described in detail by Reggio et al. (1983) were used. Briefly, cells were fixed on the culture dish with 1% glutaraldehyde in PBS for 15 min at room temperature. Then, the samples were washed twice with PBS and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After washing with PBS, the cells were incubated with primary antibodies for 1 h at room temperature. The secondary antibodies were then added and the samples were incubated for an additional 1 h. The samples were then washed with PBS and mounted with glycerol (50%) and DABCO (2%) on glass slides. The slides were observed using a microscope equipped with a fluorescence filter set.

Immunocytochemistry

Ultrastructural Immunocytochemistry. Human small intestine and colon samples were immediately fixed with 3% paraformaldehyde in PBS or with periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974).

Tissue culture cells were fixed in vitro using 1% glutaraldehyde in PBS for 1 h at room temperature. The monolayer was then scraped from the petri dish and embedded in 3% agar preheated at 37°C. Little blocks (1 mm³) of samples were cut and dehydrated in graded series of ethanol at low temperature. Samples were then embedded in Lowicryl K4M resin at −35°C (Armbruster et al., 1982). The polymer was cured under UV light for 24 h at −35°C and for 2 d at room temperature. Thin sections (500-1,000 nm) were cut with an ultracat ultramicrotome (Reichert Scientific Instruments, Div. Warner-Lambert Technologies, Inc., Buffalo, NY) and picked up on nickel grids.

Grids were incubated overnight at 4°C in affinity-purified rabbit anti-villin IgG solution (concentration 10-20 μg/ml in PBS supplemented with 0.5% BSA). Sections were then soaked in distilled water and incubated in a 10 nm gold-conjugated goat anti-rabbit IgG solution, dilution 1:25, (Janssen Pharmaceutica, Beerse, Belgium). After 1 h incubation at room temperature, grids were washed with distilled water and dried. The specificity of the immunoassays checked by three types of controls: omitting the first antibody showed an unlabeled section using the gold conjugated goat anti-rabbit IgG solution; in the tissue, no labeling was observed in cells but enterocytes; and finally nuclei, which are known as a sticky cellular compartment, were devoid of gold particles. Sections were stained for 5 min with uranyl acetate (2% uranyl acetate in 0.05 M acetate buffer, pH 5.5) and 30 s with lead citrate (Reynolds, 1963).

Results

Properties of the Monoclonal Antibody against Villin and Mapping of the Recognized Epitope

The monoclonal antibody (BDID2C3) hybridoma was selected by Western blotting analysis for its wide cross-reaction with villin of several unrelated species: human, pig, rat, chicken, and Xenopus laevis. It therefore recognizes a well-conserved epitope of villin during evolution. This monoclonal antibody specifically recognized human villin as shown by immunoblotting analysis of a total cell extract of HT29-18/glu (Fig. 1 A) as well as by immunoprecipitation using a total cell extract of these cells labeled with [35S]methionine (Fig. 1 B). Therefore the epitope is recognized in both experimental conditions. The epitope was mapped on the villin molecule using Western blot analysis with well-defined peptides obtained after proteolysis of purified chicken villin (generous gift of Dr. P. Matsudaira). The following fragments were used: the core (90 kD) and the head piece (8.5 kD).

Figure 1. Specificity of the villin monoclonal antibody. Villin was detected in HT29-18/glu cultures by the monoclonal antibody BDID2C3 using (A) immunoreplica or (B) immunoprecipitation procedures and analyzed on SDS-PAGE gels (7%). (Lanes 1 and 2) Total cell extract from HT29-18/glu cultures were analyzed on SDS-PAGE and visualized by Coomassie Blue staining (lane 1) and transferred to nitrocellulose filters and revealed by Western blotting with the monoclonal antibody BDID2C3 (lane 2). (Lanes 3 and 4) Nearly confluent cultures of HT29-18/glu were labeled for 30 min with [35S]methionine. (Lane 4) Villin was immunoprecipitated by BDID2C3 monoclonal antibody from total labeled proteins. Total (lane 3) and immunoprecipitated labeled proteins (lane 4) were revealed on SDS-PAGE by fluorography.
Figure 2. Mapping of the epitope recognized by the monoclonal anti-villin antibody. (A) Different fragments of purified chicken villin were analyzed by SDS-PAGE gel (10%), transferred to nitrocellulose filters, and stained with Ponceau S. (B) The same filter was then incubated with the specific anti-villin monoclonal antibody BDID2C3 and processed as usual. (1) Villin-trypsin fragments, (2) villin head piece, (3) villin core, or (4) purified chicken villin were used. The high sensitivity of the technique allowed the visualization of intact villin (B, lane 1) contaminating the villin trypsin fragments that are not seen by protein staining (A, lane 1).

kD) generated by limited staphylococcal V8 protease digestion; the amino-terminal peptide (45 kD) and the carboxy-terminal peptide (51 kD) generated by trypic digestion of the intact molecule. As shown in Fig. 2, BDID2C3 recognized the head piece itself and all the fragments that contained the head piece, but it did not recognize the other fragments. This indicates that the epitope is found only in the head piece. A large sequence homology has been found between human and chicken villin head pieces (Pringault et al., 1986). This result is consistent with the cross-reaction observed between human and chicken villin for this monoclonal antibody.

Villin Expression in Differentiated and Undifferentiated HT29-18 Cells

In a recent paper, Robine et al. (1985) reported that villin could be detected by immunoblotting in both the differentiated and undifferentiated HT29-18 cells. This technique indicated that the level of villin expressed in HT29-18/glu cells was much lower than the one expressed in the HT29-18/gal cells (Fig. 3, lanes 1 and 2). In contrast, in the differentiated polarized HT29-18-C1 subclones described by Huet et al. (1987 [companion paper]), the level of villin was similar when the cells were grown either in glucose medium or in galactose medium (Fig. 3, lanes 3 and 4). Polyclonal antiactin antibodies (Reggio et al., 1982) were used in the same experiment, mixed with villin antibodies, and the level of actin detected was identical for all cell lines tested and independent of the hexose in the culture medium. Although villin expression is enhanced during the differentiation of HT29-18 cells, the level of actin, to which villin is bound in vivo, remains unchanged. Furthermore, the difference of villin expression observed in HT29-18/glu and HT29-18/gal clones is found whether the cells are tested during the exponential phase of growth or after confluency (Fig. 4).

Villin Quantification

To quantify the changes of villin expression in the course of cellular differentiation, we have used an ELISA developed in our laboratory. As described in detail in Materials and Methods, we first determined the optimum conditions for villin solubilization from total cell homogenate. As shown in Fig. 5, a linear response was observed for a villin concentration from 0.5 to 12.5 ng/ml. No villin could be detected in this very sensitive assay using an epithelial cell line unrelated to the digestive tract such as HeLa cells. The ability of our assay to detect villin quantitatively in a total cell extract was tested using HeLa cells extracts to which the samples of the standard curve were added. The results obtained were completely superimposable with the standard curve observed with purified villin. Therefore, in this assay there is no interference with cellular proteins unrelated to villin. Typical linear responses are represented in Fig. 5 using HT29-18/glu and HT29-18/gal cells. The assay also has high specificity, since the specific epitope can be blocked by an excess of antibody (50 μg/ml) added to the cell extract before its incubation with the antibody-coated wells. Under these conditions, >98% inhibition of the specific response is observed for villin content between 1 and 20 ng/ml.

In Table I we have summarized the values obtained using this enzyme immunoassay. In normal enterocytes, we observed that the content of villin per cell in the colon was ~1.5 of the value detected in the small intestine. Considering that villin is found essentially in the brush border of differentiated cells, these results are in agreement with ultrastructural observations showing that microvilli are less numerous and shorter in colonic cells than in cells from the small intestine (see Fig. 10). For the different cell lines, cultures confluent for 7 d were used. We observed a 10-fold increase in villin cellular content in HT29-18/gal cells relative to HT29-18/glu
Figure 4. Comparative villin expression during growth phase. Total cell extracts (100 μg protein) from (A) HT29-18/glu or (B) HT29-18/gal cultures in (1) exponential growth phase, (2) at confluency, or (3) in stationary phase were analyzed by SDS-PAGE gels (7%) and transferred to nitrocellulose filters. Villin was revealed using polyclonal rabbit anti-villin antiserum diluted 1:1000 and protein A labeled with 125I.

cells. In HT29-18-C1, we observed a small but significant and reproducible increase (~20%) in villin content in HT29-18-C1/gal over HT29-18-C1/glu. This change is in agreement with the enhanced cell differentiation indicated by morphological criteria reported in the companion paper (Huet et al., 1987).

Villin Synthesis

To explain the origin of differences observed in cellular villin content between differentiated (HT29-18/gal) and undifferentiated (HT29-18/glu) cells, we examined the synthesis and stability of this protein in preconfluent HT29-18 cultures. Total protein synthesis was monitored after a 30–60-min incorporation of [35S]methionine. Under these conditions, the rate of protein synthesis per cell was nearly equivalent in HT29-18/glu and HT29-18/gal. After separation by SDS-PAGE, the total synthesized protein pattern looked very similar (compare lanes 1 and 4, Fig. 6). This observation suggests that the synthesis of no major protein increased significantly during terminal differentiation of HT29-18 cells when visualized by this procedure. After immunoprecipitation under standardized conditions with polyclonal antibodies against villin, the immunoprecipitates were analyzed by SDS-PAGE. The results in Fig. 6 (compare lanes 2 and 5) clearly demonstrate that the rate of synthesis of villin is much greater in the differentiated HT29-18/gal cells than in the undifferentiated HT29-18/glu. Densitometric analysis of autoradiograms indicates a 10-fold difference when standardized on a cell basis (mean of three experiments). These results indicate that the difference in steady-state villin levels in cultured HT29-18 cells can be attributed to a large difference in the rate of villin synthesis. This conclusion was further confirmed after investigation of villin stability in preconfluent cultures. After a 60-min pulse with [35S]methionine, separate cultures were incubated with a large excess of cold methionine for 1–24 h and villin was immunoprecipitated. The immunoprecipitates were analyzed as previously indicated. The data in Fig. 7 show that, once synthesized, villin is remarkably stable in both differentiated and undifferentiated cells. We conclude from the results in Figs. 6 and 7 that villin content is controlled at the level of its synthesis rather than its stability in the HT29-18 cell line.

Table I. Villin Detected by ELISA in Various Cell Types

| Cell Type                | μg cellular proteins/10⁶ cells | ng villin/10⁶ cells | % of cellular proteins |
|--------------------------|-------------------------------|--------------------|------------------------|
| Normal enterocytes       |                               |                    |                        |
| Rat small intestine      | 780                           | 2,200              | 0.28                   |
| Rat large intestine      | 690                           | 620                | 0.09                   |
| Human colonic adenocarcinoma cell line HT-29 |                       |                    |                        |
| Subclone HT29-18/glu     | 680                           | 50                 | 0.007                  |
| Subclone HT29-18/gal     | 690                           | 470                | 0.068                  |
| Subclone HT29-18-C1/glu  | 680                           | 420                | 0.062                  |
| Subclone HT29-18-C1/gal  | 640                           | 500                | 0.078                  |
| Other cell lines         |                               |                    |                        |
| HeLa (human cervix uterus) | Undetectable                |                    |                        |
| Hep-2 (human larynx)     | Undetectable                  |                    |                        |

Figure 5. Quantification of villin in cell extracts using the ELISA. A standard curve was constructed using serial dilutions of purified porcine villin. Villin was diluted in (solid circles) PBS–Tween–BSA or in a total cell extract of (open squares) Hela cells. Typical linear responses using (solid triangles) HT29-18/gal, (open triangles) HT29-18/glu, or (solid squares) Hela cell extracts are represented. From the standard curve obtained with purified villin, the concentration of villin in these cell extracts may be calculated. When an excess of antibody (50 μg/ml) is added to the cell extract before incubation with the wells coated with antibody, >98% inhibition of the specific response is observed (open circles).
Figure 6. Villin biosynthesis in HT29-18/glu and HT29-18/gal. Nearly confluent cultures were labeled for 30 min with [35S]methionine. (Lanes 1 and 4) Total newly synthesized protein in (lane 1) HT29/glu and (lane 4) HT29-18/gal. (Lanes 2 and 5) Villin was immunoprecipitated with polyclonal antibodies from newly synthesized proteins of (lane 2) HT29-18/glu and (lane 5) HT29-18/gal. (Lanes 3 and 6) Immunoprecipitation with a nonimmune serum from newly synthesized proteins of (lane 3) HT29-18/glu and (lane 6) HT29-18/gal. Total newly synthesized protein as well as immunoprecipitates were analyzed on SDS-PAGE gels (7%) and revealed by fluorography.

Cellular Localization of Villin at the Light and Electron Microscope Level

Light Microscopy. We first examined the distribution of villin using immunofluorescence techniques on semi-thin frozen sections of HT29-18/glu and HT29-18/gal cultures fixed with paraformaldehyde. As shown in Fig. 8 a, a diffuse and uniform distribution throughout the cytoplasm was observed in undifferentiated HT29-18/glu. This weak cytoplasmic staining can be completely abolished if the antibody has been previously incubated with an excess of purified villin. In contrast, in the polarized monolayer of differentiated cells grown in galactose, labeling is strong at the apical membrane although diffuse cytoplasmic label remains; (c) indirect immunofluorescence; (d) the same field in phase contrast. In all panels the upper part of the pictures corresponds to the surface facing the medium. Bar, 10 μm.

Figure 7. Stability of villin in HT29-18/glu and in HT29-18/gal. Cultures of HT29-18/glu in A and HT29-18/gal in B were labeled for 60 min with [35S]methionine and chased with cold methionine for 4, 15, or 24 h, respectively. Villin was immunoprecipitated with polyclonal antibodies after the different chase times and analyzed on SDS-PAGE gels (7%) and revealed by fluorography.
Figure 9. Ultrastructural localization of villin in the HT29-18 cultures. (Top) HT29-18/glu cells. In the undifferentiated cells, a low amount of villin is detected by the immunocytochemical method. Villin is seen randomly distributed in the cytoplasm (open squares) and is not associated with any kind of subcellular structure. The low intracellular labeling is considered significant as virtually no background is seen with the affinity-purified antibody (see Materials and Methods). Desmosomes are the only kind of intercellular junctions (arrows).

(Bottom) HT29-18/gal cells. These differentiated cells are morphologically characterized by occluding junctions isolating the apical domain from the basolateral membrane domain (arrows) and by the presence of numerous microvilli at the apical pole of the cell. Villin is clearly concentrated in the microvilli and in the underlaying cytoplasm where long microfilaments rootlets are anchored (bracket). However few gold particles are also seen randomly distributed within the cytoplasm underneath (open squares). Bars, 0.5 μm.
Figure 10. Ultrastructural localization of villin in the small and large human intestine. (Top) Small intestine. This electron micrograph shows two adjacent absorptive cells joined by a typical occluding, or tight, junction. The apical domain of these cells is characterized by long microvilli. Villin is detected by the gold particles in large amounts in each microvillus along the axial bundles of microfilaments. The rootlets anchoring the microvilli into the underlying cytoplasm contain also villin (bracket). Few gold particles indicating the presence of villin are seen dispersed within the underlying cytoplasm (open squares). (Bottom) A human colonic cell in the midcrypt area is shown here. These relatively immature cells show microvilli less developed than those seen at the apex of small intestinal cells of the upper part of the villi. Notice the striking resemblance of the apical membrane in this cell with differentiated HT29-LB/gal. Gold particles are not so numerous and not so axially well distributed compared with the small intestine. The apical domain of the cytoplasm where the microvilli rootlets are plunging also contains villin (bracket). In the underlying cytoplasm, a very small amount of villin can be detected (open squares). Bars, 0.5 μm.
HT29-18/gal cells (Fig. 8 c), a strong apical labeling was observed together with a faint diffuse cytoplasmic staining. The distribution of villin in mature HT29-18/gal cells is therefore very similar to villin localization in mature enterocytes in vivo (Breitscher et al., 1981; Reggio et al., 1982). While the monoclonal antibody BDID2C3 was used for these experiments, it is noteworthy that the same results were obtained using polyclonal antibodies against villin.

Electron Microscopy. To further investigate villin distribution in intestinal cells we used a higher resolution immunocytochemical method. When preliminary experiments showed that villin detection by the monoclonal antibody was not sensitive enough for ultrastructural studies, affinity-purified polyclonal anti-villin antibodies and colloidal gold–conjugated goat anti-rabbit immunoglobulins were used and applied to ultrathin sections of Lowicryl-embedded material.

HT29-18 Cultures

In the HT29-18/glu cells (Fig. 9 top) no differentiated phenotype is expressed and no microvilli like those observed in intestinal absorptive cells can be seen. However villin is detected in the cell and is randomly distributed all over the cytoplasm. Its distribution cannot be attributed to any subcellular structure when observed by this method.

In the HT29-18/gal culture (Fig. 9 bottom) the differentiated phenotype is well expressed and is morphologically characterized by the presence of microvilli like those observed in the brush border of colonic cells. Villin is abundant in the microvilli and in the underlying domain of the cytosol in association with the microfilament bundles plunging into the cytoplasm. However a few gold particles are also observed to be randomly distributed in the cytoplasm without any clear relationship to cytoplasmic structures.

Human Intestine

We also compared the ultrastructural localization of villin in small intestine and in the colon with that in HT29-18 cells. In the small intestine (Fig. 10 top) and in the midcrypt area of large intestine (Fig. 10 bottom) villin is located primarily in the brush border of the columnar cells. Gold particles are also found in the underlying cytoplasm closely associated with the rootlets of the microfilament bundles. Very little villin is detected in the cytoplasm in the lower part of the cells. These observations on human tissues are similar to those previously reported by Drenchkahn et al. (1983), who used immunoperoxidase procedures to localize villin in rat small intestine.

However, two clear differences between the small and large intestine were observed here. First, the small intestine contains a higher density of gold particles. Second, the labeling in the small intestine is distributed axially on the microfilament bundles of the microvillus core but in the colonic cells from the midcrypt zone, villin distribution in the microvillar space is clear but its axial distribution is not obvious.

Discussion

In contrast to most other actin-binding proteins that are expressed in most cell types, we have previously reported that villin displays a sharp tissue-specific expression (Robine et al., 1985). We have taken advantage of the human cell line HT-29 which can be manipulated in vitro to investigate the expression of villin during its differentiation. Since villin is a structural component found in the brush border of mature enterocytes, it can be anticipated that study of the synthesis and localization of this protein will provide a useful approach to monitoring the assembly of the brush border. Furthermore, understanding the molecular mechanisms involved in the assembly of the brush border, an organelle that appears during terminal differentiation of intestinal cells, will enhance our knowledge of organelle biogenesis and cellular differentiation.

To pursue these questions, we describe the isolation and characterization of a monoclonal antibody BDID2C3 against villin. This monoclonal antibody allows us to measure villin accurately in total cell extracts with an ELISA. Although villin has been detected previously by immunoreplica procedures, our ELISA is up to 500-fold more sensitive than standard Western blot analysis. Therefore villin can be determined in cells expressing low levels of villin or, alternatively, in cases where a limited number of cells is available (for example, during embryogenesis). With such an assay, we have confirmed our previous observations related to tissue-specific expression. For example, in Hela cells, villin level is found below the detection limit. However, in the cell line LLCPK1, which is believed to derive from proximal tubular cells of the kidney (Rabito and Ausiello, 1980) (the other organ synthesizing villin), low but detectable amounts of villin are found. In contrast, in Madin–Darby canine kidney cells, which are thought to be derived from the distal tubule (Herzlinger et al., 1982), no villin can be measured (Coudrier et al., 1987). Antibody BDID2C3 is able to recognize villin in numerous unrelated species (Xenopus, rat, human, pig) and has been used to screen Escherichia coli expression libraries containing cDNAs encoding for villin (Pringault et al., 1986). Furthermore it provides a useful reagent to purify villin in one step by immunoabsorption for future studies (Lupi, N., and S. Robine, unpublished results).

In comparing the expression of villin in HT-29 cells with intestinal cells isolated from small and large intestine, it is striking to note that levels expressed by these tumor cells in culture are similar to that of normal cells in vivo. In particular, the villin level in differentiated HT29-18/gal cells is close to that seen in normal differentiated colonic cells. This observation is consistent with the colon carcinoma origin of the tumor from which HT-29 was established and with the similar morphological features of HT29-18/gal cells and normal large intestinal cells. The three- to fourfold difference in villin expression between enterocytes of large and small intestine may be attributed to a less-developed brush border with less numerous and shorter microvilli in the large intestine where villin immunogold labeling is also lower compared with small intestine. However, this simple interpretation may only partially explain our results. Although villin is always found in cells displaying a brush border, we have also reported that differentiated adult cells lacking an organized brush border such as the epithelial cells lining pancreatic and bile ducts accumulate villin at their luminal faces (Robine et al., 1985). Therefore the presence of villin is also indicative of the cellular origin of cells forming simple epithelia.

In the course of HT-29 enterocytic-like differentiation, the rate of villin synthesis is dramatically increased. We have re-
recently shown using a cDNA probe for villin that this change is controlled at the RNA level either by an increase in transcrip-
tion or by mRNA stabilization. The level of mRNA en-
coding for villin is 10-fold higher in HT29-18/gal relative to
HT29-18/glu (Pringault et al., 1986). This result is directly
comparable to the 10-fold difference in the rate of villin syn-
thesis observed here.

Morphological studies on HT29-18 cells have indicated that assembly of brush border occurs ~1 wk after the cells reach confluency and stop their division. In contrast, the steady state villin level is independent of cell growth since even during exponential growth HT29-18/gal have a 10-20-
fold higher level of villin than exponentially growing HT29-
18/glu cells. Therefore, the activity of the villin gene is con-
grolled by the glucose supply in this particular system and
may be controlled in general by growth conditions. The self
assembly of structural components of the brush border can occur only when culture media are permissive for terminal differentiation and when cell division is arrested. This as-
sembly could indeed require the presence of stoichiometric
amounts of each individual protein contributing to the struc-
ture in addition to other currently unknown factors.

There are several analogies between our results and the
observations reported by Lazarides and co-workers on eryth-
roid differentiation and spectrin assembly. For example, the
expression of spectrins precedes phenotypic differentiation
of the precursors of red blood cells, but posttranslational
modification and differential catabolism of alpha and beta
spectrins play an essential role in spectrin network assembly
(Blikstad et al., 1983; Lazarides and Moon, 1984; Moon and
Lazarides, 1984; Woods and Lazarides, 1985). A difference
in the two systems is that villin appears to be very stable in
undifferentiated cells, in contrast to what has been reported
for spectrins on erythroid cells (Woods and Lazarides,
1985). Whether or not posttranslational modifications such
as phosphorylation of villin play a role in this stability re-
 mains to be studied.

A major subcellular change in villin distribution is ob-
erved between HT29-18/glu and HT29-18/gal. As expected
by comparison with normal intestinal cells, villin is recruited
in the brush border area of differentiated cells but remain
diffusely distributed in undifferentiated ones. Villin has not
been found associated with any particular membranes or
cytoskeleton structures in the cytoplasm. Since actin micro-
filaments or stress fibers are not clearly visible at the electron
microscopic level nor visualized by immunofluorescence
techniques (Louvard, D., unpublished observations), associ-
ation of cytosolic villin with these structures remains a possi-
bility. Carefully controlled cell-fractionation studies in
which redistribution is prevented are necessary to establish
the interaction of villin with actin oligomers or other struc-
tures containing actin. Our results demonstrate that in the
course of HT29-18 differentiation actin content (in contrast
to villin) remains essentially unchanged. This result argues
against new actin synthesis in parallel to villin synthesis
when HT29-18 cells differentiate and suggests that the actin
pool is sufficiently large in these cells to build new struc-
tures.

The uniform distribution of villin in undifferentiated
HT29-18/glu is quite different from the one reported for im-
mature intestinal cells in the region of the crypts where villin
is localized underneath the apical membrane. However in the
crypts, cells devoid of an organized brush border are
nevertheless polarized cells with tight junctions in contrast
to HT29-18/glu. A distribution equivalent to that seen in
HT29-18/glu may be expected in stem cells or in their first
progeny. These early progenitor cells are so few in adult in-
testinal mucosa that they would be difficult to identify in
cryosections. In this regard, Shibayama et al. (1987) recently
reported the distribution of villin in developing chick entero-
cytes. They show that villin is first detected throughout the
cyttoplasm at day 6.5-7 and then becomes localized at the apic-
al borders at day 8, before the cells display typical brush
borders. In this respect one may propose that villin distribu-
tion in HT29-18/glu is equivalent to that seen in embryonic
cells. Work in our laboratory is in progress to describe the
sequence of events for villin expression in the embryonic in-
testine of rats as an example of intestinal development in
mammals.

The HT-29 clones described in this report appear to be
useful tools to study enterocyte differentiation and organelle
assembly in polarized epithelial cells. We were able to
demonstrate that while villin is synthesized in undifferentiated
cells, its higher levels in differentiated cells are regulated by
the rate of synthesis and localized to specific subcellular
structures. Regulation of villin gene expression together with
villin posttranslational modification are now under investiga-
tion with molecular tools available in our laboratory. The
main objective is to obtain information on the important but
still unresolved questions of the role of villin in vivo and its
contribution to structure and function in intestinal microvilli.

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