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Can villin be used to identify malignant and undifferentiated normal digestive epithelial cells?

(actin/brush border/differentiation marker/development/tumor classification)

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ABSTRACT **We have investigated the presence of villin (a Ca^{2+}-regulated actin binding protein) in various tissues (normal or malignant) and in established cell lines by using sensitive immunochemical techniques on cell extracts and immunofluorescence analysis on frozen sections. Our results show that villin is a marker that can be used to distinguish normal differentiated epithelial cells from the simple epithelia lining the gastrointestinal tract and renal tubules. Villin is found in the absorptive cells of the small and large intestines, in the duct cells of pancreas and biliary system, and in the cells of kidney proximal tubules. Furthermore, undifferentiated normal and tumoral cells of intestinal origin *in vivo* and in cell culture express villin. Therefore, expression of villin is seen in cells that do not necessarily display the morphological features characteristic of their terminally differentiated state, such as the microvilli-lined brush border. We suggest the possible clinical implications of using villin as a marker in the diagnosis of metastatic adenocarcinomas.**

The identification and characterization of cell-type specific structural protein markers has been valuable in the study of cell differentiation in higher eukaryotes. Antibodies against intermediate filament proteins (e.g., vimentin, keratins, and neurofilament proteins) are used to identify cell types *in vivo* and *in vitro* (1, 2). The detection of these cell-type specific protein markers during embryogenesis and terminal cell differentiation has allowed investigators to define the developmental stage or the developmental commitment of cells (3, 4). In addition to the available markers, such as the keratins, which are especially useful in discriminating between epithelial and nonepithelial cells, it would be useful to identify other structural proteins exclusive to one cell type or subtype of differentiated epithelial cells (2).

The major epithelial cell type found in the intestinal mucosa is the absorptive enterocyte. This epithelia has a brush border, a specialized domain of the plasma membrane composed of microvilli facing the lumen of the intestine that is assembled during the terminal differentiation of enterocytes. The axial-microfilament bundle of a microvillus contains a Ca^{2+}-regulated actin-binding protein, villin (M_r 95,000), which is the best-characterized protein of the cytoskeleton of intestinal microvilli (5, 6). Indirect immunofluorescence using anti-villin antisera on frozen sections localizes villin at the apical pole of columnar cells in the intestine and at the apical pole of proximal tubule cells in the kidney, which is the location of the brush border. In contrast, villin was not detected in microvilli of other epithelia that lack a highly organized brush border (7, 8). Thus, the presence of villin may be correlated with the existence of a mature brush border. With this in mind, we investigated villin as a marker for mature, differentiating, and transformed enterocytes using specific anti-villin antisera. Villin was detected on immunoblots of cell extracts that had been electrophoresed and transferred to nitrocellulose and by indirect immunofluorescence cytochemistry on frozen tissue sections.

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MATERIALS AND METHODS

Reagents. Peroxidase-labeled sheep anti-rabbit IgG were obtained from Biosys (Compiègne, France) and iodinated protein A was from New England Nuclear. Rhodamine-conjugated sheep anti-rabbit IgG was from Nordic (Tilburg, Netherlands).

Cell lines, Tumors, and Tissues. The human adenocarcinoma-derived cell lines HT-29 and Caco-2 were obtained from J. Fogh (9) and maintained as described by Pinto *et al.* (10, 11). Other cell lines used were the porcine kidney proximal tubule cell line, LLC-PK_1 (12, 13); the canine kidney distal tubule cell line, MDCK (14); mouse embryo line 3T3; human larynx epidermoid carcinoma-derived HEp-2; human cervix epithelial carcinoma-derived HeLa; human colonic adenocarcinoma-derived HRT18; human ileocecal adenocarcinoma-derived HCT8R; human colonic adenocarcinoma-derived SW480; and *Potorous tridactylis* kidney epithelial-like Pt K2 cell lines were grown using standard conditions. Tumors in nude mice were obtained as reported (15). The methods used to isolate intestinal epithelial cells have been described by Raul *et al.* (16) and Hauri *et al.* (17).

Immunochemical Experiments. Proteins from cultures or tissues were extracted according to Garrels and Gibson (18). Electrophoresis was performed according to Laemmli (19) by using 7.5% $NaDodSO_4$/polyacrylamide slab gels. Electrotransfer of the proteins to nitrocellulose and antigen detection was performed according to Burnette (20) as modified by Coudrier *et al.* (21).

Antisera. Using purified porcine villin as immunogen (a gift from V. Gerke and K. Weber; ref. 22), antibodies were raised as described (23). The specificity of the antisera was checked on immunoreplicas of intestinal mucosa homogenate or by indirect immunofluorescence on intestinal frozen sections. The antibodies used in the present work did not cross-react with the 110-kDa protein of the microvilli (23).

Immunocytochemistry. Large and small rat intestines were fixed and prepared for cryosectioning according to Brown and Farquhar (24). Other tissues were cut without prior aldehyde fixation, and sections were fixed with cold (−20°C) acetone. Immunofluorescent labeling was performed as described by Reggio *et al.* (25).

RESULTS

Villin Expression in Epithelia. Frozen sections of rat or human small intestines showed strongly labeled brush bor-

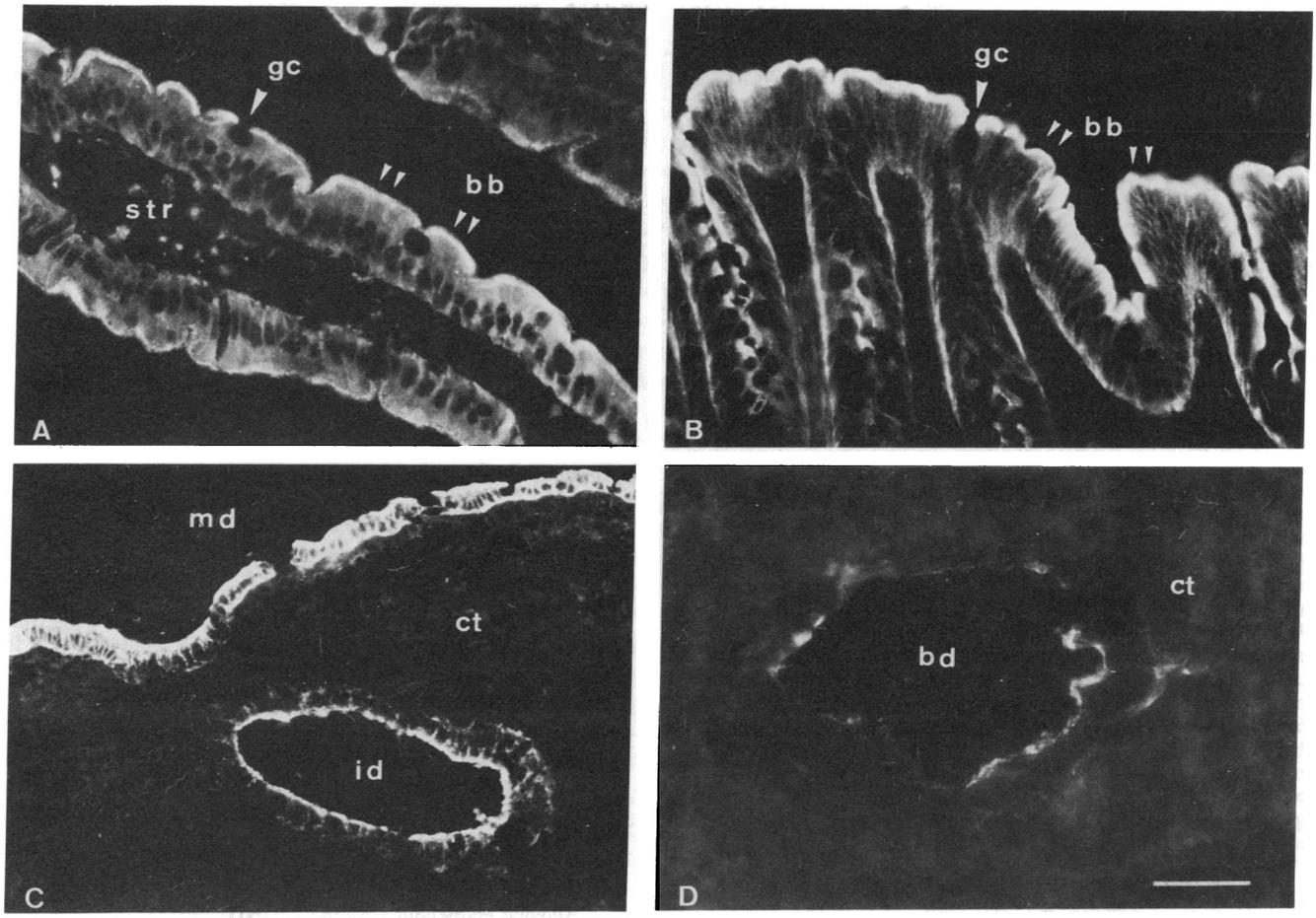

FIG. 1. Villin localization on frozen sections. (*A*) Rat small intestine. (*B*) Rat large intestine. The brush borders (bb) are heavily labeled and a significant diffuse staining is seen in the cytoplasm of the adsorptive cells. By this technique, villin was not detected in goblet cells (gc). The stroma (str) shows a strong autofluorescence presumably due to histamine (26). Tissue was fixed by the paraformaldehyde/lysine/periodate procedure (24). (*C*) Human pancreas. Villin could be observed only in cells from the main duct (md) or from interlobular ducts (id). It was absent in connective tissue (ct). (*D*) Human liver. Large bile ducts (bd) contained villin. Liver connective tissue (ct) were negative. Cells were fixed with cold (−20°C) acetone. (Bar = 0.5 μm.)

ders by indirect immunofluorescence (Fig. 1*A*). We observed a similar pattern of decoration in cells of the large intestine (Fig. 1*B*). Intestine, when prepared as described above (24), exhibited a diffuse labeling in the cytoplasm, but when the technique of Bretscher *et al.* (7) was used, this diffuse labeling was not seen. Other cells of the simple epithelia of the human digestive tract were also found to contain villin. Labeling was observed on the apical side of cells in the main and interlobular ducts of the pancreas (Fig. 1*C*), but no significant labeling of the acini was observed. Anti-villin antisera decorated the large bile ducts (Fig. 1*D*) whereas small bile ducts and bile canaliculi were usually not labeled.

By immunoreplica techniques, large amounts of villin were found in cells of the large and small intestines (Fig. 2, lanes 2b and 3). By quantitative analysis (27), an equivalent amount of villin was detected in both tissues. In extracts of stomach mucosa (Fig. 2, lane 4) villin could not be detected. Negative results (i.e., less than 5 ng of villin in 200 μg of cell protein) were also obtained in other tissues containing epithelial cells such as lung, skin, ovary, bladder, and endometrium. Our results and those of others (Table 1) demonstrate that villin can be detected either by immunofluorescence or by immunoreplica procedures in simple epithelia of the gastrointestinal and urogenital tracts.

Villin Expression in Cell Cultures. We investigated the presence of villin in an established intestinal cell line, Caco-2, which was derived from a human colonic adenocarcinoma and has been shown to express high levels of digestive enzymes (e.g., alkaline phosphatase, aminopeptidase, and sucrase). In addition, electron microscopy studies have shown a brush border at the upper cell surface (11). As shown in Fig. 2 (lane 6), Caco-2 cells expressed a high level of villin (about 0.1% of total protein as measured by an ELISA developed in our laboratory).

The relationship between terminal differentiation and villin expression in cells derived from intestinal epithelium was further studied by using a subclone of the cell line HT-29 (9), isolated in our laboratory. In this cell line, the control of enterocytic differentiation is under nutritional control. When grown in standard culture medium, Dulbeccos–Vogt modified Eagle's medium containing 25 mM glucose, cells remain undifferentiated. However, when glucose is removed or replaced by 5 mM galactose, these cells become polarized and express an enterocyte-like phenotype (10, 29). Fig. 2 (lanes 7 and 8) shows that villin was detected in differentiated and undifferentiated HT-29 cells, although the undifferentiated HT-29 cells contain only about 10% of the villin found in differentiated cells. Morphological studies of the cells grown in galactose (5 mM) or in the absence of hexose have shown a well-defined brush border; each microvillus contains a core bundle of microfilaments that extends into the cytoplasm (10). Ultrastructural examination of the cells grown in glucose shows that the cells remain undifferentiated; microvilli occur irregularly and are not organized into a brush

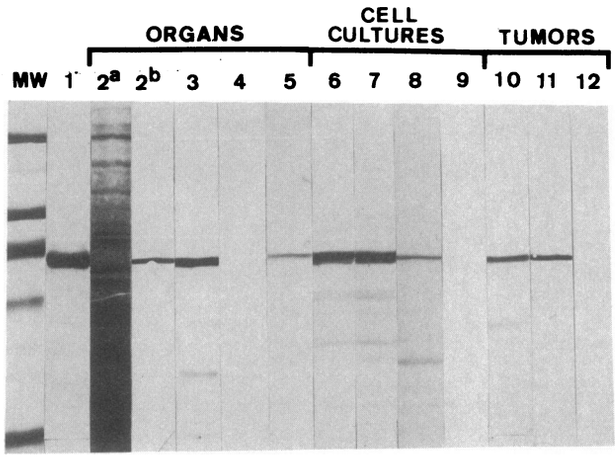

FIG. 2. Immunodetection of villin. Lanes: 1, purified pig villin (Coomassie blue); 2a, protein pattern of human colon mucosal extract (Coomassie blue). Lanes 2b–12, villin revealed after immunoreplica with anti-villin antiserum and peroxidase-conjugated Fab fragment of sheep anti-rabbit IgG (both at 20 μg/ml)—2b, extracts from adult human colon; 3, human small intestine; 4, rat stomach; 5, intestinal tube of an 8-week-old human fetus; 6, Caco-2; 7, differentiated HT-29 cells grown in 5 mM galactose medium; 8, undifferentiated HT-29 grown in 25 mM glucose medium; 9, Hela cells; 10, human colonic primary tumor; 11, a tumor induced in nude mice with an SW480 cell line; 12, a human epidermoid oesophageal carcinoma. The total amount of protein used in each experiment was 100–200 μg. In these experimental conditions, the intensity of the reaction can be correlated to the extent of expression of villin in the extract. Molecular size markers (MW): myosin (180 kDa), β-galactosidase (116 kDa), phosphorylase b (92 kDa), bovine serum albumin (68 kDa), and actin (43 kDa).

border (ref. 10 and unpublished results). Furthermore, sucrase is not detected (10). Therefore, our results show that morphologically undifferentiated HT-29 cells produce a significant amount of villin, even before they construct a typical brush border.

The presence of villin in extracts of established cell lines derived from kidney epithelia was also tested. Cells that exhibited characteristic enzymatic markers or transport activities corresponding to the proximal tubule (LLC-PK_1) (12, 13) or the distal tubule (MDCK) (14) were chosen. The presence of a low but measurable amount of villin in LLC-PK_1 cells and its absence in MDCK cells are consistent with the origin of these cells. We have summarized results obtained by using established cell lines of various origins (Table 2). Negative results were consistently obtained when the cells tested were not of intestinal or renal epithelial origin.

Villin Expression in Undifferentiated Intestinal Cells. To determine if villin is generally found in undifferentiated cells capable of differentiation into cells with a well-organized brush border, we studied villin expression along the crypt/villus axis in adult intestine. Frozen sections of rat small intestine cut along the crypt/villus axis showed labeling of epithelial cells but not the underlying mucosa. Mature enterocytes displayed strong apical decoration (the brush border) as well as diffuse cytoplasmic labeling (Fig. 3*B*). In the undifferentiated cells of the crypts, villin primarily was located near the apical plasma membrane; additionally, weak, diffuse labeling was seen in the cytoplasm. The existence of a gradient of villin expression along the crypt/villus axis was shown by using the quantitative immunoreplica procedure (27). Populations of cells enriched in differentiated cells (tip of the villi) or in undifferentiated cells (crypts) were separated. Differentiated cells at the tips of the villi (25% of the isolated cells) were characterized by a high level of alkaline phosphatase, a common marker for enterocytes. Crypt cells (25% of the isolated cells) were characterized by a high level of [^{3}H]thymidine incorporation, a marker for proliferative cells. The amount of villin in mature enterocytes was at least 10-fold higher than the amount in immature crypt cells (Fig. 3*A*).

We also studied the expression of villin during embryonic development. Villin was detected in a cell extract prepared from the intestinal tube of an 8-week-old human fetus (Fig. 2, lane 5). At this stage of development, the organization and differentiation of intestinal tissue has just been initiated (30). Between 9 and 11 weeks of gestation, many epithelial cells begin to exhibit microvilli at their apical membranes and at 16 weeks of gestation they display a differentiated brush border (31). In a 16-week-old fetus, villin was found in the small and the large intestine (data not shown). These results demonstrate the specificity of villin expression *in vivo* in a few structurally and/or embryologically related epithelia. In

Table 1. Villin expression in epithelial tissues

Tissue	Immunochemical detection	Immunolocalization
Gastrointestinal tract		
Small intestine	+ (7)	+ (7, 8)
Large intestine	+	+
Gall bladder	ND	+
Pancreas		
Duct	ND	+
Acini	ND	–
Tissue homogenate	+ (28)	
Liver		
Large bile duct	ND	+
Hepatocytes	ND	–
Tissue homogenate*	–	
Stomach	–	–
Oesophagus	ND	–
Urogenital tract		
Kidney		
Proximal tubule	ND	+ (7)
Distal tubule	ND	–
Cortex homogenate	+	
Epididymis†		
Ductus epididymidis	ND	–
Ductuli efferentes	ND	+
Tissue homogenate	–	
Testis	–	–
Oviduct	–	–
Uterus endometrium	–	–
Bladder urothelium	ND	–
Prostate glands	–	–
Other tissues		
Lung		
Bronchial epithelium	ND	–
Alveolar epithelium	ND	–
Tissue homogenate	–	
Skin		
Epidermis	ND	ND
Dermis	ND	ND
Sweat glands	ND	–
Tissue homogenate	–	
Chloroid plexus	ND	–

These results were obtained by two different methodologies: (*i*) Immunolocalization of villin by immunofluorescence techniques [results from the literature (7, 8) and our observations] and (*ii*) immunodetection of villin by using immunoreplica procedures [data from the literature (refs. 7, 8, and 28) and from our study]. ND: not determined.

*The small number of positive cells in the liver do not allow immunochemical detection of villin in an extract of this organ.

†Ductus epididymidis does not have a brush border; ductuli efferentes does.

Table 2. Villin expression in established cell lines

Cell line	Immunochemical detection	Immunolocalization
From the large intestine		
Caco-2 (human colonic adenocarcinoma)	+	+
HT-29 (human colonic adenocarcinoma)	+	+
From kidney		
LLC-PK_1 (derived from pig kidney proximal tubule)	+	ND
MDCK (derived from dog kidney distal tubule)	−	−
From other origins		
HEp-2 (human esophagus)	−	ND
Hela (human cervix)	−	−
Chicken embryo fibroblasts	−	−
3T3 (murine fibroblasts)	ND	−
Pt K2 (rat kangoroo epithelial cell)	−	−

See legend to Table 1. ND: not determined.

addition, our results emphasize the early expression of villin during development.

Villin Expression in Malignant Tissues of Human Intestinal Origin. To determine whether the specific tissue expression of villin also extends to malignant tissues of gastrointestinal origin, we investigated its occurrence in various tumors. Eleven of the 12 human colonic adenocarcinomas tested were found to express villin (see Fig. 2, lane 10), whereas villin was not detected in tumors of other origins, such as an epidermoid esophageal carcinoma (Fig. 2, lane 12), a mesenteric node lymphoma, a kidney carcinoma, and an adenocarcinoma of unknown origin metastasized in spleen (data not shown). This suggests that the tissue-specific expression of villin is maintained in primary human colonic tumors. In addition, we screened tumors in nude mice for villin expression after the mouse was injected with one of five different cell lines of colonic origin. These tumors were selected for the presence (HT-29 and Caco-2) or absence (HRT18, HCT8R, and SW480) of an enterocyte-like pattern of enzymatic differentiation (e.g., sucrase expression) (15). Villin was detected in all five tumors. The amount of villin was apparently similar from one tumor to another and was independent not only of the pattern of enterocytic differentiation (e.g., digestive enzymes) but also of the degree of ultrastructural expression of brush border microvilli, which are numerous in Caco-2 and HT-29 tumors (15) but very scarce in the other three tumors (unpublished results). These results show that the maintenance of villin expression during tumorigenesis is independent of morphological differentiation or enzymatic expression.

DISCUSSION

In this paper, we have substantiated data showing the presence of villin in brush borders. It is also of note that villin is used to construct the microvillar microfilaments both in enterocytes and the epithelia of the kidney proximal tubule, cells which have a different embryological origin. Further-

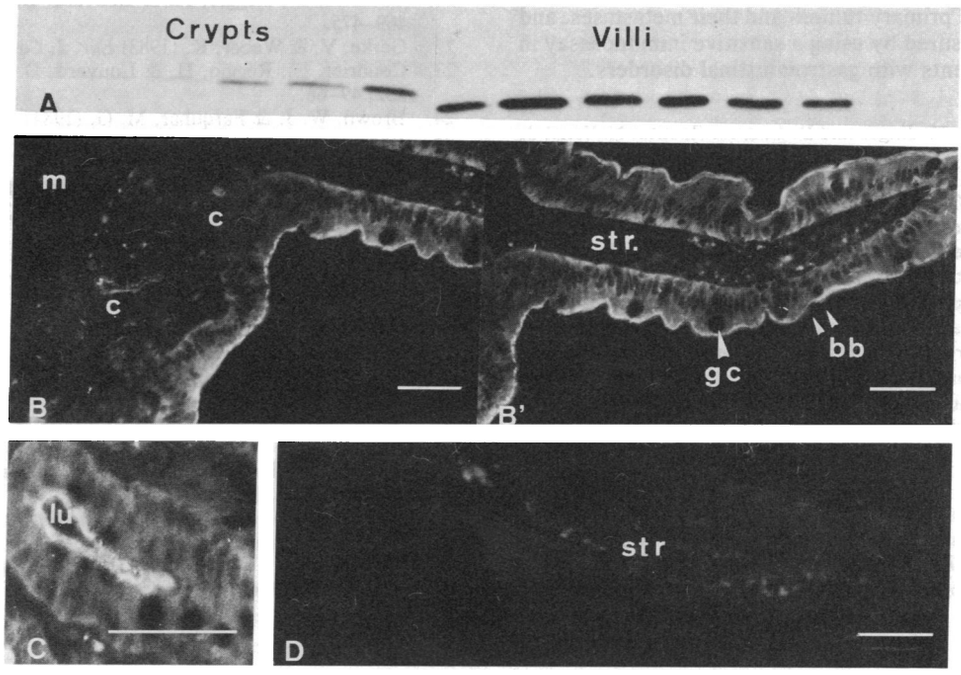

FIG. 3. Distribution of villin along the crypt/villus axis in rat small intestinal mucosa. (*A*) Autoradiograms of immunoreplicas using protein extracts from cell populations isolated stepwise from the tip of the villi to the crypts. Immunodetection of villin was with rabbit anti-villin antiserum and I^{125}-labeled protein A (6×10^5 cpm/ml). Radioactivity was quantitated according to Howe and Hershey (27). The third fraction from the right (the villi), contains 16,340 cpm whereas the ninth fraction from the right (crypt cells) contains 1950 cpm. (*B* and *B'*) Two adjacent fields of an axial section of the mucosa from the tip of the villus to the crypt zone (c) and muscular layers (m). The brush borders (bb) of the absorptive cells are intensely labeled and their cytoplasm shows a lower, diffuse staining. The goblet cells (gc) are negative, whereas the stroma (str) of the villus shows some autofluorescence (see *D*). In the crypt area (c), the apical pole of the immature cells is also labeled. (Bars = 50 μm.) (*C*) High magnification of a longitudinal section of a crypt. The apex, facing the lumen (lu) of the immature cells, is labeled. The cytoplasm shows a very faint diffuse staining. (*D*) Control. Tissue sections were incubated with preabsorbed villin antiserum. Antiserum (400 μl, diluted 1:800) was incubated with 10 μl of villin (10 mg/ml) for 3 hr at room temperature. Samples were processed as in *B*. Only autofluorescent spots are seen in the stroma (str). The same pattern is seen when the specimen is observed using a fluorescein filter set, indicating an endogenous autofluorescence of cells within the stroma (see Fig. 1).

more, our study suggests that in a given cell type the presence of villin may not necessarily be correlated with the existence of an organized brush border.

We report here the expression of villin in the undifferentiated dividing cells of the adult intestinal mucosa and of embryonic intestine. Also, well-differentiated epithelial cells lining the pancreatic and liver ducts, which lack brush borders, have significant amounts of villin localized at their apical borders. These simple epithelia and intestinal epithelia with a brush border are derived from a common epithelial sheet, the gut, during embryogenesis (32). In the simple epithelia, the presence of villin is indicative of the cell's origin rather than of the existence of a cellular structure.

Our observations emphasize that villin is found in some simple epithelia and that its distribution is restricted to organs of the gastrointestinal and urogenital tracts. However, the absence of villin in stomach mucosa and especially in its columnar cells is surprising. It is also striking to notice that exocrine glandular cells found in these organs do not express significant amounts of villin. In this respect our results are not in agreement with those reported by Drenckhahn and Mannherz (28) who have observed, by using similar approaches, villin in pancreatic and prostatic acini.

In conclusion, our observations demonstrate the utility of villin as a marker for undifferentiated enterocytes in tissue culture and *in vivo*. In contrast to differentiated enterocytes, no well-characterized protein(s) marker has been identified for crypt cells of the intestinal mucosa. Villin would appear also to be a useful marker in establishing, without reference to morphological criteria, the origin of malignant tumors such as colonic tumors [one of the most frequent cancers in Western countries (33)] or tumors arising from other villin-containing epithelia. Villin antibodies may be used clinically to classify colonic primary tumors and their metastases, and villin may be measured by using a sensitive immunoassay in the serum of patients with gastrointestinal disorders.

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