Enterophilin-1 Interacts with Focal Adhesion Kinase and Decreases β₁ Integrins in Intestinal Caco-2 Cells*

Véronique Pons‡, Christine Pérès, Jeanne-Marie Teulié, Michel Nauze, Marianne Mus, Corinne Rolland, Xavier Collet, Bertrand Perret, Ama Gassama-Diagne, and Françoise Hullin-Matsuda§

From the Institut Fédératif de Recherche Claude de Préval, IFR30, INSERM Unité 563, Département Lipoprotéines et Médiateurs Lipidiques, Hôpital Purpan, 31059 Toulouse Cedex, France

Intestinal cell growth and differentiation are tightly regulated by growth factors and extracellular matrix components along the crypt-villus axis. We previously described enterophilin-1 (Ent-1) as a new intestinal protein associated with growth arrest and enterocyte differentiation. Ent-1 interacted with sorting nexin 1 and decreased cell surface epidermal growth factor receptor. Because β₁ integrins are mostly found in vivo in the proliferative crypt cells, we investigated the role of Ent-1 in the fate of β₁ integrin subunits. In undifferentiated intestinal Caco-2 cells, overexpression of Ent-1 induces a marked decrease of α₂β₁ integrin pools, whereas αoβ₁ integrin is weakly affected. Conversely, overexpression of sorting nexin 1 has no effect on integrin levels despite its ability to interact with Ent-1. Interestingly, we identified focal adhesion kinase as a new Ent-1 partner using yeast two-hybrid screening and co-immunoprecipitation experiments. Furthermore by confocal microscopy, we observed that Ent-1 and β₁ integrins partly co-localize on vesicular structures, suggesting a role for Ent-1 in integrin trafficking. Because focal adhesion kinase is able to bind both Ent-1 and β₁ integrins, the kinase might act as a molecular bridge between the two proteins. Altogether, these results support a role of Ent-1 in regulating β₁ integrin expression that could favor intestinal differentiation.

The intestinal epithelium undergoes continuous and rapid renewal, with proliferation of the multipotent stem cells limited to the crypts of Lieberkühn. At the top of the crypt, the cells lose their proliferative ability and complete differentiation during a highly organized migration on the basement membrane, a specialized extracellular matrix consisting of a complex association of various molecules, including collagen IV, laminin, and fibronectin (1). At the tip of the villus, terminally differentiated cells are extruded to the lumen (2–4). Intestinal epithelial cell differentiation requires two major events: the transition from stem cells to committed proliferative cells and the irreversible loss of proliferative potential as the committed cells start to differentiate. However, the cellular and molecular mechanisms responsible for the fine coordination between proliferation, migration, and differentiation along the crypt-villus axis are still largely unknown.

The regulation of intestinal cell growth and differentiation is a multifactorial process, susceptible to various influences along the crypt-villus axis such as growth factors and basement membrane components. Indeed, in vivo and in vitro studies indicate that the expression of cell surface epidermal growth factor receptor (EGFR) dramatically decreases during differentiation (5, 6). Additionally, several studies have provided evidence for an important role for integrins during this process. Integrins are a family of heterodimeric cell surface adhesion receptors consisting of α and β subunits and mediating cell-extracellular matrix interactions (for a review see Ref. 7). In epithelia, such interactions are mainly achieved by integrins belonging to the β₁ and β₂ classes. Interestingly, basement membrane components and β integrins display a differential distribution along the crypt-villus axis. Briefly, fibronectin is abundant in the proliferative crypt compartment, whereas laminins are enriched at the villus tip (8, 9). Similarly, β₁ and β₂ integrins show differential patterns of expression in concert to the distribution of their respective ligands. α₁β₂, α₂β₂, and αoβ₁ integrins are expressed in proliferative crypt cells and decrease in differentiated villus cells. In fact, β₁ integrin classes are largely involved in the control of cell proliferation and particularly the specific fibronectin receptor, α₁β₂ integrin, because of its link to the Ras-mitogen-activated protein kinase signaling pathway (10). Conversely, αoβ₁, αoβ₂, and αoβ₄ integrins appear to be related to the process of intestinal cell differentiation (11, 12). Nevertheless, the underlying mechanisms of such basement membrane and integrin compositional changes remain to be precisely defined.

We have previously cloned and characterized a new family of intestinal proteins named enterophilins (13). We have shown that enterophilin-1 (Ent-1) expression was positively correlated with growth arrest and terminal differentiation program in human colon adenocarcinoma cells. Furthermore, overexpression of Ent-1 inhibited cell growth and promoted intestinal differentiation (13). Using a yeast two-hybrid screen, we recently identified the sorting nexin 1 (SNX1) as an Ent-1-binding partner (14). The SNXs are an emerging family of proteins

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§ To whom correspondence should be addressed: Present address: RIKEN Frontier Research System, Supra-Biomolecular System Research Group, Sphingolipid Functions Laboratory, 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan. Tel.: 81-48-467-9628; Fax: 81-48-467-9626; E-mail: hullin-matsuda@riken.jp.

1 The abbreviations used are: EGFR, epidermal growth factor receptor; Ent-1, enterophilin-1; GFP, green fluorescent protein; RPE, R-phycocerythrin; SNX, sorting nexin; FAK, focal adhesion kinase; HNF, hepatic nuclear factor; PBS, phosphate-buffered saline; FAT, focal adhesion targeting signal.

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Enterophilin-1, β1 Integrins, Intestinal Differentiation, and FAK

Plasmids—Full-length Ent-1 cDNA was cloned in pEGFP-C2 (Clontech Laboratories) or pcDNA3/Myc-His (Invitrogen) plasmid to encode a N-terminally GFP-tagged protein or a C-terminally Myc-tagged protein, respectively, as previously described (13, 14). The C-terminal GFP-tagged SNX1 (pEGFP-C1-SNX1) was generously provided by Gordon N. Gill (University of California at San Diego, La Jolla, CA).

Yeast Two-hybrid Screening—First, full-length Ent-1 cDNA containing vector pAS2-1 (Clontech Laboratories) was used as a bait to screen a HaLa cell cDNA library constructed in pGAD GH vectors at EcoRI-XhoI restriction sites (Clontech Laboratories) as already described (14). To pinpoint the interacting domain of Ent-1, the leucine zipper region or the B30.2 domain were used in a one to one interaction test with recombinant proteins from Saccharomyces cerevisiae (25).

Immunoprecipitation—Caco-2 cells were transfected with pcDNA3/Myc-His-Ent-1 for 48 h. All of the primers were designed using Primer Express™ software (Applied Biosystems), and 100 ng of the reverse transcribed cDNA were used for each PCR with 300 ng of the human β1 integrin specific primers (forward, 5′-TGGCGGTTTACCTTGTG-3′, bases 940–957; reverse, 5′-GTGACATCTGCATCATTTGAAA-3′, bases 985–1009 (19)). The ampiclon was 70 bp and was in the β3 coding region of the human β1A integrin cDNA (20). To normalize the difference in loading amounts, haematopoietic nuclear factor 4α (HNF4α), which is expressed along the entire length of the crypt-villus axis (21), was used at 100 ng (forward primer, 5′-GAGGAAAGCTGGGGTGCTCTGCT-3′; reverse primer, 5′-CATCTCGAGAGGCTCCCTGCT-3′). The PCR program with specific primers was as follows: 50 °C for 2 min, then 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The threshold cycle (Ct) values were obtained for the reaction by determining the quantity of the template in the samples. β1 integrin ΔCt was calculated by subtracting the HNF4α Ct value from the β1 integrin Ct value and thus represented the relative quantity of the target molecule after normalizing with the internal standard HNF4α. The β1 integrin ΔCt values of Caco-2 cells transfected with pEGFP-C2-Ent-1 or pEGFP-C1-SNX1 were expressed as percentages of β1 integrin ΔCt values of GFP control cells.

Cell Culture and Transfection—Human colon carcinoma Caco-2 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0.1% nonessential amino acids (Invitrogen), and 100 μg/ml penicillin/streptomycin (Invitrogen). The cell monolayer was exposed to a humidified atmosphere containing 5% CO2. They were transfected with a cationic lipid (LipofectAMINE; Invitrogen) according to the manufacturer’s protocols.

Confluence-induced Differentiation of Caco-2 Cells—Caco-2 cells were seeded at 18 000 cells/cm2 and grown in 60-mm dishes in the complete medium. The cells were washed twice in phosphate-buffered saline (PBS) and scraped at 4 °C in PBS containing protease and phosphatase inhibitors (1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 2 mM NaF, 2 mM Na3VO4) at various times up to 23 days after plating. The samples were sonicated, and the protein concentration was determined according to the method of Bradford (22).

Immunoprecipitation Experiments—Caco-2 cells were transfected with pcDNA3/Myc-His-Ent-1 for 48 h. All of the primers were done at 4 °C. The cells were washed twice with PBS, harvested, and then lysed for 30 min in lysis buffer containing 0.1% Nonidet P-40, 10% glycerol) containing protease and phosphatase inhibitors as described above. After sonication, insoluble debris was removed by centrifugation at 14,000 × g for 15 min, and the supernatant was subjected to a preclearing with 30 μl of protein G-Sepharose (Amersham Biosciences) for 30 min. Ent-1 was immunoprecipitated with 5 μg of monoclonal anti-Myc antibody, followed by 50 μl of protein G-Sepharose for 1 h. After two washes in lysis buffer containing 0.1% Nonidet P-40, protease, and phosphatase inhibitors, followed by one wash in lysis buffer without detergent, the bound proteins were eluted by boiling the beads in Laemmli buffer (23). The precipitated proteins were then submitted to SDS-polyacrylamide gel electrophoresis and detected by immunoblotting.
Enterophilin-1, β₁ Integrins, Intestinal Differentiation, and FAK

Fig. 1. Ent-1 expression correlates with the decrease of β₁ integrin expression during Caco-2 cell spontaneous differentiation. Caco-2 cells were grown in 60-mm dishes and harvested by scraping at various times up to 23 days after plating. A, alkaline phosphatase activity was determined at the indicated times as described under “Experimental Procedures.” The data are expressed as IU (μmoles of substrate hydrolyzed per min/gram of proteins. B, equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Ent-1 was detected with rabbit polyclonal anti-Ent-1 peptide antibody and β₁ integrin with rabbit polyclonal anti-β₁ integrin antibody (anti-cyto-β₁) as described under “Experimental Procedures.”

RESULTS

Enterophilin-1 Expression Is Correlated to the Decrease of β₁ Integrins during Confluence-induced Differentiation of Caco-2 Cells—Because β₁ integrin expression was described to decrease in vivo during intestinal differentiation along the crypt-villus axis, we first investigated the β₁ integrin variations in human colon carcinoma Caco-2 cells. These cells have proven to be the most useful in vitro model because of their unique ability to spontaneously differentiate at confluence, displaying morphological and functional characteristics of enterocytes (26).

We thus analyzed the β₁ integrin expression pattern during the confluence-induced differentiation of Caco-2 cells. After 7 days, the cells reached confluence and began to express the intestinal differentiation marker, alkaline phosphatase (Fig. 1A). As expected, Western blot analysis showed a marked decrease of β₁ integrin subunits at day 14 (Fig. 1B), which correlated with the appearance of the 65-kDa Ent-1 protein. As previously reported (13, 14), the human ortholog of Ent-1 was weakly expressed at day 9, with a high level of expression from days 12 to 23 of culture. Interestingly, we had previously observed a similar correlation between Ent-1 expression and EGFR decrease during Caco-2 cell differentiation (14).

Enterophilin-1 Overexpression Induces β₁ Integrin Decrease in Intestinal Caco-2 Cells—Undifferentiated Caco-2 cells were transfected with pEGFP-C2-Ent-1, pEGFP-C1-SNX1, or empty vector. Then cell surface β₁ integrin subunits were immunolabeled and specifically measured in the transfected cell population by flow cytometry. Interestingly, after 72 h of transfection, overexpression of Ent-1 induced a significant decrease of ~25% of plasma membrane β₁ integrin subunits, compared with the GFP-transfected cell population used as control (Fig. 2A). Conversely, overexpressed SNX1 was unable to decrease plasma membrane β₁ integrin subunit pool (Fig. 2A). Thus, our results indicate that Ent-1 could down-regulate not only the cell surface EGFR as we previously demonstrated (14) but also cell surface β₁ integrin subunits.

To further document the β₁ integrin diminution, transfected Caco-2 cells were also analyzed by immunoblotting after 72 h of transfection (Fig. 2B). Consistent with the flow cytometry data, we observed that Ent-1 induced a marked decrease of β₁ integrin subunit expression compared with the GFP-transfected control cells, whereas SNX1 did not. Altogether, these results suggest that Ent-1 contributed to the down-regulation of β₁ integrin subunits in intestinal cells.

Enterophilin-1 Induces α₂β₁ and α₅β₁ Integrin Variations in Caco-2 Cells—α₂β₁ integrin is largely involved in the proliferative status of intestinal crypt cells. However, other changes in β₁ integrins such as α₂β₁ integrin were also monitored in vivo during epithelial intestinal differentiation. We thus checked for the effect of Ent-1 on both α₂β₁ and α₅β₁ integrins in Caco-2 cells. We first verified that in the Caco-2 cell model, both integrins displayed the same variations of expression as reported during in vivo intestinal differentiation. Equal amounts of proteins from undifferentiated (day 4) and fully differentiated (day 23) Caco-2 cells were analyzed by immunoblotting with an anti-α₂ or anti-α₅ subunit antibody (Fig. 3A). We thus confirmed that α₂ and α₅ subunits, like the β₁ subunits (Fig. 1B), were highly expressed in undifferentiated proliferative Caco-2 cells, but they were strongly decreased in differentiated cells.

Consequently, we investigated the effect of Ent-1 overexpression on the cell surface integrin pool by flow cytometry using an anti-α₂β₁ or anti-α₅β₁ integrin antibody. Undifferentiated Caco-2 cells were transfected with pEGFP-C2-Ent-1, pEGFP-C1-SNX1, or empty vector for 72 h, and cell surface integrins were immunolabeled and analyzed by flow cytometry as described above. The results showed a significant 30% decrease of the plasma membrane pool of α₂β₁ integrins in Ent-1-trans-
**Enterophilin-1, β1 Integrins, Intestinal Differentiation, and FAK**

9273

![Image](https://example.com/image.png)

**Fig. 2. Ent-1 enhances the decrease of β1 integrin subunits in Caco-2 cells.** A, undifferentiated Caco-2 cells were transfected with pEGFP-C2-Ent-1 (stippled bar), pEGFP-C1-SNX1 (hatched bar), or empty vector (control, black bar) and grown for 72 h. The cells were then labeled with monoclonal anti-β1, integrin (K20) primary antibody and RPE-conjugated secondary antibody. The relative density of cell surface β1 integrin subunit was specifically evaluated in the transfected cell population by flow cytometry. The percentage of cell surface β1 integrin was measured by RPE fluorescence mean and compared with the GFP-transfected cell population used as a control. The values represented the mean and standard error of at least three independent experiments. The results were analyzed by a paired t test and were considered significantly different from control cells (***, p < 0.00005) or nonsignificantly different (ns). B, equal amounts of proteins from transfected cell extracts were separated and analyzed by immunoblotting using rabbit polyclonal anti-β1 integrin antibody (anti-cyo-β1) and monoclonal anti-β1 actin antibody for loading control. Lane 1, empty vector; lane 2, GFP-Ent-1; lane 3, GFP-SNX1.

We previously demonstrated that Ent-1 induced a marked decrease of β1 integrin protein levels (Fig. 2A), we observed a significant reduction (~50%) in the amount of β1 integrin mRNA in Ent-1-transfected Caco-2 cells compared with the control cells after 72 h of transfection (Fig. 4). By contrast, SNX1 overexpression did not diminish β1 integrin mRNA, even inducing a statistically nonsignificant increase (Fig. 4). This is in good agreement with the lack of effect on β1 integrin protein levels (Fig. 2). Altogether, these results suggest that Ent-1 overexpression modifies the expression of β1 integrin both at the protein and mRNA levels.

**FAK Is a New Partner of Enterophilin-1**—Using full-length Ent1 cDNA as a bait in a HeLa cell cDNA library yeast two-hybrid screening, we identified FAK as an Ent-1 partner. Interestingly, the tyrosine kinase FAK is largely involved in integrin signaling pathways, and its N-terminal region is known to bind β-integrin cytoplasmic domains (for reviews see Refs. 7 and 18). Two positive clones encoding FAK were isolated in a first screening (Fig. 5A). We then performed the two-hybrid one to one interaction assay with the leucine zipper part or the B30.2 domain as bait to pinpoint the domain of Ent-1 interacting with FAK. The results showed that the B30.2 domain of Ent-1 was sufficient for interaction with FAK. Furthermore, analysis of FAK clones led us to conclude that the C-terminal focal adhesion targeting signal (FAT) domain is the minimal region of FAK interacting with Ent-1. Interestingly, the FAT domain is necessary and sufficient for recruiting FAK to focal adhesion contact sites and also contains binding sites for a number of signaling molecules and cytoskeletal proteins such as talin and paxillin (27, 28) that can associate with β-integrin cytoplasmic domains. Thus, in addition to SNX1 involved in the plasma membrane receptor trafficking (14), FAK, the major integrin signaling transducing element, is a new partner of Ent-1.

We further investigated the interaction between Ent-1 and FAK by co-immunoprecipitation experiments in intestinal mammalian cells. Caco-2 cells were transfected with pcDNA3/Myc-His-Ent-1 vector, and Ent-1 was immunoprecipitated with an anti-Myc antibody after 48 h of transfection. The results revealed that FAK was co-precipitated with Ent-1, confirming the interaction of these two proteins in intestinal cells (Fig. 5B). Therefore FAK, which is able to bind both Ent-1 and β1 integrins, could act as a molecular bridge between the two proteins.

**Enterophilin-1 Down-regulates β1 Integrin mRNA Expression**—Because Ent-1 induced the down-regulation of β1 integrin mRNA, we investigated whether this was related to changes in β1 integrin gene expression. We thus analyzed β1 integrin mRNA amounts by real time quantitative reverse transcription-PCR. Undifferentiated Caco-2 cells were transfected with pEGFP-C2-Ent-1, pEGFP-C1-SNX1, or empty vector, and β1 integrin mRNA were quantified. Consistent with the decrease of β1 integrin protein levels (Fig. 2A), we observed a significant reduction (~50%) in the amount of β1 integrin mRNA in Ent-1-transfected Caco-2 cells compared with the control cells after 72 h of transfection (Fig. 4). By contrast, SNX1 overexpression did not diminish β1 integrin mRNA, even inducing a statistically nonsignificant increase (Fig. 4). This is in good agreement with the lack of effect on β1 integrin protein levels (Fig. 2). Altogether, these results suggest that Ent-1 overexpression modifies the expression of β1 integrin both at the protein and mRNA levels.

**Enterophilin-1, β1 Integrins, and FAK Partly Co-localize in Caco-2 Cells**—We previously demonstrated that Ent-1 co-localizes with SNX1 on vesicular structures and suggested the involvement of these proteins in the intracellular trafficking of EGFR (14). Because Ent-1 was also able to decrease the cell surface β1 integrin pool and to interact with FAK, we further analyzed by confocal microscopy the cellular distribution of the three proteins in intestinal Caco-2 cells. Each horizontal column of images represents a 0.2-μm optical Z section of the same cells (Fig. 6). As previously observed after 48 h of transfection (13, 14), Ent-1-transfected Caco-2 cells displayed an intracellular punctate distribution (Fig. 6, column A, green). β1 integrin pools showed membrane staining as well as a strong intracellular patched staining (Fig. 6, column B, blue) as already shown in Caco-2 and breast carcinoma cells (29, 30). Interestingly, some of Ent-1-containing vesicles merged with part of the intracellular β1 integrin signal (Fig. 6, column D, white arrowheads), suggesting that Ent-1 could play a role in β1 integrin trafficking in intestinal cells. Furthermore, FAK exhibited typical staining at the cell membrane periphery in addition to adhesion complexes throughout the cell (Fig. 6,
column C, red). We noticed that part of the three protein staining co-located at the cell membrane periphery as well as on intracellular patched structures (Fig. 6, column D, white arrowheads). In fact, Ent-1 and FAK could be both found in the same location, confirming their possible interaction in intestinal cells.

**DISCUSSION**

Little is known regarding the mechanisms involved in the regulation of cell growth and differentiation in the human intestinal epithelium. A key question in intestinal development is what triggers the differentiation process. Determination of integrin expression patterns along the crypt-villus axis has provided valuable information relative to the involvement of each of these extracellular matrix receptors in proliferative or differentiated cell status. Indeed, cell surface $\beta_1$ integrin pools are mainly found in proliferative crypt cells and markedly decrease during enterocyte differentiation (9, 12, 31). Among $\beta_1$ integrin classes, $\alpha_5\beta_1$ integrin, the specific fibronectin receptor, is particularly involved in cell growth by activating mitogen-activated protein kinases (10). Interestingly, we showed that Ent-1 overexpression induced a significant decrease of cell surface $\beta_1$ integrin pools in undifferentiated Caco-2 cells. This was

![Fig. 3.](image)

Ent-1 induces a significant decrease of $\alpha_5\beta_1$ integrin and a slighter diminution of $\alpha_2\beta_1$ integrin in Caco-2 cells. A, equal amounts of proteins from undifferentiated (lanes 1 and 3, day 4 after plating) and differentiated (lanes 2 and 4, day 23 after plating) Caco-2 cells were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. $\alpha_5$ or $\alpha_2$ integrin was detected with monoclonal anti-$\alpha_5$ or anti-$\alpha_2$ integrin antibody. B, nonconfluent Caco-2 cells were transfected with pEGFP-C2-Ent-1 (stippled bar), pEGFP-C1-SNX1 (hatched bar), or empty vector (control, black bar) and grown for 72 h. The cells were then labeled with monoclonal anti-$\alpha_5\beta_1$ integrin (P1D6) or anti-$\alpha_2\beta_1$ integrin (P1E6) primary antibody and RPE-conjugated secondary antibody and then analyzed by flow cytometry. The percentage of cell surface $\alpha_5\beta_1$ or $\alpha_2\beta_1$ integrin in the transfected cell population was measured by RPE fluorescence mean and compared with the GFP-transfected cell population used as control, as explained in Fig. 2. The values represented the means and standard errors of three independent experiments. The results were analyzed by paired t test and were considered significantly different from control cells ($*, p < 0.05$; $**, p < 0.01$) or nonsignificantly different (ns). C, equal amounts of proteins from transfected cell extracts were subjected to SDS-polyacrylamide gel electrophoresis. $\alpha_5$ or $\alpha_2$ integrin subunits were detected with monoclonal anti-$\alpha_5$ or anti-$\alpha_2$ integrin antibody. Lanes 1, 3, and 5, empty vector; lanes 2 and 6, GFP-Ent-1; lanes 4 and 7, GFP-SNX1.
Enterophilin-1, β, Integrins, Intestinal Differentiation, and FAK

**Fig. 4. Ent-1 decreases β1 integrin mRNA expression.** Undifferentiated Caco-2 cells were transfected with pEGFP-C2-Ent-1, pEGFP-C1-SNX1, or empty vector for 72 h, and β1 integrin mRNA expression was analyzed by real-time quantitative reverse transcription-PCR as described under “Experimental Procedures.” The values represent the relative quantity of the β1 integrin mRNA after normalizing with the internal standard HNF4α. The β1 integrin mRNA of Caco-2 cells transfected with pEGFP-C2-Ent-1 (stippled bar) or pEGFP-C1-SNX1 (hatched bar) were expressed as percentages of β1 integrin mRNA of GFP control cells (black bar). The values were analyzed by paired t test and were considered significantly different from control cells (**, p < 0.01) or nonsignificantly different (ns, p > 0.1). The results are the means of triplicate values from three independent experiments.

**Fig. 5. Ent-1 interacts with FAK.** A, the Ent-1 interacting clones isolated from the yeast two-hybrid screen were sequenced, and their predicted amino acid sequences were aligned below the schematic representation of human FAK (Swissprot accession number Q05397). The structure of FAK analyzed with the SMART program (52) includes the N-terminal band 4.1 or FERM domain (residues 206–401) predicted to bind cytoplasmic regions of integrins, the tyrosine kinase domain (residues 422–676) and the C-terminal FAT domain (residues 914–1052) sufficient for targeting to focal adhesions that contains binding sites for cytoskeleton proteins such as paxillin and talin. B, Caco-2 cells were transfected with pcDNA3/Myc-His-Ent-1 vector for 48 h, and cell homogenates (H) were then subjected to an Ent-1 immunoprecipitation (IP) with a monoclonal anti-Myc antibody (+) or without antibody to verify the specificity of the interaction (−). The immunoprecipitates were submitted to SDS-polyacrylamide gel electrophoresis, and FAK and Myc-tagged Ent-1 were detected by immunoblotting (IB) with monoclonal anti-FAK (H-1) and anti-Myc (9E10) antibodies, respectively.

Consistent with the observed correlation between the increased Ent-1 expression and the diminution of β1 integrins during spontaneous Caco-2 cell differentiation. In addition, we observed that the Ent-1-induced cell surface β1 integrin decrease led to a subsequent diminution of β1 integrin mRNA. Thus, Ent-1 will participate in the integrin changes occurring during the intestinal differentiation process by triggering cell surface β1 integrin removal, and also, this will induce the down-regulation of β1 integrin gene expression, thus sustaining the differentiation process as already noticed in Caco-2 cells (29, 32).

Interestingly in human epidermal keratinocytes, commitment to terminal differentiation first results in modulation of pre-existing β1 integrin subunits at the cell surface, and later the receptor is lost from the cell surface and the level of the subunits mRNA declines (33).

Furthermore, investigating the fate of β1 integrin subunits enriched in proliferative crypt cells, we reported a dramatic decrease of α5β1 integrin in Ent-1-transfected undifferentiated cells. Additionally, we observed that overexpressed Ent-1 caused a slight decrease of α2β1 integrin. These data can be correlated with our previously published results demonstrating a role of Ent-1 on cell surface EGFR removal (14).

Growth factor receptors and integrins display not only physical association (34) but also functional cooperation (35). However, the interconnection between growth factor receptor and integrin signaling pathways in the control of cell functions is a complex phenomenon. Although both EGFR and α5β1 integrin can independently stimulate Ras/mitogen-activated protein kinase signaling pathway, an emerging concept suggests a minimum activation threshold to drive cell proliferation that would require input from both EGFR and integrins (36–38). In Caco-2 cells, Kuwada and Li (39) described that α5β1 integrin potentiates the EGFR-dependent cell proliferation. Additionally, α2β1 integrin, which is proposed to be a collagen receptor, has also been reported to function in EGFR activation (35, 40). The extensive cross-talk occurring between integrins and growth factor receptors is particularly relevant in intestinal dynamic processes. In this context, the Ent-1-induced decrease of EGFR as well as α5β1 and α2β1 integrins could be functionally important in the early stages of growth arrest leading to enterocyte differentiation. This is particularly interesting because in vivo Ent-1 expression at the mid-villus could be related to the reported decrease of EGFR and β1 integrins (5, 9). Furthermore, we previously showed a synergistic effect of Ent-1 and SNX1 on EGFR cell surface removal. Conversely, we found that SNX1 had effect on neither α5β1 nor α2β1 integrin expression. We hypothesized that Ent-1 could interact with another SNX protein or another yet unidentified Ent-1 partner to mediate β1 integrin variations.

Moreover, seeking for Ent-1 cellular partners to precisely determine the molecular mechanisms involved in Ent-1-induced cell surface β1 integrin removal, we demonstrated that Ent-1 interacted with FAK. This interaction required the C-terminal B30.2 domain of Ent-1 and the C-terminal FAT region of FAK. The B30.2 domain, which characterizes the B30.2 protein family, is found in proteins involved in oncogenesis, development, and differentiation processes (for a review see Ref. 41). The physiological importance of the B30.2 domain has been revealed by the discovery of two genetic diseases where mutations in the B30.2 domain have been correlated with the disease development (42–44). However, the exact molecular function of the B30.2 domain remains to be defined. Thus, identification of FAK as a new binding partner of this domain...
will open perspectives to precisely define the B30.2 domain function and then the role of proteins belonging to the B30.2 domain family of proteins.

It is well documented that FAK plays important functions in integrin-initiated signaling events (for reviews see Refs. 7 and 18). The FAT domain of FAK is necessary and sufficient for recruiting the kinase to focal adhesion contact sites. It contains binding sites for cytoskeletal proteins such as talin and paxillin (27, 28) that can associate with β-integrin cytoplasmic domains (45, 46). In addition, the group of Schlaepfer (47) has recently demonstrated that the N-terminal domain of FAK can associate, through indirect interaction, with an activated EGFR complex, emphasizing the critical position of FAK as a connecting component to both integrin and growth factor receptors. Interestingly, the authors suggested that such interaction may be mediated by one or more intermediary bridging proteins. Therefore FAK, which is able to bind both Ent-1 and β1 integrins, could act as a common and important connection between the two proteins. Furthermore, identification of both SNX1 and FAK as Ent-1 partners indicates that Ent-1 could be part of a multiprotein complex involved in the cross-talk between integrins and growth factor signaling pathways.

In addition, by confocal microscopy, we observed a partial co-localization of Ent-1 and β1 integrin subunits on vesicular structures, suggesting that Ent-1 could regulate the integrin trafficking. Indeed, we previously reported that Ent-1 and SNX1 co-localized on vesicular and tubulovesicular structures, which were different from early endosome antigen 1-containing endosomes (14). However, more studies are needed to determine the identity of the transport vesicles carrying Ent-1 and β1 integrins as well as its association with the other endocytic markers.

The exact mechanisms regulating the intracellular integrin trafficking are still poorly understood. It has been postulated that cell migration is favored by internalizing integrins at the rear of the cells and transporting them forward within vesicles at the leading edge to form new contacts with the extracellular matrix (48). However, those studies were mainly done with migrating fibroblasts that behave differently than polarized epithelial cells. In fact during their differentiation process and their cell life, the intestinal cells are continuously moving in coherent sheets along the villus, each cell tightly linked to its neighbors via lateral adhesive complexes. The Ent-1-induced β1 integrin removal from the cell surface of intestinal Caco-2 cells will contribute to the rapid switch in the integrin repertoire that accompanies intestinal differentiation and allows cell migration along the crypt-villus axis (9, 49). Thus, our study is providing new insight into the β1 integrin trafficking in polarized intestinal cells.

To summarize, the Ent-1-induced-β1 integrin decrease in intestinal cells adds to our understanding of the role of Ent-1 in the regulation of growth arrest in intestinal epithelium that we previously reported (13, 14). Moreover, early changes in α5β1 and α3β1 integrin variations have a decisive impact on the behavior of committed crypt cells toward terminal differentiation. Alterations in controlled integrin expression could be a crucial step in colorectal tumorigenesis, as previously reported in epidermal squamous cell carcinoma (50) and in breast cancer (51). Because we previously reported that Ent-1 in cooperation with its binding partner, SNX1, was able to regulate EGFR vesicular trafficking (14), we can hypothesize that Ent-1 may similarly link integrin-containing vesicles to molecular motors involved in vesicle transport along the cytoskeleton network. The next challenge will be to explore the molecular mechanisms involving Ent-1 in both EGFR and integrin variations during intestinal cell differentiation.

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