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RhoA-dependent Switch between α2β1 and α3β1 Integrins Is Induced by Laminin-5 during Early Stage of HT-29 Cell Differentiation

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Integrin-mediated interactions between the basement membrane and epithelial cells control the differentiation of epithelia. We characterized the modulation of adhesive behaviors to basement membrane proteins and of integrin function in the human colon adenocarcinoma HT-29 cell line, which differentiates into enterocytes after the substitution of galactose for glucose in the medium. We demonstrate an increased capability of these cells to adhere to collagen type IV during the early stage of differentiation. This effect occurs without any changes in integrin cell surface expression but rather results from an α2β1/α3β1 integrin switch, α3β1 integrin becoming the major collagen receptor. The increase in laminin-5 secretion and deposit on the matrix is a key factor in the mechanism regulating cell adhesion, because it is responsible for the activation of α3β1 integrin. Furthermore, down-regulation of RhoA GTPase activity occurs during HT-29 cell differentiation and correlates with the activation of the integrin α3β1. Indeed, C3 transferase, a RhoA GTPase inhibitor, induces a similar α2β1/α3β1 switch in undifferentiated HT-29 cells. These results indicate that the decrease in RhoA activation is the biochemical mechanism underlying this integrin switch observed during cell differentiation. The physiological relevance of such modulation of integrin activity in the functioning of the crypt-villus axis is discussed.

INTRODUCTION

Epithelial cells are characterized by particular structural features, including polarized morphology and specialized cell-cell contacts. They lie on a basement membrane, which is organized into a complex structure containing collagen type IV (CO IV), various laminin isoforms, and proteoglycans (Simon-Assmann *et al*., 1995; Aumailley and Krieg, 1996; Beaulieu 1997). Interactions between cells and this specialized extracellular matrix are crucial for essential biological processes such as migration, proliferation, differentiation, and cell survival. The cell–cell or cell–extracellular matrix interactions are mediated through various transmembrane receptors, which are linked intracellularly to cytoskeleton components and signal transduction molecules (Miyamoto *et al.*, 1995; Schwartz *et al.*, 1995). The first receptor family identified was the integrin family (Ruoslahti, 1991; Hynes, 1992). Integrins are heterodimeric transmembrane glycoproteins composed of an α- and a β-subunit. The nature of both the α- and the β-subunit in the heterodimers determines its extracellular matrix ligand specificity.

Laminins include a family of T-shaped heterotrimeric molecules composed of an α, β, and γ chain. At least 12 different isoforms of laminin have been described. These different isoforms can trigger distinct cell responses (Vachon and Beaulieu, 1995; Baker *et al.*, 1996; De Arcangelis *et al.*, 1996; Green and Jones, 1996; Lampe *et al.*, 1998). Epithelial cells adhere to laminin-5 (LN 5) of the basement membrane via at least two adhesive structures, focal adhesions and hemidesmosomes, that involve α3β1 and α6β4 integrins, respectively (Carter *et al.*, 1990a; DiPersio *et al.*, 1995; Dogic *et al.*, 1998). These differences in cellular localization also reflect differences in adhesion-related functions and activation of distinct signal transduction pathways (Carter *et al.*, 1990b; Jewell *et al.*, 1995; Mainiero *et al.*, 1995; Xia *et al.*, 1996). Not only is the integrin α3β1 a receptor for LN 5, but also it recognizes a variety of other ligands with a lower affinity, including fibronectin, CO IV, entactin/nidogen, and thrombospondin (Wayner and Carter, 1987; Elices *et al.*, 1991;

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Abbreviations used: CO IV, collagen type IV; LN 5, laminin-5.

Dedhar *et al.*, 1992; Kühn and Eble 1994; DeFreitas *et al.*, 1995). The existence of different binding sites on the $\alpha3\beta1$ integrin and of different isoforms of $\alpha3$ (Tamura *et al.*, 1991) could explain the broad binding specificities of the $\alpha3\beta1$ integrin. The physiological significance of these weaker interactions is poorly understood, nevertheless they may reflect different adhesion-related functions, themselves correlated with distinct subcellular localizations. The $\alpha3\beta1$ integrins can be targeted to cell-cell junctions, where they may have a role in maintaining cell-cell adhesion or in promoting gap junctions (Carter *et al.*, 1990b; Lampe *et al.*, 1998). Otherwise, this integrin is found along the basolateral membrane of many epithelial cell types, suggesting that it functions as a basement membrane receptor. Interestingly, in keratinocytes the integrin $\alpha3\beta1$ is not detectable in focal adhesions on LN 5 (DiPersio *et al.*, 1995). Formation of focal adhesions and the closely associated actin stress fibers requires the activation of the small GTP-binding protein RhoA (Ridley and Hall, 1992). RhoA is a member of the Ras superfamily, which cycles between a GDP-bound inactive state and a GTP-bound active state. This suggests that $\alpha3\beta1$-mediated interactions may differ from other integrins by signaling through RhoA. However, studies of cells and tissues deficient in $\alpha3\beta1$ indicate a more complex role of this integrin in modulating adhesion, migration, and cytoskeleton organization (Wang *et al.*, 1999; Kreidberg, 2000). Indeed, $\alpha3\beta1$ has been described as a trans-dominant inhibitor of fibronectin, CO IV, and laminin integrin receptors (Lichtner *et al.*, 1998; Dogic *et al.*, 1998; Hodivala-Dilke *et al.*, 1998; Laplantine *et al.*, 2000). This regulatory function may be relevant for the multiple roles that have been ascribed to the integrin $\alpha3\beta1$, in particular in cell growth and in keratinocyte differentiation (Symington and Carter, 1995; Gonzales *et al.*, 1999).

The intestinal epithelium is in constant and rapid renewal, thus representing an attractive system to study the influence of cell-basement membrane interactions in the control of cell differentiation. Indeed, the crypt-villus axis is a functional unit where proliferative, differentiated, and senescent cells are topologically restricted to two distinct compartments: the crypts that contain stem cells and dividing cells, and the villi that protrude into the lumen and are composed of differentiated cells that migrate to the villus tip where they are extruded. (Leblond, 1981; Louvard *et al.*, 1992). Originating from a human colon adenocarcinoma, HT-29 cells appear to be a useful in vitro model to study the different aspects of cell differentiation, because their degree of differentiation and polarization can be modulated in vitro under specific culture conditions. The establishment of a differentiated state of HT-29 cells is obtained by a simple change of the carbon source in the culture medium (Pinto *et al.*, 1982; Zweibaum *et al.*, 1985; Wice *et al.*, 1985). By analogy with the human crypt-villus axis, it has been proposed that 1) HT-29 cells cultured in glucose have properties of undifferentiated multipotent cells located at the lower half of intestinal crypts, 2) proliferating HT-29 cells cultured in inosine or galactose resemble committed cells located at the upper half of the crypt, and 3) confluent HT-29-inosine or HT-29-galactose cells have features of terminally differentiating villus-associated enterocytes (Huet *et al.*, 1987).

The homeostasis of this highly specialized and actively proliferating epithelium depends on a tightly controlled cellular microenvironment. Both basement membrane molecules and growth factors trigger signaling pathways, which are closely interconnected. Among the basement membrane molecules, laminins present the highest variability in their spatial and temporal expression either during intestinal development or in the adult (Timpl, 1996; Simon-Assmann *et al.*, 1994, 1998; Simoneau *et al.*, 1998; Ekblom *et al.*, 1998). In the present work we have found that modifications of LN 5 expression control integrin receptor-mediated cell adhesion of human HT-29 cells during the early stage of differentiation.

MATERIALS AND METHODS

Antibodies and Cell Adhesion Substrates

The function-blocking anti-integrin monoclonal antibodies (mAbs) used were GoH3 against $\alpha6$ integrin, P1B5 and ASC1 against $\alpha3$ integrin, and BHA2.1 against $\alpha2\beta1$, all from Chemicon (Euromedex, Souffelweyersheim, France); and P4C10 and K20 against $\beta1$ integrin (Life Technologies, Cergy Pontoise, France). Other mAbs were anti-laminin $\alpha3$ chain, BM165 (Rousselle *et al.*, 1991; Rousselle and Aumailley, 1994); anti-major histocompatibility complex (MHC) class II molecules, L243 was a gift of Dr P. Bénaroche (Paris, France); and goat anti-IgG2a, U7.27 (Immunotech, Marseille, France). Alexa- or horseradish peroxidase-conjugated goat antimouse antibodies from Molecular Probes (Eugene, OR) and Bio-Rad (Hercules, CA), respectively, were used as secondary antibodies in most experiments. Human collagen type IV from placenta was obtained from Life Technologies. Bovine plasma fibronectin was purified according to the method of Engvall and Ruoslahti (1977). Laminin 5 purified from the culture medium of human SCC25 was kindly provided by Dr P. Rousselle (Institut de biologie et chimie des proteines, Lyon, France).

Cell Culture

The human colonic adenocarcinoma HT-29 cell line (kindly provided by Pr. Marvaldi, Marseille, France) was routinely cultured at 37°C in a 5% CO_2 atmosphere in DMEM containing 25 mM glucose (Life Technologies) supplemented with 10% fetal calf serum, and penicillin-streptomycin (Glu medium or standard medium). The medium was changed every day to avoid glucose exhaustion, which could induce differentiation (Pinto *et al.*, 1982). The differentiation of HT-29 cells was initiated by replacing Glu medium with glucose-free DMEM (Life Technologies) supplemented with 10% dialyzed fetal calf serum, 5 mM galactose, 15 mM HEPES, selenous acid (10^{-2} μg/ml), penicillin, and streptomycin. The medium (Gal medium or differentiating medium) was changed every day. The cells were harvested with phosphate-buffered saline (PBS) supplemented with 1 mM EDTA and 0.05% trypsin (wt/vol).

Isolation of Membrane and Enzymatic Assay

The membrane fractionation procedure was adapted from Stieger *et al.* (1986). Briefly, the cells were resuspended in buffer B (5 mM Na_2SO_4, 1 mM Tris/HCl, pH 7.6, 40 μg/ml phenylmethylsulfonyl fluoride) and sonicated for 30 s at 4°C. Then the homogenate was centifuged for 7 min at 1060 $\times$ *g*. The pellet was resuspended in buffer B by shaking the tubes very gently and recentrifuged for 7 min at 2200 rpm. The pellet was resuspended in buffer B and Percoll was added to a final concentration of 10% (vol/vol). Spinning the tubes for 20 min at 37,000 $\times$ *g* formed the gradients. The supernatant was discarded and fractions containing the brush-border–enriched membrane were collected from the surface of the glassy Percoll pellet by careful resuspension in water. Alkaline phosphatase was measured in 0.05 M glycine pH 10.5, 0.2 mM $MgCl_2$, 5 mM $CaCl_2$ and 2 mM zinc acetate with 10 mM *p*-nitrophenylphosphate diso-

dium salt as substrate. The reaction was carried out in a volume of 1.2 ml in a water bath at 37°C for 2 h and terminated by the addition of 200 μl of 10 N NaOH. The production of *p*-nitrophenol was estimated by measuring the optical density at 420 nm.

Cell Adhesion and Inhibition Assays

Microtiter plates (96-well, Nunclone; Nunc, Roskilde, Denmark) were coated overnight at 4°C with CO IV (Sigma, St. Louis, MO) at a concentration of 5 μg/ml in PBS. Plates were subsequently saturated with 3% (wt/vol) bovine serum albumin (BSA) in PBS for 2 h at 37°C to block nonspecific adhesion. HT-29 cells and HT-29 cells cultured 10 days in Gal medium (HT-29 Gal) were preincubated in serum-free DMEM for 3 h at 37°C. Then 5×10^4 cells/well were plated in triplicate in coated 96-well microtiter plates and incubated for 60 min at 37°C. Nonadherent cells were removed by washing three times with PBS, and cell adhesion was estimated by a colorimetric cell proliferation assay (CellTiter 96 AQ_{ueous} Nonradioactive Cell Proliferation Assay; Promega, Madison, WI). In inhibition assays, the cells were preincubated with the appropriate dilution of antibodies for 30 min before plating onto coated plates for a 60-min adhesion assay. Some adhesion assays were performed after treatment with various RhoA protein modulators. Lysophosphatidic acid (LPA; Sigma), an activator of Rho protein, was added to cell samples 15 min before adhesion at concentrations ranging from 0–10 mM. The C3 transferase assay (gift from Dr. P. Boquet, INSERM 652, UFR de Medecine, Nice, France) was added to the culture medium at $10^{-}8$ M overnight before the cell adhesion. LPA and C3 transferase were removed before cell adhesion assays.

Flow Cytometry

Cell monolayers were harvested with trypsin-EDTA solution, washed twice in PBS, and fixed for 10 min at 37°C with 4% (wt/vol) paraformaldehyde in PBS. The cells were first incubated under gentle rotation with negative control mAb (IgG2A isotype) or specific mAb, for 1 h at room temperature. Then the cells were incubated with Alexa 488-goat antimouse IgG for 45 min at room temperature before fluorescence-activated cell sorting (FACS) analyses (FACScan; Becton Dickinson Biosciences, Mountain View, CA, and the FACStar research software program, Lysis II). Washing with PBS containing 0.05% (wt/vol) Tween 20 eliminated the excess antibodies. Fluorescence with negative control mAb was subtracted to give specific mean fluorescence intensity units.

Quantification of Secreted Laminin-5, Embedded into Insoluble Matrix

HT-29 cells cultured in standard medium or HT-29 cells cultured for 2–10 days in Gal medium (5×10^4 cells/well) were incubated in a Microtiter plate (96-well, Nunclone). The medium was changed every day. After 48 h the cells were lyzed in 20 mM NH_4OH for 15 min at 37°C. Then the plate was washed three times with distilled water and twice with PBS. After saturation with PBS-3%BSA, the LN 5 secreted onto the matrix was estimated by an enzyme-linked immunosorbent assay with the use of BM165 mAb (1/1000 of hybridoma supernatant from culture medium) for 60 min at 37°C. Detection was performed with a rabbit antimouse conjugated with horseradish peroxidase and 2,2′-azino-di (3-ethyl-benzothiazoline-6 sulfonic acid) (Zymed Laboratories, South San Francisco, CA) as substrate. Nothing has been detected in the soluble phase by this method. All experimental conditions were performed in triplicate.

Immunocytochemical Detection of Secreted Laminin-5, Embedded into Insoluble Matrix

HT-29 cells or HT-29 Gal cells (cultured 10 days in Gal medium) were incubated 48 h on glass coverslips. Immunodetection of LN 5 deposited on the glass coverslip was performed after removal of the cells after a 1-h treatment with a solution containing 1% Triton X-100, 10 mM EDTA, and 25 mM Tris-HCl, pH 7.5. Then coverslips were washed three times with PBS and fixed with 3% (wt/vol) paraformaldehyde, 2% (wt/vol) sucrose for 10 min at 37°C. After incubation for 1 h at room temperature with blocking solution (10% [vol/vol] goat serum in PBS) to reduce background signal, LN 5 was detected with mAb BM165 diluted (1/50) in blocking solution (1 h at 37°C). After washing in PBS-0.05% (wt/vol) Tween 20, detection was continued with an anti-mouse conjugated to Alexa 488 for 45 min at 37°C. Coverslips were permanently mounted with Mowiol (Calbiochem, Meudon, France). Fluorescence photomicrographs were made with the use of a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) equipped with epifluorescence and a Zeiss 63× objective, coupled to a Hamamatsu (Bridgewater, NJ) charge-coupled device camera C4880.

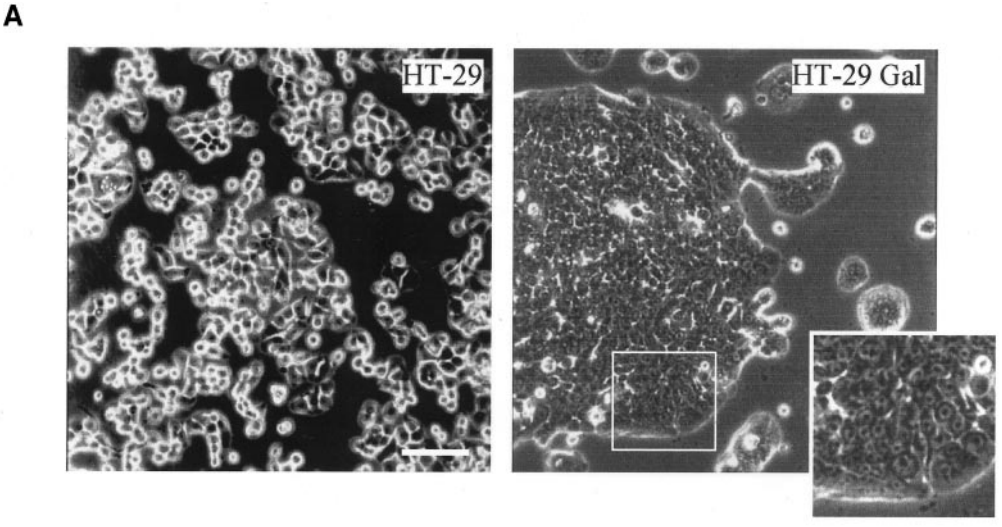
RhoA Activity Assay

RhoA activity was estimated with the use of the Rho-binding domain of Rhotekin as described (Ren *et al.*, 1999). Briefly, HT-29 and HT-29 Gal cells (5×10^6) were lyzed with RIPA buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1% SDS, 10 mM $MgCl_2$, and a cocktail of proteases inhibitors). In some experiments HT-29 cells were adhered on LN 5 (20 μg/ml) 30 min before cell lysis. After centrifugation at 14,000 × *g* for 5 min, the extracts were incubated for 45 min at 4°C with glutathione beads coupled with bacterially expressed recombinant GST-RBD (Rho-binding domain of Rhotekin) fusion protein (kindly provided by Martin Schwartz, Scripps Research Institute, La Jolla, CA) and then washed three times with Tris buffer, pH 7.2, containing 1% Triton X-100, 150 mM NaCl, and 10 mM $MgCl_2$. The RhoA content in these samples was determined by immunoblotting samples with the use of rabbit anti-RhoA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Culture in Glucose-free Medium (Gal Medium) Initiated Differentiation of HT-29 Cells

Substitution of galactose for glucose in the culture medium (Gal medium) of HT-29 cells has been described to induce a reversible enterocytic differentiation of these cells. The acquisition of the epithelial phenotype by HT-29 cells induced by Gal medium took several days and was evaluated by two approaches: the comparison of morphological changes by phase contrast microscopy (Figure 1A) and the determination of the specific activity of alkaline phosphatase in membrane-enriched fractions (Figure 1B). The microscopic observation showed that in standard medium, the cells appeared disorganized and grew in multilayers at confluence. In contrast, cells cultured in Gal medium (HT-29 Gal cells) were committed to differentiation: they became flattened and grew in monolayer (Figure 1A). Transmission electron microscopy indicated that HT-29 Gal cells developed microvilli that were partially organized into brush-border structures (data not shown). This phenotype corresponds to the early phase of cell differentiation (10 days after seeding). During cell differentiation, it has been described that the maturation of brush-border hydrolases, such as alkaline phosphatase parallels the exhibition of an apical domain covered with microvilli. An increase in the alkaline phosphatase activity was observed during the time course of the culture in Gal medium. After 10 days, HT-29 cells showed a threefold higher alkaline phosphatase activity compared with control cells (Figure 1B). Taken together, our data indicate that in Gal medium, HT-29 cells initiate a process of epithelial differentiation.



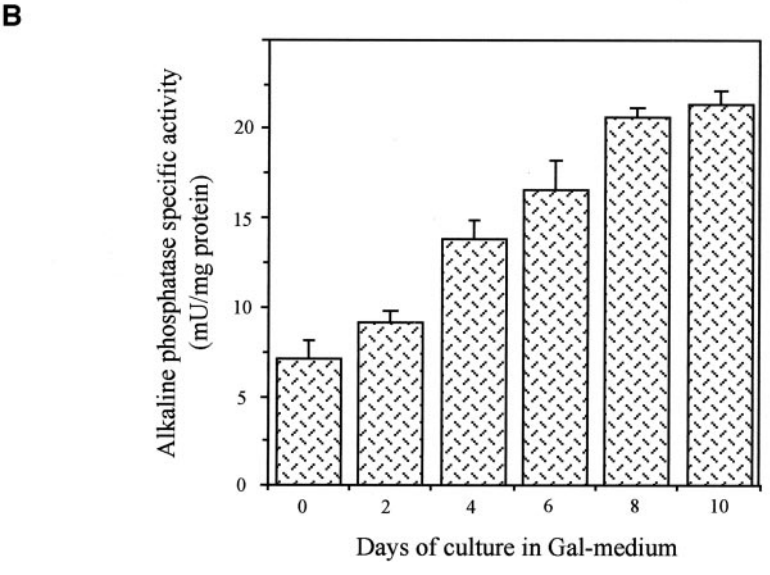


Figure 1. Induction of HT-29 cell differentiation. (A) Phase-contrast images of HT-29 cells grown in standard medium (left, HT-29) or in Gal medium for 10 days (right, HT-29 Gal). Bar, 100 μm. (B) Correlation between alkaline phosphatase activity and the day of culture in Gal medium. Membrane-enriched fractions were isolated from HT-29 cells grown for 2, 4, 6, 8, and 10 days in Gal medium and assayed for alkaline phosphatase activity as described in MATERIALS AND METHODS. The specific activity, which is reported in milli-unit per milligram (mU/mg) of protein, increased with the time of culture in Gal medium. The data were compiled from three independent assays; error bars represent SE values of the mean.

Increased Adhesion to CO IV Occurs during HT-29 Cell Differentiation

A modulation of cell basement membrane interactions has been described during the differentiation of intestinal cells that occurs along the crypt-villus axis. Because CO IV is an important component of the basement membrane, we analyzed the ability of HT-29 cells to adhere to this matrix protein depending on the time of culture in Gal medium up to the early differentiation stage. Figure 2 shows a time-dependent increase in cell adhesion to CO IV. Although HT-29 cells cultured in standard medium adhered poorly to CO IV, 69% of cells cultured for 10 days in the differentiating medium adhered to this protein. The adhesion capacity of HT-29 cells increased twofold after 6 days of culture in Gal medium, in correlation with the appearance of differentiation markers such as hydrolases (Figure 1B) and of carcinoembryonic antigen (Fantini et *al*., 1989).

Switch from $\alpha 2\beta 1$ to $\alpha 3\beta 1$-mediated Cell Adhesion to CO IV in Early State of HT-29 Differentiation

Many $\beta 1$ integrins were described as putative collagen receptors, including $\alpha 1\beta 1$, $\alpha 2\beta 1$, and with a lower affinity, $\alpha 3\beta 1$. To determine which integrins were predominantly involved in CO IV-mediated adhesion of HT-29 and HT-29 Gal cells, we performed adhesion assays in the presence or

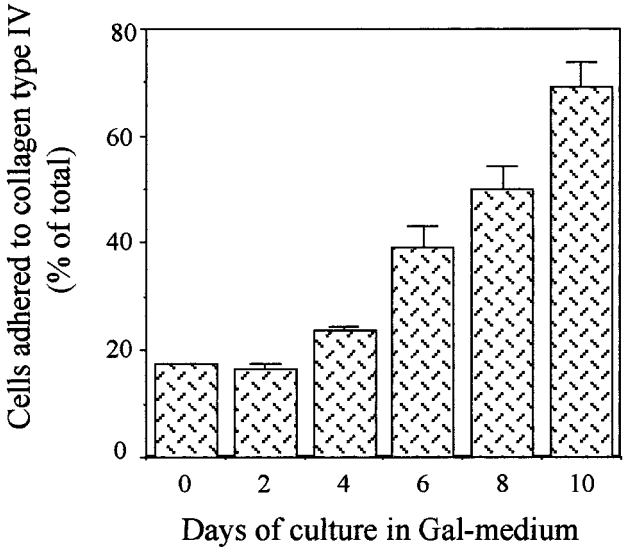


Figure 2. Adhesive behavior on Co IV of HT-29 cells grown in Gal medium. Cells grown for 2, 4, 6, 8, and 10 days in Gal medium were allowed to attach to 5 μg/ml CO IV. Adhesion was estimated with the use of a Cell Titer Aqueous MTT reagent kit and expressed as a percentage of adherent cells. Depending on the time of culture in Gal medium, the percentage of HT-29 cells adhering to CO IV increased. The experiment was done in triplicate and repeated four times.

absence of inhibitory mAbs against specific integrins (Figure 3A). Incubation with antibodies directed against the β1 subunit resulted in an 80% inhibition of both HT-29 and HT-29 Gal cell adhesion to CO IV, confirming that β1 integrins are the major receptors for this protein. Similar experiments were carried out with antibodies specific for $\alpha2\beta1$ and/or the $\alpha3\beta1$ integrins. In the presence of anti-$\alpha2\beta1$ antibodies only 4.9% of HT-29 cells adhered to CO IV (adhesion was blocked by 77%), whereas 65% of HT-29 Gal cells remained adherent (adhesion was blocked by 9%). Conversely, when anti-$\alpha3\beta1$ antibodies were used, 16.5% of HT-29 cells adhered to CO IV (adhesion was blocked by 12%) versus 22.5% for HT-29 Gal cells (adhesion was blocked by 68%). Combination of antibodies directed against both the $\alpha2\beta1$ and the $\alpha3\beta1$ integrins gave similar results to those obtained with anti-β1, i.e., ~80% inhibition for both culture conditions. Finally, antibodies directed against the α6 integrin, which is predominantly involved in cell adhesion to LN 5, had no significant effect on cell adhesion to CO IV.

The fact that $\alpha3\beta1$ integrin contributes to HT-29 Gal but not to HT-29 cell adhesion to CO IV suggested that a switch in collagen receptors occurs during HT-29 cell differentiation. To test this hypothesis, we used function-blocking antibodies to identify the integrin receptor involved in CO IV adhesion depending on the days of culture in Gal medium (Figure 3B). During the first 2 d of culture in the differentiation medium, adhesion of HT-29 cells to CO IV was mostly mediated by $\alpha2\beta1$ (70% inhibition with anti-$\alpha2\beta1$ compared with 5% with anti-α3). After 6 days of culture in Gal medium, both integrins were equally involved in HT-29 cell adhesion to CO IV. Beginning with the 8th days of culture, $\alpha3\beta1$ integrin became the major receptor of HT-29 involved

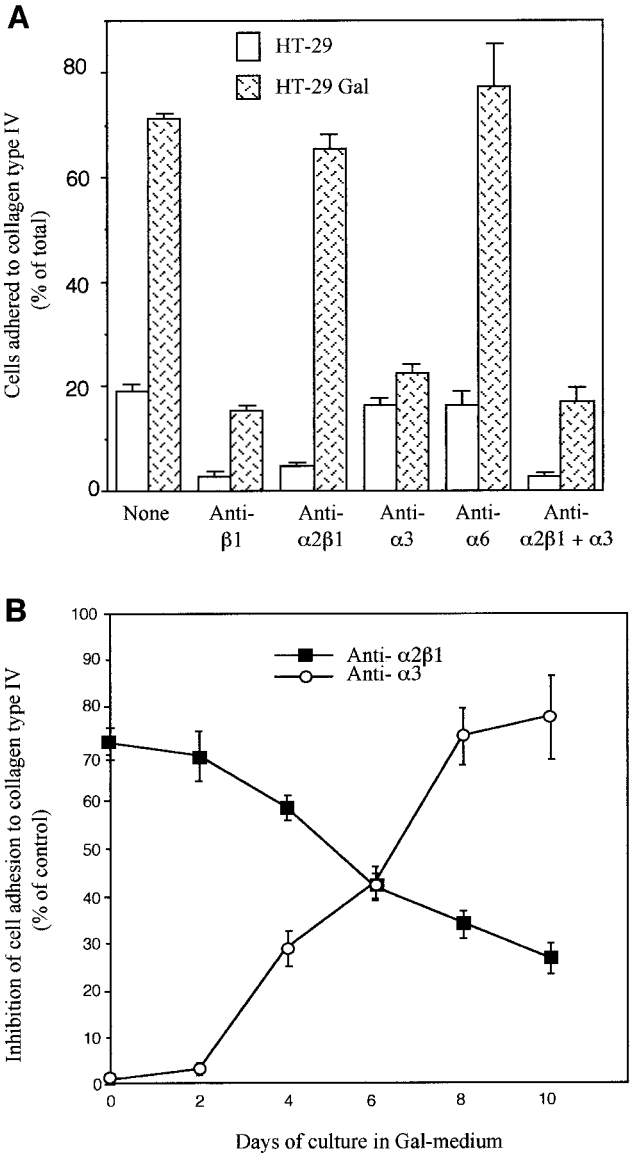


Figure 3. Inhibition of HT-29 and HT-29 Gal cell adhesion to Co IV by anti-integrin antibodies. (A) Before adhesion to CO IV (5 μg/ml), HT-29 and HT-29 Gal cells were incubated for 45 min with various mAbs directed against integrin subunits at a concentration of 10 μg/ml: P4C10 (anti-β1), BHA2.1 (anti-$\alpha2\beta1$), P1B5 (anti-α3), and GoH3 (anti-α6). Although HT-29 cell adhesion was mostly mediated by $\alpha2\beta1$, a collagen receptor switch to $\alpha3\beta1$ is observed in HT-29 Gal cells. (B) Before adhesion assays, cells grown for 2, 4, 6, 8, and 10 days in Gal medium were incubated for 45 min with 10 μg/ml anti-$\alpha2$ $\beta1$ or anti-α3 blocking mAbs. The switch from $\alpha2\beta1$ to $\alpha3\beta1$ as collagen receptor occurred after 6 days of culture in Gal medium. The experiment was performed in triplicates and repeated three times.

in cell adhesion to CO IV (80% inhibition with anti-α3 compared with 30% with anti-$\alpha2\beta1$ after 10 days of culture in Gal medium). Thus, it appeared that the differentiation of HT-29 cells was indeed associated with a switch in the

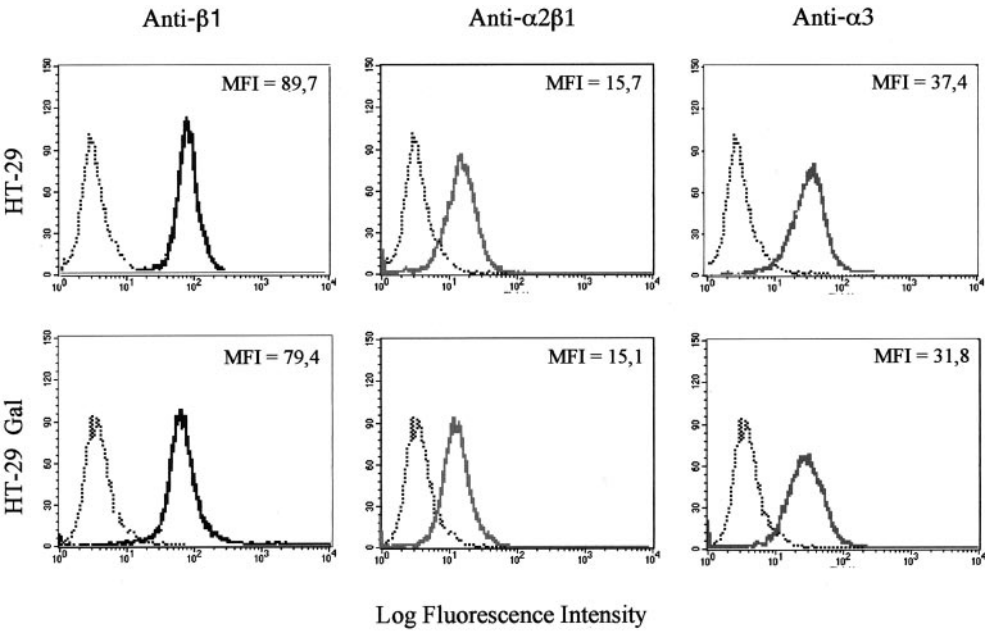


Figure 4. FACS analysis of expression levels of $\beta 1$, $\alpha 2$, and $\alpha 3$ integrin subunits in HT-29 and HT-29 Gal cells. FACS profiles of HT-29 and HT-29 Gal cells stained with anti-integrin antibodies at a concentration of 10 μg/ml: P4C10 (anti-$\beta 1$), BHA2.1 (anti-$\alpha 2\beta 1$), P1B5 (anti-$\alpha 3$), or goat anti-IgG2a (negative control). The negative controls (dotted line) indicated the staining background levels. Specific mean fluorescence intensity (MFI) values are indicated. HT-29 and HT-29 Gal cells expressed similar level of $\beta 1$, $\alpha 2$, and $\alpha 3$ integrin subunits.

collagen receptors from integrin $\alpha 2\beta 1$ to $\alpha 3\beta 1$, which was accompanied by an overall increase in cell adhesion to CO IV.

$\alpha 3\beta 1$ Surface Expression Is not Modified During Early Stage of HT-29 Differentiation

To investigate whether the differences in adhesion to CO IV between HT-29 and HT-29 Gal cells could result from changes in the surface expression levels of integrins, the cells were stained with anti-integrin antibodies and analyzed in flow cytometry assays (Figure 4). Concerning the $\beta 1$, $\alpha 2\beta 1$, or $\alpha 3\beta 1$ integrins, no significant changes in the levels of surface expression were found, indicating that HT-29 cell differentiation did not modify the integrin expression profiles.

$\alpha 3\beta 1$ Integrin Becomes Major CO IV Receptor after Being Activated

Because $\alpha 3\beta 1$ mediated-adhesion of HT-29 Gal cells to CO IV did not result from a modulation of its surface expression, we hypothesized that in HT-29 Gal cells $\alpha 3\beta 1$ had been activated. The presence of activated $\alpha 3\beta 1$ at the cell surface of HT-29 Gal cells was tested by an adhesion assay on LN 5, the major ligand for this integrin. As shown in Figure 5A, the adhesion to LN 5 of HT-29 cells cultured 8 days in Gal medium was twofold higher than that of HT-29 cells cultured in standard medium. To rule out a possible contribution of other integrin-mediated adhesion to LN 5, we performed adhesion assays to LN 5 in presence of blocking function mAbs against specific integrins (Figure 5B). Although HT-29 cells adhesion to LN 5 was mediated by both $\alpha 3$ and $\alpha 6$ integrins (40 and 60% inhibition, respectively), the adhesion of HT-29 Gal cells was mostly mediated by $\alpha 3\beta 1$ integrins (72% inhibition). Thus, it appears that during differentiation of HT-29 cells, the $\alpha 3\beta 1$ integrin becomes activated and increases the capacity of HT-29 cells to adhere to both CO IV and LN 5.

The mAb P1B5 has been reported to have various "activating" or "inhibitory" effects according to its concentration on $\alpha 3\beta 1$ function such as tyrosine phosphorylation (Jewell *et al.*, 1995) or cell-cell localization and differentiation (Takeuchi *et al.*, 1994; Symington and Carter., 1995). Thus, we investigated whether triggering of $\alpha 3\beta 1$ by low concentrations of P1B5 (0.5–5 μg/ml) could lead to its activation and consequently modulate adhesion to CO IV matrix, but inhibit the integrin at high concentrations (10–20 μg/ml). HT-29 and HT-29 Gal cells were preincubated for 1 h with different concentrations of P1B5, before adhesion to CO IV (Figure 6A). In the absence of the mAb P1B5, only 23.5% of HT-29 cells adhered to CO IV (Figure 6A, top). At low concentrations of P1B5, the percentage of adherent cells increased progressively to a maximum of 42.8% (at 2 μg/ml P1B5). At higher concentrations of P1B5, the percentage of HT-29 cells adherent to CO IV decreased to 20%, which corresponded to their basal adhesion on this matrix. In HT-29 Gal cells (Figure 6A, bottom), low concentrations of P1B5 already induced an inhibition of cell adhesion (61.6% of cell adhered in the control vs. 30% with 2 μg/ml P1B5), indicating that most $\alpha 3\beta 1$ molecules on these cells were already activated. In contrast to HT-29 cells, high concentrations of P1B5 (20 μg/ml) completely inhibited adhesion of HT-29 Gal cells to CO IV (around 10% of HT-29 Gal cells adhered). Our data indicate that $\alpha 3\beta 1$ became the major collagen receptor (Figure 3). To test whether these mAb-mediated effects on cell adhesion to matrix were specific for P1B5, we performed similar experiments with control antibodies used at the same concentration. With the use of another anti-$\alpha 3$ mAb, ASC1, we found the same results as for P1B5 (Figure 6A). In competition experiments we observed that these two antibodies have overlapping epitopes (data not shown). With the use of two anti-$\beta 1$ mAbs, we

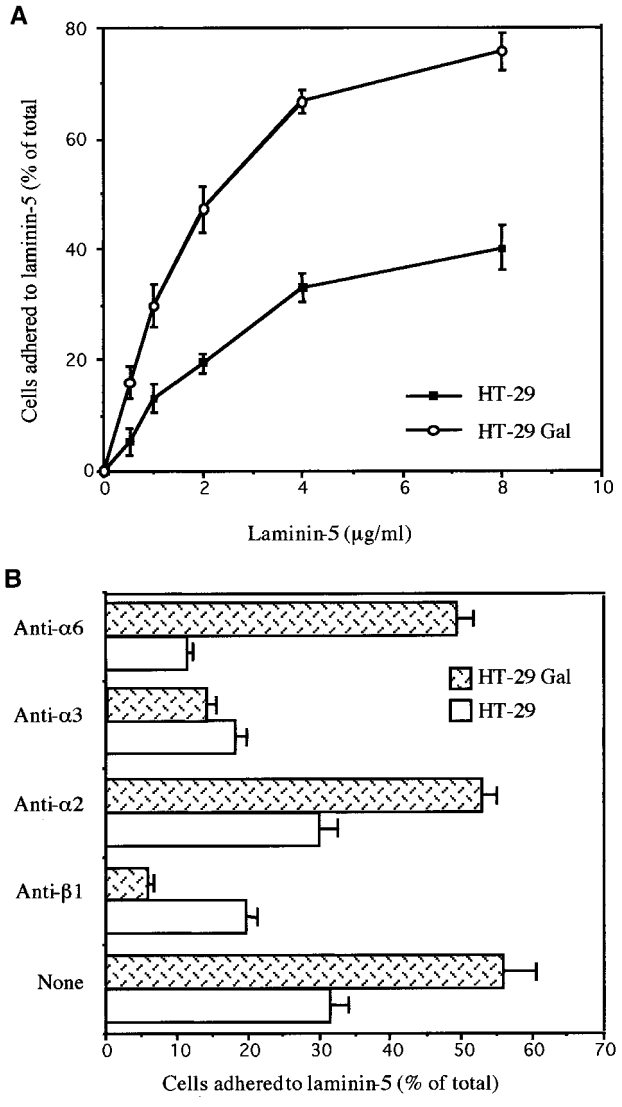


Figure 5. Adhesive behavior of HT-29 and HT-29 Gal cells on laminin-5. (A) Multiwell plates were coated with different concentrations of LN 5, ranging from 0 to 8 μg/ml. After a saturating step with 3% BSA in PBS, HT-29 and HT-29 Gal cells were allowed to attach for 1 h at 37°C. Cell adhesion was measured and expressed as described in MATERIALS AND METHODS. The data were compiled from three independent experiments; error bars represent SE values of the mean. (B) Before adhesion to LN 5 (4 μg/ml), HT-29, and HT-29 Gal cells were incubated for 45 min with various mAbs directed against integrin subunits at a concentration of 10 μg/ml: P4C10 (anti-β1), BHA2.1 (anti-α2β1), P1B5 (anti-α3), and GoH3 (anti-α6). Although HT-29 cells adhesion to LN-5 was mediated by both α3 and α6 integrins, the adhesion of HT-29 Gal cells was essentially mediated by α3β1 integrins.

observed no effect on HT-29 cell adhesion to CO IV, whatever the concentration of the nonblocking mAb K20 used, whereas cell adhesion was progressively decreased by increasing concentrations of the function-blocking mAb P4C10 (Figure 6B). Thus, the effect of anti-α3 mAbs appeared to be specific to the epitope of this integrin.

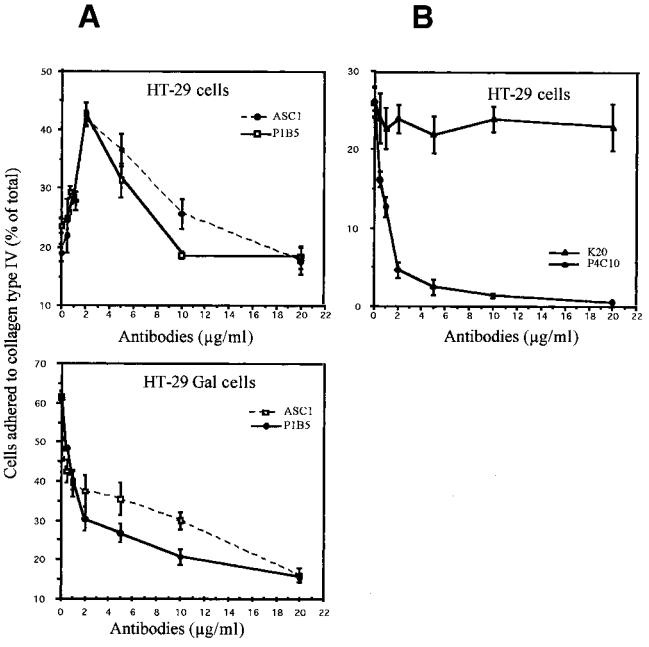


Figure 6. Effect of the P1B5 antibody on HT-29 cell adhesion to CO IV. (A) When adhesion to CO IV was carried out in the presence of low concentrations of P1B5 or ASC1 (0.5–2 μg/ml; activation concentrations), the adhesion of HT-29 cells was increased. At higher concentrations (10–20 μg/ml; blocking concentrations) P1B5 and ASC1 had no effect on HT-29 cells, in fact both antibodies inhibited HT-29 Gal cell adhesion in a dose-dependent manner. P1B5 and ASC1 share overlapping epitopes on the α3 integrin subunit. (B) Adhesion assays were performed with control antibodies (anti-β1 mAbs) used at the same concentration. Adhesion of HT-29 cells to CO IV was not modified regardless of the concentration of the mAb K20 used; in contrast, cell adhesion decreased progressively upon increasing the concentration of the function blocking mAb P4C10.

Laminin-5 Secretion Is Responsible for Activation of α3β1 during Differentiation of HT-29 Cells

In situ, LN 5 is mostly found in the basement membrane lining the differentiated enterocytes (Simon-Assmann *et al.*, 1998). Furthermore, it has been shown that human colon carcinoma cells synthesize LN 5 (Orian-Rousseau *et al.*, 1998). Therefore, we hypothesized that elevated expression of LN 5 in HT-29 cells cultured in Gal medium could be responsible for the activation of α3β1 integrins.

To test this hypothesis, levels of LN 5 deposited into insoluble matrix from undifferentiated HT-29 cells and HT-29 cells cultured for different time periods in Gal medium were determined by a quantitative solid phase assay. As shown in Figure 7A, the relative deposit of LN 5 increased with the time of culture in Gal medium. After 10 days in Gal medium, the amount of LN 5 synthesized was fivefold higher than in HT-29 cells cultured in standard medium. The immunocytochemical detection of LN 5 deposited on the culture wells confirmed the differential expression of LN 5 in HT-29 and HT-29 Gal cells (Figure 7B). HT-29 Gal cells displayed a regular labeling of LN 5 in basal patches. Conversely, the HT-29 cells deposited only tiny dispersed spots.

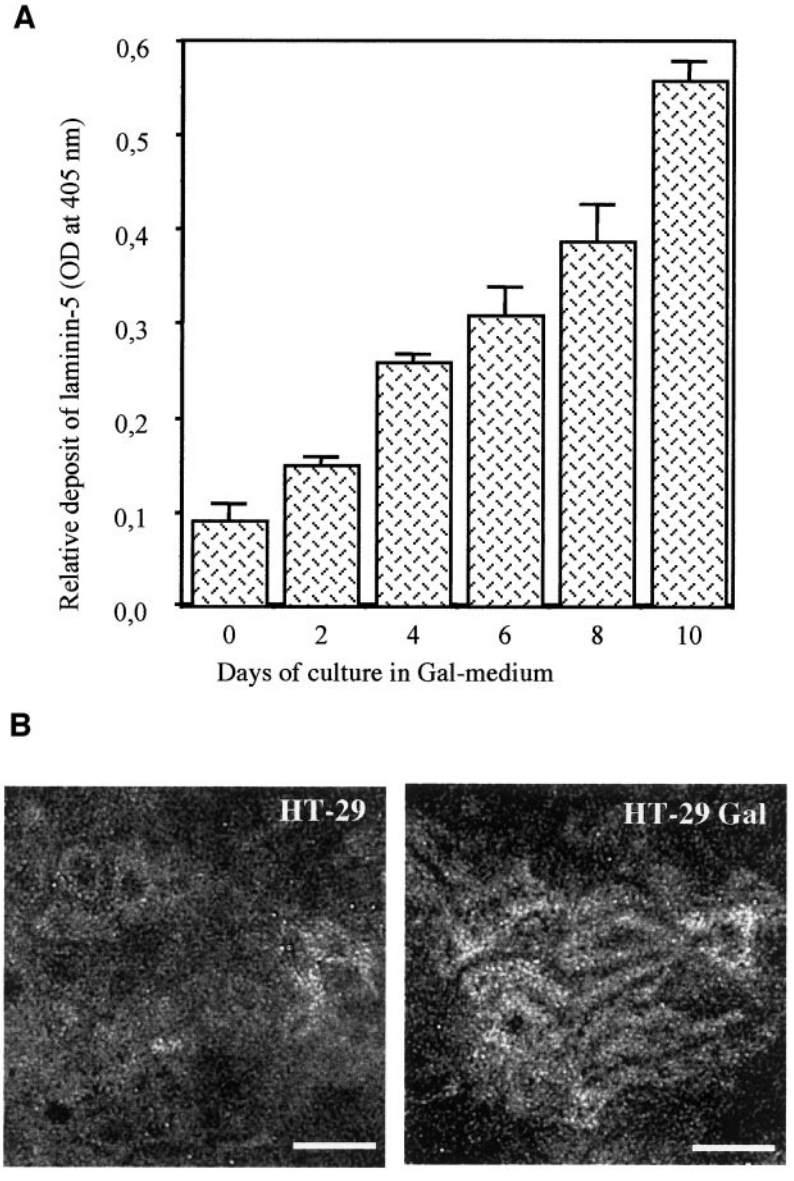


Figure 7. Laminin-5 secretion into insoluble form by HT-29 and HT-29 Gal cells. (A) Quantification of LN 5 secretion into insoluble form by HT-29 cells and HT-29 Gal cells cultured for 2, 4, 6, 8, and 10 days in Gal medium, in a solid phase assay. Laminin-5 was revealed by the BM165 mAb, followed by a goat antimouse mAb coupled to horseradish peroxidase. A time-dependent increase in LN 5 secretion and deposit into the matrix was observed for HT-29 cells cultured in Gal medium. (B) Immunochemical detection of secreted LN 5 by HT-29 and HT-29 Gal cells. HT-29 cells or HT-29 Gal cells (10 days in Gal medium) were cultured 48 h on glass coverslips. After cell removal and fixation as described in MATERIALS AND METHODS, LN 5 deposited on the glass coverslips was immunodetected with a 1/50 diluted BM165 mAb (from cell culture supernatant). Laminin-5, which is organized in strips (parallel lines), is clearly detected in deposits of HT-29 Gal cells, whereas it is barely observed after HT-29 cell cultures. Bar, 35 μm.

To determine whether this differential expression of LN 5 was responsible for the activation of $\alpha 3\beta 1$ integrins, we performed adhesion assays on CO IV after a variable preculture period of HT-29 cells on various extracellular matrix proteins, including LN 5. As shown in Figure 8A, the preculture on fibronectin or plastic did not modulate HT-29 cell adhesion, and ~18% of the HT-29 cells adhered to CO IV. In contrast, preculture on CO IV for 1–16 h lead to a twofold increase in the number of HT-29 cells adherent to CO IV. Finally, a time-dependent increase in HT-29 adhesion to CO IV was only observed in HT-29 cells precultured on LN 5: after 1 h on LN 5, 50% of cells were able to adhere to CO IV versus 78% after 16 h. Analysis of the integrin receptor(s) involved in collagen adhesion after 16 h of culture on the different matrix proteins was performed with the use of inhibitory antibodies directed against $\beta 1$, $\alpha 2$, and $\alpha 3$ (Figure 8B). Whatever the matrix used during the preculture, cell adhesion to COIV was mostly mediated by the $\beta 1$ subunit. However the α subunit associated with $\beta 1$ to constitute the CO IV receptor differed depending on the nature of the matrix used for preculture of HT-29 cells. After an overnight preculture on plastic or fibronectin, adhesion of HT-29 cells to CO IV was mediated by $\alpha 2\beta 1$ integrin. Similar experiments performed with a CO IV matrix during the preculture step indicated a participation of one-third for $\alpha 2\beta 1$ (31% inhibition with anti-$\alpha 2\beta 1$ mAbs) and two-thirds for $\alpha 3\beta 1$ (62% inhibition with anti-$\alpha 3$ mAbs) in cell adhesion to collagen. Finally, when HT-29 cells were precultured overnight on LN 5, the major CO IV receptor switched to $\alpha 3\beta 1$ integrin. Altogether, our results indicate that LN 5 and to a lesser extent CO IV are able to activate $\alpha 3\beta 1$, which becomes the major collagen receptor, whereas $\alpha 2\beta 1$ is switched off.

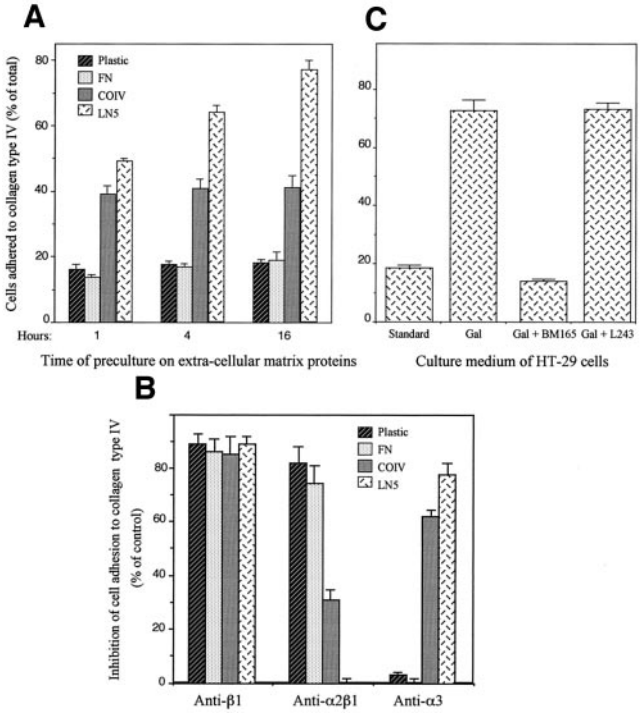


Figure 8. Role of LN 5 in $\alpha 3\beta 1$ activation. (A) Modulation of HT-29 cell adhesion to CO IV after preculture on various extracellular matrix proteins. HT-29 cells were seeded on plates coated with various extracellular matrix proteins at 20 μg/ml (FN, fibronectin; CO IV, collagen type IV; LN 5, laminin-5) for 1, 4, or 16 h and then tested for their adhesive behavior to CO IV as described in MATERIALS AND METHODS. Preincubation with CO IV stimulated a twofold increase in cell adhesion. Cell adhesion remained constant during the time of the experiment. Conversely, preincubation with LN 5 led to a time-dependent and stronger increase in adhesion. Results are expressed as the mean ± SE for three wells. (B) Effects of inhibitory mAbs on HT-29 cell adhesion to Co IV after overnight preculture on various extracellular matrix proteins. Cells were precultured overnight on plastic coated with 20 μg/ml various extracellular matrix proteins (FN, CO IV, LN 5) and then tested for adhesion to CO IV after an incubation of 45 min with mAbs directed against integrin subunits as described above. (C) Cell adhesion to CO IV after culture of HT-29 cells in Gal medium in presence of anti-laminin-5 antibody. After 10 days, 5×10^4 living cells were tested for their adhesive capacity to CO IV, as described above. Adhesion of HT-29 Gal cells cultured in the presence of the BM165 mAb was dramatically reduced, whereas culture in presence of the L243 mAb had no effect. Standard: HT-29 cells grown in standard medium. Results are expressed as the mean ± SE for three wells.

However, because both $\alpha 3$ and $\alpha 6$ integrins are LN 5 receptors on HT-29 cells (Figure 5), the initiation of the integrin switch by LN 5 could be due either to $\alpha 3$ or $\alpha 6$ integrins.

To further demonstrate that LN 5 is able to provoke a switch in collagen receptors, HT-29 cells were cultured for 10 days in Gal medium, in the presence or absence of mAbs directed against the $\alpha 3$ subunit of LN 5 (mAbs BM165) or the MHC class II molecules (mAbs L243) before testing their adhesive properties on CO IV. Figure 8C indicates that adhesion of HT-29 cells cultured in Gal medium with mAb L243 was similar to that of HT-29 Gal cells (~73%). Conversely, only 14% of cells cultured in Gal medium with mAb BM165 had adhered to CO IV, a percentage similar to that of HT-29 cells cultured in standard medium (18%).

Switch from $\alpha 2\beta 1$ to $\alpha 3\beta 1$-mediated Cell Adhesion to CO IV Depends on RhoA Activity

To address the mechanism of the integrin switch during colon epithelial cell differentiation, we examined the differences between HT-29 and HT-29 Gal cell signaling pathways in correlation with CO IV adhesive properties. The $\alpha 2\beta 1$-mediated adhesion to CO IV has recently been described as Rho dependent (Nguyen *et al.*, 2000), so we compared the effect of a modulator of RhoA GTPase activity on the adhesion of both cell lines to CO IV. As a result of treatment for 15 min before adhesion with various concentrations of LPA (an activator of RhoA proteins), adhesion of HT-29 cells cultured in Gal medium to CO IV was inhibited in a dose-dependent manner, suggesting that $\alpha 3\beta 1$ mediating adhesion to COIV is sensitive to the level of active RhoA proteins (Figure 9A). Conversely, the adhesion of HT-29 cells was unaffected by treatment with LPA. In contrast, when C3 transferase was added to the culture medium of HT-29 cells, we noticed an increase in the percentage of cells adhered to CO IV from 17 to 61.8% (Figure 9B). This increased adhesion was mediated by $\alpha 3\beta 1$ integrin because the effect was eliminated by P1B5 mAb. C3 transferase specifically ADP-ribosylates RhoA proteins at Asn41, thereby rendering them inactive (Sekine *et al.*, 1989). Our results strongly suggested that $\alpha 3\beta 1$-mediated adhesion required a low level of active RhoA. With the use of a Rho-GTP pull-down assay, we compared the levels of active RhoA-GTP in HT-29 and HT-29 Gal cells (Figure 9C). It appeared that the RhoA-GTP level was 10-fold higher in HT-29 cells than in differentiated cells. Therefore, our results indicated that the balance of RhoA-GTP levels was responsible for the switch from $\alpha 2\beta 1$- to $\alpha 3\beta 1$-mediated cell adhesion to CO IV. Because LN 5 was responsible for this switch, we investigated the effect of adhesion to LN 5 on RhoA-GTP levels (Figure 9D). Thirty minutes of adhesion to LN 5 is sufficient to induce a decrease (factor 2.5) of RhoA activity.

DISCUSSION

Integrin-mediated interactions between epithelial cells and the basement membrane play a crucial role in various biological processes, such as cell proliferation, migration, and differentiation. Studies carried out with both cell cultures and tissues have determined the expression and localization of basement membrane molecules and integrins. The next step is to dissect the precise signaling pathways involved in the cellular response to basement membrane proteins. In the present work we have addressed this basic question by studying, during an early stage of epithelial cell differentiation, the correlation between matrix protein expression and integrin activation and function. Taken together, our data indicate that the initiation of epithelial cell differentiation leads to LN 5-mediated activation of $\alpha 3\beta 1$ integrins, which increases adhesion of HT-29 cells to CO IV; the $\alpha 3\beta 1$ integrin becoming the major collagen receptor, whereas the $\alpha 2\beta 1$ integrin is switched off. This integrin switch depends on the cellular level of activated RhoA.

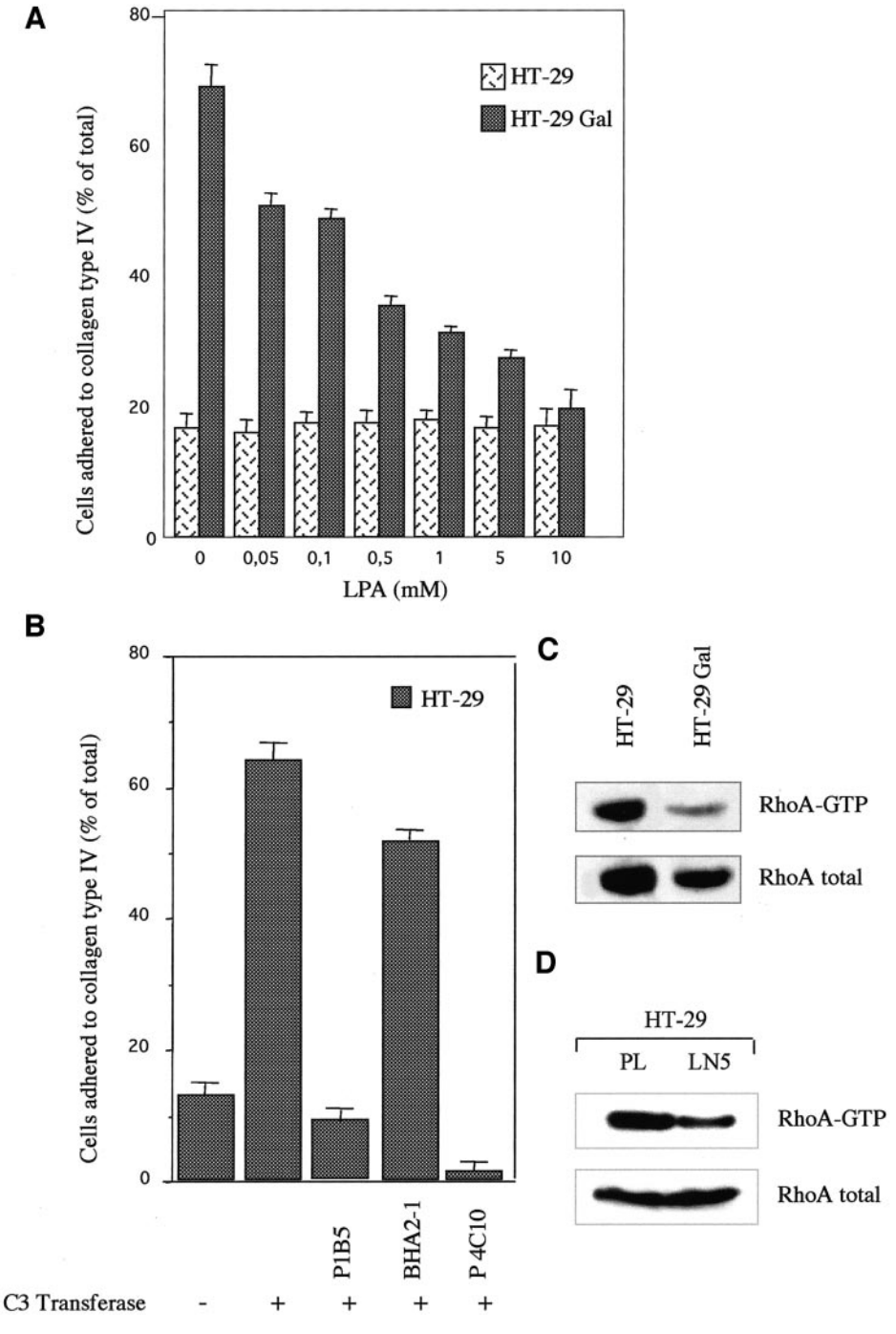


Figure 9. RhoA activity controls the switch from $\alpha 2\beta 1$- to $\alpha 3\beta 1$-mediated cell adhesion to CO IV (A and B). Before adhesion to CO IV (5 μg/ml), HT-29 and HT-29 Gal cells were incubated for 3 h, either with LPA at concentration ranging from 0 to 10 mM (A) or overnight with C3 transferase (10^{-8} M) (B). The cells were then washed with PBS and allowed to attach for 1 h at 37°C. Cell adhesion was measured and expressed as described in MATERIALS AND METHODS. The data were compiled from three to five independent experiments; error bars represent SE values of the mean. In competition experiments cells were incubated with various mAbs directed against integrin subunits at a concentration of 10 μg/ml: P4C10 (anti-$\beta 1$), BHA2.1 (anti-$\alpha 2$ $\beta 1$), and P1B5 (anti-$\alpha 3$). (C and D) Western blots showing RhoA activity in HT-29 and HT-29 Gal cells. The levels of RhoA-GTP were also compared for HT-29 cells after 30 min adhesion on LN 5 (20 μg/ml). Colon epithelial cell differentiation is characterized by a decrease in RhoA-GTP levels, even than adhesion to LN 5.

HT-29 Cells Mimic Enterocyte Differentiation

In our experiments, we induced HT-29 cell differentiation by switching to culture media containing galactose as the source of carbon. The induction of a differentiation process is not due to galactose per se, but rather to the substitution of galactose for glucose, which results in a reduction of hexose consumption and lactic acid production (Eagle *et al.*, 1958). During the process of differentiation, a number of biochemical markers become polarized to the apical membrane such as alkaline phosphatase and the carcinoembryonic antigen (Le Bivic and Arsanto, 1987; Fantini *et al.*, 1989) and could be used as markers of cell differentiation in concert with morphological changes. In our culture conditions (10 days of culture in Gal medium), HT-29 cells were only partially differentiated as judged by morphological and ultrastructural observations but were committed to differentiation

according to biochemical analysis showing an increase in the level of alkaline phosphatase activity in membrane-enriched fractions. Furthermore, studies from Fantini *et al.* (1989) indicated that in a HT-29-derived cell clone, apical carcinoembryonic antigen release into the culture medium starts after 6 days of culture in Gal medium and increases in a time-dependent manner. Full differentiation of HT-29 cells required 21 days of culture in Gal medium.

Switch between α2β1 and α3β1 Integrins Is Associated with a Signaling Pathway Involving RhoA-GTPase Proteins

Previous studies on HT-29 cell adhesion indicated that these cells adhere well to laminin isoforms 1 and 5 and to a lesser extent to CO IV (Schreiner *et al.*, 1991; Orian-Rousseau *et al.*, 1998). The integrins involved in cell adhesion to CO IV have not been precisely defined although they belong to the β1 integrin family (Schreiner *et al.*, 1991). Our functional assays reveal that only 20% of HT-29 cells adhere to CO IV. However, when these cells start a differentiation process, the proportion of cells adhering to this protein can reach 70%. These changes in HT-29 cell adhesion are not due to an increase in the surface expression of integrins but rather to a switch in the nature of the integrins involved in collagen binding. Indeed, our data indicate that during differentiation α3β1 becomes the major CO IV receptor instead of α2β1.

The possibility that CO IV constitutes a ligand for the α3β1 integrin has been discussed. It has been shown previously that integrins α1β1 and α2β1 are high-affinity receptors for type IV collagen, whereas α3β1 is at best a low-affinity receptor (Kühn and Eble, 1994). Furthermore, in direct ligand binding assays with the use of soluble recombinant α3β1, Eble *et al.* (1998) showed that α3β1 specifically binds laminin isoforms 5 and 10, but not collagen. This discrepancy with our data suggests that activation of α3β1 is a prerequisite for the recognition of collagen by this integrin. Indeed, melanoma and ovarian carcinoma cells have been shown to use the α3β1 integrin to bind to a sequence in the α1 chain of basement membrane CO IV (amino acids 531–543) (Miles *et al.*, 1995; Lauer *et al.*, 1998). Moreover, an anti-α3 antibody inhibits melanocyte migration on CO IV as well as cell motility (Morelli *et al.*, 1993; Melchiori *et al.*, 1995).

A regulatory role for α3β1 has already been pointed out by other groups and may also apply to other integrins bound to fibronectin and laminin (Dogic *et al.*, 1998). In these studies, the inhibition of α3 integrin function or the deficiency in α3 integrin expression enhanced both the adhesion and the migration of keratinocytes on fibronectin and CO IV (Kim *et al.*, 1992; Hodivala-Dilke *et al.*, 1998, Lichtner *et al.*, 1998). In contrast, we found that activation of α3β1 favored the adhesion of human colon carcinoma cells to CO IV. Thus, it can be postulated that α3β1 generates different effects in the regulation of both adhesion and migration processes, depending on the cell type and the environment. To date, the mechanism by which α3β1 exerts its regulatory role is not clearly unraveled. One hypothesis is that α3β1 may interfere with the formation of typical adhesion complexes by segregating and/or redistributing focal adhesion-associated proteins to another subcellular localization. Alternatively, α3 may interfere directly with the binding of integrins to intracellular proteins present in typical adhesion complexes, by masking or inducing a conformational change in the β1-cytoplasmic domain. Surface plasmon resonance studies revealed that the cytoplasmic domain of the β1 integrin subunit interacts with low affinity with the cytoplasmic tail of the α3 integrin subunit, but not with those of several other α subunits (Laplantine *et al.*, 2000). Another hypothesis is that α3β1 integrin may also regulate the function of other integrins by changing signaling events involved in transduction pathways associated with other integrins. Our results indicated that a RhoA-mediated signaling pathway was responsible for the α2β1/α3β1 integrin switch. The differentiation of HT-29 cells was associated with a decrease in active RhoA-GTP proteins. Active Rho-GTPases may be a limiting factor in epithelial cells. Hence, regulation of their activities and/or subcellular localization by cell-substrate interactions may affect important processes such as intercellular adhesion, differentiation, and migration.

Key Role of Laminin-5 in α3β1 Activation

In HT-29 cells, activation of α3β1 is not constitutive but rather depends on LN 5 secretion. Recent work indicates that the globular module G3 of the LN 5 α3-chain is required for this activation and for rapid cell adhesion and migration (Hirosaki *et al.*, 2000). We found that during the early stage of cell differentiation, HT-29 cells produce more LN 5 that activates α3β1 and switches off α2β1. Both α3 and α6 integrins, which are involved in LN 5 interaction on HT-29 cells, could trigger this switch. Furthermore, once activated the α3β1 integrin becomes a major receptor for collagen. The possibility that the increased adhesion to CO IV is mediated by LN 5 located at the cell surface can be excluded. Indeed, the γ2 chain of LN 5 cannot bind nidogen (Mayer *et al.*, 1995) and therefore LN 5 alone cannot associate with CO IV (Yurchenco *et al.*, 1985; Rousselle *et al.*, 1997). Furthermore, FACS experiments performed with the anti-LN 5 mAb indicated that no LN 5 was detected at the cell surface of HT-29 Gal cells before adhesion (data not shown). The mechanism by which LN 5 induces the integrin switch is probably associated with its effect on signaling pathways and in particular on the level of RhoA-GTP. Similar effects have been described in keratynocytes; Nguyen at al. (2000) showed that deposition of LN 5 onto CO IV substratum induced a change in signaling from a Rho-dependent to a PI3-kinase–dependent pathway.

The effect of LN 5 on integrin activation, which in turn modulates cell adhesion and/or migration, appears to be associated with the stage of epithelial cell differentiation. Treatment of HT-29 cells with anticancer drugs, 5-fluorouacil or methotrexate, generated the clones HT-29FU and HT-29MTX, which are committed to differentiate into specific phenotypes (Lesuffleur *et al.*, 1990, 1991). These differentiated subclones also produce more LN 5 than parental HT-29 cells. In contrast, highly differentiated HT-29 cells or the differentiated colon carcinoma Caco-2 cells produce almost no LN 5 (Orian-Rousseau *et al.*, 1998 and our personal observations). These observations suggest that LN 5 is not involved in the later stages of cell differentiation.

The activation of α3β1 integrin by LN 5 could be mimicked in vitro by low concentrations of P1B5 or ASC1. The mechanism by which these antibodies induced the α3β1

integrin activation is not clear. P1B5 treatment was neither associated with a modification of integrin clustering nor with integrin redistribution as observed in Takeuchi *et al.* (1994). In HT-29 cells, although $\alpha3\beta1$ was localized at the cell surface the integrin did not participate in homotypic adhesion (data not shown). Therefore, we favor the view that PIB5 binding to $\alpha3$ integrins induces a conformational change that is responsible for their activation. One possible explanation is that at low concentrations of P1B5, this antibody stimulates outside-in signaling of a subpopulation of $\alpha3\beta1$ that then activates the remaining $\alpha3\beta1$ to bind CO IV. High concentrations of P1B5 may also stimulate these activating signals but would fail to result in increased binding to CO IV, because all $\alpha3\beta1$ is bound to P1B5 and unavailable for CO IV binding.

Physiological Implications for Trans-dominant Inhibition Mediated by α3 β1

The switch between $\alpha2\beta1/\alpha3\beta1$ integrins may participate in the differentiation processs that occurs along the crypt-villus unit in at least two ways. First, the substitution for RhoA by $\alpha3\beta1$-mediated cell-substrate adhesion may favor E-cadherin–based cell-cell adhesion, a step in the differentiation process that required RhoA proteins (Braga *et al.*, 1997). Second, the activation of $\alpha3\beta1$ by LN 5 present in the basement membrane could trigger cell migration along the crypt-villus unit. Indeed, the mechanism of epithelial cell migration in the adult intestine remains obscure, this process probably does not involved a classical cell migration implicating focal adhesions, as well as the detachment of the cell surface receptors as previously described (Lauffenburger and Horwitz, 1996). Probably, it involves instead a sliding of the differentiated cells along the basement membrane of the crypt-villus axis, pushed by the lower proliferative cells. This possibility is emphasized by the fact that most $\alpha3\beta1$ integrins are not concentrated into focal adhesions (DiPersio *et al.*, 1995) and that $\alpha3\beta1$ is a low-affinity receptor for CO IV.

The involvement of integrin switching during colon cell differentiation is supported by analysis of the expression of integrins and basement membrane molecules in the human intestine (reviewed by Beaulieu, 1999). LN 5 displays a concentration gradient increasing from the base to the tip of the villus (Leivo *et al.*, 1996; Orian-Rousseau *et al.*, 1996). Furthermore, this spatial distribution of LN 5 is associated with a differential crypt-villus pattern of expression for laminin-binding integrins: $\alpha2\beta1$ and $\alpha3\beta1$ displayed complementary staining patterns for the lower crypt ($\alpha2^+$, $\alpha3^-$) and upper crypt-villus regions ($\alpha2^-$, $\alpha3^+$) (Beaulieu 1992).

The $\alpha3\beta1$ integrin-dependent migration on LN 5 could also be relevant to tumor invasion. Studies of LN 5 expression in various human cancers indicate that this protein is specific to cells of epithelial origin (Pyke *et al.*, 1995). It is noteworthy that in colon adenocarcinomas, the LN 5 staining was invariably associated with budding cancer cells located at the invasive front of the malignant epithelium.

In this study we provide new insight into the key role of $\alpha3\beta1$ integrin as a "regulator" integrin of epithelial cell differentiation. Clearly, $\alpha3\beta1$ function is more complex than that of a simple adhesion receptor. It appears to modulate events that are mediated by other transmembrane receptors, including integrins and cadherins. A next decisive step will be to unravel the signal transduction cascade involved in $\alpha3\beta1$ regulation and function.

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