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Involvement of Laminin Binding Integrins in Human Intestinal Epithelial Cell Functions

By

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Département d'anatomie et de biologie cellulaire

Thesis presented to the Faculté de médecine in partial Fulfilment of the requirements for a Philosophae Doctor (Ph.D) in Cellular Biology

June 1998

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ABSTRACT

In this study we sought to determine the expression of three laminin binding integrins, previously poorly or uncharacterized, in intestinal epithelial cells: α9β1, α7β1 and α6β4. The expression and distribution of each of these integrins was characterized in the developing and mature, human small and large intestine. Complementary studies were carried out using two intestinal epithelial cell models, HIEC, which are crypt-like cells, and Caco-2/15 cells ,which are villus-like. These experiments allowed us to associate each individual integrin to a particular cellular function.

α9β1 was shown to be absent in mature intestinal epithelium. It was however, associated with highly proliferative cells, such as those found in the developing crypts in fetal intestine, as well as in HIEC and Caco-215 cells where, in the latter, α9β1 expression was down-regulated as the cells stopped proliferating and undertook their differentiation. This integrin was also found to be reexpressed in an onco-fetal like pattern of expression in a subset of colon cancers.

α7Bβ1was present in the intestine and was found to be restricted to the crypt-villus junction, which suggested a correlation with the onset of differentiation. This pattern of expression was reproduced in Caco-2/15 cells where α7B protein levels peaked between confluence and five days post-confluence coinciding almost perfectly with laminin-1 deposition, which was shown to be critical for triggering terminal differentiation in these cells.

We determined that α6 associates predominantly with β4 in HIEC and Caco-2/15 cells and have identified a novel β4 variant expressed by HIEC, confirming the pattern of expression of β4 in crypt cells observed in vivo. Moreover, we have determined that the α6β4 receptor in HIEC is inactive in terms of adhesion to laminin-5, contrary to Caco-2/15 cells which use α6β4 to bind this ligand.

Taken together, these results show that intestinal epithelial cells express a number of laminin binding integrins and that each is associated with a distinct cell function.

Implication des intégrines liant la laminine sur les fonctions cellulaires de l'épithélium intestinal humain

Par

Nuria Basora

Thèse présentée à la Faculté de médecine en vue d'obtention du grade Philosophae Doctor (Ph.D) en biologie cellulaire, Université de Sherbrooke, Sherbrooke, Qc.

RÉSUMÉ

Le but de cette étude était de caractériser l'expression et la distribution des intégrines capables de lier la laminine dans l'intestin humain. La laminine-1 a été identifiée comme étant un élément critique pour l'induction de la différenciation entérocytaire mais la ou les intégrine(s) responsable(s) de médier ces effets demeure(nt) inconnue(s). Trois intégrines ont étés choisis dû au fait qu'elles étaient, jusqu'à présent, peu ou pas caractérisées dans ce modèle: α9β1, α7β1 et α6β4. L'expression et la distribution de chacune des ces intégrines ont été étudiées dans l'intestin fœtal and adulte ainsi que dans nos modèles cellulaires, les cellules HIEC, qui se comportent comme des cellules de la crypte, et les Caco-2/15, qui exhibent les caractéristiques des cellules de la villosité.

α9β1 est exprimé dans l'épithélium intestinal au niveau des cryptes au stade fœtal, mais est absente chez l'adulte. Cette distribution suggère que cette intégrine puisse être associée avec un état prolifératif, ce qui à été confirmé dans nos modèles in vitro. De plus, une réexpression d'α9β1 a été observée au niveau d'adénocarcinomes de côlon, suggérant une expression de type onco-fœtale.

α7Bβ1 est aussi exprimée au niveau de l'intestin fœtal et adulte mais son expression est restreinte à la jonction crypte-villosité, une distribution qui corrèle bien avec l'initiation de la différentiation. Cette intégrine est présente chez les cellules Caco-2/15 et une augmentation de son expression est observée lors de l'atteinte de la confluence, soit au même moment où les cellules commencent à déposer la laminine-1 à leur base.

Concernant α6β4, nous avons confirmé, dans un premier temps, que α6 s'associe principalement avec β4 dans les cellules épithéliales intestinales. Dans un deuxième temps, nous avons identifié une nouvelle forme de la sous-unité β4 présente dans les cellules indifférenciées, confirmant le patron d'expression observé in vivo. De plus, le récepteur α6β4 exprimé dans les cellules HIEC est inactif en terme d'adhésion à son ligand, la laminin-5.

Les résultats obtenus dans ce travail démontrent que malgré le fait que plusieurs intégrines soient capables de lier le même ligand, dans ce cas la laminine, elles sont associées avec des fonctions cellulaires distinctes.

CONTENTS

LIST of ABBREVIATIONS

BM	basement membrane
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular related kinase
FAK	focal adhesion kinase
HIEC	human intestinal epithelial cells
MAPK	mitogen activated protein kinase
MCMD	merosin-deficient congenital muscular dystrophy
TGF	tumour growth factor
SI	sucrase-isomaltase

I. INTRODUCTION

1. Morphogenesis of the human intestine

In man, the intestine begins to develop early in gestation. At 7 weeks, the intestine consists of a tube containing stratified epithelial cells surrounded by concentric mesenchymal cell layers. By 9 weeks, villus formation begins and is covered by a simple epithelial cell layer. At this stage, morphological development is accompanied by functional development and markers such as sucrase-isomaltase (SI) and lactase are already expressed (Ménard 1989; Ménard and Beaulieu, 1994) The crypts form at approximately 16 weeks, and by mid-gestation the fetal small intestine morphologically and functionally resembles the adult intestine. Colon development parallels small intestinal morphogenesis. Surprisingly, between 9-20 weeks, the formation of villi, as well as expression of all small intestinal brush border hydrolases, is seen in the fetal colon, and strikingly resembles the fetal small intestine. These features are transitory, and disappear during the second half of gestation. At birth, the colon has acquired its mature glandular architecture after a substantial morphogenic rearrangement (Ménard, 1989; Ménard and Beaulieu, 1994)

1.1 The Adult Crypt-Villus Axis

The adult intestinal epithelium is in constant and rapid renewal. Within its functional unit, the crypt-villus axis, are two distinct cell populations, the

proliferative and poorly differentiated cells located in the crypts and the functionally differentiated enterocytes of the villus (Leblond, 1981; Gordon, 1989; Louvard et al., 1992). The intestinal epithelium contains five main cell populations: i) absorptive cells, or enterocytes, which are, by far, the principal cell type, ii) mucus secreting goblet cells, iii) endocrine cells and iv) Paneth cells which are differentiated but remain in the crypts and v) stem cells. The stem cells, which are located at the base of the crypts, generate proliferative, immature cells which migrate up the axis and begin to differentiate as they reach the crypt-villus junction (i.e. the top third of the crypt and base of the villi). These differentiated enterocytes continue to migrate up the villus until they reach the tip, at which point these cells are exfoliated into the lumen (Leblond, 1981; Potten and Loeffler, 1990.) The segregation of these two distinct populations consequently results in the segregation of genes associated with proliferation and differentiation, in the crypt and villus, respectively, resulting in the establishment of a differentiation gradient regulating the proliferation, migration, differentiation and exfoliation essential in the dynamic renewal of the intestinal epithelium (Louvard et al., 1992; Ménard and Beaulieu, 1994). This gradient is defined by cell state specific morphological and functional characteristics which distinguishes the crypt cells from the villus cells (Gordon, 1989; Traber et al., 1991; Louvard et al., 1992; Sykes and Weiser, 1992; Ménard and Beaulieu, 1994) . For example, MIM-1/39, an antigen found only in the secretory granules of proliferative cells, is not detected in villus cells (Beaulieu et al., 1992). Functional enterocytes are mainly and easily identified by the restricted expression of digestive enzymes,

including mature sucrase-isomaltase (SI) and lactase (Koldovski, 1981; Louvard et al., 1992; Ménard and Beaulieu, 1994). Processes controlling the expression of genes specifically associated with proliferation or differentiation are assumed to be tightly and precisely regulated along the crypt-villus axis (Boyle and Brenner, 1995; Podolvsky, 1993).

1.2. The Basement Membrane

An important influence in the maintenance and/or regulation of cell phenotype has been attributed to the local micro-environment. For epithelial cells, this is largely defined by the composition of the basement membrane, with which they are in intimate and constant contact. Basement membranes (BM) are specialized regions of the extracellular matrix (ECM) located at the epithelial-mesenchyme interface. Intestinal BM contains ubiquitous molecules such as type IV collagen, heparin sulfate proteoglycans and laminins, as well as other ECM molecules including fibronectin, tenascin and decorin (reviewed in Beaulieu, 1997 and ref. therein). Both epithelial and mesenchymal cells directly contribute to the production and deposition of the various constituents of the basement membrane (Louvard et al., 1992; Adams and Watt, 1993; Simon-Assmann et al., 1993; Beaulieu, 1997). Some of these molecules such as type IV collagen, are uniformly distributed throughout the BM of the crypt-villus axis, while others such as fibronectin and tenascin are reciprocally expressed (Probstmeier et al., 1990; Beaulieu, 1992; Beaulieu et a., 1991; 1993).

Accumulating evidence has demonstrated that molecules found in the basement membrane can play not only permissive, but instructive, roles in directing various critical cellular functions including adhesion, migration, proliferation, differentiation and cell survival (Adams and Watt, 1993; Simon-Assmann et al., 1993). A number of heritable disorders containing genetic mutations in ECM molecules have been identified. For example, Alport's syndrome is due to mutations in the α5 chain of type IV collagen (Weber et al., 1992; Tryggvason et al., 1993), certain forms of Junctional Epidermal Bullosa are caused by mutations in laminin-5, and autosomal recessive (or merosin-deficient) congenital muscular dystrophy (MCMD) is caused by mutations to the α2 chain of laminin-2.

Many of the biological effects exerted by the BM have been attributed to laminins, the most abundant non-collagenous proteins in the BM (Timpl, 1989; Yurchenco and Schittany, 1990; Timpl and Brown, 1996). For example, laminins have been shown to promote migration, adhesion, proliferation and differentiation (Adams and Watt, 1993; Timpl and Brown, 1996). Laminins are a growing family of heterotrimeric glycoproteins each composed of an α, β and γ chain (Beck et al., 1990; Engel, 1992; Paulsson, 1992; Engvall, 1993; Burgeson et al., 1994;). Genetically distinct variants exist for each chain and so far eleven different structurally related proteins (laminin-1 to 11) have been identified (reviewed in Burgeson et al., 1994). The distribution of laminin variants is tissue specific and

a single tissue can express different laminins (Engvall et al., 1990; Beaulieu and Vachon, 1994; Wewer and Engvall, 1994).

In the adult small intestine three laminins have been identified: laminin-1 ($\alpha1\beta1\gamma1$), 2 ($\alpha2\beta1\gamma1$), and 5 ($\alpha3\beta3\gamma2$). Laminin-1 and 5 are located in the BM underlying the villus cells while laminin-2 is restricted to the crypts (Beaulieu and Vachon, 1994; Simon-Assmann et al., 1994; Aberdam et al., 1994; Leivo et al., 1996). The mutually exclusive distribution of laminin-1 and 2 was subsequently shown to be functionally significant. Indeed, it was clearly demonstrated that laminin-1 could precociously induce functional differentiation as determined by increased expression of SI and lactase in Caco-2/15 cells, while laminin-2, although highly similar to laminin-1, did not induce these effects (Vachon and Beaulieu, 1995). Both molecules, however, increased expression of other enzymes such as alkaline phosphatase, aminopeptidase N and dipeptidylpeptidase IV, which are expressed by all intestinal epithelial cells. These studies concluded that laminin-1 and laminin-2 were functionally distinct and that laminin-1 was critical for triggering terminal differentiation of intestinal epithelial cells, an observation which was later confirmed (Simon-Assmann et al., 1995).

2. Integrins

The primary mediators of ECM-cell interactions are a family of receptors known as integrins. These are heterodimeric, $\alpha\beta$, proteins and, to date, 16α chains and 8β chains have been identified, which can combine to form twenty

different receptors (Humphries, 1990; Hynes, 1992) Those integrins which use ECM molecules as ligands are the β1 family, (α1-9 β1), and β4 which associates only with α6. Some integrins, for example α5β1 and α6β1, bind single ligands, fibronectin and laminin respectively, while others, such as α2β1, can bind either collagen or laminin according to cell type. Similarly, molecules can be recognized by various integrins: laminin is a ligand for α1β1, α2β1, α3β1, α6β1, α7β1 and α6β4 (Juliano, 1993; Kuhn and Eble, 1994; Mercurio, 1995). Although some ligands are recognized by more than one integrin, numerous studies have made it apparent that each individual integrin is responsible for markedly distinct functions in mediating crucial cell processes including adhesion, migration, proliferation, differentiation and survival (Hynes, 1992; Giancotti, 1997; Meredith and Schwartz, 1997).

2.1 Integrin Mediated Cell Growth

It has long been observed that cell growth is anchorage-dependent and that several molecules can regulate proliferation, the best known examples being vitronectin via αvβ3 and fibronectin via α5β1, the latter in particular has been extensively documented (reviewed in Varner and Cheresh, 1996). The involvement of α5β1 in the regulation of cell growth was shown by transfecting α5 into CHO cells (Giancotti and Ruoslahti, 1990). Expression of α5β1 promoted cell adhesion to its ligand and significantly slowed proliferation, re-establishing normal growth characteristics. Moreover, these cells displayed a loss of

tumourgenicity. A similar result was obtained when α5 was introduced into HT-29 cells, an intestinal epithelial cell line (Varner et al., 1995). In the absence of fibronectin, α5β1 inhibited proliferation but if the cells where plated onto this substrate inhibition was reversed raising the possibility that ligated and unligated α5β1 send different signals affecting the cell's decision to proliferate or not.

A role for the regulation of cell growth has also been defined for a splice variant of the β1 subunit, β1c (Languino and Rouslahti, 1992; Meredith et al., 1995). Overexpression of this subunit inhibited cell cycle progression, apparently in a ligand independant manner, although the exact mechanism involved in growth arrest has not been elucidated.

2.2 Integrin Mediated Differentiation

A second critical role of integrins is their participation in the induction of cell specific gene expression. The key role that BM plays in triggering terminal differentiation has been demonstrated for several different cell types and examples include neurite outgrowth by thrombospondin, vitronectin and tenascin, albumin synthesis in hepatocytes by laminin and tubule formation of endothelial cells by laminin and collagens (for review see Adams and Watt, 1993). These effects can either be indirect or direct. One of the best characterized systems is induction of milk proteins in mammary epithelial cells. These cells become polarized and secrete milk proteins apically when cultured on reconstituted BM (i.e. Matrigel) or collagen gels (Hall et al., 1982, Lee et al., 1985). Later studies

showed that collagen was directly responsible for observed morphogenesis, including formation for acini and cell polarization, but that acquisition of a differentiated phenotype causing secretion of milk proteins was actually regulated by the endogenous production of laminin stimulated by the exogenous collagen (Streuli et al., 1991). The direct regulation of laminin on gene expression was demonstrated by studying activation of the β-casein promoter (Streuli et al., 1995) and this was determined to be integrin dependent with the use of β1-inhibitory antibodies. Simultaneous exposure to lactogenic hormones are nonetheless required suggesting that integration of signals sent by both soluble factors and ECM are necessary for complete morphological and functional differentiation.

Collagen has similar morphogenic effects on intestinal cell lines. Human colon carcinoma cell lines formed well-organized glandular structures when grown in 3-D collagen gels, Matrigel or on normal rat mesenchymal cells (Pignatelli et al., 1992; Pignatelli and Bodmer, 1989; Liu et al., 1994). This morphogenetic differentiation was later shown to involve α2β1, but not α3β1 (Liu et al., 1994). Glandular structure formation required cell migration which was also mediated by α2β1. These effects, i.e. migration and morphogenic differentiation, were enhanced by growth factors TGFα and TGFβ whose morphogen activity was dependent on cell adhesion (Liu et al., 1994) and was independant of cell proliferation.

2.3 Integrin Mediated Adhesion/Maintenance

Epithelial cells are characterized by attachment to an underlying BM, polarization and specialized cell-cell contacts. Loss of epithelial characteristics are a hallmark of neoplastic transformation which leads to altered phenotype (e.g. dedifferentiation), anchorage-independent growth, usually associated with a resistance to apoptosis and increased invasiveness. Changes in BM composition and integrin profiles have been observed in various types of cancer, including colon cancers, and are, therefore, assumed to contribute to tumor progression (Juliano, 1993; Sheppard, 1996; Varner and Cheresh, 1996; Beaulieu, 1997).

Cell adhesion to BM is orchestrated and maintained through distinct complexes interacting with the actin and intermediate filaments. The β1 family is directly linked to the actin cytoskeleton and upon ligation they cluster into focal adhesions (Clarke and Brugge, 1995; Williams et al., 1994). α6β4, on the other hand, localizes to hemidesmosomes upon binding laminin-5 and the association of a variety of proteins links it to the intermediate filaments (Giancotti, 1996; Borradori and Sonnenberg, 1996). The formation of either type of adhesion complex requires the participation of kinases and phosphatases (Clarke and Brugge, 1995; Giancotti, 1996)

Integrins play a structural role in linking the ECM with the cellular actin cytoskeleton and regulate cell shape, cell migration and tissue architecture. Although integrins have no intrinsic kinase activity, integrin mediated adhesion to ECM induces calcium influx, changes in phosphoinositoside metabolism and stimulates the increase of tyrosine and serine phosphorylation of signaling

proteins. Integrin signaling is coordinated via a non-receptor tyrosine kinase known as focal adhesion kinase, or FAK, which, upon ECM recognition, is capable of phosphorylating actin binding proteins, subsequently resulting in important actin reorganization. Activated FAK also recruits a number a signaling molecules via SH2/SH3 interactions, resulting in the activation of signaling cascades (reviewed in Clarke and Brugge, 1995; Giancotti, 1997; Schlaepfer and Hunter, 1998), and has been reported to participate in various cell functions such as cell growth and, more recently, in apoptosis (Frisch et al., 1996; Khwaja et al., 1997). The final cell response is regulated by the combination and integration of integrin and growth factor signaling events (Miyamoto et al., 1996, Sastry et al., 1997).

α6β4, as mentioned above, is associated with the intermediate filaments, and does not induce FAK activation. The unique cytoplasmic tail confers distinct properties to the β4 subunit (Suzuki and Naithoh, 1990; Hogervost et al., 1990; Tamura et al., 1990), the best characterized of which is its role in mediating hemidesmosome formation (Dowling et al., 1996; VanderNeut et al., 1996). Upon binding its ligand, laminin-5, α6β4 becomes phosphorylated by an integrin-associated kinase (Mainiero et al., 1995) and subsequently recruits adaptor molecule Shc, then Grb2-mSOS, which activates Ras signaling pathways. Besides adhesion, α6β4 also regulates cell growth (Clarke et al., 1995; Mainiero et al., 1997;) and is strongly suspected to offer protection against anoikis (Dowling et al., 1996).

Although many of the pathways and functions mediated by integrins appear to be highly redundant, it has become quite apparent that each integrin is responsible for markedly distinct functions. Epithelial cells simultaneously and constitutively express a number of laminin binding integrins at their cell surface (Mercurio, 1995; Sheppard, 1996; Beaulieu, 1997). Any attempt to associate an individual, or a given set of integrins, with a specific cell function must begin with the identification of the integrin population present in the cell type studied.

3. Experimental Outline

In this work we sought to characterize laminin-binding integrins and their individual involvement in intestinal epithelial cells in order to better understand how crucial cell functions are regulated in these cells. We have focused on three integrins whose distribution in intestinal epithelium were poorly or uncharacterized: $\alpha 9\beta 1$, $\alpha 7\beta 1$ and $\alpha 6\beta 4$.

$\alpha 9\beta 1$ is a poorly characterized integrin. It has been localized in various cell types and has been reported to be expressed in fetal colonic epithelium (Palmer et al., 1993; Dieckgraeffe, et al., 1996). This integrin recognizes tenascin-C (Yokosaki et al., 1994; Weinacker et al., 1995), osteopontin (Smith et al., 1996) and one report has identified laminin as a possible ligand (Forsberg et al., 1994). Further studies have demonstrated that $\alpha 9\beta 1$ can directly stimulate proliferation upon binding to tenascin-C and can also activate MAPK signaling pathways.

α7β1 is a specific laminin receptor which binds laminin-1 and 2, but not laminin-5 (Echtermeyer et al., 1996; Yao et al., 1996a; Yao et al., 1996b). This integrin is largely responsible for laminin induced differentiation of striated muscle (Von der Mark et al., 1991; Song et al., 1993; Vachon et al., 1996). Moreover, α7 exists as one of three isoforms, α7A, α7B and α7C, and these variants are developmentally regulated during myogenesis suggesting distinct functions, consistent with their differential location in myofibers (Collo et al., 1993; Song et al., 1993; Ziober et al., 1993; Wang et al., 1995). Although considered as a muscle specific integrin, expression of α7B had been detected in non-muscle cell types (Collo et al., 1993).

α6β4 distribution and function have been extensively characterized and our group has previously reported the expression of the β4 subunit in intestinal epithelium (Beaulieu and Vachon, 1994; Perreault et al., 1995). Lack of classical hemidesmosomes in these cells suggests alternative functions for this receptor. Routine screening of adult small intestine with two different antibodies directed against the β4 subunit surprisingly resulted in two distinct patterns of expression indicating the presence of at least two different β4 proteins (this study). This differential expression was studied in depth to determine if the presence of two distinct β4 subunits was associated with function or with cell state.

In this work we have characterized the expression of these three integrin receptors in human small and large intestine, during development and in the adult. The association of spatio-temporal patterns of expression with a morphogenic

event or with a specific cell population should provide clues as to their possible function. The biochemical characterization, as well as functional analysis, of these integrins were carried out in two intestinal cell models. The first in vitro cell model was the HIEC (Perreault and Beaulieu, 1996), human normal intestinal epithelial cells which are poorly differentiated and proliferative, i.e. crypt-like. The second cell model is the Caco-2/15 cell line. These cells are capable of spontaneously undertaking an enterocyte-like differentiation program upon reaching confluence (Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1992; Vachon et al., 1996).

II- ARTICLE

Regulated expression of the integrin $\alpha 9\beta 1$ in the epithelium of the developing human gut and in intestinal cell lines: relation with proliferation*

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Regulated expression of the integrin α9β1 in the epithelium of the developing human gut and in intestinal cell lines: relation with cell proliferation

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ABSTRACT

The integrin α9β1 is one of the recently identified integrins whose expression is restricted to specialized tissues. Its exact function is still unknown. In the present study, we have analyzed the expression of the α9 subunit in human fetal and adult small intestinal and colonic epithelia as well as in intestinal cell lines by indirect immunofluorescence, immunoprecipitation, Western blot and Northern blot. In intact tissues, the antigen was restricted to the basolateral domain of epithelial cells in intestinal crypts at the fetal stage and was absent in the adult. The α9β1 integrin was also detected in the intestinal cell lines HIEC-6 and Caco-2/15. The presence of α9β1 in HIEC-6 was found to be consistent with their proliferative crypt-like status. In Caco-2/15 cells, the integrin was present at high levels in proliferating cells but was down-regulated when cells cease to grow and undertake their differentiation. EGF treatment, which is known to maintain Caco-2/15 cells in a proliferative state, resulted in higher levels of α9 as compared to control cells. Taken together, these observations suggest a relation between integrin α9β1 expression and proliferation in human intestinal cells.

INTRODUCTION

Integrins are a superfamily of cell surface receptors that mediate cell-cell and cell-extracellular matrix (ECM) interactions. These αβ heterodimer glycoproteins provide a structural and functional bridge between extracellular molecules and cytoskeletal components, and are involved in mediating signal transduction processes (Clark and Brugge 1995; Rosekelly et al., 1995). So far, 16 α and 8 β subunits have been identified, but it is mainly the integrins of the β1 family that are responsible for ECM-cell interactions. In epithelia, β1 integrins known to be widely expressed include α1β1, α2β1, α3β1, α5β1 and α6β1 (Mercurio 1995; Sheppard 1996; Beaulieu 1997a). Some of them are ligand-specific such as α5β1 and α6β1, which bind exclusively to fibronectin and laminins, respectively, while others such as α2β1 and α3β1 can use collagens and/or laminins depending on the cell type. The presence of multiple integrins on the cell surface allows cells to recognize and respond to a variety of different ECM molecules in the promotion and regulation of adhesion, migration, growth, apoptosis and differentiation (Akiyama et al., 1990; Hynes 1992; Rosekelly et al., 1995; Assoian and Zhu, 1997; Meredith and Schwartz 1997).

The intestinal epithelium is an advantageous system for analysis of cell-matrix interactions in relation to the cell state in the intact organ (Beaulieu 1997a). Indeed, this epithelium, which is in constant and rapid renewal, consists of spatially confined proliferative and differentiated cell compartments, located respectively, in the crypts and on the villi . Functionally, the crypt-villus axis is further defined by typical morphological and functional properties displayed by the mature enterocyte, which clearly distinguish it from the crypt cell (Leblond 1981; Louvard et al., 1992; Ménard and Beaulieu 1994; Beaulieu 1997b). The regulation of intestinal cell growth and differentiation is susceptible to various influences along the crypt-villus axis (Boyle and Brenner 1995; Podolsky and Babyatsky 1995), including differential cell-matrix interactions (Beaulieu 1997a, 1997b). Indeed, analysis of β1 integrins in the human small intestine has demonstrated peculiar patterns of expression for many of them. For instance, in the adult, the integrin α2β1 is

predominantly expressed by crypt cells and α3β1 is mainly expressed by villus cells, whereas the α6 subunit is present throughout the epithelium (MacDonald et al., 1990; Beaulieu 1992; Beaulieu and Vachon 1994). Furthermore, the α7Bβ1 integrin, which has been recently identified in the human intestinal epithelium, is primarily located at the crypt-villus junction, a distribution that coincides with the onset of epithelial cell differentiation (Basora et al., 1997). Interestingly, patterns of expression for these integrins were found to be already established by mid-gestation (Perreault et al., 1995; Basora et al., 1997; Beaulieu 1997a).

The integrin α9β1 is one of the most recently discovered integrins (Palmer et al., 1993). In the mouse, it has been identified in smooth and skeletal muscles as well as in a few other specialized tissues such as squamous and airway epithelia (Palmer et al., 1993; Wang et al., 1995). A well-characterized ligand for the α9β1 integrin is tenascin-C (Yokosaki et al., 1994; Weinacker et al., 1995). Recent observations suggest that α9β1 may also serve as a receptor for osteopontin (Smith et al., 1996). The expression of α9β1 has not yet been studied in the small intestine but this integrin is absent from the normal adult colonic epithelium (Palmer et al., 1993; Basora et al., 1998). However, the α9 integrin subunit has been reported in the fetal colonic epithelium (Dieckgraefe et al., 1996) and, interestingly, was also found in adenocarcinomas of the human colon (Basora et al., 1998), suggesting that the expression of this integrin may be related to extensive remodeling/cell proliferation-related events such as those occurring in the intestinal epithelium during fetal development and tumorigenesis. This possibility prompted us to examine the expression of the α9β1 integrin in relation to intestinal cell proliferation. Using the intact fetal small intestine and colon as well as intestinal cell lines, we demonstrate in the present study that the expression of the α9β1 integrin is predominantly associated with proliferative intestinal cells both in vivo and in vitro.

MATERIALS AND METHODS

Tissues

Specimen of small intestine and colon from 15 fetuses ranging in age from 15 to 18 weeks (post-fertilization) were obtained after legal abortion. Ten samples of adult small intestine (jejunum) and colon were obtained from non-diseased parts of resected segments (resection margins). The project was in accordance with protocols approved by the Institutional Human Research Review Committee for the use of human material. Only tissues obtained rapidly (in less than 40 minutes) were used in the present study.

In some experiments, fetal small intestinal and colonic epithelia were separated from mesenchymes by using a procedure derived from the method described for the isolation and production of HIEC cell lines (Perreault and Beaulieu 1996), which allows high rates of recovery of pure intestinal epithelial cell preparations (Perreault and Beaulieu 1998).

Cell culture

The Caco-2/15 cell line, a stable clone of the parent Caco-2 cell line (Pinto et al., 1983), has been characterized elsewhere (Beaulieu and Quaroni 1991; Vachon and Beaulieu 1992; Vachon et al., 1996). These cells are unique in that upon confluence they spontaneously undergo a gradual enterocytic differentiation process, similar to that observed in the epithelium of the intact fetal small and large intestine (Ménard and Beaulieu 1994, Zweibaum and Chantret 1989). Cells between passages 54 and 72 were cultured in plastic dishes as described (Vachon and Beaulieu 1992). In this study, Caco-2/15 cells were analyzed 2 days before confluence (50-60 % confluence) and at 0-24 days of post-confluence. In some experiments, EGF (Gibco-BRL, Life Technologies, Burlington, Ont) was added to the culture medium at a final concentration of 20 ng/ml from the day after seeding (day -5) to 4 days after confluence. It is noteworthy that Caco-2/15 cells produce negligible amounts of endogenous transforming growth factor-α/EGF-like activity (Beaulieu and Quaroni 1991).

The HIEC-6 cell line has been generated from the normal fetal human small intestine (Perreault and Beaulieu 1996). These cells express a number of crypt cell markers but no villus cell markers and are thus considered as poorly differentiated crypt-like cells (Perreault and Beaulieu 1996, Quaroni and Beaulieu 1997). The HIEC cells were used between passages 5-10 and were grown as described (Perreault and Beaulieu 1996).

Primary antibodies

The antibodies used in this work were the monoclonal mAb13 (Akiyama et al., 1989) directed against the human β1 integrin subunit (kindly provided by Dr. S.K. Akiyama, Howard University Cancer Center, Washington, DC), HSI-14 (Beaulieu et al., 1989) directed against the human sucrase-isomaltase complex and CY90 (Sigma Chemicals Co, St-Louis, MO) specific to the human keratin 18 (Vachon et al., 1995). The production of the rabbit specific anti-serum directed against the α9 integrin subunit and the procedure to affinity purify the anti-α9 antibodies have been described previously (Palmer et al., 1993).

Immunofluorescence staining

The preparation and Optimum Cutting Temperature embedding compound (OCT; Tissue Tek, Miles Laboratories, Elkart, IN) embedding of specimens for cryo-sectioning was performed as previously described (Beaulieu et al., 1991). Cryosections 2-3 μm thick were cut on a Jung Frigocut 2800N cryostat (Leica Canada Inc., Saint-Laurent, Qué.), spread on silane-coated glass slides then air-dried for 1 h at room temperature before storage at -80 C. Tissue sections were fixed in fresh 2% paraformaldehyde (60 min, 4 C) before immunostaining as described previously (Beaulieu 1992; Beaulieu et al., 1991). Anti-α9 subunit affinity-purified antibodies were diluted 1:200 in PBS (pH 7.4) containing 10% Blotto. FITC-conjugated goat anti-rabbit IgG (Boehringer Mannheim Canada, Laval, Qué.) were used as secondary antibodies at a working dilution of 1:25. Sections were then stained

with 0.01% Evan's blue in PBS. Preparations were mounted in glycerol-PBS (9:1) containing 0.1% paraphenylene diamine and viewed with a Reichert Polyvar 2 microscope (Leica Canada) equiped for epifluorescence. In all cases, no immunofluorescent staining was observed when primary antibodies were omitted or replaced by appropriate non-immune sera.

Western blot analysis

Sodium dodecyl sulfate (SDS) /12% PAGE and Western blotting were performed as described previously (Vachon and Beaulieu 1995; Beaulieu et al., 1989). Briefly, proteins from the various cell lines were directly solubilized in sample buffer containing 5% ß-mercaptoethanol. Separated proteins (150 µg/lanes) were transferred onto nitrocellulose (ImmunoSelect, Gibco/BRL, Burlington, Ont) and stained with Ponceau red to localize molecular weight markers (44-220 kD range, BioRad, Mississauga, Ont.). Membranes were blocked overnight at room temperature in PBS (pH 7.4) containing 10 % Blotto and incubated with the primary antibody diluted in the blocking solution (anti-α9, 1:500; HSI-14, 1:500; mAb13, 1:1000; and CY90, 1:2000). After washing in PBS, membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse, -rabbit or -rat IgG (BioRad, Mississauga, Ont; Cedar Lane, Hornby, Ont), further washed and finally incubated with a chromogenic substrate for alkaline phosphatase detection according to the instructions of the manufacturer.

Immunoprecipitation

HIEC and Caco-2/15 cells at different stages of confluence were analyzed for the presence of the α9β1 complex according to a procedure described previously (Basora et al., 1998). Briefly, the cells were washed twice in ice-cold PBS and solubilized in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 2 uM PMSF, 50 ug/ml leupeptin, 50 ug/ml pepstatin, 100 ug/ml aprotinin) for 20 min on ice, then centrifuged for 15 min at 13,000 x g. Five ug of

mAb13 or a 1/200 dilution of the affinity purified anti-α9 was added to the samples for 15 h at 4°C followed by the addition of protein-G Sepharose (Gibco/BRL) for a 1 h incubation at 4°C. Immunoprecipitates were then processed for analysis by SDS-PAGE and Western blotting (see above).

Northern blot analysis

RNA was isolated from cell lines or tissue homogetates using Trizol reagent (Gibco/BRL). Total cellular RNA was electrophoresed on a 1%:1.8% agarose:formaldehyde gel (30-50 µg/lane) and blotted onto nylon membrane (Hybond-N, Amersham, Oakville, Ont.) as described previously (Beaulieu and Quaroni 1991). Radiolabeled probes were synthesized from a 2.1 kb fragment of α9 cDNA (Palmer et al., 1993) and a 1.3 kb fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA used as a control using the Multiprime Labeling System (Amersham, Oakville, Ont) in the presence of [α-^{32}P]dCTP. Prehybridization (1h) and hybridization (12h) were performed at 68°C. Kodak (New Haven, CT) Biomax MS films were exposed with intensifying screens for 2 h (GAPDH) and 42 h (α9) at -80°C.

RESULTS

Expression of the integrin α9 subunit in the human gut

The expression of the α9 subunit was first analyzed by indirect immunofluorescence in fetal and normal adult human small intestine and colon. In the fetal samples, the antigen was mainly detected at the basolateral surface of the epithelial cells located in the developing crypts in both the jejunum (Fig. 1A) and colon (Fig. 1C). The staining observed in the colon was found to be consistently stronger than in the small intestine. The antigen was also faintly detected in the cytoplasm of most epithelial cells located on both the villus and the crypt (Fig. 1A,C). Furthermore, a predominant staining in muscle layers was observed at all stages studied. The immunostaining in the epithelium was considered specific as it was not seen with a negative control affinity-purified rabbit antibody directed against another integrin subunit not expressed in the intestinal epithelium (the α7A subunit; 18). In adult specimens, the α9 subunit was not detected in the epithelium (Fig. 1B,D) but was present in the contractile network of the small intestinal mucosa (Fig. 1B) as well as in subepithelial myofibroblasts surrounding the lower two thirds of the colonic glands (Fig. 1D) and in the muscularis mucosa (Fig. 1 B,D).

Expression of the integrin α9 subunit in human intestinal cells

The expression of the α9 subunit by fetal intestinal epithelial cells was confirmed by Northern blot analysis. The epithelium was dissociated from its underlying mesenchyme and muscle layers in both small intestine and colon with a non-enzymatic method and the total RNA was extracted from each of these preparations. The purity of the preparations was confirmed by using different approaches including Western blot analysis for keratin 18 and vimentin contents (not shown; Perreault and Beaulieu 1998). As expected from immunofluorescence studies, high levels of α9 subunit transcripts were observed in the mesenchyme (Fig. 2, lanes 1,2) while a signal in the

corresponding isolated epithelia was consistently detected although much weaker in the jejunum (Fig. 2, lane 3) than in the colon (Fig. 2, lane 4).

The expression of the α9 subunit by intestinal cells was further investigated at the protein and mRNA levels in our two current in vitro models: the enterocyte-like Caco-2/15 cell line and the normal crypt-like cell line HIEC-6. Interestingly, the two forms of the α9 transcript (Palmer et al., 1993) were detected in both proliferating/poorly differentiated HIEC-6 (Fig.3, lane 1) and Caco-2/15 cells (Fig. 3, lane 2). However, in post-confluent Caco-2/15 cells, which gradually loose their ability to proliferate as they undertake their enterocytic differentiation program, a marked reduction in the relative amounts of the α9 transcripts (both forms) was consistently observed over time (Fig.3, lanes 3-7). Analysis of the α9 subunit at the protein level revealed similar findings. The α9 subunit was detected in both proliferating HIEC-6 and Caco-2/15 cells after immunoprecipitation with anti-α9 affinity purified antibodies and Western blot analysis with the same antibody (Fig. 4A, lanes 1 and 2). As expected from the transcript levels observed above, the relative amounts of α9 decreased substantially in post-confluent differentiating Caco-2/15 cells (Fig. 4A, lanes 3-5). The analysis of α9 immunoprecipitates for the β1 subunit showed that it co-precipitates with α9 in approximately the same proportion over the culture period (Fig. 4A) confirming that intestinal cells express α9 as a typical α9β1 complex. Comparison of the relative amounts of the β1 subunit associated with α9 complex (Fig. 4A) with the total β1 population (Fig. 4B) allows two observations. First, that quantitatively, α9β1 is not a major integrin in intestinal cells when considering that lysates used for α9 immunoprecipitation (Fig.4A) were five times more concentrated (10 mg protein/sample) than those used for total β1 immunoprecipitation (Fig. 4B; 2 mg protein/sample). Second, that in comparison with the β1 population (Fig. 4B), which is relatively stable over the culture period studied, α9β1 is indeed predominantly expressed in the proliferating subconfluent Caco-2/15 cells, as opposed to post-confluent quiescent cells (Fig. 4A).

Modulation of the integrin α9 subunit expression by EGF

The potential relation between expression of the α9 subunit and intestinal cell proliferation was further investigated in EGF-treated Caco-2/15 cells. EGF has been shown previously to inhibit sucrase-isomaltase expression and stimulate both DNA synthesis and proliferation of Caco-2/15 cells in postconfluent cultures (4-10 days after confluence) when added to the standard culture medium at a concetration of 20 ng/ml (Cross and Quaroni 1991). By using a similar experimental procedure, we found that the expression of the α9 subunit was significantly higher in lysates of EGF-treated Caco-2/15 cells than in the control cells grown under exactly the same conditions but without EGF, as determined by Western blot analysis (Fig. 5) and densitometry (Relative amounts of α9/K18: -EGF, 0.351 ± 0.045; +EGF, 0.576 ± 0.014; $p < 0.01$, n=4). As expected, a corresponding ~50% reduction in immunoreactive sucrase-isomaltase was observed (Fig. 5; relative amounts of SI/K18: -EGF, 0.937 ± 0.025; +EGF, 0.428 ± 0.144; $p < 0.025$, n=4), while the β1 subunit appeared to remain constant (data not shown, β1/K18: -EGF, 0.410 ± 0.042; +EGF, 0.508 ± 0.044; n=4).

DISCUSSION

The precise function of the α9β1 integrin still remains to be elucidated. Analysis of α9 subunit expression during murine embryogenesis has suggested that this integrin probably plays a role in the maturation and/or maintenance of a variety of differentiated tissues (Wang et al., 1995), a suggestion that fits well with the constitutive expression of the α9 subunit in contractile cells of both the developing and adult intestinal mucosa (Palmer et al., 1993; Dieckgraefe et al., 1996; this work). However, in some tissues, α9 expression is restricted to cells known to retain the capacity to proliferate, such as the basal layer of some squamous epithelia, which is indicative of other function(s) for this integrin (Palmer et al., 1993; Wang et al., 1995). Furthermore, in the mouse cornea, the pattern of expression for the α9 integrin suggests that it may be important in helping to maintain a proliferative epithelial cell phenotype (Stepp et al., 1995). Herein, we demonstrate that the expression of the α9β1 integrin is related to proliferation in human immature intestinal epithelial cells.

In the developing fetal small intestinal and colonic epithelia, the α9 subunit was detected according to a typical basolateral staining pattern predominantly confined to cells located in the crypt, which represent the proliferative compartment (Boyle and Brenner 1995; Podolsky and Babyatsky 1995). In contrast, the α9 subunit remained undetectable in the epithelium of the adult gut. These observations, which are in agreement with previous studies (Palmer et al., 1993; Wang et al., 1995), suggest that the α9 subunit is downregulated in mature small intestinal and colonic epithelia. This pattern of expression for an integrin in the gut is unusual as most other integrins present in the fetal intestinal epithelium remain at the adult stage (Beaulieu 1997a). However, the lack of α9 subunit expression in the proliferative compartments of adult intestinal epithelia is of interest as it may suggest that the presence of this integrin may not be required in these highly ordered renewing epithelia, and may even not be desirable for their regulation. In support of this later possibility, we have recently demonstrated that the α9 subunit is re-expressed in human colon adenocarcinomas (Basora et al., 1998). Such a pattern of expression for the α9 subunit in the gut appears consistent

with a possible relation between the presence of the integrin α9β1 and an onco-fetal cell proliferation status. In the support of this, Yokosaki et al. (1996) demonstrated recently that α9β1 expression in colon carcinoma cells and interaction with its ligand tenascin-C can promote proliferation in vitro.

To further investigate the potential relation between α9β1 and intestinal cell proliferation, we have analyzed its expression in two well characterized human intestinal cell lines: HIEC-6, which have been derived from normal human fetal small intestine (Perreault and Beaulieu 1996) and Caco-2/15 cells, an adenocarcinoma cell line that is unique in its ability to spontaneously undertake a program of fetal intestinal cell differentiation at confluence (Beaulieu and Quaroni 1991; Vachon and Beaulieu 1992; Vachon et al., 1996). Interestingly, both cell lines were found to express α9β1 as determined by immunoprecipitation and Northern blot analyses. The presence of the α9β1 integrin in HIEC-6 cells was consistent with their proliferative crypt-like status (Perreault and Beaulieu 1996; Quaroni and Beaulieu 1997) along with the fact that the integrin was also present in their in situ counterpart, the crypt. The pattern of α9β1 expression in differentiating Caco-2/15 cells revealed additional interesting findings. The α9β1 complex was present at relatively high levels in growing cells but declined rapidly over the first ~10 days of confluence, a culture period characterized by a substantial reduction in cell proliferation, DNA synthetic activity and expression of the proliferation-associated nuclear antigen Ki-67 (Cross and Quaroni 1991; Vachon et al., 1996). The sharp reduction seen for immunoreactive α9 was closely paralleled to a substantial decrease of its corresponding transcript, suggesting that the down-regulation occurs primarily at the mRNA levels. Interestingly, EGF was found to reverse the phenomenon of α9 reduction in newly confluent Caco-2/15 cells. It is noteworthy that considerable experimental evidence supports a role for EGF/TGFα in the control of intestinal epithelial cell proliferation (Ménard and Beaulieu 1994; Podolsky 1993; Drucker 1997). In a detailed study on the Caco-2/15 clone, which produce negligible amounts of endogenous TGF-α/EGF-like activity (Beaulieu and Quaroni 1991), Cross and Quaroni (1991) have shown that when added to the culture medium, even at a relatively low concentration (20 ng/ml),

EGF partly abolishes the reduction of cell proliferation occurring after confluence in control cultures and inhibits the expression of the differentiation marker sucrase-isomaltase. The higher levels of the α9 subunit observed in EGF-treated Caco-2/15 cell cultures fit well with the possibility of a functional relation between α9β1 expression and cell proliferation.

The transitory expression of the α9β1 integrin mostly restricted to proliferating Caco-2 cells seems unique in regard to other β1 integrins expressed in these cells. For example, the α2β1 and α7β1 integrins are expressed at significant levels in both subconfluent and postconfluent Caco-2/15 cells (Basora et al., 1997). It is noteworthy that downregulation of gene expression in differentiating Caco-2 cells is an unusual phenomenon. For instance, most functional brush border membrane markers and extracellular matrix molecules studied in these cells have been found to be either constitutively expressed or induced after reaching confluence (Beaulieu and Quaroni 1991; Vachon and Beaulieu 1995; Vachon et al., 1996; Simoneau et al, 1997). In fact, to our knowledge, cellular fibronectin is the only other molecule known to be clearly downregulated at both the protein and transcript levels in differentiating Caco-2 cells (Vachon et al., 1995), a phenomenon that, like for the α9β1 integrin, also reflects the normal in vivo situation since the molecule is predominantly associated with the proliferative epithelial cells of the crypt compartment (Beaulieu et al., 1991). Incidentally, fibronectin levels were found significantly increased by EGF in Caco-2/15 cells (data not shown). However, a functional relevance for these two closely related patterns of expression is unlikely since α9β1 does not seem to bind to fibronectin (Yokosaki et al., 1994; 1996). On the other hand, it has been clearly demonstrated that α9β1 can mediate functional adhesion to the third fibronectin type III repeat of tenascin-C (Yokosaki et al., 1994; 1996; Weinacker et al., 1995) leading to an activation of the mitogen-activated kinase Erk-2 and stimulation of cell proliferation (Yokosaki et al.,1996). However, the pattern of tenascin-C expression in the developing and adult human small and large intestines (Beaulieu et al., 1991; Beaulieu 1992; Beaulieu et al., 1993; Desloges et al., 1994; Beaulieu 1997a) differs considerably to that observed herein and elsewhere for the α9 integrin

subunit (Palmer et al., 1993; Wang et al., 1995; Basora et al., 1998), suggesting that other ligands must exist for α9β1 (Wang et al., 1995), as recently demonstrated for the N-terminal domain of osteopontin (Smith et al., 1996). Considering the requirement of integrin-ligand interactions in the growth-promoting activity of α9β1 (Yokosaki et al., 1996), it would be of importance to identify the ligand used by this integrin in the human intestinal epithelium to further investigate its relation with intestinal cell proliferation.

In summary, these observations demonstrate that the α9 subunit is present in the fetal small intestinal and colonic epithelia as well as in their in vitro counterparts, the HIEC-6 and Caco-2/15 cell lines, and that the α9β1 integrin is predominantly associated with proliferative cells. The integrin has not been detected in the epithelium of the adult small intestine and colon suggesting that its expression is restricted to immature proliferating intestinal cells.

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FIGURE LEGENDS

FIGURE 1. Expression and distribution of the α9 integrin subunit in the developing and adult intestine. Representative immunofluorescence micrograph of human jejunum at 17 weeks of gestation (A) and adult (B) and colon at 17 weeks of gestation (C) and adult (D) stained with an affinity-purified antibody directed against the α9 integrin subunit. In both the fetal small intestine (A) and colon (C), the α9 subunit is present at the basolateral surface in the glandular epithelium (arrows) and detectable in the cytoplasm of epithelial cells in both crypt (c) and villus (v). Some staining is also apparent at the tip of the villus core (arrowheads) as well as in muscle layers (ml). In the adult small intestine (B), the α9 subunit was exclusively detected in association with smooth muscle cells of the lamina propria (lp) and muscularis mucosa (mm). The crypt (c) and villus (v) epithelium was found to be negative. In the adult colon (D), the antigen is detected in subepithelial myofibroblasts (arrowheads) surrounding the lower third of the glands and in the muscularis mucosa (mm) while the crypt (c) and surface epithelium (se) remained consistently negative both in the cytoplasm and at their basolateral surface (arrows).
A-C: X90, D: X165.

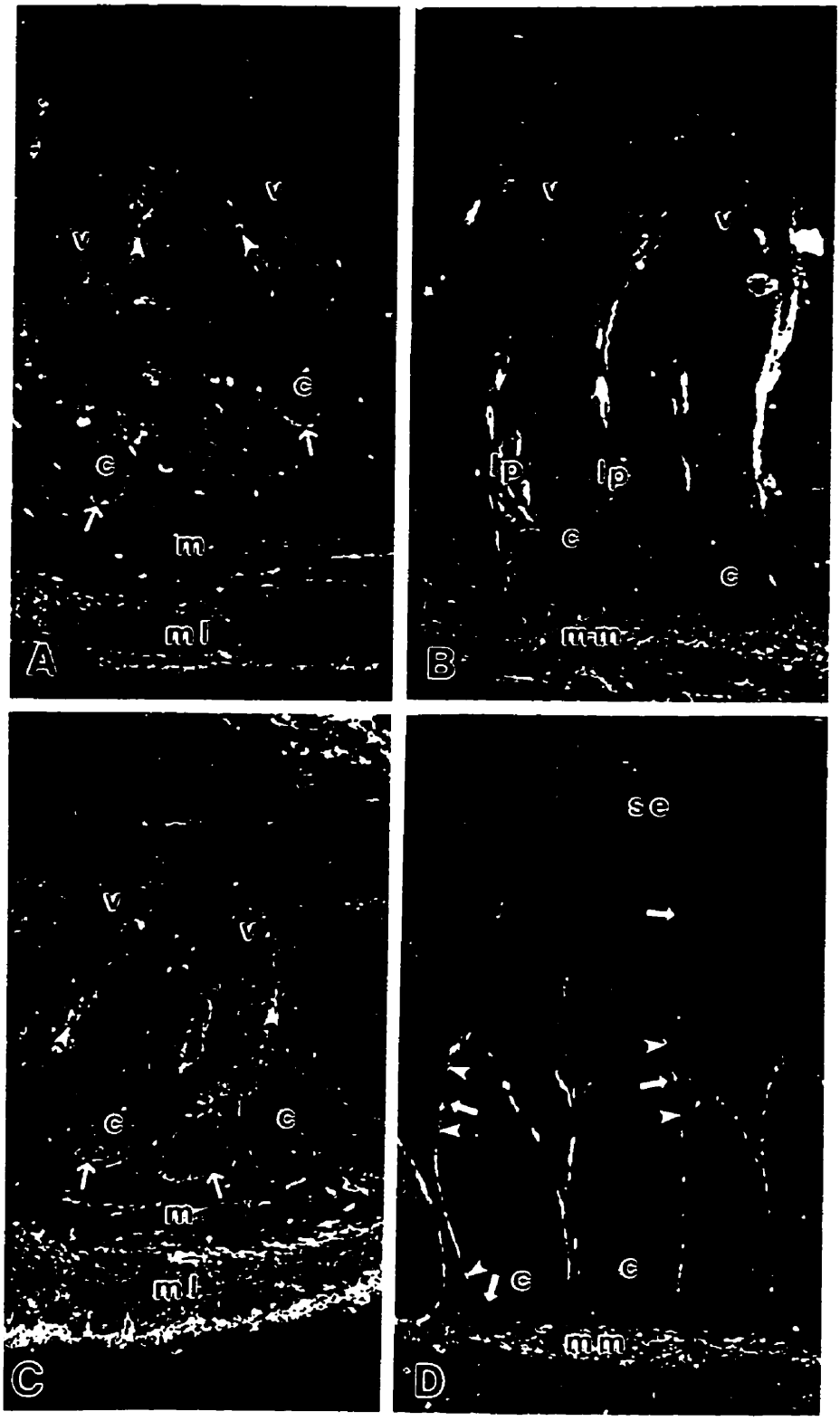
FIGURE 2. Representative Northern blot analysis for the detection of the α9 mRNA in the fetal small intestinal and colonic epithelium. Epithelia (E) from both jejunum (J) and colon (C) were dissociated from their corresponding underlying mesenchyme and muscle (M) by a non-enzymatic method and total RNA was isolated for each of these preparations and analyzed with ^{32}P-labeled DNA probes for the presence of α9 and GAPDH transcripts.

FIGURE 3. Expression of the α9 subunit mRNA in intestinal cell lines. Representative Northern blot analysis. Total RNA was isolated from HIEC and Caco-2/15 cells at -2, 0, 2, 6, 12 and 18 days of

confluence and analyzed with ^{32}P-labeled DNA probes for the presence of α9 and GAPDH transcripts.

FIGURE 4. Expression of the integrin α9 and β1 subunits in intestinal cell lines. Representative Western blot analysis of the α9β1 complex detected with anti-α9 and mAb 13 antibodies after immunoprecipitation with an affinity-purified anti-α9 subunit (A) as well as corresponding total β1 subunit immunoprecipitated and detected with the mAb 13 antibody (B) from HIEC and Caco-2/15 cells at -2, 2, 13 and 24 days of confluence. Lysates used for α9 immunoprecipitation were five times more concentrated than those used for β1.

FIGURE 5. Modulation of the integrin α9 subunit in Caco-2/15 cells by EGF. Representative Western blot analysis of Caco-2/15 cell lysates harvested 4 days after confluence and treated with 0 or 20 ng/ml of EGF from the day after the seeding. Sucrase-isomaltase (SI) and keratin 18 (K18) were used as control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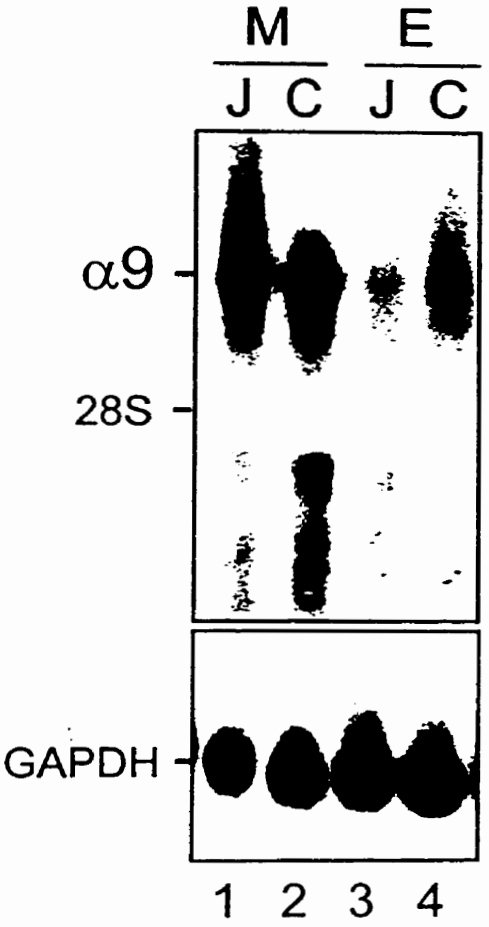
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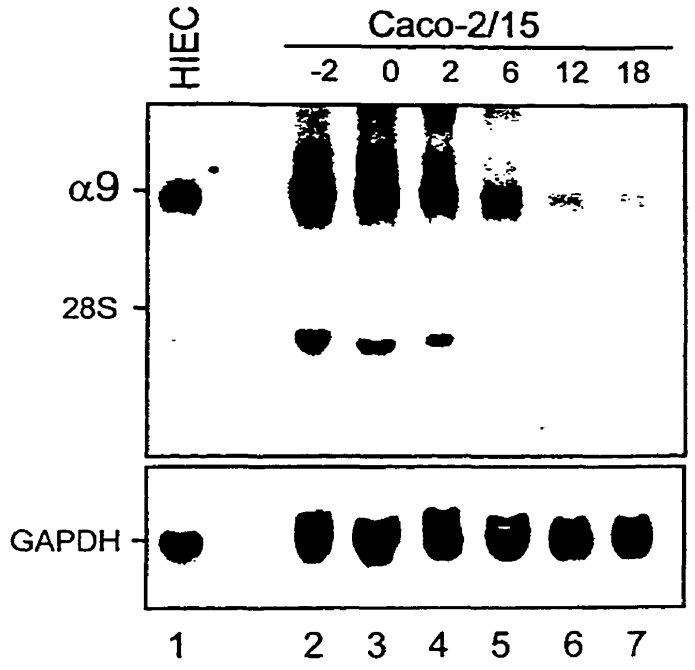
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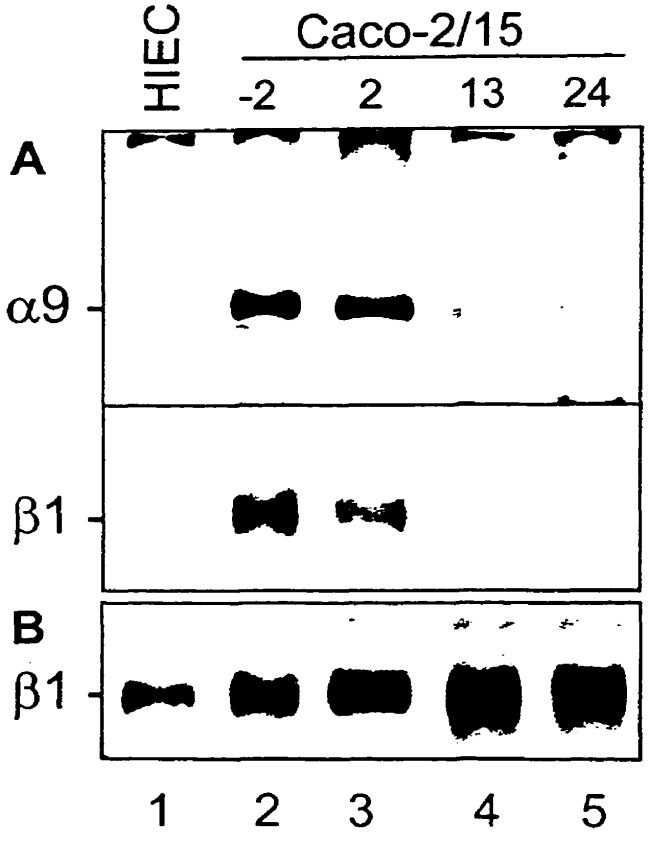
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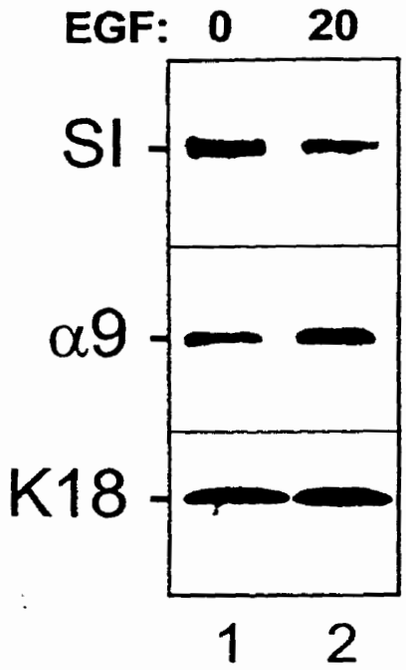
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Relation Between Integrin α7Bβ1 Expression in Human Intestinal Cells and Enterocytic Differentiation

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Background & Aims: **Cell-laminin interactions are principally mediated by specific membrane receptors of the integrin family. The integrin α7β1 is one of them. Its expression in the intestine has not yet been investigated although it appears to be a key element in muscle cell differentiation. In this study, the expression of its three known isoforms has been analyzed in developing and adult small intestine and in intestinal cell lines.** ***Methods:*** **The expression of the integrin α7β1 was analyzed by indirect immunofluorescence, Western blotting, immunoprecipitation, and reverse-transcription polymerase chain reaction.** ***Results:*** **The α7B isoform, but not the α7A and C isoforms, was detected in intestinal epithelial cells. In vivo, the presence of the α7B subunit was closely paralleled with (1) acquisition of differentiation characteristics during development and along the crypt-villus axis in the adult small intestine and (2) loss of enterocytic functions in the re-differentiated colonic epithelium. In vitro, the expression of α7B was also shown to correlate with the acquisition of enterocytic functions. In Caco-2 cells, the α7Bβ1 integrin was found transiently up-regulated at the onset of sucrase-isomaltase expression.** ***Conclusions:*** **Taken together, these results suggest that α7Bβ1 expression is correlated with human intestinal cell differentiation.**

Epithelia are in direct contact with the basement membrane (BM), a specialized extracellular matrix (ECM) of which the composition is recognized to define the necessary microenvironment required for tissue-specific gene expression.[1–5] A major biologically active BM component appears to be the laminins, a family of heterotrimeric glycoproteins composed of genetically distinct α, β, and γ chains, which may each be expressed as various isoforms.[6,7] Laminins have been shown to mediate several cellular activities, namely the promotion of adhesion, growth, polarization, and differentiation, depending on the cell type studied.[4,5,7,8] These diverse functions are themselves mediated by various cell membrane receptors, many of which are members of the integrin superfamily.[9–13]

Integrins are transmembrane αβ heterodimers, which are known as the primary mediators of ECM-cell interactions and signaling.[10,14] So far, 16 α and 8 β subunits have been identified. Furthermore, variants of some of these subunits are known to arise through differential splicing, presumably increasing the diversity as well as the specificity of both the ligand-binding and the signal-transduction properties of integrins.[10–12,14] Known laminin-binding integrins expressed in epithelial cells include α1β1, α2β1, α3β1, α6β1, and α6β4.[9,10,12,13,15]

The intestinal epithelium is a useful model for the in vivo analysis of cell-matrix interactions in relation to cell state.[5] In the adult small intestine, the epithelium is in constant and rapid renewal. Within its functional unit, the crypt-villus axis, it contains two main distinct cell populations: the proliferative and poorly differentiated crypt cells and the mature enterocytes of the villus.[16–18] Processes of epithelial cell growth and enterocyte functional differentiation should be tightly regulated along the crypt-villus axis.[19,20] Analysis of integrins and laminins in the human small intestine has shown particular patterns of expression for many of these molecules.[5] Of interest is the reciprocal expression of laminin-1 (α1β1γ1) and laminin-2 (α2β1γ1) that has been found along the crypt-villus axis[21,22] with laminin-1 occurring as a villus form and laminin-2 as a crypt form, indicating a relationship between laminin expression and functional intestinal cell differentiation. Such a relationship was found to be of functional relevance, because we have shown recently that state-specific enterocyte gene expression is differentially regulated by laminin-1 and laminin-2.[23] In fact, laminin-1 appears crucial for the establishment and maintenance of the enterocyte-differentiated state.[5,23,24]

Accordingly, the laminin-binding integrins α1β1 and

Abbreviations used in this paper: BM, basement membrane; cDNA, complementary DNA; ECM, extracellular matrix; mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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α2β1 are expressed predominantly by crypt cells, α3β1 is mainly expressed by villus cells, whereas α6β1 and α6β4 are expressed throughout the epithelium.[21,25–27] Because of its distribution, α3β1 may at first appear to be a major candidate for the mediation of the effects of laminin-1 in intestinal cell differentiation. However, the recent demonstration that epithelial cells—including intestinal ones—use primarily α3β1 to bind to laminin-5,[12,28,29] a laminin isoform (α3β3γ2) also present at the BM of villus epithelial cells,[30,31] makes this unlikely. However, the role of the α6 integrins remains unclear because of their apparent ubiquitous expression along the crypt-villus axis[5] and a potential to bind various laminins including laminin-1 and laminin-5.[12] Therefore, the laminin-binding integrin(s) involved in the mediation of the effects of laminin-1 in enterocyte differentiation still remains to be identified.

The α7β1 integrin is another laminin receptor that was first identified as a laminin/E8 fragment-binding protein expressed by skeletal muscle myoblasts and malignant melanocytes.[32,33] Further analysis of the α7 subunit later led to the identification of three alternate cytoplasmic domains (α7A, α7B, α7C) that arise through alternative splicing of the α7 messenger RNA (mRNA) and that are differentially expressed as a function of striated muscle cell state.[34–37] These α7 variants are presumed to mediate laminin functions in myogenesis and myogenic differentiation.[34,35,38] Recent data showing a key role for α7β1 in the promotion of adhesion and motility on laminin-1, but not on laminin-5, strengthened its potential importance in muscle development and regeneration.[39,40]

In this study, we have analyzed the expression of the α7 integrin in epithelia of the developing and adult small and large intestine. Our results indicate that the α7B variant is expressed as a α7Bβ1 complex in epithelial cells and that its distribution is time and site specific, coinciding with enterocytic differentiation both in vivo and in vitro. Furthermore, its distribution pattern along the crypt-villus axis correlates with that of laminin-1. Therefore, this integrin may play a key role in the mediation of laminin-1–induced intestinal cell differentiation.

Materials and Methods

Tissues

Specimens of small intestine and colon from fetuses ranging from 8 to 20 weeks postfertilization were obtained after legal abortion. Samples of adult small intestine (jejunum) and colon were obtained from nondiseased parts of resected segments. Between four and seven samples were studied for each segment and each age group. Only specimens obtained rapidly (<60 minutes) were used. The project was in accordance with the protocol approved by the Institutional Human Research Review Committee for the use of human material.

Primary Antibodies

Monoclonal antibodies against the integrin β1 subunit (mAb 13)[41] and sucrase-isomaltase (HSI-14)[42] were obtained from Drs. S. K. Akiyama and A. Quaroni, respectively. Human cytokeratin 18 was detected using the monoclonal CY90 (Sigma Chemical Co., St. Louis, MO). The rabbit antiserum directed to the human α2 integrin cytoplasmic domain was obtained from Dr. Ruoslahti.

To generate antibodies specific for individual α7 integrin subunit variants, peptides corresponding to the specific cytoplasmic domains of α7A, α7B, and α7C were synthesized.[34,35] Sequences used were the following: NH_2-CGWDSSSGRSTP-RPPCPSTTQ for α7A, NH_2-CDWHPELGPDGHPVSVTA for α7B, and NH_2-CKCAVPAQRPLSIY for α7C. Immunization and affinity purification of the peptide antisera were performed as described.[43,44] Specificity of the antibodies was verified by Western blot analysis, immunofluorescence, and immunoprecipitation using striated muscle tissues and cells (not shown).

Cell Culture

The human colon carcinoma Caco-2/15 cell line, a stable clone of the parent Caco-2 cells (HBT 37; ATCC, Rockville, MD) has been characterized elsewhere.[45–47] These cells are unique in that upon reaching confluence they spontaneously undergo a gradual enterocytic differentiation process, similar to that observed in the epithelium of the intact fetal small and large intestine.[45–48] Cells between passages 53 and 70 were cultured as described.[46]

The HIEC-6 cell line has been generated from normal fetal human small intestine.[49] These cells express a number of crypt cell markers but no villus cell markers and are thus considered as poorly differentiated crypt cells.[49,50] Cells were used between passages 5 and 10 and were grown as described.[49]

The human colon adenocarcinoma cell lines Colo 201 and LoVo (CCL224 and CCL229, respectively; ATCC) were grown as suggested by the supplier. These cells are considered as poorly differentiated and do not undergo enterocytic differentiation.[51,52]

Indirect Immunofluorescence

The preparation and embedding of tissue samples for cryosectioning was performed as described previously.[26] Frozen tissue sections were fixed in ethanol (10 minutes, −20°C) before immunostaining, as described elsewhere.[21,26] Primary antibodies were diluted 1:100 (anti-α7A, B, and C antisera and corresponding neutralized antisera with 20 μg/mL of the synthetic peptide used for immunization) or 1:100 (affinity-purified antibodies) in 10% nonfat powdered milk in phosphate-buffered saline (PBS). The secondary antibody was a fluorescein isothiocyanate–conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim Canada, Laval, Québec, Canada) used at 1:25 in 2% bovine serum albumin in PBS.

Sections were then stained with 0.01% Evan's blue in PBS, mounted in glycerol-PBS (9:1) containing 0.1% paraphenylenediamine and viewed with a Reichart Polyvar 2 microscope (Leica, St. Laurent, Canada) equipped for epifluorescence. In all cases no specific immunofluorescent staining was observed when primary antibodies were omitted or replaced by rabbit nonimmune serum.

Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide gels and immunoblotting were performed as described previously.[23,45,49] Intestinal and colon cancer cells grown to different stages of confluence were washed twice in PBS and were harvested in 1× solubilization buffer (2.3% SDS, 10% glycerol, 0.001% bromophenol blue in 62.5 mmol/L Tris-HCl pH 6.8) containing 5% β-mercaptoethanol. Samples were boiled for 5 minutes, cleared by centrifugation (13,000*g*, 5 minutes), and aliquoted for storage at −80°C. Separated proteins were transferred onto nitrocellulose (Bio-Rad, Mississauga, Ontario, Canada) and blocked in PBS containing 10% powdered skim milk, then incubated overnight at room temperature with primary antibodies (anti-α7A and anti-α7B affinity-purified antibodies, 1:500; anti-α2, 1:400; HSI-14–conditioned medium, 1:5; CY90, 1:2000; mAb13, 1:1000). Alkaline phosphatase (Bio-Rad) or Western Light chemiluminescence (Tropix, Lexington, KY) detection was used according to the manufacturer's instructions. Band intensities were quantified using an LKB Ultroscan XL densitometer (Pharmacia, Piscataway, NJ).

Immunoprecipitation

Caco-2/15 cells grown to 5 days postconfluence were washed twice in ice-cold PBS. Cells were solubilized in lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L sodium orthovanadate, 2 μmol/L phenylmethylsulfonyl fluoride, 50 μg/mL leupeptin, 50 μg/mL pepstatin, and 100 μg/mL aprotinin) for 20 minutes on ice, then centrifuged for 15 minutes at 13,000*g*. Ten micrograms of mAb13 or a 1:300 dilution of affinity-purified α7B was added to the samples, and they were incubated for 15 hours at 4°C followed by the addition of protein-G Sepharose (Gibco-BRL, Burlington, Ontario, Canada) for a 1-hour incubation at 4°C. Immunoprecipitates were then processed for analysis by SDS-PAGE and immunoblotting as previously described.[42]

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from cell lines or tissue homogenates using TriZOL (Gibco-BRL). Reverse transcriptase Superscript (Gibco-BRL) and 0.5 μg of oligo-(dT)12-18 primer (Pharmacia, Baie d'Urfe, QU, Canada) were added to 5 μg of total RNA, as described elsewhere.[33] For the α7 subunit, we used the sense primer 5′-GTCTCTGGAACAGCACTTTC-3′ and the antisense primer 5′-GTCCGACGAGGAAGAGATCAA-3′ specific for humans, designed according to the method of Song et al.,[34] who described primers common to both α7A and α7B rat isoforms, and modified according to Wang et al.[47] for the human sequence; these primers were expected to generate two products corresponding to α7A (998 base pairs) and α7B (885 base pairs). Single-stranded complementary DNAs (cDNAs) were amplified in PCR buffer (Pharmacia) containing 1 μmol/L of both sense and antisense primers for 25 cycles of denaturation (1 minute at 93°C) and annealing/extension (1 minute at 59°C and 2 minutes at 72°C) in a thermal cycler (Perkin-Elmer DNA Thermal cycler model 480; Branchburg, NJ) in the presence of 250 μmol/L deoxyribonucleotide triphosphate and 2 μCi [^{32}P]deoxycytidine triphosphate (Amersham, Oakville, Ontario, Canada) and 2.5 U of Taq (Roche; obtained from Pharmacia).

Conditions for amplification of sucrase-isomaltase and S14, used as endogenous controls, have been described previously.[53,54] The products were separated on a 5% acrylamide/TBE gel and visualized by autoradiography. Band intensities were quantified using an LKB Ultroscan XL densitometer (Pharmacia).

Sequence Analysis

The α7A and α7B PCR products were cloned into pGEm-T (Promega, Madison, WI), and the inserts were sequenced using the T7 and Sp6 sequencing primers (Sheldon Biotechnology Centre, McGill University, Montréal, Québec, Canada). The sequences were analyzed using BLAST programs developed by the National Center for Biotechnology Information at the National Library of Medicine.[55]

Statistical Analysis

Student's unpaired two-tailed *t* test was used as a statistical test. The results expressed as means ± SE were considered significant when P was <0.05.

Results

Expression of the α7B Integrin Subunit Correlates With Intestinal Epithelial Cell Differentiation In Vivo

The expression and distribution of the three α7 integrin subunit isoforms was first studied on cryosections of fetal and adult small intestine by indirect immunofluorescence, using affinity purified antisera directed against the cytoplasmic domains of α7A, α7B, and α7C. No specific staining was detected with the anti-α7C antibody. The α7A isoform was detected in smooth muscles, in both the muscularis propria (circular and longitudinal layers) and the muscularis mucosa, whereas the epithelium remained unstained at all fetal stages studied as well as in the adult (Figure 1).

The α7B variant was detected in both epithelial cells and smooth muscle (Figure 2). In the epithelium, α7B was not detected at early stages (7–10 weeks; Figure 2*A* and *B*). At 11–12 weeks, specific staining was detected at the base of most epithelial cells on the villus, whereas

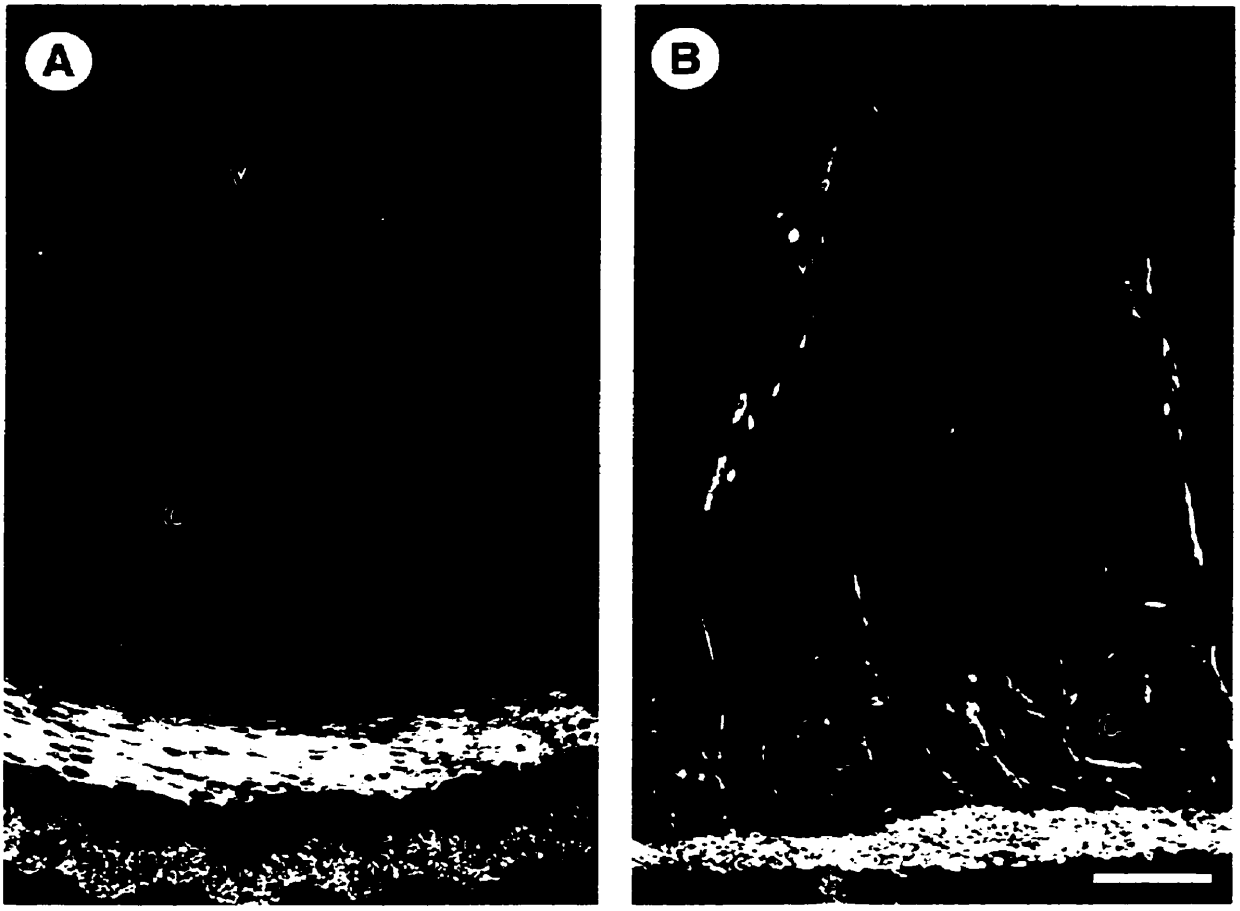

Figure 1. Expression of the α7A integrin subunit in the fetal and adult human small intestine. Representative immunofluorescence micrographs of jejunum stained with the affinity-purified antibody directed against the cytoplasmic domain of α7A (*A*) at 18 weeks' gestation and (*B*) in the adult. The α7A subunit was exclusively detected in association with smooth muscle cells at all stages studied. The epithelium in both crypts (C) and villi (V) was found to be negative (*bar* = 10 μm).

the intervillous area remained unstained (Figure 2*C*). At 18 weeks, a relatively intense staining was observed in the upper part of the crypts and lower half of the villi (Figure 2*D*). The staining in the upper half of the villi was consistently found to be weaker, whereas the lower two-thirds of the crypts were negative. This site-specific distribution of α7B was also found in the adult small intestine where the staining, although weaker than that observed at midgestation, was found restricted at the base of those enterocytes lining the upper third of the crypt and the lower half of the villi (Figure 2*E* and *F*). At both extremities of the axis, the expression of α7B decreased quickly. Controls used for immunofluorescence staining with the anti-α7B included (1) absence of epithelial staining with the anti-α7A antibody raised under the same conditions (see Figure 1); (2) abolishment of all epithelial staining by using the anti-α7B serum neutralized with the peptide used for immunization; and (3) use of affinity-purified anti-α7B antibody (illustrated in Figure 2). In smooth muscles, the anti-α7B antibody stained the circular and longitudinal muscle layers at all ages studied, from 8 weeks (presumptive muscularis propria, Figure 2*A*) to 20 weeks' gestation, stained some isolated smooth muscle cells in the lamina propria from 12 weeks onward (Figure 2*CBF*), and stained the muscularis mucosa (Figure 2*E*).

Transient Expression of α7B in the Developing Colonic Epithelium

The early developing colon (8–16 weeks' gestation) presented a similar time- and site-specific expression of α7B. At 18–20 weeks, the fetal colon morphologically resembles the small intestine at the same stage, due to the presence of relatively well formed villi and the presence of functional enterocytic differentiation markers[56] (reviewed by Ménard and Beaulieu[18]). The distribution of α7B at this stage was comparable with that observed in the small intestine. In the adult colon, however, all epithelial cells were consistently devoid of any specific staining for α7B. Finally, as in the small intestine, expression of neither α7A nor α7C was observed in the colonic epithelium (data not shown).

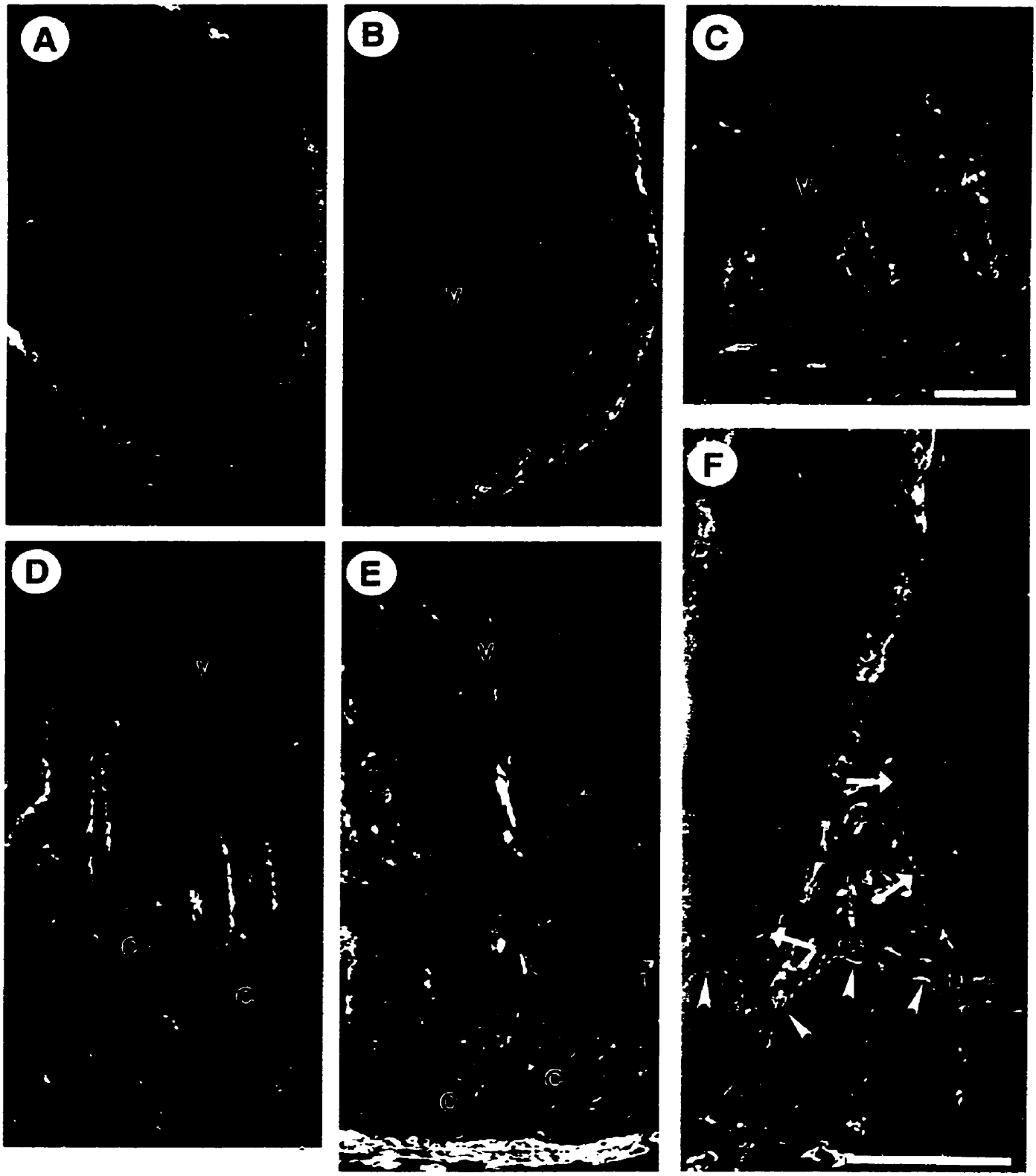

Figure 2. Expression and distribution of the α7B integrin subunit in the developing and adult small intestinal mucosa. Representative immunofluorescence micrographs of human jejunum stained with an affinity-purified antibody directed against the cytoplasmic domain of α7B at (*A*) 8, (*B*) 10, (*C*) 12, and (*D*) 18 weeks' gestation and (*E* and *F*) in the adult. In the epithelium, the α7B subunit was first detected at 12 weeks at the base of villus (v) cells (*C*). Maximum staining intensity was consistently observed at 18–20 weeks (*D*). The α7B subunit was found at the base of all villus epithelial cells, although predominant in the lower half of villi but remained absent in the lower half of developing crypts (c). In the adult (*E* and *F*), the α7B staining was weaker than that observed at midgestation and was restricted to a region extending from the upper crypt to the lower half of the villus. The antigen was also found in association with smooth muscles cells at all stages. *Arrows* and *arrowheads* in *F* point to the base of epithelial cells located at the lower portion of the villus and the upper part of the corresponding crypts, respectively (*bars* = 10 μm).

Expression of α7 Variants in Intestinal Cells In Vitro

Four different intestinal epithelial cell lines were screened using Western blot analysis to detect the presence of the α7 subunit (Figure 3). These included the normal crypt-like cell line HIEC-6, the enterocyte-like Caco-2/15 cell line, and the Colo 201 and LoVo cell lines, which are, like the Caco-2 cell line, of cancerous origin but cannot differentiate.[51,52] As shown in Figure 3*A*, affinity purified antibodies against α7A revealed no specific bands. The use of chemiluminescence as a more sensitive approach also failed to reveal the α7A subunit in these cells. In contrast, α7B was detected but only in the Caco-2/15 cells (Figure 3*B*). The major band at 38 kilodaltons corresponds to the carboxyl terminal portion of the mature polypeptide.[34] A second specific band was found at 121 kilodaltons, and this band represents the entire length of the immature polypeptide.[34] Immunostaining of both the 38- and 121-kilodalton bands was abolished by prior incubation of the antibody with the immunizing peptide (not shown). Hence α7B was expressed only in enterocytes that have the potential to differentiate.

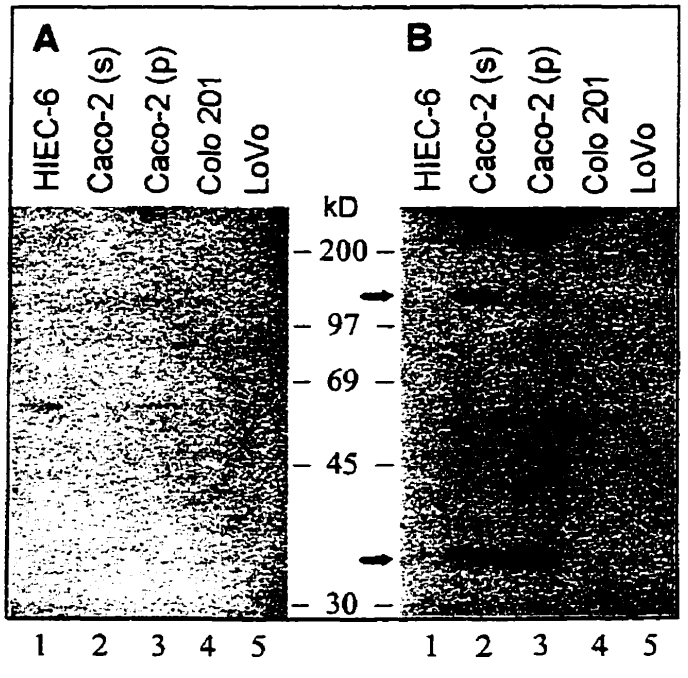

Figure 3. Expression of α7 subunits in intestinal epithelial cell lines. Total proteins were analyzed by Western blotting with (*A*) an affinity-purified anti-α7A or (*B*) an anti-α7B antibody. HIEC-6 cells (*lane 1*), Caco-2/15 cells (*lane 2*) as well as Colo 201 (*lane 4*) and LoVo cells (*lane 5*) were 80%–90% confluent; Caco-2/15 cells (*lane 3*) were confluent for 12 days. Molecular weight markers are indicated in thousands. *Arrows* in *B* point to the 35- and 121-kilodalton bands corresponding to the processed and unprocessed forms of the α7B subunit, respectively.

Enterocytes Express the α7Bβ1 Complex

In muscle, α7B associates with the β1 subunit to form the functional laminin receptor.[33,34] Immunoprecipitation of the β1 complex from 3–5-day postconfluent Caco-2/15 cells, followed by Western blot analysis for the detection of this subunit with the mAb 13 antibody, revealed the presence of a broad 118-kilodalton band (Figure 4, lane 1) corresponding to β1, although of a slightly smaller molecular weight than the 130 kilodaltons expected,[57] probably because of differences in composition and/or number of the carbohydrate chains, a phenomenon also observed for sucrase-isomaltase in Caco-2/15 cells.[58] The same immunoprecipitate analyzed for the presence of α7B by immunoblotting showed that this subunit coprecipitated with β1, as evidenced by the presence of the characteristic 121- and 38-kilodalton bands (Figure 4, lane 3). Immunoprecipitation with the anti-α7B antibody followed by Western blot analysis for α7B showed the presence of this subunit (Figure 4, lane 4; the intact 121-kilodalton form, the 38-kilodalton

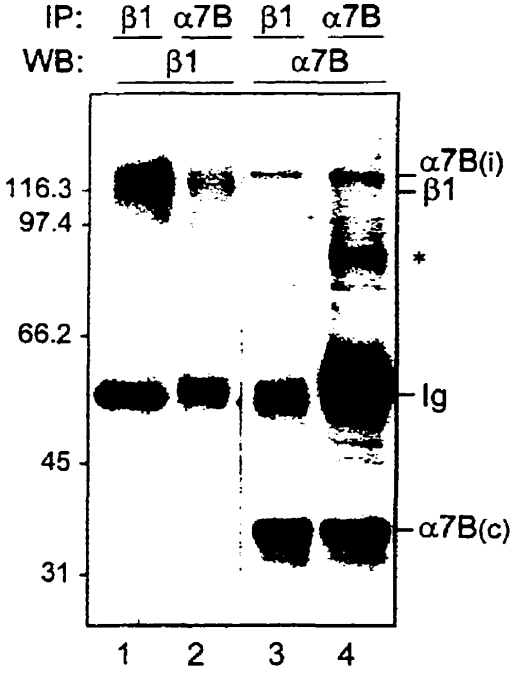

Figure 4. The α7B subunit complexes with the β1 subunit in Caco-2/15 cells. α7Bβ1 complexes were immunoprecipitated with the rat monoclonal mAb13 (anti-β1; lanes 1 and 3) and the affinity-purified anti-α7B antibody (*lanes 2* and *4*) from identical 5d postconfluent Caco-2/15 cell lysates and analyzed for the detection of β1 (*lanes 1* and *2*) and α7B (*lanes 3* and 4) by Western blotting. Molecular weight markers in the left margin are indicated in kilodaltons. The specific antigens detected in *lanes 1–4* were the intact α7B subunit (α7B(i), 121 kilodaltons; *lanes 3* and *4*), the β1 subunit (~118 kilodaltons, *lanes 1* and *2*), a presumed α7B proteolytic cleavage product (*asterisk*; ~80 kilodaltons, *lane 4*), rat (*lanes 1* and *3*) and rabbit (*lanes 2* and *4*) immunoglobulins (Ig), and the cleaved α7B subunit cytoplasmic tail (α7B(c), 38 kilodaltons; *lanes 3* and *4*).

cleaved domain, and a presumed 80-kilodalton proteolytic cleavage product),[34] whereas analysis of the α7B immunoprecipitate for the detection of the β1 subunit revealed a faint band at 118 kilodaltons (Figure 4, lane 2) consistent with the fact that β1 is associated with a number of α-integrin subunits in intestinal cells.[5] Hence these data show that enterocytes express α7B as a typical α7Bβ1 laminin receptor complex.

Transient Up-regulation of α7B in Differentiating Enterocytes

The Caco-2 cell line is currently used as a model for enterocytic differentiation. The gradual acquisition of morphological and functional characteristics of mature intestinal epithelial cells by postconfluent Caco-2 cells is well documented.[18,48] To determine whether the expression of α7Bβ1 is correlated with epithelial differentiation, the expression of the α7B subunit was analyzed throughout the enterocytic differentiation process of Caco-2/15 cells. As illustrated in Figure 5*A*, α7B was detected at all stages, whereas the brush border enzyme sucrase-isomaltase, used as a differentiation marker, was only barely detectable 3 days after confluence but accumulated substantially at later stages. Protein expression levels of α7B and sucrase-isomaltase were compared with those of cytokeratin 18, which in Caco-2/15 cells remains constant relative to total cellular proteins during enterocytic differentiation.[59] As shown in Figure 5*B*, a peak of α7B protein expression occurs in newly differentiating Caco-2/15 cells, and this peak correlates with the onset of functional intestinal differentiation, as determined by sucrase-isomaltase expression. To determine whether this transient up-regulation was specific to α7B, the expression of two other integrin subunits, α2 and β1, was also analyzed in parallel (Figure 5*A*). The levels of these integrin subunits did not vary significantly during Caco-2/15 cell differentiation (Figure 5*C*).

The expression of the α7B subunit was further investigated at the RNA level. Specific primers for the amplification of α7B were first designed. Although the complete human sequence for either α7 variants has not been determined, a partial sequence for α7B is available.[37] Based on previous studies in the rat,[34] we were able to design a corresponding set of primers specific for human α7, which, similar to the situation in the rat, were expected to amplify both α7A and α7B cDNAs (Figure 6*A*). This was confirmed by amplifying the two variants from human fetal skeletal muscle (Figure 6*B*), as shown by the presence of two products, one at 885 base pairs (α7B) and a second at 998 base pairs (α7A). PCR amplification of DNA complementary to the RNAs extracted from newly confluent Caco-2/15 cells yielded a single band corresponding to the α7B cDNA fragment (Figure 6*B*),

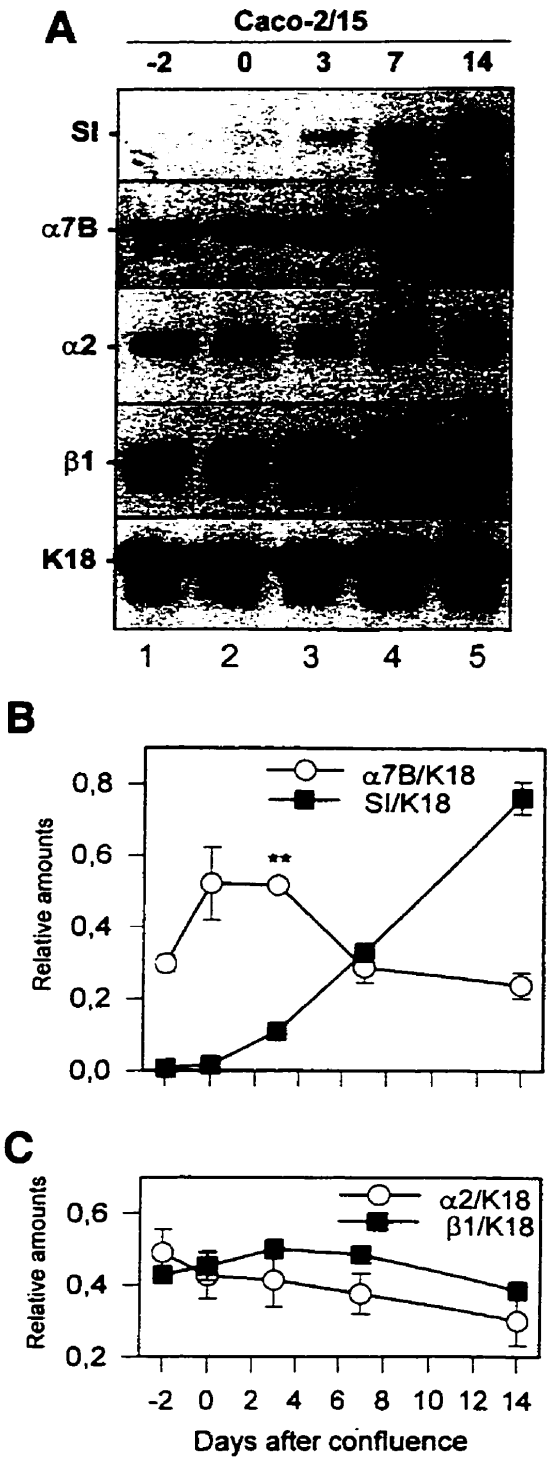

Figure 5. Modulated expression of the α7B subunit during enterocytic differentiation. (*A*) Representative Western blot analysis of total proteins from cell lysates of Caco-2/15 cells at −2 (*lane 1*), 0 (*lane 2*), 3 (*lane 3*), 7 (*lane 4*), and 14 (*lane 5*) days of confluent culture analyzed for the expression of sucrase-isomaltase (SI), the α7B, α2, and β1 integrin subunits, and keratin 18 (K18). (*B, C*) Estimation of the relative abundance of (*B*) α7B and SI and (*C*) α2 and β1 integrin subunits. Proteins were analyzed as in *A* and then scanned by laser densitometry. Relative amounts of SI and integrin subunits were determined as ratios relative to K18. Data represent means ± SE from three separate experiments. **Statistically significant difference in relative amounts of α7B at day 3 vs. days −2 and 10.

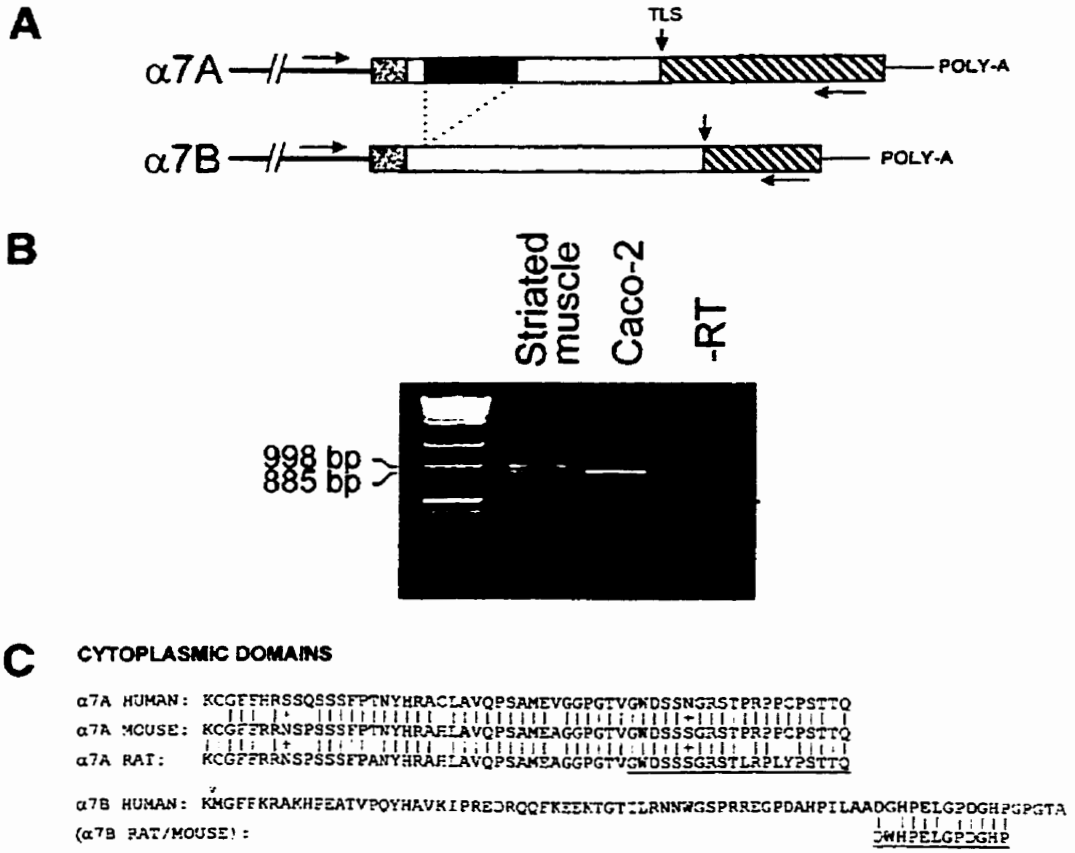

Figure 6. Identification of human α7A and α7B mRNA variants by RT-PCR and characterization. (*A*) Organization of the region encoding the cytoplasmic domain of human α7. (*B*) RT-PCR amplification of Caco-2/15 cells and fetal skeletal muscle RNA using paired primers specific for the cytoplasmic domain of human α7 integrin. In the skeletal muscle, two species of amplified products observed were 998– and 885–base pair bands corresponding to α7A and α7B mRNA. In Caco-2/15 cells, only the 885–base pair band corresponding to α7B was detected. The sites of PCR primers used to detect the alternate RNAs are indicated by horizontal arrows in *A*. ■, 113 deleted nucleotides in α7B. TLS, translation stop codons; –RT, reverse transcriptase omitted. (*C*) Amino acid sequences of the cytoplasmic domains of the human α7A and B integrin subunits as determined from the nucleotide sequences of the three PCR products obtained in (*B*). The amino acid sequence of the human α7A integrin cytoplasmic domain was aligned with the mouse[36] and rat[34] α7A sequences. Identical residues (|) and conserved changes (+) are indicated. Rodent amino acid sequences used to generate the anti-α7A and B antibodies are underlined.

confirming that these epithelial cells express only the α7B isoform. The identity of the PCR products amplified using these primers was confirmed by sequencing. The bands at 885 base pairs in both the muscle and the Caco-2/15 cells were found to align perfectly with the reported human α7B sequence[37] (EMBL/GenBank Accession X74295). The 998–base pair band in muscle showed 88% identity (89.7% homology at the amino acid level) with the reported mouse α7A sequence[36]; it was thus determined to be the human α7A. The deduced amino acid sequences for α7A and B cytoplasmic domains are provided (Figure 6*C*).

Reverse transcription-polymerase chain reaction (RT-PCR) was then performed on total RNA extracted from Caco-2/15 cells at various stages of confluence to determine the pattern of α7B mRNA expression in relation to the sucrase-isomaltase messenger (Figure 7*A*). Expression of the S14 transcript was also determined to ensure cDNA integrity and to compare amounts of starting RNA material. Densitometric analysis of the α7B and sucrase-isomaltase amplification products were then assessed relative to S14. The results show that a transient increase of α7B mRNA occurs in newly differentiating Caco-2/15 cells (Figure 7*B*), which parallels that of the α7B protein (Figure 5*B*). Therefore, α7B protein and mRNA are transiently up-regulated at the onset of enterocyte differentiation.

Discussion

In this report, we have studied the expression of α7 integrins in the human intestine as well as in cultured cells derived from the human intestinal epithelium. The integrin α7β1 has been described as being essentially muscle cell specific,[32,33,35] although nonmuscle locations have been recently identified.[60] We have shown here that the α7B subunit is expressed by epithelial cells as well.

In intestinal cells, it is present as α7Bβ1, and its expression is site and time specific and correlates particularly well with the onset of enterocytic differentiation.

In the skeletal muscle, a switch between the α7A and B variants observed at the time proliferating myoblasts underwent fusion and myotube formation, in concert with the fact that the α7β1 complex is the principal integrin on skeletal myoblasts that bind laminin, suggests a key role for this integrin in laminin-mediated muscle cell differentiation.[53-55,61,62] Recent studies have clearly established that the α7β1 integrin can promote both adhesion and motility on laminin-1 but not on laminin-5.[39,40] In intestinal epithelial cells, there is clear evidence that laminin-1 is critical in triggering functional differentiation.[21,23,24] However, the identification of a particular integrin involved in the mediation of these effects is complicated by the fact that besides α7Bβ1, a number of other laminin-binding integrins, including α3β1, α6β1, and α6β4, as well as α1β1 and α2β1, are present.[5] However, few, if any, of these integrins appear to be good candidates in mediating the effects of laminin-1 on intestinal functional cell differentiation. Indeed, the α1 and α2 integrin subunits have been found to be confined primarily to the crypts, which contain the proliferative and less differentiated cells[25,26] and are devoid of laminin-1 in the adult.[21,22] However, the α6, β1, and β4 subunits have been found to be expressed by all intestinal epithelial cells during fetal development as well as along the crypt-villus axis in the adult.[21,22,26,27] This widespread expression at the base of all intestinal cells, including endodermal cells in the 6-week-old fetal intestine and proliferative crypt cells in the fetal and adult small intestine, suggests that α6 integrin(s) may not be responsible for triggering terminal differentiation in the intestinal epithelium. It must be stressed that knowledge about α6 integrin organization in human intestinal cells is still limited,[12] because the distribution of variants for the α6, β1, and β4 subunits has not been yet determined[5] and the existence of a hypothetical α6β1 complex in these cells appears controversial.[63,64] Concerning the α3β1 integrin, its spatio-temporal pattern of expression in the human small intestine is of interest. This integrin first appears in the epithelium of the developing villi and remains predominantly associated with villus epithelial cells including in the adult,[26,27] a pattern of expression that appears to closely correlate with that of laminin-5,[30,31] its predominant ligand.[12,28,29] Thus, α3β1 may be involved in the regulation and/or the maintenance of differentiated intestinal cells but, most likely, acts in concert with laminin-5 rather than with laminin-1 as a ligand.[5] It is also noteworthy that the Caco-2/15 cells, which depend on laminin-1 for their functional differentiation,[23,24] lack the α3 integrin subunit (Basora et al., unpublished data, June 1996) further supporting the hypothesis that another receptor for laminin-1 must be involved. The findings presented herein suggest that α7Bβ1 could be this receptor.

By analyzing the spatiotemporal pattern of distribution of this integrin in epithelia of the intact developing and adult small intestine and colon, we have shown a tight parallel between α7B expression and enterocytic differentiation. Indeed, in the small intestinal epithelium, its restricted presence at the crypt-villus junction overlaps an undifferentiated but committed population and the first appearance of functional enterocytes, being perfectly compatible with the expression of functional

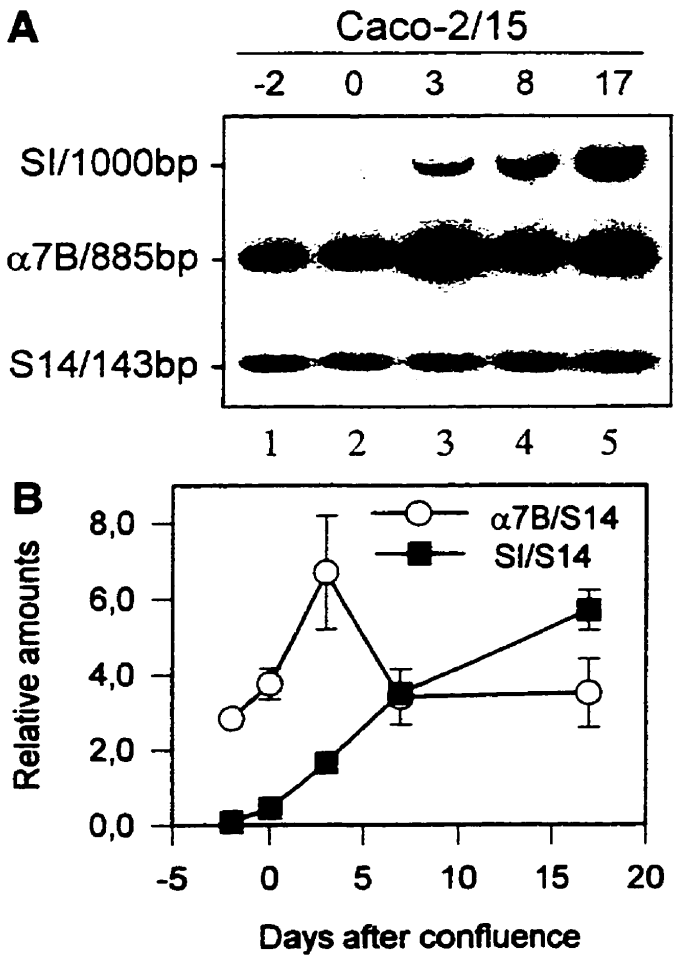

Figure 7. Expression of α7B transcripts during enterocytic differentiation. (*A*) Representative RT-PCR analyses of α7B integrin and sucrase-isomaltase mRNA in Caco-2/15 cells at −2 (*lane 1*), 0 (*lane 2*), 3 (*lane 3*), 8 (*lane 4*), and 17 (*lane 5*) days of confluent culture. (*B*) Estimation of the relative abundance of α7B and SI transcript levels during Caco-2/15 cell differentiation. Amplified materials were analyzed as in (*A*) and then scanned by laser densitometry. Relative amounts of SI and α7B were determined as ratios relative to S14. Data represent means ± SE from three separate experiments. *Statistically significant difference in relative amounts of α7B at day 3 vs. days −2 and 8.

differentiation markers such as the mature form of sucrase-isomaltase, lactase-phlorizin hydrolase, and the maltase-glucoamylase complex.[17,18]

Interestingly, patterns of staining observed in the fetal colon were similar to those observed in the fetal small intestine at comparable ages, whereas, in contrast, in the adult colon none of the specimens examined expressed detectable levels of this integrin subunit in the epithelium. This observation is most intriguing in view of the fact that the human fetal colon during the first half of the gestation is known to transiently display features of small intestine, including villi and enterocyte-like hydrolytic activities such as sucrase and lactase. Then, towards the end of the gestation, the villi disappear, as well as most of the hydrolytic activities, so that at birth the colon displays both an adult-like morphology and epithelium.[18,19] Because specimens from the second half of gestation were unavailable, it has not been possible to study directly the α7B expression during the process of redifferentiation of the colonic epithelium. Nevertheless, the fact that the integrin was not detected in the adult normal colonic epithelium suggests that α7Bβ1 could be specifically involved in the regulation of the expression of small intestinal enterocytic functions.

To further investigate the potential relation between the α7β1 integrin and small intestinal epithelial cell differentiation, we have analyzed its expression on various intestinal epithelial cell lines. The Caco-2/15 was the only cell line tested that expressed the α7 subunit as an α7Bβ1 complex. The Caco-2/15 cells have been extensively characterized[45B47] and, as the uncloned Caco-2 cells,[48] are the only cells identified so far that can spontaneously undertake, in vitro, a program of enterocyte-like differentiation.[51] For instance, the Colo 201 and LoVo cell lines, also derived from human colon adenocarcinomas, do not express functional small intestinal cell markers such as sucrase and lactase[51,52] and, as shown herein, also lack the α7 integrin subunit. The HIEC-6 cells are normal human intestinal epithelial cells that are considered as poorly differentiated crypt cells.[49,50] The lack of α7 subunit in HIEC-6 cells is therefore consistent with their proliferative crypt-like status, because the integrin is also missing in their in situ counterpart, the lower two thirds of the crypt compartment. Taken together, the observations with intestinal cell lines suggest a direct relationship between integrin α7B subunit expression and a potential to display functional small intestinal cell markers.

This assumption is reinforced by the behavior of α7B in differentiating Caco-2/15 cells. Although these cells express and secrete laminin-1, the accumulation of insoluble trimeric laminin-1 at the base of the cell begins only at confluence.[23] The transient increase of α7B at precisely this moment coincides both with the appearance of laminin-1 as a functional ligand, followed quickly by the onset of sucrase-isomaltase expression. Furthermore, this peak of α7B in newly differentiating Caco-2/15 cells is particularly intriguing in light of the similarity of the pattern of expression along the crypt-villus axis in vivo, reinforcing the hypothesis that α7Bβ1 plays a key role in the regulation of laminin-1–mediated enterocytic differentiation although the participation of other integrins in this process cannot be excluded.

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III- ARTICLE

Expression of the α9β1 integrin in human colonic epithelail cells: Resurgence of the fetal phenotype in a subset of colon cancres and adenocarcinoma cell lines

N. Basora, N. Desloges Q. Chang, Y. Bouatrouss, J. Gosselin, J. Poisson, D. Sheppard and J.-F. Beaulieu. (*Int J Cancer* 75:738-743, 1998).

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EXPRESSION OF THE α9β1 INTEGRIN IN HUMAN COLONIC EPITHELIAL CELLS: RESURGENCE OF THE FETAL PHENOTYPE IN A SUBSET OF COLON CANCERS AND ADENOCARCINOMA CELL LINES

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Cell–matrix interactions are thought to be of critical importance in the regulation of various cell functions, including proliferation, migration and control of gene expression. The integrins, a large family of specific receptors for the macromolecules of the extracellular matrix, are important mediators of these interactions. The integrin α9β1 is one of the integrins whose expression is restricted to specialized tissues. Its exact function is unknown. In the present study, we have analyzed expression of the α9 subunit in human colonic epithelial cells by indirect immuno-fluorescence and Western and Northern blots. In normal intact tissues, the antigen was detected at the basolateral domain of epithelial cells in colonic glands at the fetal stage but was absent in adults. Strong staining was detected constitutively in contractile cells at both stages. In adenocarcinomas, the α9 subunit was detected at the basolateral domain of epithelial cells in 6 of the 10 tumors tested, while a reduction of the staining was observed in the subepithelial myofibroblasts in parallel with peri-glandular stroma disorganization. The potential for colon adenocarcinoma cells to express the integrin α9 subunit was confirmed at both the protein and transcript levels in Caco-2 and T84 cell lines, 2 well-characterized cell lines known to exhibit polarization features. The 5 other cell lines tested were negative for expression of the α9 subunit. Taken together, our observations suggest that the α9 integrin subunit is subject to an onco-fetal pattern of expression in human colonic epithelium. ***Int. J. Cancer* 75:738–743, 1998.**
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Cell proliferation, migration and regulation of gene expression depend on various influences, including communication with the extracellular matrix (ECM). Integrins are the major family of cell-surface receptors that mediate cell interactions with the ECM. They are transmembrane αβ heterodimer glycoproteins that provide a structural and functional bridge between ECM molecules and cytoskeletal components and are involved in mediating signal-transduction processes (Clark and Brugge 1995; Rosekelly *et al.*, 1995). So far, 16 α and 8 β subunits have been identified, as well as variants for some of these subunits which arise through differential splicing, presumably increasing the diversity as well as the specificity of both the ligand-binding and the signal-transduction properties of integrins. ECM-binding integrins known to be widely expressed in epithelia include α1β1, α2β1, α3β1, α5β1, α6β1 and α6β4 (Mercurio, 1995; Sheppard, 1996; Beaulieu, 1997). Some of these are ligand-specific, such as α5β1, which binds exclusively to fibronectin, and α6β4, which is specific for the laminins, while others, such as α2β1 and α3β1, can use the collagens and/or laminins depending on the cell type. Thus, the presence of different integrins on cell surfaces allows cells to recognize and respond to a variety of different ECM molecules.

In contrast to normal cells, which are dependent on anchorage for their growth and survival, transformed cells are characterized by an anchorage-independent growth and their ability for invasion and motility. Analysis of integrin expression in cancer cells and accumulating complementary experimental evidence strongly suggest that integrins play important roles in the progression of solid tumors (Heino, 1993; Juliano, 1993; Sheppard, 1996; Varner and Cheresh, 1996; Meredith and Schwartz, 1997). In colon cancers, the alteration in the pattern of integrin expression is also relatively well documented (Juliano, 1993; Agrez and Bates, 1994; Beaulieu, 1997). In general, there is a consensus that there is a reduction of the integrin α3 and α5 subunits in adenomas and a diminished expression of α2, α6 and β4 in carcinomas, while the β1 and αv subunits appear to be maintained (Koretz *et al.*, 1991; Stallmach *et al.*, 1992; Falcioni *et al.*, 1994; Agrez and Bates, 1994).

The integrin α9 subunit is an additional partner for β1 (Palmer *et al.*, 1993). In mice, α9 is expressed in a few highly specialized tissues, such as squamous epithelia, smooth and skeletal muscles and airway epithelium (Palmer *et al.*, 1993). During development, it is associated with the onset of tissue differentiation, suggesting a role in the maturation and/or the maintenance of these tissues (Wang *et al.*, 1995). A well-characterized ligand for the α9β1 integrin is tenascin-C (Yokosaki *et al.*, 1994; Weinacker *et al.*, 1995).

Interestingly, one of the few cell types identified so far to express the α9 subunit are Caco-2 cells (Palmer *et al.*, 1993), a cell line derived from an adenocarcinoma of the human colon (Zweibaum and Chantret, 1989). At least in mice, the normal colonic epithelium lacks the α9 subunit (Palmer *et al.*, 1993). Moreover, since over-expression of its ligand, tenascin-C, in human colonic neoplasms is well documented (Sakai *et al.*, 1993; Hauptmann *et al.*, 1995), these observations taken together suggest a relation between integrin α9β1 and progression from normal to malignant epithelium. In the present study, as a first step to test this hypothesis, we have analyzed expression of the α9 subunit in human fetal and adult colon, in colon adenocarcinomas and in adenocarcinoma cell lines derived from the colon. The presence of the α9 subunit in fetal but not adult human colonic epithelium and its expression in 6 of the 10 adenocarcinomas analyzed and in 2 of the 7 cell lines tested suggest that it is subject to a onco-fetal pattern of expression.

MATERIAL AND METHODS

Tissues and cell lines

Samples of adult colon were obtained consecutively from 16 patients who underwent surgical treatment for colon carcinoma or, in some instances, other pathologies. Staging of colon tumors was

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TABLE I – CLINICAL DATA OF HUMAN COLONIC SPECIMENS STUDIED AND RESPECTIVE IMMUNOSTAINING PATTERNS FOR THE INTEGRIN α9 SUBUNIT DETECTION AND SUCRASE-ISOMALTASE EXPRESSION

Patient	Age (years)	Sex	Diagnosis[3]	Integrin α9 expression[1]					
				Tumor		Resection margin		SI expression[2]	
				E	SEMF	E	SEMF	T	RM
1	42	M	Diverticulosis			−[4]	− −		±
2	74	M	Carcinoma (small intestine)			−	− −		±
3	65	M	Diverticulosis			−	− −		±
4	43	M	Diverticulosis			−Fig 1b	+		±
5	34	M	Diverticulosis			−	+		±
6	71	M	Adenocarcinoma (rectum)			−	− −		±
7	49	F	Adenocarcinoma WD B2			−	− −		±
8	65	M	Adenocarcinoma WD			−	− −		−
9	71	M	Adenocarcinoma MD B2	+/−Fig 1c	±			−/−	
10	45	M	Adenocarcinoma MD B2	+/−	−			−/−	
11	77	F	Adenocarcinoma MD B2	+/−Fig 1d	−			−	
12	72	F	Adenocarcinoma MD B2	+/−	±			−	
13	86	M	Adenocarcinoma MD B2	−	±			−	
14	71	M	Adenocarcinoma PD B2	−	−	−	− −	−	±
15	67	F	Adenocarcinoma PD C2	+/−	−	−	− −	+/−	±
16	73	M	Adenocarcinoma PD C2	+/−	±			−	
17	72	M	Adenocarcinoma PD C2	−Fig 1e	−	−	+	−	±
18			Adenocarcinoma PD C2	−	±			nd	

[1]Immunostaining for the α9 subunit at the basolateral domain of epithelial cells (E) and in sub-epithelial myofibroblasts (SEMF) at the tumor site and/or resection margin.–[2]Immunostaining for sucrase–isomaltase (SI) in epithelial cells at the tumor site (T) or the resection margin (RM).–[3]As determined by pathologists; staging of primary colon tumors was according to Astler and Coller (1954). WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; nd, not determined.–[4]Immunostaining for α9 and SI was expressed on a scale from − (negative) to ++ (maximum reaction); +/− indicates that only a subpopulation of cells was stained with a + intensity.

according to Astler and Coller (1954). As detailed in Table I, samples were from non-diseased parts (at least 10 cm distant from the lesion) corresponding to the resection margin and/or from the primary tumor. Specimens of colon from 15 fetuses ranging in age from 14 to 19 weeks (post-fertilization) were obtained after legal abortion. The project was in accordance with protocols approved by the Institutional Human Research Review Committee for the use of human material. Only tissues obtained rapidly (in less than 40 min) were used. The preparation and optimum cutting temperature (OCT; Tissue Tek; Miles, Elkhart, IN) for embedding of specimens for cryo-sectioning were as previously described (Beaulieu *et al.*, 1990).

The Caco-2/15 cell line, a clone of the parent Caco-2 cell line (Zweibaum and Chantret, 1989), has been characterized elsewhere (Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1992). Cells between passages 54 and 72 were cultured in plastic dishes as described previously (Vachon and Beaulieu, 1992). In this study, Caco-2/15 cells were analyzed at subconfluence (50–60% confluence) and at 15 days post-confluence (sucrase–isomaltase-positive). The other colon carcinoma cell lines—HT29(G), T84, LS123, HCT116, LoVo and Colo201—were grown according to instructions provided by the ATCC (Rockville, MD) and used at 80–90% confluence. HT29(I) cells (sucrase–isomaltase-positive) were obtained after selection in a glucose-free medium containing 2.5 mM inosine for 6 passages, as described previously (Beaulieu *et al.*, 1989).

Primary antibodies

The antibodies used in this work were the monoclonal antibody MAb 13 (Basora *et al.*, 1997) directed against the human β1 integrin subunit (kindly provided by Dr. S.K. Akiyama, Washington, DC), HSI-14 (Beaulieu *et al.*, 1989) directed against the human sucrase–isomaltase complex, 1A4 and CY90 (Sigma, St. Louis, MO) specific for human α-smooth muscle actin (Sappino *et al.*, 1989; Beaulieu *et al.*, 1993), keratin 18 (Vachon and Beaulieu, 1995), and clone 36 (Transduction Laboratories, Lexington, KY) against human E-cadherin. Production of the rabbit specific anti-serum directed against the α9 integrin subunit and the procedure to affinity-purify the α9 antibodies have been described previously (Palmer *et al.*, 1993).

Immuno-fluorescence staining

Cryosections 2 to 3 μm thick were cut on a Jung Frigocut 2800N cryostat (Leica, Saint-Laurent, Canada), spread on silane-coated glass slides then air-dried for 1 hr at room temperature before storage at −80°C. Tissue sections were fixed in fresh 2% paraformaldehyde (60 min, 4°C) before immuno-staining, as described previously (Beaulieu *et al.*, 1990; Beaulieu, 1992). Anti-α9 subunit affinity-purified antibodies were diluted 1:200 in PBS (pH 7.4) containing 10% Blotto. Anti-α-smooth muscle actin 1A4 antibodies were used at a 1:2,000 dilution. FITC-conjugated goat anti-mouse or rabbit IgG (Boehringer-Mannheim, Laval, Canada) were used as secondary antibodies at a working dilution of 1:25. Sections were then stained with 0.01% Evan's blue in PBS. Preparations were mounted in glycerol-PBS (9:1) containing 0.1% paraphenylene diamine and viewed with a Reichert Polyvar 2 microscope (Leica) equipped for epifluorescence. In all cases, no immuno-fluorescent staining was observed when primary antibodies were omitted or replaced by appropriate non-immune sera.

Western blot analysis

SDS/12% PAGE and Western blotting were performed as described previously (Beaulieu *et al.*, 1989; Vachon and Beaulieu, 1995). Briefly, proteins from the various cell lines were solubilized directly in sample buffer containing 5% β-mercaptoethanol. Separated proteins (150 μg/lane) were transferred onto nitrocellulose (ImmunoSelect; GIBCO BRL, Burlington, Canada) and stained with Ponceau red to localize m.w. markers (44–220 kDa range; Bio-Rad, Mississauga, Canada). Membranes were blocked overnight at room temperature in PBS (pH 7.4) containing 10% Blotto and incubated with the primary antibody diluted in the blocking solution (anti-α9, 1:500; HSI-14, 1:500, MAb13, 1:1,000; anti-E-cadherin, 1:1,000; and CY90, 1:2,000). After washing in PBS, membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse, -rabbit (Bio-Rad) or -rat IgG (Cedar Lane, Hornby, Canada), further washed and finally incubated with a chromogenic

substrate for alkaline phosphatase detection according to the instructions of the manufacturer.

Northern blot analysis

Cells from 2 100 mm dishes were used for each RNA extraction using Trizol reagent (GIBCO BRL). Total cellular RNA was electrophoresed on a 1%:1.8% agarose:formaldehyde gel (30 to 50 µg/lane) and blotted onto nylon membrane (Hybond-N; Amersham, Oakville, Canada) as described previously (Beaulieu and Quaroni, 1991). Radiolabeled probes were synthesized from a 2.1 kb fragment of Qa9 cDNA (Palmer *et al.*, 1993) and a 1.3 kb fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, used as a control, using the Multiprime Labeling System (Amersham) in the presence of [α-^{32}P]dCTP. Pre-hybridization (1 hr) and hybridization (12 hr) were performed at 68°C. Kodak (New Haven, CT) XAR-5 films were exposed with intensifying screens for 2 hr (GAPDH) and 42 hr (α9) at −80°C.

RESULTS

Expression of the integrin α9 subunit in human fetal and adult colon

Expression of the α9 subunit was analyzed first by indirect immuno-fluorescence in fetal and normal adult human colon. In the fetal samples, antigen was detected in the cytoplasm of epithelial cells and at the basolateral surface of the glandular epithelium (Fig. 1*a*), as well as in muscle layers, at all stages studied. Immunostaining was considered specific as it was not seen with a negative control affinity-purified rabbit antibody directed against another integrin subunit not expressed in the intestinal epithelium (the α7A subunit; Basora *et al.*, 1997). In adult colon, the α9 subunit was not detected in the epithelium (Fig. 1*b,c*) but was present in sub-epithelial myofibroblasts surrounding the lower two-thirds of the glands (Fig. 1*b*, Table I) as well as in the smooth musculature.

We then screened a number of human colon adenocarcinomas. For 3 of the specimens (patients 6 to 8), only the resection margin

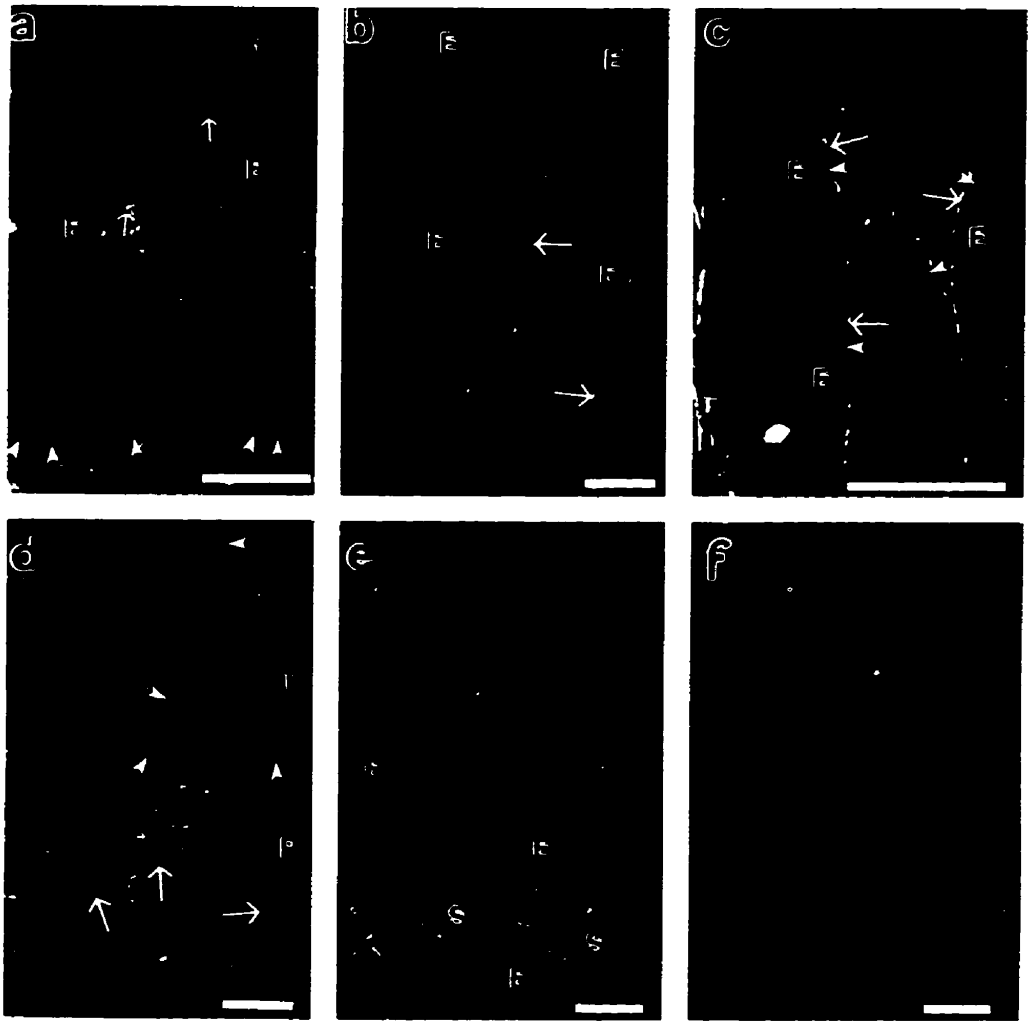

FIGURE 1 – Immuno-fluorescence staining of frozen tissue sections from the human colon for detection of the integrin α9 subunit. (*a*) Fetal colon at 18 weeks of gestation. The α9 subunit is detectable in the cytoplasm of epithelial cells (E) and at the basolateral surface in the glandular epithelium (arrowheads). Some staining is also apparent at the tip of the villus core (arrows). (*b,c*) Normal adult colonic mucosa. Antigen is detected in sub-epithelial myofibroblasts (arrows) surrounding the lower two-thirds of the glands. The epithelium (E) remains consistently negative at its base (arrowheads). (*d*) A moderately differentiated adenocarcinoma of the colon (grade B2). As in most of the tumors of this type, antigen expression is patchy. In the stained areas of the tumor (T), the α9 subunit is localized at the basolateral surface of epithelial cells (arrowheads), while it is not detected in the stroma. In the adjacent peri-tumoral region (P), the distribution of antigen is comparable to normal, being restricted to the sub-epithelial myofibroblast layer (arrows). (*e*) Another moderately differentiated adenocarcinoma of the colon (grade B2). The basolateral distribution of the α9 subunit in epithelial cells (E) is more evident at higher magnification. Note also the lack of staining in the stroma (S). (*f*) A poorly differentiated adenocarcinoma (grade C2). As in 3/5 tumors of this type, antigen was not detected in any structure. Scale bar: 10 µm.

was available. The staining pattern of α9 subunit expression in these tissues was comparable to that observed in control tissues (Table I) as well as in peri-tumoral tissues (*e.g.*, see the P region in Fig. 1*d*). For the remaining 10 specimens, primary tumors were obtained and expression of the integrin α9 subunit was observed in 6 of them (patients 9–12, 15, 16), 4 of which were diagnosed as moderately differentiated adenocarcinomas. In these tumors, staining was heterogenous, varying from a bright basolateral staining at some sites, namely, at the bottom of the glandular structures, to below detection level at other sites (Fig. 1*d,e*). The other 4 tumors (patients 13, 14, 17, 18) were devoid of any staining for the α9 subunit (Fig. 1*f*). Three of them were diagnosed as poorly differentiated adenocarcinomas (Table I). We found no correlation between expression of the α9 subunit and sucrase–isomaltase, a differentiation marker for colon cancer cells (Table I).

Expression of the integrin α9 subunit in human adenocarcinoma cell lines

Expression of the α9 subunit was analyzed by Western blot in 7 well-characterized cell lines derived from human colon adenocarcinomas. Three of these cell lines (Caco-2/15, HT29 and T84) are well known for their potential to polarize *in vitro* and exhibit morphological and/or functional characteristics of normal intestinal cells, while the other 4 are considered to be poorly differentiated and unpolarized under standard culture conditions (Zweibaum and Chantret, 1989; Rutzky and Moyer, 1990). Interestingly, the α9 subunit was detected exclusively in the Caco-2/15 and T84 cell lines (Fig. 2, lanes 1–3), which have the potential to polarize. In contrast, the β partner for the integrin α9 subunit, β1, was detected in all cell lines tested (Fig. 2). Similarly, E-cadherin and keratin 18 also were expressed ubiquitously by all of these cell lines. Finally, the relative amounts of the α9 subunit in Caco-2/15 cells were invariably higher in subconfluent cells (lane 1) as compared to confluent Caco-2/15 cells, which expressed sucrase–isomaltase (lane 2).

Analysis of the α9 subunit transcripts by Northern blot in the same cell lines confirmed that expression of the α9 subunit is restricted to the Caco-2/15 and T84 cell lines (Fig. 3, lanes 1, 3). A very weak signal for the α9 subunit transcripts in post-confluent Caco-2/15 cells (lane 2) relative to their subconfluent counterpart (lane 1) was observed consistently.

DISCUSSION

In this study, we provide evidence that the integrin α9β1 can be expressed according to an onco-fetal-like pattern in human colonic epithelium. During development, by using specific antibodies directed to the α9 subunit in indirect immuno-fluorescence, this integrin was detected in fetal colonic epithelium at all stages studied (14 to 19 weeks), while it remained undetectable in its adult counterpart. In contrast, the α9 subunit was found in α-smooth muscle actin-expressing cells in both developing and adult mucosa. These observations, which are in good agreement with previous results (Palmer *et al.*, 1993; Dieckgraefe *et al.*, 1996), suggest that the α9 subunit is down-regulated in mature colonic epithelium, while its expression is maintained in contractile cells, such as sub-epithelial myofibroblasts and smooth muscle cells. Such a pattern of expression for an integrin in the gut is unusual as most other integrins present in fetal intestinal epithelium remain at the adult stage (Beaulieu, 1997). It is nevertheless not unique in this regard as the α7Bβ1 integrin also disappears from colonic epithelium in the transition from fetal to adult stages (Basora *et al.*, 1997). One other peculiarity worth mentioning for the α9 subunit is the evident discrepancy between its pattern of expression in colonic epithelium and that of its ligand, tenascin-C (Yokosaki *et al.*, 1994; Weinacker *et al.*, 1995), which is absent at the epithelium–mesenchyme interface in fetal colon but is expressed in its adult counterpart (Beaulieu, 1992; Desloges *et al.*, 1994). Such differences also have been reported for other developing organs in mice,

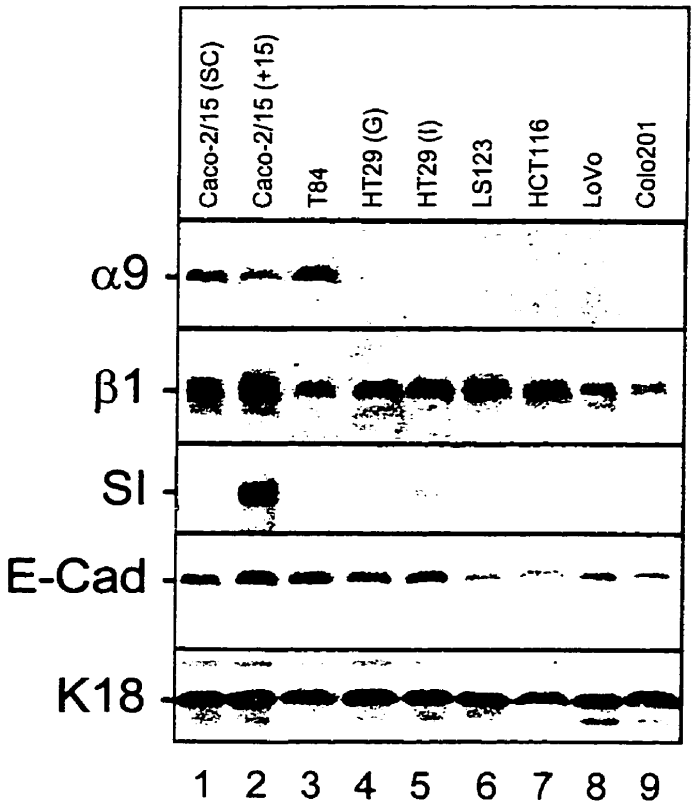

FIGURE 2 – Representative Western blot analysis of adenocarcinoma cell lines for expression of the integrin α9 subunit in relation to other intestinal cell markers. Total proteins from cell lysates (150 μg/lane) were separated on SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes for the detection of integrin α9 and β1 subunits, sucrase–isomaltase (SI), E-cadherin (E-cad) and keratin 18 (K18). The α9 subunit was detected in Caco-2/15 cells (both subconfluent [SC] and at 15 days after confluence [+15]) and in T84 cells. Other cell lines, which include HT29—both undifferentiated (glucose: G) and differentiated (inosine: I)—LS123, HCT116, LoVo and Colo201 were negative. In comparison, the 7 cell lines were positive for the integrin β1 subunit, the β partner for the α9 subunit. E-cadherin and keratin 18. Sucrase–isomaltase, a differentiation marker for intestinal cells, was detected only in confluent Caco-2/15 (lane 2) and glucose-depleted HT29 cells (lane 5).

suggesting the existence of other ligands for α9β1 integrin (Wang *et al.*, 1995).

The exact function of α9β1 remains unknown. Analysis of the α9 subunit during murine embryogenesis has suggested that this integrin is not likely to contribute to the early steps of organ formation but probably plays a role in the maturation and/or maintenance of a variety of differentiated tissues (Wang *et al.*, 1995), a suggestion which fits well with the constitutive expression of α9 in normal colonic α-smooth muscle actin–expressing cells (Palmer *et al.*, 1993; Dieckgraefe *et al.*, 1996; this work) and its substantial reduction seen in adenocarcinomas, where α-smooth muscle actin-positive sub-epithelial myofibroblasts tend to disappear (Sappino *et al.*, 1989). However, the fact that in some tissues α9 expression is restricted to cells known to retain the capacity to proliferate, *i.e.*, basal layer of several squamous epithelia, is indicative of an additional function(s) for this integrin (Palmer *et al.*, 1993; Wang *et al.*, 1995).

Interestingly, the original characterization of the α9 subunit revealed expression of the α9β1 integrin in Caco-2 cells (Palmer *et al.*, 1993), a cell line derived from an adenocarcinoma of the human colon, suggesting a possible relation between α9 subunit expres-

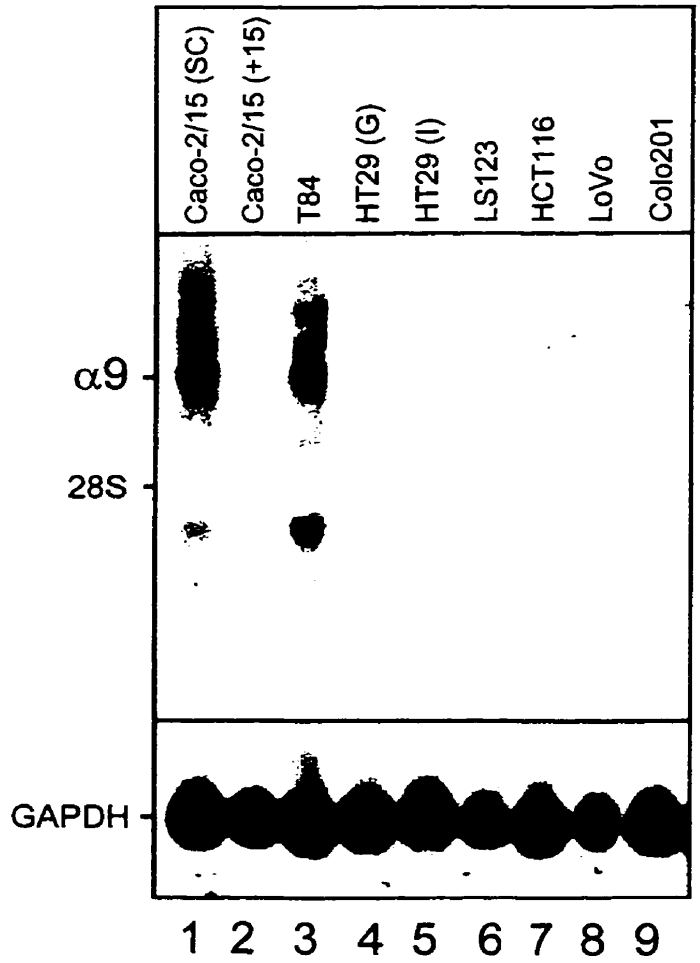

FIGURE 3 – Representative Northern blot analysis of adenocarcinoma cell lines for expression of the α9 subunit transcript. Total RNA (30 to 50 μg/lane) isolated from the various cell lines was separated in a 1%:1.8% agarose:formaldehyde gel, transferred to a nylon membrane and hybridized with ^{32}P-labeled α9 cDNA (top panel) and GAPDH cDNA (lower panel). The position of the 28-S ribosomal band is indicated on the left. The transcript for the α9 subunit was observed in sub-confluent (SC) Caco-2/15 cells (lane 1) and in T84 (lane 3). A weak signal also can be seen in post-confluent Caco-2/15 cells (lane 2), but the transcript was not detected in the other cell lines tested (lanes 4–9). Transcripts consisting of a major band at approx. 7.0 kb and a second one at approx. 4.0 kb were observed in both the Caco-2/15 and T84 cell lines.

sion and the neoplastic nature of the cells. In this context, Caco-2 cells have been characterized extensively for their ability to express a number of cell-surface markers also present in primary colorectal tumors (Zweibaum and Chantret, 1989; Ménard and Beaulieu, 1994), including integrins (Beaulieu, 1997). To test this hypothesis, we first analyzed a number of adenocarcinomas of the human colon for expression of the α9 subunit by indirect immuno-fluorescence. Our observations revealed that more than half of the tumors tested were immunoreactive for the α9 subunit. Staining was heterogeneous in these tumors. In the positive areas, the distribution of the antigen at the basolateral domain of cancer cells was comparable to that observed in the fetal colonic glands. Analysis of α9 subunit expression in a panel of human colon adenocarcinoma cell lines confirmed the potential of adenocarcinoma cells to express this integrin. In addition to its presence in Caco-2 (Palmer *et al.*, 1993) and Caco-2/15 cells, the α9 subunit was identified in the T84 cell line at both the protein and mRNA levels. Caco-2 and T84 cell lines are among those few intestinal cell lines known for their ability to reach a level of cellular polarization *in vitro* comparable to that exhibited by the differentiated cells in the intact intestine, while the 5 other cell lines tested, which remain poorly polarized under standard culture conditions—HT29(G), LS123, HCT116, LoVo and Colo201 (Zweibaum and Chantret, 1989; Rutzky and Moyer, 1990)—were negative for α9 subunit expression. However, the lack of α9 expression in HT29(I) cells, which can polarize and express sucrase–isomaltase, rules out a direct requirement of this integrin for the expression of these cellular characteristics. Interestingly, the lack of correlation between the presence of the α9 integrin subunit and sucrase–isomaltase expression also was observed in primary tumors.

Taken together, our observations indicate that the α9 subunit is present in the fetal colonic epithelium but absent in the normal adult, while it can be expressed in some human colon cancer cell lines and primary colonic tumors. This is not unique to this protein as it also has been observed for CEA (Gold and Freeman, 1965), a very well-characterized onco-fetal antigen, and CCA (Quaroni *et al.*, 1986) as well as for a number of brush border hydrolases, namely, sucrase–isomaltase, aminopeptidase N and dipeptidylpeptidase IV (Ménard and Beaulieu, 1994). As described here for the integrin α9 subunit, these markers occur transiently in fetal colon during the second trimester, while they are absent or expressed only weakly in the epithelium of the normal adult colonic mucosa. Thus, as suggested for these other markers, we propose that expression of the α9 subunit in colonic neoplasia may represent the resurgence to a fetal pattern of epithelial gene expression. In a context where over-expression of its ligand, tenascin-C, at the tumor–stroma interface appears common in colorectal tumors (Sakai *et al.*, 1993; Hauptmann *et al.*, 1995; data not shown), the resurgence of α9β1 expression may be of importance in the process of tumor progression.

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IV- ARTICLE

Relation between integrin $\alpha 7B\beta 1$ expression in human intestinal cell relation and enterocytic differentiation

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V- ARTICLE

Expression of functionally distinct integrin β4 subunit variants in relation to the differentiation state in human intestinal cells

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Expression of functionally distinct integrin β4 subunit variants in relation to the differentiation state in human intestinal cells

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ABSTRACT

Integrins are important mediators of cell-laminin interactions. In the small intestinal epithelium, which consists of spatially separated proliferative and differentiated cell populations located respectively in the crypt and on the villus, laminins and laminin-binding integrins are differentially expressed along the crypt-villus axis. One exception to this is the integrin α6β4, which is thought to be ubiquitously expressed by intestinal cells. However, in this study, a re-evaluation of the β4 subunit expression with different antibodies revealed that two forms of β4 may exist in the human intestinal epithelium. By using two well characterized intestinal epithelial cell models, the crypt-like HIEC cells and the villus-like Caco-2/15 cells, we show that differentiated enterocytes express a full-length 205 kD β4A subunit, whereas undifferentiated crypt cells express a novel β4A subunit that does not contain the COOH-terminal segment of the cytoplasmic domain (β4Atcd-). This new form was not found to arise from alternative β4 mRNA splicing. Moreover, we found that these two β4A forms can associate into α6β4A complexes; however, the β4Atcd- integrin expressed by the undifferentiated crypt cells is not functional for adhesion to laminin-5. Hence, these studies identify a novel α6β4Atcd- integrin expressed in undifferentiated intestinal crypt cells that is functionally distinct.

INTRODUCTION

Epithelial cells are characterized by special structural features such as polarized morphology, specialized cell-cell contacts and their attachment to an underlying basement membrane (Rodriguez-Boulan and Nelson, 1989). This attachment mediates various crucial cell functions including adhesion, migration, proliferation, differentiation and cell survival . The biological effects of basement membranes are largely mediated by laminins, a growing family of structurally related molecules (Burgeson et al., 1994; Wewer and Engvall, 1994), which are expressed in a tissue specific manner (Engvall et al., 1990). Epithelia bind to laminins via various cell membrane receptors, many of which are members of the integrin superfamily (Ruoslahti, 1991; Hynes, 1992; Sonnenberg, 1992; Mercurio, 1995; Sheppard, 1996) which can subsequently initiate intracellular signaling cascades upon ligation to their ligand (Clarke and Brugge, 1995; Miyamoto et al., 1995). Some integrins, such as $\alpha 2\beta 1$ and $\alpha 3\beta 1$, are considered quite promiscuous (Wayner and Carter 1987) and bind various molecules including laminins, while others such as $\alpha 6\beta 1$, $\alpha 7\beta 1$ and $\alpha 6\beta 4$ are specific for laminins (Mercurio, 1995; Beaulieu, 1997a and ref. therein).

The integrin $\alpha 6\beta 4$ has a number of features which distinguish it structurally and functionally from the other laminin receptors of the $\beta 1$ family (Suzuki and Naitoh, 1990; Hogervost et al., 1990; Tamura et al., 1990). It has an unusually long cytoplasmic domain having no homology with other integrin β subunits. Upon binding to laminin-5, this integrin becomes concentrated in hemidesmosomes and is associated with intermediate filaments, making it essential for the organization and maintenance of the epithelial structure (Mainiero et al., 1995; Borradori and Sonnenberg,1996; Weaver et al., 1997). This role for $\alpha 6\beta 4$ was confirmed using transgenic mice in which the $\beta 4$ subunit had been knocked out (Dowling et al., 1996;Vanderneut et al., 1996), causing the loss of hemidesmosomal structures. Moreover, tissues that do not form hemidesmosomes were more susceptible to degeneration that, in some cases, lead to apoptosis suggesting that $\beta 4$ coordinates hemidesmosome assembly but may be responsible for additional signals, some of which may be essential for cell survival. $\alpha 6\beta 4$, like other integrins, can mediate signal transduction (Giancotti, 1996). Indeed, upon ligation to laminin-5, $\alpha 6\beta 4$ has been shown to become phosphorylated and to cause

recruitment of Shc and Grb2-mSOS, which go on to activate Ras/MAP kinase pathways (Mainiero et al., 1995; Mainiero et al., 1997).

The intestinal epithelium, which is in constant and rapid renewal, represents an interesting model for the study of mechanisms involved in the determination of the cell state (Beaulieu, 1997b). Within its functional unit, the crypt-villus axis, are two main distinct cell populations: the proliferative and poorly differentiated crypt cells and the mature enterocytes of the villus (Leblond,1981; Louvard et al., 1992; Ménard and Beaulieu, 1994). Processes of epithelial cell growth and functional differentiation need to be tightly regulated along the crypt-villus axis (Poldovsky and Babyatsky,1995; Boyle and Brenner, 1995). Analysis of integrins and basement membrane molecules in the human intestine has revealed particular patterns of expression for many of these molecules, namely those involved in laminin-cell interactions (Beaulieu, 1997a, 1997b). Of interest is the reciprocal expression of laminin-1 and laminin-2 that has been found along the crypt-villus axis, with laminin-1 occuring as a villus form and laminin-2 as a crypt form, suggesting a relation between laminin form expression and cell differentiation (Beaulieu and Vachon, 1994; Simon-Assmann et al., 1994). The functional relevance of these observations was provided by the demonstration that laminin-1, but not laminin-2, can precociously induce differentiation in intestinal Caco-2/15 cells (Vachon and Beaulieu, 1995) suggesting that laminin-1 is critical in triggering terminal differentiation in the intestinal epithelium (Vachon and Beaulieu, 1995, De Archangelis et al., 1996). A differential crypt-villus pattern of expression for laminin-binding integrins was also reported in the intestinal epithelium. The distribution of $\alpha 2\beta 1$ was mainly restricted to the crypt while $\alpha 3\beta 1$ was found predominantly in the villus (Beaulieu, 1992; Perreault et al., 1995). More recently, $\alpha 7B\beta 1$, a specific laminin receptor largely involved in muscle development in response to laminin (Vachon et al., 1997) has been identified in the intestinal epithelium. It was found to be located at the crypt-villus junction in the intact intestine while its expression was transiently up-regulated in differentiating Caco-2/15 (Basora et al., 1997). In contrast, the integrin $\alpha 6\beta 4$ has been reported to be present at the base of intestinal epithelial cells all along the crypt-villus axis suggesting that this laminin receptor is ubiquitously expressed by intestinal cells (Beaulieu and Vachon, 1994; Simon-Assmann et al., 1994; Perreault et al., 1995; Leivo et al., 1996).

In this study, the expression of the β4 subunit in intestinal cells was re-evaluated on the basis of the observation that an antibody directed to the COOH-terminal segment of the cytoplasmic domain of the molecule (Giancotti et al., 1992) detected the β4 subunit only in the villus cells of the intact small intestine, suggesting that two distinct forms of β4 may exist in the intestinal epithelium. To further investigate this, we used two well characterized intestinal cell models which allow to some extent the recapitulation of the crypt-villus axis in vitro: the crypt-like HIEC cells, which are proliferative and undifferentiated (Perreault et al., 1996), and the Caco-2/15 cells which have the ability to differentiate into fully functional villus-like enterocytes (Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1992; Vachon et al., 1996). Our data show that differentiated enterocytes express a full-length 205 kD β4 subunit while undifferentiated crypt cells express a novel β4 subunit which does not contain the COOH-terminal segment of the cytoplasmic domain. Moreover, we found that these two β4 forms can associate with the α6 subunit, constituting α6β4 complexes that are functionally distinct with regard to adhesion activity, the undifferentiated crypt cells expressing an inactive α6β4 receptor.

MATERIALS AND METHODS

Tissues

Specimens of adult small intestine (jejunum) were obtained from non-diseased parts of resected segments. Only specimens obtained rapidly were used; the overall period required before freezing the tissue after surgery never exceeded 60 minutes. The project was in accordance with the protocol approved by the Institutional Human Research Review Committee for the use of human material.

Cell Culture

The human colon carcinoma Caco-2/15 cell line, a stable clone of the parent Caco-2 cell line (HBT 37; ATCC, Rockville, MD) has been characterized elsewhere (Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1992; Vachon et al., 1996). These cells are unique in that upon reaching confluence they spontaneously undergo a gradual enterocytic differentiation process, similar to that observed in the epithelium of the intact fetal small and large intestine (Pinto et al., 1983; Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1992; Vachon et al., 1996). Cells between passages 53 and 70 were cultured in plastic dishes (100 mm Falcon, Becton-Dickenson Labware, Mississauga, Ont.) at 37°C in an atmosphere of 95% air - 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, CELLect Gold, ICN/Flow, Costa Mesa, CA), 4 mM glutamine and 20 mM HEPES and cultures were refed every 48 h.

The HIEC-6 cells have been generated from the normal fetal human small intestine. These cells express a number of crypt cell markers but no villus cell markers and are thus considered as poorly differentiated crypt cells (Perreault and Beaulieu, 1996; Quaroni and Beaulieu, 1997). They were grown in DMEM supplemented with 4 mM glutamine, 20 mM HEPES, 5 ng/ml recombinant epidermal growth factor (Gibco-BRL), 0.2 IU/ml insulin (Connaught Novo Laboratories, Willowdale, Ont.) and 5% FBS in 100 mM plastic culture dishes (Perreault and Beaulieu, 1996). Cells were used between passages 5 and 15.

Primary Antibodies

Antibodies used in this study were the monoclonals GoH3 (Sonnenberg et al., 1988; Pharmingen, Mississauga, Ont.) against the extracellular domain of the α6 integrin subunit and 3E1 (Henssle et al., 1984; Gibco-BRL, Burlington, Ont) and 439-9B (Pharmingen) both against the extracellular domain of the β4 integrin subunit; mAb13 against integrin β1 and mAb16 against integrin α5 were kind gifts from Dr. S.K. Akiyama (National Institute of Dental Research, Bethesda, MD; Akiyama et al., 1989).; P1E6 against integrin α2 (Wayner and Carter, 1987; Gibco); P1B5 against integrin α3 (Wayner and Carter, 1987; Oncogene Science, Uniondale, NY). An anti-β4 antiserum (Giancotti et al., 1992) against the cytoplasmic terminal domain of the β4 subunit (referred hereafter as the anti-β4c antibody) was generously provided by Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA); Purified laminin-5 was a generous gift fror Dr. Burgeson (Cutaneous Biology Research Center, Charlestown, MA).

Indirect Immunofluorescence

The preparation and OCT (Optimum Cutting Temperature embedding compound; Tissue Tek, Miles laboratories, Elkhart, IN) embedding of tissue samples for cryosections was performed as described previously (Beaulieu, 1992). Frozen sections 3 µm thick were cut on a Jung Frigocut 2800N cryostat (Leica Canada, St. Laurent, Qué), spread on silane-coated glass slides and air-dried 1 h at room temperature. Tissue sections were fixed in methanol or ethanol (10 min, -20°C) before immunostaining, as described elsewhere (Beaulieu, 1992; Beaulieu and Vachon, 1994). Primary antibodies were diluted 1/100 (anti-β4cyto) or used at 5µg/ml (3E1 and 439-9B). The secondary antibody was an FITC-conjugated goat anti-rabbit or anti-mouse IgG (Boehringer Mannheim Canada, Laval, Qué.) or anti-rat IgG (Caltag, Cedarlane Laboratories, Hornby, Ont.) used 1/25 in 2% bovine serum albumin in PBS. Sections were stained with 0.01% Evan's blue in PBS, mounted in glycerol-PBS (9:1) containing 0.1% paraphenylenediamine and viewed with a Reichart Polyvar 2 microscope (Leica Canada) equipped for epifluorescence. In all cases no specific immunofluorescent staining was observed when primary antibodies were omitted or replaced by the corresponding non-immune serum.

Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels and immunoblotting were performed as described previously (Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1995, Perreault and Beaulieu, 1996). Cells were washed twice in PBS and harvested in 1x solubilization buffer (2.3% SDS, 10% glycerol, 0.001% bromophenol blue in 62.5 mM Tris-HCl pH 6.8) containing 5% β-mercaptoethanol. Samples were boiled for 5 min, cleared by centrifugation (13,000xg, 5 min) and aliquoted for storage at -80°C. Separated proteins (100 µg/lane) were transferred onto nitrocellulose (BioRad, Mississauga, Ont.) and blocked in PBS containing 10% powdered skim milk, then incubated overnight at room temperature with primary antibodies (anti-β4c, 1/500) diluted in the blocking solution. Alkaline phosphatase-conjugated secondary antibodies (BioRad) were used according to the manufacturers instructions.

Metabolic Cell Labeling and Immunoprecipitation

Cells were metabolically labeled using Promix [^{35}S]-methionine and cystine (Amersham, Oakville, Ont) 100 µCi/ml for HIEC and 200 µCi/ml for Caco-2/15 for the indicated times. For certain points, cells were labeled as described above and after the labeling period the radioactive medium was removed, cells were washed once with complete DMEM and chased with 10 ml of complete DMEM containing 10X methionine and 10X cystine for the indicated times. Cells were solubilized in ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5mM EDTA, 0.5% Triton X-100, 2 µM PMSF, 50 µg/ml leupeptin, 50 µg/ml pepstatin, 100 µg/ml aprotinin) for 20 minutes on ice, then centrifuged for 15 minutes at 13,000xg. Samples were pre-cleared using 100 µl heparin-agarose (Bio-Rad) followed by 50 µl protein-G Sepharose (Gibco-BRL) each for 1 hour at 4°C. Antibodies were added to the samples and were incubated for 15 h at 4°C followed by the addition of protein-G Sepharose for 1 hour at 4°C. Radioactive samples were analyzed under non-reduced conditions by SDS-PAGE, the gels were fixed (10% acetic acid:45% methanol:45% deionized water) for 1 hour and soaked for 15 minutes in Amplify (Amersham), dried and exposed using Hyperfilm ECL (Amersham).

RT-PCR

Total RNA was isolated from cell lines or tissue homogenates using TriZOL (Gibco-BRL). The integrity of the RNA was verified by ethidium bromide staining and the quantities were determined spectrophotometrically. Reverse transcriptase Superscript (Gibco-BRL) and 0.5 µg of oligo-(dT)12-18 primer (Pharmacia) were added to 5 µg of total RNA, as described elsewhere (Beaulieu et al., 1994). The integrin β4 subunit was amplified using three different sets of primers. B1/B2: B1 is 5'-GAATTCGTTCTACGCTCTCC-3' and B2 is 5'-GAATTCTGAGAGATGTGGGC-3' and amplify a band of 373 bp which spans the region 4182 to 4565 of β4A (Suzuki and Naitoh, 1989). B3/B4: B3 is 5'-CCCGGGGATATCGTCGGCTAC-3' and B4 is 5'-CCCGGGGCTGTCTCCATCCAC-3' and amplify a band of 93 bp spanning the region of 4918 to 5011 bp of β4A (Clarke et al, 1994); B5/B6: B5 is 5'-GAATTCTTCCTAGTGGATGG-3' and B6 is 5'-GAATTCCTAGTGGGACAT-3' and amplify a band of 185 bp spanning the region of 5235 to 5420 of β4A. Single stranded cDNA was amplified in PCR buffer (Pharmacia) containing 0.25 µM of both sense and antisense primers for 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C for B1/B2, 65°C for B3/B4 and 51°C for B5/B6) and extension (1 min at 72°C) in a thermal cycler (Perkin-Elmer DNA Thermal cycler model 480) in the presence of 250 µM dNTP's and 2.5 U of Taq (Roche; obtained from Pharmacia). Conditions for S14 amplification and sequence analysis of the β4 B3/B4 PCR products were described elsewhere (Basora et al.,1997).

Cell Adhesion Assays

The adhesion of HIEC and Caco-2/15 cells on laminin-5 was carried out in 96 well plates based on a procedure described previously (Niessen et al., 1994). Bovine serum albumin (BSA) 1% was used to set background binding. Purified laminin-5 was used at 10 ug/ml. The wells were coated for 1 hour at 37 °C and were subsequently blocked with BSA 0.25% for 1 hour at 37 °C. Cells harvested using 10mM EDTA, were washed twice and 5×10^4 cells were plated per well for 30 min. Unbound cells were removed by gently washing twice with PBS and bound cells were fixed in 1% formaldehyde, colored with a 1% crystal violet solution and

solubilized using 2% SDS. The plates were read at 595 nm with a Microplate Reader (model 3550, BioRad). For inhibition studies, neutralizing antibodies were added to cells before plating and incubated for 30 min, each at a final concentration of 10 ug/ml.

RESULTS

As shown previously with the 3E1 antibody (Beaulieu and Vachon, 1994; Perreault et al., 1995), the use of 439-9B, another antibody directed to the extracellular domain of the β4 subunit, was found to uniformly stain the base of the epithelium throughout the entire crypt-villus axis (Fig. 1A). The use of another antibody, the anti-β4c, raised against the last thirty one amino acids of the COOH terminal of the β4 subunit, produced a distinct pattern of expression, strongly labeling the basal surface of villus cells but only weakly staining the crypt (Fig. 1B). This observation suggests that at least two immunologically distinct forms of β4 exist in the intestinal epithelium and that they are associated with different cell populations along the crypt-villus axis.

Expression of β4 in intestinal epithelial cells

This hypothesis was tested by analyzing the expression of the β4 subunit in undifferentiated (HIEC) and differentiated (Caco-2/15) cells. Western blot analysis cell lysates using anti-β4c (Fig. 2A) detected the β4 subunit in Caco-2/15 (lane 2) cells but not in HIEC cells (lane 1). However, RT-PCR amplification using different sets of primers, all located in the region encoding for the cytoplasmic domain of β4, revealed that both cell models express the β4 transcript (Fig. 2B, lanes 2 and 4). The B1/B2 primer set spans the region which is alternatively spliced to produce isoforms β4A, β4B or β4C (Tamura et al., 1990; Hogervost et al., 1990; Giancotti et al., 1992). This set of primers amplifies a band of 373 bp which corresponds to β4A, suggesting that this is the major β4 isoform in both cell lines. The primer set B3/B4 has been used previously to identify the β4D isoform which contains a 21 bp deletion (Clarke et al., 1994). The amplification of a single band of 93 bp with this set of primers in HIEC and Caco-2/15 cells indicated that neither cell line expresses this isoform.

Undifferentiated crypt cells contain a novel form of the β4A subunit

The differential expression of β4 subunits between differentiated and undifferentiated intestinal cells was further investigated by immunoprecipitation using the 3E1 antibody,

followed by Western blot detection using the anti-β4c antibody (Fig. 3A). As expected from Western blot analyses (previous section), the β4 subunit was only detected in Caco-2/15 cells (lane 2) with this procedure, suggesting that the β4c epitope is absent in HIEC. To verify this, HIEC and Caco-2/15 cells were metabolically labeled using ^{35}S-methionine and cystine for six hours, then β4 was immunoprecipitated using either 3E1 or the anti-β4c antibody. As shown in Fig. 3B, both antibodies immunoprecipitated comparable amounts of the β4 subunit in Caco-2/15 cells, demonstrating that both 3E1 and anti-β4c epitopes are expressed in these cells (Fig. 3B, lanes 3-4). In HIEC, however, the β4 subunit was detected as a 205kD band using 3E1 (lane 2) while the anti-β4c failed to immunoprecipitate the protein (lane 1), indicating that HIEC express the β4 subunit, but under a form which lacks the β4c epitope, thus distinct from the β4 subunit found in Caco-2/15 cells.

It has been previously reported that the β4 subunit can undergo proteolytic cleavage in its cytoplasmic tail (Kajiji et al, 1989; Giancotti et al., 1992; Potts et al., 1994). The possibility that this occured in undifferentiated crypt cells was evaluated by pulse-chase experiments and sequential immunoprecipitation. HIEC and Caco-2/15 cells were metabolically labeled with ^{35}S-methionine and cystine for two hours and chased for 0-8 h before lysis (Fig. 4A). For each sample, the β4 subunit was first immunoprecipitated using anti-β4c antibody. Then, after two additional rounds of immunoprecipitation with anti-β4c to ensure complete depletion of the immunoreactive β4c form in the samples, the remaining β4 was isolated with a last immunoprecipitation using the 3E1 antibody. This sequential immunoprecipitation procedure showed that in Caco-2/15 cells, the anti-β4c identified a major band at 205 kD at all time points corresponding to the β4 subunit (Fig. 4A; upper left hand panel). Subsequent immunoprecipitation of the β4c-depleted lysates using 3E1 indicated that the majority of the β4 subunit contains the anti-β4c epitope since very little material was recovered with 3E1 (Fig. 4A; upper right hand panel). In contrast, in undifferentiated HIEC cells, β4 was barely detected after immunoprecipitation using the anti-β4c (Fig. 4A; lower left hand panel), even with a chase time of zero minutes, the almost complete majority of the β4 subunit being recognized only by 3E1(Fig. 4A; lower right hand panel). Shortening the labeling period down to 30 minutes (Fig.

4B) did not allow the detection of the β4 protein containing the anti-β4c epitope in these cells (Fig. 4B; left hand panel), again the majority of the β4 subunit present being recognized only by 3E1 (Fig. 4B; right hand panel). This suggests that if the protein is cleaved, it occurs extremely rapidly. The possibility that the β4 transcript may not code for the COOH-terminal domain in undifferentiated crypt cells was verified by designing primers (B5/B6; see material and methods) for RT-PCR that would amplify the region overlapping the nucleotides encoding the last thirty amino acids against which the anti-β4cyto was raised. As shown in Fig. 2C, both cell lines expressed, in significant amounts, a β4 transcript that contains this sequence, ruling out the possibility that undifferentiated intestinal cells produce an alternatively spliced mRNA lacking the anti-β4c epitope. Hence, altogether these results indicate that undifferentiated crypt cells express a novel form of the β4A integrin subunit which is distinct from that found in differentiated intestinal cells.

The α6β4 complex is not functional in the undifferentiated HIEC cells

As shown in Fig 4A, the co-immunoprecipitation of α6 with its β4 partner in both HIEC (with 3E1) and Caco-2/15 cells (with the anti-β4c) indicates that β4 is expressed in association with α6 in both cell lines. The α6β4 integrin serves as a specific receptor for laminins, namely laminin-5 (Lee et al., 1992; Sonnenberg et al., 1993; Rousselle and Aumailley, 1994; Niessen et al., 1994; Mainiero et al., 1995; Borradori and Sonnenberg,1996). Adhesion of HIEC and Caco-2/15 cells to laminin-5 was thus analyzed (Fig. 5). Adhesion of Caco-2/15 cells was partially inhibited by the G0H3 (anti-α6) or 3E1 (anti-β4) antibodies. When both of these antibodies were used in combination, they significantly inhibited cell adhesion, indicating that the α6β4 complex modulates substantially Caco-2/15 cell adhesion to laminin-5 in these cells. The mAb13 (anti-β1) antibody also significantly impaired laminin-5 binding, suggesting that Caco-2/15 cells may use a β1 integrin in cooperation with α6β4, but one other than α2β1 or α3β1 since the neutralizing antibodies P1E6 (anti-α2) and P1B5 (anti-α3), alone or in combination with G0H3, failed to affect significantly Caco-2/15 cell adhesion to laminin-5. In contrast, HIEC cell binding to laminin-5 was unaffected in the presence of GoH3 or 3E1, either

alone or in combination, suggesting that α6β4 is not functional in these cells. However, HIEC cells appear to use α3β1 instead, another laminin-5 receptor, since adhesion is markedly inhibited with P1B5 or mAb13. In combination, P1B5 and GoH3 did not increase inhibition, supporting the interpretation that α6β4 is not involved in the binding of undifferentiated intestinal cells to laminin-5.

DISCUSSION

Epithelial cell proliferation, migration and differentiation are tightly regulated along the crypt-villus axis of the small intestine and evidence that cell-laminin interactions play a role in their regulation is strengthening (Vachon and Beaulieu, 1995; De Archangelis et al., 1996). As in other systems, cell-laminin interactions are highly dynamic in the renewing intestinal epithelium, a differential expression of both receptors and ligands having been observed along the crypt-villus axis (Beaulieu, 1997a, 1997b). In the present study, we have examined the function of the α6β4 integrin in the mediation of intestinal cell-laminin interactions. We have identified two distinct forms of the β4 subunit expressed according to the cell state in the intestinal epithelium: a previously undescribed proteolytic variant of β4A, the major β4 isoform in intestinal cells, that lacks a small terminal fragment of the cytoplasmic domain (β4A$_{tcd-}$), which is expressed by crypt cells and their normal in vitro counterpart, the HIEC cells, and the full-length β4A subunit, which is expressed by Caco-2/15 cells and by villus cells in the intact intestine. Our results provide clear evidence that both variants of β4A can associate with the α6 subunit to form a stable α6β4 complex. However, in contrast to the α6β4A present in differentiated intestinal cells, the α6β4A$_{tcd-}$ expressed by undifferentiated crypt cells was found to be non-functional for cell adhesion to laminin-5.

The β4 subunit can exhibit a high degree of structural complexity resulting from alternative splicing of its mRNA as well as proteolytic cleavage. Up to now, four distinct mRNA variants of the human β4 integrin have been identified, each altering the cytoplasmic domain, and designated (Neissen et al., 1997a) β4A, which is the most common form (Suzuki et Naitoh, 1990), β4B (Hogervost et al., 1990), β4C (Tamura et al., 1990) and β4D (Clarke et al., 1994). Information about the distribution of these variants is still limited (Tamura et al.,1990; Clarke et al., 1994) and the implication of their expression has only begun to be investigated at the functional level (Neissen et al., 1997a; 1997b). Analysis of these variants in intestinal epithelial cells by RT-PCR using specific primer sets revealed that both undifferentiated and differentiated cells express mainly, if not exclusively, the β4A form. Furthermore, RT-PCR with

primers designed to amplify the 3' end of the β4 transcript encoding the anti-β4c epitope ruled out the possibility that the β4tcd- form observed in undifferentiated cells was the product of a deletion arising from an alternative splicing mechanism. However at the present time, the possibility that the β4tcd- form is the result of a still unidentified alternative splice site which would produce a 3'-end reading frameshift, cannot be excluded.

The expression of a β4A subunit lacking the immunoreactive terminal portion of the cytoplasmic domain may thus result from alteration(s) of the protein itself. Conformation changes are unlikely since the anti-β4c antibody used in this study is a polyclonal serum raised against a 31 amino acid stretch (Giancotti et al., 1992), and lack of the anti-β4c epitope was observed under both denaturing and non-denaturing conditions (see Fig. 2 and 3 for comparison). However, proteolytic cleavage in the cytoplasmic domain of the β4 subunit has been previously reported yielding a characteristic pattern of bands migrating at 205, 165 and 125/130 kD (Kajiji et al., 1989; Giancotti et al., 1992; Potts et al., 1994). Such proteolytic fragments of β4 were not detected in either HIEC or Caco-2/15 cell extracts prepared under conditions in which proteolysis was inhibited (Giancotti et al., 1992; Potts et al., 1994; see Material and Methods). Furthermore, immunofluorescent cytoplasmic staining of the β4 subunit, used as an indicator of endogenous proteolysis (Giancotti et al., 1992), was not observed in the intact intestinal epithelium. These observations suggest that proteolytic processing of β4 does not occur significantly in the normal human intestinal epithelium. If the lack of the anti-β4c epitope is the result of proteolysis, it is likely to take place by a distinct mechanism. First, the actual portion of the cytoplasmic tail that appears to be deleted in β4Atcd- would be very short since no significant difference was observed in the apparent size of β4Atcd- from HIEC cells as compared to the full length β4A isolated from Caco-2/15 cells. Second, its proteolysis should occur very rapidly in the biosynthetic pathway, i.e. co-translationally, since a β4 bearing the anti-β4c epitope was not detected in HIEC even after only 30 min of labeling. Taken together, these data indicate that undifferentiated intestinal cells constitutively express β4Atcd-, a form of β4 that lacks a short portion of the terminal cytoplasmic domain. The exact mechanism involved in the generation of the β4tcd- form

remains to be elucidated but our observations suggest that it arises from a co-translational processing of the integrin subunit.

A second interesting observation is that even though the β4 subunit was able to associate with its α6 partner in the two cell lines studied, the α6β4Atcd- complex expressed by undifferentiated HIEC cells was found to be inactive in terms of laminin-5 adhesion. The HIEC do bind to laminin-5 but use the α3β1 integrin, which has also been reported to have a high affinity for this ligand (Carter, 1991; Delwel et al., 1994; Mercurio, 1995). Interestingly in these cells, α3β1 appears to function without the cooperation of α6β4 since the combination of neutralizing antibodies to α3 and α6 did not inhibit adhesion of laminin-5 significantly more than anti-α3 alone. In a recent study, it has been suggested that α3β1 and α6β4 can function cooperatively to mediate adhesion (Dipersio et al., 1998). This was not observed in HIEC adhesion to laminin-5 even when incubation times were extended to over one hour (not shown) which is consistent with the apparent inability of α6β4Atcd- to bind to laminin-5. This lack of activity was surprising in view of the fact that tail-less β4 mutants (Clarke et al., 1995; Spinardi et al., 1995) can still bind to laminin-5. The relation between a apparently minor alteration in the COOH terminal domain of the β4Atcd- subunit in undifferentiated intestinal crypt cells and the lack of activity of its α6β4 complex remains to be explored.

The cytoplasmic domain of the β4 subunit is composed of distinct structural domains namely two pairs of type III fibronectin-like modules separated by a region referred to as the connecting segment. The first pair of type III fibronectin-like modules (Spinardi et al 1995), and in particular the second domain and the N-terminal amino acids of the connecting segment, appear to be essential to hemidesmosome assembly including recruitment of HD-1 and association with the keratin cytoskeleton (Niessen et al., 1997a; Niessen et al., 1997b). The connecting segment contains many target amino acids, many of which are phosphorylated including tyrosine, serine and threonine (Mainiero et al.,1995). Two of the tyrosines are located in a tyrosine activation motif and have been shown to participate in hemidesmosome formation (Mainiero et al., 1995). Finally, a novel protein of unknown function, which associates with the two N-terminals of the fibronectin type III modules, may be involved in linking β4 to the

intermediate filaments (Biffo et al., 1997). As yet, no specific functional role for the COOH terminal domain of β4 has been identified. However, a number of interesting features pertaining to the cytoplasmic portion of the β4 subunit have not yet been mapped. These include ligand dependant phosphorylation of β4, causing the recruitment of Shc/Grb2-mSOS and subsequently activating the Ras/MAP kinase pathways (Mainiero et al., 1995;1997), the ablity to induce, in a ligand independent manner, p21/WAF/Cip1 expression and cell-cycle withdrawal in a rectal carcinoma cell line overexpressing the wild-type, but not tail-less β4 (Clarke et al., 1995), and, more recently, a distinct association of the α6β4 integrin with the actin cytoskeleton and PI3K activation in migrating cells on laminin-1 (Rabinovitch and Mercurio, 1997; Shaw et al., 1997).

What would be the biological relevance for undifferentiated crypt cells to express a α6β4A$_{tcd-}$ complex unable to mediate adhesion to laminin? While further investigations will be required to clearly answer this question, it is interesting to note that the epithelial basement membrane in the crypt region is negative for laminin-5 in the human small intestine while it is expressed in the villus basement membrane (Leivo et al., 1996; Orian-Rousseau et al., 1996; Basora and Beaulieu, unpublished), and therefore coincides almost perfectly with the pattern of β4A$_{tcd+}$ expression. Furthermore, the expression of HD1/plectin, another key component related to the formation of type II hemidesmosomes, has also been reported to be exclusively expressed by villus intestinal cells (Orian-Rousseau et al., 1996). The repression of laminin-5 and HD1/plectin expression and the production of an inactive form of α6β4 may thus represent two distinct control mechanisms ensuring that type II hemidesmosomes will not form in undifferentiated intestinal cells. The expression of an inactive form of a molecule in human crypt cells is not without precedent. For instance, sucrase-isomaltase, a brush border hydrolytic enzyme, is the subject of a posttranslational regulatory mechanism that depends on the state of differentiation for the acquisition of its mature fully active form (Beaulieu et al., 1989). On the other hand, the lack of laminin-binding activity of the α6β4A$_{tcd-}$ complex in the intestinal crypt may be of some importance, because of the presence of laminin-2 which is distributed according to an increasing gradient from the upper half to the bottom of the gland

(Beaulieu and Vachon, 1994; Simon-Assmann et al., 1994). Indeed, in addition to laminin-1 and laminin-5, which are restricted to the villus, laminin-2 can also serve as a ligand for α6β4 (Mercurio, 1995; Spinardi et al., 1995; Giancotti, 1996). Therefore, it could be speculated that the inactivity of this receptor in terms of laminin binding is required to allow enterocytes to migrate upward to the villus. Finally, in the light of the recent evidence that α6β4 may exert biological activities in conditions where the interaction with its ligand does not occur (Clarke et al., 1995; Xia et al., 1996), it is conceivable that the α6β4A$_{tcd-}$ complex present in crypt cells may exert ligand-independent activities of functional relevance for undifferentiated epithelial cells.

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FIGURE LEGENDS

Figure 1. Different patterns of expression for the β4 subunit using two specific antibodies. Indirect immunofluorescence on normal adult human small intestine using (A) 439-9B against the extracellular domain and (B) an anti-β4c against the cytoplasmic domain of the β4 subunit. Both antibodies were detected using FITC-conjugated secondary antibodies.

Figure 2. Detection of the β4 subunit in HIEC and Caco-2/15 cells. (A) Immunoblot analysis using anti-β4cyto on HIEC (lane 1) and Caco-2/15 cell (lane 2) lysates (100 µg/lane) separated on a 10% SDS-PAGE detected the presence of the β4 subunit, at its known molecular weight of 205 kD, only in the Caco-2/15 cells. (B) RT-PCR analysis using three different primer sets (See materials and methods) B1/B2 (373 bp), B3/B4 (93 bp) and B5/B6 (185 bp) amplified identical bands in reverse transcripted mRNA (+) from both HIEC (lane 2) and Caco-2/15 cells (lane 4). Identity of the band amplified with B3/B4 primers was confirmed by sequencing and was 100% identical to the human mRNA encoding β4. S14 was used to ensure comparable quantities of starting material.

Figure 3. HIEC contain a novel form of the β4A subunit. (A) Immunoprecipitation of the β4 subunit using 3E1 from equal quantities (3mg of protein) of HIEC (lane 1) and Caco-2/15 cells (lane 2) followed by separation on a 10% SDS-PAGE and immunodetection using the anti-β4c. (B) Immunoprecipitation using either the anti-β4c (lanes 1 and 3) or 3E1 (lanes 2 and 4) from HIEC (lanes 1 and 2) and Caco-2/15 cells (lanes 3 and 4) cells metabolically labeled using ^{35}S-methionine and cystine for 6 hours. Samples were separated on a 10% SDS-PAGE and the gel was dried and exposed for 48 hrs.

Figure 4. Identification of the major form of β4 expressed in HIEC and Caco-2/15 cells. (A) HIEC (lower panels) and Caco-2/15 cells (upper panels) were metabolically labeled using ^{35}S-methionine and cystine for 2 hours and chased for 0 min (lanes 1 and 5), 1 hour (lanes 2 and 6), 3 hours (lanes 3 and 7) or 8 hours (lanes 4 and 8). Cells were lysed in a non-denaturing

buffer and immunoprecipitated using the anti-β4c (lanes 1-4). After two successive rounds of depletion with the anti-β4c, the remaining β4 was then immunoprecipitated from the same lysates using 3E1 (lanes 5-8). Samples were migrated on a 10% SDS-PAGE under non-reducing conditions. The β4 subunit migrates at 205KD and the α6 subunit migrates at 150 kD. (B) HIEC were labeled using ^{35}S-methionine and cystine for 30 min with chase times of 0 min (lanes 1 and 4), 30 min (lanes 2 and 5) or 1 hour (lanes 3 and 6). Cells were lysed in a non-denaturing buffer and immunoprecipitated using anti-β4cyto (lanes 1-3). After immunodepletion with the β4c, the remaining lysates were then immunoprecipitated for a last round using 3E1 (lanes 4-6). All samples were separated on a 10% SDS-PAGE under non-reducing conditions and the gels were dried and exposed for 72 hours.

Figure 5. The α6β4A integrin is not a functional receptor for laminin-5 in undifferentiated HIEC cells. Adhesion of HIEC and Caco-2/15 cells on purified laminin-5 (10 µg/ml) in the presence of neutralizing antibodies to α2 (P1E6), α5 (mAb16), α3 (P1B5), α6 (GoH3), β1 (mAb13) and β4 (3E1) integrin subunits either alone or in the following combinations α2+α6, α3+α6 and α6+β4. All antibodies were added to the cells at 10 µg/ml and incubated for 30 min before plating. Results are from 4 separated experiments and are expressed as the percentage of cells bound after 30 min, with 100% being adhesion on laminin-5 in the absence of antibodies (n-=4).

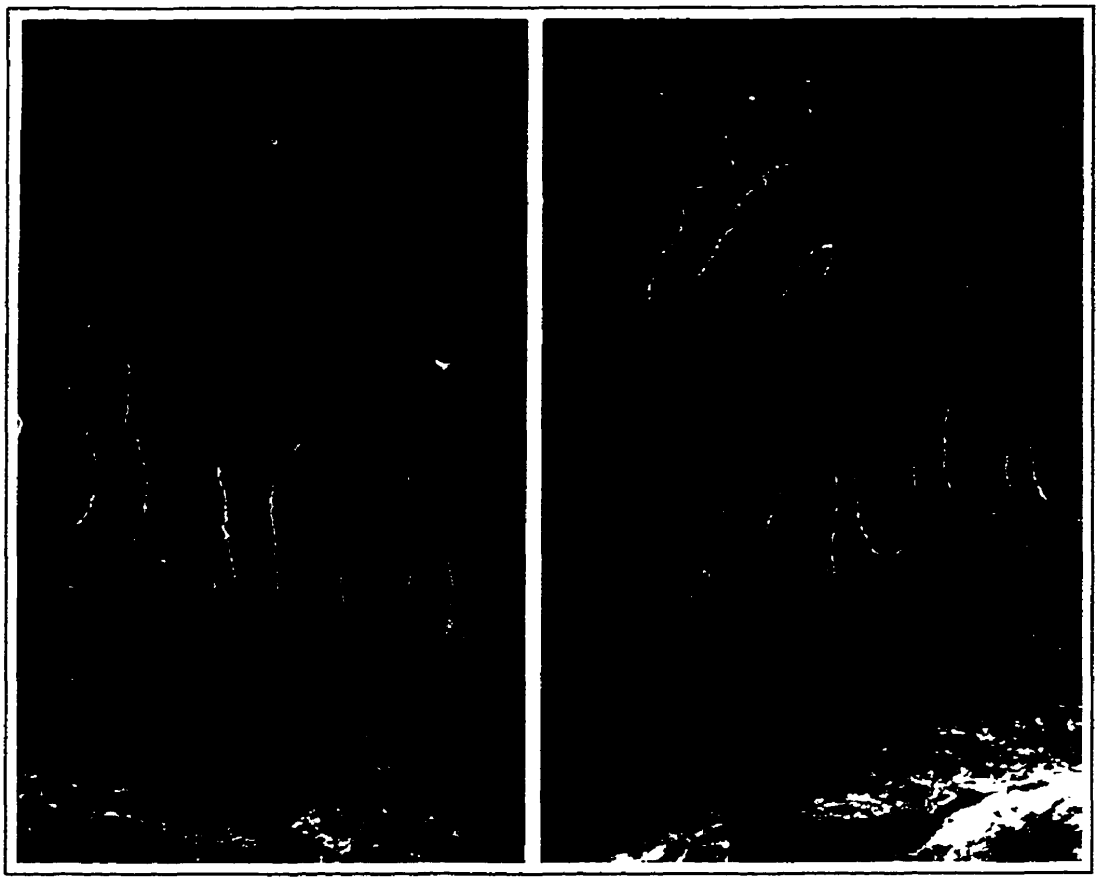

Figure 1

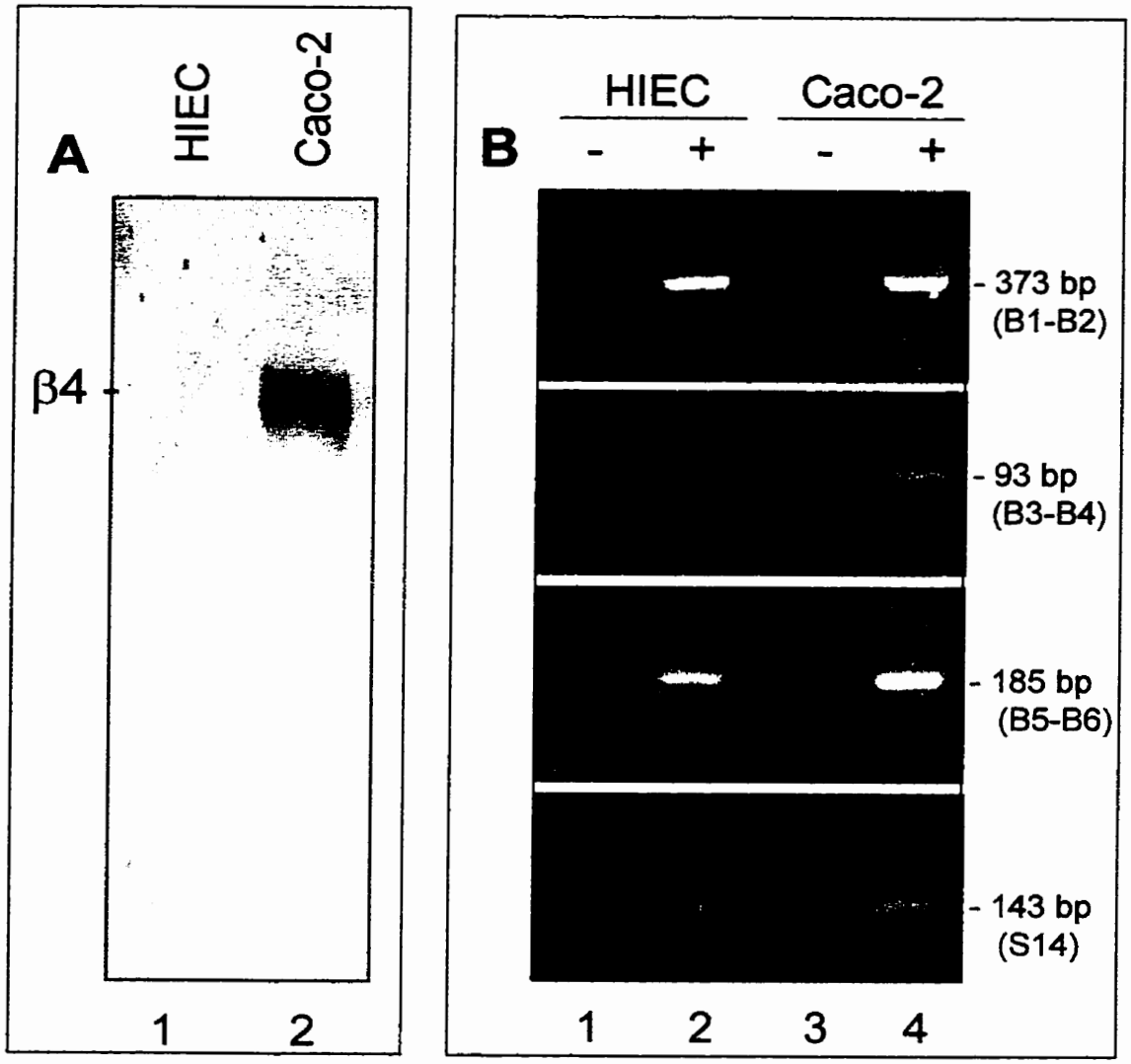
A
HIEC
Caco-2
β4
1
2
B
HIEC
Caco-2
-
+
-
+
- 373 bp
(B1-B2)
- 93 bp
(B3-B4)
- 185 bp
(B5-B6)
- 143 bp
(S14)
1
2
3
4

Figure 2

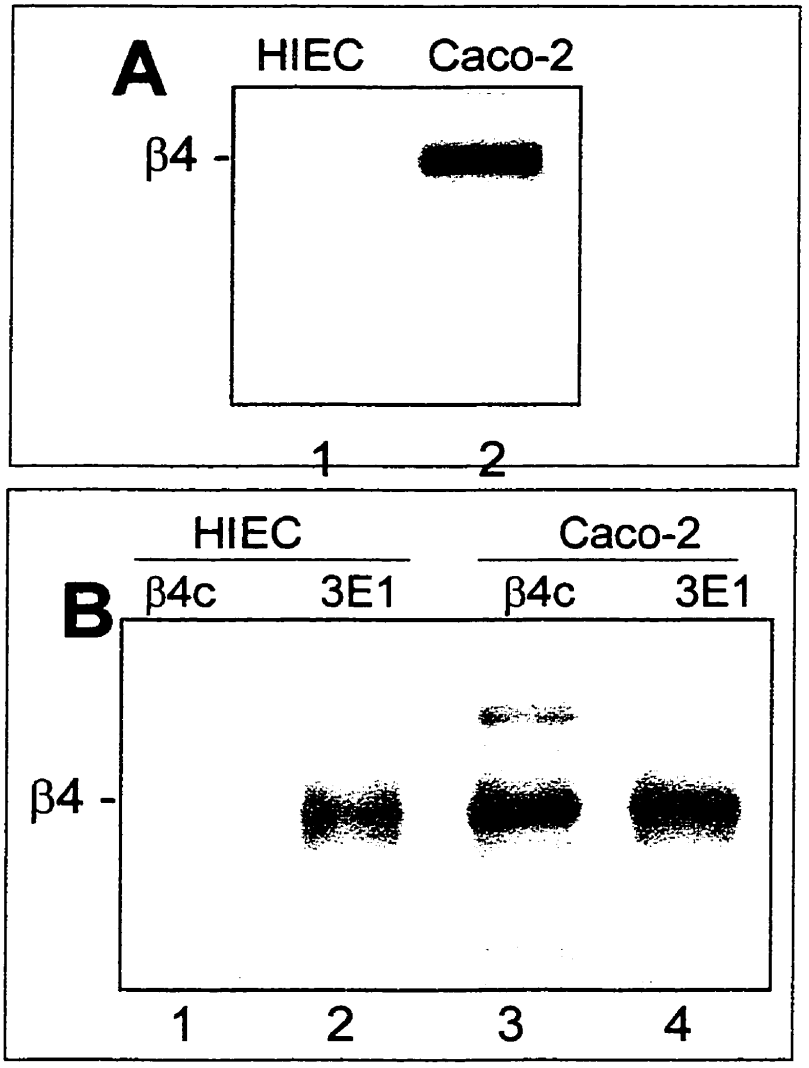

Figure 3

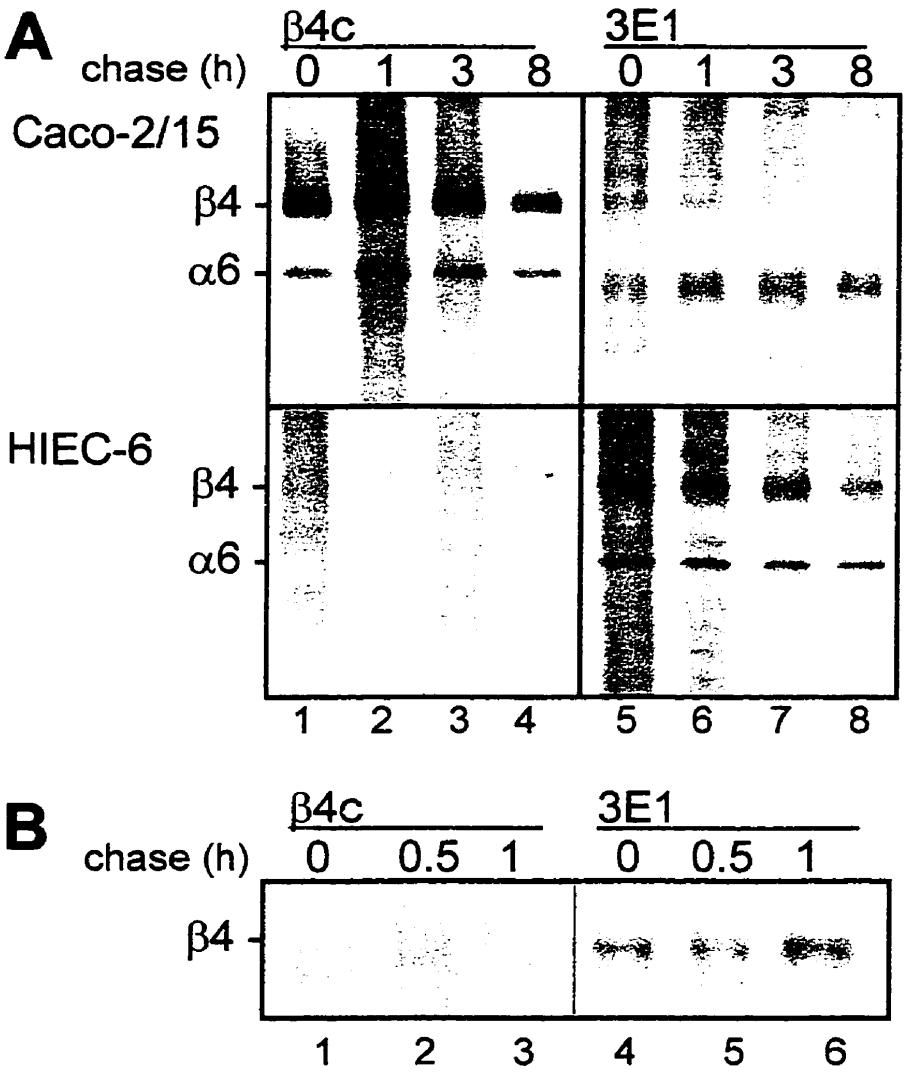
A
β4c
3E1
chase (h)
0 1 3 8
0 1 3 8
Caco-2/15
β4
α6
HIEC-6
β4
α6
1 2 3 4 5 6 7 8
B
β4c
3E1
chase (h)
0 0.5 1
0 0.5 1
β4
1 2 3 4 5 6

Figure 4

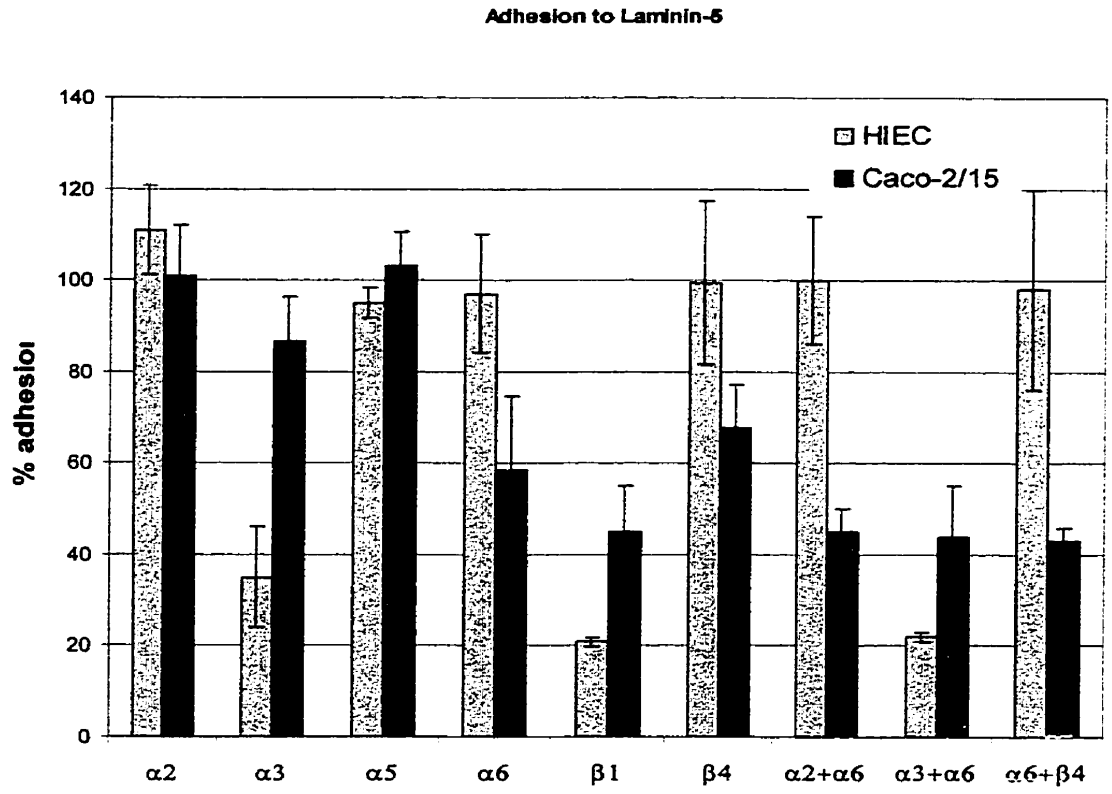
Adhesion to Laminin-5
% adhesion
140
120
100
80
60
40
20
0
HIEC
Caco-2/15
α2
α3
α5
α6
β1
β4
α2+α6
α3+α6
α6+β4

Figure 5

VI- DISCUSSION

The crypt villus axis is an excellent model for the study of the mechanisms responsible for the regulation of cell state (Beaulieu, 1997). The proliferative immature cells, which rapidly give rise to differentiated functional enterocytes, are spatially restricted to distinct compartments. Consequently, the expression of genes associated either with proliferation or differentiation are also segregated to either the crypt or villus, respectively. The main objective of this work was to identify integrins in intestinal epithelial cells and study their possible roles in mediating important cellular functions. We focussed on the three distinct integrins and demonstrated possible roles for $\alpha 9\beta 1$ in proliferation, $\alpha 7B\beta 1$ in enterocytic differentiation and $\alpha 6\beta 4$ in mediating adhesion/maintenance.

The integrin $\alpha 9\beta 1$ is a receptor for tenascin-C (Yokosaki et al., 1994; Weinacker et al., 1995), osteopontin (Smith et al., 1996) and laminin (Forsberg et al., 1994). We have characterized the distribution of $\alpha 9$ in the fetal and in the adult intestine (chapter II). This receptor is expressed in small intestinal and colonic epithelium during development, and perhaps more significantly, is found restricted to the developing crypts from 15 weeks to at least mid-gestation. This temporal and spatial pattern of expression is in accordance with the hypothesis that $\alpha 9$ is associated with proliferation (Wang et al., 1995). Absence of $\alpha 9\beta 1$ in the adult intestinal mucosa, however, suggests that proliferative cells are not

regulated via this integrin in the mature epithelium. An intriguing observation is that α9β1 and tenascin-C expression in normal intestine, either fetal or adult, are uncoordinated (Beaulieu, 1992; Desloges et al., 1994; Wang et al., 1995), suggesting that α9β1 interacts with other ligands in this tissue. Complementary experiments in our in vitro cell models indicated that α9 is associated with cells at a proliferative state since this receptor is detected in both HIEC and subconfluent Caco-2/15 cells, while protein levels of α9 drop quickly in differentiating Caco-2/15 cells. Moreover, if these cells were treated with EGF, which has been shown to maintain an artificially proliferative state, consequently retarding differentiation (Cross and Quaroni, 1989), α9 protein levels remained significantly higher after confluence than in control cells, suggesting that this subunit may be regulated by EGF. The α2 subunit is also upregulated by EGF in Caco-2 cells, which results in increased migration of these cells on collagen and laminin via α2β1 (Basson et al., 1992).

An interesting observation was the reappearance of epithelial staining for α9 in a subset of colon adenocarcinomas (chapter III). Heterogeneous expression was detected only in well to moderately differentiated tumors (4/5), where glandular structures were still present. Furthermore, of the various human colon carcinoma cell lines screened for α9, this subunit was detected only in those which displayed polarized features under normal culture conditions (i.e. plastic) consistent with the pattern observed in colon cancers in vivo. Poorly differentiated tumors and cell lines with no morphological or differentiated characteristics did not

express α9. It is interesting to note that overexpression of tenascin-C occurs in colon cancer (Sakai et al., 1993; Hauptmann et al., 1995). α9β1, when transfected in colon carcinoma SW480 cells, was reported to directly stimulate proliferation upon adhesion to tenascin-C. Since this BM molecule is often overexpressed in colon cancer, it is, therefore, possible that, in this context, interaction of α9β1 and tenascin-C contributes to tumor progression.

Recently a subset of integrins, α1β1, α5β1 and αvβ3, has been shown to induce recruitment, most likely through calveolin, and activation of Shc, with subsequent activation of Ras/ERK pathways (Wary et al., 1996). Initiation via this signaling cascade was shown to be necessary for progression through the G1 phase of the cell cycle and also offered protection from apoptosis. α2β1, α3β1 and α6β1 did not induce these events, but cause growth arrest even in the presence of high concentrations of mitogens. α6β4 also activates Ras/ERK via Shc upon ligation to laminin-5 stimulating keratinocyte proliferation in response to mitogens. In apparent contrast, transfection of the β4 subunit into RKO cells, which only express α6β1, induced partial cell-cycle arrest caused by an increase in p21Waf/Cip levels, and apoptosis (Clarke et al., 1995). However, this effect was independent of ligand binding consistent with the observation that occupied or unoccupied α6β4 directs distinct intracellular pathways (Xia et al., 1996). α9β1 has been shown to stimulate proliferation by activating MAPK pathways upon ligation to tenascin-C, although whether or not via Shc and Ras has not yet been determined. It will be interesting to determine what ligand is recognized by α9β1

in the intestinal epithelium and whether or not this receptor stimulates cell growth upon ligation. Preliminary data using α9 neutralizing antibodies suggest that HIEC and Caco-2/15 cells do not use α9β1 to bind to laminin-1, 2 or 5 (N. Basora and J.-F. Beaulieu, unpublished results).

Our studies have identified α7Bβ1, a specific receptor for laminin-1 and 2, but not for laminin-5 (Yao et al., 1996), as an additional integrin expressed by intestinal epithelial cells, and its expression is correlated with cell state (Chapter IV). Its restricted localization at the crypt-villus junction in vivo, as well as its regulated expression in newly confluent Caco-2/15 cells, are consistent with a possible role for this integrin in initiating terminal differentiation. This receptor is associated with the population of cells that are undergoing transition from immature, proliferative cells to functional, differentiated enterocytes.

An interesting observation is the presence of α7B in fetal colonic epithelium and its absence in adult colon. Fetal colon is known to transiently display morphological and functional small intestinal features, such as the presence of villi and enterocytic-like hydrolytic activities, such as sucrase and lactase. These characteristics, along with α7B expression, are lost during the second half of gestation suggesting that α7Bβ1 could be specifically involved in the regulation of the expression of small intestinal enterocytic functions.

In intestinal epithelial cells there is clear evidence that laminin-1 is critical for triggering functional differentiation (Beaulieu and Vachon, 1994; Vachon and Beaulieu, 1995; deArcangelis et al., 1996). Identification of integrin(s) which can

initiate enterocytic differentiation has been complicated by the fact that intestinal epithelial cells express a number of laminin binding integrins at their surface. Few, if any, of the previously identified integrins found in intestinal epithelial cells appeared to be good candidates in mediating functional differentiation for various reasons. First, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are restricted to crypt cells, being completely absent in villus cells (Beaulieu, 1997). Several reports have demonstrated that $\alpha 2\beta 1$, in combination with growth factors, mediates migration and glandular formation of colon cell lines (e.g. SW480 and HT-29 cells) grown in 3-D collagen gels (Pignatelli et al., 1992; Pignatelli and Bodmer, 1989; Del Buono et al., 1991; Liu et al., 1994) consistent with its association with crypts in the crypt-villus axis. This integrin also mediates EGF stimulated migration on laminin in Caco-2 cells (Basson et al., 1992).

Second is $\alpha 3\beta 1$, which was the most interesting due to its co-localization with laminin-1 in the villus, in vivo. However, the absence of this receptor in Caco-2/15 cells (N. Basora and J.-F. Beaulieu, unpublished observations), which depend on laminin-1 for their differentiation, argues against any involvement of $\alpha 3\beta 1$. Recently, $\alpha 3\beta 1$ has been identified as a high affinity receptor for laminin-5, which is also found associated with the villus BM (see Chapter V; N. Basora and J.-F. Beaulieu, unpublished results; Aberdam et al., 1994; Leivo et al., 1996). Furthermore, a recent report (Dipersio et al., 1998) using $\alpha 3$ knockout mice has demonstrated a postattachment requirement for $\alpha 3\beta 1$ as well as a novel role in

the establishment/maintenance of basement membrane integrity in collaboration with α6β4.

Third, are the α6 and β1/β4 subunits, which can exist as either α6β1 or α6β4. All three subunits are uniformly expressed throughout the intestinal epithelium, and this, at all stages of development (Beaulieu, 1992; Beaulieu and Vachon, 1994; Perreault et al., 1995). Although this type of expression is not, at first glance, supportive of a role for α6 in mediating differentiation, this assumption must be made with caution as the exact nature of α6β1 or α6β4 has not been properly characterized in intestinal cells. Studies using intestinal cell lines show that α6 appears to associate almost exclusively with β4 (Lotz et al., 1990; Schreiner et al., 1991). Moreover, isoforms exist for both α6 and β4. For example, preliminary data from our lab indicate that the two α6 isoforms, α6A and α6B, have a differential expression in the crypt-villus axis which must be taken into consideration since these two proteins have different signaling capacities (Shaw and Mercurio, 1993; Shaw et al., 1995).

The spatial and temporal pattern of expression of α7Bβ1 in the adult intestinal epithelium, as well as its regulated expression in Caco-2/15 cells, correlates almost perfectly with the first appearance of laminin-1 both in vivo and in vitro. Interestingly, α7B expression is increased in newly differentiating Caco-2/15 cells between 0 and 4 days post-confluence. This peak of expression was specific to α7B and was not observed for other integrins. Sastry and co-investigators transfected α5 or α6 into primary quail skeletal muscle cells to determine

individual integrin participation in differentiation (Sastry et al., 1996). A major conclusion drawn from this work was that the decision of whether to proliferate/differentiate, or not, depends on different integrin ratios and their ligation state. This may suggest that alteration of α7Bβ1 levels in initial stages of differentiation may be of functional significance.

The third, and last, laminin-binding integrin characterized in this study is α6β4. We have identified a novel form of the β4 integrin subunit in intestinal epithelial cells. HIEC express a β4 subunit (3E1+/β4cyto-) which is different from the full length β4 expressed in Caco-2/15 cells (3E1+/β4cyto+) (chapter V). Our experiments have demonstrated that Caco-2/15 cells use α6β4 to bind to laminin-5, but not to laminin-1 or 2 (data not shown). Interestingly enough, the resulting α6β4 receptor in HIEC was inactive in terms of adhesion on laminin-5, nor did it bind laminin-1 or 2.

An essential role for α6β4 in assembling and maintaining hemidesmosomes was recently deduced from β4 knockout mice (Dowling et al., 1996; Vanderneut et al., 1996). These animals displayed no discernible hemidesmosomes in stratified epithelia (e.g. skin). Loss of adhesion in these tissues, and surprisingly in simple epithelia as well, resulted in increased susceptibility to degeneration and, in some cases, this led to apoptosis. More importantly, even cells which could still attach to the BM were also more susceptible to degeneration. This observation implies that α6β4 may mediate a signal important for cell survival, distinct from its function in promoting hemidesmosome assembly. The existence of numerous isoforms for

the β4 subunit is suggestive of distinct functions although, to date, none have been identified.

Some epithelia form type II hemidesmosomes which contain laminin-5, α6β4 and cytokeratins but not all of the associated proteins (e.g. BPG180 or BPG230). This results in adhesion complexes which are less stable, i.e. more dynamic, but which are probably better suited to simple epithelium. For example, in the small intestine enterocytes must remain firmly attached to the BM, but must also be able to migrate up the crypt-villus axis. This organ does not, in fact, form type I hemidesmosomes but does express laminin-5, α6β4 and HD-1, an important hemidesmosome component which binds to α6β4 and mediates attachment to the intermediate filaments (Orian-Rousseau et al., 1996). Expression of all three of these molecules overlap in the villus. In vivo, differentiated epithelial cells also express α3β1, another high affinity receptor for laminin-5 (Neissen et al., 1994; Delwel et al., 1994). It was shown in α3 knockout mice that both α6β4 and α3β1 work cooperatively, using distinct, but overlapping functions in ensuring cell adhesion (Dipersio et al., 1997). A novel role was proposed for α3β1 requirement in the establishment and/or maintenance of basement membrane integrity while α6β4 mediates initial and stable adhesion. Taken together, these observations suggest that differentiated intestinal epithelial cell adhesion is coordinated by laminin-5 and integrins α6β4 and α3β1.

Adhesion to the BM is important for maintaining differentiated cell phenotype. For example, Perreault and co-workers have recently developed a technique for

the isolation of primary cultures of differentiated epithelial cells from human fetal small intestine (N. Perreault and J.-F. Beaulieu, 1998). On plastic these cells die rapidly (2-3 days) while culturing these cells on type I collagen dramatically improves their survival (10-12 days) and all cell specific markers remain expressed, without stimulation of cell growth. Whether collagen directly or indirectly promotes morphological and function cellular integrity in the intestine epithelium is unknown. However, collagen has been reported to directly regulate morphological features in colon carcinoma cell lines (Pignatelli et al., 1992; Pignatelli and Bodmer, 1989; Del Buono et al., 1991; Liu et al., 1994). In mammary epithelial cells, collagen directly affects cell polarization and formation of acini (Streuli et al., 1991). The production and maintenance of functional features in these cells was actually a result of the endogenous production of laminin-1 by exogenous collagen (Streuli et al., 1991). A more complete example of ECM requirement for maintenance of tissue and cellular integrity is provided by striated muscle. Vachon and co-workers (1996) showed that laminin-2 is critical in promoting the survival and function of differentiated muscle cells. Absence of laminin-2 leads to apoptosis and muscle degeneration and, as mentioned above, is the cause of MCMD. The same group, in a second study, also provided evidence for a role of $\alpha 7\beta 1D$ integrins in mediating laminin-2 directed survival and, consequently, tissue integrity and function (Vachon et al., 1997).

Expression of $\alpha 6\beta 4$ in HIEC is intriguing. Extrapolation of results obtained with this normal crypt-like cell model would suggest that $\alpha 6\beta 4$ is also inactive in

crypt cells, in vivo. However, a number of studies have shown that removal of the entire cytoplasmic domain, by directed mutagenesis, disrupts hemidesmosome assembly and interferes with intracellular signaling, but does not effect binding to laminin-5 (Spinardi et al., 1995; Clarke et al., 1995). Expression of a truncated β4 subunit may represent a novel regulation mechanism of β4 function. It is possible that α6β4 is kept inactive, in terms of adhesion, in HIEC and that these cells rely on signals sent from an unoccupied β4 (Xia et al., 1996), whose signaling pathways are distinct from those activated via ligated α6β4 (Mainiero et al., 1995, 1997). The observation that different β4 proteins are expressed by HIEC and Caco-2/15 cells, as well as their in vivo counterparts the crypt and villus cells, respectively, may imply that the β4 expression, and in this particular case, function, are regulated according to cell state. The HIEC do bind to laminin-5 but use α3β1. This interaction is unlikely to occur in vivo, however, since α3β1 is located in the lateral membrane of epithelial cells in the crypt a suggestion consistent with the observation that laminin-5 is not present in crypts (N. Basora and J.-F. Beaulieu, unpublished observations; Aberdam et al., 1994; Leivo et al., 1996). α3β1 is relocalized to the basolateral membrane in villus cells corresponding exactly with the appearance of laminin-5.

Finally, it is interesting to note that the pattern of β4 expression is reminiscent of sucrase isomaltase expression in the crypt-villus axis (Beaulieu et al., 1989). SI accumulates in significant amounts of an inactive form in immature crypt cells. Upon differentiation, an active form of SI quickly appears and this regulation was

shown to be posttranslational. A general concept would be that immature crypt cells possess a particular mechanism which allows the transcription and translation of proteins which are crucial to differentiated cell function but, at the same time keeps them inactive, until the cell receives the necessary signal. One could easily envision that an additional modification step of an inactive protein population already being actively synthesized would allow a more efficient and rapid accumulation of protein than de novo transcription.

VII- CONCLUSIONS and PERSPECTIVES

The main objective of this work was to analyze the expression and possible function of three, previously uncharacterized, laminin-binding integrins in intestinal epithelial cells.

In the first part of this study we have identified the presence of the α9β1 integrin in intestinal epithelial cells during fetal development, as well as in our cell models, HIEC and Caco-2/15 cells and have demonstrated an association of its expression with a proliferative cell state. Given that in normal fetal intestine tenascin-C is not always detected and that our cell models do not appear to bind laminin via α9β1, it would be pertinent to verify if osteopontin, the only other known ligand for α9β1, is expressed in the intestine. Furthermore, the co-expression of α9β1 and tenascin-C in colon cancers, combined with the fact that this receptor-ligand interaction can stimulate proliferation, suggests a role for this integrin in tumor progression. The intestinal cell lines, HIEC, Caco-2/15 and T84, three cell lines which express α9β1, could prove to be useful models for studying α9β1 implication in colon cancers in response to tenascin-C and osteopontin, which has also been reported to be upregulated in cancer. Consequently, whether or not α9β1 is a member of the newly identified subset of β1 integrins, which activate Ras/ERK pathways via Shc recruitment in the regulation of cell growth, remains to be determined. The absence of α9β1 in normal adult mucosa, however, implies that cell growth is not regulated via this integrin. Integrins

associated with proliferation in crypt cells, therefore remain to be determined. The HIEC model faithfully reproduces the integrin population found in fetal crypt cells in vivo and should prove to be particularly useful in these studies.

In the second part of this work we identified the presence and distribution of α7Bβ1, in fetal and adult intestinal epithelial cells located at the crypt-villus junction. It is in this region that immature crypt cells are induced to begin their differentiation. Unlike striated muscle, α7Bβ1 does not appear necessary for cell survival by intestinal epithelial cells since it is not expressed, in vivo, after differentiation is properly established (i.e. upper two thirds of the villus). Whether this integrin is directly responsible for the observed laminin induced effects in Caco-2/15 cells remains to be determined. Caco-2/15 cells are particularly well suited for the study of mechanisms involved in enterocytic differentiation due to the fact that their differentiation has been shown to be laminin-1 dependent and, of all the colon carcinoma cell lines screened, they were the only cell line which expressed α7Bβ1. Although α7β1 is the only known specific laminin-2 receptor, it is not co-localized with this molecule in vivo. Quite interestingly, using adhesion assays, we determined that HIEC, which do not express α7, bind laminin-2 via a β1 integrin, although neutralization of all the α subunits known to be expressed (i.e. α2, α3, α6 and α9) did not inhibit adhesion, suggesting that HIEC may contain a novel α subunit.

In the third part of this study we have confirmed that in intestinal epithelial cells α6 is almost exclusively associated with the β4 subunit, although we cannot

exclude the possibility that α6β1 exists in very low quantities. We have also identified a novel form of the β4 subunit expressed in crypt cells and in their in vitro counterpart, HIEC, which is different from that found in villus cells, suggests that β4 variant expression is cell state specific. The differential distribution of the two β4 subunits identified in intestinal epithelial cells may be indicative of functionally distinct roles. Determination of the α6β4 signaling pathways in HIEC vs Caco2/15 cells should provide useful insights into the role of this receptor in intestinal epithelial cells. Recent reports have suggested that α6β4 offers protection against anoikis, since removal of β4 increases cell susceptibility to degeneration. This mechanism is independent of hemidesmosome assembly. Expression of α6β4 may ensure cell adhesion and survival of differentiated enterocytes, until they are exfoliated into the lumen upon reaching the villus tip.

The identification of the complete integrin repertoire expressed by a given cell type is essential in understanding the mechanisms which are involved in mediating BM influence on intestinal epithelial cells. The fact that each integrin has a distinct function, and that most cells express several integrins suggest that cellular functions are a result of coordinated signaling which is cell and tissue context specific. This context is defined by a combination of BM composition and soluble factors (i.e. growth factors or hormones) and the final cell response most likely results from an integration of both signaling pathways. The cell is constantly monitoring its external environment and the simultaneous presence of various

integrins allows the cell to respond rapidly to any detected modifications encountered.

Our in vitro cell models, HIEC and Caco-2/15 faithfully reproduce the integrin patterns for their respective in vivo counterparts and promise to be extremely useful in the determination of the integrins and the mechanisms involved in proliferation, differentiation and adhesion/maintenance of intestinal epithelial cells.

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