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Research Article

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Cell Polarity of the Insulin-like Growth Factor System in Human Intestinal Epithelial Cells

Unique Apical Sorting of Insulin-like Growth Factor Binding Protein-6 in Differentiated Human Colon Cancer Cells

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Abstract

In this study, we have used enterocyte-like differentiated HT29-D4 human colonic carcinoma cells cultured in a glucose-free medium (HT29-D4-GAL cells) on semi-permeable supports in order to investigate the polarity of the insulin-like growth factor (IGF) system. We report that these cells secrete endogenous IGF-II predominantly (66%) from the basolateral cell surface where type I IGF receptors are almost all (> 96%) localized. HT29-D4-GAL cells also secrete IGF-binding protein (IGFBP) -2, -4, and -6 as evidenced by Western ligand and immunoblot analyses of conditioned medium. IGFBP-2 and IGFBP-4 are secreted primarily into the basolateral side (71 and 87%, respectively), whereas IGFBP-6 is targeted to the apical surface (76%) as a possible consequence of an active sorting. Finally, HT29-D4-GAL cells are found to display responses to IGF-II added to the basolateral but not the apical membrane side in terms of intracellular tyrosine phosphorylation and long-term stimulation of amino acid uptake. This study indicates (a) that IGF-II is potentially capable of autocrine regulation on the basolateral side of HT29-D4-GAL cell, and (b) that IGFBP-6 has a unique pattern of secretory polarity. It supports the concept that a differential sorting of the various forms of IGFBPs might play a modulatory role in the maintenance of a functional polarity in the differentiated HT29-D4-GAL cells. (*J. Clin. Invest.* 1995. 96:192–200.) Key words: cell polarity • insulin-like growth factor • insulin-like growth factor binding protein • intestine epithelial cell • autocrine

Introduction

Both morphological and functional integrity of epithelia requires a high degree of coordination between the processes of cell proliferation, cell differentiation and cell death (apoptosis) (1). This is particularly essential in the intestinal epithelium which is characterized by one of the most rapid cell turnover

rates of any tissue in the body (2). Indeed, it is by uncoupling of these processes that tumors arise.

The differentiation of an intestinal epithelial cell involves the establishment and the maintenance of a spatial polarization that is fundamental to achieve its function. A basic aspect of this polarity is that the cell plasma membrane is divided into distinct apical and basolateral specialized domains. These domains are separated by tight junctions which integrate the cells into a sheet forming a cellular barrier across which vectorial processes take place between the external milieu, i.e., the lumen of the intestine, and the internal milieu, i.e., the underlying connective tissue (for review see reference 3).

Among the signals whose the appearance as well as the withdrawal regulate the cell behavior are those mediated by the growth factors (1). It is now widely accepted that autocrine/paracrine stimulation of cell membrane receptors by growth factors constitutes an essential physiological mechanism to ensure functional maintenance and wound healing in most organized tissues (1). In epithelial cells, such autocrine signaling processes might be also involved in both acquirement and maintain of the functional cell polarity (4).

The HT29-D4 human colonic carcinoma cell line is a particularly useful model to study the relationship between the intestine epithelial cell differentiation process and the secretion of endogenous growth factors. On the one hand, HT29-D4 cells can be induced to differentiate in an enterocyte-like phenotype by culture in a glucose-free, galactose-containing medium (HT29-D4-GAL cells) (5, 6). These differentiated cells are highly polarized with mature junctional complexes, well-organized microvilli, and functional specialized apical and basolateral membrane domains which are characterized by an asymmetric distribution of plasma membrane markers (7–9). On the other hand, we have previously presented evidence that suggests the involvement of a regulatory insulin-like growth factor-II (IGF-II) autocrine loop in the control of the differentiation state of HT29-D4 cells (10, 11). The inability of these cells to differentiate when grown in a standard culture medium (HT29-D4-GLU cells), would be due, in part, to their incapacity to use the regulatory potential of endogenous IGF-II because of its complete sequestration in the extracellular medium by different molecular species of IGF binding proteins (IGFBPs)¹ (12). The shunt of this IGFBPs inhibitory activity indeed allows IGF-II to interact with cell surface type I IGF receptors and to induce a differentiated phenotype in HT29-D4 cells (10, 11). Six differ-

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1. Abbreviations used in this paper: CM: serum-free conditioned medium; HT29-D4-GAL cells, HT29-D4 cells cultured in glucose-free, galactose-containing medium; HT29-D4-GLU cells, HT29-D4 cells cultured in glucose-containing medium; IGFBPs, IGF-binding proteins.

ent IGFbps have been identified by molecular cloning and sequencing (13) that have been shown to modulate either positively or negatively the accessibility of the IGFs to their specific receptors at the cell surface (14). Thus, in the HT29-D4 cell model, the molecular pattern of secreted IGFbps constitutes an essential parameter in the fate of a putative regulatory IGF-II autocrine loop.

A further constraint that applies to the IGF system to be operative in a functional polarized intestine epithelial cell is the coherent localization of its different molecular partners in order to ensure both secretion and binding of the growth factor. We have undertaken in this study to determine the polarity of the "IGF system" in the differentiated HT29-D4-GAL cell, i.e., the polarity of IGF-II and IGFbps secretion, and of IGF receptors distribution into the plasma membrane. We report that IGF-II is secreted predominantly from the basolateral membrane domain where the type I IGF receptors are also localized. In accordance with this polar distribution, IGF-II is found to induce biological cell responses only when added to the basolateral side of the HT29-D4-GAL cell monolayer. We also show that IGFbps secretion is differentially polarized: IGFBP-2 and IGFBP-4 are secreted primarily from the basolateral side whereas secretion of IGFBP-6, the distinctive property of which is an elevated preference for IGF-II over IGF-I (15–17), is markedly polarized towards the apical cell surface. These findings provide a rationale for an ability of IGF-II to function as a potential autocrine factor in a functional polarized intestine epithelial cell.

Methods

Materials. Tissue culture flasks, multi-well plates and Cyclo-pore transparent microporous polyester filters (25-mm diameter; 0.45 μ m pore size, and 10 mm diameter, 1.0 μ m pore size) mounted on cell culture inserts were purchased from Falcon (Lincoln Park, NJ). Dulbecco's modified Eagle's medium (DME), RPMI containing 20 mM *N*-2 hydroxyethyl piperazine-*N'*-2 ethane sulfonic acid (RPMI-Hepes), fetal calf serum (FCS), and other cell culture reagents were from Eurobio (Les Ulis, France). Bovine serum albumin (BSA) was obtained from Sigma (L'Isle d'Abeau, France). Recombinant human IGF-I and IGF-II were purchased from Bachem (Bubendorf, Switzerland). 125 I-IGF-I and 125 I-IGF-II (~ 2,000 Ci/mmol), 14 C-2-aminobutyric acid (AIB) (50 mCi/mmol) and [3 H]thymidine (2.0 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Des(1–3)IGF-I and des(1–6) IGF-II were obtained from GroPep (Adelaide, Australia). Des(1–3)IGF-I was labeled with 125 I by the chloramin-T procedure to a specific radioactivity of 150–200 μ Ci/ μ g. Electrophoresis reagents and molecular weight standards were obtained from BioRad (Hercules, CA). Nitrocellulose sheets (Hybond-C extra) were purchased from Amersham. Rabbit polyclonal antibodies against IGFbps were obtained either from Upstate Biotechnology (Lake Placid, NY) (anti-IGFBP-2, and -4) or from Austral Biologicals (San Ramon, CA) (anti-IGFBP-6). A monoclonal antibody against rat IGF-II was also purchased from Upstate Biotechnology. The type I IGF receptor monoclonal antibody (α IR-3) was from Oncogene Science (Uniondale, NY). The monoclonal anti-phosphotyrosine antibody (PY 20) was obtained from ICN Biomedicals (Aurora, Ohio). Horseradish-peroxidase-coupled anti-rabbit and anti-mouse secondary antibodies, and enhanced chemiluminescence (ECL) detection reagents were purchased from Amersham. All other reagents were of analytical grade.

HT29-D4 cells and cell culture conditions. The HT29-D4 human colon adenocarcinoma cell line was routinely cultured in DME containing 25 mM glucose and 10% FCS (HT29-D4-GLU cells) as previously described (6). To induce cell differentiation, HT29-D4 cells were cultured in glucose-free DME supplemented with 5 mM galactose and 10% dialyzed FCS (HT29-D4-GAL cells), and the medium was

changed daily as reported elsewhere (6). For culture on permeable supports, HT29-D4 cells were plated at a density of 4.0×10^5 cells/cm² (7, 8) on the inside of either a 25-mm diameter/0.45- μ m pore size or a 10-mm diameter/1.0- μ m pore size Cyclo-pore microporous filter. The membrane mounted in a cell culture insert allowed independent access to both sides of the cell monolayer when the insert was placed in either a 6- or a 12-multiwell culture plate. Both compartments on each side of the cell layer were filled with DME, containing either glucose or galactose, and supplemented with 10% FCS with daily medium changes. Cells were grown at 37°C under 5% CO₂ and monitored daily by light microscopy. In addition, HT29-D4-GAL differentiated cell monolayers were periodically analyzed by electron microscopy as reported elsewhere (6–8) in order to check the maintenance of the polarized morphological phenotype.

Collection of serum-free conditioned media (CM). This was done with HT29-D4-GAL cells ~ 15–20 d after confluency. Serum-free CM was collected as previously reported (18). Briefly, the culture medium was removed and the cells were washed three times with serum-free medium, i.e., the same medium as before but without FCS, and with 0.005% BSA and glutamine increased to 5 mM. The first collection of CM, made after 24 h was discarded since FCS-derived growth factors were still expected to be present. The cultures were then refed with fresh appropriate serum-free medium, and further incubated for 48 h. CM from apical and basolateral culture compartments were then collected. Cell debris was removed by centrifugation at 3,000 *g* for 10 min and CM was stored at –20°C until use.

Evaluation of the epithelial barrier function in HT29-D4-GAL cell monolayers grown on semi-permeable supports. The ability of HT29-D4-GAL cell monolayers to function as an epithelial barrier was assessed by determining the electrical resistance across the cell monolayer and the permeability of the cell monolayer towards a radiolabeled macromolecular marker.

The transepithelial resistance of the confluent cell monolayer grown on each filter was determined in a sterile manner using a two electrode model Millicell-ERS voltammeter (Millipore, Bedford, MA) which measures resistance by passing alternating current across the cell monolayer. Filters without cells were used as controls. Readings on the filters alone were < 30 $\Omega \cdot \text{cm}^2$.

The epithelial cell monolayer permeability was also assessed by using either 125 I-IGF-I or 125 I-des(1–3)IGF-I as a probe. Confluent cell monolayers were first washed three times in fresh serum-free DME, then 0.25 ng of radiolabeled tracer (~ 100,000 cpm) premixed in the appropriate culture medium was added either in the upper or in the lower chamber while the opposite chamber was filled with medium alone. After 24 h at 37°C, the medium was removed from each chamber and the radioactivity in the total volume of each chamber was counted in a gamma counter.

All these measurements were made both before and after culturing the confluent cell monolayers in serum-free medium containing 0.005% BSA as described above.

IGF binding to HT29-D4 cells. This was carried out at 4°C essentially as previously described (19). HT29-D4-GAL cell monolayers cultured on permeable filters were first washed three times with binding medium (RPMI-Hepes containing 0.1% BSA, pH 7.2), then either 125 I-IGF-I or 125 I-IGF-II (0.2 nM) with or without cold competitors or α IR-3 antibody was applied in a total volume of 1 ml of binding medium to either the upper (apical) or the lower (basolateral) side of the monolayer while the opposite side contained 1 ml of binding medium alone. The binding was then carried out at 4°C for 4 h (equilibrium conditions). At the end of the incubation period, the cells were washed three times with cold PBS containing 0.1% BSA, then the cell-covered permeable filters were directly cut from the plastic inserts and cell-associated radioactivity was counted in a gamma counter. Nonspecific binding, determined as the radioactivity bound to the cells in the presence of 1 μ M of unlabeled IGF, was subtracted from total binding to obtain specific binding. Nonspecific binding represented ~ 2.5 and 15% of total binding of 125 I-IGF-I and 125 I-IGF-II, respectively. To determine apical vs. basolateral IGF binding, each determination was made in triplicate on cell-

covered filters identically and simultaneously cultured and treated. Replicate wells were used to determine cell number.

Western ligand blotting. The method used was essentially as previously described by Hossenlopp et al. (20). Briefly, aliquots of CM were concentrated ~30–50-fold by ultrafiltration in Centricon-10 microconcentrators, then mixed with sample buffer (final concentration: 62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, without reducing agents), heated at 100°C for 3 min and finally submitted to SDS-PAGE on 12.5% polyacrylamide slab gels. The proteins were then electroblotted onto a nitrocellulose sheet for 1 h at 100 V. After quenching with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% nonfat dry milk, and 0.05% Tween-20, the nitrocellulose strips were incubated for 24 h at 4°C with about 100,000–300,000 cpm of ¹²⁵I-IGF-II with or without 1 μg/ml of either unlabeled IGF-II or IGF-I. IGF-BPs molecular forms were visualized by autoradiography using RX Fuji x-ray film placed in contact with the nitrocellulose strips between two intensifying screens at –80°C for 2–4 d.

Western immunoblotting. CM samples were concentrated, electrophoresed and transferred to nitrocellulose sheets under conditions identical to those described for ligand blotting analysis. The blots were then blocked to prevent nonspecific binding with 5% nonfat dry milk in Tris-buffered saline as described above for ligand blots. The blots were then probed with a 1:2,000 dilution of anti-IGFBP-2 antiserum, 1:500 dilution of anti-IGFBP-4 antiserum, 1:1,000 dilution of anti-IGFBP-6 antiserum or 1 μg/ml of anti-IGF-II monoclonal antibody. The IGF-BP-2 antibody shows < 0.1% cross-reactivity with IGF-BP-1, -3, or -4. The IGF-BP-4 antibody shows < 0.1% cross-reactivity with IGF-BP-3, and 1% with IGF-BP-1. It shows 50% cross-reactivity with IGF-BP-2. The IGF-BP-6 antibody has only minor cross-reactivity with IGF-BP-5 at 1/100 dilution. The mouse anti-rat IGF-II monoclonal antibody (IgG1) was specific to rat and human IGF-II. It shows < 10% cross-reactivity to human IGF-I. Dilutions of the rabbit polyclonal antisera and of the mouse monoclonal antibody were made in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 containing 0.5% nonfat dried milk), and incubation was carried out for 60 min at room temperature. The membranes were then rinsed three times with Tris-buffered saline and then incubated for 60 min with either anti-rabbit or anti-mouse horseradish-peroxidase antibody at a final dilution of 1:5,000 in Tris-buffered saline, then rinsed three times in the same buffer, and immunoreactive proteins were visualized using the Amersham ECL System.

Both ligand blots and immunoblots were quantitated by scanning autoradiographs with a Sebia laser densitometer (Sebia, Issy les Moulineaux, France).

Aminoisobutyric acid (AIB) uptake. HT29-D4-GAL cells were cultured on microporous filters (10 mm diameter and 1.0 μM pore size) in glucose-free, galactose-containing DME with 10% FCS as described above. Confluent cell monolayers were washed three times with the same medium, but without FCS and containing 0.1% BSA, then incubated in this medium for 20 h at 37°C in the presence or not of 25 nM of IGF-I, IGF-II, des(1–3)IGF-I or des(1–6)-IGF-II added to the chamber either above (apical) or below (basolateral) the cell monolayer. ¹⁴C-AIB uptake (0.3 μCi/ml in 8.0 μM unlabeled AIB) was then measured for 12 min as described previously (21). After extensive washing with cold PBS containing 0.1% BSA, the cell-covered filters were cut, and counted in a liquid scintillation counter.

Thymidine incorporation. HT29-D4-GAL cells were cultured on microporous filters (10 mm diameter and 1.0 μM pore size) in glucose-free, galactose-containing DME with 10% FCS as described above. Confluent cell monolayers were washed three times with serum-free, glucose-free, galactose-containing DME with 0.1% BSA, then incubated in the same medium for 24 h. After extensive washing, cells were incubated in fresh serum-free culture medium with or without 25 nM of IGF-I, IGF-II, des(1–3)IGF-I, or des(1–6)-IGF-II added to either the apical or the basolateral chamber, and the cell monolayers were further incubated for 24 h. 1.0 μCi/well of [³H]thymidine was then added for the last 5 h of culture. The cells on the filters were then rinsed twice with ice-cold PBS, twice with ice-cold 5% trichloroacetic acid, and

twice with ethanol. The cell-covered filters were then counted in a liquid scintillation counter.

Intact cell tyrosine phosphorylation. Tyrosine phosphorylation of cellular proteins was analyzed by immunoblotting with a monoclonal anti-phosphotyrosine antibody essentially as described by Kato et al. (22). Confluent HT29-D4-GAL cells in 60-mm dishes were washed three times with serum-free culture medium containing 1% BSA and incubated in this medium for 16 h at 37°C. Since HT29-D4-GAL cells constitute a leakproof polarized cell monolayer, the access to the basolateral membrane domain of cells cultured on a plastic support requires a pretreatment with Ca²⁺-free Eagle's medium for 1–2 h at 37°C in order to disrupt the tight junctions (19). Cells were then stimulated with either IGF-I or IGF-II (100 nM) for 15 min at 37°C, then washed rapidly with cold PBS on ice and frozen on liquid nitrogen, and then thawed on ice. Cells were then lysed with 250 μl/well of RPMI-Hepes containing 1% Triton X-100, 1 mg/ml bacitracin, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 100 mM NaF, and 10 mM EDTA. The lysate was clarified by centrifugation (12,000 g for 3 min) and equal amounts of protein (~20 μl of the lysates) were submitted to SDS-PAGE electrophoresis on 7.5% polyacrylamide slab gels, then electrophoretically transferred onto a nitrocellulose sheet for 1 h at 100 V. Tyrosine-phosphorylated proteins were probed with a monoclonal anti-phosphotyrosine antibody at 1:2,000 dilution and detected by the Amersham ECL System.

Statistical methods. Results are expressed as the mean ± SD of the number of determinations indicated in the legend of figures. Significance was determined using the two tailed Student's test, *P* < 0.05 being taken as the level of significance.

Results

HT29-D4-GAL cells form a functional polarized epithelial cell monolayer

To study the polarity of the IGF system in the differentiated HT29-D4-GAL cells, the functional integrity of the cell monolayers requires to be systematically assessed before use. First, it was verified that HT29-D4-GAL cells grown on plastic supports formed domes after ~6–7 d at confluency. Dome formation is indeed well characterized as a marker of transporting epithelia (6). Second, for HT29-D4-GAL cells grown on microporous filters, the formation of a functional epithelial monolayer was monitored by the development of a transepithelial resistance. Whereas undifferentiated HT29-D4-GLU cells displayed a low transepithelial resistance ($R_T = 32.5 \pm 3.2 \Omega \cdot \text{cm}^2$), polarized HT29-D4-GAL cell monolayers gave resistance which increased at confluence and reached a plateau ($R_T = 242.8 \pm 16.3 \Omega \cdot \text{cm}^2$) ~3–4 d postconfluency. Cell-covered filters that had a low resistance, i.e., < 220 $\Omega \cdot \text{cm}^2$, were therefore not used for polarity studies. Third, the functionality of the tight junctions was assessed by their ability to prevent macromolecular flow through the cell monolayer. Filter-grown HT29-D4-GAL cells were pulsed with 0.25 ng ¹²⁵I-IGF-I which was added to either the apical or the basolateral medium compartment, then radioactivity present in the medium bathing the opposite monolayer surface was counted after 24 h at 37°C. As shown in Fig. 1, only 3.9% passed to the basolateral compartment and 4.8% passed to the apical compartment. Since ¹²⁵I-IGF-I added to the medium was found to be totally complexed with the secreted IGF-BPs within 2 h of incubation at 37°C, it was concluded that no significant transcytosis of IGF-IGF-BPs complexes takes place across the HT29-D4-GAL cell monolayer. This was also valuable for IGF itself since no transcellular pathway was detectable when ¹²⁵I-des(1–3)IGF-I, a NH₂-terminally truncated IGF analog which was previously found to be unable to bind

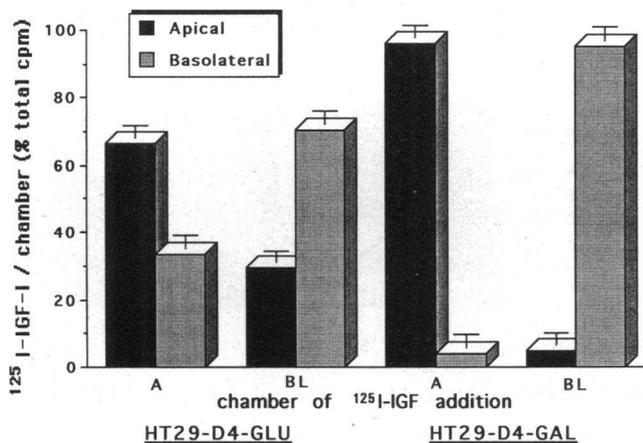


Figure 1. Evaluation of the epithelial barrier function for macromolecular transfer in HT29-D4-GAL cell monolayers grown on semi-permeable filters. Confluent HT29-D4-GLU cells and HT29-D4-GAL cells were pulsed either in the upper (apical; A) or the lower (basolateral; BL) compartment with approximately 100,000 cpm of ^{125}I -IGF-I. After 24 h at 37°C, the media in the apical (solid bar) and basolateral (shaded bar) chambers were recovered and counted in a gamma counter. Values are expressed as the percent of cpm recovered in each chamber as compared with the total cpm added. Shown values are the means \pm SD from three independent experiments performed in duplicate.

to HT29-D4 cell-secreted IGF-BPs (11), was used as a tracer (not shown). When filters covered with confluent undifferentiated HT29-D4-GLU cells were similarly assayed, about 30–35% of exogenously added ^{125}I -IGF-I was transferred across the cell layers (Fig. 1). It should be noted that both permeability and electrical resistance data determined with HT29-D4-GAL cell monolayers cultured either in FCS-containing medium or in serum-free medium for 48-h as described in Methods section were quite similar.

Polarity of the IGF-II release by HT29-D4-GAL cells

Previous studies (18) have indicated that HT29 cells secrete high amounts ($\sim 6 \text{ ng}/10^6 \text{ cells}/24 \text{ h}$) of IGF-II, 70% occurring as a higher molecular weight form (15–20 kD). Using HT29-D4-GAL cells cultured on porous membrane supports, we determined the accumulation of secreted IGF-II into each side of the cell monolayers cultured for 48 h in serum-free medium. IGF-II secreted into the apical and basolateral compartments was assayed by an immunoblot procedure with an anti-IGF-II monoclonal antibody. This procedure had the further advantage to visualize the molecular weight heterogeneity of the secreted IGF-II forms. Fig. 2 shows that HT29-D4-GAL cells released immunoreactive IGF-II predominantly into the basolateral medium ($66.4 \pm 5.2\%$; $n = 4$). The figure also shows that $\sim 70\%$ of the secreted IGF-II exhibited, whatever the secretory compartment, an apparent molecular weight of $\sim 18 \text{ kD}$ rather than 7.5 kD corresponding to the molecular weight of the mature IGF-II.

Type I IGF receptors are polarized into the basolateral membrane domain of HT29-D4-GAL cells

The presence of type I, but not of type II, IGF receptors at the cell surface of both HT29-D4-GLU cells and HT29-D4-GAL cells has been previously reported by us (19). Here, we determined the distribution of these IGF receptors between apical

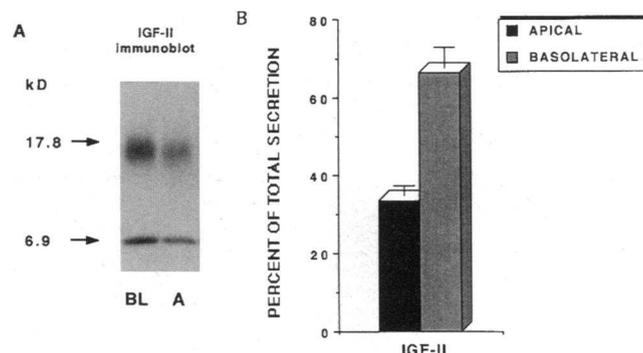


Figure 2. Polarized secretion of IGF-II by HT29-D4-GAL cell monolayers. (A) Confluent cell monolayers grown on permeable filters were washed three times, then incubated in serum-free medium as described in Methods. After a 48-h period, apical (A) and basolateral (BL) CM were recovered, concentrated and submitted to electrophoresis on 12.5% SDS-polyacrylamide gels in the absence of reducing agents, then transferred to nitrocellulose and immunoblotted with a monoclonal antibody to IGF-II followed by visualization with the ECL detection system as described in Methods. Note that under these conditions, standard IGF-II migrated with the front of migration. (B) Densitometric quantitation of IGF-II (both standard and big forms) secreted into apical and basolateral CM. The data (densitometric units) from IGF-II immunoblots as shown in (A) are expressed as a percentage (mean \pm SD) of the total IGF-II secretion (apical + basolateral) determined in four independent experiments made in triplicate ($P < 0.001$).

and basolateral membrane domains in HT29-D4-GAL cells grown on semi-permeable filters. This culture system allows to ^{125}I -IGF tracer to access directly and selectively to either the apical or the basolateral surface of the polarized cell monolayer. Fig. 3 shows that the specific binding of both ^{125}I -IGF-I and ^{125}I -IGF-II was barely detectable when the radioligand was added in the apical compartment only. It represented only 3.4 and 3.8% of the specific binding measured when ^{125}I -IGF-I or ^{125}I -IGF-

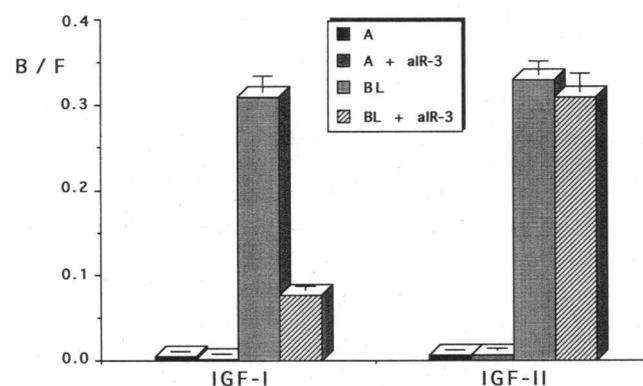
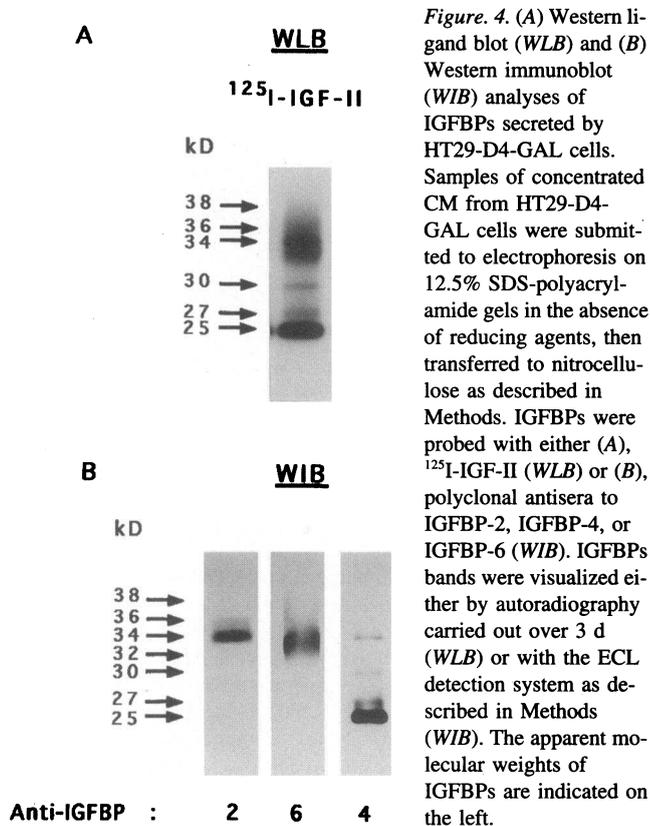


Figure 3. Polarized distribution of IGF receptors in HT29-D4-GAL cell monolayers. Confluent cell monolayers grown on semi-permeable filters were washed three times, then incubated for 4 h at 4°C with 0.2 nM of either ^{125}I -IGF-I or ^{125}I -IGF-II with or without 10 $\mu\text{g}/\text{ml}$ of $\alpha\text{IR-3}$ monoclonal antibody added to the apical (A) or the basolateral (BL) side of the cell monolayer whereas the opposite side received binding medium alone. Nonspecific binding was determined as the radioactivity bound in the presence of 1.0 $\mu\text{g}/\text{ml}$ of either unlabeled IGF-I or IGF-II. Shown values are the means \pm SD from three independent experiments made in triplicate, and expressed as bound to free ratio (B/F).



II, respectively, was added in the basolateral compartment of cell-covered filters that were simultaneously and identically treated (Fig. 3). Thus, the specific binding of both IGF-I and IGF-II was highly concentrated to the HT29-D4-GAL basolateral cell surface (> 96%). This evidences a strong polarization of the membrane IGF receptors to the basolateral aspect of the cell with a basolateral to apical ratio of 28:1 and 25:1 for ^{125}I -IGF-I and ^{125}I -IGF-II, respectively. Unlabeled IGF-I (1 $\mu\text{g}/\text{ml}$) was able to prevent more than 95% of ^{125}I -IGF-I binding and > 80% of ^{125}I -IGF-II binding to the HT29-D4-GAL cells (not shown). Moreover, $\alpha\text{IR-3}$, a monoclonal antibody to the type I IGF receptor (23), was able to block the binding of ^{125}I -IGF-I by ~ 75% confirming the presence of true type I IGF receptors in HT29-D4-GAL cells as previously reported (19). In contrast, ^{125}I -IGF-II binding to HT-29-D4-GAL cells was not significantly inhibited by the presence of $\alpha\text{IR-3}$ (Fig. 3). Such an inability of this monoclonal antibody to prevent IGF-II binding to type I IGF receptors is a well known data (24).

IGFBPs are released in a differential polarized way by HT29-D4-GAL cells

We next identified IGFBPs secretion by HT29-D4-GAL cells. Western ligand blots with radiolabeled IGF-II, shown in Fig. 4 A, revealed the presence of several IGFBPs molecular species in conditioned medium. Immunoblot analysis (Fig. 4 B) allowed to identify the bands at 30-, 27-, and 25-kD as isoforms of IGFBP-4. IGFBP-4 antiserum also recognized the bands at 34- and 36-kD due to an elevated cross-reactivity with IGFBP-2 (see Methods). IGFBP-2 was effectively present in HT29-D4-GAL cell conditioned medium appearing as two species of 36- and 34-kD (Fig. 4 B). Quantitation of the immunoblots indicated that IGFBP-2 and IGFBP-4 molecular isoforms were secreted

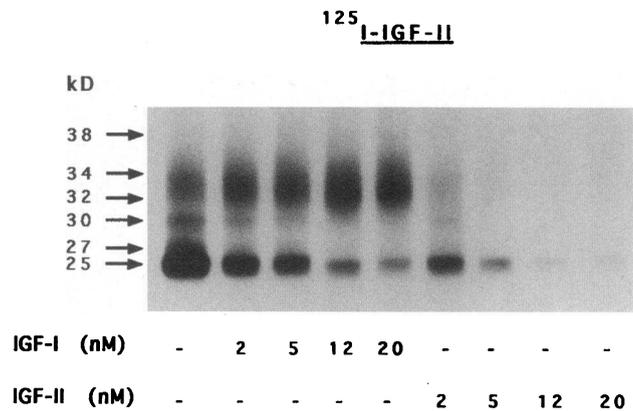


Figure 5. Competitive inhibition by either unlabeled IGF-I or IGF-II for ^{125}I -IGF-II binding to IGFBPs secreted by HT29-D4-GAL cells. Samples of concentrated CM from confluent HT29-D4-GAL cells were submitted to WLB analysis as described in Fig. 4. IGFBPs were then probed with ^{125}I -IGF-II in the absence (-) or the presence of the indicated concentrations of either unlabeled IGF-I or IGF-II.

with a ratio of ~ 1:1 depending on the experiment. However, the bands corresponding to IGFBP-2 were masked in part by a third diffuse IGFBP species migrating between 32- and 38-kD in Western ligand blot analysis (Fig. 4 A). This band represented IGFBP-6 as determined by Western immunoblot (Fig. 4 B). As judged by densitometry, this species represented ~ 40–60% of total secreted IGFBPs according to the experiments. Experiments of competition of unlabeled IGF-I and IGF-II for ^{125}I -IGF-II binding to IGFBPs secreted by HT29-D4-GAL cells revealed that all species preferentially bound IGF-II than IGF-I (Fig. 5). However, the most marked preferential affinity for IGF-II over IGF-I was exhibited by the 32–38-kD IGFBP species. As shown on Fig. 5, 20 nM unlabeled IGF-I was unable to compete for radiolabeled IGF-II; this was also true up to 100 nM (not shown). Such an elevated specificity for IGF-II was an expected result for IGFBP-6 (15–17).

HT29-D4-GAL cells were further cultured on semi-permeable filters in FCS-containing medium for up to 15–20-d after confluency. As described in Methods, cells were further washed and cultured for 48 h in serum-free medium, then apical and basolateral CM were collected and analyzed for their IGFBPs contents by both Western ligand blotting and Western immunoblotting. As illustrated in Fig. 6, significant differences in the polarity of secretion of the different IGFBP classes were observed. IGFBP-6 was secreted primarily (75.7±8.1%) out of the apical side of the cell as indicated by both its ligand binding activity (Fig. 6 A) and its immunoreactivity (Figs. 6 D). In contrast, IGFBP-4 was secreted predominantly (87.3±5.4%) in the medium of the basolateral chamber (Figs. 6, A and C). IGFBP-2 was difficult to evaluate by Western ligand blot since the bands were obscured by the IGFBP-6 broad migration (Fig. 6 A). However, its specific quantitation by Western immunoblot (Fig. 6 B) evidenced a basolateral polarity of its secretion (71.2±6.5%). The quantitative analysis of the polarized distribution of the various classes of IGFBPs into apical and basolateral CM from 10 independent experiments is shown in Fig. 7.

HT29-D4-GAL cells are responsive towards IGF-I and IGF-II

In vivo tyrosine phosphorylation. Intracellular tyrosine phosphorylation of the type I IGF receptors and their intracellular

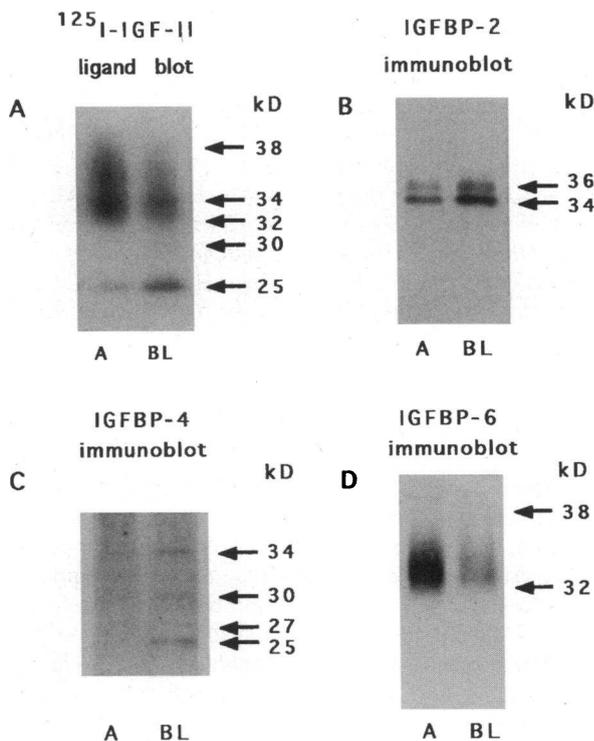


Figure 6. Secretory polarity of IGF-BPs in HT29-D4-GAL cell monolayers. Confluent cell monolayers grown on semi-permeable filters were washed three times, then incubated in serum-free medium as described in Methods. After a 48-h period, apical (A) and basolateral (BL) CM were recovered, concentrated and submitted to electrophoresis on 12.5% SDS-polyacrylamide gels in the absence of reducing agents, then transferred to nitrocellulose as described in Methods. IGF-BPs were then probed with either (A), ¹²⁵I-IGF-II or polyclonal antisera to (B) IGF-BP-2, (C) IGF-BP-4 and (D) IGF-BP-6. IGF-BPs bands were visualized either by autoradiography carried out over 3 d (ligand blot) or with the ECL detection system (immunoblot) as described in Methods. The apparent molecular weights of IGF-BPs are indicated on the right.

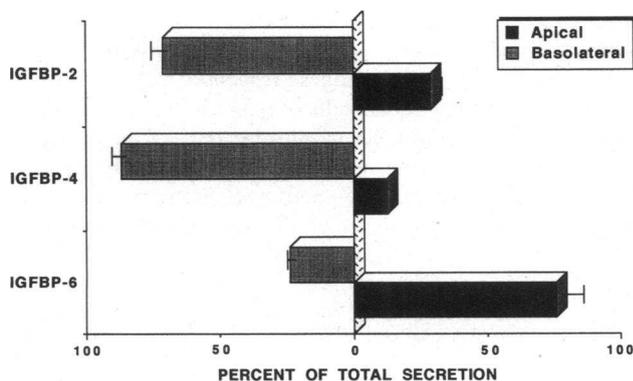


Figure 7. Densitometric quantitation of the polarity of IGF-BP-2, IGF-BP-4 and IGF-BP-6 secretion from HT29-D4-GAL cell monolayers cultured on semi-permeable filters. The data (densitometric units) from IGF-BPs immunoblots as shown in Fig. 8 are expressed as a percentage (mean \pm SD) of the total IGF-BP secretion (apical + basolateral) determined in 10 independent experiments made in duplicate ($P < 0.0005$).

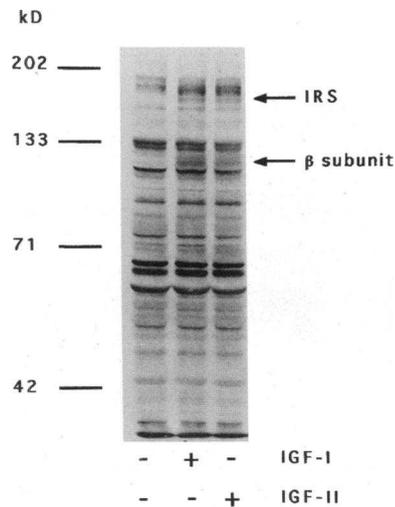


Figure 8. Stimulation of tyrosine phosphorylation of type I IGF receptors and their substrates by IGF-I and IGF-II. Lysates of confluent HT29-D4-GAL cells either not treated (-/-) or treated with 100 nM IGF-I or IGF-II for 15 min after disruption of the tight junctions (see Methods) were directly applied to SDS-PAGE, and proteins containing phosphotyrosine were detected by Western immunoblotting using an anti-phosphotyrosine monoclonal antibody (PY 20) and ECL detection system as indicated in Methods. The positions and sizes of prestained molecular weight markers are indicated (kD). Each lane contained equal amounts of protein.

substrates after stimulation of HT29-D4-GAL cells with IGF-I or IGF-II was examined by Western immunoblotting using an anti-phosphotyrosine antibody. Fig. 8 shows a typical immunoblot of a whole lysate from cells that were treated with 100 nM of either IGF-I or IGF-II for 15 min. The level of tyrosine phosphorylation of the β subunit of the type I IGF receptor (97-kD) and of a 185-kD protein (presumably a protein belonging to IRS family) (22) was significantly increased over the basal level. At this IGF concentration, tyrosine phosphorylation in response to IGF-I was slightly more potent than that seen in response to IGF-II.

It should be noted that the necessity to measure here short term effects of IGFs, i.e., for 15 min, did not allow to use cell-covered filters because in such conditions the interaction of IGFs added in the basolateral chamber with the membrane receptors cannot be instantaneous. Thus, we have used HT29-D4-GAL cells cultured on a plastic support. Because the differentiated cells constitute a leakproof monolayer (6), it was necessary to first disrupt the tight junctions, as described in Methods, in order to allow exogenously added IGFs to interact with the IGF receptors in the basolateral membrane domain. When this experimental step was omitted, i.e., when IGFs could interact only with the apical cell surface, no significant increase in tyrosine phosphorylation was noticeable (not shown).

Amino acid uptake. We next examined the effects on ¹⁴C-AIB uptake by HT29-D4-GAL cells of the addition of IGF-I and IGF-II, and their NH₂-terminally truncated analogs, des(1-3)IGF-I and des(1-6)IGF-II, to either the apical or the basolateral side of the cell monolayer. As shown in Fig. 9, exposure of basolateral monolayer surfaces to 25 nM of either IGF-I or IGF-II for 20 h increased ¹⁴C-AIB uptake over the basal level by 269 and 247%, respectively. Both des(1-3)IGF-I and des(1-6)IGF-II were more potent than their natural counterpart, inducing a stimulation of 304 and 294%, respectively. In contrast, a similar exposure of the apical HT29-D4-GAL cell surface had no significant effect on AIB uptake (Fig. 9).

Thymidine uptake. Whatever the experimental conditions, especially the use of a wide spectrum (10-130 nM) of IGFs or des-IGFs concentrations added to either the apical or basolateral

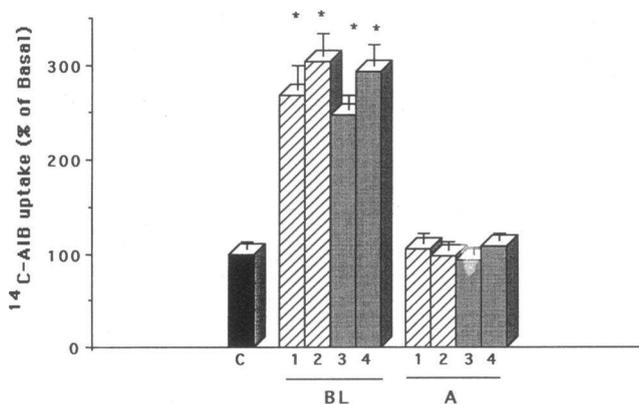


Figure 9. Effect of IGFs and desIGFs on long term AIB uptake. Confluent HT29-D4-GAL cells cultured on semi-permeable supports were treated for 20 h with 25 nM of IGF-I (1), des(1-3)IGF-I (2), IGF-II (3), or des(1-6)IGF-II (4) added to either the apical (A) or basolateral (BL) chamber. C, control cells. ¹⁴C-AIB uptake during 12 min was measured as described in Materials and Methods. Data are expressed as the percent increase \pm SD over basal uptake. All assays were carried out in triplicate. * $P < 0.005$.

cell surface, we were unable to detect a significant effect, either stimulatory or inhibitory, on [³H]thymidine incorporation by HT29-D4-GAL cells. Also, no IGFs-induced effect on the HT29-D4-GAL cell number was observed.

Discussion

IGFs cellular actions have been shown to be dependent on the specific interaction of IGF-I or IGF-II with cell surface type I IGF receptors (14, 25). However, many recent studies underline the pivotal role of locally produced IGFBPs that can modulate either in a positive or a negative manner the IGFs targeting to receptors (14). Because a functional intestine epithelial cell monolayer constitutes a polarized permeability barrier, a further regulatory constraint applies to IGF-II to trigger biological responses that is an adequate polarity of the IGF system, i.e., the apical vs. basolateral distribution of IGF receptors, and of IGF-II and IGFBPs secretion.

In the present study, this latter point is examined by using HT29-D4-GAL enterocyte-like differentiated human colonic cancer cells grown on a semi-permeable support. This cell differentiation model, i.e., the induction of HT29-D4 cells in an enterocyte-like morphology simply by culturing the cells in a glucose-free, galactose-containing medium, has been widely used as a unique model of polarized epithelial cell for studying protein expression during enterocyte differentiation (5-9). However, it is yet unknown how the IGF system may contribute in part to this process. Previous work done in our laboratory has shown that induction of enterocyte-like differentiation in HT29-D4-GLU cells, i.e., cultured in a standard glucose-containing medium, is associated, at least in part, with the establishment of an IGF-II regulatory autocrine loop (10, 11).

The following observations suggest that this might be also possible in the HT29-D4-GAL cell differentiation process induced by glucose starvation. The levels of secretion of IGFBP-6 and the number of a "variant" type I IGF receptor expressing an elevated affinity for IGF-II over IGF-I are up-regulated (both ~ 5-fold) during the course of the process, whereas the levels of

secreted IGF-II do not appear to be grossly altered (unpublished observation). Although only correlative, such an alteration lead us to speculate that the IGF system might also participate in the glucose-free medium-induced HT29-D4 cell differentiation. To test this hypothesis, we have also investigated for and do not observe a preventing effect of α IR-3, a monoclonal antibody against the type I IGF receptor, on the HT29-D4-GAL cell differentiation process (unpublished observation). However, this does not disprove the above mentioned hypothesis since α IR-3 has been previously found to favor HT29-D4 cell differentiation itself (10, 26), presumably via its IGFs-mimetic activities (22).

Analysis of ¹²⁵I-IGFs binding in HT29-D4-GAL cells shows that these cells express IGF receptors which are strongly polarized (> 96%) towards the basolateral surface. The basolateral to apical polarity ratio is similar (~ 28:1) whatever the radioligand used, either IGF-I, or IGF-II. This accurate sorting of IGF receptors to the basolateral membrane agrees with the general view that receptors for hormones, growth factors and neurotransmitters are distributed to the basolateral membranes of epithelial cells (27). Both ¹²⁵I-IGF-I and ¹²⁵I-IGF-II basolateral binding in HT29-D4-GAL cells are inhibited by unlabeled IGF-I and IGF-II. In addition, the α IR-3 monoclonal antibody is able to inhibit ¹²⁵I-IGF-I binding (it is a well known data that this antibody does not prevent IGF-II binding to type I IGF receptors (24)). These observations are in agreement with previous findings showing that all the IGF receptors expressed at the surface of either HT29-D4-GLU cells or HT29-D4-GAL cells belong to the type I IGF and not to the type II IGF/mannose-6-phosphate receptor (19). Constitutive secretion of endogenous IGF-II (both standard and big forms) is also observed to be predominantly driven from the basolateral cell surface. This basolateral secretory polarity (~ 66%) is, however, lower than that reported for several endogenous secretory proteins in Caco-2 cells (~ 80-90%), another human colonic carcinoma cell line displaying at confluency a spontaneous enterocyte-like differentiation morphology (28). We think that this does not reflect the presence in IGF-II of a structural sorting signal for apical secretion but merely the consequence of the differential sorting observed for the different classes of secreted IGFBPs (discussed below). Exogenous added IGF-II, as IGF-I, is effective to stimulate autophosphorylation of the IGF receptors, IRS phosphorylation and amino-acid uptake when selectively added to the basolateral membrane side of HT29-D4-GAL cells, whereas an apical exposure results in no change in these parameters. IGF-I is more potent than IGF-II to induce these responses that confirms the presence in HT29-D4-GAL cells of type I IGF receptors which are known to have a higher affinity for IGF-I than for IGF-II (19, 24-25). It is also noticeable that the N-terminally truncated IGFs are more potent than their native counterpart revealing that HT29-D4-GAL cell-produced IGFBPs constitute a functional barrier to IGFs bioavailability (see below). Although both localization and functional data make clear that endogenous IGF-II has the potential to interact with and to activate type I IGF receptors in the basolateral membrane domain of HT29-D4-GAL cell, it is not possible at present to assess the precise biological function that it could fulfill in a differentiated intestine epithelial cell. It is obvious from the present data that this is not a mitogenic effect since no enhancement of thymidine incorporation or cell number is observed in IGFs-stimulated HT29-D4-GAL cells whatever the experimental conditions used. This absence of growth effects of IGFs has been pre-

viously reported by us and others in the nondifferentiated HT29-D4-GLU cells (11, 18, 29). A possibility is that IGF-II acts as a survival factor as it does in other cell types (30, 31), thus contributing to the acquisition and/or the maintain of a functional cell polarity. Further experiments to detail such a function are pending. How IGF-II may function like this in vivo has yet to be determined.

Whatever may be, the simultaneous presence of both IGF-II and type I IGF receptors on the basolateral side of the HT29-D4-GAL cell is not sufficient to trigger a biological signal systematically. As indicated above, the presence of several species of IGFBPs modulates in a critical way IGFs-receptor interaction and subsequently IGFs cellular responses (14). In HT29-D4-GAL cells, the pattern of IGFBPs secretion comprises IGFBP-2, IGFBP-4, and IGFBP-6, this latter being up-secreted in HT29-D4-GAL cells as compared to HT29-D4-GLU cells (unpublished observation). IGFBP-6 has been reported to inhibit IGF-II-induced myoblast differentiation (32). However, it has been also reported to be induced by retinoic acid, a differentiation agent, in fibroblasts (33) and breast cancer cells (34). We think that the marked binding affinity of IGFBP-6 for IGF-II over IGF-I, which is a distinct feature among the human IGFBPs (15–17), might be significant in modulating endogenous IGF-II bioavailability during the course of HT29-D4 cell differentiation. In IEC-6 intestinal epithelial cells derived from rat jejunal crypts, IGFBP-2 has been shown to damp the biological effects of IGFs (35), and IGFBP-4 found to inhibit the mitogenic effects of IGF-I in HT29 cells (29). However, the precise mechanisms by which IGFBPs modulate either positively or negatively IGFs biological responses being yet not well known (14), it is difficult actually to assign a precise modulatory function to any of the HT29-D4-GAL cell-secreted IGFBPs.

Our understanding of the mechanisms involved in the regulation of IGF signaling is shown here to be further complicated in a polarized epithelial cell by a differential secretory polarity of IGFBPs. Both IGFBP-2 and IGFBP-4 exhibit a marked polarity for basolateral secretion (71 and 87%, respectively). Such a basolateral direction of the secretion pattern corresponds to the default (or constitutive) secretory pathway which has been determined to be basolaterally directed in Caco-2 cells (28). The default pathway has been defined as the route taken by proteins lacking a particular sorting signal and carried to the cell surface by bulk flow. By contrast, up to 76% of IGFBP-6 is secreted from the apical cell surface in HT29-D4-GAL cells. Although we have no information from the literature on the existence of structural determinants within IGFBPs that might be involved in an active sorting, this predominant delivery of IGFBP-6 to the apical surface of HT29-D4-GAL cells suggests that such determinants should exist in this IGFBP species specifying its preferential apical delivery. The distinctive sorting of IGFBP-6 as compared with IGFBP-2 and IGFBP-4 may be drawn together with the fact that IGFBP-6 is the most structurally distinct among the six known IGFBPs. A distinctive behavior of IGFBP-6 has been also noticed by Martin et al. (33) that report a pattern of regulation by cAMP and TGF- β unique among the IGFBPs. Human IGFBP-6 lacks 2 amino-terminal cysteine residues resulting in the loss of the invariant GCGCC sequence present in all the other five IGFBPs (36), that undoubtedly confers to this species an unique tertiary structure. Moreover, IGFBP-6 is *O*-glycosylated (37). Although this does not interfere or interfere slightly with its IGF-binding properties (33, 37), *O*-glycosylation may alter physicochemical properties of IGFBP-6. *O*-gly-

cosylation indeed affects protein folding (38) and has been reported to be required for the correct intracellular sorting of several glycoproteins (39). Finally, the physiological significance of the IGFBPs differential secretory sorting in HT29-D4-GAL cells remains to be determined. In particular, the active sorting of IGFBP-6 towards the apical side of the cell monolayer, i.e., the lumen of the intestines in vivo where no IGF receptor appears to be located, is a striking finding. Although there is actually no experimental data to suggest an IGFBP-6 apical function, if any, it might be speculated that it might exert direct IGF-independent effects on the apical membrane domain. Such a possibility has been postulated for other species of IGFBP (29, 40–43). The observed differential sorting of IGFBPs may also represent a mean for the cell to regulate the molecular profile of IGFBPs actually present in the basolateral cellular environment thus allowing a fine tuning of IGF-II-biological signaling. However, much work remains to be done on clarifying this mechanism, and especially identifying the putative signals involved in an active apical secretory sorting of IGFBP-6.

In summary, this study establishes that HT29-D4-GAL cells have all prerequisite for IGF-II to function as an autocrine peptide by acting on the basolateral side of the polarized cell where are localized all the type I IGF receptors. However, such an IGF-II bioactivity appears to be tightly regulated by multiple IGFBPs, each one exerting undoubtedly unique roles in the cellular environment. In addition, a differential secretory polarity of IGFBPs may work towards controlling the amount of IGF-II actually available for the IGF receptors in the basolateral membrane domain of the cell. IGFBP-6 might be especially significant in modulating endogenous IGF-II bioavailability since it binds preferentially IGF-II, and appears unique in its active apical cellular sorting.

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