Human intestinal epithelial cell survival: differentiation state-specific control mechanisms

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Gauthier, Rémy, Charlène Harnois, Jean-François Drolet, John C. Reed, Anne Vézina, and Pierre H. Vachon. Human intestinal epithelial cell survival: differentiation state-specific control mechanisms. Am J Physiol Cell Physiol 280: C1540–C1554, 2001.—To investigate whether human intestinal epithelial cell survival involves distinct control mechanisms depending on the state of differentiation, we analyzed the in vitro effects of insulin, pharmacological inhibitors of Fak, MEK/Erk, and PI3-K/Akt, and integrin (β1, β4)-blocking antibodies on the survival of the well-established human Caco-2 enterocyte-like and HIEC-6 cryptlike cell models. In addition, relative expression levels of six Bcl-2 homologs (Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, and Bad) and activation levels of Fak, Erk, and Akt were analyzed. Herein, we report that 1) the enterocyte differentiation process results in the establishment of distinct profiles of Bcl-2 homolog expression levels, as well as p125Fak, p42Erk-2, and p57Akt activated levels; 2) the inhibition of Fak, of the MEK/Erk pathway, or of PI3-K, have distinct impacts on enterocyte cell survival in undifferentiated (subconfluent Caco-2) cells; 3) exposure to insulin and the inhibition of Fak, MEK, and PI3-K resulted in differentiation state-specific modulations in the expression of each Bcl-2 homolog analyzed; and 4) Fak, β1 and β4 integrins, as well as the MEK/Erk and PI3-K/Akt pathways, are distinctively involved in cell survival depending on the state of cell differentiation. Taken together, these data indicate that human intestinal epithelial cell survival is regulated according to differentiation state-specific control mechanisms.

anoikis; apoptosis; gut; intestine; signal transduction

PROGRAMMED CELL DEATH, or apoptosis, is a complex and tightly regulated process that performs crucial functions in tissue homeostasis (1, 25). The Bcl-2 family of proteins constitutes a critical decisional checkpoint in cell survival, regulating the common downstream effector pathway of apoptosis (1, 47). At least 15 family members have been identified so far, functioning either as anti-apoptotic (e.g., Bcl-2, Bcl-XL, Mcl-1) or pro-apoptotic (e.g., Bax, Bad, Bak) regulators (1, 20, 48). It is now well acknowledged that regulation of cell survival in different cell types does not depend on the activity of single Bcl-2 homologs but, rather, on a balance of anti- and pro-apoptotic activities from multiple homologs (1, 47). To this effect, extracellular signals determine in large part if a cell lives or dies, involving signaling events that ultimately affect the expression and/or functions of multiple anti- and pro-apoptotic Bcl-2 homologs (1, 4, 13, 15, 19–21). Such survival signals originate from growth factors (e.g., insulin) and cell adhesion, often implicating similar signaling pathways (4, 13, 15, 19–21, 25). In this respect, the roles of focal adhesion kinase (p125Fak), the phosphatidylinositol 3’-kinase (PI3-K/Akt (PKB; p57Akt) pathway, and the MEK [mitogen-activated protein kinase (MAPK) kinase]/Erk (extracellular regulated kinases 1 and 2; p42Erk-2/p44Erk-1 MAPK) pathway have received much attention in recent years (4, 11, 13, 15, 21). However, it is becoming increasingly evident that the involvement of these signaling molecules/pathways in cell survival varies depending on the cell type that is receiving such signals, thus underlying the complexity in the regulation of apoptosis among various tissues (1, 4, 11, 13, 15, 19–21, 25).

The intestinal epithelium is a useful model for the study of the working mechanics of tissue renewal processes, including apoptosis. Its rapid, continuous cell renewal consists of spatially separated stem cells, proliferative and differentiated compartments located, respectively, in the lower regions of the crypts and on the villi (26, 45). Such a “gradient” of cell differentiation is further defined by functional properties of the fully differentiated villus enterocytes, which distinguish them from crypt cells (26, 37, 41, 45). Hormonal responses as well as cell adhesion components, such as integrins, underlie additional distinctions between

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Crypt and villus enterocytes (7, 16, 37, 41, 43–45). Although the predominant means to remove obsolete enterocytes is through apoptosis and shedding at the villus apex, “spontaneous” crypt cell apoptosis is a less frequent process that serves to remove defective/injured progeny cells (22, 24, 26, 40, 44, 45, 53). In addition to this apparent “duality of fate” between undifferentiated and differentiated intestinal cells, some Bcl-2 homologs have been shown individually to exhibit gradients of expression along the crypt-villus axis (29–32, 38–40, 44, 45, 59, 60). By analyzing six homologs at the same time in the proximal-distal axis of the developing human gut (jejunum, ileum, and colon), we have previously shown that villus cells gradually come to exhibit a Bcl-2 homolog expression profile that clearly differs from the one observed in crypt cells by midgestation, once the crypt-villus axis has matured (52, 53). These observations altogether suggest that intestinal epithelial cell survival may be regulated distinctively according to the state of cell differentiation.

In the present study, we investigated this hypothesis by using the human enterocyte-like cell line Caco-2, a widely used model of intestinal epithelial cell maturation and functions (8, 27, 37, 41, 42, 50). Although cancerous in origin, these cells are unique in their property to undergo a gradual differentiation process that takes place spontaneously once confluence has been reached and that is completed after 25–30 days of postconfluent culture (8, 27, 37, 41, 42, 50). Consequently, Caco-2 cells acquire a morphological polarity and show enzymatic activities as well as protein and mRNA levels of brush-border membrane enzymes that are highly comparable to those of mature enterocytes (8, 27, 37, 41, 42, 50). In this respect, Caco-2 cells as a single cell culture system have provided an important tool to study and further understand the regulation of various human intestinal epithelial cellular processes, such as enterocyte differentiation (2, 12, 37, 41, 42, 50, 51), proliferation (2, 17, 41–43), digestive functions (8, 12, 27, 37, 41), and cell adhesion (5–7, 35, 41, 51, 61). We also used the normal human fetal cryptlike HIEC-6 cells (41), which do not differentiate even when in postconfluent culture, as an additional model of undifferentiated intestinal epithelial cells to complement the one represented by undifferentiated Caco-2 cells.

Herein, Caco-2 and HIEC-6 cells were exposed to growth factors (namely, insulin) or pharmacological compounds that inhibit signal transduction molecules (namely, tyrosine kinases, Fak, PI3-K/Akt, and/or MEK/Erk) to evaluate their impact on cell survival and Bcl-2 homolog expression. We find that undifferentiated and differentiated cells display strikingly distinct Bcl-2 homolog expression profiles, such profiles being established gradually during the enterocytic differentiation process. In addition, we find that exposure to insulin, or the inhibition of signaling molecules/pathways, modulates the expression of Bcl-2 homologs regardless of the state of differentiation but that the modulatory effects and homologs affected vary between undifferentiated and differentiated cells. Furthermore, we find that β1 and β4 integrins, as well as the PI3-K/Akt and MEK/Erk pathways, are distinctively involved in the survival of undifferentiated (subconfluent Caco-2, confluent HIEC-6) and differentiated (30 days postconfluent Caco-2) enterocytes. Altogether, these data indicate that human intestinal cell survival is subject to distinct regulatory mechanisms according to the state of differentiation.

Materials and Methods

Cell culture. The Caco-2/15 cell line, a stable clone of the parent Caco-2 cells (42), has been characterized elsewhere (8, 50). Caco-2/15 cells are known to be poor secretors of growth factors (2, 8, 12, 17). Cells between passages 53 and 70 were cultured in plastic dishes (60 or 100 mm; Falcon Plastics, Los Angeles, CA), or on 13-mm coverslips, at 37°C in an atmosphere of 95% air-5% CO2. The medium used was Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Burlington, ON, Canada), 4 mM glutamine, 20 mM HEPES (pH 7.5), 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultures were refed every 48 h and subcultured serially as described previously (8, 50). Studies were performed on cultures at ~2 days (subconfluence; 50–70% confluence), 0 days (confluence), and/or 5–30 days postconfluence. The expression of suraceomalastase, a major enterocytye differentiation marker, was monitored by Western blot to ensure proper differentiation of cultures (not shown) as previously described (8, 50, 51). In some experiments, the intestinal cryptlike human fetal HIEC-6 cells (41) were used in parallel with undifferentiated Caco-2 cell cultures; HIEC-6 cells do not differentiate on reaching confluence, and their undifferentiated “cryptlike” status does not change with postconfluence (41).

For treatments, cell cultures were rinsed with DMEM without serum and then maintained 48 h in DMEM without serum with one of the following: 10 μg/ml human recombinant insulin (GIBCO BRL); 150–300 μM genistein (Sigma, St. Louis, MO), a wide-spectrum tyrosine kinase activity inhibitor (4); 0.3–1 μM cytochalasin D (Sigma), which inhibits Fak within the concentration range used (36, 58); 20–60 μM PD-98059 (Calbiochem, San Diego, CA), a specific inhibitor of MEK, the upstream activator of Erk-1/Erk-2 (2, 3, 10, 14, 33–35, 48, 49, 61); or 5–30 μM Ly-294002 (Calbiochem), a specific inhibitor of PI3-K (9, 14, 33, 34, 48, 49, 57). In some experiments, treatments consisted instead of inhibiting the binding activities of the β1 β4 integrin subunits with the monoclonal antibodies P4C10 and 3E1, respectively, for 24 h as previously described (5, 6, 55). For undifferentiated and differentiated cells, determination of the working concentrations of the compounds used led us to settle with the following: 300 μM genistein, 1 μM cytochalasin D, 20 μM PD-98059, and 30 μM Ly-294002. Note that maintenance of cells in absence of serum for 48 h did not impact on their survival compared with exposure to 10% FBS (undifferentiated cells: see Fig. 2A, lane 1 vs. 2; differentiated cells: see Fig. 2B, lane 1 vs. 2; Table 1) and did not influence significantly their expression levels of Bcl-2 homologs (not shown; Table 1). Henceforth, cultures maintained in DMEM without serum were considered as “controls” for the experimental treatments used.

Antibodies. Primary rabbit polyclonal antibodies used in the present study were Ab1682, directed against human Mcl-1 (29); Ab1695, directed to human/mouse Bcl-XL (28, 32); Ab1701 (29, 31) and AbPC68 (Calbiochem), both directed against human Bcl-2; Ab1712 (31) and AbPC66 (Calbiochem), both directed to human Bax; Ab1764, directed against...
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Table 1. Differentiation state-specific controls of intestinal epithelial cell survival

<table>
<thead>
<tr>
<th>Treatment</th>
<th>State</th>
<th>Apoptotic Index</th>
<th>Bcl-2</th>
<th>Bcl-X&lt;sub&gt;L&lt;/sub&gt;</th>
<th>McI-1</th>
<th>Bax</th>
<th>Bak</th>
<th>Bad</th>
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<tr>
<td>Control (no serum)</td>
<td>U</td>
<td>7.9 ± 1.8</td>
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<td></td>
<td>D</td>
<td>11.3 ± 2.4</td>
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<td>+FBS (10%)</td>
<td>U</td>
<td>4.1 ± 1.5</td>
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<td></td>
<td>D</td>
<td>5.9 ± 1.7</td>
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<td>+I (10 μg/ml)</td>
<td>U</td>
<td>5.5 ± 1.9</td>
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<td></td>
<td>D</td>
<td>8.2 ± 2.0</td>
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<td>+G (300 μM)</td>
<td>U</td>
<td>68.3 ± 7.7*</td>
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<tr>
<td></td>
<td>D</td>
<td>63.1 ± 8.1†</td>
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<tr>
<td>+PD (20 μM)</td>
<td>U</td>
<td>3.8 ± 1.7</td>
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<td></td>
<td>D</td>
<td>39.5 ± 5.1†</td>
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<tr>
<td>+Ly (30 μM)</td>
<td>U</td>
<td>85.7 ± 5.4*</td>
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<tr>
<td></td>
<td>D</td>
<td>67.2 ± 7.1†</td>
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<tr>
<td>+CD (1 μM)</td>
<td>U</td>
<td>58.5 ± 9.9*</td>
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<tr>
<td></td>
<td>D</td>
<td>85.3 ± 12.7*†</td>
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Values for the apoptotic indexes are means ± SE expressed as the percentage of apoptotic cells over the total number of cells counted. Caco-2/15 cells were maintained 48 h in DMEM medium without serum (control) or in the presence of serum (fetal bovine serum (FBS)), growth factors (insulin (I)), or inhibitors of signaling molecules/pathways (+G, genistein; +PD, PD-98059; +Ly, Ly-294002; +CD, cytochalasin D) as specified; U, undifferentiated cultures; D, differentiated cultures. In situ TdT-mediated dUTP nick-end labeling (ISEL) was performed on coverslip-grown cell cultures to establish the apoptotic indexes after treatments. A minimum of 300 cells was counted in at least 3 (n ≥ 3) separate experiments and/or cultures. Homolog, qualitative assessment of significant modulations in the expression of Bcl-2 homologs (Bcl-2, Bcl-X<sub>L</sub>, McI-1, Bax, Bak, Bad) in treated cultures, in comparison to control cultures, as observed from Western blot analyses (see Figs. 7 and 8); †, downmodulation; ‡, upregulation; lack of symbol indicates no significant modulation or control steady-state levels.

*Statistically significant (0.001 < P ≤ 0.01) differences between treatments and their respective control cultures. †Statistically significant (0.001 < P ≤ 0.01) differences between differentiated and undifferentiated cells.

Note that antibodies Ab1682, Ab1695, Ab1701, Ab1712, and Ab1764 were developed in the laboratory of one of the authors (J. C. Reed) of the present study; these, as well as the commercial antibodies used herein, have been characterized in previous studies (10, 23, 28–32, 39, 52, 53).

In situ detection of apoptosis-associated DNA strand breaks. For some experiments, in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (ISEL) was carried out as already described (52, 54, 55). DNA extraction uses Triton rather than SDS, thus leaving behind intact genomic DNA (18); consequently, nonapoptotic cell cultures produce near-empty lanes on the gel as a result (for example, see Fig. 2, A and B, lanes 1 and 2).

Protein expression levels. For analyses of protein expression levels of Bcl-2 homologs, total protein from cell lysates was obtained after medium removal, PBS washes, and scraping of cells in 1× solubilization buffer (2.3% SDS, 10% glycerol, and 0.01% bromophenol blue in 62.5 mM Tris·HCl (pH 6.8) containing 5% β-mercaptoethanol). Samples were then boiled (105°C, 5 min), cleared by centrifugation (15,000 g, 5 min, room temperature), and processed for storage as described previously (51–55). SDS-PAGE on 15% acrylamide Tris·HCl gels (Bio-Rad, Hercules, CA) was performed as described previously (51–55). Broad-range molecular mass markers (6.8–209 kDa range; Bio-Rad) were used as standards. Total proteins (50 μg/well) were separated by electrophoresis and then electrotransferred to nitrocellulose membranes (Support NitroCellulose-1; GIBCO BRL) as standard. Note that the method used for DNA extraction uses Triton rather than SDS, thus leaving behind intact genomic DNA (18); consequently, nonapoptotic cell cultures produce near-empty lanes on the gel as a result (for example, see Fig. 2, A and B, lanes 1 and 2).

To analyze the expression profiles of Bcl-2 homologs in relation to the enterocytic differentiation process, relative expression levels were determined by comparison with cytokeratin 18 (K18) as a reference protein. This epithelial cytoskeletal component is expressed at stable levels throughout the enterocytic differentiation process (Fig. 1A) (51–53). Total peak areas [arbitrary units (AU) × mm] were determined at −2, 0, 5, 10, 15, 20, and 30 days postconfluence to establish the ratios of homolog to K18 for each molecule studied. For...
treatments, the relative expression levels of molecules analyzed were evaluated compared with that of control cultures (DMEM without serum) by determining total peak areas (AU × mm) to establish the ratios of treatment to control × 100 (expressed as “% of control”). In all cases, values shown represent means ± SE for at least three (n ≥ 3) separate experiments and/or cultures.

**Immunoprecipitation.** For some experiments, cell cultures were harvested in cold RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.7 μg/ml pepstatin, 40 mM β-glycerophosphate, and 10 mM Na2P2O7] and lysed in the buffer (30 min on ice). Lysates were cleared by centrifugation (15,000 rpm, 15 min, 4°C) and aliquoted for storage as previously described (6, 54). Immunoprecipitation of Fak was carried out according to the protocol already described (54), using 1 μg of the rabbit polyclonal AbC-903 (Santa Cruz Biotechnology) directed against human/mouse/rat p125Fak.

**Assay of Fak, Erk-1/Erk-2, and Akt relative activation levels by immunoblotting.** Total proteins were resolved by SDS-PAGE and electrotransferred as described above. Assays of p125Fak, p42Erk-2/p44Erk-1, or p57Akt relative activation levels were performed as described elsewhere (2, 10, 34, 61). For Fak assays, membranes were first probed with the rabbit polyclonal Ab07-012 (Upstate Biotechnology, Lake Placid, NY) directed to the activated phospho-Tyr-397 form of Fak (11, 21, 61) and then reprobed with the AbC-903 for normalization purposes. For some experiments, Fak immunoprecipitates were used instead (see above). For Erk-1/Erk-2 assays, membranes were probed with the rabbit polyclonal Ab9101a (New England Biolabs) directed to the doubly phosphorylated (activated) forms of Erk-1/Erk-2 (2, 4, 10, 34) and then reprobed with the rabbit polyclonal Ab9102 (New England Biolabs) directed to total Erk-1/Erk-2. Finally, assays of p57Akt relative activation levels were performed by first probing membranes with the rabbit polyclonal Ab9272 (New England Biolabs) directed to the activated phospho-Ser-473 form of Akt (36, 39, 45, 71) and then reprobed with the rabbit polyclonal Ab9272 (New England Biolabs) directed to total Akt.

The relative activation levels of p125Fak, p42Erk-2/p44Erk-1, or p57Akt were evaluated by determining the total peak areas (AU × mm) for the phosphorylated forms and for the corresponding total protein, to establish the ratios pp125Fak/pp42Erk-2/pp44Erk-1, and pp57Akt/pp57Akt. In the case of Erk-1/Erk-2, analyses focused on p42Erk-2 (2, 10). Ratios were in turn compared with those of control cultures, × 100 (expressed as % of control). Values shown represent means ± SE for at least three (n ≥ 3) separate experiments and/or cultures.

**RESULTS**

**Establishment of distinct Bcl-2 expression profiles during enterocytic differentiation.** To determine whether human intestinal epithelial cell survival is subject to differentiation state-specific regulatory mechanisms, we first investigated the steady-state expression levels of six Bcl-2 homologs (Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, Bad) during the enterocytic differentiation process of Caco-2/15 cells. Immunoblot analyses of cell lysates demonstrated the protein expression of all molecules analyzed herein (Fig. 1A). Thus Bcl-2 (~26 kDa), Bcl-XL (~28–30 kDa), Mcl-1 (~39–42 kDa), Bak (~21 kDa), Bad, and K18 steady-state expression levels from Caco-2/15 cell differentiation. Total proteins were separated, probed, and scanned by laser densitometry to assess the relative expression levels of each culture stage studied.

**Comparison of Bcl-2 homolog expression profiles.** To establish the ratios of treatment to control × 100 (expressed as “% of control”). In all cases, values shown represent means ± SE for at least three (n ≥ 3) separate experiments and/or cultures.
B (25–28 kDa), Bak (~28–32 kDa) were detected at all differentiation stages studied as protein bands migrating at their previously reported relative molecular weights (1, 10, 28–32, 39, 47–49, 50, 52, 53).

To examine the modulations of Bcl-2 homolog expression in relation to Caco-2/15 cell enterocytic differentiation, the relative expression levels of Bcl-2 homologs were determined by comparison with a reference protein, K18. The densitometric data presented in Fig. 1, B and C, show that the relative expression levels of all Bcl-2 homologs analyzed were distinctively modulated in parallel to the differentiation process of Caco-2/15 cells. In the case of anti-apoptotic homologs (Fig. 1B), the steady-state levels of Bcl-2 gradually increased between 0 and 15 days postconfluence, to stabilize more or less thereafter as the cells completed their differentiation (Fig. 1A, Bcl-2; Fig. 1B, open squares). Similarly, Mcl-1 levels gradually increased throughout the differentiation process of Caco-2/15 cells (Fig. 1A, Mcl-1; Fig. 1B, filled triangles). On the other hand, Bcl-XL levels decreased during enterocyctic differentiation, particularly between the 10- and 30-day postconfluent stages (Fig. 1A, Bcl-XL; Fig. 1B, filled circles).

In the case of proapoptotic homologs (Fig. 1C), the steady-state levels of Bak were found to increase gradually during Caco-2/15 cell differentiation (Fig. 1A, Bax; Fig. 1C, filled squares). On the other hand, Bak levels decreased sharply between the 2- and 0-day postconfluent stages, to more or less stabilize at low levels thereafter (Fig. 1A, Bax; Fig. 1C, open triangles), whereas Bad levels decreased sharply between 5 and 10 days postconfluence, also to remain more or less stable at low levels thereafter (Fig. 1A, Bad; Fig. 1C, filled circles).

These gradual changes in Bcl-2 homolog steady-state levels were found not to be a product of postconfluent culture but, instead, to be clearly associated with the enterocytic differentiation process of Caco-2/15 cells. First, these changes in Bcl-2 homolog expression levels were parallel to the appearance and gradual increase of sucrase-isomaltase expression (not shown; 8, 12, 27, 37, 41, 42, 50, 51), as well as gradual changes in the activation levels of signaling molecules (see below), during the differentiation of Caco-2/15 cells. Second, the steady-state levels of all Bcl-2 homologs analyzed herein did not change significantly in the undifferentiated crypthead-like human HIEC-6 cells while maintained in postconfluent culture (not shown); these cells remain undifferentiated regardless of their state of postconfluent culture (41).

It is noteworthy that the Caco-2/15 cell undifferentiated-to-differentiated expression profiles of the six Bcl-2 homologs analyzed herein do not correspond to the in vivo crypt-villus gradients already reported for these homologs in the adult (26, 28–32, 39, 45) and/or midgestation (52, 53) human small intestine but correspond instead to those already observed along the fully functional and jejunal-like crypt-villus axis of the midgestation human fetal colon (52). Nonetheless, our observations altogether indicate that the steady-state expression levels of Bcl-2 homologs undergo major modulations during the enterocytic differentiation process, resulting in expression profiles of differentiated cells that are distinct from those of undifferentiated ones.

Enterocytes display distinct susceptibilities to apoptosis according to their differentiation state. Because undifferentiated and differentiated Caco-2/15 cells exhibit distinct Bcl-2 homolog expression profiles, we then sought to ascertain whether this translated into differential susceptibilities to apoptosis as well. Using DNA laddering assays to visualize the internucleosomal DNA fragmentation associated with apoptosis, we evaluated the presence of apoptosis in undifferentiated and differentiated cultures maintained for 48 h with or without FBS, or without serum but with the addition of insulin or pharmacological inhibitors of signal transduction molecules/pathways. Apoptotic indexes in treated cultures were also determined using the ISEL method.

We first found that maintenance of Caco-2/15 cells in the absence of serum for 48 h did not impact significantly on their survival compared with their maintenance in the presence of FBS. Indeed, no DNA laddering was evidenced in either culture condition for undifferentiated (Fig. 2A, lane 1 vs. 2) and differentiated cells (Fig. 2B, lane 1 vs. 2), whereas the “basic” apoptotic indexes found in the presence of FBS (Table 1, +FBS) did not differ significantly from those obtained in absence of serum (Table 1, control) for either differentiation states. Incidentally, we (51) and others (27, 41) have previously reported that absence of serum for 24–48 h was not detrimental to enterocytic functions of Caco-2 cells.

As expected from the lack of differences between the absence and presence of serum, the exposure to insulin for 48 h had no significant influence on the survival of either undifferentiated (Fig. 2A, lane 3) or differentiated (Fig. 2B, lane 3) Caco-2/15 cell cultures. In contrast, the inhibition of tyrosine kinase activities greatly impacted on cell survival in both differentiation states (~64% apoptosis in undifferentiated cells and ~83% apoptosis in differentiated cells; Table 1, +G) (Fig. 2, A and B, lane 4). Likewise, the specific inhibition of PI3-K (Fig. 2, A and B, lane 6; Table 1, +Ly) and Fak (Fig. 2, A and B, lane 7; Table 1, +CD) resulted in significant increases in apoptosis in both states of differentiation.

However, stark differences were noted on the respective impacts of some of these treatments on cell survival between undifferentiated and differentiated Caco-2/15 cells (Table 1). For instance, the inhibition of tyrosine kinase activities qualitatively resulted in more abundant DNA laddering in differentiated Caco-2/15 cells than in undifferentiated ones (Fig. 2B, lane 4 vs. A, lane 4), which translated into significant differences in the corresponding apoptotic indexes (Table 1, +G). Likewise, the inhibition of Fak produced more DNA laddering in differentiated Caco-2/15 cells than undifferentiated ones (Fig. 2B, lane 7 vs. A, lane 7), which translated as well into a significantly greater apoptotic index in differentiated cells than undifferen-
Differentiation state distinct involvement of Fak and integrins in enterocytic cell survival. To verify whether the observed differentiation state distinctions in apoptosis susceptibility were linked to a differential involvement of signaling pathways, we first investigated specifically the role of cell adhesion signaling in Caco-2/15 cell survival, focusing on p125Fak activation (Fig. 3). As previously reported in the parental Caco-2 cell line (35), we found that levels of activated Fak (pp125Fak) were downregulated while those of the protein (p125Fak) increased during the differentiation process (Fig. 3A). This resulted in distinct enterocytic p125Fak activation profiles, whereby Fak activation was at minimal levels in differentiated cells as opposed to undifferentiated ones (Fig. 3, A and B). Nonetheless, relative p125Fak activated levels were equally inhibited in the two differentiated states when either genistein (Fig. 3C, +G) or cytochalasin D (Fig. 3C, +CD) was used as a treatment. The nonspecific inhibition of p125Fak activation by genistein, a wide-spectrum inhibitor of tyrosine kinases, was expected (35, 36, 58, 61), whereas cytochalasin D can act as a more specific inhibitor of p125Fak at the concentration range used (36, 58). We also observed that the inhibition of the downstream MEK/Erk pathway (Fig. 3C, +PD) or PI3-K (Fig. 3C, +Ly), as well as exposure to insulin (Fig. 3C, +I), did not impact on the relative activation levels of p125Fak itself in either undifferentiated or differentiated Caco-2/15 cells, as expected from previous studies in other cell types and tissues (4, 11, 33, 34, 36, 58).

Because Fak is largely responsible for β1 integrin signaling (11, 19, 21), we then analyzed the impact of the inhibition β1 integrin activity on Caco-2/15 cell survival and immunoprecipitated p125Fak relative activation levels. In parallel, the potential involvement of β4 integrins was analyzed as well; it is noteworthy that enterocytes, including Caco-2/15 and HIEC-6 cells, express the β4 integrin subunit (5, 7). As expected from other studies (11, 18, 19, 21, 33, 55, 58), the blockage of β1 integrins induced apoptosis/anoikis in undifferentiated (Fig. 4A, lane 3) and differentiated (Fig. 4A, lane 7) Caco-2/15 cells, in addition to causing sharp reductions in immunoprecipitable p125Fak activated levels in both differentiation states (Fig. 4B, lanes 5 and 6; Fig. 4C, +P4C10). However, and as already noted when Fak is inhibited by cytochalasin D (Table 1, +CD), the impact of β1 integrin inhibition on cell survival was significantly greater in differentiated Caco-2/15 cells (~78% apoptosis; Table 2, +P4C10) than in undifferentiated ones (~55% apoptosis; Table 2, +P4C10); this was likewise apparent from the intensity of the DNA laddering observed (Fig. 4A, lane 3 vs. 7). Our β4 integrin inhibition experiments produced even more striking results between undifferentiated and differentiated Caco-2/15 cells. Although the inhibition of the β4 subunit did not influence p125Fak relative activation levels (Fig. 4B, lanes 7 and 8; Fig. 4C, +3E1), as expected (11, 21), it did nonetheless impact significantly on the survival of differentiated Caco-2/15 cells (Fig. 4A, lane 8; Table 2, +3E1) but not

Fig. 2. Differentiation state-distinct susceptibilities to apoptosis in intestinal epithelial cells. A and B: representative DNA laddering assays from undifferentiated (~2 days postconfluence; A) and differentiated (30 days postconfluence; B) Caco-2/15 cell cultures maintained 48 h without serum (control, C, lane 1) or with the addition of 10% fetal bovine serum (+FBS; lane 2), 10 μg/ml insulin (+I; lane 3), 300 μM genistein (+G; lane 4), 20 μM PD-98059 (+PD; lane 5), 30 μM Ly-294002 (+Ly; lane 6), or 1 μM cytochalasin D (+CD; lane 7). DNA (20 μg/lane) were isolated from treated cultures and then separated by agarose gel electrophoresis and stained with ethidium bromide for the visualization of apoptosis/anoikis-associated internucleosomal DNA fragmentation (“DNA laddering”). Results illustrated here for undifferentiated Caco-2/15 cells were similar, if not identical, to those obtained with the undifferentiated cryptlike HIEC-6 cells (not shown). L, 100-bp DNA size markers.
on undifferentiated ones (Fig. 4A, lane 4; Table 2, +3SE). To this effect, blockage of β1, but not β4, induced apoptosis/anoikis in the undifferentiated crypt-like HIEC-6 cells, as observed for undifferentiated Caco-2/15 cells (Table 2; HIEC-6 cells vs. undifferentiated Caco-2/15 cells). These data, together with the observation that undifferentiated and differentiated Caco-2/15 cells exhibit distinct profiles of activated Fak levels, thus indicate that Fak and β1 and β4 integrins are distinctively involved in intestinal epithelial cell survival depending on the state of differentiation.

Differentiation state-specific involvement of MEK/Erk signaling in enterocytic cell survival. To further dissect the observed distinctions in apoptosis susceptibility between undifferentiated and differentiated Caco-2/15 cells, we then examined more closely the involvement of the MEK/Erk pathway by focusing on p42Erk-2 activation (Fig. 5). As in the case of p125Fak (see above and Ref. 35) and as previously reported (2, 35), we found that levels of activated Erk-2 (pp42Erk-2) levels were downregulated while those of the Erk-2 protein (p42Erk-2) increased during the differentiation process (Fig. 5A). This resulted in distinct enterocytic p42Erk-2 activated profiles, whereby Erk-2 activation was at minimal levels in differentiated Caco-2/15 cells as opposed to undifferentiated ones (Fig. 5, A and B). Nonetheless, the inhibition of upstream MEK with PD-98059 significantly lowered the activated levels of p42Erk-2 in the two differentiated states (Fig. 5C, +PD) as expected (2, 3, 10, 34, 61), while the inhibition of PI3-K had no effect (Fig. 5C, +Ly). Also as expected (4, 11, 13, 21, 34), the inhibition of p125Fak (Fig. 5C, +CD) and of tyrosine kinase activities (Fig. 5C, +G) significantly lowered the relative activation levels of p42Erk-2 in both undifferentiated and differentiated Caco-2/15 cells. Conversely, exposure to insulin produced similar results (Fig. 5C, +I).

Although the regulation of MEK/Erk pathway activation did not exhibit distinctions in relation to the differentiation state of Caco-2/15 cells, we found that the inhibition of this pathway impacted nonetheless distinctively on the survival of undifferentiated and differentiated cells. Indeed, and as already mentioned, the inhibition of MEK/Erk induced apoptosis significantly in differentiated Caco-2/15 cells but not in undifferentiated ones (Fig. 2B, lane 5 vs. A, lane 5; Table 1, +PD). Similarly to that in undifferentiated Caco-2/15 cells, the inhibition of MEK did not significantly
induce apoptosis in the undifferentiated cryptlike HIEC-6 cells (not shown). These data, together with the observation that undifferentiated and differentiated Caco-2/15 cells exhibit distinct profiles of acti-

Table 2. Differentiation state-specific involvement of β1 and β4 integrins in intestinal epithelial cell survival

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HIEC-6 cells</th>
<th>Undifferentiated Caco-2/15 cells</th>
<th>Differentiated Caco-2/15 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.7 ± 4.7</td>
<td>8.6 ± 2.0</td>
<td>5.1 ± 1.3</td>
</tr>
<tr>
<td>+IgGs</td>
<td>10.2 ± 3.1</td>
<td>9.4 ± 2.7</td>
<td>6.1 ± 2.1</td>
</tr>
<tr>
<td>+P4C10</td>
<td>47.9 ± 10.8a</td>
<td>55.1 ± 4.5a</td>
<td>78.0 ± 6.4a†</td>
</tr>
<tr>
<td>+3E1</td>
<td>13.9 ± 5.9</td>
<td>7.7 ± 2.3</td>
<td>28.3 ± 9.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE for percentage of apoptotic cells over the total number of cells counted. Caco-2/15 (subconfluent, undifferentiated; 30 days postconfluent, differentiated) and HIEC-6 (confluent, undifferentiated) cells were maintained 24 h in DMEM medium without serum (control) or in the presence of nonimmune mouse IgGs (100 μg/ml), a human β1 integrin subunit-blocking mouse monoclonal antibody (+P4C10; 50 μg/ml), or a human β4 integrin subunit-blocking mouse monoclonal antibody (+3E1; 100 μg/ml). ISEL was performed on coverslip-grown cell cultures to establish the apoptotic indexes after treatments. A minimum of 300 cells was counted for at least 3 (n = 3) separate experiments and/or cultures. *Statistically significant (0.001 ≤ P ≤ 0.01) differences between treatments and their respective controls. †Statistically significant (0.001 ≤ P ≤ 0.01) differences between differentiated and undifferentiated Caco-2/15 cells.

activated Erk-2 levels, thus indicate that the MEK/Erk pathway is distinctively involved in intestinal epithelial cell survival, depending on the state of differentiation.

Differentiation state-distinct regulation of the PI3-K/ Akt pathway and involvement in enterocytic cell survival. Because the MEK/Erk pathway displayed differentiation state distinctions in its involvement in enterocytic cell survival but not in its regulation of activation, we then verified whether this was also the case for the PI3-K/Akt pathway by focusing on p57Akt activation (Fig. 6). As in the case of p125Fak (see above and Ref. 35) and p42 Erk-2 (see above and Refs. 2 and 35), we found that levels of activated Akt (pp57Akt) were downregulated while those of the Akt protein (p57Akt) increased during the differentiation process of Caco-2/15 cells (Fig. 6A). This resulted in distinct p57Akt activated profiles, whereby Akt activation was at minimal levels in differentiated Caco-2/15 cells as opposed to undifferentiated ones (Fig. 6, A and B). Conversely, we found that the inhibition of p125Fak (Fig. 6C, +CD) and tyrosine kinase activities (Fig. 6C, +G) lowered p57Akt relative activation levels in both undifferentiated and differentiated Caco-2/15 cells, also as observed for p42Erk-2 (see above) and as expected from previous studies in other cell types and tissues (11, 15, 21, 56).

Although the inhibition of MEK/Erk did not impact on p57Akt activated levels (Fig. 6C, +PD), insulin was found to exert distinct influences on p57Akt activation depending on the state of differentiation: hence, relative p57Akt activated levels were increased by insulin in undifferentiated Caco-2/15 cells but were not affected significantly in differentiated ones (Fig. 6C, +I). Further distinctions were noted in the regulation of the PI3-K/Akt pathway between undifferentiated and dif-
differentiated Caco-2/15 cells. Indeed, the specific inhibition of PI3-K with Ly-294002 resulted in a significant decrease of downstream p57Akt activation in differentiated Caco-2/15 cells (Fig. 6C, +Ly), as usually expected (15, 33, 34, 48, 57), but did not affect p57Akt activation in undifferentiated ones (Fig. 6C, +Ly). Likewise, the inhibition of PI3-K also failed to affect significantly p57Akt activation in HIEC-6 cells (not shown), as in the case of undifferentiated Caco-2/15 cells. To this effect, it is noteworthy that there is increasing evidence from other cell systems, which indicates that Akt activation can be PI3-K independent (15, 57).

In addition to such complex but differentiation state-specific regulation of PI3-K/Akt pathway activation, we also found that the inhibition of PI3-K had a significantly greater impact on cell survival in undifferentiated Caco-2/15 cells than in differentiated ones, as already noted (Table 1, +Ly). These data, together with the observation that undifferentiated and differentiated Caco-2/15 cells exhibit distinct profiles of activated Akt levels, thus indicate that the PI3-K/Akt pathway is not only subject to a differentiation state-specific type of regulation but is furthermore distinctively involved in the survival of undifferentiated and differentiated intestinal epithelial cells.

Differentiation state-specific regulation of Bcl-2 homolog steady-state levels in enterocytes. To ascertain whether the distinctions in susceptibility to apoptosis and involvement of signaling pathways between undifferentiated and differentiated Caco-2/15 cells are linked with differentiation state-specific regulatory mechanisms of Bcl-2 homolog expression, we then investigated the steady-state expression levels of six Bcl-2 homologs (Bcl-2, Bcl-X\(_L\), Mcl-1, Bax, Bak, Bad) following the various treatments. The densitometric analyses presented in Figs. 7 and 8 show that the relative expression levels of each Bcl-2 homolog analyzed were distinctively modulated in both undifferentiated and differentiated Caco-2/15 cells.

In undifferentiated Caco-2/15 cells, inhibition of tyrosine kinase activities resulted in a significant downregulation of Bcl-2 (Fig. 7A, +G), Bcl-X\(_L\) (Fig. 7B, +G), Bax (Fig. 8A, +G), and Bad (Fig. 8C, +G), while only Bak expression was increased (Fig. 8B, +G) and Mcl-1 remained unaffected (Fig. 7C, +G). On the other hand, the inhibition of Pak led to a significant downmodulation of Mcl-1 (Fig. 7C, +CD), whereas Bax (Fig. 8A,
obtained with the undifferentiated cryptlike HIEC-6 cells (not shown).

controls. Results illustrated here for undifferentiated Caco-2/15 cells were similar, if not identical, to those of control cultures. *Statistically significant (0.001 < P < 0.01) differences between treatments and their respective controls. Results illustrated here for undifferentiated Caco-2/15 cells were similar, if not identical, to those obtained with the undifferentiated cryptlike HIEC-6 cells (not shown).

In the case of differentiated Caco-2/15 cells, the inhibition of tyrosine kinase activities led to a significant downregulation of Bcl-2 (Fig. 7A, +PD) and Bcl-XL (Fig. 5B) and to an increase of Bak (Fig. 8B, +G) and Bad (Fig. 8C, +G). The inhibition of Fak resulted in a significant decrease of all anti-apoptotic homologs analyzed (Fig. 7, A–C, +CD) and of Bad (Fig. 8C, +CD), while increasing Bax (Fig. 8A, +CD) and Bak (Fig. 8B, +CD). The inhibition of the MEK/Erk pathway led to a significant decrease of Bcl-2 (Fig. 7A, +PD), Bcl-XL (Fig. 7B, +PD) and all proapoptotic homologs analyzed (Fig. 8, A–C, +PD). Last, the inhibition of PI3-K resulted in a decrease of Bcl-XL (Fig. 7B, +LY), Mcl-1 (Fig. 7C, +LY), and Bak (Fig. 8A, +LY) without affecting the other homologs analyzed.

In addition to such complex modulations of Bcl-2 homolog expression in both undifferentiated and differentiated Caco-2/15 cells, we also observed stark differences between the two differentiation states (summarized in Table 1). For instance, although insulin increased Mcl-1 levels in both undifferentiated and differentiated cells (Fig. 7C, +I), the same treatment further resulted in an increase of Bcl-2 (Fig. 7A, +I) and decrease of Bax (Fig. 8A, +I) and Bak (Fig. 8B, +I) in undifferentiated cells (Table 1, +I), while further resulting instead in a decrease of Bcl-XL (Fig. 7B, +I) and Bad (Fig. 8C, +I) in differentiated cells (Table 1, +I). Similarly, the inhibition of tyrosine kinase activities affected Bcl-2 (Fig. 7A, +G), Bcl-XL (Fig. 7B, +G), Mcl-1 (Fig. 7C, +G), and Bak (Fig. 8B, +G) in the same manner in both differentiation states (Table 1, +G), while having opposing effects on Bad expression (Fig. 8C, +G) and causing a decrease of Bax (Fig. 8A, +G) in differentiated cells only (Table 1, +G). Further examples of differentiation state-distinct modulations of individual Bcl-2 homologs were noted as well when Fak, MEK/Erk, or PI3-K were inhibited (Table 1, Figs. 7 and 8).

It is noteworthy that the relationship between the balance of anti- vs. proapoptotic homologs and the impact on enterocytic cell survival was not always clear from the experiments performed herein (Table 1).
For instance, the inhibition of MEK/Erk in differentiated Caco-2/15 cells resulted in a balance seemingly in favor of anti-apoptotic homologs (Table 1, +PD), even if the treatment induced apoptosis (Fig. 2B, lane 5; Table 1, +PD). Considering that cell survival implicates not just one homolog but rather complex interactions between numerous Bcl-2 homologs, as well as posttranslational modifications to further modulate their func-

Fig. 7. Differentiation state-specific modulations of anti-apoptotic Bcl-2 homologs in Caco-2/15 cells. Relative expression levels of Bcl-2 (A), Bcl-XL (B), and Mcl-1 (C) in undifferentiated (open bars) and differentiated (solid bars) Caco-2/15 cell cultures maintained 48 h without serum (control) or with the addition of 10 μg/ml insulin (+I), 300 μM genistein (+G), 20 μM PD-98059 (+PD), 30 μM Ly-294002 (+Ly), or 1 μM cytochalasin D (+CD). Total proteins were separated and probed as represented in Fig. 1, and then scanned by laser densitometry. For each treatment of cultures, the relative expression levels of each homolog were compared with those of control cultures, ×100 (expressed as % of control). Columns represent means ± SE for at least 3 (n = 3) separate experiments. *Statistically significant (0.001 ≤ P ≤ 0.01) differences between treatments and their respective controls.

Fig. 8. Differentiation state-specific modulations of proapoptotic Bcl-2 homologs in Caco-2/15 cells. Relative expression levels of Bax (A), Bak (B), and Bad (C) in undifferentiated (open bars) and differentiated (solid bars) Caco-2/15 cell cultures maintained 48 h without serum (control) or with the addition of 10 μg/ml insulin (+I), 300 μM genistein (+G), 20 μM PD-98059 (+PD), 30 μM Ly-294002 (+Ly), or 1 μM cytochalasin D (+CD). Total proteins were separated and probed as represented in Fig. 1, and then scanned by laser densitometry. For each treatment of cultures, the relative expression levels of homolog were compared with those of control cultures, ×100 (expressed as % of control). Columns represent means ± SE for at least 3 (n = 3) separate experiments. *Statistically significant (0.001 ≤ P ≤ 0.01) differences between treatments and their respective controls.
tions (1, 4, 20, 25, 47), such apparent discrepancies are likely due to the involvement of additional homologs other than those analyzed herein (e.g., Bcl-w, Bid, etc.) and/or to Bcl-2 homolog phosphorylation events (1, 20, 25, 45, 47, 59) not focused on in the present study. Nonetheless, these data altogether indicate that the steady-state expression levels of Bcl-2 homologs are not only subject to complex regulatory mechanisms in both undifferentiated and differentiated intestinal epithelial cells but that such mechanisms exhibit distinctions related to the state of differentiation.

**DISCUSSION**

In this study, we investigated the question of whether the regulation of human intestinal epithelial cell survival involves distinct control mechanisms depending on the state of differentiation, using the well-established human Caco-2/15 enterocyte-like and HIEC-6 cryptlike in vitro models. We found that the enterocytic differentiation process of Caco-2/15 cells results in the gradual establishment of differentiation state-distinct profiles of 1) Bcl-2 homolog steady-state levels and 2) p125 Fak, p42 Erk-2, and p57 Akt activated levels. Accordingly, we found that the inhibition of tyrosine kinase activities, Fak, the MEK/Erk pathway, or PI3-K have distinct impacts on enterocytic cell survival in undifferentiated (subconfluent Caco-2/15, confluent HIEC-6) and differentiated (30 days postconfluent Caco-2/15) cells. We also observed that exposure to insulin and the inhibition of these various signaling molecules/pathways modulated distinctively the expression of each Bcl-2 homolog analyzed in both undifferentiated and differentiated Caco-2/15 cells; however, sharp distinctions were noted between the two states of differentiation in the resulting effects of the same treatments on the expression of Bcl-2 homologs. Furthermore, we found that the PI3-K/Akt pathway is distinctively regulated in undifferentiated and differentiated enterocytes. Finally, we have shown that Fak and β1 and β4 integrins, as well as the MEK/Erk and PI3-K/Akt pathways, are distinctively involved in enterocytic cell survival, depending on the state of differentiation. Therefore, these data altogether indicate that human intestinal epithelial cell survival is characterized by differentiation state-specific susceptibilities to apoptosis, which in turn are linked with distinctions in both the regulation of Bcl-2 homologs and the involvement of signaling molecules/pathways.

The intestinal crypt-villus axis is defined by proliferative and functional properties of the crypt cells that distinguish them from the fully differentiated villus cells (26, 37, 41, 44, 45). Previous in vivo studies in the adult rodent (26, 29–32, 38–40, 44, 45, 60) and human (26, 28–32, 39, 44, 45, 52, 53, 59) intestine have reported that Bcl-2 homologs exhibit gradients of expression along this crypt-villus axis of enterocytic differentiation. To this effect, the data reported herein show for the first time that such distinct Bcl-2 homolog expression profiles are gradually established during the enterocytic differentiation process. Because Bcl-2 homologs constitute a critical checkpoint in the regulation of apoptosis, it has been suggested that intestinal epithelial cell survival may be regulated distinctively according to the state of cell differentiation (22, 24, 26, 40, 44–46, 53). In support of this, it is now well established that the predominant means to remove obsolete differentiated enterocytes is through apoptosis and shedding at the villus apex, whereas spontaneous crypt cell apoptosis, a rarer (less frequent) process, serves to remove defective/injured progeny cells (22, 26, 40, 44, 45, 53). Hence, in vivo studies have consistently reported a dramatic increase of apoptosis in crypt cells, but little or no increase in villus cells, after irradiation or chemotherapeutic drug exposure (24, 38, 44–46). Likewise, analyses of enterocytic apoptosis in bcl-2−/− and bax−/− knockout mice have reported differential consequences for crypt and villus cells, with regard to resistance and/or susceptibility to apoptosis after irradiation (44–46, 60). Incidentally, the present study provides further documentation on the distinct susceptibilities to apoptosis between undifferentiated and differentiated enterocytes in vitro. In addition, our data demonstrate for the first time that such distinctions are linked with a differentiation state-specific regulation of Bcl-2 homolog expression and involvement of integrins and signal transduction pathways. Finally, it has been recently reported (22) that villus cells exhibit distinct expression and activation profiles of caspases, the major components of the apoptotic effector machinery (1, 25, 47). Consequently, and in light of these considerations, our data altogether clearly establish that human intestinal epithelial cell survival is subject to differentiation state-specific control mechanisms in vitro.

External stimuli responsible for the promotion of cell proliferation, differentiation, and/or survival largely come from hormones/growth factors and cell adhesion (1, 4, 11, 13, 15, 19–21, 56). As with other cell types (4, 10, 13, 19, 21, 25, 54, 55), the differentiation process of enterocytes is accompanied by the establishment of differentiation state distinctions in hormonal responses (12, 16, 17, 27, 37, 43–45) as well as in the expression of cell cycle regulators (2, 17) and cell adhesion components, such as integrins (7, 35, 37, 41, 43–45), in addition to morphological and functional differentiated characteristics. For example, while both crypt and villus cells express β1 integrins, differentiation state-distinct profiles of specific αβ1 integrins are nonetheless expressed (6–7, 33, 35, 51); also, the β4 integrin subunit is functional in differentiated enterocytes but not in crypt cells (5, 7). In addition, signaling molecules/pathways have been shown to play dual roles in enterocytes: for instance, the MEK/Erk pathway is required for proliferation of undifferentiated cells and the onset of enterocytic differentiation (2). Similarly, PI3-K performs a major role in enterocytic de novo DNA synthesis and differentiation (9, 35). Taking these observations into consideration, it therefore follows that 1) undifferentiated and differentiated intestinal cells exhibit distinct profiles of activated levels of Fak (this study and Ref. 35), Erk-1/Erk-2 (this...
study and Refs. 2 and 35) and Akt (this study) which, in the case at least of the PI3-K/Akt pathway (this study and Refs. 9 and 35), underlie as well distinct modes of regulation depending on the differentiation state; and 2) Fak and integrins β1 and β4, as well as the MEK/Erk and PI3-K/Akt pathways, exert distinct influences on intestinal epithelial cell survival depending on the state of cell differentiation (this study). In this respect, the establishment of differentiation state-specific profiles of MEK/Erk and PI3-K/Akt activation has been observed in other cell types (10, 13, 15, 56). Furthermore, a differentiation state-distinct involvement of integrins and/or signaling pathways in cell survival has been demonstrated in other cell types as well (4, 11, 13, 15, 19, 21), such as neural (4, 13) and skeletal muscle (54, 55) cells.

In the past few years, it has become increasingly evident that the regulation of individual Bcl-2 homologs can involve numerous pathways acting in synergy or independently and that the specific pathways involved in the regulation of a single homolog can differ depending on the cell type and the differentiation state studied (1, 4, 10, 11, 13–15, 19–21, 47, 49, 56). It now appears that intestinal epithelial cells are no exception to this (this study and Refs. 23, 33, 39, 48). Indeed, exposure to butyrate induces apoptosis in undifferentiated intestinal cells by decreasing Bcl-2 levels without affecting those of Bax or Bak (48), whereas the same treatment induces apoptosis in differentiated cells by increasing Bak levels (39, 48). Similarly, ras transformation of rat crypt cells stimulates the PI3-K/Akt pathway but not the MEK/Erk pathway, resulting in decreased Bak levels (33). In addition to these, our findings that Fak, MEK/Erk, and PI3-K/Akt play distinct roles in the modulation of individual Bcl-2 homolog expression either within undifferentiated and differentiated intestinal cells or between the two differentiation states illustrate well the complexity that is required in the regulation of the expression and functions of Bcl-2 homologs, depending on the cell type and state of cell differentiation. This in turn further stresses the concept that cell survival is not regulated by the activity of single Bcl-2 homologs but, rather, by a balance of activities from numerous homologs that arises from the input of multiple stimuli and pathways (1, 4, 13, 14, 21, 25, 34, 47, 49).

It is now well acknowledged that signaling pathways can cross talk with each other to act in tight coordination, although the exact mechanisms involved remain unclear (4, 13, 21, 56). For example, a close cooperation has been reported between the MEK/Erk and PI3-K/Akt pathways in suppressing apoptosis in some cell types, even to a point where both pathways can be required to effectively sustain cell survival (4, 13, 14, 21, 49, 56). On the other hand, it is becoming increasingly evident that cell survival is furthermore regulated through cell type-specific machineries of integration and fine-tuning of signaling pathways, which remain to be fully understood (1, 4, 13, 15, 20, 21, 56). For instance, recent observations indicate that the MEK/Erk and PI3-K/Akt pathways can also act independently in maintaining cell survival, with one being able to compensate for the inhibition of the other (4, 13–15, 34, 49, 56). To this effect, several lines of evidence now indicate that the relationship between the MEK/Erk and PI3-K/Akt pathways, as well as their integration and fine-tuning, are distinct depending on the state of enterocytic differentiation: 1) the inhibition of Fak impacts equally on the MEK/Erk and PI3-K/Akt pathways in undifferentiated and differentiated enterocytes, and yet Fak and these two pathways nonetheless display striking differentiation state-specific distinctions in their involvement in enterocytic cell survival (this study); 2) the inhibition of the MEK/Erk pathway does not impact on the survival of undifferentiated enterocytes (this study and Ref. 33) but does so for differentiated cells (this study); 3) the inhibition of PI3-K causes apoptosis in both undifferentiated (this study and Refs. 9 and 33) and differentiated (this study) cells, albeit to a greater extent in the former differentiation state (this study); 4) the inhibition of β1 integrins causes a downactivation of Fak in both undifferentiated and differentiated enterocytes but, as for the inhibition of Fak (this study), causes apoptosis to a greater extent in the latter cell state (this study); 5) the β1 integrin-mediated survival signaling in undifferentiated enterocytes appears to be PI3-K/Akt dependent but not MEK/Erk dependent (33); 6) overexpression of ras protects crypt cells from anoikis by stimulating the PI3-K/Akt pathway and not the Ras/MEK/Erk pathway (33), even though ras is well known for its role in usually stimulating the latter pathway (2, 4, 11, 13, 21, 34); 7) β4 integrins, which are known for their role in maintaining the activation of the MEK/Erk pathway through the recruitment of Shc and Grb2-SOS (7, 11, 21), are involved in enterocytic cell survival only in the differentiated state (this study); and 8) p57Akt activation appears to be PI3-K dependent in undifferentiated enterocytes only (this study), an uncoupling situation already shown to exist in other cell systems (13, 15, 56). Consequently, these considerations altogether suggest that both the MEK/Erk and PI3-K/Akt pathways are required for the survival of differentiated enterocytes, whereas PI3-K (with or without p57Akt) is sufficient and able to compensate for the inhibition of the MEK/Erk pathway in the survival of undifferentiated enterocytes. Alternatively, the MEK/Erk pathway may not play any role in the survival of undifferentiated intestinal cells.

In conclusion, the present findings provide new insights into the complex regulatory mechanisms that are responsible for the survival of human intestinal epithelial cells. Such mechanisms are likely to vary somewhat in their specifics along the proximal-distal axis of the gut, considering the differences in Bcl-2 homolog expression profiles (28–32, 38, 40, 52, 53, 60), susceptibilities to apoptosis (24, 26, 38, 40, 44–46), and regulation of cellular functions (7, 16, 37, 41, 43) between jejunum, ileum, and colon enterocytes in vivo and in vitro. Nonetheless, it is now clear that intestinal epithelial cells exhibit a differentiation state-specific susceptibility to apoptosis through distinctions in the
involvement of signaling molecules/pathways, such as Fak, MEK/Erk, and PI3-K/Akt, which in turn impact distinctively on the expression of Bcl-2 homologs. Accordingly, cell adhesion components such as β1 and β4 integrins also participate in the regulation of intestinal cell survival in a differentiation state-specific manner. However, the exact molecular processes responsible for such distinct control mechanisms of survival between undifferentiated and differentiated cells remain to be fully understood. For example, the question is open as to why Akt is seemingly independent of PI3-K for its activation in undifferentiated intestinal cells. Conversely, further analyses are required to dissect the exact molecular relationships and functions of the MEK/Erk and PI3-K/Akt pathways in the regulation of enterocytic cell survival and Bcl-2 homolog expression. Increasing our knowledge on the specific roles of these pathways in intestinal epithelial cell survival should provide a better understanding of the role of apoptosis/anokisis in the maintenance and repair of the intestinal epithelium, as well as in the pathogenesis of intestinal disorders with dysregulation of apoptosis, such as cancer.

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