Human Intestinal Epithelial Cell Survival and Anoikis

DIFFERENTIATION STATE-DISTINCT REGULATION AND ROLES OF PROTEIN KINASE B/Akt ISOFORMS*

We have shown previously that human intestinal epithelial cell survival and anoikis are distinctively regulated according to the state of differentiation. Here we analyzed the roles of protein kinase B/Akt isoforms in such differentiation state distinctions. Anoikis was induced in undifferentiated and differentiated enterocytes by inhibition of focal adhesion kinase (Fak; pharmacologic inhibition or overexpression of dominant-negative mutants) or β1 integrins (antibody blocking) or by maintaining cells in suspension. Expression/activation parameters of Akt isoforms (Akt-1, Akt-2, and Akt-3) and Fak were analyzed. Activity of Akt isoforms was also blocked by inhibition of phosphatidylinositol 3-kinase or by overexpression of dominant-negative mutants. Here we report the following. 1) The expression/activation levels of Akt-1 increase overall during enterocyte differentiation, and those of Akt-2 decrease, whereas Akt-3 is not expressed. 2) Akt-1 activation is dependent on β1 integrins/Fak signaling, regardless of the differentiation state. 3) Akt-2 activation is dependent on β1 integrins/Fak signaling in undifferentiated cells only. 4) Activation of Akt-1 is phosphatidylinositol 3-kinase-dependent, whereas that of Akt-2 is not. 5) Akt-2 does not promote survival or apoptosis/anoikis. 6) Akt-1 is essential for survival. 7) Akt-2 cannot substitute for Akt-1 in the suppression of anoikis. Hence, the expression and regulation of Akt isoforms show differentiation state-specific distinctions that ultimately reflect upon their selective implication in the mediation of human intestinal epithelial cell survival. These data provide new insights into the synchronized regulation of cell survival/death that is required in the dynamic renewal process of tissues such as the intestinal epithelium.

Programmed cell death (or apoptosis) is a tightly regulated process that performs crucial functions in tissue homeostasis (1, 2). Maintenance of cell survival requires constant regulation through various stimuli originating from growth factors, cytokines, and/or cell adhesion (1–5). The disruption or loss of integrin-mediated cell adhesion induces apoptosis by a process that is termed “anoikis” (4–7). Integrins are a family of transmembrane heterodimeric (αβ) receptors. Those integrins belonging to the β1 subfamily are largely responsible for the establishment of extracellular matrix-cytoskeleton linkage as well as cell adhesion-mediated activation of signaling pathways (4–6). To this effect, signaling originating from β1 integrins to promote cell survival often implicates Fak1 (p125Fak) and/or the MEK/Erk-1 and -2 (p42Erk2/p44Erk1 MAPK) pathway, depending on the tissue and cellular contexts (3–8). Conversely, the induction of apoptosis and/or anoikis can be driven by other signaling pathways (3, 6–9), such as that of p38 stress-activated MAPK (9–12).

The PI3K/PKB (Akt) pathway is another signaling pathway implicated in growth factor- and integrin-driven cell survival, in addition to other roles in cell proliferation and/or differentiation (3, 5, 8, 13–16). Indeed, the serine/threonine kinase Akt has been identified as a major effector of PI3K in the promotion and maintenance of cell survival, being able to act at various strategic points of the common apoptosis/anoikis pathway and thus suppressing the process (2–5, 13–18). Three isoforms of Akt have been characterized to date in mammalian cells, namely Akt-1 (PKBα), Akt-2 (PKBβ), and Akt-3 (PKBγ) (13–17). Studies on the expression patterns of these isoforms have revealed that Akt-1 and Akt-2 are ubiquitous, whereas Akt-3 is found predominantly in brain, heart, and kidney (13–17). The phosphorylation of the serine 473 residue in the tail domain of Akt-1 is required to allow for its maximal activation. Although the corresponding phosphorylation sites in Akt-2 (serine 474) and Akt-3 (serine 472) have been identified, the question of the functional redundancy between Akt isoforms currently remains unclear (13–17). To this effect, the expression and/or

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1 The abbreviations used are: Fak, focal adhesion kinase; Ab, antibody; CASP, caspase; CD, cytochalasin D; CMV, cytomegalovirus; Erk, extracellular signal-regulated kinase; FFC, fluorochrome isothiocyanate; FRNK, Fak-related nonkinase; HA, hemagglutinin; IF, indirect immunofluorescence; ISEL, in situ terminal deoxynucleotidyltransferase–dUTP-mediated nick-end labeling; K18, cyto-keratin 18; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEG, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PC, days post-confluence; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; RHD, lissamine-rhodamine; RT, reverse transcriptase; SI, sucrase-isomaltase; WB, Western blot.

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regulation of activation of Akt isoforms has been reported to vary depending on the external stimulus involved and the tissue studied (13–17, 19). Furthermore, there is mounting evidence that Akt isoforms may perform distinct functions in cellular processes depending on the cell type being analyzed (13–18, 20–23).

The human intestinal epithelium is a useful biological model for the study of the cell dynamics involved in tissue homeostasis. Its continuous renewal essentially consists of the production of enterocytes in the crypts, which differentiate and then migrate toward the apex of the villi in order to enter anoikis (24–28). In addition, proliferative crypt cells may enter "spontaneous" apoptosis, a less frequent process that serves to remove defective or injured undifferentiated progeny cells (24–26). Although both crypt and villus cells are susceptible to anoikis, it is now becoming well established that human intestinal epithelial cell survival and death are subjected to differentiation state-specific control mechanisms (12, 24–27, 29–33).

For instance, undifferentiated/crypt cells exhibit a susceptibility to apoptosis and/or anoikis that is distinct from the differentiated/villus cells as follows: 1) their expression and regulation of cell death regulators/effectors (e.g. Bel-2 homologs and caspases) (27, 29–34); 2) a differential involvement of the PI3K/Akt and MEK/Erk pathways in their survival (30, 31, 34); 3) distinct roles enacted by integrins and Fak in the suppression of anoikis (12, 30, 31, 34); and 4) a differentiation state-selective involvement of p38 stress-activated MAPK isoforms in the induction of apoptosis/anoikis (12, 34). This in turn fits well with the fact that intestinal epithelial cells express differential integrin profiles and interact with specific basement membrane components along the crypt-villus axis, depending on their state of differentiation (35).

Considering that the regulation of human enterocytic cell survival involves distinct mechanisms according to the state of differentiation, the question as to the specific implication of individual Akt isoforms in this process therefore remains open. In the present study, we investigated the expression, regulation, and roles of Akt-1, Akt-2, and Akt-3 in the suppression of human intestinal epithelial cell anoikis within the context of undifferentiated versus differentiated cells. By using several approaches, we provide evidence that the expression and regulation of Akt isoforms by cell adhesion and cell adhesion signaling show differentiation state-specific distinctions in human intestinal epithelial cells. Furthermore, we identify the β1 integrin/Fak/PI3K/Akt-1 signaling pathway as a crucial determinant in the suppression of enterocytic anoikis, whereas Akt-2 is independent of PI3K and does not play a role in enterocytic cell survival or death.

**EXPERIMENTAL PROCEDURES**

**Materials**—The antibodies used are as follows: mAb4.47 (Upstate Biotechnology, Inc., Lake Placid, NY) directed against p125Akt-1, Ab07-012 (Upstate Biotechnology, Inc.) directed to the phosphotyrosine 397-activated form of p125Akt (p Tyr397)/p125Akt-1; Ab07-372 (Upstate Biotechnology, Inc.) and Ab1240002 (Calbiochem), both directed against p57Akt-1; α-Akt (Research Diagnostics, Flanders, NJ), directed to phosphoserine 473-activated form of p125Akt-2; Ab9271 (Cell Signaling Technology, Inc.), directed to p57 Akt isoform /K18, Ab9271 (Cell Signaling Technology, Beverly, MA), directed to the phosphotyrosine 473-activated form of p57Akt-1 (p Tyr473)/p57Akt-1; Ab07-372 (Upstate Biotechnology, Inc.) and Ab1240002 (Calbiochem), both directed against p57Akt-1; Ab5b5 (Research Diagnostics, Flanders, NJ), directed to phosphoserine residues; mAbM5 (Sigma), directed against human K18. Anti-epitope tag sequences were provided by the manufacturer’s instructions. Band intensities were scanned by using an Astra 1200S scanner (Umax Technologies, Fremont, CA) and semi-quantified using an Alpha Imager 1200 Documentation and Analysis system (Alpha Innotech, San Leandro, CA), in order to establish the ratios of Akt isoforms by cell adhesion and cell adhesion signaling as described previously (10–12, 30, 31, 34, 47).

**RNA Purification and Reverse Transcription (RT)-PCR**—Total RNA extraction and subsequent RT-PCR were carried out as described previously (12, 38, 46, 48). Amplification products were resolved on agarose gels (1.5%) and stained with ethidium bromide. A 100-bp ladder was used as standard (New England Biolabs). Primers for the amplification of human sucrase-isomaltase (SI), Akt-1, Akt-2, and Akt-3 have been published previously (19, 38, 42, 50, 51) and were purchased from Invitrogen. Primers for human S14 (Invitrogen) were also used. Rel. expression levels of Akt isoforms and SI mRNAs were determined by comparison with S14 mRNA as a reference. Band intensities of amplified fragments were scanned and semi-quantified using an Alpha Imager 1200 Documentation and Analysis system (Alpha Innotech, San Leandro, CA), in order to establish the ratios “Akt isoform/S14” and “S14.”

**Western Blotting (WB) and Protein Expression**—Cell cultures were lysed in sample buffer (2.3% SDS, 10% glycerol, and 0.001% bromphenol blue) to a final protein concentration of 2 mg/ml. Proteins were resolved by SDS-PAGE on 4–12% polyacrylamide gels (1.5%) and stained with Coomassie blue in 62.5 mM Tris-HCl (pH 6.8) and processed as described previously (12, 30, 34). Total proteins (50 μg/well) were resolved by SDS-PAGE on 4–15% gels (Bio-Rad), electrotransferred onto nitrocellulose membranes (Hybond-ECL; Amersham Biosciences), and probed for S14, Akt isoforms, and SI, as described previously (12, 30, 31, 34). Broad range molecular mass markers (Bio-Rad) were used as standards. Immunoreactive bands were visualized by the enhanced chemiluminescence method (Amersham Biosciences), according to the manufacturer’s instructions. Band intensities were scanned by using an Astra 1200S scanner (Umax Technologies, Fremont, CA) and semi-quantified with Scion Image (Scion, Frederick, MD). K18 was used as a control as an internal standard, in order to establish the ratios “p125Akt-1/K18,” “p57Akt isoform/K18,” “p57Akt isoform/K18,” and “p220/K18” as measures of both steady-state expression levels (12, 30, 31, 34).

**Immunoprecipitation (IP) and Relative Kinase Activation Assays**—Cell cultures were lysed in cold IP buffer (50 mM Tris-HCl (pH 7.2), 1 mM dithiothreitol, 0.5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxy-
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cholate, 0.1% SDS, 100 μM NaVO₃, 1 mM phenylmethylsulfonyl fluoride, 10 mM p-nitrophenylphosphate, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotonin, 0.7 μg/ml peptatin, 40 μg β-glycerophosphate, and 10 mM Na₃P0₄) and processed as described previously (10, 12, 29, 33, 47). Immunoprecipitation of Fak, Akt isoforms was carried out according to the protocol already described (10, 12, 30, 34, 48). Immunoprecipitates were solubilized in sample buffer, resolved by SDS-PAGE, and probed by WB (see above). Relative kinase activation assays were performed as described previously (10, 12, 30, 34, 47). These essentially consisted of probing for the phosphorylated (active) form of each kinase analyzed (Fak, pstry397/p125Fak; Akt-1, pser473/p57Akt-1; and Akt-2, pser473/p57Akt-2), followed by reprobing for each of the immunoprecipitated ("total") kinases and then semi-quantification of the immunoreactive bands (see above) in order to establish the ratios of the phosphorylated and total forms (48). Consequently, nonapoptotic cell culture conditions often produce near-empty lanes on the gel as a result. For the in situ detection of DNA ladder, treated (see above) or transfected (see below) coverslip-grown cells were washed twice in cold phosphate-buffered saline (PBS) and fixed in 2% formaldehyde/PBS for 30 min at 4 °C. Following permeabilization with 0.1% Triton X-100 in PBS for 5 min, free aldehyde groups were quenched with 100 mM glycine/PBS (pH 7.4). ISEL was then carried out as described previously (10, 12, 27, 30, 31, 34, 46, 48), using the fluorescein isothiocyanate (FITC)-ApopTag apoptosis detection kit (Intergen, Purchase, NY). Negative staining controls included omission of the terminal deoxyribonucleotidyltransferase enzyme in all experiments performed. Preparations were viewed with a Light Microscopy (Leica) microscope (Leica, Inc., Buffalo Grove, IL), and evaluation of ISEL-positive cells was performed as described previously (12, 27, 30, 31, 34, 46, 48). Apoptotic indices were expressed as the percentage (%) of apoptotic (ISEL-positive) cells over the total number of cells counted (n ≥ 300 cells). Alternatively, counts were compared with those of control cultures, ×100 (expressed as % of control).

Transfections and cDNA Constructs—Coverslip-grown cell cultures were transfected transiently as described previously (10–12, 30, 46, 48), using LipofectAMINE 2000 (Invitrogen). Cultures were switched to serum-free medium following transfection and were thereafter maintained as such for a duration of 24 h, before being processed for ISEL (see above) or IP double-labeling (see below). The cDNA constructs used were as follows: myc-C. elegans wild type human Fak (52); myc-FakY397F, coding for a Myc-tagged, nonactivatable, dominant-negative mutant of human Fak (52); myc-Fak-related kinase (FRNK; p57FRNK), coding for a Myc-tagged, dominant-negative, kinase domain-lacking isoform of human Fak (52); Flag-Akt-IWT, coding for Flag-tagged wild type human Akt-1 (18); Flag-Akt-IDN, coding for a Flag-tagged, dominant-negative, kinase-dead mutant of human Akt-1 (18); hemagglutinin (HA)-Akt-2W, coding for HA-tagged wild type human Akt-2 (20, 23); HA-myAkt-2, coding for an HA-tagged, constitutive-active mutant of human Akt-2 (20, 23); and HA-Akt-2DN, coding for an HA-tagged, dominant-negative, kinase-dead mutant of human Akt-2 (20, 23). Fak constructs were the generous gifts from J. T. Parsons (Health Science Center, University of Virginia, Charlottesville, VA). Mammalian expression vectors used were pCMV for Fak constructs, pCMV-Akt, pCMV-Flag-Akt-1 constructs, and pCDNA3 for Akt-2 constructs. In any event, transfection controls ("sham-transfected") consisted of transfections of the expression vectors without cDNA inserts. Indirect Immunofluorescence Double Labeling—ISEL was performed on transfected coverslip-grown cells (see above) was immediately followed by incu- bation (45 min, room temperature) with an anti-Flag (for Akt-1 constructs) or anti-HA (for Akt-2 constructs) primary antibody diluted in 10% powdered skim milk/PBS (pH 7.4). After washing with PBS, a secondary antibody coupled to lissamine-rhodamine (RHD) was likewise incubated. Preparations were then washed, mounted, and viewed with a Leica DMRB microscope (Leica). Negative staining controls included incubation without primary antibody in all experiments performed. Transfected (RHD-stained/FLAG-or HA-positive) cells that were induced to enter apoptosis (FITC-stained/ISEL-positive) as a consequence show up in shades of yellow when the RHD and FITC stainings are overlaid.

Data Processing—Results and values shown represent the mean ± S.E. for at least three (n ≥ 3) separate experiments and/or cultures. Statistically significant differences were determined with the Student’s t test. Data were compiled, analyzed, and processed with Excel (Microsoft, Redmond, WA) and Cricket Graph (Computer Associates, Islandia, NY). Unless specified otherwise, images from blots, gels, and scans were processed with Vistascan (Umax), Photoshop (Adobe, San Jose, CA), and PowerPoint (Microsoft).

RESULTS

Establishment of Distinct Expression/Activation Profiles of Akt Isoforms during Human Enterocytic Differentiation—We first analyzed the mRNA and protein expression levels of Akt-1, -2, and -3 by RT-PCR and Western blot, in relation to the human enterocytic differentiation process. Undifferentiated Caco-2/15 cells undergo a gradual differentiation process that takes place spontaneously once confluence (0 PC) has been reached and that is completed after 25–30 PC (36, 37, 41). This is well evidenced by a sharp drop of proliferation at confluence (not shown; see Refs. 36–38, 40–42, and 73) coincident with the post-confluent appearance and/or gradual increase in the expression of brush border membrane hydrolases such as SI (Fig. 1B, filled columns; Fig. 1D, dark-gray columns), lactase-phlorizin hydrolase, aminopeptidase N, and dipetidylpeptidase IV (not shown; see Refs. 36–38, 40–42, and 73).

The relative levels of Akt-1 mRNA were found to decrease overall throughout the enterocytic differentiation process (Fig. 1, A, Akt-1, and B, gray columns). However, such patterns of Akt-1 mRNA expression did not reflect the relative expression levels of the protein; to this effect, Akt-1 steady-state protein levels remained more or less stable throughout the enterocytic differentiation process (Fig. 1, C, p57Akt-1, and D, gray columns). Akt-2 mRNA levels were also found to decrease overall as a function of the state of differentiation (Fig. 1, A, Akt-2, and B, open columns). Furthermore, and in contrast to Akt-1, such patterns of Akt-2 mRNA expression reflected the relative expression levels of the protein (Fig. 1, C, p57Akt-2, and D, open columns). Finally, Akt-3 mRNA was not detected by RT-PCR in Caco-2/15 cells, regardless of their state of differentiation (data not shown).

It is well acknowledged that the expression of a kinase is not necessarily a reflection of its activation/activity levels (3, 8, 9, 14, 17). Fak is a good example of this in human intestinal epithelial cells, and although its protein expression levels increase according to the state of differentiation (Fig. 1, C, p125Fak, and D, filled columns; see also Refs. 12, 28, and 51), its relative activation levels (as assessed by phosphorylation on its tyrosine 397 residue) are nonetheless significantly lower in differentiated cells than in undifferentiated ones (Fig. 2, B, IP: Fak, and C, gray columns). Therefore, we investigated whether human intestinal epithelial cells also display variations of activation levels of Akt-1 and Akt-2 in relation to their state of differentiation. In contrast to Fak, the relative activation levels of Akt-1 (as assessed by phosphorylation on its serine 473 residue) were found significantly higher in differentiated cells than in undifferentiated ones (Fig. 2, B, IP: Akt-1, and C, open columns). On the other hand, and similarly to Fak, the activation levels of Akt-2 (as assessed by phosphorylation on its serine residues) were significantly higher in undifferentiated cells than in their differentiated counterparts (Fig. 2, B, IP: Akt-2, and C, filled columns). Altogether, these results indicate that human intestinal epithelial cells display differentiation state-specific profiles of expression and activation of Fak, Akt-1, and Akt-2.

Distinct Regulation of Akt-1 and Akt-2 Activation within the Context of Human Intestinal Epithelial Apoptosis/ Anotikis—We then analyzed the regulation of activation of Akt-1 and Akt-2 in intestinal epithelial cells, specifically within

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Fig. 1. Expression of Akt isoforms, SI, and Fak during human enterocytic differentiation. A, representative RT-PCR analyses of the expression of Akt-1 and Akt-2 mRNA in Caco-2/15 cells at 2 (subconfluent, undifferentiated; lane 1), 0 (confluent; lane 2), 5 (lane 3), 10 (lane 4), 20 (lane 5), and 30 (differentiated; lane 6) PC. Total RNA was extracted and reversed-transcribed, and then 20 cycles of amplification were performed using primers specific for human Akt-1/PKBα and Akt-2/PKBβ. S14 mRNA expression was likewise analyzed for normalization purposes. L, 100-bp DNA size markers. B, same as in A except that the mRNA expression of SI, an enterocytic differentiation marker, was also analyzed. Furthermore, amplified bands were semi-quantified, and the relative levels of Akt-1 (grey columns), Akt-2 (open columns), and SI (filled columns) were determined as ratios relative to S14. C, representative WB analyses of the expression of p57Akt-1, p57Akt-2, and p125Fak in Caco-2/15 cells at the same time points as in A. Total proteins (50 μg/well) were separated by SDS-PAGE under reducing conditions, electrotransferred onto nitrocellulose membranes, and then probed with specific antibodies for the detection of Akt-1, Akt-2, and Fak. Detection of K18 was likewise performed using specific antibodies. D, same as in C except that the expression of p220Fak was also analyzed. Furthermore, amplified bands were semi-quantified, and the relative levels of Akt-1 (grey columns), Akt-2 (open columns), Fak (filled columns), and SI (dark-grey columns) were determined as ratios relative to K18. B and D, statistically significant (0.001 ≤ p ≤ 0.01) differences between -2 (Undifferentiated) and 30 (Differentiated) PC Caco-2/15 cells are indicated by *.

the context of loss of cell adhesion signaling. In contrast to adherent/control cultures (Fig. 3, A, lanes 1 and 5, and B, lanes 1, 2, 4, and 5), apoptosis/anoksis-associated internucleosomal DNA laddering was observed in both undifferentiated (-2 PC) and differentiated (30 PC) cells when Fak was inhibited with CD (Fig. 3A, lanes 2 and 6), when PI3K activity was inhibited with Ly294002 (Fig. 3A, lanes 3 and 7), when β1 integrin binding activity was blocked with the P4C10 antibody (Fig. 3B, lanes 3 and 6), or when cells were kept in suspension (Fig. 3A, lanes 4 and 8). Likewise, elevated apoptotic indices (Fig. 3C), as well as high cleavage/activation of CASP-3 (see Fig. 3D as example) and CASP-7 (not shown), were noted when undifferentiated and differentiated cultures were exposed to the same treatments, as opposed to controls.

To confirm the efficiency of the treatments, the relative activation levels of Fak were first analyzed (Fig. 4). As expected (12, 30, 31, 34), the pharmacological inhibition of PI3K did not impact on Fak activation (Fig. 4A, lanes 5 versus 1 and 6 versus 2; Fig. 4B, Cytochalasin D), the inhibition of β1 integrins (Fig. 5B, P4C10), and/or forced cell suspension (Fig. 5A, lanes 7 versus 1 and 8 versus 2; Fig. 5B, Suspension). As in the case of Fak (see above), poor amounts of Akt-1 were immunoprecipitated from cells undergoing anoksis (Fig. 5A, lanes 7 and 8). Indeed, Akt-1 is also a choice target of caspases (see above) when true anoksis is induced (6, 7, 12, 18). Also as expected, the inhibition of PI3K impacted negatively on Akt-1 activation (Fig. 5A, lanes 5 versus 1 and 6 versus 2; Fig. 5B, Ly294002). Therefore, these results indicate that Fak activation is primarily dependent on β1 integrin-mediated cell adhesion in human intestinal epithelial cells, regardless of their state of differentiation (12, 30, 31, 34, 53, 55).

We then analyzed the impact of the treatments on Akt-1 activation (Fig. 5). In both undifferentiated and differentiated cultures, significant drops of Akt-1 activated levels were noted following the inhibition of Fak (Fig. 5A, lanes 3 versus 1 and 4 versus 2; Fig. 5B, Cytochalasin D), the inhibition of β1 integrins (Fig. 5B, P4C10), and/or forced cell suspension (Fig. 5A, lanes 7 versus 1 and 8 versus 2; Fig. 5B, Suspension). As in the case of Fak (see above), poor amounts of Akt-1 were immunoprecipitated from cells undergoing anoksis (Fig. 5A, lanes 7 and 8). Indeed, Akt-1 is also a choice target of caspases (see above) when true anoksis is induced (6, 7, 12, 18). Also as expected, the inhibition of PI3K impacted negatively on Akt-1 activation (Fig. 5A, lanes 5 versus 1 and 6 versus 2; Fig. 5B, Ly294002). Therefore, these results indicate that Akt-1 activation is dependent on cell adhesion/β1 integrins, Fak, and PI3K in human intestinal epithelial cells, regardless of the state of differentiation.

In contrast to Akt-1, the regulation of Akt-2 activation was found to be more complex (Fig. 6). The inhibition of PI3K failed to affect significantly the relative activated levels of Akt-2 in both undifferentiated and differentiated cells (Fig. 6A, lanes 5
versus 1 and 7 versus 1; Fig. 6B, −2 PC: Cytochalasin D and Suspension) but was not affected significantly in differentiated cells following these same treatments (Fig. 6A, lanes 4 versus 2 and 8 versus 2; Fig. 6B, 30 PC: Cytochalasin D and Suspension). It is also noteworthy that the amounts of Akt-2 immunoprecipitated from cells undergoing true anoikis were barely affected as compared with those from control cultures (Fig. 6C, lanes 7 versus 1 and 8 versus 2), in sharp contrast to Fak and Akt-1 (see above). In any event, these results show that Akt-2 activation is dependent on cell adhesion/β1 integrins and Fak in undifferentiated human intestinal epithelial cells only. Furthermore, these indicate that Akt-2 activation is not dependent on PI3K in human intestinal epithelial cells, regardless of their state of differentiation. Consequently, these data altogether show that the activated levels of Akt-1 and Akt-2 are distinctively regulated by cell adhesion/β1 integrins, Fak, and PI3K in human enterocytes.

Distinct Roles for Akt-1 and Akt-2 in the Survival of Human Intestinal Epithelial Cells—To verify whether Akt-1 and/or Akt-2 contribute in the promotion of human intestinal epithelial cell survival, Caco-2/15 cells were transiently transfected with wild type or mutant cDNA constructs of these kinases and assayed for apoptosis in comparison to control cells (i.e. sham-transfected or transfected with an empty vector). Fak cDNA constructs were also tested. The forced expression of the non-kinase, dominant-negative Fak isoform p45FRNK significantly induced anoikis, as opposed to cells transfected with wild type Fak and/or control cells (Fig. 7, pCMV-myc-p45FRNK versus pCMV-myc-FakWT). Likewise, the forced expression of the nonactivable, dominant-negative mutant FakY397F also induced anoikis in a significant manner (Fig. 7, pCMV-myc-FakY397F versus pCMV-myc-FakWT). Hence, these data confirm that Fak is crucial for the survival of human intestinal epithelial cells (12, 30, 31, 34).

As in the case of Fak, Akt-1 was identified as a major promoter of the suppression of apoptosis/anoikis in human intestinal epithelial cells. Indeed, the forced expression of a kinase-dead, dominant-negative mutant of Akt-1 significantly induced apoptosis, as opposed to cells transfected with wild type Akt-1 and/or control cells (Fig. 7, pCMV-2-Flag-Akt-1DN versus pCMV-2-Flag-Akt-1WT). Double IF studies were then performed on cultures transfected with either the Flag-Akt-1WT or Flag-Akt-1DN constructs, in order to verify any correlation between apoptotic (i.e. ISEL-positive, in green) and construct-expressing (i.e. FLAG-positive, in red) cells (Fig. 8). For cells transfected with the Flag-Akt-1WT construct (Fig. 8, A–C), we found that the ISEL-positive (Fig. 8A) and FLAG-positive (Fig. 8B) stainings correlated poorly when overlaid (Fig. 8C). On the other hand, for cells transfected with the Flag-Akt-1DN construct (Fig. 8, D–F), the ISEL-positive (Fig. 8D) and FLAG-positive (Fig. 8E) stainings exhibited a near-perfect correlation when overlaid (Fig. 8F). Therefore, these results demonstrate a role for Akt-1 in the survival of human enterocytes.

In sharp contrast to Akt-1, Akt-2 was not found to play a role in human enterocytic cell survival. Indeed, the forced expression of a kinase-dead, dominant-negative mutant of Akt-2 did not induce apoptosis, as compared with cells transfected with wild type Akt-2 and/or control cells (Fig. 7, pCDNA3-HA-Akt-2DN versus pCDNA3-HA-Akt-2WT). Likewise, the forced expression of a constitutive-active mutant of Akt-2 had no significant effect on cell survival/death (Fig. 7, pCDNA3-HA-myraAkt-2 versus pCDNA3-HA-Akt-2WT). Double IF studies were also performed on cultures transfected with either the HA-Akt-2WT, HA-Akt-2DN, or HA-myraAkt-2 constructs, in order to verify any lack of correlation between apoptotic (i.e. ISEL-positive) and construct-expressing (i.e. HA-
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We investigated the question whether the three known Akt isoforms (Akt-1, Akt-2, and Akt-3) perform distinct roles in the regulation of survival and apoptosis/anoikis in human intestinal epithelial cells. The roles of \( \beta_1 \) integrins/Fak and PI3K in human enterocytic cell survival were also analyzed in order to verify any linkage between cell adhesion, PI3K, and the activation of Akt isoforms. Here we report the following. (i) Human enterocytes display differentiation state-distinct profiles of expression and/or activation of Akt-1 and Akt-2, whereas the Akt-3 isoform is not expressed. (ii) Regardless of the state of enterocytic differentiation, Akt-1 activation is dependent on PI3K, whereas Akt-2 activation is not. (iii) Akt-1 activation is dependent on cell adhesion/\( \beta_1 \) integrins and Fak, regardless of the state of enterocytic differentiation. (iv) Akt-2 activation is dependent on cell adhesion/\( \beta_1 \) integrins and Fak in undifferentiated cells only. (v) As in the case of Akt-1, Akt-3 is not expressed in human enterocytes. (vi) Akt-2 is not essential for survival nor is it involved in the induction of apoptosis/anoikis. (vii) Akt-2 cannot substitute for Akt-1 in the maintenance of intestinal epithelial cell survival. Altogether, these data indicate that the expression and regulation of Akt isoforms show both isoform-specific and differentiation state-specific distinctions in human enterocytic cells.

**DISCUSSION**

Here we investigated the question whether the three known Akt isoforms (Akt-1, Akt-2, and Akt-3) perform distinct roles in the regulation of survival and apoptosis/anoikis in human intestinal epithelial cells. The roles of \( \beta_1 \) integrins/Fak and PI3K in human enterocytic cell survival were also analyzed in order to verify any linkage between cell adhesion, PI3K, and the activation of Akt isoforms. Here we report the following. (i) Human enterocytes display differentiation state-distinct profiles of expression and/or activation of Akt-1 and Akt-2, whereas the Akt-3 isoform is not expressed. (ii) Regardless of the state of enterocytic differentiation, Akt-1 activation is dependent on PI3K, whereas Akt-2 activation is not. (iii) Akt-1 activation is dependent on cell adhesion/\( \beta_1 \) integrins and Fak, regardless of the state of enterocytic differentiation. (iv) Akt-2 activation is dependent on cell adhesion/\( \beta_1 \) integrins and Fak in undifferentiated cells only. (v) As in the case of Akt-1, Akt-3 is not expressed in human enterocytes. (vi) Akt-2 is not essential for survival nor is it involved in the induction of apoptosis/anoikis. (vii) Akt-2 cannot substitute for Akt-1 in the maintenance of intestinal epithelial cell survival. Altogether, these data indicate that the expression and regulation of Akt isoforms show both isoform-specific and differentiation state-specific distinctions in human enterocytic cells.

**DISCUSSION**

Here we investigated the question whether the three known Akt isoforms (Akt-1, Akt-2, and Akt-3) perform distinct roles in the regulation of survival and apoptosis/anoikis in human intestinal epithelial cells. The roles of \( \beta_1 \) integrins/Fak and PI3K in human enterocytic cell survival were also analyzed in order to verify any linkage between cell adhesion, PI3K, and the activation of Akt isoforms. Here we report the following. (i) Human enterocytes display differentiation state-distinct profiles of expression and/or activation of Akt-1 and Akt-2, whereas the Akt-3 isoform is not expressed. (ii) Regardless of the state of enterocytic differentiation, Akt-1 activation is dependent on PI3K, whereas Akt-2 activation is not. (iii) Akt-1 activation is dependent on cell adhesion/\( \beta_1 \) integrins and Fak, regardless of the state of enterocytic differentiation. (iv) Akt-2 activation is dependent on cell adhesion/\( \beta_1 \) integrins and Fak in undifferentiated cells only. (v) As in the case of Akt-1, Akt-3 is not expressed in human enterocytes. (vi) Akt-2 is not essential for survival nor is it involved in the induction of apoptosis/anoikis. (vii) Akt-2 cannot substitute for Akt-1 in the maintenance of intestinal epithelial cell survival. Altogether, these data indicate that the expression and regulation of Akt isoforms show both isoform-specific and differentiation state-specific distinctions in human enterocytic cells.
intestinal epithelial cells (Fig. 9). Furthermore, we demonstrate that the β1 integrin/Fak/PI3K/Akt-1 signaling pathway is a crucial determinant in the suppression of human enterocytic apoptosis/anooikis, whereas Akt-2 does not intervene in the survival or death of human enterocytes (Fig. 9).

Restrictions and Distinctions in the Expression/Activation of Akt Isoforms in Human Intestinal Epithelial Cells—Although Akt isoforms are widely expressed, and often overexpressed in cancers (13–17, 19, 23, 50, 51), Akt-3 is recognized as being more restricted in its expression patterns than Akt-1 and Akt-2 (13–17, 19, 56). This is also the case for human intestinal epithelial cells, because Akt-3 is not detected in such cells regardless of their state of differentiation. However, previous studies (19, 56) have shown the presence of Akt-3 mRNA in the human small intestinal and colonic mucosae, as well as in colon cancers. This apparent discrepancy is explained by the fact that whole-tissue extracts were used in these studies and therefore included not only intestinal epithelial cells as a source of biological material but also cells from the underlying stromal tissue. To this effect, Akt-3 mRNA is indeed detected in pure cultures of human intestinal mesenchymal cells and yet absent in pure cultures of human crypt or villus cells, thus confirming a lack of Akt-3 expression in intestinal epithelial cells.

Aside from such restriction in the expression of Akt isoforms in human enterocytes, our study also showed that the specific expression and/or activation profiles of Akt-1 and Akt-2 are distinct according to the state of enterocytic differentiation. Indeed, the activation of Akt-1 increases with differentiation whereas that of Akt-2 decreases sharply. It is noteworthy that these expression/activation profiles of Akt-1 and Akt-2 are somewhat complementary in relation to the state of differentiation and corroborate our previous findings concerning the expression/activation of Akt in human enterocytes, using nonisofrom-specific antibodies (30). It is germane that differentiation state-distinct profiles of expression and activation of Akt isoforms have been observed as well in skeletal muscle cells, although not in similar patterns as we noted here for intestinal epithelial cells. Specifically, Akt-1 expression/activation remains more or less stable during myogenic differentiation, whereas that of Akt-2 gradually increases to even surpass that of Akt-1 (57, 58). Consequently, it is now becoming

\* P. H. Vachon, G. Dufour, and A. B. Dydensborg, unpublished data.
increasingly evident that the expression/activation of Akt isoforms may not only vary from one tissue type to the next but may also show distinct profiles according to the state of differentiation within the same given cell type.

**Distinctions in the Regulation of Akt-1 and Akt-2 Activation by PI3K and β1 Integrins/Fak in Human Intestinal Epithelial Cells**—In addition to differences in the expression/activation profiles between Akt isoforms, there is now mounting evidence that the regulation proper of their activation may also involve isoform-specific mechanisms (13–17). As an example, one Akt isoform and two or all three known isoforms may be stimulated alone or simultaneously by epidermal growth factor or insulin, depending on the cell type being analyzed (13–17, 19, 59–61). Furthermore, several studies (14, 17, 62, 63, 74) have reported that Akt isoforms may be independent of PI3K for their activation, again depending on the tissue or cell type context being studied. To this effect, our observation that the activation of Akt-1, but not that of Akt-2, is dependent on PI3K in human intestinal epithelial cells not only provides another example of PI3K independence for an Akt isoform (Fig. 9) but also corroborates our previous findings (30, 31) concerning the apparent PI3K independence of Akt in enterocytes by using nonisoform-specific antibodies. More surprising was our finding that β1 integrins/Fak regulate Akt-1 activation regardless of the state of enterocytic differentiation, whereas such β1 integrins/Fak dependence for Akt-2 activation occurred in undifferentiated enterocytes only (Fig. 9). Much remains to be understood of the mechanisms implicated in the regulation of the activation of Akt isoforms. In addition to other PI3K effectors such as 3-phosphoinositide-dependent kinase 1 or integrin-linked kinase, several signaling molecules have been identified as being susceptible to also influence Akt activation (13–17, 42, 64, 65). To this effect, such additional players are thought to be potentially responsible for the isoform-selective and/or cell type-specific differences in the regulation of Akt activation (13–17). In addition, several cell types such as intestinal epithelial cells are known to express differentiation state-distinct profiles of integrins, which in turn are thought to provide yet another level of complexity and specificity in the regulation of the activation of signaling pathways (4–6, 30, 34–35, 38, 40–41, 46, 66, 67). Consequently, further studies will be required to identify which integrin receptors and upstream kinases selectively contribute to the differentiation state-distinct stimulation of Akt isoforms in human enterocytes. Nonetheless, our data herein provide a striking example of isoform-selective, as well as differentiation state-specific, distinctions in the regulation of the expression and activation of Akt-1 and Akt-2.

**Selective Roles of Akt-1 and Akt-2 in Human Enterocyte Cell Survival and Anoikis**—Akt isoforms have been found to perform diverse functions in the control of various cellular processes such as cell proliferation, differentiation, and survival (13–17, 68). There is now mounting evidence that this diversity of function may be defined by the cell type and differentiation state contexts in which stimuli influence Akt activation. For example, Akt-1 drives the proliferation of myoblasts but hinders myogenic differentiation and does not play a role in the...
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Fig. 8. Akt-1 is crucial for the survival of human enterocytes. Representative indirect IF double-labeling color micrographs of subconfluent Caco-2/15 cells transfected with either wild type Akt-1 (pCMV-2-Flag-Akt-1WT, A–C) or a dominant-negative, kinase-dead mutant of Akt-1 (pCMV-2-Flag-Akt-1DN, D–F). After 24 h in serum-free medium following transfection, ISEL was performed and immediately followed by an incubation with an anti-FLAG antibody. The ISEL reaction was visualized using a filter for FITC (green, A and D), whereas the staining for FLAG was visualized with a filter for RHD (red, B and E). When the corresponding images are overlaid (yellow, F), the transfected (FLAG-positive) cells that were induced to enter apoptosis (ISEL-positive) show up in shades of yellow. Bar equals 25 μm.

Fig. 9. Distinct roles and regulation of Akt isoforms in human enterocytic cell survival and apoptosis/anoikis. Schematic drawing of an undifferentiated, proliferating enterocyte (left) and its nonproliferating, differentiated counterpart (right), illustrating how integrin-mediated cell adhesion can stimulate the Fak/PI3K/Akt-1 pathway to promote cell survival and suppression of enterocytic apoptosis/anoikis, whereas Akt-2 does not play a role in either the survival or death of enterocytes. The drawing also summarizes the main results of the present study concerning the differentiation state distinctions in the regulation of the activation of Fak, Akt-1, and/or Akt-2 by cell adhesion, as well as the independence of Akt-2 activation with regard to PI3K. The low levels of expression and activation of Akt-2 in differentiated enterocytes is shown by parentheses. Finally, note that Akt-3 is not expressed by enterocytes, regardless of their state of differentiation.

In conclusion, this study has provided evidence that Akt-1 is crucial for the survival of human enterocytes, regardless of their state of differentiation. Akt-2 plays a major role in driving myogenic differentiation and promoting survival during myoblast fusion but does not play a role in myoblast proliferation (21, 22, 58). In addition, recent studies using single and double gene knock-out mice have revealed that Akt isoforms can at best compensate partially for each other and therefore perform specific, crucial roles depending on the tissue type (68–72). To this effect, our demonstration that Akt-1 is crucial for the survival of human intestinal epithelial cells, whereas Akt-2 does not play a role in either cell survival or induction of apoptosis/anoikis (Fig. 9), therefore provides a novel instance whereby the functions of Akt isoforms within a given cell type are not redundant and cannot be compensated/substituted by one or another. Given this, our study nonetheless raises the question as to what roles Akt-2 may play in human intestinal epithelial cellular processes, other than promoting survival or apoptosis/anoikis.

In conclusion, this study has provided evidence that the expression and regulation of Akt isoforms by cell adhesion and cell adhesion signaling show differentiation state-specific distinctions in human intestinal epithelial cells. Furthermore, we identify the β1 integrin/Fak/PI3K/Akt-1 signaling pathway as a crucial determinant in the suppression of enterocytic anoikis, whereas Akt-2 is independent of PI3K and does not play a role in enterocytic cell survival or death. However, the exact molecular processes responsible for such differentiation state-distinct controls on the roles and regulation of Akt-1 and Akt-2 remain to be understood. For example, the question is open as to why Akt-2 does not participate in the regulation of enterocytic cell survival, although Akt-1 does. Likewise, and considering that human intestinal epithelial cell survival and death are subjected to differentiation state-distinct control mechanisms (this study and see Refs. 12, 24–27, and 29–34), the survival-promoting functions specifically enacted by Akt-1 remain to be fully elucidated in both undifferentiated and differentiated cells. Such further studies, in addition to the present findings, will provide a greater insight into the complex, synchronized governance of cell survival and death that is required for the maintenance and renewal of tissues such as the intestinal epithelium.

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