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Regulation and role of brush border-associated ERK1/2 in intestinal epithelial cells

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Abstract

We have recently shown that elevated ERK activities stimulate proliferation of intestinal cells whereas low sustained levels of ERK activities correlate with Gl arrest and are required for expression of several enterocyte differentiation proteins. In an attempt to clarify how ERK1/2 regulates intestinal differentiation, the present study assessed the subcellular distribution and regulation of ERK proteins and activities in differentiated enterocytes. We report that (1) ERK1/2 and their upstream modulators Ras, p85 (PI-3K), Rac1, and MEK1 are found in the brush border; (2) brush border-associated ERK1/2 are stimulated by EGF and feeding; (3) immunoblotting of proteins phosphorylated on SP/K motif suggests the presence of ERK substrates in the brush border, one of which could be actin; and (4) pharmacological inhibition of ERK alters microvilli architecture. Our results suggest that ERK may play important roles in the control of microvilli structure and possibly, in brush border-associated responses in differentiated intestinal epithelial cells.

Keywords: ERK; Brush border; Junctions; Intestinal epithelial cell differentiation

Adult small intestinal enterocytes are highly polarized columnar cells with structural components adapted to the digestive function. These epithelial cells are endowed with a highly ordered apical brush border specifically designed for providing the enterocyte with maximal absorptive capacity for nutrient assimilation. Each of the 1000–2000 microvilli of a fully mature brush border is supported by a bundle of actin filaments, the basal end of which descends into the apical cytoplasm forming a rootlet approximately 1 μM below the membrane. Here, a dense and complex meshwork of filaments in this so-called terminal web region interdigitates between the rootlets serving most likely to stabilize the overall brush border architecture while conferring a contractile capacity, modulating transepithelial resistance and nutrient transport [1,2].

Intestinal epithelial cells are continuously exposed to various stimuli such as bacteria and associated products (endotoxins), dietary components, luminal growth factors, and inflammatory mediators (cytokines). Enterocytes react to these stimuli by rapidly adjusting the microvillus length, and thus their absorptive capacity, in response to changing needs incurred by gut activity during digestion and absorption of various dietary nutrients [3–5].

The response of intracellular signaling pathways within the intestine to septic insult has been studied extensively. Binding of endotoxin, growth factors or cytokines to membrane receptors activates a series of intracellular signaling cascades, including families of several PKC isozymes and MAP kinases such as extracellular signal-regulated kinase (ERK), Jun amino-terminal kinase (JNK), and p38, ultimately resulting in various gene expression and biological responses [5]. These mechanisms are designed to allow the enterocyte to maintain intestinal homeostasis.

To achieve the specificity of each signaling pathway, cells often employ compartmentalization. Information as to the compartmentalization of specific signaling on

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microvilli of intestinal epithelial cells remains limited. Phosphatidylinositol 3-kinase (PI-3K) [4], cPLA2 [6], and myosin light chain kinase (MLCK) [2,7] have been reported to be localized at the apical surface or within the brush border of enterocytes. In the present study, we demonstrate that the ERK1/2 are localized in the brush border and may act locally to modulate microvilli architecture and brush border-associated function in human enterocytes.

Materials and methods

Materials. Monoclonal antibody HIS-14 against sucrase-isomaltase was provided by Dr A. Quaroni (Cornell University, Ithaca, NY, USA). Antibody recognizing the Na^+/K^+ ATPase pump was a kind gift from Dr. D.M. Fambrough (Johns Hopkins University, Baltimore, MD, USA). Dr. J. Pouysségur (University of Nice, France) provided the rabbit antibody that specifically recognizes MEK1. Antibodies raised against villin, E-cadherin, Rac1, and PKCα were purchased from Transduction Laboratories (Mississauga, Ontario, Canada). Antibody directed against the p85 regulatory subunit of PI-3K was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-AKT and anti-phospho-ERK were purchased from Cell Signaling (Mississauga, Ont., Canada) and anti-ERK1 (C16) was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Antiserum OP40 specifically recognizing p21Ras was from Oncogene Sciences (Calbiochem, Mississauga, Ont., Canada). The antibody specifically recognizing phosphoserine with a proline or lysine at their C-terminal was purchased from Biomol Cedarlane Laboratories (Hornby, Ont., Canada). EGF was from Collaborative Biomedical (Bedford, MA, USA). TNFα, IL1β, and IL6 were purchased from R&D Systems (Minneapolis, MN, USA). All other materials were obtained from Sigma–Aldrich (Oakville, Ont., Canada) unless stated otherwise.

Animals. Sprague–Dawley male rats (170–190 g) were purchased from Charles River Laboratories (St-Constant, QC, Canada). Animals were fed Purina chow and water ad libitum until the start of the experiments and kept in a controlled temperature and light cycle environment (21 °C; 12 h light, 12 h darkness). All studies were conducted in agreement with the principles and procedures outlined in the Canadian Guidelines for Care and Use of Experimental Animals. Eight rats were fasted during 18 h. Thereafter, 4 fasted rats were sacrified by decapitation. Of the remaining 4 fasting rats, 2 were re-fed for 1 h and two others re-fed for 3 h. The animals were then sacrificed and the jejunum was removed for preparation of brush border membrane vesicles (BBMV), as described below.

Human tissues and cell culture. Tissues from human fetuses varying in age from 18 to 20 weeks of gestation (post-fertilization fetal ages estimated according to Streeter [8]) were obtained from normal elective pregnancy terminations. No tissue was collected from cases associated with a known fetal abnormality or fetal death. Studies were approved by the Institutional Human Subject Review Board (University of Sherbrooke, Sherbrooke, QC, Canada). The human adenocarcinoma Caco-2/15 cell line was obtained from A. Quaroni (Cornell University, Ithaca, NY, USA) and cultured as previously described [9,10]. These cells undergo morphological and functional differentiation to an enterocyte phenotype several days after they have reached confluency [9,10].

Preparation of brush border membrane vesicles. The mucosae of rat jejunum and human fetal jejunum were scraped and homogenized (HO) or differentiated Caco-2/15 cells (at least 25 days post-confluent) were lysed (HO) in Tris–Hepes 2 mM containing 50 mM mannitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin. BBMV were prepared by the calcium chloride precipitation method as previously described [11]. $CaCl_2$ was added to a final concentration of 10 mM for 10 min. Centrifugation of the Ca^{2+}-treated homogenate at 5000*g* for 15 min yielded a P1 fraction which contains the total recovered DNA and more than 90% of mitochondrial, microsomal, and basolateral membranes. The P1 fraction was then resuspended in Laemmli's buffer (62.5 mM Tris–HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue, and 1 mM PMSF). The first supernatant (S1) was centrifuged at 30,000*g* for 30 min. The second supernatant (S2) which contains lysosomes and some microsomal membranes and the second pellet (P2) which contains BBMV were then lysed in Laemmli's buffer.

Western blotting. Western blot analyses were performed essentially as described previously [9].

Immunofluorescence. Fully differentiated Caco-2/15 cells (30 days post-confluence) were embedded in optimum cutting temperature compound, and quickly frozen in liquid nitrogen. Frozen sections of 3 μm were spread onto silane-coated glass slides and air-dried. Samples were immunostained for 1 h with primary antibody and 30 min with secondary antibody. Negative controls (no primary antibody) were included in all experiments.

Electron microscopy. Electron microscopy was performed as described previously [10]. All reagents were purchased from Electron Microscopy Sciences (Cedarlane, Hornby, Ont., Canada).

Isolation of cytoskeleton-associated proteins. Cells were first washed twice with ice-cold PBS and then soluble proteins were extracted on ice with cold lysis/cytoskeleton stabilization buffer (0.5% Triton X-100, 50 mM NaCl, 10 mM PIPES, pH 6.8, 300 mM sucrose, and 3 mM $MgCl_2$). Cytoskeleton-associated proteins (insoluble fraction) were harvested by centrifugation (13,000 rpm at 4 °C for 10 min) and solubilized in Laemmli's buffer. Finally, ERK and E-cadherin levels were determined by immunoblot of the cytoskeleton and soluble fractions.

Results

Subcellular distribution of ERK1/2 and other signaling proteins in differentiated enterocytes

In an attempt to analyze the subcellular distribution of ERK proteins in differentiated enterocytes, homogenates from intestinal fetal mucosae and from 25-day post-confluent Caco-2/15 cells were fractionated and brush border vesicles were prepared by Ca^{2+} precipitation, as described previously [11]. As expected, the brush border-enriched fraction (P2) was found to be enriched in villin and sucrase-isomaltase while the P1 fraction contained the bulk of the basolateral membranes as visualized by protein expression of the Na^+/K^+ ATPase pump (Fig. 1A). E-cadherin was distributed throughout the P1 and P2 fractions, consistent with previous observation that E-cadherin is expressed along the lateral membrane of intestinal epithelial cells [10]. As shown in Fig. 1B, expression of ERK1 and ERK2 was detected in all fractions including the brush border-enriched P2 fraction. Using a polyclonal antibody specific for the detection of phosphorylated ERK1 and ERK2, ERK activity was mostly detected in the P1 and P2 fractions derived from the human fetal intestinal mucosae but uniformly detected in all fractions derived from differentiated Caco-2/15 cells. On longitudinal sections, immunostaining of differentiated Caco-2/15 cells (30 days post-confluency) confirmed that ERK1/2 were distributed uniformly.

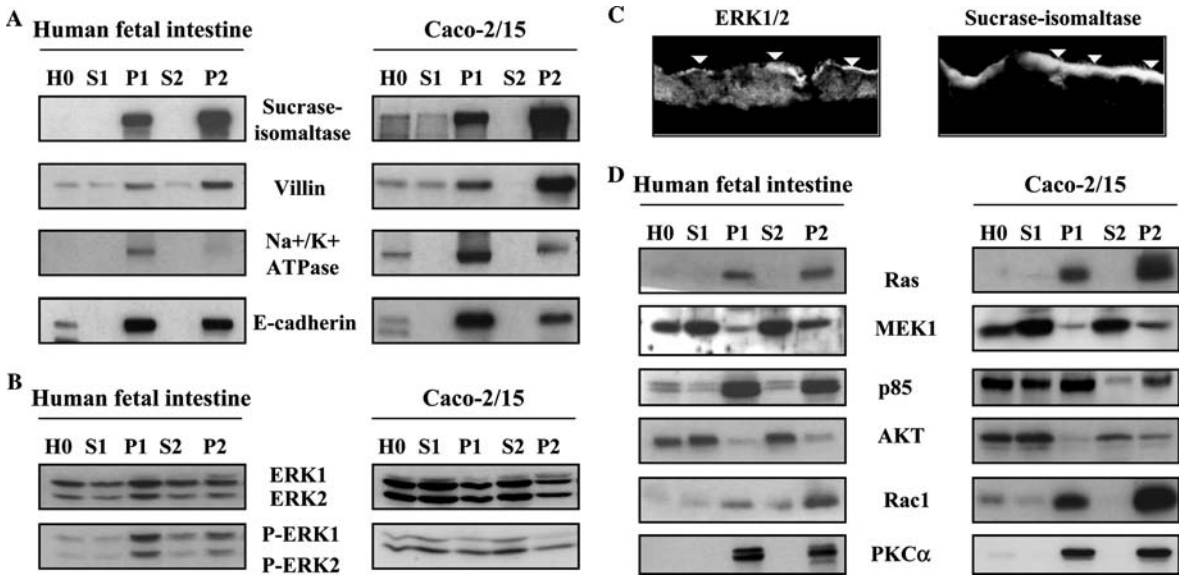

Fig. 1. Subcellular distribution of ERK1/2 and other signaling proteins in differentiated enterocytes. BBMV were prepared from mucosae of human fetal intestine or 25-day post-confluent Caco-2/15 cells, as described in Materials and methods. Equal amounts of proteins from each fraction were separated by SDS–PAGE and protein expression was analyzed by Western blotting using antibodies against sucrase-isomaltase, villin, Na^+/K^+ ATPase, and E-cadherin (A) or ERK1/2 and phosphorylated ERK1/2 (B) or Ras, MEK1, p85/PI-3K, AKT, Rac1, and PKCα (D). H0, homogenate; S1, supernatant 1; P1, pellet 1; S2, supernatant 2; P2, and pellet 2 containing BBMV. P-ERK, phosphorylated and activated ERK. (C) Fully differentiated Caco-2/15 cells were embedded in OCT freezing medium and sectioned longitudinally, fixed, and labeled for ERK1/2 and sucrase-isomaltase proteins.

Interestingly, a sharp staining of the apical surface of the epithelial cells was also observed (Fig. 1C, see arrowheads). As expected, sucrase-isomaltase, an enterocyte differentiation marker, was detected at the apex of the cells (Fig. 1C, see arrowheads).

The compartmentalization of ERK activity in several fractions and particularly in the brush border fraction prompted us to investigate whether known upstream regulators of ERK cascade were also expressed in these fractions in both human fetal intestine and differentiated Caco-2/15 cells. The expression of Ras, MEK1, the p85 regulatory subunit of PI-3K, Akt, Rac1, and PKCα, all known to potently influence the ERK signaling cascade in different cell types, was therefore analyzed [12]. As shown in Fig. 1D, the small G proteins Ras and Rac as well as p85 (PI-3K) and PKCα were mostly detected in the P1 and P2 fractions from both fetal intestine and differentiated Caco-2/15 cells. The dual specificity kinase MEK1 and the kinase Akt were also detected in the P1 and P2 fractions but were predominantly found in the supernatant fractions.

Effect of EGF, TNFα, IL-6, IL-1β, and feeding on ERK1/2 activity in enterocytes

In an attempt to characterize the regulation of ERK1/2 in differentiated intestinal epithelial cells, enzymatic activation of ERK1/2 by various agonists was assessed. Cell fractions were prepared from 3 week-post-confluent cells initially stimulated for 15 min with EGF (50 ng/ml) and for 20 min with TNF-α (30 pg/ml), IL-6 (10 pg/ml) or IL-1β (10 ng/ml). As shown in Fig. 2A, control differentiated Caco-2/15 cells exhibited minimal ERK activities in all recovered fractions. Addition of EGF dramatically increased ERK activities in all fractions, including the brush border-enriched fraction (P2). By contrast, treatment with TNF-α, IL-6 or IL-lβ enhanced ERK1/2 phosphorylation levels, moderately in S1 and S2 fractions and slightly in P1 and P2 fractions. These modulations occurred without any variation in total amount or subcellular distribution of ERK-1/2 (Fig. 2B). There was no significant induction of ERK activities detected in any of the fractions following 20 min of LPS or butyrate treatment (data not shown).

As the primary function of the intestinal epithelium is the terminal digestion and absorption of water and nutrients, ERK1/2 activities were analyzed in rat gut epithelium under conditions of fasting and of re-feeding. Rats were fasted overnight and re-fed for 1 or 3 h. One hour after feeding, pancreatic enzymes and biliary acid have now reached the duodenum, initiating the process of terminally digesting the chyme emanating from the stomach. After 3 h of feeding, digestive and absorptive processes are fully initiated [13]. In rat intestinal mucosae, total and phosphorylated ERK1/2 were mostly detected in P1 and P2 fractions (Fig. 2C). Re-feeding of fasted rats markedly enhanced ERK phosphorylation levels, especially after 3 h when terminal digestion and absorption were well initiated [13]. These modulations occurred without any variation in total amount or

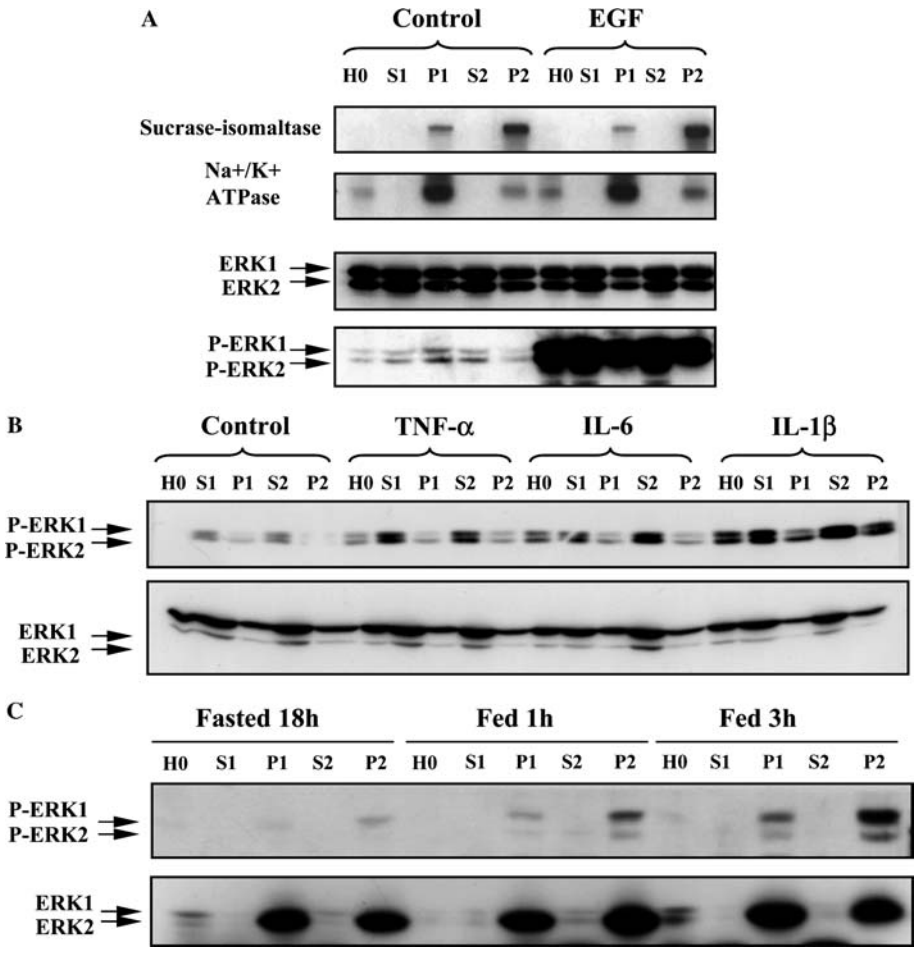

Fig. 2. Effect of EGF, TNFα, IL-6, IL-1β, and feeding on ERK1/2 activity in enterocytes. (A) Twenty day post-confluent Caco-2/15 cells seeded on a Transwell polycarbonate filter were stimulated basolaterally with EGF (50 ng/ml) for 15 min. (B) Twenty-five-day post-confluent Caco-2/15 cells were untreated (control) or treated for 20 min with TNF-α (30 pg/ml), IL-6 (10 pg/ml) or IL-lβ (10 ng/ml). (C) Rats were sacrificed by decapitation after 18 h of starvation or after 1 and 3 h of re-feeding. (A–C) BBMV were prepared, equal amounts of proteins from each fraction were separated by SDS–PAGE, and protein expression was analyzed by Western blotting using antibodies against ERK1/2 and phosphorylated ERK1/2 (P-ERK).

subcellular distribution of ERK-1/2 (Fig. 2C). Of note is that the highest ERK1/2 activation levels were observed in BBMV fractions (P2) suggesting that, in a physiological context of normal differentiated enterocytes, ERK1/2 proteins may regulate brush border-associated functions.

Evidences of ERK1/2 substrates in brush border of enterocytes

In a first attempt to identify brush border-associated ERK substrates, differentiated Caco-2/15 cells were treated with UO126, followed by preparation of brush border-enriched fractions, and by an immunoblot with an antibody which specifically recognizes proteins phosphorylated on a serine localized immediately ahead of either a proline or lysine. ERK1/2 are proline-directed serine/threonine kinases recognizing the consensus sequence PX(T/S)P [12]. As shown in Fig. 3A, treatment of differentiated Caco-2/15 cells with UO126 drastically inhibited ERK1/2 activities in all fractions studied. Immunoblotting with phosphoserine antibodies revealed that one S1-associated protein (lane 4) and several P2-associated proteins (lane 10) were less phosphorylated following UO126 treatment. UO126 reduced phosphorylation of P2-associated proteins with approximate molecular masses of 31, 36, 46, and 48 kDa (Fig. 3A, lane 10, see arrows). When immunoblots were performed using the anti-actin antibodies, actin aligned precisely with p46 (Fig. 3B). Therefore, these data reveal the presence of ERK substrates in the brush border of enterocytes, one of which could be actin.

The major components of the intestinal brush border involve actin cytoskeleton-associated proteins and hence cannot be extracted with a 0.5% solution of the non-ionic detergent Triton X-100, but are found in the insoluble fraction. Experiments were therefore conducted to analyze whether ERK1/2 are associated with the cytoskeleton in differentiating intestinal epithelial cells. As shown in Fig. 3C, differentiation of Caco-2/15 cells

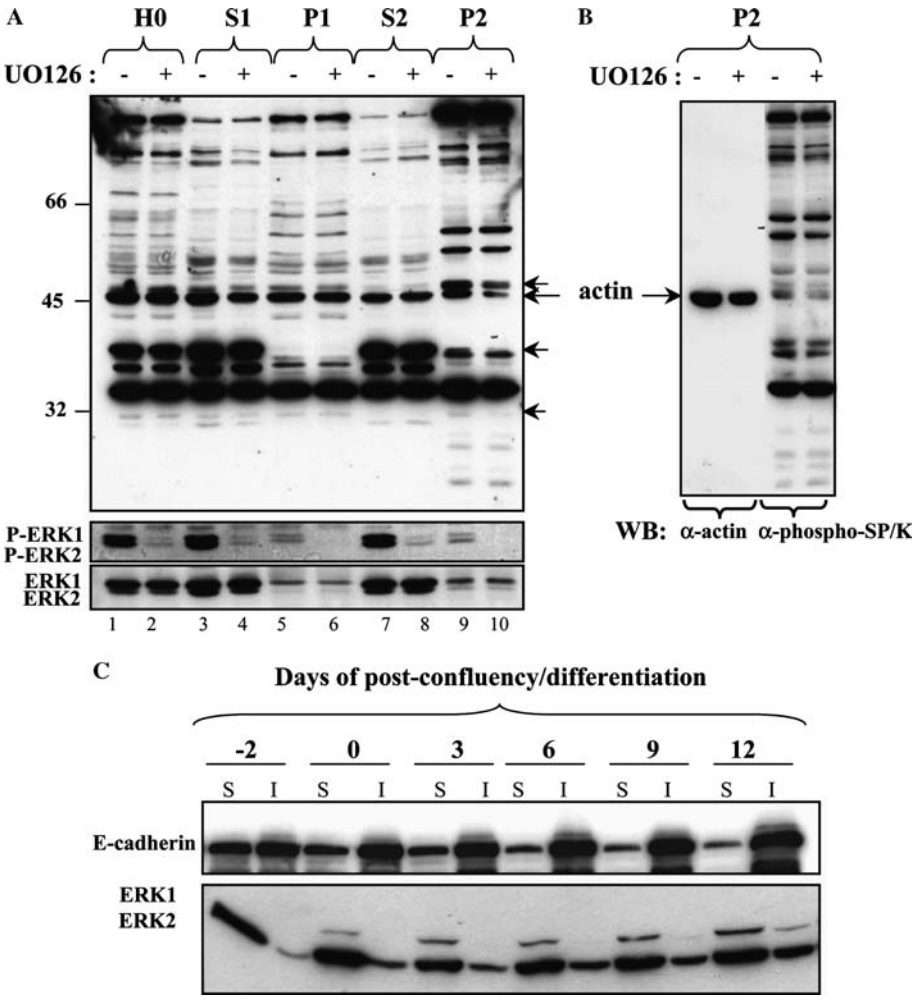

Fig. 3. Evidences of ERK1/2 substrates in enterocyte brush border. (A,B) Twenty-five-day post-confluent Caco-2/15 cells were treated for 4 h with UO126 (10 μM) and BBMV were prepared as described in Materials and methods. Equal amounts of proteins from each fraction were separated by SDS–PAGE. Proteins phosphorylated on serine localized immediately ahead of either a proline or lysine as well as expression of ERK1/2 and phosphorylated ERK1/2 were analyzed by Western blotting. (B) Equal amounts of proteins from P2 fraction were separated by SDS–PAGE. Actin and proteins phosphorylated on serine localized immediately before either a proline or a lysine were analyzed by Western blotting. (C) Caco-2/15 cells were harvested at subconfluence (day −2), confluence (day 0), and at day 3, 6, 9, and 12 of post-confluence. Cells were extracted on ice with cold lysis/cytoskeleton stabilization buffer. Soluble (S) and cytoskeleton-associated proteins (I) were separated by SDS–PAGE and subjected to immunoblotting for E-cadherin and ERK1/2.

resulted in a significant increase in the proportion of E-cadherin associated with the cytoskeleton, correlating with the assembly of adherens junction components with cortical F-actin [10]. Of special interest was the progressive increased association of ERK1 and ERK2 with the cytoskeleton during differentiation of Caco-2/15 cells, suggesting that ERK may regulate locally cytoskeletal-associated processes in enterocytes.

Role of ERK1/2 in differentiated intestinal epithelial cells

To investigate the role of ERK in intestinal epithelial cell differentiation, the impact of its inhibition was evaluated on morphological differentiation of Caco-2/15 cells. Cell cultures were characterized by transmission electron microscopy 4 days after confluence. As shown in Fig. 4, at 4 days post-confluence, Caco-2/15 cells began to exhibit certain ultrastructural characteristics similar to that found in the intact villus epithelium, including presence of microvilli (panel 2, see arrowheads) as well as junctional complexes (panel 2, see arrows). Addition of the pharmacological inhibitor of MEK1/2, UO126, at day 0 to 4 post-confluence did not alter cell polarization or the assembly and maturation of junctional complexes (panels 3 and 4, see arrows). This is consistent with the fact that inhibition of ERK in these cells did not influence transepithelial resistance (data not shown). However, several microvilli had an irregular, slightly swollen shape (Fig. 4, panel 4, see arrowheads) while others were fragmented, suggesting that ERK may be implicated in the control of microvilli architecture.

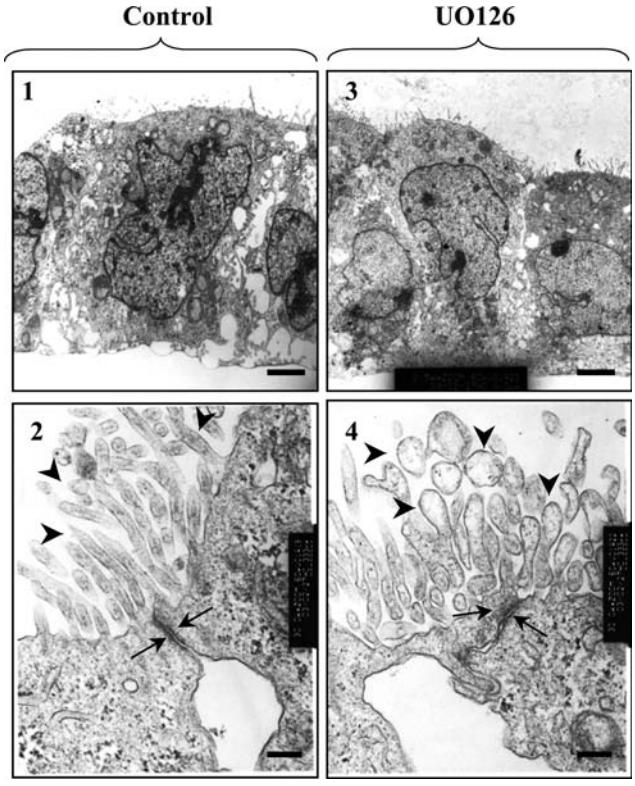

Fig. 4. Role of ERK1/2 activity in differentiated intestinal cells. Caco-2/15 cells were treated from day 0 to day 4 of post-confluence with or without 10 μM UO126. Cells were fixed in glutaraldehyde and osmium tetroxide prior to epoxy embedding for electron microscopy analysis. Bars = 4 μm for panels 1 and 3 and 333 nm for panels 2 and 4.

Discussion

We have recently shown that elevated ERK activities stimulate proliferation of intestinal cells whereas low sustained levels of ERK activities correlate with G1 arrest and enterocyte differentiation. Addition of the MEK1 inhibitor, PD98059, during differentiation interfered with sustained activation of ERK and sucrase-isomaltase expression, suggesting a role of ERK signaling in differentiated intestinal epithelial cells [9]. In a first attempt to clarify how ERK1/2 regulate intestinal epithelial cell differentiation, the present study assessed the subcellular distribution of ERK proteins and activities in differentiated enterocytes. The prominent localization of ERK1/2 and their upstream modulators Ras, p85 (PI-3K), Rac1, and MEK1 in the brush border of human enterocytes suggests that one of the sites of action of ERK signaling is at the apex of these cells. This is consistent with the observation that inhibition of ERK alters microvillus structure and that stimulation of ERK activity by feeding occurs predominantly at the brush border of rat intestinal epithelial cells. Therefore, we propose that ERK may contribute to the regulation of some brush border-associated functions.

The cellular localization of ERK in the brush border-associated enriched fraction of Caco-2 cells and intestinal epithelium places it at location where it can actively participate in apical signal transduction. In this regard, it is of interest that some of the known substrates of ERK1/2 have locations similar to those observed herewith for ERK proteins in human enterocytes. Among the potential substrates, $cPLA_2$ has been localized at the apical surface of the plasma membrane and can be phosphorylated and activated by ERK1/2 [6]. The MLCK present in the intestinal brush border [2,7] has been shown to be phosphorylated and activated by ERK1/2 in FG carcinoma cells [14]. In addition, caldesmon and cortactin, two actin-binding proteins previously described as localized in the terminal web of enterocytes [15,16], are similarly phosphorylated by ERK1/2 [17,18]. This apical localization of ERK has also been reported in chicken [19] and human [9] intestinal epithelium. In addition, it has been recently reported that Ras, Raf, MEK, and ERK1/2 were all associated with microvillar microfilaments of ascite cells isolated from rat mammary adenocarcinoma [20]. Therefore, the presence of ERK1/2 at a site involved in regulating microfilament and cell surface dynamics suggests that these enzymes may play important roles in brush border-associated responses or in the control of microvilli architecture.

The brush border of intestinal epithelial cells, an evolutionary adaptation designed to increase the surface area for digestion and absorption of ions and nutrients, appears to be a primary site of action of EGF and gastrointestinal hormones in the small intestine [1,2]. For instance, EGF and diet have been shown to regulate transport function in differentiated intestinal cells [21,22]. This effect is associated with increases in both brush border surface area and total absorptive surface area, primarily due to an increase in microvillus height [22,23]. Of particular interest is the fact that ERK inhibition in enterocytes alters microvilli architecture. Indeed, we observed that many microvilli appeared somewhat swollen and even fragmented following treatment of cells with the MEK inhibitor. Such microvillus phenotype has been recently demonstrated in duodenal and jejunal villus epithelial cells in fasted rats [24]. Interestingly, we found that feeding markedly enhanced brush border-associated ERK activities. Taken together these findings indicate that ERK1/2 may exert some biological effects via alterations in microvilli shape. This is consistent with our results demonstrating the localization of ERK1/2 proteins in the microfilament-rich apical region of enterocytes and their association with the cytoskeleton during differentiation. However, the mechanism by which ERK1/2 may alter microvilli structure remains to be determined. Our data do suggest however that brush border-associated proteins like actin could be phosphorylated on serine residues by ERK1/2

or in a ERK1/2-dependent manner. Such serine phosphorylation of actin has been reported following EGF stimulation in A431 and HER14 cells [25]. It has been suggested that a possible function of actin phosphorylation is to create a specific binding site for other proteins. Furthermore, there is evidence to suggest that actin phosphorylation facilitates actin polymerization [26]. In addition an association of ERK with contractile filaments of actin has been previously reported in vascular smooth cells stimulated with phenylephrine [27]. As such, it is plausible that the ERK-induced phosphorylation of actin is involved in increased actin polymerization observed in microvilli in response to EGF [3,23] or feeding [22,28]. It should be mentioned that caldesmon is another potential cytoskeletal target for brush border-associated ERK1/2. Indeed, caldesmon is a major actin-, myosin-, tropomyosin-, and calmodulin-binding protein which exhibits inhibitory function toward actin-tropomyosin-activated myosin ATPase activity. Phosphorylation of caldesmon by ERK1/2 has been shown to attenuate its interaction with actin [17] and to release actomyosin interaction [29].

In summary, our findings demonstrate that the ERK signaling pathway is present in the brush border of human intestinal epithelial cells. Moreover, ERK1/2 present in the brush border are stimulated by EGF and feeding, suggesting a role in nutrient, ion transport or other brush border-associated functions. Our findings indicate that ERK1/2 may exert some biological effects via alterations in microvilli shape of enterocytes. However, a potential activation of these enzymes cannot be excluded following epithelial cell injury since ERK1/2 were significantly activated after mechanical wounding of IEC-6 cells [30]. Current investigations are presently underway to further clarify the physiological role of brush border-associated ERK1/2 in differentiated intestinal epithelial cells.

Acknowledgments

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