Requirement of the MAP kinase cascade for cell cycle progression and differentiation of human intestinal cells

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Requirement of the MAP kinase cascade for cell cycle progression and differentiation of human intestinal cells. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G631–G641, 1999.—The intracellular signaling pathways responsible for cell cycle arrest and establishment of differentiated cells along the gut axis remain largely unknown. In the present study, we analyzed the regulation of p42/p44 mitogen-activated protein kinase (MAPK) in the process of proliferation and differentiation of human intestinal cells. In vitro studies were done in Caco-2/15 cells, a human colon cancer cell line that spontaneously differentiates into an enterocyte phenotype. In vivo studies were performed on cryostat sections of human fetal intestinal epithelium by indirect immunofluorescence. We found that inhibition of the p42/p44 MAPK signaling by the PD-98059 compound or by ectopic expression of the MAPK phosphatase-1 strongly attenuated E2F-dependent transcriptional activity in Caco-2/15 cells. p42/p44 MAPK activities dramatically decreased as soon as Caco-2/15 cells reached confluence. However, significant levels of activated p42 MAPK were detected in differentiated Caco-2/15 cells. Addition of PD-98059 during differentiation interfered with sustained activation of p42 MAPK and sucrase-isomaltase expression. Although p42/p44 MAPKs were expressed in both the villus tip and crypt cells, their phosphorylated and active forms were detected in the undifferentiated crypt cells. Our results indicate that elevated p42/p44 MAPK activities stimulate cell proliferation of intestinal cells, whereas low sustained levels of MAPK activities correlated with G1 arrest and increased expression of sucrase-isomaltase.

The control of cell division and differentiation is mediated by interactions of signaling molecules at the cell surface, which ultimately lead to long-term changes in gene expression. In most cell types, the mitogen-activated protein kinase (MAPK) cascade, a relay of cytoplasmic protein kinases expressed in organisms as diverse as yeast and mammalian cells, transmits the mitogenic (8, 37) or the differentiating signals (37, 42). p42 and p44 MAPKs, also termed ERK2 and ERK1 for extracellular signal regulated kinases, the most widely studied members, are ubiquitously expressed (1, 37, 58). An interesting feature of this family of kinases is that they require dual phosphorylation on specific threonine and tyrosine residues for their activation (1). MAPK activation is mediated by a dual specificity kinase termed MAP kinase kinase (MEK), which in turn is activated by Raf oncoproteins (1, 8). Raf proteins appear to be regulated by both the Ras family of oncoproteins (35, 66) and the recently described 14-3-3 proteins (24). Cellular activation of Ras is mediated by the guanine nucleotide exchange factor Sos, which, when associated in a complex with the adaptor protein Grb2, binds to activated receptor tyrosine kinases (21). Hence, the cascade leading from receptors with intrinsic tyrosine kinase activity to MAPK activation is relatively complete.

On stimulation, p42/p44 MAPKs translocate to the nucleus where they may phosphorylate nuclear transcription factors and thus regulate gene expression (17, 33). Whereas the mechanisms of MAPKs regulation are relatively well understood, the precise physiological roles of these enzymes remain to be established. However, activated MAPKs can phosphorylate and regulate many downstream targets, including additional kinases, receptors, and transcription factors such as E1K-1, ATF-2, c-jun, and CHOP (25, 36, 42, 52). Strong evidence exists for the critical involvement of MAPKs in the regulation of cell proliferation; indeed, a close correlation was established between MAPK activation and DNA synthesis (11, 20, 39), and inhibition of cellular MAPK activity was shown to block cell cycle progression (10, 46). Recently, it has been demonstrated that l-glutamine, tumor necrosis factor-α, and epidermal growth factor (EGF) stimulate proliferation of intestinal crypt cells (IEC-6) by activating the MAPK pathway (26, 45, 54). Furthermore, studies with constitutively active and dominant-negative mutants of MEK-1 (20) together with pharmacological blockade experiments (2) also demonstrated the absolute requirement for the MAPK pathway in neuronal differentiation.

The intestinal epithelium remains a model of choice to study regulation of signal transduction pathways during differentiation because it is a constant differentiating system with a rapid and orderly turnover of cells (62). Differentiation of cells starts with a sudden loss of their proliferative ability when they reach the upper third of the crypts; this process is characterized by marked changes in cell ultrastructure and by the expression of several new cell products that include the...
expression of the gut disaccharidase sucrase-isomaltase (51, 62, 65). Evers et al. (22) have recently suggested that Caco-2 cell differentiation may be dependent on the induction of the cell cycle inhibitor p21Cip. However, the intracellular signaling pathways responsible for cell-cycle arrest and establishment of differentiated cells occupying specific positions along the gut axis remain largely unknown.

In the present study, we analyzed the regulation of p42/p44 MAPK in the process of proliferation and differentiation of the human fetal intestinal epithelium (18–20 wk) and of the gut-derived Caco-2 cell line. Caco-2, a human colon cancer cell line, provides a unique and well-characterized model system for the evaluation of gut differentiation because these cells undergo differentiation to a small bowel-like phenotype in culture with microvilli, dome formation, and expression of sucrase-isomaltase occurring several days after the cells have reached confluence (5–7, 22, 49, 50). Moreover, our interest in intestinal development led us to also examine IEC-6 intestinal epithelial cells, a nontransformed rat jejunal cell line that shares characteristics of undifferentiated small intestinal crypt cells (4, 26, 53). In this study, we established that p42/p44 MAPK activities are necessary for both cell-cycle progression and differentiation of the intestinal cells. Indeed, elevated p42/p44 MAPK activities correlated with E2F-dependent transcriptional activity and DNA synthesis. In contrast, low sustained levels of MAPK activities correlated with G1 arrest and increased expression of sucrase-isomaltase.

**MATERIALS AND METHODS**

Materials. [γ-32P]ATP, [methyl-3H]thymidine, and the enhanced chemiluminescence (ECL) immunoassay system were obtained from Amersham (Montreal, PQ). Antiserum E1B, which specifically recognizes p42 and p44 MAPK on Western blots (38), and antisera against MKP-1 and MKP-2 (9), were kind gifts from Drs. Fergus McKenzie and Jacques Pousségur (Université de Nice, Nice, France). Rabbit polyclonal antibodies against phosphorylated and active forms of p42/p44 MAPK were from Promega (Madison, WI). Antisense OP40 specifically recognizing p21ras was from Oncogene Sciences (Calbiochem, San Diego, CA). Monoclonal antibody HSI-14 (6) against sucrase-isomaltase was kindly provided by Dr. Andrea Quaroni (Cornell University, Ithaca, NY). Goat anti-rabbit IgG-FITC, goat anti-mouse IgG-FITC, and sheep anti-rabbit IgG (1:1,000) IgG in blocking solution for 1 h. The blots were then denatured with four changes of 60 min each of 120 times at 60 min) of 250 ml renaturing buffer containing 50 mM Tris, pH 8.0, 10 mM MgCl2, 5 mM mercaptoethanol, 50 mM MgCl2, and 50 mM mercaptoethanol. The enzymes on gel were then renatured with four changes (2 times at 60 min, 1 time overnight, and 1 time at 20 wk of gestation, postfertilization fetal ages were estimated according to Streeter (61), were obtained from normal elective pregnancy terminations. No tissue was collected from cases associated with known fetal abnormality of fetal death. Specimens of adult intestinal jejunum were also analyzed. Studies were approved by the Institutional Human Subject Review Board. Segments of fetal small intestine were rinsed with 0.15 M NaCl, cut into small fragments, embedded in optimum cutting temperature compound, and quickly frozen in liquid nitrogen (64, 65). Frozen sections 2 to 3-µm thick were spread on silane-coated glass slides and then air dried 1 h at room temperature before storage at −80°C. For indirect immunofluorescence, sections were fixed with methanol (10 min, −20°C) in PBS (pH 7.4, 45 min, 4°C) before immunostaining. Negative controls (no primary antibody) were included in all experiments.

Cell culture. The rat intestinal epithelial crypt cell line IEC-6 (53) and the cell line Caco-2/15 were obtained from Dr. Andrea Quaroni (Cornell University, Ithaca, NY). This clone of the parent Caco-2 cell line (HTB-37; American Type Culture Collection, Manassas, VA) has been extensively characterized elsewhere (5, 7, 64). Both cell lines were cultured in plastic dishes in DMEM (GIBCO) containing 10% FCS, as described previously (64). Caco-2/15 cells were used between passages 53 and 78. Studies were performed on culture at subconfluence (50–70% confluence), confluence, and between 2 and 20 days postconfluence. Primary culture of human differentiated enterocytes prepared with specimens of small intestines from fetuses ranging from 18 to 20 wk of age were cultured as described (47) in DMEM supplemented with 4 mM glutamine, 20 mM HEPES, 50 µg/ml streptomycin, and 10 mM sodium pyruvate (all obtained from GIBCO BRL), 0.2 IU/ml insulin, and 5% FCS. When tested after 5–7 days, these primary cultures of differentiated enterocytes remained well preserved, and both goblet and absorptive cells exhibit all the main characteristics of intact villus intestinal cells (47).

Protein expression and immunoblotting. Cells were lysed in SDS sample buffer (62.5 mM Tris·HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromphenol blue, 1 mM phenylmethylsulfonyl fluoride; proteins (40 µg) from whole cell lysates were separated by SDS-PAGE in 10% gels. Proteins were detected immunologically following electrotransfer onto nitrocellulose membranes. The blots were then incubated with different antibodies in blocking solution for 2–4 h at 25°C and then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:1,000) IgG in blocking solution for 1 h. The blots were visualized by the Amersham ECL system. Protein concentrations were measured using a modified Lowry procedure with BSA as standard (48). MAPK in gel assays. MAPK activity present in IEC-6 cells was determined in renatured SDS polyacrylamide gels according to the method of Kameshita and Fujisawa (30). Briefly, cell extracts (20 µg proteins) were resolved on a 10% SDS-PAGE gel copolymerized with 0.25 mg/ml myelin basic protein. After electrophoresis, gels were washed with four changes of 50 mM Tris, pH 8.0, containing 20% propanol. The gels were then denatured with two changes of 60 min each of 120 ml denaturing buffer containing 6 M guanidine hydrochloride, 50 mM Tris, pH 8.0, and 5 mM mercaptoethanol. The enzymes on gel were then renatured with four changes (2 times at 60 min, 1 time overnight, and 1 time at 20 min) of 250 ml renaturing buffer containing 50 mM Tris, pH 8.0, 0.4% Tween 20, and 5 mM mercaptoethanol at 4°C. The renatured gels were then incubated in an assay buffer containing 40 mM HEPES, pH 8.0, 10 mM MgCl2, 2 mM dithiothreitol, and 0.1 mM EGTA at room temperature for 30 min. The MAPK activities were determined by incubating gels into 20 ml of the assay buffer containing 20 µM ATP and 100 µCi [32P]ATP at room temperature for 2 h. The reaction was then stopped by adding 250 ml of a solution containing 5% TCA and 10 mM sodium pyrophosphate, followed by washing with the same solution nine times over a period of 1.5 h to eliminate
nonspecific radioactivity in the gels. Gels were exposed to Kodak X-OMAT film overnight at −70°C before development.

DNA synthesis reinitiation. Subconfluent and 1-wk confluent IEC-6 cells were serum-starved for 24 h in DMEM. Cells were then stimulated for 20 h with 50 ng/ml of EGF in fresh DMEM medium (without serum) containing 0, 20, or 40 µM of the MEK-1/2 inhibitor PD-98059. At the end of the incubation, 0.25 M NaCl and 3 M (methyl-3H)-ethymidine were added for an additional 1 h. Cells were then washed three times with ice-cold TCA (5%), harvested with 0.1 N NaOH, and the radioactivity incorporated was counted as previously described (55).

p21\textsuperscript{ras} Activation assays. The assay to measure the activity status of p21\textsuperscript{ras} was as described (67). Briefly, Caco-2/15 cells were harvested at 70% confluence, 100% confluence (day 0), and 4, 8, and 14 days postconfluence in lysis buffer A (50 mM Tris-HCl, pH 7.5, 15 mM NaCl, 20 mM MgCl\textsubscript{2}, 5 mM EDTA, 0.1 mM phenylmethylsulfonil fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1% Triton X-100, 1% N-octyl glucoside) for 15 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 2 min at 4°C. Proteins from lysates (900 µg) were incubated with 30 µg of GST-RBD fusion protein, where RBD is amino acids 81–131 of Raf-1 and is the minimal domain required for binding of Ras-GTP (29), preadsorbed to glutathione-agarose beads for 2 h at 4°C. Precipitates were washed three times with buffer A. The presence of p21\textsuperscript{ras} was detected by resuspending the final pellet in 25 µl of Laemmli buffer, followed by protein separation on 12.5% polyacrylamide gels, and Western blotting with antisera OP40 recognizing p21\textsuperscript{ras}.

Expression vectors and reporter constructs. The sucrase-isomaltase reporter construct (SI-luciferase) used for luciferase assays contains the human sucrase-isomaltase promoter from residues −201 to +139 cloned upstream of the luciferase gene of the pGL2 reporter construct as described previously (Dr. P. G. Traber, Univ. of Pennsylvania, Philadelphia, PA) (63). Plasmid E2F SV40-luciferase, which contains a high-affinity E2F binding site from the dihydrofose reductase domain (DHFR) promoter coupled to a luciferase gene (60), was a kind gift of Dr. P. Farnham (Univ. of Wisconsin, Madison, WI) (27). Human MKP-1 constructs (kindly provided by Dr. N. Tonks, Cold Spring Harbor, NY) was described previously (10). Human MKP-3 (GenBank accession number X93920) construct was a gift from Dr. S. Meloche (Université de Montréal, Montréal, PQ) (27).

Northern blots analysis. Total RNA from subconfluent and postconfluent Caco-2/15 cells were extracted after homogenization in 4 M guanidine thiocyanate prepared as suggested by Chirgwin et al. (18) and subjected to electrophoresis on 1% agarose-2.2 M formaldehyde for quality control. For Northern blots, 20 µg of total RNA, quantified by measuring absorbency at 260 nm, were size fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Keene NH). For RNA probes, the membranes were prehybridized at 65°C for 2–4 h in solution containing 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM HEPS (pH 6.8), 1% SDS, 5× Denhardt’s, 5 mM EDTA, 50% formamide, and sonicated salmon sperm DNA (100 µg/ml). Hybridization was performed at 65°C for 16–20 h in the above solution containing [32P]UTP-labeled cRNA probes (1 × 10\textsuperscript{6} counts per min/ml); an 18S ribosomal [32P]UTP-labeled cDNA probe was used as a control probe to evaluate RNA loading and transfer. The sizes of the RNA transcripts were estimated according to the position of the 18S and 28S rRNAs. Autoradiograms were quantified using a laser densitometer (Bio-Rad Imaging densitometer; Bio-Rad, Mississauga, ON). The relative densities of the bands were expressed as arbitrary absorbency units; to correct for differences in loading of total RNA on Northern blots, a ratio of the relative density of each band to the relative density of the 18S ribosomal band was calculated before comparisons were made. The templates (for human M KP-3 cRNA probe) were linearized with Not I and labeled with [32P]UTP using T3 RNA polymerase (Promega transcription system; Promega, Madison, WI). Transient transfections and luciferase assays. Subconfluent and 3 days postconfluent Caco-2/15 cells were seeded in a 24-well plate and cotransfected by the lipofectine technique with 0.1 µg of E2F-SV40 or SI-luciferase reporter and 0.1 µg of the relevant expression vector (pECE or pcDNAneo) containing epoetin-tagged hyperactivated MEK-1 (S218D/S222D) or wild-type MEK-1 (11) or dominant-negative mutant of p44 (p44MAPK-T192A) (46) or MKP-1 (10). Two days after transfection, luciferase activity was measured according to the Promega protocol.

Determination of brush-border lactase-phlorizin hydrolase activity. Five-day postconfluent Caco-2/15 cells were scraped in water and sonicated. The homogenates were used for enzymatic determinations of lactase, assayed according to Dahlqvist as modified by Ménard and Arsenault (40). Protein content of the homogenates was determined using a modified Lowry procedure with BSA as standard (48). Data were expressed in international units [µmol of substrate hydrolyzed per minute] per gram of protein.

Data presentation and statistical analysis. Assays were performed in either duplicate or triplicate. The data presented are from representative experiments performed at least twice. Results were analyzed by Student’s t-test. Results were considered significantly different at P < 0.05.

RESULTS

Requirement of p42/p44 MAPK activities for DNA synthesis in IEC-6 and Caco-2/15 cells. To investigate the role of the p42/p44 MAPK pathway in intestinal cell proliferation, we first examined the enzymatic activation of p42/p44 isoforms by growth factors in IEC-6 cells. Cell lysates were prepared from subconfluent and 1-wk postconfluent cells, serum starved for 24 h, and stimulated for the indicated time periods with serum. As previously reported in other cell types (9, 20, 39, 46), serum stimulation of p42/p44 MAPK in subconfluent IEC-6 cells was rapid and maximal within 5 min. p42/p44 MAPK activities then declined slowly to reach their minimal activities at 3 h (Fig. 1A). Interestingly, activity of the p44 isoform detected was always superior to that of p42 at any given time point. The same kinetic of MAPK activation was observed in the postconfluent IEC-6 cells. However, the level of stimulation by
serum was significantly less than observed in subconfluent cells, suggesting that cell density might regulate the enzymatic activity of p42/p44 MAPK.

IEC-6 cells are highly dependent on growth factor addition for reinitiation of DNA synthesis (26, 45, 53, 54). We measured the effect of PD-98059, a specific inhibitor of MEK-1/2, on induction of DNA synthesis by EGF. Figure 1B shows that treatment of IEC-6 cells with PD-98059 dose dependently inhibited the significant stimulatory effect of EGF on [3H]thymidine incorporation. Complete inhibition was reached at 40 µM of inhibitor. Interestingly and as expected (4), the ability of EGF to stimulate the reinitiation of DNA synthesis was completely abolished in 1-wk postconfluent IEC-6 cells (Fig. 1B).

To evaluate the role of p42/p44 MAPK in Caco-2/15 cell proliferation, we used both the PD-98059 inhibitor and a series of previously characterized expression constructs known to modulate either positively or negatively the endogenous p42/p44 MAPK activities. Cell proliferation was monitored by transfecting Caco-2/15 cells with a plasmid construction containing the E2F-responsive element of the DHFR promoter linked to a luciferase reporter gene (see MATERIALS AND METHODS). The results shown in Fig. 2A confirm that the p42/p44 MAPK activities are also required for Caco-2/15 cell proliferation. When the MAPK cascade was blocked by inhibition of MEK-1/2 with the PD-98059 compound (40 µM), by expression of the dominant-negative p44 MAPK (p44DN = p44 MAPK-T192A) or by expression of MKP-1, E2F-dependent luciferase expression was significantly inhibited by 76, 48, and 80%, respectively (Fig. 2A). In marked contrast, the expression of a constitutively active form of MEK-1 (MEKCA = S218D/S222D) increased more than twofold the E2F-regulated reporter gene expression compared with control. Taken together, these findings on

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Fig. 1. Regulation and role of mitogen-activated protein kinase (MAPK) cascade in the control of DNA synthesis in IEC-6 cells. A: 50 µg of IEC-6 cell extracts were harvested at different time periods (0–3 h after serum addition) and MAP kinase activity present in the extracts was determined in renatured SDS polyacrylamide as described in MATERIALS AND METHODS. B: subconfluent and 1-wk confluent IEC-6 cells were arrested for 24 h in serum-free DMEM medium. Reinitiation of DNA synthesis in response to 50 ng/ml epidermal growth factor (EGF) was measured as described in MATERIALS AND METHODS. Results are means ± SE of at least 3 separate experiments. *Significantly different from control at P < 0.05 (Student’s t-test). PD, PD-98059.  

Fig. 2. Role of MAPK cascade in control of DNA synthesis in Caco-2/15 cells (A) and modulation of sucrase-isomaltase (SI) expression (B), p42/p44 MAPK expression (C), and activities (D) in differentiating Caco-2/15 cells. A: Caco-2/15 cells were transfected with 0.1 µg of reporter vector E2F-luciferase and 0.1 µg of relevant expression vector (pECE or pcDNAneo) alone or containing epitope-tagged hyperactivated MAP kinase kinase (MEK)-1 (SS/DD) or wild-type MEK-1 or dominant-negative mutant of p44 (p44MAPK-T192A) or MAPK phosphatase-1 (MKP-1). One day after transfection, cells were exposed to 20 and 40 µM of PD-98059 (PD) for 24 h and luciferase activity was measured. Increase in luciferase activity was calculated relative to the basal level of SI-luciferase set at 100% and corrected for the empty vector effects. Results are means ± SE of at least 3 separate experiments. *Significantly different from control at P < 0.05 (Student’s t-test). B–D: Caco-2/15 cells were harvested at 70% (subconfluent), 100% confluence (day 0), and 3, 6, 9, 12, and 15 days postconfluence. Cell extracts (50 µg) were separated by 10% SDS, and proteins were analyzed by Western blotting as described in MATERIALS AND METHODS. B: expression of sucrase-isomaltase was analyzed with monoclonal antibody HSI-14. C: expression of p42 and p44 MAPK was visualized with the polyclonal antibody E1B, which recognizes the phosphorylated and unphosphorylated forms. D: p42/p44 MAP kinase activities were visualized with an antibody specifically recognizing p42 and p44 phosphorylated on TEY motif.
both IEC-6 and Caco-2/15 cells indicate that the MAPK cascade plays a central role in the regulation of S phase entry and hence in cell proliferation of intestinal cells.

Repression of p42/p44 MAPK activities in postconfluent Caco-2/15 cells. Caco-2/15 cells, which differentiate spontaneously to small bowel phenotype when they reach a postconfluent state (7, 50), were harvested at 70% (subconfluent), 100% confluence (day 0), and 3, 6, 10, 12, and 15 days postconfluence and analyzed by Western blot to confirm the timing of induction of the sucrase-isomaltase protein expression. Consistent with previous observations (7, 64), sucrase-isomaltase protein levels significantly increased 6 days postconfluence (Fig. 2B). Within this time course, we next analyzed the protein expression of p42/p44 MAPK. Neither p42 nor p44 abundance changed with differentiation as indicated with the p42/p44 MAPK antibody (E1B), recognizing the phosphorylated and unphosphorylated forms of the enzymes (see MATERIALS AND METHODS; Fig. 2C). However, differential regulation of their kinase activities was observed during the differentiation of Caco-2/15 cells. Western blot analysis with an antibody recognizing the biphosphorylated and active MAPK isoforms revealed that p42/p44 MAPK activities dramatically decreased as soon as Caco-2/15 cells reached confluence to become almost inactive by day 6 postconfluence (Fig. 2D).

Caco-2/15 cell differentiation is associated with progressive decrease in GTPase activity of Ras. Although alternative pathways have been suggested, the principal mechanism of p42/p44 MAPK activation requires active p21Ras (8, 21, 29). Attempts to measure p21Ras activation using the standard immunoprecipitation technique following metabolic cell labeling with 32Pi (Fig. 2E) were unsuccessful (not shown). Hence, we used a novel assay to detect p21Ras activity based on the Ras-binding domain of Raf as a specific “trap” to selectively precipitate p21Ras only in its GTP-bound state and hence activated form (see MATERIALS AND METHODS). Figure 3A shows that p21Ras activity of Caco-2/15 cells was significantly reduced when the cells reached confluence (day 0), activation which progressively decreased during their differentiation. However, as previously observed (14), Western blot performed on cell extracts using antibodies to p21Ras revealed that expression of p21Ras was significantly increased during differentiation of these cells (Fig. 3B). Our data suggest that an impairment to p21Ras function might be required for the repression of p42/p44 MAPK activities in confluent Caco-2/15 cells.

Role of MKPs in regulation of p42/p44 MAPK activities during Caco-2/15 cell differentiation. The observation that p21Ras remained significantly activated during the early days of postconfluence led us to investigate whether the activity of p42/p44 MAPK can be negatively regulated by some protein phosphatases in these cells. To address the potential role of protein serine-threonine phosphatases in the control of MAPK activities, 2-day confluent Caco-2/15 cells were treated with okadaic acid, a potent inhibitor of PP2A and PP1 (19). As shown in Fig. 4A, treatment of confluent Caco-2/15 cells with okadaic acid decreased p42/p44 MAPK activities, indicating that PP2A or PP1 is unlikely to play a major role in the downregulation of p42/p44 MAPK activities in these cells. We next examined the hypothesis that p42/p44 MAPK might be negatively regulated by a protein tyrosine phosphatase by treating confluent cells with the potent inhibitor vanadate (28). Figure 4A also demonstrates that vanadate treatment alone resulted in activation of p42/p44 MAPK in confluent Caco-2/15 cells. Moreover, treatment of these cells with a combination of okadaic acid and vanadate had no additive effect on p42/p44 MAPK activities compared with vanadate alone (data not shown). Thus these experiments support the existence of a vanadatesensitive mechanism that negatively regulates MAPK isoforms activities in confluent Caco-2/15 cells.

In light of the observation that vanadate increased p42/p44 MAPK activities in confluent Caco-2/15 cells, it was of interest to examine the expression of dual-specificity MKPs, known to be sensitive to this inhibitor (15, 31). Western blot analysis with a specific antibody to MKP-1 and MKP-2 (see MATERIALS AND METHODS) showed that MKP-1 is induced during Caco-2/15 cell differentiation. The expression of MKP-1 was slightly detected in subconfluent cells, reached a peak of expression 6 days postconfluence, and declined slowly thereafter (Fig. 4B). MKP-2 was not detected in Caco-2/15 cells (Fig. 4B). Northern blot analysis confirmed the lack of MKP-2 expression in these cells (not shown). Interestingly, the expression of the MKP-3 transcript was found to be markedly induced in newly confluent Caco-2/15 cells (Fig. 4C). These results suggest that the induction of MKP-3, a newly described cytoplasmic MKP (41), could be involved in the rapid repression of cytoplasmic p42/p44 MAPK activities during the first days of confluence. Moreover, the significant induction of MKP-1, a nuclear MKP (10), observed at day 6 postconfluence could reduce the MAPK activity still present into the nucleus, a process that may be required for these cells to assume a differentiated phenotype.

Fig. 3. Modulation of p21Ras expression and activity in differentiating Caco-2/15 cells. Caco-2/15 cells were lysed at 70% (subconfluent), 100% confluence (day 0), and 4, 8 and 14 days postconfluence. A: p21Ras-GTP specifically precipitated was also analyzed by Western blotting as described in MATERIALS AND METHODS. B: 50 µg of cell extracts were separated by 10% SDS and expression of p21Ras was visualized by Western blotting as described in MATERIALS AND METHODS. Data represent a single experiment performed twice with identical results.
Sucrase-isomaltase gene and protein expression are regulated by MAPK cascade. To determine whether MAPK activation plays a significant role in differentiation, we examined the consequences of blocking or activating specific components of the MEK/MAPK signaling pathway on sucrase-isomaltase expression in both subconfluent and confluent Caco-2/15 cells. The luciferase gene driven by the human sucrase-isomaltase promoter represents a sensitive reporter of intestinal cell differentiation (63). In transiently transfected subconfluent Caco-2/15 cells, sucrase-isomaltase gene expression was significantly stimulated by more than twofold above control with the MEK-1/2 inhibitor, PD-98059 (Fig. 5A, left). In contrast, expression of the hyperactivated MEK-1 (MEK-1 SS/DD), but not wild-type MEK-1, significantly reduced below control values sucrase-isomaltase gene expression by 75%. Interestingly, this inhibitory effect of active MEK-1 can be overcome by treatment with PD-98059 (data not shown). These results suggest that the high p42/p44 MAPK activities observed in subconfluent Caco-2/15 cells negatively regulate sucrase-isomaltase gene expression. However, activated p42 MAPK was detectable in differentiated Caco-2/15 cells, suggesting that sustained MAPK activation may be involved in enterocyte differentiation. When the MAPK cascade was blocked in 5-day postconfluent cells by the PD-98059 compound (20 µM), by expression of the dominant-negative p44 MAPK (p44ΔN = p44 MAPK-T192A), or by expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited by 49, 44, and 81%, respectively (Fig. 5A, right). Conversely, addition of PD-98059 at day 7–12 postconfluence dose dependently reduced both p42/p44 MAPK activities and sucrase-isomaltase expression (Fig. 5B) observed at day 12 postconfluence. However, treatment with 20 µM PD-98059 was shown to significantly reduce the enzymatic activity of lactase-phlorizin hydrolase [control cells (8.02 ± 1.01 IU) vs. PD-98059-treated cells (6.48 ± 0.09 IU), P < 0.05 on day 6 postconfluency]. Moreover, higher concentrations of PD-98059 were able to completely block p42 MAPK activity, sucrase-isomaltase expression, and lactase-phlorizin hydrolase activity but protein synthesis was also reduced (unpublished observations). However, this inhibitor when used at optimal concentrations (20–40 µM) allows us to conclude that p42/p44 MAPK cascade activation is required for maximal induction of sucrase-isomaltase in postconfluent Caco-2/15 cells.

Furthermore, we analyzed the effect of PD-98059 on sucrase-isomaltase expression in primary culture of human differentiated enterocytes (see MATERIAL AND METHODS). As shown in Fig. 5C, treatment of these primary cultures with 2 and 10 µM PD-98059 significantly and dose dependently reduced expression of sucrase-isomaltase by 22 and 55%, respectively. Over-all, these data support the hypothesis that the p42/p44 MAPK cascade is involved in the regulation of sucrase-isomaltase expression in intestinal cells.

Regulation of p42/p44 MAPK activities in human fetal intestinal epithelium. MAPKs were further investigated in the intact intestinal epithelium. We first
localized p42 and p44 MAPK isoforms in the human fetal small intestine by immunofluorescence staining. P44 MAPK was seen mostly localized in the nuclei of the cells present in the crypt and lower third of the villus and in cytoplasm and nucleus of the villus cells (Fig. 6A). Interestingly, p42 MAPK was found predominantly at the luminal surface of all enterocytes, according to an increasing crypt-villus gradient of expression (Fig. 6B). The use of a specific antibody to the p42 and p44 isoforms phosphorylated on their TEY motif revealed that the active forms of MAPKs were detected primarily in the nuclei of epithelial cells present in the crypts and lower third of the villi (Fig. 6C).

**DISCUSSION**

The mucosal lining of the gut is a constantly renewing system with multiple and diverse functions. As the enterocytes progress from crypt to villus, they lose their ability to proliferate and acquire differentiated characteristics that include among others the expression of the gut disaccharidase sucrase-isomaltase (62). Specific gene expression changes and proteins that mediate growth arrest and induction of differentiation in the gut remain to be fully elucidated. Factors that determine whether cells continue to proliferate or cease dividing and begin to differentiate appear to operate during the first gap phase (G1) of the cell cycle. Activation of p42/p44 MAPKs is necessary for growth factor-dependent proliferation of fibroblasts (10, 46). The two enzymes are coordinately activated during the G0 to G1 transition, and their activity remained elevated up to S phase entry, implicating this family of protein kinases in the control of G1 progression (11, 20, 39). However, in another cell model, the PC-12 cells, it is differentiation that is promoted by a sustained activation of MAPKs in response to nerve growth factor (20, 37). These observations raised the important question of whether activation of the MAPK cascade is involved in the proliferation and differentiation processes of intestinal epithelial cells.

To answer this question, we employed a variety of separate but complementary experimental approaches, using the specific inhibitor of MEK activity PD-98059 (2) and transfection of specific MEK or MAPK constructs. Based on our observations, we can conclude that MAPKs are clear positive regulators of the intracellular pathways leading to intestinal epithelial cell proliferation and differentiation. As previously suggested in other cell systems (20, 37, 57, 58), the intensity and the duration of p42/p44 MAPK activities seem critical for cell-signaling decisions. We provided several evidences that there is a direct relationship between elevated p42/p44 MAPK activities, particularly p44 MAPK activity, and intestinal cell proliferation:

1. p44 MAPK activity is markedly stimulated by serum in IEC-6 cells;
2. inhibition of p42/p44 MAPK activities by treatment of IEC-6 cells with PD-98059 lead to G1 arrest;
3. in subconfluent Caco-2/15 cells, when the MAPK cascade was blocked by PD-98059, by expression of dominant-negative p44 MAPK (p44 MAPK-T192A) or by expression of MKP-1, the E2F-
dependent transcriptional activity was significantly repressed; 4) p42/p44 MAPK activities, particularly p44 MAPK, were markedly reduced in postconfluent Caco-2/15 cells, suggesting that MAPK are selectively inactivated during enterocyte differentiation; 5) the p44 isoform is primarily localized in the nucleus of intestinal crypt cells; 6) the phosphorylated and active forms of MAPKs were detected only in the nuclei of undifferentiated crypt cells; 7) the MEK inhibitor PD-98059 significantly increased transcriptional activity of sucrase-isomaltase gene promoter. Furthermore, the forced expression of the constitutive active mutant of MEK-1 in Caco-2/15 cells significantly inhibited transcriptional activity of sucrase-isomaltase gene promoter.

Previous studies reported that transfection of Caco-2 cells with an activated human Val-12 Ha-ras gene repressed the expression of sucrase-isomaltase and villin, suggesting that this oncoprotein and its downstream effectors, which include MAPK (8, 21, 42, 66), may exert a general antagonizing effect on the enterocyte-like differentiation of Caco-2 cells (14). These observations are in agreement with those of Mamajewalla and Burgess (34), who recently demonstrated that although p42 MAPK was expressed in both crypt and villus cells of adult chicken epithelium it was phosphorylated on tyrosine and active only in the crypt cells (34).

As various Caco-2 cell lines (7, 49, 50), Caco-2/15 cell line has been extensively characterized for their ability to differentiate gradually between day 0 and day 20 (5–7, 64). For instance, the sucrase-isomaltase transcript begins to increase as soon as Caco-2/15 cells reached confluency (day 0) (5). As shown herein, significant levels of activated MAPK were detected in differentiated Caco-2/15 cells, predominantly p42 MAPK. This suggests that sustained p42 MAPK activation could be involved in enterocyte differentiation. Using the PD-98059 inhibitor and an antibody recognizing the active forms of p42/p44 MAPKs, we demonstrated that a certain level of MAPK activity is required for maximal increases of sucrase-isomaltase protein levels. Indeed, inhibition of MEK activation during differentiation interfered with sustained activation of p42 MAPK and sucrase-isomaltase protein expression, consistent with the conclusion that p42 MAPK are involved in the regulation of sucrase-isomaltase expression in Caco-2/15 cells. Moreover, when the MAPK cascade was blocked in 5-day postconfluent cells with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited. Most importantly, treatment of primary culture of human differentiated enterocytes with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited. Most importantly, treatment of primary culture of human differentiated enterocytes with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited. Most importantly, treatment of primary culture of human differentiated enterocytes with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited. Most importantly, treatment of primary culture of human differentiated enterocytes with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited. Most importantly, treatment of primary culture of human differentiated enterocytes with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited. Most importantly, treatment of primary culture of human differentiated enterocytes with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited. Most importantly, treatment of primary culture of human differentiated enterocytes with the PD-98059 compound, by ectopic expression of the dominant-negative p44MAPK, or by ectopic expression of MKP-1, the sucrase-isomaltase gene expression was significantly inhibited.
The enzymatic activity of MAPK isoforms is positively regulated by the upstream cascade Ras > Raf > MEK-1/2 and negatively regulated by ill-defined protein phosphatases. In the colon, Ras is probably the most well-studied upstream activator of MAPK. Unfortunately, virtually nothing is known on how upstream activators of MAPKs are regulated during intestinal differentiation. Although the Ki-ras gene is frequently mutated in human colon cancer (12, 44, 59), the Ha-ras protooncogene is highly expressed in most differentiated cells of the intestinal mucosa (14). However, our data indicate that an impairment to p21Ras functions might be required for the maintenance of a differentiated phenotype since a progressive decrease in p21Ras activity was observed during differentiation of the Caco-2/15 cells.

In the present report, we demonstrated that a significant Ras activity remained detectable at the time Caco-2/15 cells reached confluency. This observation implies that downregulation of the MAPK pathway in newly confluent Caco-2/15 cells does not result from a major failure to activate the upstream protein kinases. The role of protein phosphatases in the regulation of MAPK activities was addressed by a combination of approaches. Treatment of confluent cells with okadaic acid had no stimulatory effect on the enzymatic activity of MAPK isoforms. However, treatment with vanadate restored MAPK activities to a level comparable to what was observed in subconfluent cells. These observations indicate that MAPK is predominantly regulated by a vanadate-sensitive mechanism in confluent Caco-2/15 cells, which most probably involves a protein tyrosine phosphatase. Our results with the inhibitor drugs also suggest that PP2A and PP1 are not major regulators of MAPK activities in Caco-2/15 cells. This is apparently different from the situation prevailing in PC-12 cells where PP2A was identified as the major MKP in cellular extracts (3).

Recently, an increasing number of "dual specificity" phosphatases have been cloned. Members of this family of dual-specificity phosphatases are capable of inactivating p42/p44 MAPK isoforms in vitro and are sensitive to vanadate (31). It was therefore of interest to determine whether one of these phosphatases was upregulated in differentiating Caco-2/15 cells. Interestingly, it was recently reported that the erp transcript (MKP-1 mRNA) was detected in the differentiated villus cells but not in the proliferating cells within the intestinal crypts of the adult intestinal epithelium (43). Consistent with these observations, an increase in the expression of MKP-1 was detected from day 6 postconfluence in Caco-2/15 cells. However, the expression of MKP-3 mRNA was greatly enhanced in cells reaching confluence. It is also of note that the expression of MKP-3 mRNA parallels reduction in p42/p44 MAPK activities during the first days of postconfluence. Interestingly, MKP-3 was localized exclusively in cytosolic compartments of a number of cell types (41). In this respect, MKP-3 appears distinct from other dual specificity phosphatases that are clearly nuclear (10). Experiments using purified proteins as well as expression in COS cells showed that MKP-3 displays clear selectivity for inactivation of ERK MAPK family members (41). This finding, together with our data, could indicate an important role for MKP-3 in inactivating selectively cytosolic p42/p44 MAPKs during induction of Caco-2/15 differentiation. Such compartmentalized regulation of MAPKs following confluence may be of fundamental importance in molecular processes underlying Caco-2/15 cell differentiation. Thus our results are consistent with the hypothesis that induction of MKP-1 and MKP-3 might be responsible for the impairment of p42/p44 MAPK activities in the cytoplasm and nucleus of postconfluent Caco-2/15 cells. The involvement of MKP-3 during the nerve growth factor-induced differentiation of PC-12 cells was also recently demonstrated (13). However, whereas MAPK activities remained low through differentiation of confluent Caco-2/15 cells and whereas it is suggested that MKP-1 and MKP-3 may be regulating the activity of MAPK, their expressions decreased significantly after day 6. This suggests that the impairment in the p21Ras function observed in differentiating Caco-2/15 cells is required for the long-term repression of p42/p44 MAPK activities.

We demonstrated here for the first time that p42 and p44 MAPK isoforms are localized in different compartments of the intestinal epithelial cells. The p44 isoform is primarily localized in the nucleus of the crypt cells and in the cytoplasm and nucleus of the villus cells. However, the p42 isoform is primarily detected at the apical surface along the crypt-villus axis. These observations are in agreement with those of Mamajiwalla and Burgess (34) who also recently reported that p42 MAPK was localized to the apex and nuclei of cells throughout the crypt-villus axis of the adult chicken intestinal epithelium.

In summary, our results indicate that p42/p44 MAPK and their regulators are tightly controlled during enterocyte differentiation and implicate the MAPK pathway as a key signaling pathway in the normal development of the intestinal epithelium.

NOTE ADDED IN PROOF

While this work was being reviewed, Taupin and Podolsky (Gastroenterology 116: 1072-1080, 1999) also reported a loss of activation of the MAP kinases p42/p44 in HT29-N2 cells on change to glucose-free growth medium, preceding the change in differentiated phenotype. However, in contrast to our data showing an inhibition of sucrase-isomaltase gene expression with the MEK inhibitor, Taupin and Podolsky demonstrated that the PD-98059 inhibitor enhanced expression of the differentiation markers sucrase-isomaltase, intestinal trefoil factor, and the mucin gene MUC2 in HT29-N2 cells.

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