Intestinal Epithelial Cell Differentiation Involves Activation of p38 Mitogen-activated Protein Kinase That Regulates the Homeobox Transcription Factor CDX2*

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The intracellular signaling pathways responsible for cell cycle arrest and differentiation along the crypt-villus axis of the human small intestine remain largely unknown. p38 mitogen-activated protein kinases (MAPKs) have recently emerged as key modulators of various vertebrate cell differentiation processes. In order to elucidate further the mechanism(s) responsible for the loss of proliferative potential once committed intestinal cells begin to differentiate, the role and regulation of p38 MAPK with regard to differentiation were analyzed in both intact epithelium as well as in well-established intestinal cell models recapitulating the crypt-villus axis in vitro. Results show that phosphorylated and active forms of p38 were detected primarily in the nuclei of differentiated villus cells. Inhibition of p38 MAPK signaling by 2–20 μM SB203580 did not affect E2F-dependent transcriptional activity in subconfluent Caco-2/15 or HIEC cells. p38 MAPK activity dramatically increased as soon as Caco-2/15 cells reached confluence, whereas addition of SB203580 during differentiation of Caco-2/15 cells strongly attenuated sucrase-isomaltase gene and protein expression as well as protein expression of villin and alkaline phosphatase. The binding of CDX2 to the sucrase-isomaltase promoter and its transcriptional activity were significantly reduced by SB203580. Pull-down glutathione S-transferase and immunoprecipitation experiments demonstrated a direct interaction of CDX3 with p38. Finally, p38-dependent phosphorylation of CDX3 was observed in differentiating Caco-2/15 cells. Taken together, our results indicate that p38 MAPK may be involved in the regulation of CDX2/3 function and intestinal cell differentiation.

The epithelium of the small intestine is a highly dynamic system continuously renewed by a process involving cell generation and migration from the stem cell population located at the bottom of the crypt to the extrusion of the terminally differentiated cells at the tip of the villus (1, 2). The crypt-villus functional axis unit, which develops relatively early during human ontogeny (being established by mid-pregnancy), can be defined by typical morphological and functional properties displayed by the mature villus enterocytes that distinguish them from crypt cells (1–3). Indeed, the villi are mainly lined by functional absorptive, goblet, and endocrine cells, whereas the crypts contain stem cells, proliferative and poorly differentiated cells, as well as a subset of differentiated secretory cells, namely Paneth cells (3). The differentiation of each cell type takes place as the cells move either upward toward the villus (absorptive, mucus and endocrine cells) or downward to concentrate at the bottom of the crypt (Paneth cells) (2). The basic mechanisms responsible for induction of cell differentiation are little understood. The decision to differentiate is taken by the committed crypt cells abruptly, while in their most rapid state of proliferation (4). The newly differentiated cells acquire their distinctive ultrastructural features and cell surface markers after leaving the proliferative cell cycle, at the top of the crypts or the base of the villi (2, 5–7). It is noteworthy that in all species studied, the crypt-villus axis junction represents a physical limit from which enterocytes acquire their final functional characteristics (1–7).

The process of cell differentiation in the intestinal epithelium has been the subject of extensive studies, for which the morphological and functional characteristics of the intestinal mucosa (8, 9), the growth kinetics of the epithelial cells (10), and the chronological changes that affect brush border enzyme activities during pre- and postnatal development (11, 12) have all been well documented. However, the basic mechanisms involved in the induction and the modulation of cell differentiation in the upper portion of the crypts, and the cellular interactions responsible for the orderly arrangement of the relative numbers of proliferative, maturing, and functional epithelial cells are still largely unknown. Hormones, such as glucocorticoids, and growth factors, such as epidermal growth factor (EGF),1 have been implicated in the regulation of intestinal growth and development (12, 13). However, little is known about the molecular signals responsible for the ontogenic changes in intestinal gene expression.

Several lines of evidence suggest that the intestinal specific, caudal-related cdx1 and cdx2/3 homeobox genes encode nu-

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1 The abbreviations used are: EGF, epidermal growth factor; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; MEK, MAPK/extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; HIEC, human intestinal epithelial cells; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; DHFR, dihydrofolate reductase; PCR, polymerase chain reaction; DPP IV, dipeptidyl peptidase IV; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; PCDE, primary cultures of differentiated enterocytes; pRB, retinoblastoma protein.

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clear transcription factors that play critical roles in intestinal cell proliferation and differentiation. CDX1 is mainly expressed in the crypt compartment although not restricted to proliferative cells (14), and its inhibition by antisense RNA reduces cell proliferation in vitro (15). The CDX2/3 homeoproteins (the protein designated CDX3 in the hamster and CDX2 in the mouse and humans) are mainly expressed in differentiating enterocytes (16), triggering growth retardation and cell differentiation by overexpression in several intestinal lines in vitro (15, 17, 18). Furthermore, genes regulated by either CDX1 or CDX2/3 generally define a functional differentiated phenotype (for example, sucrase-isomaltase (18), glucagon (19), intestinal phospholipase A2/lysophospholipase (20), carbonic anhydrase (21), and lactase (22)). However, little is known about the intracellular signaling pathways that positively regulate the activities of CDX transcription factors, especially those involved in receiving and transducing extracellular cues.

In eukaryotic cells, the mitogen-activated protein kinase (MAPK) family has been shown to play various important roles in regulating gene expression via transcription factor phosphorylation (23–26). In mammals, two distinct classes have been identified to date as follows: p42–p44 (extracellular signal-regulated kinase) MAPKs inducible by growth factors, and SAPKs (stress-activated protein kinases), which include p38 MAPKs and p46–p54 JNKs, inducible by cytokines and cellular stress (27). Unique structural features, specific activation pathways, and varying substrate specificities support the contention that different MAPKs are independently regulated and control different cellular responses to extracellular stimuli (28, 29). We (30) and others (31) recently analyzed the role and regulation of p42/p44 MAPKs in the process of proliferation and differentiation of human intestinal cells. Our results demonstrated that elevated p42/p44 MAPK activities stimulated cell cycle progression of intestinal epithelial cells, whereas low sustained levels were correlated with G1 arrest and differentiation. However, the intracellular pathways responsible for establishment of differentiated cells occupying specific positions along the gut axis still remain largely unknown.

Several recent studies have demonstrated that p38 MAPK is involved in various vertebrate cell differentiation processes, namely adipocytic (32) and myogenic differentiation (33). The role of p38 MAPK in intestinal cell differentiation is, however, not known. In the present work, we analyzed the regulation of p38 MAPK in the process of proliferation and differentiation of human intestinal cells. Our results demonstrated that elevated p42/p44 MAPK activities stimulated cell cycle progression of intestinal epithelial cells, whereas low sustained levels were correlated with G1 arrest and differentiation. However, the intracellular pathways responsible for establishment of differentiated cells occupying specific positions along the gut axis still remain largely unknown.

p38 MAPK Assay—The cells were lysed for 10 min on ice with 1 ml/dish of lysis buffer (150 mM NaCl, 1 mM EDTA, 40 mM Tris, pH 7.6, 1% Triton X-100) supplemented with protease inhibitors (0.1 mM PMSF, 10 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), Proteins (40 μg) from whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 7.5 or 10% gels. Proteins were detected immunologically following electrotransfer onto nitrocellulose membranes (Amersham Pharmacia Biotech). Protein and molecular weight markers (Bio-Rad) were used for staining with Ponceau Red. Membranes were blocked for 3 h at 25 °C in phosphate-buffered saline containing 10% powdered milk. Membranes were then incubated overnight with primary antibodies in blocking solution and with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:1000) IgG for 1 h. The blots were visualized by the Amersham Pharmacia Biotech ECL system. Protein concentrations were measured using a modified Lowry procedure with bovine serum albumin as standard (44).
phosphate, 40 mM β-glycerophosphate). Lysates (400 µg) cleared by centrifugation (10,000 × g, 10 min) were incubated for 2 h at 4 °C with protein A-Sepharose (Amersham Pharmacia Biotech) that had been preincubated for 1 h with anti-p38 antibody. Immunocomplexes were then washed four times with ice-cold lysate buffer and three times with ice-cold PBS (1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na3VO4). The beads were resuspended in 1.5 ml of SDS-PAGE and autoradiographed. Incorporation of 32P by MBP was linear over the course of the kinase assay.

Expression Vectors and Reporter Constructs—The sucrase-isomaltase reporter construct used for luciferase assays contained the human sucrase-isomaltase promoter from residues −183 to +54 cloned upstream of the luciferase gene of the pGL2 reporter construct as described previously (20). The expression vector was transfected into Caenorhabditis elegans (C. elegans) (45). Plasmid E2F SV40-luc, which contains a high affinity E2F-binding site from the dihydrofolate reductase (DHFR) promoter immobilized fusion proteins by end-over-end rotation. The beads were extensively in SB buffer and used for in vitro binding assays, as described (57).

GST Pull-down Assays—Caco-2/15 cells were grown in 60-mm dishes to confluence in DMEM supplemented with 10% FBS. The cells were lysed in 700 µl of lysis buffer (150 mM NaCl, 1 mM EDTA, 40 mM Tris (pH 7.6, 1% Triton X-100) supplemented with protease inhibitors (0.1 mM PMSF, 10 µg/ml leupeptin, 1 µg/ml pepstatin A) and phosphatase inhibitors (0.1 mM orthovanadate, 20 mM para-nitrophenol, 40 µg/ml β-glycerophosphate). Lysates were centrifuged at 15,000 g for 10 min. 5 µg of GST-CDX3 or GST proteins were coupled to 50 µl of glutathione-Sepharose (Amersham Pharmacia Biotech). The Caco-2/15 lysates were incubated for 2 h at 4 °C with the immobilized fusion proteins by end-over-end rotation. The beads were washed four times with the lysis buffer. Lysates were added to the beads, and the mixture was boiled for 5 min. Bound proteins were visualized by SDS-PAGE (9% acrylamide gels) and immunoblotting as described above. In other experiments, the beads were washed four times with lysis buffer followed by three times with ice-cold kinase buffer before performing the kinase assay in the presence or absence of 0.1–20 µM SB203580.

Data Presentation and Statistical Analysis—Luciferase assays were performed in triplicate, and results were analyzed by the Student’s t test and were considered significantly different at p < 0.05. Typical Western blots, representative of two or three independent experiments, are shown. Densitometric analyses were carried out for each Western blot.

RESULTS

Activity of p38a MAPK in the Human Fetal Intestinal Epithelium—Phosphorylation and activity of p38 MAPK were investigated in intact fetal intestinal epithelium (20 weeks of gestation). It is generally agreed that by 16–18 weeks of gestation, the overall morphological appearance of the small intestine and the expression of most of the functional markers including sucrase-isomaltase are comparable to those in adult intestine (3, 58). However, lactase represents an exception, with its activity increasing between 36 and 40 weeks of gestation (58). The use of a specific antibody against p38, phosphorylated on the TGY motif, revealed that phosphorylated and active p38 MAPKs were mostly localized in the nuclei of all villus cells (Fig. 1A, see arrows), whereas the intensity of staining was significantly decreased in the crypt.

Activation of p38 MAPK during Differentiation of Intestinal Cells—Caco-2/15 cells that differentiate spontaneously to a small bowel phenotype after confluence (30, 34–37) were harvested at 70 (day −2) and 100% confluence (day 0), and 3, 6, 10, 16, 25, and 31 days post-confluence, and analyzed by Western blot to confirm timing of induction of sucrase-isomaltase pro-
Fig. 1. Expression of phosphorylated p38 MAPK in the human fetal intestinal epithelium. Frozen sections of fetal intestine at 20 weeks of gestation were stained with antibodies to phospho-p38 MAPK (A). Nuclei were stained with propidium iodide (B). The crypt-villus axis is oriented perpendicular to figures in both panels, with the crypt at the bottom. Along the crypt-villus axis, phosphorylated p38 staining was mostly detected in the nuclei of all villus epithelial cells (see arrows). Original magnification, ×97.

protein expression. Consistent with previous observations (30, 34, 37), sucrase-isomaltase protein levels significantly increased at 3 days post-confluence (Fig. 2A). Expression and kinase activity of p38α MAPK were also analyzed by immunoprecipitation. As shown in Fig. 2B, p38α abundance did not change with differentiation of Caco-2/15 cells. However, differential regulation of p38α kinase activity was observed during the differentiation of Caco-2/15 cells. As demonstrated in Fig. 2B, immunoprecipitated p38α exhibited very low basal activity in phosphorylating MBP in subconfluent growing Caco-2/15 cells, in contrast to a dramatic induction of p38α activity when cells reached confluence (day 0). This activation persisted during cell differentiation. Furthermore, Western blot analysis with an antibody recognizing the biphosphorylated and active p38 MAPK isoforms revealed that p38α phosphorylation significantly increased as soon as Caco-2/15 cells reached confluence (Fig. 2C). These results imply that p38α activation precedes the induction of sucrase-isomaltase, a differentiation marker. Of note, p38β protein was never detected in Caco-2/15 cells by Western blotting, and very low levels of RNA were detected by reverse transcriptase-PCR analysis.2 Thus, p38α MAPK activation may be functionally linked to intestinal differentiation.

Because Caco-2/15 cells are derived from a human colonic adenocarcinoma (59), we wanted to support our results in normal human small intestine-derived cells. We then analyzed the phosphorylation of p38 MAPK in normal human intestinal cell models as follows: the crypt-like HIEC cells that are proliferative and undifferentiated (38), and the PCDE cells that are primary cultures of differentiated and non-proliferative villus enterocytes (39). Cell lysates were prepared from subconfluent growing HIEC cells and from PCDE cells. As shown in Fig. 2D, phosphorylation of p38 was significantly lower in subconfluent growing HIEC cells compared with that found in PCDE cells. Hence, p38 MAPK exhibits similar patterns of activity if we compare pre- and post-confluent Caco-2/15 cells versus normal HIEC and PCDE cells that together allow the in vitro repro-duction of the normal crypt-villus axis (9).

p38 MAPK Is Not Involved in Cell Cycle Progression of Intestinal Epithelial Cells—An important early event in the terminal differentiation of cells, especially in tissues exhibiting a rapid turnover such as the intestinal epithelium, is their withdrawal from the cell cycle (60, 61). To evaluate the role of p38 MAPKs in intestinal cell cycle progression, SB203580 compound, a specific inhibitor of p38α/β MAPKs (62), was tested on dihydrofolate reductase (DHFR) expression in subconfluent Caco-2/15 and HIEC cells. The DHFR gene, which is required for DNA synthesis and is transcribed at the G1/S transition, contains E2F-dependent binding sites in the promoter. In addition, microinjection of E2F into quiescent fibroblasts provokes S phase re-entry, underscoring the importance of E2F in cell growth control (47). Therefore, the plasmid construction containing the E2F-responsive DHFR promoter linked to a luciferase reporter gene represents a sensitive reporter of cell cycle progression and S phase entry (46). When the p38α/β MAPKs were blocked with the SB203580 compound (2–20 μM), E2F-dependent luciferase expression was not significantly affected in either cell line (Fig. 3A). In contrast, the MEK1 inhibitor PD98059 significantly reduced E2F-regulated reporter gene expression by 50% in Caco/15 cells and by 81% in HIEC compared with control untreated cells. These results confirm our previous observations (30) whereby the MEK>p42/
p38 MAPK and Intestinal Epithelial Cell Differentiation

A Subconfluent Caco-2/15 and HIEC cells

B Confluent Caco-2/15

Fig. 3. Inhibition of p38 MAPK has no effect on cell cycle progression and survival of Caco-2/15 cells. A, subconfluent Caco-2/15 and HIEC cells (40–50% of confluence) were transfected with 0.1 μg of DHFR-luciferase reporter. One day after transfection, cells were exposed to 2–20 μM SB203580 (SB) or 20 μM PD98059 (PD) for 24 h, lysed (the cells were still not confluent), and luciferase activity measured. The increase in luciferase activity was calculated relative to the MeSO (DMSO) level of DHFR-luciferase, which was set at 1. Results are the mean ± S.E. of at least three separate experiments. *, significantly different from control at p < 0.05 (Student’s t test). B, confluent Caco-2/15 cells (day 0) were treated with or without 20 μM SB203580 for 3 and 6 days and lysed, and proteins were separated by 10% SDS-PAGE. Phosphorylation of pRb proteins was analyzed with specific antibodies against the active (lower band) as well as the inactive (upper bands) forms of the proteins. PARP cleavage was analyzed by Western blotting as described under “Experimental Procedures.” Similar results were obtained in two different experiments.

p44 MAPK cascade is required for intestinal cell cycle progression.

We recently demonstrated that Caco-2/15 cells slowed their cell cycle at confluence to become almost completely arrested in the G1 phase by day 6 post-confluence. Indeed, decreased phosphorylation of retinoblastoma proteins and reduced Cdk2 activity correlated with the induction of differentiation markers, namely sucrase-isomaltase and villin (61). To determine whether p38 MAPK activation plays a significant role in cell cycle arrest of confluent Caco-2/15 cells, the consequences of blocking p38 MAPK with SB203580 were examined on pRb phosphorylation by using specific antibodies detecting the active hypophosphorylated form of p105Rb protein (lower band) as well as the inactive hyperphosphorylated forms of the protein (upper bands). As shown in Fig. 3B, addition of SB203580 at days 0–6 post-confluence had no significant effect on the decrease in pRb phosphorylation observed at days 3 and 6 post-confluence suggesting that p38 MAPK is not involved in the loss of proliferative potential as committed intestinal cells begin to differentiate.

p38 MAPKs appear to play important roles in the regulation of cell survival in other cell types (63, 64). To verify the potential effect of the inhibition of p38 MAPKs on Caco-2/15 cell survival, expression of PARP, a well known substrate for caspase-3 (65), was measured in cells treated with SB203580. As shown in Fig. 3B, treatment of confluent Caco-2/15 cells with SB203580 had no effect on PARP cleavage, suggesting that persistent inhibition of p38 MAPK did not affect Caco-2/15 cell survival.

Inhibition of p38 MAPK Activity Prevents Enterocyte Differentiation—The dramatic induction of p38 MAPK activity led us to verify whether this MAPK was associated with differentiation of Caco-2/15 cells. Daily addition of SB203580 at confluence repressed sucrase-isomaltase protein expression in confluent Caco-2/15 cells (Fig. 4A). In addition, induction of villin expression was also decreased by 2–3-fold at days 3, 7, and 12 post-confluence. Equal protein loading was confirmed by using an anti-actin antibody (Fig. 4A). Enzymatic assays were also performed in order to verify the induction pattern of other differentiation markers, namely lactase, DPPIV, and alkaline phosphatase, in control and in SB203580-treated cells. As shown in Table I, treatment of confluent Caco-2/15 cells with 20 μM SB203580 did not significantly affect the induction of DPPIV at post-confluence, whereas induction of lactase and alkaline phosphatase was significantly attenuated (Table I).

The effect of p38 on sucrase-isomaltase gene expression was further analyzed by transiently transfecting newly confluent Caco-2/15 cells with the luciferase gene driven by the human sucrase-isomaltase promoter (45). As shown in Fig. 4B, sucrase-isomaltase gene expression was inhibited in a dose-dependent manner by the p38 inhibitor, SB203580, with maximal effect observed at 20 μM (72% inhibition). Furthermore, in contrast to wild-type p38a, ectopic expression of the dominant-negative mutant of p38a significantly reduced sucrase-isomaltase gene expression by 61%. Collectively, these results indicate that p38 activation is an early and necessary event for activation of the intestinal differentiation program, preceding the induction of various differentiation markers.

Of note, addition of SB203580 to differentiating Caco-2/15 cells at days 6–12 post-confluence still reduced sucrase-isomaltase expression by 47%, as observed at day 12 post-confluence (Fig. 4C). In addition, treatment of primary cultures of normal differentiated enterocytes (see “Experimental Procedures”) with 20 μM SB203580 also significantly reduced the expression of sucrase-isomaltase by 22 and 55% and villin by 38 and 57% after 2 and 4 days of treatment, respectively (Fig. 4D). Equal protein loading was confirmed by using an anti-keratin-18 antibody. These data suggest that p38 MAPK activity is required for maximal expression of sucrase-isomaltase in differentiating Caco-2/15 cells and differentiated normal enterocytes.

Transactivation Activity of CDX2/3 Is Down-regulated by Inhibition of the p38 Pathway—The dramatic effect of the p38 inhibitor on the expression of sucrase-isomaltase prompted us to investigate whether p38 also affected the activity of the transcription factor CDX2/3 (the protein designated CDX3 in the hamster and CDX2 in the mouse and humans (66)), a key activator of sucrase-isomaltase transcription (45) and an inducer of intestinal epithelial cell differentiation (18). We first examined whether inhibition of p38 in confluent Caco-2/15 cells affected the expression of CDX2. Northern blot analysis demonstrated that addition of SB203580 at days 0–6 post-confluence had no effect on CDX2 mRNA expression in confluent Caco-2/15 cells (Fig. 5A). Lysates from COS cells were used as negative control.
Previous studies revealed that p38 increases the transcriptional activity of various transcription factors by phosphorylation of the transactivation domain (67). To determine whether p38 had a similar effect on CDX2/3, fusion proteins were used containing the transactivation domain of CDX3 (amino acids 1–180) fused to Gal4 (DBD) (see “Experimental Procedures”). To assess the transcriptional activity of Gal4-CDX3 proteins, Caco-2/15 cells were co-transfected with a luciferase reporter gene containing five copies of a Gal4 DNA-binding site upstream of a minimal promoter and Gal4-CDX3 expression plasmids. As demonstrated above, inhibition of the p38 pathway by SB203580 significantly reduced CDX3-dependent reporter gene expression (Fig. 5B, upper panel) suggesting that p38 modulates the transcriptional activity of CDX3. In contrast, inhibition of the p42/p44 MAPK pathway with the PD98059 inhibitor had no effect on CDX3-dependent reporter gene expression (Fig. 5B, lower panel).

Effect of SB203580 on the DNA-binding Capacity of Transcription Factors Involved in Sucrase-Isomaltase Expression—There are three positive regulatory elements for transcription within the sucrase-isomaltase promoter region in intestinal epithelial cells known as sucrase-isomaltase footprint (SIF-1), SIF-2, and SIF-3. SIF-1 binds the intestine-specific homeodomain transcription factor CDX2/3 (17), whereas SIF-2 and SIF-3 bind the transcription factors HNF-1α and HNF1-β (68). In addition, a negative cis-acting element SIF-4 may be a binding site for the E4BP4 transcriptional repressor protein (11). Electrophoretic mobility shift experiments were performed to determine whether the DNA-binding capacity of these transcription factors was affected by SB203580. As shown in Fig. 6, binding of nuclear proteins to SIF-1 was not affected by using extracts prepared from SB203580-treated cells except for increased binding observed at day 6 post-confluence, which was reproducibly blocked by SB203580. Furthermore, binding of nuclear proteins to the SIF-3 and SIF-4 oligonucleotides was unchanged with extracts prepared from SB203580-treated cultures.

**CDX3 Specifically Interacts with p38 MAPK**—To determine whether CDX2/3 can directly associate with p38 MAPK, pull-down assays were performed using the GST-CDX3 fusion protein to absorb naïve newly confluent Caco-2/15 cell lysates. The absorbed material was analyzed by Western blot with the p38 antibody. Immunoprecipitated p38 from newly confluent Caco-2/15 cells was used as positive control. As shown in Fig. 7A, a significant amount of p38 MAPK bound to the GST-CDX3 fusion protein was detected. GST protein alone did not pull down the p38 protein (data not shown). Interestingly, the GST-CDX3 fusion did not pull down other MAPKs such as p42 MAPK or JNK1 (data not shown). To determine whether CDX3-p38 association may have some functional relevance, the capacity of pulled down p38 to phosphorylate the GST-CDX3 fusion protein was evaluated in a kinase assay. As shown in Fig. 7B, pull-down p38 efficiently phosphorylated the GST-CDX3 protein. More importantly, this phosphorylation was inhibited in a dose-dependent fashion by the addition of low concentrations of the specific p38 inhibitor SB203580 (50% inhibition with 0.5 μM SB203580). In this regard, an amino acid sequence analysis revealed that CDX2/3 contains putative phosphorylation sites for p38 (17, 67). An in vitro kinase assay using bacterially expressed GST-CDX3 protein or MBP as substrates and p38 immunoprecipitated from newly confluent Caco-2/15 cells revealed that p38 MAPK was able to potently phosphorylate MBP and, more importantly, phosphoryl-
ate GST-CDX3 to a significant level (Fig. 7C). This suggests that CDX2/3 may indeed be a specific target for p38 MAPK.

We further verified whether CDX3-p38 association could be detected in vivo. CDX3 was co-transfected into 293T cells with a plasmid encoding wild-type HA-p38α, and co-immunoprecipitations were performed on total cell lysates. As shown in Fig. 7D (lane 2), CDX3-p38α association was easily detected upon immunoprecipitation of HA-p38α in 293T cells. Parallel control experiments using non-transfected cells did not precipitate the CDX3 protein under similar conditions (Fig. 7D, lane 1).

EGF Represses p38 MAPK Activity and Sucrase-Isomaltase Expression in Differentiating Caco-2/15 Cells—Sucrase-isomaltase expression has been reported to be down-regulated by growth factors such as keratinocyte growth factor (69) and EGF (70). However, the mechanism involved in the repression of sucrase-isomaltase expression by growth factors is unknown. The effect of EGF was therefore examined on both sucrase-isomaltase protein expression and p38 activity. As shown in Fig. 8, chronic treatment (from days 0 to 15) of confluent Caco-2/15 cells with 100 ng/ml EGF repressed sucrase-isomaltase protein expression compared with untreated cells (over 95% inhibition). Of interest, treatment with EGF significantly and persistently down-regulated p38 MAPK activity with maximal effect observed after 2 and 4 h of treatment (Fig. 8).

Finally, the inhibitory action of EGF on sucrase-isomaltase expression was not attributable to the re-activation of the p42/p44 MAPK pathway since the specific MEK inhibitor, PD98059, blocked p42/p44 MAPK activation but did not interfere with the repressive effect of EGF on sucrase-isomaltase expression.

**DISCUSSION**

The molecular mechanisms orchestrating cellular transitions and changes in gene expression during intestinal epithelial differentiation are largely unknown. In this report, we suggest for the first time the possible involvement of p38 MAPK in intestinal cell differentiation. We show that the activity of p38 was induced as soon as Caco-2/15 cells reached confluence and began differentiating into a small bowel-like phenotype with microvilli formation and expression of disaccharidases. Furthermore, the nuclear localization of p38 MAPK activity in the villi, which is indicative of its functional role in transcription, reflects the distribution of differentiated cells. Inhibition of p38 activity by the specific inhibitor SB203580 did not interfere with cell cycle progression of committed cells but inhibited intestinal cell differentiation and expression of various differentiation markers (namely sucrase-isomaltase, alkaline phosphatase, villin, and lactase). The effects of p38 MAPK on sucrase-isomaltase transcription revealed a regulation of sucrase-isomaltase expression, an effect mediated by CDX2/3. The latter was demonstrated previously to be an important modulator of enterocyte differentiation (17–18, 68).

A key issue in intestinal development is what triggers the differentiation process. Members of the CDX family have been shown to be involved in enterocyte lineage specification (18, 71). Once specified, intestinal cells continue to proliferate until they receive a differentiation signal that has yet to be identified. However, in vitro cell culture experiments have shown that cell-cell contact can trigger differentiation and therefore substitute the in vivo signal. In Caco-2 cells, the establishment of cell-cell contact is a critical step initiating both cell cycle exit and induction of the differentiation process (30, 31, 34–37, 59, 61, 72). Indeed, as with various clones of Caco-2 cell line (35, 59, 72), the Caco-2/15 clone has been extensively characterized for its ability to differentiate gradually between days 0 and 20 of post-confluence (34, 36, 37, 41). For instance, sucrase-isomaltase transcription increases as soon as Caco-2/15 cells reach confluency (73). In this regard, junctional cell interactions play an important role in the control of cell differentiation during intestinal ontogeny and the continuous cell renewal of the mature organ (74, 75). Our results illustrate a pathway by which cell-cell contacts can modulate enterocyte differentiation through activation of a distinct MAPK, the p38 MAPK. With respect to this, it has been observed that the p38 pathway is more efficiently activated in confluent muscle cells than in subconfluent myocytes cultured under the same conditions (33). Furthermore, we have recently shown that in contrast to p38 MAPK, p42/p44 MAPK (30) and p46/p54 JNK3 activities dramatically decreased as soon as Caco-2/15 cells reached confluence and began to differentiate. Hence, persistent activation of p38 in differentiating Caco-2/15 cells, in the absence of a parallel JNK activation, distinguishes this pathway from those activated in response to stress or cytokines (24, 27–29, 76). Similar observations were recently reported in differentiating muscle cells (33) and in PC12 (77). Recent data have shown that assembly of E-cadherin-mediated adherens junctions is sufficient to trigger the activation of the PI3-kinase/Akt (78) and p42/p44 MAPK cascades in renal epithelial cells (79). However, the exact mechanisms through which cell-cell contacts activate the p38 MAPK pathway in intestinal epithelial cells remain to be determined. Cell-cell contacts may stimulate the p38 pathway by silencing the activity of a mitogen-dependent factor (e.g. a phosphatase). All together, these observations underscore the importance of cell density in the activation of p38 during cell differentiation.

Members of the CDX family act within a regulatory network that establishes the differentiated phenotype of intestinal epithelial cells. Indeed, these homeobox proteins activate the expression of many intestine-specific genes (68). p38 MAPK appears to be a potential activator of CDX2/3 and could control intestinal differentiation. The fact that CDX2/3 plays a role in mediating p38 function in the activation of sucrase-isomaltase transcription and enterocyte differentiation is suggested by the

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**TABLE I**

Modulation of cell differentiation by SB203580 in Caco-2/15 cells

| Treatment | Day 0 | Day 3 | Day 6 | Day 9 |
|-----------|-------|-------|-------|-------|
| Alkaline phosphatase | ME_SO | 13.1 ± 2.3 | 22.4 ± 1.9 | 31.5 ± 2.9 | 35.6 ± 3.1 |
| SB203580 | 14.2 ± 1.7<sup>a</sup> | 18.5 ± 3.1<sup>a</sup> | 21.1 ± 1.5<sup>a</sup> | |
| Lactase | ME_SO | 3.9 ± 0.8 | 5.0 ± 1.2 | 7.1 ± 1.6 | 7.6 ± 1.4 |
| SB203580 | 4.1 ± 1.3 | 5.9 ± 1.1<sup>a</sup> | 6.1 ± 0.9<sup>a</sup> | |
| DPPIV | ME_SO | 402 ± 51 | 567 ± 84 | 689 ± 58 | 789 ± 74 |
| SB203580 | 549 ± 49 | 671 ± 47 | 759 ± 38 | |

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<sup>a</sup> Significantly different from control (ME_SO) at p < 0.1 (Student’s t test).

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<sup>3</sup> M. Houde, P. Laprise, and N. Rivard, unpublished data.
1) the inhibition of p38 activity with the SB203580 inhibitor repressed CDX3-induced sucrase-isomaltase gene transcription in a dose-dependent manner; 2) the transcriptional activity of a Gal4-CDX3 fusion protein was reduced by SB203580; 3) similar to CDX2 (11), p38 MAPK activity was primarily localized in the nucleus of villus cells; 4) CDX3 specifically associated with p38 MAPK both in vitro and in vivo. p38 MAPK could be targeted to the transcription factor CDX2/3 by a docking domain that is distinct from the phosphoacceptor motifs. In fact, we have found a docking domain for p38 homologous to the general consensus sequence (Arg/Lys)-Xaa-Xaa-Xaa-Xaa-(Leu/Ile)-Xaa-(Leu/Ile) (67) and localized between amino acids at positions 19–26 of the transactivation domain of CDX2 (17) and CDX3 proteins (48).

Recently, we reported that p42/p44 MAPK activity was repressed during the differentiation of Caco-2/15 cells (30). How-
Fig. 7. Specific interaction of CDX2/3 proteins with p38 MAPK.
A, CDX3 associates with p38 kinase in vitro. Lysates from newly confluent Caco-2/15 cells were prepared and incubated with 4 μg of p38 antisera bound to protein A-Sepharose (Ip α-p38) or 5 μg of GST alone (not shown) or with purified GST-CDX3 bound to glutathione-Sepharose (pull-down GST-CDX3). The beads were washed and resuspended in SDS sample buffer, and the bound material was transferred onto nitrocellulose after SDS-PAGE. Immunological detections were performed using antibodies recognizing p38α MAPK, B, p38 phosphorylates CDX3 in a pull-down assay. Lysates from newly confluent Caco-2/15 cells were prepared and incubated with 5 μg of GST alone (not shown) or with purified GST-CDX3 bound to glutathione-Sepharose. The beads were washed four times with lysis buffer followed by three times with ice-cold kinase buffer before performing the kinase assay in the presence or in the absence of 0.1-20 μM SB203580. The kinase activity is demonstrated by the phosphorylation of GST-CDX3. Similar results were obtained in three different experiments. C, phosphorylation of CDX3 by immunoprecipitated p38 MAPKs in an in vitro kinase assay. Kinase assays were performed for 30 min at 30 °C with 1 or 5 μg of GST-CDX3 or 2 μg of MBP, as described under Experimental Procedures. Similar results were obtained in three different experiments. D, co-immunoprecipitation of CDX3 with HA-p38. Co-transfection of CDX3 and HA-p38ex expression vectors was performed in 293T cells. 24 h after transfection, cells were lysed. Immunoprecipitations (Ip) of HA-p38ex were performed using antibody against HA and were analyzed by SDS-PAGE followed by electrotransferred onto nitrocellulose. Immunological detections were performed with an antibody against CDX3 or an antibody against HA. Immunoblots of total lysates show the levels of HA-p38 and CDX3 expression. Similar results were obtained in three different experiments.

ever, significant levels of activated MAPK were detected in differentiated Caco-2/15 cells, predominantly p42 MAPK. We demonstrated that 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 is involved in the regulation of sucrase-isomaltase expression in Caco-2/15 cells. However, our data suggest that the p42/p44 and p38 MAPK pathways also exhibit distinct activities. First, p42/p44 MAPK and p38 activi-
MEF2, and CDX2/3 in adipocyte, muscle, and intestinal cell precursors. Although further studies are needed to pinpoint the upstream pathways activating p38 in committed cells induced to differentiate, our study provides novel fundamental insights into the function of p38 in the early events of intestinal epithelial differentiation.

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