The CDX2 transcription factor regulates furin expression during intestinal epithelial cell differentiation

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The CDX2 transcription factor regulates furin expression during intestinal epithelial cell differentiation. Am J Physiol Gastrointest Liver Physiol 290: G310–G318, 2006. First published October 20, 2005; doi:10.1152/ajpgi.00217.2005.—CDX2, a member of the caudal family of transcription factors, is involved in enterocyte lineage specification. CDX2 activates many intestine-specific genes, such as sucrase-isomaltase and lactase-phlorizin hydrolase (LPH), and adhesion proteins, namely, E-cadherin and claudin-2. In this study, we show that the proprotein convertase furin, involved in proteolytic maturation of proprotein substrates including LPH and cell surface proteins, is a CDX2 target. Indeed, expression of the rat furin homolog was induced 1.5-fold, as determined by microarray experiments that compared control with CDX2-expressing intestinal epithelial cells (IEC-6). As determined by transient transfection assays in Caco-2/15 cells, the furin P1 promoter 1.3-kb fragment between SacI and NheI was essential for CDX2 transcriptional activation. Electrophoretic mobility shift/supershift assays followed by site-specific mutagenesis and chromatin immunoprecipitation identified the CDX DNA-binding site (CBS)2 sequence from nt −1827 to −1821 as the major CBS involved in furin P1 promoter activation. Increased furin mRNA and protein expression correlated with both CDX2 expression and intestinal epithelial cell differentiation. In addition, furin mRNAs were detected predominantly in differentiated epithelial cells of the villus, as determined by in situ hybridization. Treatment of Caco-2/15 cells with a furin inhibitor led to inhibition of LPH activity. Morphological differentiation of enterocyte-like features in Caco-2/15 such as epithelial cell polarity and brush-border formation were strongly attenuated by furin inhibition. These results suggest that CDX2 regulates furin expression in intestinal epithelial cells. Furin may be important in modulating the maturation and/or activation of key factors involved in enterocyte differentiation.

enterocyte; intestinal epithelium; convertase

THE CDX2 AND CDX3 HOMEO PROTEINS (the protein designated CDX3 in the hamster and CDX2 in the mouse and humans) are members of the caudal-related homeobox gene family of transcription factors. A growing number of studies have suggested that CDX2/3 is important in a broad range of functions from early differentiation to maintenance of the intestinal epithelial lining of both the small and large intestine. In this regard, CDX2/3 is involved in the transcriptional regulation of multiple genes expressed in the intestinal epithelium including sucrase-isomaltase and lactase-phlorizin hydrolase (LPH), among others (16). In vitro, CDX2/3 expression negatively regulates the proliferation of intestinal epithelial and colon cancer cells while promoting the acquisition of a mature enterocyte phenotype consisting of a polarized, columnar shape with apical microvilli and tight junctions (36, 51). In support of in vitro observations, CDX2+/− heterozygous mice develop hamartomas or polyps containing heterotopic intestinal tissue in the colon (5). However, despite the obvious role of CDX2 in the induction and regulation of enterocyte differentiation, little is known about the molecular mechanisms by which CDX2 achieves such functions.

Furin, a calcium-dependent serine protease, belongs to a family of mammalian processing enzymes called proprotein convertases (PCs). These Ca++-dependent serine endoproteases cleave mostly COOH terminal to R-R or K-R pairs of basic amino acids and share overlapping cleavage site specificity and tissue distribution (46, 49). Furin is mostly concentrated in the trans-Golgi network and can recycle from the cell membrane to endosomes (46). Furin processes many proproteins including transforming growth factor-β (TGF-β) (10), BMP-4 (8), the insulin receptor (3), the Notch1 receptor (35), and the cell adhesion protein E cadherin (42, 43) as well as several metalloproteases (40, 58), among others. Furin catalyzes the proteolytic maturation of intestinal specific proteins such as LPH (39) and mucin 2 (57). The fur gene, which encodes furin, is expressed in all tissues and cell types examined to date but in variable amounts (49). In most cell types, furin expression is driven by three distinct promoters known as P1, P1A, and P1B (1). The fur transcripts differ in their 5′-end but are all translated from the same AUG, giving rise to identical furin proteins. The P1A and P1B promoters resemble housekeeping genes with multiple Sp1 binding sites. On the other hand, the P1 promoter bears inducible gene features, with the presence of TATA and CAAT elements in the proximal region, and is transactivated by C/EBPβ, GATA-1, SMADs, and HIF-1 (1, 9, 31, 37).

In this report, we show that furin is a novel target of CDX2 in intestinal epithelial cells. Indeed, the fur gene P1 promoter is induced by CDX2 through a specific CDX2-DNA binding element. Furin expression is significantly enhanced during differentiation of intestinal epithelial cells. Finally, inhibition of proteolytic activity of furin alters the maturation of LPH and severely impairs morphological differentiation of intestinal epithelial cells.

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MATERIALS AND METHODS

Cell culture. The rat intestinal epithelial crypt cell line IEC-6 (44) was grown in DMEM (Invitrogen, Burlington, ON, Canada) supplemented with 5% FBS. The human colonic carcinoma cell line Caco-2/15 (4) (A. Quaroni, Cornell University, Ithaca, NY) was cultured in DMEM containing 10% FBS. This cell line provides a unique and well-characterized model for the study of gut epithelial differentiation because these cells undergo differentiation to a small bowel-like phenotype with microvilli, dome formation, and the expression of sucrase-isomaltase several days after reaching confluence (4, 32).

Human embryonic kidney (HEK)-293 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% FBS.

Retroviral constructs and infection. The coding sequence for CDX2 (51) was subcloned in the retroviral vector pBabe-puro. HEK-293 cells were transfected by lipofection (Lipofectamine 2000, Invitrogen) with the retroviral vectors and helper amphotropic DNA, as described previously (15). IEC-6 cells at 60% confluence were infected for 24 h with the control (empty vector) or the CDX2 retroviral supernatants in the presence of 8 μg/ml polybrene (Sigma-Aldrich Canada, Oakville, ON, Canada). One day after infection, IEC-6 cells were divided and selected in medium containing 2 μg/ml puromycin (Sigma-Aldrich Canada), as described previously (15).

Microarray analysis. Total RNAs were extracted from IEC-6 cells stably expressing empty vector (pBabe-puro) or CDX2 with the RNeasy kit (Qiagen, Mississauga, ON, Canada). For microarray analysis, 10 μg of RNA were used for cDNA synthesis, followed by in vitro transcription to generate biotin-labeled cDNAs with a T7 promoter primer having a poly(T) tail for subsequent hybridization. The resulting product was hybridized and processed with the Rat Genome RAE230 2.0 Array GeneChip system (Affymetrix). Three independent experiments were done for each condition. Data analysis, normalization, average difference and expression for each feature on the chip were performed using Affymetrix Microarray Suite 5.0 (MAS5) with default parameters (Microarray platform, McGill University and Genome Quebec Innovation Centre). Gene classification according to cellular processes was performed with the “Database for Annotation, Visualization, and Integrated Discovery” (DAVID: http://david1.nci.nih.gov/david/).

DNA constructions. The human fur promoter luciferase constructs pGL2-P1, pGL2-P1-SacI, pGL2-P1-NheI, pGL2-P1-KpnI, pGL2-P1-A, and pGL2-P1-B were generously provided by Dr. T. A. Y. Ayoubi (University of Leuven and Flanders Interuniversity). A PCR-amplified P1-truncated promoter between the SacI and NheI restriction sites was subcloned in the KpnI and NheI sites of the pGL3 promoter vector (Promega, Madison, WI), with the following oligonucleotides: FurKpnI 5'-TGG GTA GGT ACC TTA GGA TCT GAA TTT CTA TCA TAA ACT GGC-3' (upstream, nt -2647 to -2615) and FurNheI 5'-GAG ACA GCT AGC AGT GAG GGA AAC TCA AAC ACT G-3' (downstream, nt -1305 to -1340). Deletions of the putative CDX2 binding motifs in the P1-Sacl/NheI pGL3 construct were done by PCR amplification. Deletion of the -2625 to -2619 region [CDX DNA-binding site (CBS)1] (–2625 CATAAAC –2619) was performed with the following oligonucleotides: FurCBS1 5'-TGG GTA GGT ACC TTA GGA TCT GAA TTT CTA TCA TAA ACT GGC-3' (upstream, nt -2654 to -2610) and FurNheI primer. Deletion of the -1827 to -1821 (CBS2) (–1827 ATTATTT –1821) and -1705 to -1699 (CBS3) (–1705 TATAAAC –1699) regions was performed by overlap extension, as described before (52). The upstream amplification was performed with the FurKpnI oligonucleotide and either the FurΔCBS2R 5'-CCA TTT CTT TCG TGA AGG GCC GCG TCG TGT AAT A-3' (nt -1807 to -1844) or FurΔCBS3R 5'-CTT TAA TTC ATC CAT GGC GTG TGT CAG TTA TAC C-3' (nt -1684 to -1721) oligonucleotides, and the downstream amplification with the FurΔCBS2F 5'-TAC TAC AGT GAC CCC TAC ATC AAA GAA ATG G-3' (nt -1844 to -1807) or FurΔCBS3F 5'-GTT ATA ACT GAC AAA CTT CTG CAA TTA GGA T-3' and the FurNheI primer. Products resulting from these two PCR were used as DNA templates for the final PCR using the FurKpnI and FurNheI oligonucleotides. The PCR fragments were then cloned into the pGL3 promoter vector upstream of the luciferase gene. The presence of the mutations was verified by sequence analysis (University of Sherbrooke sequencing services, Sherbrooke, QC, Canada).

Transient transfections and luciferase assays. Caco-2/15 cells, at 70% of confluence, were seeded in 24-well plates for 24 h. The next day, 1 h before transfection, complete DMEM was aspirate, cells were washed twice with warm PBS, and 500 μl of Opti-MEM media (Invitrogen), free of antibiotic and antimycotic, were added to each well. Cells were cotransfected using Lipofectamine 2000 with 0.1 μg pBabe (control) or 0.1 μg pBABI/CDX3 expression vector (14, 21) and 0.1 μg of the different P1 furin promoter constructs. After 8 h of transfection, the Opti-MEM media was replaced with the complete DMEM described above. Two days after transfection, luciferase activity was measured as described before (21). Results are expressed as fold induction compared with control (empty vector) values.

Electrophoretic mobility shift assays. Nuclear extracts were prepared as previously described (41). Samples were separated in a 4% polyacrylamide gel containing 0.5% Tris borate and 2% glycerol, as described previously (41). Four potential CDX2 binding sites were identified by comparison of the P1–SacI/NheI promoter sequence to CDX2 consensus DNA-binding sites (A/C)TTTAT(A/G) (13). The following double-stranded oligonucleotides were tested for their ability to bind CDX2: fur CBS1, 5'-GAG AAC ACC TCT TTA TCA AAG GAA A-3' (nt –1836 to –1810); fur CBS3, 5'-GAG TCA CAC ATC ATA AAC GGC GAT TGA T-3' (nt –1715 to –1689); and fur CBS4, 5'-GCA AAT ACG TCT TTA ATG CTA CAA GGA C-3' (nt –1541 to –1515). Murine monoclonal CDX2 antibody (BioGenex Laboratories, San Ramon, CA) or mouse IgG (Upstate, Charlottesville, VA) were used for supershift assays, as described previously (41).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit protocol (Upstate). Confluent Caco-2/15 cells were cross linked with 1% formaldehyde for 10 min at 37°C. Chromatin was immunoprecipitated without or with a rabbit polyclonal antibody against CDX2 (BioGenex Laboratories). Ten percent of the lysate was kept to verify the amount of DNA used for each immunoprecipitation. Immunoprecipitated DNA was purified and diluted 1:50 before PCR amplification with furin 5'–(−1515 to −1932), 5'-CTTCTTACGGCATTITTTT-3'; furin 3'–(−1784 to −1764), 5'-TGCTTGAAGGGCATTATTTT-3'; SI 5'–(−219 to −195), 5'-AATGTCTCAGCCTTGAGAATAC-3'; SI 3'–(−23 to −3), 5'-GATCAAGGAAAGCTGTTGAGG-3'; IL-8 5'–(−121 to −103), 5'-GGGCCATCGATGGCAATC-3'; or IL-8 3'–(−62 to −42), 5'-CTTCTTACGGCATTITTTT-3'. The program used for amplification was first cycle of 94°C for 5 min (hot start) followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final cycle of 72°C for 10 min. Amplified PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Northern blot analysis. Total cellular RNAs were prepared with the TRizol reagent (Invitrogen) from IEC-6 pBabe or pBabe/CDX2-infected cell populations or from Caco-2/15 cells at −2, 0, 6, and 15 days postconfluence. RNAs were subjected to agarose gel electrophoresis with formaldehyde and transferred to nylon membranes (Biodyne B, Pall, Mississauga, ON, Canada). Equal RNA loading was confirmed by hybridization to a glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe. Hybridizations were performed with a random-primed 32P-labeled probe (Amersham Biosciences, Baie d’Urfé, QC, Canada) of a PCR-amplified rat and human furin fragment (nt 579 to 1158, NM_002569). Quantification of band intensity
was performed using Bio-Rad Quantity One software (Bio-Rad, Mississauga, ON, Canada).

**Immunoblotting.** IEC-6 cells stably expressing CDX2 and Caco-2/15 cells were lysed in Laemmli buffer. Proteins (40 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. CDX2 immunoblotting was performed with a 1:1,000 dilution of a murine monoclonal antibody against CDX2 (BioGenex Laboratories). This was followed by a 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as the secondary antibody. Furin immunoblotting was performed with a 1:1,000 dilution of a rabbit polyclonal antibody against furin (Santa Cruz Biotechnology). Poly (ADP-ribose) polymerase (PARP) immunoblotting was performed with 1:1,000 dilution of a mouse monoclonal antibody recognizing the 89-kDa apoptotic fragment and the 113-kDa noncleaved fragment of PARP (Dr. G. G. Poirier, Université Laval, Quebec, QC, Canada). In some experiments, subconfluent proliferating Caco-2/15 cells were treated with 50 μM R-roscovitine (Sigma-Aldrich Canada) for 1, 2, 4, 6, and 8 h before lysis. Chemiluminescence associated with specific protein bands was visualized with the enhanced chemiluminescence system (Amersham Biosciences). For normalization of the signal, the membranes were stripped and probed with a 1:5,000 dilution of a mouse monoclonal actin antibody (Chemicon International, Temecula, CA) and a 1:1,000 dilution of HRP-conjugated anti-mouse IgG as the secondary antibody. Densitometric analysis was performed using Bio-Rad Quantity One software.

**Human specimens.** Jejunums from human fetuses varying in age from 18 to 20 wk of gestation [postfertilization fetal ages were estimated according to Streeter (50)] 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.

**In situ hybridization.** T3 sense and T7 antisense oligonucleotides were used to amplify 238-bp fragments of human furin (nt 3309 to 3546, X17094.1). RNA was synthesized in vitro using T3 and T7 polymerase (Promega) according to specifications of the manufacturer. In situ hybridization was performed according to the Wilkinson procedure (56) modified by Lantz et al. (30). Signal detection was achieved using the digoxin (DIG) system following the instructions of the manufacturer (Roche Applied Sciences, Laval, QC, Canada). Briefly, jejunum from human fetuses varying in age from 18 to 20 wk of gestation were dissected and fixed 4 h in 4% paraformaldehyde at 4°C. Tissues were embedded in paraffin, from which 5-μm sections were generated and spread onto glass slides. The sections were rehydrated, deparaffinized, treated with 10 mg/ml proteinase K for 4 min, postfixed in 4% paraformaldehyde for 5 min, acetylated in 1 mM triethanolamide, 0.25% acetic anhydride for 10 min, and hybridized overnight at 50°C in hybridization solution containing 54% formamide, 5× SSC, 5× Denhardt’s solution, 0.25 mg/ml yeast tRNA, 0.5 mg/ml herring sperm DNA, and 300 ng/ml DIG-labeled riboprobe. Subsequent to hybridization, the first wash was performed at 50°C in 0.2× SSC for 1 h and 5 min at room temperature. Sections were blocked for 1 h in blocking buffer (1% blocking solution; Roche Applied Sciences) in 0.1 M maleic acid and 0.2 M NaCl (pH 7.5) followed by incubation with anti-DIG (1:1,500; Roche Applied Sciences) for 3 h. Slides were exposed to 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride (Roche Applied Sciences) until color reactivity was fully developed.

**LPH and alkaline phosphatase enzymatic activity.** Furin inhibitor I (decanoyl-RVKR-CMK) (EMD Biosciences, La Jolla, CA) was added to subconfluent Caco-2/15 cells for 24 and 48 h at a concentration of 50 μM. Cells were washed twice with ice-cold PBS and lysed in Triton buffer [150 mM NaCl, 1 mM EDTA, 40 mM Tris (pH 7.6), 1% Triton X-100, 0.1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 0.1 mM orthovanadate, and 40 mM β-glycerophosphate]. Proteins were quantified, and enzymatic activity was determined for LPH according to the method of Dahlqvist as modified by Lloyd and Whelan (34). Alkaline phosphatase activity was assayed with paranitrophenylphosphate as a substrate (11) as previously described (38).

**Electron microscopy.** Cell cultures were rinsed with PBS, prefixed for 15 min with a 1:1 mixture of culture medium (DMEM) and freshly prepared 2.8% glutaraldehyde in cacodylate buffer (0.1 M cacodylate-7.5% sucrose), and then fixed for 30 min with 2.8% glutaraldehyde at room temperature. After two rinses, specimens were postfixed for 60 min with 2% osmium tetroxide in cacodylate buffer. The cells were then dehydrated using increasing ethanol concentrations (40%, 70%, 90%, 95%, and 100%), three times each, and then covered twice for 3 h with a thin layer of Araldite 502 resin (for ethanol substitution). Finally, the resin was allowed to polymerize at 60°C for 48 h. The specimens were detached from the plastic vessels, inverted in embedding molds, covered with Araldite 502, and reincubated at 60°C for 48 h. Thin sections were prepared using an ultramicrotome, contrasted with lead citrate and uranyl acetate, and observed in a blind fashion on a JEOL 100 CX transmission electron microscope. All reagents were purchased from Electron Microscopy Sciences (Cedarlane, Hornby, ON, Canada).

**Statistical analysis.** Typical Western and Northern blot representatives of three independent experiments performed are shown. Denitometric analyses performed on each blot were performed using Bio-Rad Quantity One software. Luciferase results are means ± SE of at least four independent experiments performed in triplicate. Data were analyzed by one-way ANOVA with Student’s post hoc test.

**RESULTS**

**CDX2 positively regulates furin expression through the P1 promoter.** To understand the role of CDX2 in intestinal epithelial cells, we analyzed by microarray the pattern of gene expression in IEC-6 cells overexpressing CDX2. We first generated IEC-6 cell populations overexpressing CDX2 by retroviral infection. This intestinal epithelial crypt cell line does not express endogenous CDX2, but ectopic expression of CDX2 is sufficient to promote the acquisition of a mature enterocyte phenotype consisting of a polarized, columnar shape with apical microvilli and sucrase-isomaltase expression (Ref. 51 and unpublished observations). Results from microarrays comparing control (empty vector) with CDX2-expressing IEC-6 cells identified furin (psck-3) as a potential target of CDX2. Indeed, furin expression (psck-3) was significantly induced more than 1.5-fold (P < 0.05) in cells overexpressing CDX2 (data not illustrated).

Computer-assisted analysis of the furin promoters uncovered the presence of several putative CDX binding elements. These observations prompted us to investigate the regulation of the fur gene by the CDX2 transcription factor. Furin expression is regulated by three distinct promoters: P1, P1A, and P1B (1). We thus assessed the transcriptional activity of CDX2 on the three furin-promoter luciferase reporter constructs in the intestinal epithelial cell line Caco-2/15 by transient transfection followed by luciferase assays. Although the P1A and P1B promoters were induced, respectively, 6-fold (P < 0.01) and 2-fold (P < 0.01), transcriptional activity from the P1 promoter was increased 11-fold by CDX2 (Fig. 1; P < 0.001). Similar to P1 and P1A promoters, sucrase-isomaltase promoter transcriptional activity was markedly induced eightfold following CDX2 expression. These results suggest that CDX2, a key activator of sucrase-isomaltase transcription (53), may also regulate furin gene expression in intestinal epithelial cells.

To determine the P1 promoter sequences required for CDX2-dependent induction, we compared the transcriptional
Fig. 1. CDX2/3 positively regulates furin (fur) expression through the P1 promoter. Subconfluent Caco-2/15 cells were transiently transfected with the P1, P1A, and P1B furin promoter-luciferase constructs, the P1 furin promoter-luciferase deletion constructs (P1SacI, P1NheI, P1KpnI), or the sucrase-isomaltase promoter-luciferase, and the empty (control) or CDX3-expressing vector. Cell extracts were assayed by luciferase activity after 48 h. Luciferase activity is expressed as the fold increase relative to the activity of the empty vector control. Results are the means ± SE of at least 4 independent experiments performed in triplicate. ***P < 0.001 and **P < 0.01 compared with control; §§§P < 0.001 compared with P1; ††P < 0.01 compared with P1SacI.

Fig. 2. The CDX DNA-binding site (CBS)2 DNA-binding site is important for CDX2-dependent regulation of furin expression. A: schematic representation of the fur P1 promoter (top) and the minimum fur P1SacI-NheI promoter (bottom). B: nuclear extracts from confluent Caco-2/15 cells were incubated with the putative 32P-labeled CDX2/3 DNA-binding sites CBS1, CBS2, CBS3, and CBS4 with normal murine IgG (–) or with antibodies against CDX2 (+) for electrophoretic mobility and supershift assays. DNA-protein complexes were separated from the free probe on a native polyacrylamide gel. The results are representative of 2 independent experiments. C: SacI to NheI fragment of the furin P1 promoter and the site-specific deletions of the P1 promoter CBS1, CBS2, and CBS3 CDX2 DNA-binding sites were cloned in the pGL3-promoter luciferase vector. Subconfluent Caco-2/15 cells were transiently transfected with the P1-SacI-NheI, ΔCBS1, ΔCBS2, and ΔCBS3 furin promoter-luciferase constructs and the empty (control) or CDX3-expressing vector. Cell extracts were assayed by luciferase activity after 48 h. Luciferase activity is expressed as the fold increase relative to the activity of the empty vector control. Results are means ± SE of at least 4 independent experiments performed in triplicate. ***P < 0.001 compared with control and ††P < 0.01 compared with P1SacI-NheI.
activity of the P1 promoter (3.7 kb upstream of exon 1) to P1SacI (2.6 kb upstream), P1NheI (1.3 kb upstream,) and P1KpnI (500 bp upstream) deletion mutants (1). Although the P1NheI and P1KpnI promoters were induced about threefold, transcriptional activity from the P1SacI promoter was increased ninefold by CDX2 (Fig. 1; \(P < 0.001\)). These data indicate that the P1 promoter 1.3-kb fragment between SacI and NheI is essential for CDX2 transcriptional activation.

The CBS2 DNA-binding site is important for CDX2-dependent regulation of furin expression. We identified by computer analysis four putative CBS with the consensus sequence (A/ C)TTTAT(A/G) within the SacI and NheI P1 promoter 1.3-kb fragment, between nt −2625 to −2619 from the furin exon 1 transcription start site (CATAAAC; CBS1), nt −1827 to −1821 (ATTATT; CBS2), nt −1705 to −1699 (TATAAAC; CBS3), and nt −1532 to −1526 (CTTATA; CBS4) (Fig. 2A). Electrophoretic mobility shift and supershift assays of 32p-labeled double-stranded oligonucleotides with nuclear extracts from Caco-2/15 cells showed that CDX2 interacted with the CBS1, CBS2, and CBS3 DNA-binding sites (Fig. 2B). To determine the importance of these CBS2 DNA-binding sites for furin expression, we cloned the SacI and NheI P1 promoter 1.3-kb fragment in the pGL3-promoter-luciferase construct and generated by PCR 6-bp deletions of the consensus CDX2-responsive elements. Although deletion of the CBS1 or CBS3 DNA-binding site did not alter significantly CDX2 transcriptional activity, deletion of the CBS2 site abolished CDX2-dependent activation, as assessed by transient transfection and luciferase assays (Fig. 2C).

To confirm whether the CBS2 region bound CDX2 in vivo, we performed ChIP assays with confluent Caco-2/15 cell cross-linked chromatin and the CDX2 antibody. Although CDX2 binding to the IL-8 promoter region was not detected, both the CDX2-binding sucrase-isomaltase promoter region and the CBS2-containing furin promoter bound CDX2 (Fig. 3). Thus CBS2 is the major CBS2 DNA-binding site involved in furin P1 promoter transcriptional activation.

Furin expression correlates with increased CDX2 expression during enterocyte differentiation. Caco-2/15 cells, which spontaneously differentiate into an enterocyte phenotype after confluence (6, 21), were harvested at different times of confluence to determine the timing of CDX2 and furin expression during cell differentiation. Consistent with previous observations (6), CDX2 protein levels were significantly increased when Caco-2/15 reached confluence (day 0). This induction was sustained during differentiation (Fig. 4A). Notably, furin protein levels were markedly enhanced in confluent and postconfluent differentiating Caco-2/15 cells (Fig. 4A) as well as furin mRNA levels with a 4.6-fold induction (\(P < 0.01\)) after 15 days of postconfluence (Fig. 4B). In addition, both furin mRNA expression (2-fold, \(P < 0.01\); Fig. 4D) and protein levels (Fig. 4C) were increased in IEC-6 cells overexpressing CDX2. Of note, significant levels of furin mRNA and protein were detected in subconfluent Caco-2/15 cells and in empty vector-transfected IEC-6 cells, indicating that factors other than CDX2 may also control and drive furin expression in undifferentiated intestinal epithelial cells.

We (6) and others (22, 26, 48) have demonstrated a gradient of CDX2 protein expression along the human and mouse small intestinal crypt-villus axis, with higher levels being detected in differentiated villus epithelial cells (6). The pattern of furin mRNA expression was therefore analyzed in normal epithelial cells along the crypt-villus axis of the midgestational human fetal jejunum. In situ hybridization revealed that furin mRNAs were detected in all enterocytes along the human crypt-villus axis (Fig. 4E, right vs. left). However, similar to CDX2 protein staining (6), the intensity of the furin mRNA staining was consistently weaker in the lower portion of the crypt than in the remaining epithelium (Fig. 4E, right). We have recently demonstrated that inhibition of Cdk2-associated complexes in Caco-2/15 cells prevented CDX2 degradation and led to an accumulation of CDX2 protein into the nucleus (6). We therefore examined the effect of the Cdk2 inhibitor R-roscovitine on CDX2 and furin expression. As shown in Fig. 4F, treatment of proliferating Caco-2/15 cells with R-roscovitine led to an increase in expression levels of CDX2 and furin proteins comparatively with nontreated Caco-2/15 cells at the same time points (data not shown). Taken together, these data show a clear correlation between levels of expression of furin mRNAs and CDX2 protein and enterocyte differentiation.

Furin regulates LPH maturation during enterocyte differentiation. Our data suggest that furin expression is induced by CDX2, a positive regulator of intestinal cell differentiation (16). We therefore hypothesized that furin may be involved in some aspects of intestinal epithelial cell differentiation. Of interest, furin is involved in the posttranslational maturation of LPH (39) and mucin 2 (57), two differentiation-specific intestinal targets. However, these experiments have been performed in heterologous cells such as COS-7 cells. We therefore analyzed the effect of an irreversible furin inhibitor, decanoyl-RVKR-CMK (17), on the enzymatic activity of LPH, induced during Caco-2/15 cell differentiation. Treatment of preconfluent Caco-2/15 cells with the furin inhibitor for 48 h significantly decreased LPH specific activity by 40% (Fig. 5). This
effect appears specific to LPH because furin inhibitor treatment did not alter alkaline phosphatase activity (Fig. 5).

Furin activity is essential for morphological differentiation of intestinal epithelial cells. To further investigate the role of furin in intestinal epithelial cell differentiation, we evaluated the impact of its inhibition on morphological differentiation of Caco-2/15 cells. Caco-2/15 cell cultures were characterized by transmission electron microscopy 12 days after confluency. As shown in Fig. 6, 1 and 3, arrows, postconfluent Caco-2/15 cells exhibited ultrastructural characteristics similar to those found in the intact villus epithelium, including well-organized brush borders, terminal webs at the luminal aspect of absorptive cells, as well as typical junctional complexes. Interestingly, treatment of confluent Caco-2/15 cells with the furin inhibitor remarkably affected cell polarization and brush border formation. Indeed, the treated cells seemed to exhibit a less-polarized phenotype compared with untreated cells (Fig. 6, 1 and 2). More obviously, the morphology of the brush border was altered markedly, as visualized by a reduction in microvilli number (Fig. 6, 1 and 2, arrows). In addition, apical junction complexes were less developed and less defined in these cells (Fig. 6, 3 and 4, arrows). Again, these effects were not due to an increase in apoptosis because neither DNA fragmentation nor detached cells were observed in decanoyl-RVKR-CMK-
treated Caco-2/15 cells (data not shown). Taken together, these results indicate that furin activity is necessary for morphological differentiation of intestinal epithelial cells.

**DISCUSSION**

Intestinal epithelial cell maturation along the crypt-villus axis involves different signals that contribute to phenotypic changes associated with enterocytes transitioning between a proliferative state (crypt and lower villus) and cells undergoing differentiation and polarization. Members of the CDX family of transcription factors have been linked to enterocyte lineage specification (13). CDX2/3, in particular, is mainly expressed in differentiating enterocytes (6, 26, 48). CDX2/3 overexpression triggers cell differentiation in several intestinal cell lines in vitro (36, 51). Furthermore, genes regulated by CDX2/3 such as sucrase-isomaltase (51), glucagon (24), LPH (12), and, more recently, LI-cadherin (19) and claudin-2 (45), generally define a functional differentiated phenotype.

In this report, we have shown that the PC furin is a novel CDX2 transcriptional target in intestinal epithelial cells. Indeed, the furin P1 promoter is transactivated by CDX2. CBS2, a P1 promoter specific CDX2 DNA-binding site is essential for P1 promoter activation. The CBS2 site is juxtaposed to a

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Fig. 5. Furin regulates lactase-phlorizin (LPH) activity during enterocyte differentiation. Subconfluent Caco-2/15 cells were treated with furin inhibitor I (decanoyl-RVKR-CMK, 50 μM) or DMSO (0 h; solid bars) for 24 h (shaded bars) or 48 h (open bars). Cells were lysed and assayed for LPH and alkaline phosphatase enzymatic activities as described in MATERIALS AND METHODS. Error bars represent the standard deviations among duplicates of 3 independent experiments. *P < 0.05 compared with control.

Fig. 6. Furin inhibition alters the morphological differentiation of human intestinal epithelial cells. Caco-2/15 cells were treated from 1 day preconfluency to day 12 postconfluency with or without 5 μM decanoyl-RVKR-CMK. Cells were fixed in glutaraldehyde and osmium tetroxide before being epoxy embedded for electronic microscopy analysis. Bars = 1–2: 2 μm; 3–4: 500 nm. Representative results from 2 independent experiments are shown.
degernrate CDX2 binding motif (5'-TATGTCACAGCAGCAGA-3') on the opposite DNA strand. Palindromic binding sites for transcription factors have been reported for other transcription factors, such as GATA-1, NFAT-1, and STAT (23, 29, 31, 47). Such an arrangement may be involved in stabilizing the ligation of CDX2 to its DNA binding site. This could explain the specific importance of the CBS2 site, as opposed to CBS1 and CBS3, in furin regulation. We have also shown that the three furin promoters, and to a larger extent P1, are transactivated by CDX2. Furthermore, ChIP assays confirmed that CDX2 is recruited to the furin promoter in confluent Caco-2/15 cells. This indicates that furin gene expression in differentiating enterocytes is regulated in part by CDX2 binding to the furin promoter. Finally, we also show that furin mRNA and protein expression correlate with CDX2 protein expression along the intestinal crypt-villus axis in vivo during Caco-2/15 cell differentiation and in IEC-6 cells overexpressing CDX2.

In our opinion, the most interesting and novel finding from this study is that furin endoprotease activity is necessary for functional and morphological differentiation of intestinal epithelial cells. The fact that furin plays a role in enterocyte differentiation is suggested by the following: 1) the inhibition of furin activity decreased the activity of the enterocyte marker LPH, the enzyme responsible for the digestion of milk lactose into the absorbable monosaccharides glucose and galactose and expressed in absorptive differentiated enterocytes on villi (55); 2) the long-term inhibition of furin activity reduced cell polarization and brush border formation. E-cadherin-mediated cell-cell adhesion plays a crucial role in the polarization and differentiation of intestinal epithelial cells. Indeed, forced E-cadherin expression suppressed proliferation, promoted apoptosis, and delayed cell migration of intestinal epithelial cells (18), whereas inhibition of cadherin function resulted in loss of the polarized columnar phenotype (18, 32, 33). Our observation that decanoyl-RVKR-CMK-treated cells exhibited a less polarized and differentiated phenotype as well as poorly defined apical junctions suggests that furin might control polarization and the assembly of apical junctions at least by promoting E-cadherin maturation in Caco-2/15 cells. Indeed, preliminary data have suggested that inhibition of furin with the decanoyl-RVKR-CMK inhibitor leads to a time-dependent accumulation of the proform of E-cadherin after 24 and 48 h. Thus the inhibition of E-cadherin maturation by furin may affect intestinal epithelial cell differentiation. Therefore, CDX2 may mediate, in part, enterocyte differentiation by increasing furin expression and, therefore, proteolytic processing/activation of proproteins. In this respect, CDX2 expression induced a mature columnar morphogenesis in the COLO 205 cell line, a poorly differentiated human adenocarcinoma cell line, associated with E-cadherin-mediated cell-cell adhesion (25). Previous reports (51) have described similar effects of CDX2 expression in rat small intestine-derived IEC-6 cells.

The TGF-β1-dependent autocrine/paracrine regulatory loop may be another furin target important for intestinal epithelial cell differentiation. Enterocytes produce TGF-β1 soon after their exit from intestinal crypts (2). Activated TGF-β1 inhibits enterocyte proliferation and stimulates differentiation and migration of intestinal epithelial cells (27). Of note, furin is involved in the maturation of TGF-β1 in prostate epithelial cells and rat hepatocytes (20, 54). In addition, furin is implicated in the maturation and differentiation of gastric surface mucous cells (28), 3T3-L1 adipocyte cells (7), prostate epithelial cells (54), rat hepatocytes (20), and human megakaryoblastic Dami cells (31).

In conclusion, our results identify furin as a novel CDX2-regulated gene target. Furin may be important in modulating the maturation and/or activation of key factors involved in functional (LPH) and morphological (E-cadherin) differentiation of intestinal epithelial cells. Hence, this CDX2-dependent regulation of furin expression could well represent one mechanism by which CDX2 controls differentiation along the crypt-villus axis of the human intestine.

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REFERENCES


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