Clusterin gene transcription is activated by caudal-related homeobox genes in intestinal epithelium

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Suh, EunRan, Zhengqi Wang, Gary P. Swain, Martin Tenniswood, and Peter G. Traber. Clusterin gene transcription is activated by caudal-related homeobox (Cdx) proteins play an important role in development and differentiation of the intestinal epithelium. Using cDNA differential display, we identified clusterin as a prominently induced gene in a Cdx2-regulated cellular model of intestinal differentiation. Transfection experiments and DNA-protein interaction assays showed that clusterin is an immediate downstream target gene for Cdx proteins. The distribution of clusterin protein in the intestine was assessed during development and in the adult epithelium using immunohistochemistry. In the adult mouse epithelium, clusterin protein was localized in both crypt and villus compartments but not in interstitial cells of the intestinal mucosa. Together, these data suggest that clusterin is a direct target gene for Cdx homeobox proteins, and the pattern of clusterin protein expression suggests that it is associated with the differentiated state in the intestinal epithelium.

Cdx2; differential display; differentiation; transactivation

Clustering genes in undifferentiated cell lines (18). Mice null for Cdx2, but it does not seem to play a primary role in directing epithelial morphogenesis previously seen in this system. Its developmental expression in the intestine suggests several potential functions.

EXPERIMENTAL PROCEDURES

Cell culture and maintenance. Small intestinal epithelial IEC-6 cells and IEC6-Cdx2L1 cells were maintained as pre-

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viously described (18). Briefly, IEC-6 cells and stably transduced IEC-6 cells with a mouse Cdx2-inducible expression vector (LacSwitch system, Stratagene, La Jolla, CA) were cultured under an atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium with 4.5 g/l glucose containing 5% fetal calf serum and 0.1 U/ml of insulin. For isolation of total RNA cells were plated at 20% confluence in 100-mm plates, and 24 h later the medium was replaced with medium with or without 4 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

**RNA preparation and differential display analysis.** Differential display was performed as previously described (11) with modifications. Total RNA was obtained from IEC-6 and IEC6-Cdx2L1 cells that were grown in the absence or presence of 4 mM IPTG for 3, 24, 48, 72, and 96 h and prepared using the guanidinium-ScCl procedure (15). In each case, cDNA was synthesized at 37°C for 1 h in the reverse transcription reaction containing 500 ng of total RNA, 2.5 μM reverse primer (T1aCG), 20 μM dNTPs, 5 mM dithiothreitol, 40 units RNase inhibitor (Promega, Madison, WI), and 5× reaction buffer with 200 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (GIBCO BRL, Gaithersburg, MD). At the end of this incubation, the temperature was increased to 95°C for 5 min. PCR amplification of a given reverse transcription reaction was prepared in 20 μl of PCR mixture containing 2 μl of the reverse transcription reaction, 2.5 μM reverse primer (T1aCG), 0.5 μM arbitrary primer (5'-CTTGATAGGCC-3'), all dNTPs, each at 100 μM, 10 mM Tris·HCl (pH 8.3), 2.5 mM MgCl2, 10 μCi of [32P]dCTP (3,000 Ci/mmol), and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). PCR was performed in a 9600 thermocycler (Perkin-Elmer, Foster City, CA) using the following parameters: 94°C for 2 min; 1 cycle of 94°C for 30 s, 42°C for 60 s, and 72°C for 45 s; 35 cycles, the last of which was followed by a 5-min extension at 72°C. Three microliters of the PCR-amplified cDNA products were resolved on a 6% DNA sequencing gel. The gels were transferred to filter paper and dried on vacuum dryer. Glogos II autoradiographic markers (Stratagene, La Jolla, CA) were adhered to the dried gels to aid in subsequent alignment. The gels were then exposed for autoradiography.

**Recovery and reamplification.** The cDNA bands of interest were excised from the dried gel on the filter paper and transferred to Eppendorf tubes. Each gel slice was rehydrated for 30 min in 100 μl of distilled water, and the supernatant was removed and 10 μl of 3 M sodium acetate and 250 μl of 100% EtOH were added. The DNA was precipitated at −70°C for 1 h and collected by centrifugation. The pellet was resuspended in 10 μl of distilled water, and 5 μl of eluted DNA were used for PCR reamplification. PCR conditions were as described in RNA preparation and differential display analysis except that all dNTPs, each at 60 μM, were added without [32P]dCTP and the concentration of both primers was 2.5 μM in a final reaction volume of 40 μl. The reamplified PCR products were cloned using the TA cloning kit (Invitrogen, San Diego, CA) and sequenced. The sequence of cDNA was analyzed using the BLAST program.

**Northern blot analysis.** Total RNA (10 μg) was separated in a 1.4% agarose-formaldehyde gel and transferred to a Hybond-N membrane (Amersham, Arlington Heights, IL). Probes were synthesized from reamplified PCR products, followed by random priming in the presence of [γ-32P]dCTP (3,000 Ci/mmol). Hybridization was performed as described previously (17).

**Immunohistochemistry.** Paraffin-embedded tissue sections were fixed with 4% paraformaldehyde as described previously (14) and treated by boiling for 6 min in a microwave oven to quench endogenous alkaline phosphatase activity. For immunostaining for clusterin, SGP-2 antibody (generous gift of Dr. Michael Griswold, Washington State University) was used in a 1:1,500 dilution. The primary antibody was visualized with goat anti-rabbit biotinylated antiserum and avidin/biotin system (ABC) according to the protocol provided by Vector Labs (Burlingame, CA). The slides were developed with 3-bromo-4-chloro-3-indolyl-phosphate-4-nitro blue tetrazolium chloride (Boehringer Mannheim). The tissue was counterstained with neutral red.

**Stable transfection.** The complete coding sequence of rat clusterin cDNA (testosterone-repressed prostate message-2; TRPM-2) was obtained from pG117H by excising with EcoRI. A 1.7-kb cDNA fragment was blunt ended and inserted with either orientation to construct sense (pMTTrpm) or antisense (pMTTrpmAS) into an EcoRI V site in pMTCB6, which contains the promoter of the sheep metallothionein I gene (6). IEC-6 and IEC6-Cdx2L1 cells were transfected as described previously (18) by electroporation with pMTTrpm or pMTTrpmAS, respectively.

**E lectrophoretic mobility shift assay.** Electrophoretic mobility shift assay (EMSA) was performed with nuclear extract from COLO DM cells as described previously (25). Oligonucleotides for making probes were as follows: upstream Cdx2 binding element (UCB) top strand: 5'-GATCCATGTTTAG-GTTTATGCTACTCA-3'; UCB bottom strand: 5'-GATCT-TGAGATGCATAAACCTAAACATG-3'; mutant UCB top strand: 5'-GATCCATGTTTAGGATGCTACTCAACCAACATG-3'; mutant UCB bottom strand: 5'-GATCT-TGAGATGCATAAACCTAAACATG-3'; TATA top strand: 5'-GATCCATGTTTAGGGATGCTACTCAACCAACATG-3'; TATA bottom strand: 5'-GATCT-TGAGATGCATAAACCTAAACATG-3'; mutant TATA top strand: 5'-GATCCATGTTTAGGGATGCTACTCAACCAACATG-3'; and mutant TATA bottom strand: 5'-GATCT-TGAGATGCATAAACCTAAACATG-3'.

**Plasmid reporter constructs and transfection analysis.** The rat clusterin gene structure was reported previously (26). The Hind III-Pvu II fragment, representing 1,297 nucleotides of the rat clusterin promoter upstream of the transcription start site and 57 nucleotides downstream of the start site, was subcloned into the luciferase reporter pLuc-Link (5). A series of unidirectional deletional mutants were generated by exonuclease III digestion of the plasmid using the Erase-a-Base system (Promega, Madison, WI). Plasmids for transfection were purified by two rounds of CsCl gradient centrifugation, and cells were transfected and analyzed as previously described (23, 27, 28).

**RESULTS**

**Clusterin mRNA is induced in intestinal cell lines that express Cdx2.** We previously showed (18) that induction of Cdx2 expression in IEC-6 cells, an undifferentiated rat intestinal epithelial cell line, results in dramatic changes in proliferation, morphology, and expression of intestinal genes. To identify other genes that are induced as part of the differentiation program, we used the cDNA differential display method to compare patterns of mRNA expression between IEC-6 cells that express Cdx2 and those that do not (see EXPERIMENTAL PROCEDURES). For these experiments, we used the IEC6-Cdx2L1 cell line that we engineered to conditionally express Cdx2 when IPTG is included in the
culture medium (18). In one condition of differential display, cDNA was generated by reverse transcription using T12CG, which anneals to a subset of polyadenylation tails. PCR amplification was then performed with T12CG and a second primer containing a sequence of 10 nucleotides that were chosen at random. A 160-bp cDNA was detected in RNA isolated from IEC6-Cdx2L1 cells that had been cultured in medium containing IPTG to induce expression of Cdx2 but not from RNA isolated from either uninduced IEC6-Cdx2L1 cells or the parental IEC-6 cell line (Fig. 1A). This cDNA fragment was recovered from the gel, reamplified, subcloned, sequenced, and identified as the 3'-untranslated region of the rat clusterin mRNA (26).

The clusterin cDNA was used to examine the expression of clusterin mRNA using Northern blot analysis (Fig. 1B). Parental IEC-6 cells express low levels of clusterin mRNA, as do IEC6-Cdx2L1 cells cultured in medium that did not contain IPTG. In contrast to the parietal cells, IEC6-Cdx2L1 cells were stimulated with IPTG to express Cdx2 as early as 2 h after induction. By 8 h after induction with IPTG there was already a small increase in clusterin mRNA, and by 20 h there was a threefold increase in clusterin mRNA that was sustained through 120 h of culture. There was a slight increase in clusterin mRNA at 120 h in the uninduced IEC6-Cdx2L1 cells caused by leaky expression of Cdx2 (Fig. 1B; Ref. 18). These results show that the expression of the clusterin gene is induced in IEC-6 cells as a result of expression of Cdx2. Thus clusterin may be a downstream gene in the regulatory network leading to the differentiated phenotype in IEC-6 cells.

Expression of clusterin in mouse intestinal tract. Clusterin is a heterodimeric glycoprotein encoded from a single mRNA that is expressed widely in tissues and is found circulating in the serum (13). Clusterin has been isolated and cloned from multiple species resulting in multiple synonyms for clusterin, including SGP-2, apolipoprotein J, and TRPM-2. Clusterin is widely expressed and is relatively abundant in testes, brain, and liver under normal conditions. It has been implicated in several biological processes such as cell-cell interactions, apoptosis, sperm maturation, membrane remodeling, lipid transport, and regulation of complement-induced cell lysis. In a number of tissues, clusterin is expressed during cellular differentiation and development. Clusterin mRNA is induced in the intestinal epithelium at embryonic day 16.5, just after the start of a marked change in the morphology of the epithelium from a stratified endoderm to a columnar intestinal epithelium (7).

To further explore the potential role of clusterin in the intestinal tract, we examined the expression of clusterin protein in the developing mouse intestinal tract and in the adult small intestine and colon. At postcoital day 13.5, a time when the endoderm of the developing intestine has a pseudostratified structure, there was very light staining of the endodermal cells (Fig. 2A). Intense immunostaining for clusterin was seen in the developing intestine immediately after the transition from endoderm to the columnar intestinal epithelium (Fig. 2, B and C). At postcoital day 14.5, when the stratified endoderm begins to form villi, much more intense cytoplasmic staining is noted in the...
nascent villi and intervillus epithelial cells (Fig. 2B). At postcoital day 15.5, when the simple columnar epithelium has been fully established, clusterin immunostaining was concentrated in the supranuclear area of epithelial cells (Fig. 2C). This pattern of protein expression at the time of the endoderm-intestinal transition is similar to the previously described pattern of expression of clusterin mRNA (7).

In the small intestinal epithelium of a 9-day-old (Fig. 2D) and an adult (Fig. 2E) mouse, clusterin protein was exclusively expressed in epithelial cells. Both villus and crypt cells were positive for clusterin staining, but there was a gradient of activity with higher expression in the villus compartment than in the crypts. A high-power view shows more detail of supranuclear staining (Fig. 2F).

Overexpression of clusterin is insufficient to initiate a differentiation program in IEC-6 cells. Early expression of clusterin in IEC-6 cells expressing Cdx2, as well as the developmental timing of clusterin gene expression in the intestinal epithelium, suggested that the clusterin protein may have a regulatory role in the differentiation process. Therefore, we made stable IEC-6 cell lines that expressed clusterin when treated with medium containing zinc sulfate. These cell lines did not show changes in proliferation or morphology on induction of clusterin protein expression (data not shown). Therefore, although clusterin is induced as a result of Cdx2 expression, expression of clusterin by itself is incapable of initiating the same differentiation program. This experiment showed that clusterin alone is insufficient for induction of differentiation, but it does not address the issue of whether clusterin is necessary for differentiation in this cell model. To answer this question, we attempted to inhibit the expression of clusterin in IEC6-Cdx2L1 cells by introducing an antisense clusterin cDNA under the control of the zinc-inducible promoter. Although we were able to isolate cell lines, we were not able to identify a cell line that showed decreased clusterin mRNA expression. Therefore, the issue of whether clusterin gene expression is required for induction of differentiation in this model remains unresolved.

Cdx2 binding elements in the rat clusterin gene 5′-flanking region. Cdx2 might induce clusterin mRNA levels either by stimulation of gene transcription or by prolongation of the mRNA half-life. Moreover, activation of gene transcription might be a direct effect of Cdx2 on the gene promoter or an effect on other genes that subsequently regulate clusterin gene transcription. We inspected the promoter of the rat clusterin...
gene and identified two elements located at \(~740\) and \(25\) nucleotides upstream of the transcriptional start site that had a consensus sequence for Cdx2 binding elements (Fig. 3A). The UCB (nucleotide \(-740\)) contains two adjacent binding elements, as in the sucrase-isomaltase promoter where the Cdx2 binding elements were originally described (17). The element located at nucleotide \(-25\) contains a TATA box consensus sequence and is located appropriately to be the binding site for TATA box binding protein (TBP) in the process of transcriptional initiation. To examine direct binding of Cdx2 protein to the two binding elements, EMSA was performed using nuclear extract from COLO DM cells, which express a high level of Cdx2 proteins (17, 18). Supershift experiments with specific Cdx2 antibodies and competition experiments with mutant oligonucleotides showed that both elements interacted specifically with Cdx2 (Fig. 3B). Previously, we showed (12, 20) that Cdx1 could bind to DNA elements similarly to Cdx2 and could activate transcription. Therefore, we performed EMSA using nuclear extracts from IEC-6Cdx1MT1 cells (12). Supershift experiments with Cdx1 antibodies demonstrated that Cdx1 was also able to bind to the UCB and the TATA element (data not shown). Thus Cdx1 and Cdx2 proteins can bind to both of these potential regulatory elements in the rat clusterin gene.

**Activation of the clusterin promoter in cell lines.** The ability of the rat clusterin promoter to drive transcription in intestinal and nonintestinal cell lines was tested using various lengths of the 5′-flanking region of the rat clusterin gene linked to the luciferase reporter gene (Fig. 4). The construct \(-771\)-luc contained the UCB Cdx binding site, and the \(-732\)-luc construct had this element deleted. In the mouse fibroblast cell line NIH/3T3, the clusterin promoter was able to activate transcription with very little difference between the three different constructs tested. Caco-2 cells were used as a model intestinal cell line; they have a small amount of endogenous Cdx2 protein and can support low-level expression of Cdx-responsive genes such as sucrase-isomaltase (17). The shortest construct \((-421-\)...
and either Cdx1 or Cdx2 on the constitutive activation. Similar results on induction by shown). Removal of the NH2-terminal domain of Cdx2 in independent transfection experiments (data not shown). Addition of the UCB Cdx binding element struct. As a control, when the clusterin promoter was inserted into the luciferase vector in the inverse orientation, there was minimal constitutive expression in either NIH/3T3 or Caco-2 cells (Fig. 4). These results suggest that there are factors in Caco-2 cells that are able to activate DNA regulatory elements located between nucleotides −421 and −771. Although not responsible for all the additional activation, these results also suggest that the 39-base element located between −771 and −732, which contains two Cdx binding sites, is able to augment clusterin gene transcription in an intestinal cell line.

Cdx proteins transactivate the rat clusterin promoter. We next examined the ability of Cdx proteins to directly activate transcription of the rat clusterin promoter. The minimal clusterin promoter, containing nucleotides −48 to +57, was not able to activate transcription in NIH/3T3 cells. As shown in Fig. 4, 771 nucleotides of the 5′-flanking sequence resulted in moderate constitutive activation of the promoter-reporter construct. Removal of the Cdx binding element between −771 and −732 did not change the constitutive activation of the promoter in NIH/3T3 cells (Figs. 4 and 5). Cotransfection of an expression vector for either Cdx1 or Cdx2 resulted in marked activation of each of the clusterin promoter constructs (Fig. 5). The level of induction over constitutive expression was greatest for the −48 to +57 construct, with the same level of induced expression for the −732 to +57 construct. Addition of the UCB Cdx binding element (−771 to −732) resulted in a further induction by both Cdx1 and Cdx2 expression vectors with no change in constitutive activation. Similar results on induction by either Cdx1 or Cdx2 on the −48, −732, and −771 clusterin promoter constructs were obtained from five independent transfection experiments (data not shown). Removal of the NH2-terminal domain of Cdx2 (HD2CD), which embodies the activation domain of the protein, eliminated the transcriptional activation of the clusterin promoter constructs (Fig. 5). This deletion construct of Cdx2 has been shown in other studies to effectively bind to DNA but is not capable of activating the sucrase-isomaltase promoter (21). As a final negative control, transfection with an expression construct for hepatocyte nuclear factor-1β (HNF-1β) failed to activate transcription of the clusterin constructs.

DISCUSSION

Our results identify the clusterin gene as a direct target for Cdx transcription factors in intestinal epithelial cells. Moreover, we found that clusterin protein is expressed in a complex pattern during intestinal epithelial cells development. These findings have several implications for the mechanism of Cdx proteins in transcriptional regulation as well as the potential functions of the clusterin protein in intestinal development and differentiation.

The rapid induction of clusterin mRNA on expression of Cdx2 in IEC-6 cells provides evidence that regulation of the clusterin gene is part of a cascade of events initiated by Cdx2 expression. Transfection experiments using the rat clusterin gene promoter show that Cdx proteins can be direct regulators of transcriptional initiation of the clusterin gene. The 5′-flanking region of the clusterin gene revealed two areas with specific binding sites for the Cdx2 protein, one located between −771 and −732 nucleotides upstream of the transcriptional start site (UCB) and one that overlaps the putative TATA box. Both of these sites appear to play a role in clusterin gene regulation by Cdx proteins.

The most robust activation of the rat clusterin gene promoter by both Cdx1 and Cdx2 appears to be mediated primarily via a short promoter region encompassing nucleotides −48 to +57. The only Cdx binding site within this region is the putative TATA box, which is positioned properly with respect to the transcriptional start site to be the site of binding for TBP, an important component of the basal transcriptional apparatus. A number of controls show that transcriptional induction from this promoter is due to Cdx2 expression. First, when the promoter region is inverted in the
clusterin expression suggest that the protein may have a role in a variety of intestinal cells during development. Therefore, the patterns of Cdx2, which leads to the development of a differentiated columnar epithelium. Thus clusterin is a direct target gene for Cdx1 and Cdx2, which are transcription factors that participate in directing differentiation of intestinal epithelial cells. Thus clusterin is likely to be one of many genes regulated by Cdx genes that are important either for the differentiated process itself or for defining differentiated cellular function.

Although these studies of clusterin expression suggest an association with intestinal differentiation, the data provide little additional insight on the function of clusterin in intestinal epithelial cells. Many functions have been ascribed to clusterin, including a prominent association with apoptosis in many tissues and cells (13). Our data suggest that in the normal intestinal epithelium clusterin is not associated with apoptosis. Apoptotic cells are normally seen in the developing epithelium at the endoderm-intestinal epithelium transition, particularly associated with false lumens in the endoderm. Apoptotic cells are also seen occasionally in normal intestinal crypts and at the villus tips in adult intestine, but this is relatively uncommon. We did not see a concentration of clusterin protein in these locations. Furthermore, a previous study in the intestine showed that clusterin mRNA is not concentrated in apoptotic intestinal epithelial cells after irradiation (1).

In other cell systems, clusterin has also been found in association with differentiation (22). Although the function of clusterin in differentiation of the intestinal epithelium is not elucidated by the current studies, it appears that expression of clusterin is associated with epithelial differentiation. Clusterin is first expressed in the intestinal epithelium during the endoderm-intestinal transition, the most important developmental transition in the morphogenesis of the intestinal epithelium. Once the mature architecture of the epithelium is attained, clusterin expression is distributed in a very specific cellular and spatial pattern. It is expressed in highest levels in villus-associated enterocytes that reside in the nonproliferating, differentiated compartment of the intestinal epithelium. Because many genes are expressed in this pattern in the epithelium, this does not suggest a specific function but only that expression is associated with the differentiated phenotype, as suggested by the expression in IEC-6 cells expressing Cdx2. In enterocytes, clusterin protein appears to be distributed throughout the cytoplasm and not only in a supranuclear Golgi distribution as might be found for an exclusively secreted protein product. In addition to enterocytes, clusterin is expressed in higher levels in goblet cells. Immunohistochemistry performed on both paraffin-fixed and frozen sections indicates that clusterin protein is present both in the apical goblet cell and in the mucous globule and luminal mucus (data not shown). Therefore, it appears that clusterin may be secreted by goblet cells into the intestinal lumen. There is no evidence from our data that clusterin is expressed in either Paneth cells or enteroendocrine cells.

Together, our results show that clusterin expression is associated with the differentiation process in the intestinal epithelium. Moreover, clusterin might be a direct target gene for Cdx1 and Cdx2, which are transcription factors that participate in directing differentiation of intestinal epithelial cells. Thus clusterin is likely to be one of many genes regulated by Cdx genes that are important either for the differentiated process itself or for defining differentiated cellular function.
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