Differential regulation of intestinal alkaline phosphatase gene expression by Cdx1 and Cdx2

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Alkhoury, Fuad, Madhu S. Malo, Moushumi Mozumder, Golam Mostafa, and Richard A. Hodin. Differential regulation of intestinal alkaline phosphatase gene expression by Cdx1 and Cdx2. Am J Physiol Gastrointest Liver Physiol 289: G285–G290, 2005. First published March 17, 2005; doi:10.1152/ajpgi.00037.2005.—We have examined the role that the caudal-related homeobox transcription factors Cdx1 and Cdx2 play in activating the enterocyte differentiation marker gene intestinal alkaline phosphatase (IAP). Human colon cancer Caco-2 cells were transiently transfected with Cdx1 and/or Cdx2, and semiquantitative RT-PCR was used to study the effects on IAP mRNA expression. Transfections with a variety of IAP-luciferase reporter constructs were used to identify a Cdx response element located within the human IAP gene promoter. Protein-DNA interactions were examined by EMSA. Results showed that Cdx1 markedly induced IAP mRNA expression, whereas Cdx2 did not, and, in fact, inhibited the Cdx1 effects. Functional analysis revealed that Cdx1 transactivates (fourfold, P < 0.05) the IAP promoter through a novel Cdx response element (GGTTTAGA) located between −2369 and −2375 upstream of the translational start site. EMSA showed that both Cdx1 and Cdx2 could bind to the cis element, but in cotransfection experiments, Cdx2 inhibited the Cdx1 effects by ~50%. Thus we have identified a previously unrecognized interaction between two important gut transcription factors, Cdx1 and Cdx2, in the context of IAP gene regulation. Cdx1 activates the IAP gene via a novel cis element, whereas Cdx2 inhibits the Cdx1 effects.

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polymerase from Promega. The list of primers used in RT-PCR is given in Table 1.

Quantitative real-time PCR. Real-time PCR was performed on cDNAs to quantitate the amount of IAP expression in cells transfected with Cdx1 or Cdx2 following the protocol as previously described (2). The primers used in real-time PCR were the same as used in semi-quantitative RT-PCR.

DNA sequencing. Plasmids were sequenced by the Sequencing Core Facility at the Department of Molecular Biology, Massachusetts General Hospital (Boston, MA), using dye-labeled dideoxynucleotide chain-terminators.

Transient transfection and luciferase reporter assays. Transient transfection and luciferase reporter assays were performed following the protocols as previously described (23). Caco-2 cells were plated at a density of 300,000 cells per well of a six-well plate in DMEM containing 10% FBS. Cells were grown overnight, and transient transfections were performed using SuperFect reagent from Qiagen. Approximately 1.5 μg of a test plasmid DNA per well was used in each transfection. The total amount of DNA was kept the same for each transfection by adding nonspecific plasmid TF12 DNA. Firefly and Renilla luciferase assays were then performed on cell lysates using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. The control Renilla luciferase activity was used to determine transfection efficiency as well as to normalize the firefly luciferase activity data.

In vitro protein synthesis. TNR T7 Quick Coupled Transcription/Translation System (Promega) was used for in vitro synthesis of human Cdx1 and Cdx2 proteins from the derivatives of pcDNA3 (Invitrogen) carrying the relevant coding sequences under the control of the T7 promoter.

EMSA. EMSAs were performed following the previously described protocol (23). Complementary oligonucleotides were annealed and radiolabeled by the kinasing reaction with T4 polynucleotide kinase in the presence of [γ-32P]ATP. The radiolabeled probe was purified twice by passing it through Micro Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA), followed by determination of the specific activity that usually measured ~10^8 cpm/μg DNA (23). Radiolabeled probe (10 ng) was incubated at room temperature for 20 min with 1 μl (~1 ng) of synthesized Cdx1 or Cdx2 in 10 μl of binding buffer containing 20 mM HEPES (pH 7.7), 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 5 μM nonspecific oligo, 10% glycerol, and 2 μg of poly(dI-dC). The samples were electrophoresed in a 5% agarose gel containing ethidium bromide. The gels were illuminated with UV light and photographed.

Table 1. Eight primers used in RT-PCR

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<th>Primer</th>
<th>Sequence</th>
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<td>hIAPcDNA2146F</td>
<td>5'-gca acc ctt cag ccc acc cca gga g-3'</td>
</tr>
<tr>
<td></td>
<td>(25 bases)</td>
</tr>
<tr>
<td>hIAPcDNA2423R</td>
<td>5'-cga gca tcc aga tgt ccc ggg ag-3'</td>
</tr>
<tr>
<td></td>
<td>(23 bases)</td>
</tr>
<tr>
<td>hbetaActin601F</td>
<td>5'-gag tct gga cct ggc cgg cgc gga cct c-3'</td>
</tr>
<tr>
<td></td>
<td>(28 bases)</td>
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<tr>
<td>hbetaActin1100R</td>
<td>5'-ggg cgc ccc agc cac acc gag tac tgt c-3'</td>
</tr>
<tr>
<td></td>
<td>(28 bases)</td>
</tr>
<tr>
<td>hCdx1 971F</td>
<td>5'-ggc tct gtc acc ttc tgg gcc atg tgt g-3'</td>
</tr>
<tr>
<td></td>
<td>(25 bases)</td>
</tr>
<tr>
<td>hCdx1 1250R</td>
<td>5'-cag gat cag tgt ggt gcc ccc cac cca c-3'</td>
</tr>
<tr>
<td></td>
<td>(25 bases)</td>
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<tr>
<td>hCdx2 1211F</td>
<td>5'-ccc acc ggc ata gac cta cag acc c-3'</td>
</tr>
<tr>
<td></td>
<td>(24 bases)</td>
</tr>
<tr>
<td>hCdx2 1550R</td>
<td>5'-ggc atg agc agg cag cag cca tc-3'</td>
</tr>
<tr>
<td></td>
<td>(23 bases)</td>
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Fig. 1. Effect of Cdx1 and Cdx2 on the endogenous intestinal alkaline phosphatase (IAP) gene in the Caco-2 cell line. Caco-2 cells were transfected with the Cdx1 and/or Cdx2 expression vector or the empty vector as a control. Total RNA was isolated 48 h after transfection from the transfected cells, and then cDNA was synthesized. A: semi-quantitative RT-PCR analysis of IAP expression. Standard PCR reactions were performed with 30 cycles for IAP (upper), Cdx1, Cdx2 (middle), and actin (lower). The PCR products were electrophoresed in 2% agarose gels containing ethidium bromide. The gels were illuminated with UV light and then photographed. Lane 1, empty vector-transfected cells; lane 2, Cdx1-transfected cells; lane 3, Cdx2-transfected cells; and lane 4, Cdx1 and Cdx2-transfected cells. B: LightCycler real-time PCR analysis of IAP expression. Using the same cDNA, LightCycler PCR was performed as described in MATERIALS AND METHODS. LightCycler real-time PCR was performed in three independent experiments.
polyacrylamide gel in a cold room (4°C), followed by drying of the gel and autoradiography.

*In vitro* mutagenesis. Site-directed PCR mutagenesis was performed to introduce mutations in the putative CdxRE for functional analysis as described previously (23). PCR primers were synthesized with specific mutations followed by PCR amplification and ligation of the PCR product into an appropriate plasmid. Each mutant plasmid was sequenced to verify the nature of the mutations.

**RESULTS**

*Cdx1 activates the endogenous IAP gene.* We first examined the effects of Cdx1 and Cdx2 on endogenous IAP gene expression. Preconfluent Caco-2 cells were transiently transfected with Cdx1 and/or Cdx2 expression vectors or the empty vector as a control, and total RNA was isolated 48 h later. Semiquantitative RT-PCR (Fig. 1A) and LightCycler real-time PCR assays (Fig. 1B) showed no Cdx1 and minimal Cdx2 levels under basal conditions in this cell line, consistent with the protein levels previously reported (30). As expected, Cdx1 and/or Cdx2 overexpression was confirmed when their expression plasmids were transfected into these cells. Basal IAP levels are low but were increased significantly by Cdx1 (five-fold, \(P < 0.05\)). In contrast, Cdx2 had no effects on IAP mRNA expression. Furthermore, cotransfection experiments revealed that Cdx2 inhibited the ability of Cdx1 to activate IAP gene expression (90% inhibition, \(P < 0.05\)).

*Cdx2 inhibits Cdx1-mediated transactivation of the IAP-luciferase reporter gene.* To determine the molecular mechanisms by which the Cdx transcription factors regulate IAP gene expression, Caco2 cells were transfected with IAP-luciferase reporter plasmids along with Cdx1 and/or Cdx2 expression plasmids. The results showed that overexpression of Cdx1 caused an approximate fourfold increase in the expression of the luciferase reporter gene in the plasmid carrying the full-length IAP promoter (pIAP-2574/-49) (Fig. 2A). Cdx2 overexpression alone had minimal effects on IAP activation. However, similar to what was seen regarding regulation of the endogenous IAP gene, Cdx2 inhibited the effects of Cdx1 on the IAP promoter (≈50% inhibition) (Fig. 2A). The effects of Cdx1 were dose dependent, as shown in Fig. 2B, and, furthermore, the Cdx2 inhibitory effects on IAP gene were also dose dependent (Fig. 2C). Specificity of Cdx2 regarding the Cdx1 effects was verified in cotransfections with the empty vector pCDNA as well as with another IAP transcription factor KLF4 (no inhibition of KLF4 by Cdx2) (Fig. 2A).

*CdxRE is located between -2369 and -2375 of the 5' regulatory region of the IAP gene.* We constructed various derivatives of pIAP-2574/-49 carrying sequential 5' deletions of the IAP promoter (see MATERIALS AND METHODS). Caco-2 cells were transiently cotransfected with these pIAP-luciferase reporter plasmids along with the Cdx1 expression plasmid. Cdx1 induced the expression of the 2.5-kb IAP reporter gene by approximately fourfold (as previously seen), but in the case of the pIAP-1875/-49 plasmid, the level of activity was substan-

Fig. 2. Cdx1 and Cdx2 regulation of the IAP-luciferase reporter gene. Caco2 cells were transiently transfected with a 2.5-kb IAP-luciferase reporter plasmid along with Cdx1 and/or Cdx2 expression vectors. Kruppel-like factor (KLF4) expression vector and the empty vector pCDNA were used for control purposes. Nonspecific plasmid DNA was used to maintain equal amounts of DNA in all transfection samples. Results are expressed in fold-activation (Cdx/Cdx- ) of relative luciferase activity after normalization with Renilla activity from 6 independent experiments, and the values are expressed as means ± SD (\(P < 0.05\)). A: Cdx1 and Cdx2 regulation of IAP-luciferase reporter gene. B: dose-dependent effects of Cdx1 on IAP gene expression. C: dose-dependent effects of Cdx2 on Cdx1-mediated activation of the IAP gene.
IAP REGULATION BY Cdx1 AND Cdx2

A

IAP 5’ Flanking Region

pl/AP-2574/-49

pl/AP-1875/-49

pl/AP-1107/-49

pl/AP-750/-49

Fig. 3. Localization of the Cdx response element in IAP promoter. Luciferase reporter plasmids carrying various IAP promoter regions were used to transfect Caco-2 cells (see MATERIALS AND METHODS). The cells cotransfected with Cdx1 expression vector. The results expressed as fold activation (Cdx+/−Cdx−) of relative luciferase activity after normalization with Renilla activity from 6 independent experiments, and the values are expressed as means ± SD (P < 0.05). A: Cdx1 regulation of the IAP-luciferase reporter gene in plasmids carrying different 5’ deletion of the IAP promoter. Various 5’ deletion mutants (pIAPs) of the plIAP-2574/-49 plasmid were constructed by deleting specific restriction fragments of the IAP promoter (see MATERIALS AND METHODS). B: Cdx1 regulation of IAP-luciferase reporter gene in plasmid carrying internal mutation of the IAP Cdx response element (see Table 2). This mutation was constructed by in vitro site-directed mutagenesis of the plIAP-2574/-49 plasmid (see MATERIALS AND METHODS).

IAP-5’ Flanking Region

B

Fold Activation

0

1

2

3

4

wt IAP

mut IAP

wt IAP

mut IAP

Cdx1

KLH4

DISCUSSION

The human IAP gene maps to chromosome 2q34–37 and produces a 528-amino acid polypeptide. IAP is a glycosylphosphatidylinositol-anchored enzyme expressed exclusively in the microvillus membrane of the intestine. A recent report on IAP knockout mice demonstrated that IAP functions to limit fat absorption during high-fat feeding (27). In the rat, either forced feeding of corn oil (6) or natural feeding of a high-fat diet (5) increases IAP expression, whereas its levels are dramatically decreased in the setting of starvation (13). Thus it seems likely that the IAP protein functions in a homeostatic manner to modulate the efficiency of dietary fat absorption according to the needs of the organism. Given the physiological importance of the IAP protein, we have been interested in understanding the molecular mechanisms that govern its regulation.

In this report, we demonstrate that the two related Cdx1 and Cdx2 transcription factors, both of which are expressed in an intestine-specific manner, participate in controlling IAP levels in the gut. Remarkably, we found that Cdx1 efficiently and specifically transactivates the IAP promoter, whereas Cdx2 has no effect alone and, in fact, inhibits the effects of Cdx1. Distinct effects of Cdx1 and Cdx2 have already been reported in the contexts of other target genes. For example, Cdx1 transactivates the human PCNA gene promoter in HepG2 and colorectal carcinoma cell lines, Colo320HSR and HCT116, whereas Cdx2 does not (28). In addition, Cdx1 was shown to inhibit the transcriptional activity of the p21 gene (26), whereas Cdx2 was found to induce this promoter (2). Gautier-Stein et al. (8) describes the direct antagonistic role of these transcription factors in the context of the glucose-6-phosphatase gene.

Cdx1 and Cdx2 exhibit strong sequence homology between their homeodomains that function in DNA binding, but they only share limited stretches of common amino acids outside the homeodomain. It can be hypothesized from EMSA and transactivation competition experiments (Figs. 4 and 2C) that the suppressor role of Cdx2 on the Cdx1 effects might be due to competition for the same DNA binding site within the IAP gene. Alternatively, the Cdx2 inhibitory effects may not be

Table 2. Sequences of wild-type and mutated IAP-CdxRE, and the TATA region of p21

<table>
<thead>
<tr>
<th>Cdx consensus</th>
<th>A/CTTTATA/G</th>
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<tr>
<td>Wild-type IAP</td>
<td>-2389</td>
</tr>
<tr>
<td>Mutant IAP</td>
<td>5′-tttttttttttGGGATCGaggtcttttgt-3′</td>
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<tr>
<td>TATA p21</td>
<td>5′-ggtgagtttttttGGGATCGaggtcttttgt-3′</td>
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IAP, intestinal alkaline phosphatase.
A repressor role, since it had no effects on basal region(s) in the protein. It is unlikely that Cdx2 by itself plays dependent on the homeodomain itself, but instead on other region(s) in the protein. It is unlikely that Cdx2 by itself plays a repressor role, since it had no effects on basal IAP gene transcription in the absence of Cdx1 (Fig. 2A).

Cdx1 and Cdx2 are well-recognized regulators of intestine-specific gene expression (7, 33, 35, 36, 39), promoting differentiation and regulating proliferation (33, 36). Most studies have indicated that Cdx2 expression is restricted to the villus, while Cdx1 is expressed only in the crypts. However, recent reports have expanded this simple model, demonstrating low but detectable Cdx1 levels in the nonproliferating surface and villus epithelial cells (31, 32). Furthermore, studies employing antibodies against phosphorylated Cdx2 have demonstrated activated Cdx2 in small intestinal crypts (29). As such, the expression of Cdx1 and Cdx2 may overlap within cells along the crypt-villus axis, allowing for interaction between these two proteins in regulating specific target genes such as IAP.

The role of Cdx1 in carcinogenesis remains a controversial topic. It has been hypothesized that Cdx1 promotes intestinal epithelial proliferation, because Cdx1 is expressed highly in the crypt compartment and because its expression is induced by oncogenic Wnt/β-catenin and Ras signaling (16, 17, 34). However, in addition to the fact that it is expressed in villus (31, 32), Cdx1 expression is generally diminished or actively silenced in human colon cancers (31, 38), as demonstrated by Northern blot analysis (20), quantitative RT-PCR (15), and immunostaining (31). The expression of Cdx1, like Cdx2, is also decreased during the transition from intestinal metaplasia to dysplasia to cancer (3, 31). In addition, Cdx1 caused a decrease in proliferation of cells in culture, and stably transfected 3T3 cell line overexpressing Cdx1 failed to grow on the soft agar (18). These findings and the present work suggest that Cdx1 can promote a more differentiated phenotype and cast doubt on the oncogenic role of Cdx1.

Mechanisms of Cdx-mediated transcriptional regulation of the IAP gene were defined by using transient transfection assays and EMSAs. These experiments demonstrated that Cdx1 is capable of causing the transcriptional activation of a reporter gene under the control of various segments of the human IAP promoter. Using a number of deletion constructs of the IAP 5′ regulatory region and in vitro site-directed mutagenesis of the IAP-CdxRE, we were able to show that the Cdx1 effect on IAP is largely dependent on an intact CdxRE (GTT-TAGA) localized between −2369 and −2375 (Fig. 3). Although EMSA demonstrated that either Cdx1 or Cdx2 is able to bind to the RE (Fig. 4), only Cdx1 actually functions as an IAP activator. Most of the known Cdx target genes contain one CdxRE in the proximal promoter region, in some cases overlapping the TATA-box such as in the calbindin-9, clusterin, and Glc6Pase genes (1, 4, 8, 37). In contrast, there are two CdxREs within the sucrose-isomaltase, LPH, and claudin-2 genes (25, 30, 36). In terms of the IAP gene, it appears that there is a single major Cdx-binding site, located far upstream from the translation start site, although other minor CdxREs could be present in other regions of the gene.

Previously, using both in vitro and in vivo models, we have demonstrated that the IAP gene is a direct target for two transcription factors, the thyroid hormone receptor (12, 14) and the gut-enriched KLF4 (or GKLF) (10). We have localized the major KLF4RE to a segment of the IAP gene between −224 and −114 bp upstream from the translational start site (10), and the TRE response element between −632 and −612 (23). The major CdxRE we have identified in the present work is located quite upstream from these other cis elements. Clearly, IAP gene regulation is a complex process that involves a variety of different transcription factors, some of which may interact in both positive and negative ways. It will be important in future studies on IAP gene regulation to delineate the precise roles played by Cdx1/Cdx2 relative to these other transcription factors. This fact is underscored by an examination of the patterns of Cdx and IAP expression, the former being highest in the distal small intestine and colon (32), whereas IAP levels are highest in the proximal small intestine (11). It will be important in future studies on IAP gene regulation to delineate the precise roles played by Cdx1/Cdx2 relative to these other transcription factors and to try and identify those factors that specifically result in the high IAP levels within the proximal gut.

In summary, the results presented here constitute the first report that the enteroocyte differentiation marker gene IAP is a direct target of the Cdx transcription factors. More specifically, Cdx1 activates the IAP gene, whereas Cdx2 inhibits the activation mediated by Cdx1. These results indicate a novel mechanism of interaction of the Cdx factors regarding the IAP gene and will likely have important implications for our understanding of how fat absorption is regulated within the mammalian gut.

<table>
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<th>Probe Cdx-RE</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
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Fig. 4. Binding of Cdx1 and Cdx2 to the putative IAP-Cdx response element (IAP-CdxRE) analyzed by EMSA. Double-stranded oligonucleotide carrying the putative IAP-CdxRE was 5′ end-labeled with 32P and used as the probe in EMSA. Cdx1 and Cdx2 proteins were synthesized in vitro, and ~3 ng of protein was used in each reaction (see MATERIALS AND METHODS). Related oligonucleotide sequences are shown in Table 2. The competitions were done with 100-fold excess of oligonucleotides from wild-type (wt) and mutant IAP and from the TATA region of P21 gene previously characterized as Cdx1 binding element. The protein-DNA complexes were separated by nondenaturing 5% PAGE at 4°C. EMSA for each experiment was repeated more than 3 times, and similar results were obtained.
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