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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 (GTTTAGA) 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.

cell cycle; enterocyte differentiation; gene regulation; gut development; homeostasis; intestinal alkaline phosphatase; tumorigenesis

INTESTINAL ALKALINE PHOSPHATASE (IAP) is one of four alkaline phosphatase (AP) isoenzymes. In humans, three of the four AP isozyme are tissue specific, i.e., the intestinal AP (IAP), placental AP, and germ cell AP, while the fourth AP gene is the tissue-nonspecific AP present in bone, liver, and kidney (9). IAP is exclusively expressed in villus enterocytes and hence serves as an excellent marker of crypt-villus differentiation (11). IAP represents a major component of the surfactant-like particles seen after fat feeding (19), and Narisawa et al. (24) recently described the phenotype of the *IAP* knockout mice, indicating that IAP functions to limit fat absorption during high-fat feeding.

IAP expression is dramatically regulated by a variety of dietary, developmental, and hormonal factors, e.g., it is induced at the time of postnatal weaning, whereas fasting in the adult rat causes a dramatic decrease in IAP levels. These alterations in IAP levels likely have important physiological consequences regarding the absorption of dietary fat, and we have, therefore, been interested in elucidating the molecular mechanisms that govern *IAP* gene regulation. Among the known transcriptional regulators of IAP expression are the thyroid hormone receptor and the gut-enriched Kruppel-like factor (KLF4) (10, 23).

Cdx1 and Cdx2 are members of the caudal-related homeobox family and appear to play critical roles in gut differentiation, proliferation, and neoplasia. The Cdx proteins are involved in the regulation of intestine-specific genes, such as *sucrase-isomaltase, intestinal phospholipase A/lysophospholipase, lactase-phlorizin hydrolase (LPH), p21,* and *claudin-2* (2, 7, 26, 32, 40). Cdx1 and Cdx2 have been shown to regulate specific gene promoters generally through binding to an A/T-rich Cdx response element (CdxRE) whose consensus sequence is A/CTTTATA/G in direct or reverse orientation (24).

Given their important role in gut epithelial development and differentiation, we examined the effects of the Cdx proteins on the IAP gene regulation. Our findings indicate that Cdx1, but not Cdx2, is an activator of IAP gene, and, furthermore, that the two factors appear to interact in a novel way in this context.

MATERIALS AND METHODS

DNA restriction enzymes and DNA modifying enzymes were obtained from New England BioLabs (Beverly, MA). TRIzol reagent for RNA preparation and Thermoscript RT were purchased from Invitrogen Life Technologies (Carlsbad, CA). *Taq* DNA polymerase and TNT^{R} T7 Quick Coupled Transcription/Translation System were bought from Promega (Madison, WI). SuperFect transfection reagent, the kit for DNA extraction from agarose gel, and the kit for large scale DNA preparation were obtained from Qiagen (Valencia, CA). Radionucleotides were purchased from PerkinElmer Life Sciences (Boston, MA), and oligonucleotides were synthesized by Sigma Genosys (The Woodlands, TX). Poly(dI-dC) was obtained from Pharmacia (Piscataway, NJ).

Plasmids. The human Cdx1 and Cdx2 expression plasmids (21) were the derivatives of the pcDNA3 vector (Invitrogen Life Technologies), and were the kind gifts of Dr. Juan Lucio Iovanna (Institut National de la Santé et de la Recherche Médicale, Marseille, France). Construction of the IAP-luciferase reporter plasmids has been previously described (22, 23). Briefly, the 2.5 kb *Sac*I-*Bam*HI fragment from the $IAP_{2.4}$CAT reporter construct (14) carrying the human IAP promoter region (−2574 to −49, relative to the translation initiation codon ATG) was subcloned into the pGL3-Basic vector (Promega) digested with *Sac*I and *Bgl*II, thus constructing the plasmid pIAP-2574/−49 (23). Different 5′ deletion mutants were constructed by digesting the plasmid pIAP-2574/−49 with *Sac*I and another appropriate restriction enzyme followed by generation of blunt-ended fragments, agarose gel purification of the desired band, and recircularization. The *IAP* fragments from the derivatives of pGL3-Basic were transferred to an improved pFRL2 promoter-detection vector that eliminates errors due to copy number variation associated with

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Table 1. *Eight primers used in RT-PCR*

Primer	Sequence
hIAPcDNA2146F	5′-gca acc ctg caa ccc acc caa gga g-3′ (25 bases)
hIAPcDNA2423R	5′-cca gca tcc aga tgt ccc ggg ag-3′ (23 bases)
hbetaActin601F	5′-ggg tct gga cct ggc tgg ccg gga cct g-3′ (28 bases)
hbetaActin1100R	5′-ggg ccg ccg atc cac acg gag tac ttg c-3′ (28 bases)
hCdx1 971F	5′-gcc ctg acc ttc tgg gac atg gtg g-3′ (25 bases)
hCdx1 1250R	5′-cag gat cag tgt ggt ccc cac aca g-3′ (25 bases)
hCdx2 1211F	5′-ccc aca gcc ata gac cta cag acc-3′ (24 bases)
hCdx2 1550R	5′-gcc atg agc agg cag cag cca tc-3′ (23 bases)

reporter assays using derivatives of pGL3-Basic and other promoter-detection vectors (22). To construct a derivative of pFRL2, the *Kpn*I-*Nar*I fragment of a derivative of pGL3-Basic carrying a specific fragment of the *IAP* promoter region was isolated and then subcloned into pFRL2 codigested with *Kpn*I and *Nar*I restriction enzymes. Thus the *IAP* reporter plasmid pFRL2-IAP1 carries the 2.5-kb full-length IAP promoter region (−2574 to −49). The plasmids pFRL2-IAP2 to pFRL2-IAP4 are the derivatives of pRFL2 and carry different 5′ deletions (from −49 to −1875, −1283, −1107, and −750, respectively) of the *IAP* promoter.

Cell culture. The human colorectal adenocarcinoma-derived Caco-2 cell line was purchased from American Type Culture Collection (Manassas, VA) and was maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (Sigma, St. Louis, MO), 2 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin (Invitrogen Life Technologies). Cells were grown at 37°C in the presence of 5% CO_2 and were split by trypsinization when they reached ~80–90% confluence.

RNA preparation and RT-PCR. Total RNA was prepared using TRIzol reagent from Invitrogen Life Technologies following the manufacturer's instructions. First-strand cDNA was synthesized using polydTn RNA from Caco-2 cells with primer and Thermoscript RT. PCR was then performed with gene-specific primers using *Taq* DNA 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. TNT^R 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-^{33}P]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%

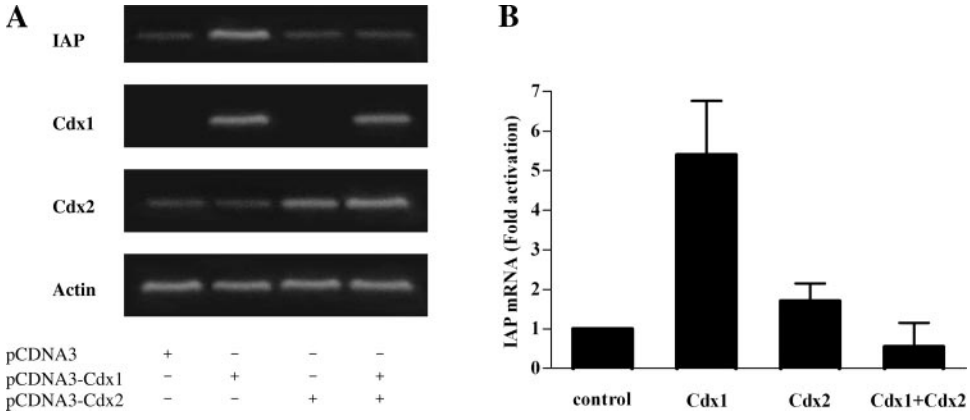

Fig. 1. Effect of Cdx1 and Cdx2 on the endogenous intestinal alkaline phosphatase (*IAP*) gene in the Caco-2 cell line. Caco2 cells were transfected with the Cdx1 and/or Cdx2 expression vector or the empty vector as a control. Total RNA was isolated 48 h after transfection from the transfected cells, and then cDNA was synthesized. *A*: semiquantitative 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*: LightCyler real-time PCR analysis of IAP expression. Using the same cDNA, LightCycler PCR was performed as described in MATERIALS AND METHODS. LightCyler 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.

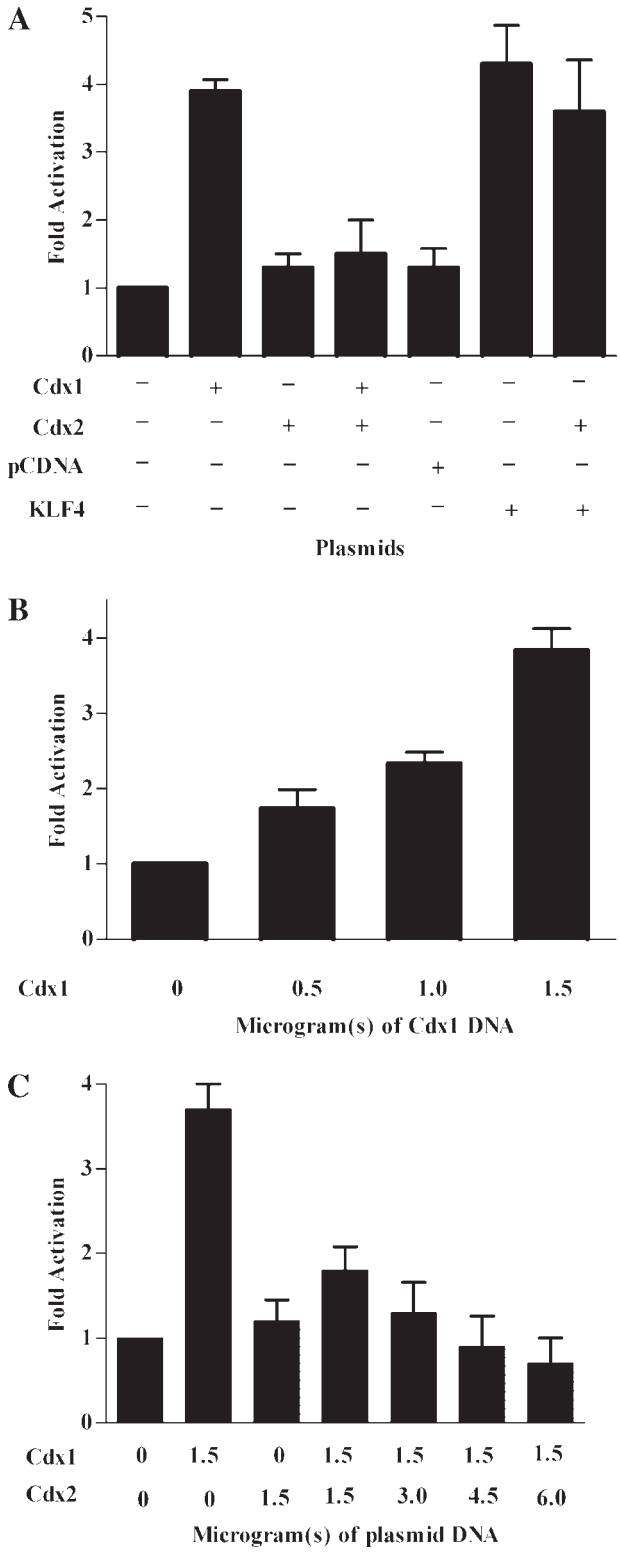

Fig. 2. Cdx1 and Cdx2 regulation of the *IAP-luciferase* reporter gene. Caco2 cells were transiently transfected with a 2.5-kb IAP-luciferase reporter plasmid along with Cdx1 and/or Cdx2 expression vectors. Kruppel-like factor (KLF4) expression vector and the empty vector pCDNA were used for control 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.

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. 1*A*) and LightCycler real-time PCR assays (Fig. 1*B*) 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 (fivefold, $P < 0.05$). 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. 2*A*). 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. 2*A*). The effects of Cdx1 were dose dependent, as shown in Fig. 2*B*, and, furthermore, the Cdx2 inhibitory effects on *IAP* gene were also dose dependent (Fig. 2*C*). 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. 2*A*).

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-

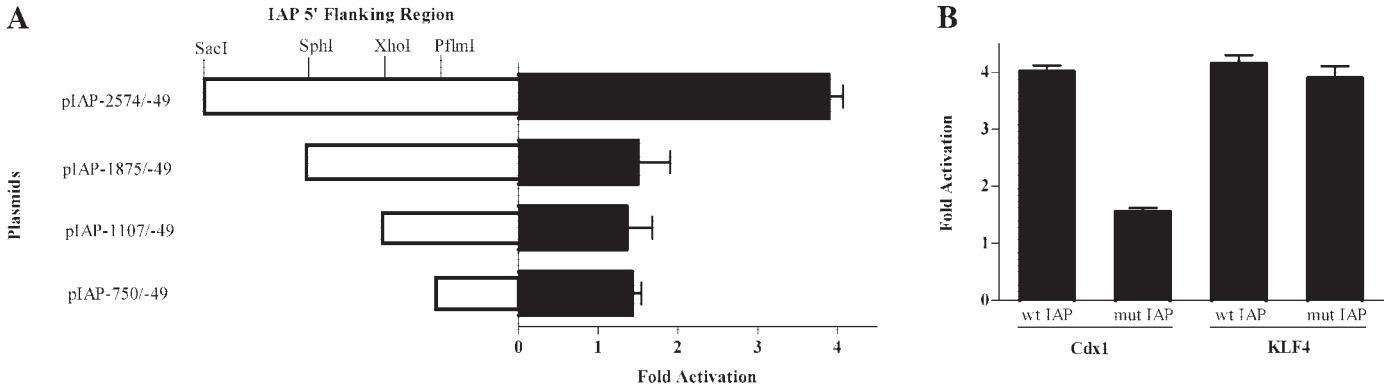

Fig. 3. Localization of the Cdx response element in *IAP* promoter. Luciferase reporter plasmids carrying various *IAP* promoter regions were used to transfect Caco-2 cells (see MATERIALS AND METHODS). The cells cotransfected with Cdx1 expression vector. The result expressed as fold activation (Cdx^+/Cdx^-) 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 pIAP-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 pIAP-2574/−49 plasmid (see MATERIALS AND METHODS).

tially decreased, suggesting that one or more CdxRE are located in the region between −2574 and −1875 (Fig. 3*A*).

Based on sequence analysis of the region between −2574 and −1875 of the *IAP* promoter, a putative CdxRE was identified. An internal substitution mutant of the pIAP-2574/-49 plasmid was constructed in which the putative CdxRE was changed (GTTTAGA between −2369 and −2375 was changed to GCGGATC). This mutation resulted in a significant loss of *IAP* promoter induction by Cdx1, indicating that the stimulatory effect of Cdx1 on the *IAP* promoter is dependent on an intact CdxRE (Fig. 3*B*).

IAP-CdxRE binds to the in vitro synthesized human Cdx1 and Cdx2 proteins. We next employed EMSA to determine whether the identified CdxRE is capable of binding Cdx proteins in vitro. We synthesized a double-stranded oligonucleotide corresponding to the putative IAP-CdxRE sequence as well as a mutant version (mCdxRE) and an oligonucleotide corresponding to previously characterized CdxRE from the *p21* gene (Table 2) (26). EMSAs were performed using the radiolabled CdxRE probe and in vitro synthesized Cdx1 or Cdx2 proteins. Figure 4 demonstrates that both Cdx1 and Cdx2 bind efficiently to the CdxRE, thus generating a shifted band (*lanes 2* and *7*). Approximately 100-fold excess of cold competitor oligonucleotides (CdxRE and TATA p21) was able to compete with the CdxRE probe, whereas the mutant mCdxRE could not compete (*lanes 3-5*, and *8-10*, respectively), thus verifying the specificity of binding. These results confirm that the CdxRE within the *IAP* gene specifically binds to the Cdx1 and Cdx2 proteins.

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

Cdx consensus	A/CTTTATA/G
	-2389 -2358
Wild-type IAP	5′-ttttttttttttGTTTAGACagggtcttgtt-3′
Mutant IAP	5′-ttttttttttttGCGGATCCagggtcttgtt-3′
TATA p21	5′-gggcggTTGTATATcagggccgc-3′

IAP, intestinal alkaline phosphatase.

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 force 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, while 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 2*C*) 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

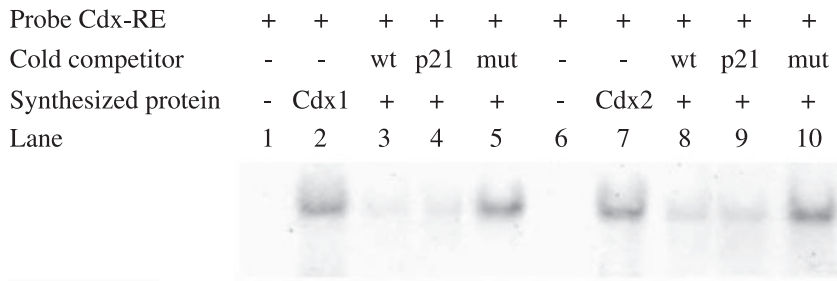

Fig. 4. Binding of Cdx1 and Cdx2 to the putative IAP-Cdx response element (IAP-CdxRE) analyzed by EMSA. Double-stranded oligonucleotide carrying the putative IAP-CdxRE was 5′ end-labeled with ^{33}P and used as the probe in EMSA. Cdx1 and Cdx2 proteins were synthesized in vitro, and ~3 ng of protein 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.

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. 2*A*).

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 (GTTTAGA) 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 *sucrase-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 enterocyte 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.

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