Repression of the Desmocollin 2 Gene Expression in Human Colon Cancer Cells Is Relieved by the Homeodomain Transcription Factors Cdx1 and Cdx2

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Abstract
Desmosomes are intracellular junctions that provide strong cell-cell adhesion in epithelia and cardiac muscle. Their disruption causes several human diseases and contributes to the epithelial-to-mesenchymal transition observed in cancer. Desmocollin 2 (DSC2) is a cadherin superfamily member and a critical component of desmosomes found in intestinal epithelium. However, the mechanism regulating DSC2 gene expression in intestinal cells is not known. Cdx1 and Cdx2 are homeodomain transcription factors that regulate intestine-specific gene expression. Cdx expression in the past has been associated with the induction of desmosomes. We now show that the DSC2 gene is a transcriptional target for Cdx1 and Cdx2. Colon cancer cell lines retaining Cdx2 expression typically express DSC2. Restoration of DSC2 expression in Colo 205 cells induced DSC2 mRNA and protein and the formation of desmosomes. The 5’-flanking region of the DSC2 promoter contains two consensus Cdx-binding sites. Electrophoretic mobility shift assays show that Cdx1 and Cdx2 bind these sites in vitro, and chromatin immunoprecipitation confirmed Cdx2 binding in vivo.

DSC2 promoter truncations established that these regions are Cdx responsive. The truncations also identify a region of the promoter in which potent transcriptional repressors act. This repressor activity is relieved by Cdx binding. We conclude that the homeodomain transcription factors Cdx1 and Cdx2 regulate DSC2 gene expression in intestinal epithelia by reversing the actions of a transcriptional repressor. The regulation of desmosomal junctions by Cdx contributes to normal intestinal epithelial columnar morphology and likely antagonizes the epithelial-to-mesenchymal transition necessary for the metastasis of colon cancer cells in humans.

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Introduction
Desmosomal junctions are sites of mechanically strong cell-cell adhesive interactions. They are found in tissues exposed to high levels of mechanical stress, such as skin, gastrointestinal epithelium, and cardiac muscle. Desmosomes are members of a class of junction known as “anchoring junctions” due to their role in coupling the stress-bearing intermediate filaments from adjacent cells (1). Intermediate filaments are anchored to the desmosomes, effectively linking intermediate filaments from adjacent cells into a “supracellular scaffolding” that is able to withstand high levels of mechanical stress. Desmosomal proteins therefore contribute to the maintenance of tissue integrity and architecture. Loss or disruption of desmosomes is recognized as the underlying cause of several human diseases, including pemphigus foliaceus and arrhythmogenic right ventricular cardiomyopathy (2, 3). Additionally, the loss of normal cell-cell adhesion mechanisms, including desmosomes, contributes to the epithelial-to-mesenchymal transitions (EMT) that is a critical feature of many epithelial cancers.

Desmocollin 2 (DSC2) is one of three desmocollin cadherins that, along with the desmoglein cadherins, form the electrodense protein core of desmosomes. The desmocollin cadherins are membrane-spanning glycoproteins that function as Ca2+-dependent cell adhesion molecules (1, 3). DSC2 has the widest tissue distribution in epithelia and desmosome-bearing nonepithelial tissues (4). DSC1 and DSC3 are restricted to stratified epithelia and cardiac muscle (5). However, in several human cancers, there is a breakdown in this tissue-restricted expression pattern. It is believed that this “desmocollin switching” could be a component of EMT, similar to the well-described “cadherin switching” (6, 7). The mechanisms regulating this change in desmocollin gene patterning are not known, although the switch from E-cadherin to N-cadherin seen with EMT is brought about by potent transcriptional repressors such as Slug or Snail that suppress E-cadherin gene expression (8, 9). In normal intestinal and colonic epithelium, only DSC2 is expressed. However, little is known about the mechanisms regulating DSC2 gene expression in this tissue. The homeobox transcription factors Cdx1 and Cdx2 are important regulators of intestinal epithelial cell differentiation, cell adhesion and columnar morphology, apoptosis, and proliferation (10). They modulate these diverse processes largely by enhancing the expression of several target genes, including sucrase isomaltase, haephestin, LI-cadherin, claudin2, Bcl2, and...
We and others have shown that Cdx1 and Cdx2 can modulate adherens and tight junction activity in intestinal epithelial cells (11, 17-19); however, regulation of desmosome formation by Cdx remains unexplored.

Colo 205 cells are an undifferentiated, unpolarized human colon cancer cell line that do not express their endogenous Cdx1 or Cdx2 genes. Restoration of Cdx expression induces Colo 205 cell differentiation, characterized by the development of a polarized morphology and numerous desmosomal junctions (18). Herein, we show that the DSC2 gene is an important transcriptional target of Cdx1 and Cdx2. DSC2 mRNA and protein levels are significantly enhanced by Cdx expression in Colo 205 and other colon cancer cell lines. In contrast, short hairpin RNA (shRNA)-mediated reduction in Cdx2 levels results in a diminishment of DSC2 protein expression. The 5′-flanking region of the DSC2 gene promoter contains two consensus binding sites for Cdx. Using several experimental approaches, including electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP), and transfection of DSC2 promoter mutants, we establish that Cdx binds these sites to enhance DSC2 gene expression. Moreover, we identify a region of the DSC2 gene promoter that is bound by a potent transcriptional repressor in Colo 205 cells. Cdx2 enhances DSC2 gene expression in part by relieving this repressor activity.

We conclude that the DSC2 gene is a transcriptional target for the intestine-specific transcription factors Cdx1 and Cdx2. Regulation of adherens, tight, and now desmosomal junctions by Cdx contributes to normal intestinal epithelial columnar morphology.

![Figure 1](image-url)

**FIGURE 1.** Induction of DSC2 expression in colon cancer cells is associated with Cdx1 or Cdx2 expression. A. Transmission electron micrograph of control MIGR1-infected and MIGR-Cdx1–treated Colo 205 cells showing induction of desmosomes only in the Cdx1-expressing cells. Cells were grown on Transwell filters for 14 d and then fixed and subjected to transmission electron microscopy. B. DSC2 mRNA and protein are reliably induced 3- to 4-fold with Cdx1 or Cdx2 expression in Colo 205 cells (n = 3). mRNA measured using quantitative real-time SYBR Green reverse transcription-PCR. X1, Colo 205 cells treated with the MIGR-Cdx1 retrovirus; M, Colo 205 cells receiving the control MIGR1 retrovirus; X2, Colo 205 cells treated with the MIGR-Cdx2 vector. C. Transient Cdx1 expression was induced in DLD1 colon cancer cells using either an adenoviral vector (AdCdx1) or electroporation, as previously described (22, 34). DSC2 mRNA levels were then quantified at 48 h by quantitative real-time SYBR Green reverse transcription-PCR. AdNull and pRC/empty served as controls for these experiments. n = 3 for each. D. Western blot analysis of Cdx1, Cdx2, and DSC2 in colorectal cancer cell lines. Actin levels are included as a loading control. E. Knockdown of Cdx2 gene expression reduces DSC2 levels in DLD1 cells. Whole-cell lysates were prepared from DLD1 cells treated with a shRNA vector targeting Cdx2 (shRNA CDX2), an empty control vector (shRNA Em), or uninfected control cells (Con). Western blot analysis for DSC2, Cdx2, and actin was carried out as before.
and likely antagonizes the process of EMT necessary for the metastasis of colon cancer cells in humans.

**Results**

**Cdx1 and Cdx2 Expression Are Associated with the Induction of Desmosomes and DSC2 Gene Products in Colon Cancer Cells**

Colo 205 cells are a poorly differentiated human colon cancer cell line that do not express detectable levels of the intestine-specific transcription factors Cdx1 or Cdx2. Using a retroviral vector (MIGR1), we were able to restore Cdx1 or Cdx2 expression to these cells. We noted a profound change in their appearance when Cdx1 or Cdx2 was expressed. Cdx expression induced tight cell clustering and enhanced cell-cell adhesion (17, 18). At the ultrastructural level, this is associated with the induction of a polarized, columnar morphology and the development of advanced cell-cell junctions, including adherens, tight, and desmosomal junctions (Fig. 1A). The induction of desmosomes by Cdx has been noted by us and others in the past (17, 20, 21). However, the mechanism by which this occurs remains unknown.

The DSC2 gene had appeared on several exploratory microarray studies as a gene whose mRNA levels were increased with Cdx1 or Cdx2 expression in Colo 205 cells (data not shown). To confirm this finding, we measured DSC2 mRNA and protein levels in our Colo 205 cells. We observed a significant 3-fold increase in DSC2 mRNA and protein levels (Fig. 1B) with expression of either Cdx1 or Cdx2. The increase seemed proportionate, suggesting that the escalation in DSC2 protein might be entirely explained by the mRNA increase. Moreover, DSC2 mRNA levels are also significantly increased when Cdx1 is overexpressed in DLD1 cells (Fig. 1C). Transient transfection of a Cdx1 expression vector or adenoviral-mediated Cdx1 expression (22, 23) both enhanced DSC2 mRNA levels, suggesting that this response is not specific to Colo 205 cells and may be generalizable to all colonocytes and colon cancer cells.

We then examined DSC2 expression in other colon cancer cell lines. In general, DSC2 gene expression correlated with Cdx2 expression. Those cell lines with abundant Cdx2 expression (DLD1 and T84) also had high levels of DSC2 protein detected (Fig. 1D). In contrast, in LoVo and SW480 cells, where Cdx2 levels are low, little DSC2 protein was observed. HT29 cells seem to be the exception to this rule because they express significant DSC2 protein despite the lack of detectable Cdx expression. Thus, Cdx2 expression seems to be important for DSC2 expression in colon cancer cells, but its expression is not absolutely required.

To further show the importance of Cdx1 or Cdx2 expression for DSC2 levels, we targeted Cdx2 expression in DLD1 cells using a shRNA retroviral vector. A Western blot analysis shows that reductions in Cdx2 levels resulted in a similar decline in DSC2 protein levels (Fig. 1E). No decline in either Cdx2 or DSC2 was observed in the empty vector control cells. Together, these findings support the hypothesis that DSC2 gene expression is regulated by the intestine-specific transcription factors Cdx1 and Cdx2 in human colon cancer cells.

**FIGURE 2.** Sequence analysis of the 5'-flanking region of the human DSC2 gene. The sequence of the DSC2 promoter and identification of the transcription-initiation site was previously described (4). Putative major consensus Cdx2-binding sites are TTTATG and TTTATA. These sequences are indicated by * in the distal promoter sequence. The identity of other weak sequence matches for the canonical Cdx-binding element is indicated by gray shading. The sites for the DSC2 promoter truncations are indicated by the arrows and designated pMDD16, FI, GI, pMDM18, HI, pMDD19, and pMDM20. EMSA oligo sequences are underlined. Primer sets for the ChIP are indicated by the underlined arrows.
Cdx1 and Cdx2 Regulate DSC2 Gene Expression

The DSC2 Gene Promoter Contains DNA Elements That Are Bound by Cdx1 and Cdx2 Proteins

The human DSC2 gene is found on chromosome 18 at q12.1 (24, 25). The cloning and description of the promoter for the human DSC2 gene has been reported (4). The DSC2 gene is found on chromosome 18 at q12.1 (24, 25). The cloning and description of the promoter for the human DSC2 gene has been reported (4). The DSC2 gene is found on chromosome 18 at q12.1 (24, 25). The cloning and description of the promoter for the human DSC2 gene has been reported (4).

Table 1. Oligonucleotide and Primer Sequences Used to Generate Mutants or for Reverse Transcription-PCR

<table>
<thead>
<tr>
<th>EMSA oligos</th>
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<tr>
<td>DC1</td>
<td>5'-AATGAAATTTATGAGATAT</td>
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<tr>
<td>DC1AS</td>
<td>5'-TTCCATATCCATTTATTTATAT</td>
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<tr>
<td>DC2</td>
<td>5'-AAGATTTGCTACCACTCATCCCTATATCA</td>
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<td>DC2</td>
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The DSC2 gene has been reported (4). The DSC2 gene might be a direct transcriptional target for the intestine-specific transcription factors Cdx1 and Cdx2. To test this hypothesis more directly, we generated double-stranded oligonucleotides for use in EMSA studies (Fig. 2; Table 1). As a positive control for these studies, we used the SIF1 sequence from the sucrase isomaltase promoter (26). Nuclear extracts were obtained from Colo 205 cells infected with the Cdx1- or Cdx2-expressing retroviruses. For a positive control, we used nuclear extracts from colon cancer cell lines DLD1 and T84 that express their endogenous Cdx2 gene (Fig. 3A). The negative controls included nuclear extracts from wild-type Colo 205 cells, as well as Colo 205 cells that received the control retrovirus, MIGR1.

Nuclear extracts from Cdx1- or Cdx2-expressing cells generated two novel EMSA bands when incubated with the SIF1 probe (Fig. 3A, arrows). The SIF1 sequence contains two consensus Cdx-binding sites and is known to yield two bands when incubated with Cdx-containing extracts (22, 26). These bands are competed by an excess of cold SIF1 competitor but not the mutant SIF1 (data not shown). Of interest, the EMSA patterns seen when using Colo-MIGR-Cdx1 and Colo-MIGR-Cdx2 nuclear extracts are very similar to the DLD1- and T84-positive controls. Next, we did the same study using radiolabeled DC1 and DC2 probes. A
new shifted band is plainly visible associated with Cdx1 or Cdx2 (arrow) expression in the test and positive control lanes but not when using the negative control nuclear extracts (Fig. 3A).

A more detailed analysis indicates that there are typically three bands visible when radiolabeled DC1 or DC2 oligos are incubated with Colo-MIGR-Cdx1 or Colo-MIGR-Cdx2 nuclear extracts (Fig. 3B; data not shown). The middle band seems to be specific for Cdx (arrow). It is effectively competed by cold DC1, cold DC2, and cold SIF1 oligonucleotides. Moreover, the intensity of this band is reduced when the extract is incubated with a supershifting Cdx2 antibody but not the control rabbit IgG (Fig. 3B). The faster-migrating band (black arrowhead) seems to be nonspecific, as its signal intensity is diminished when any of the unlabeled competitors are included, including mutant DC oligos. In contrast, the slower-migrating band (gray arrowhead) may represent another specific DNA-protein interaction. The intensity of this band is diminished by the unlabeled DC competitor but not by the SIF1 or mutant DC competitors (Fig. 3B). This suggests that the protein-DNA complex is not simply bound Cdx protein because SIF1 should have competed it away. The complex must contain another protein that does not recognize the SIF1 sequence. However, this band is lost when supershifting Cdx2 antibodies are incubated with the nuclear extract. Thus, Cdx must also be present in some fashion, either bound directly to the DNA probe or bound to the unknown protein recognizing specific sequences in DC1 and DC2. A similar supershift activity was

**FIGURE 3.** Interaction of Cdx1 and Cdx2 with the putative Cdx-binding sites of the human DSC2 promoter. A, Standard reaction EMSA was done using 10 µg of nuclear extracts from several colon cancer cell lines. Labeled oligonucleotide probes included DC1 and DC2 from the DSC2 promoter. SIF1 was used as a positive control. Three protein-DNA complexes are observed with SIF1. The Cdx-specific complexes are indicated by the arrows, and a nonspecific complex is indicated by the black arrowhead. For DC1 and DC2, the Cdx-specific complex is indicated by the arrows; nonspecific complexes by black arrowheads. A specific complex that is not Cdx is indicated by gray arrowheads. Western blot analysis of Cdx2 and Cdx1 with the nuclear extracts. B, Competition assays using the labeled DC1 and DC2 oligos as probes. The Cdx-specific complex is indicated by the arrow; other specific and nonspecific complexes are indicated by the gray and black arrowheads, respectively. The competitors included unlabeled DC1 and DC2 as well as their mutants (MutDC1 and MutDC2). A specific competitor from Cdx binding, SIF1, was used as an additional control. DC1, DC2, and SIF1 competitors were used in a 50- or 200-fold excess. Colo-MIGR-Cdx2 cells were the source for the nuclear extracts used in this study. Cdx2-bound probe was supershifted using the Cdx2-88 monoclonal antibody or an anti-actin monoclonal antibody assays. The supershifted bands are indicated by white arrows.
observed using Colo-Cdx1 nuclear extract and an anti-Cdx1 polyclonal antibody CPSP (data not shown). In summary, the two consensus Cdx-binding sites identified in the DSC2 promoter are specifically recognized and bound by Cdx1 and Cdx2 protein

in vitro.

ChIP Assays Establish Cdx2 Binds the DSC2 Promoter

in vivo

We have identified putative Cdx-responsive elements within the human DSC2 promoter. Moreover, we established by EMSA that these elements are specifically recognized by Cdx1 and Cdx2 protein. To further show the importance of these DNA elements, we used ChIP to determine if these sites were occupied by Cdx binding in vivo. We used Colo 205 cells expressing a wild-type Cdx2 or Cdx2 with an NH2-terminal Flag tag and compared it with Colo 205 cells receiving the control empty retrovirus MIGR1. PCR primer sets were designed to flank either DC1 or DC2 and were designated Primer1 (P1) or Primer2 (P2; Fig. 2). P1- and P2-amplified regions were separated by only 140 nucleotides, limiting our ability to distinguish between occupancy of the individual sites by Cdx2. In Colo 205 cells, Cdx2 binding was detected in the region of the DSC2 promoter that contains the DC1- and DC2-binding sites (P1 and P2), but only in Colo-MIGR-Cdx2 cells and not in control Colo-MIGR cells (Fig. 4A). This was a specific effect, as there was no enrichment of α-actin on the DSC2 promoter. Moreover, similar results were obtained using the Flag antibody to pull down Flag-tagged Cdx2 (Fig. 4B). We extended our study to DLD1 cells and immunoprecipitated the endogenous Cdx2 using the Cdx2-88 antibody (Fig. 4C). Here again, DNA fragments containing DC1 and DC2 regions were recovered in the ChIP assay. No fragments were detected when the control α-actin antibody was used. Taken together, both in vitro and in vivo observations support the conclusion that Cdx2 binds to the DC1 and DC2 sequence elements within the DSC2 promoter.

Cdx2 Expression Enhances DSC2 Promoter Activity

We next obtained DSC2 promoter-reporter constructs to directly test for a functional response to Cdx2. Deletion
constructs of the DSC2 promoter, pMDM16 (−1,697/−9), pMDM18 (−525/−9), pMDM19 (−332/−9), and pMDM20 (−134/−9), were kindly provided by Dr. Roger S. Buxton (Division of Membrane Biology, National Institute for Medical Research, London, United Kingdom; ref. 4). We then generated two additional truncation constructs for the DSC2 promoter. We called them pMDMFI (−1,223/−9), in which DC1 is deleted, and pMDMGI (−1,119/−9), which deletes both DC1 and DC2 (Fig. 2). The full-length promoter construct, pMDM16, contained both consensus sites and was very responsive to Cdx2. Luciferase activity from the pMDM16 reporter increased in a dose-dependent fashion as the amount of the cotransfected Cdx2 expression vector was increased (Fig. 5A). Truncation of the promoter to remove the DC1 site significantly reduced pMDMFI responsiveness to Cdx2 by ~70% when compared with pMDM16 (Fig. 5B). When the pMDMGI reporter construct was used, in which both DC1 and DC2 sites are deleted, Cdx2 responsiveness was completely abrogated. The luciferase activity was no different than that observed with the pGL2-Basic construct alone. This finding strongly supports the hypothesis that Cdx2 regulates DSC2 gene expression by binding the DC1 and DC2 sites in the distal promoter to enhance DSC2 gene expression.

Further truncating the DSC2 promoter yielded some surprising results. Unexpectedly, removing the region −1,119 to −525 restored significant Cdx2 responsiveness. The pMDM18 construct in fact had double the Cdx2 responsiveness of the full-length pMDM16 construct (Fig. 5B). Because this finding was so unusual, we generated a new truncation construct from pMDM16, called pMDMHI. It is essentially the same as pMDM18 in sequence; however, it was made the same way as pMDMFI and pMDMGI using PCR cloning techniques. Functionally, the pMDMHI and pMDM18 constructs were indistinguishable in their response to Cdx2 (Fig. 5B). Truncating the promoter further to −323 or −134 (pMDM19 and pMDM20) completely removed any remaining Cdx2 responsiveness. The results with the pMDM18 and pMDMHI reporters suggested two possibilities. First, there is likely a potent transcriptional repressor suppressing DSC2 gene expression. It seems to bind within the region −1,119 and −525. The repression is relieved only by Cdx1 or Cdx2 binding the DC1 and DC2 sites. Second, it suggests that there may be other Cdx-responsive elements within the DSC2 promoter between −525 and −323 that are active only when the repressor complex is not present.

FIGURE 6. Cdx2 binds cryptic responsive elements in the proximal promoter with less efficiency. Cryptic Cdx-binding sites were identified in the proximal promoter region and tested for Cdx2 binding in vitro and in vivo. A. EMSA using Colo-MIGR-Cdx2 nuclear extract and labeled oligos for DC1 and DC2 (the canonical Cdx-binding sites) as well as DC3, DC4, and DC5 (imperfect matches to the consensus sequence). Labeled probes were competed with unlabeled probe or unlabeled SIF1. White arrowhead, specific Cdx2-binding complex. B. DC oligos were tested for their ability to compete with a labeled SIF1 probe for Cdx2 binding. Specific Cdx shift complexes are denoted by the black arrows. A nonspecific complex is indicated by the black arrowhead. C. ChIP assay for Cdx2 binding the proximal DSC2 promoter (P3) in DLD1 cells. ChIP was done as before. D. Direct comparison of ChIP from the DSC2 promoter for three regions: the region containing the consensus Cdx site DC1 (P1), the segment containing the second consensus site DC2 (P2), both in the distal promoter region, and the third is from the proximal promoter region and contains imperfect Cdx-binding sites (P3) and is Cdx responsive based on transient transfection assays (Fig. 5). ChIP was done in the indicated cells using the same antibodies and methods as before.
Several Cryptic Cdx2-Binding Sites Identified within the DSC2 Promoter

We therefore restudied the promoter sequence and identified several sites with a close match to the canonical Cdx sequence (Fig. 2). To determine if these sites had any Cdx activity, we generate oligonucleotides for EMSA studies. DC1 and DC2 were served as controls for these studies. Two of these sequences, DC3 and DC5, yielded shifted bands that were competed by cold specific competitor as well as unlabeled SIF1 (white arrowheads), suggesting that Cdx2 may interact with these sites (Fig. 6A). In contrast, the DC4 shifted complex was not competed by unlabeled SIF1. In the reverse experiment, labeled SIF1 oligonucleotide was competed by either unlabeled SIF1 or unlabeled DC oligonucleotides. DC5 was the only oligonucleotide that did not efficiently compete off Cdx2 binding from the labeled SIF1 oligos (Fig. 6B, black arrows).

We next used ChIP assays to determine if the proximal DSC2 promoter region was bound by Cdx2. This PCR primer set, P3, is at least 500 nucleotides away from the P2 set and overlaps with the DC5 oligo site (Fig. 2). A weakly positive ChIP using the P3 primer set was observed in DLD1 and Colo-MGR-Cdx2 cells (Fig. 6C; data not shown). To directly compare the primer sets, a PCR amplification was done using all three primer sets on the same Cdx2 immunoprecipitation products. In each case, the P3 signal was obviously weaker than P1 and P2 (Fig. 6D). We conclude that the proximal DSC2 promoter is bound by Cdx2 in vivo, but this interaction is not as prominent as the interaction with the distal canonical Cdx-binding sites.

Mutation of the DC1 or DC2 Elements Reduces Cdx2 Responsiveness and Disrupts the Actions of the Transcriptional Repressor

To further establish the central role played by the Cdx-responsive elements DC1 and DC2, we used site-directed mutagenesis to destroy the Cdx-binding sites. Rather surprisingly, mutation of either DC site seemed to completely disrupt the actions of the repressor, as the new promoter-reporters were all as Cdx2 responsive as the truncated pMDM18 construct (Fig. 7A). This was especially surprising, as the deletion of these sites by truncation, as in the construct pMDMGI, preserved repressor function.

We then isolated the Cdx2-responsive elements away from the repressor and subcloned them separately into the pGL2-Promoter vector (Promega). The pGL2-Promoter construct contains an SV40 promoter and is used to evaluate the function of putative enhancer and repressor elements away from their native promoter context. In this context, the DC1 and DC2 elements behaved as predicted. Cdx2-responsive activity from the chimeric promoter is reduced by more than half when the DC1 and DC2 sites are mutated (Fig. 7B). The remaining Cdx2 responsiveness of the 16Gmut mutated construct seems to be either from the inherent Cdx responsiveness of the pGL2-Promoter construct or from cryptic Cdx2 sites within the DSC2 promoter fragment (Fig. 2).

Equally interesting, the transcriptional repressor located within the 600 nucleotides between −1,119 and −525 was able to inhibit the pGL2-Promoter. In fact, both basal promoter activity as well as Cdx2-responsive activity were essentially abolished by the DNA fragment containing the repressor element (Fig. 7B). Of note, the pGL2-Promoter construct alone is very responsive to Cdx2 expression. We have observed this before and believe it is real. We think it has to do with the AT-rich sequences frequently upstream of these reporters to prevent RNA polymerase read through. Alternatively, it can be due to cryptic Cdx-binding sites within the SV40 promoter itself. Due to this effect, we do not use SV40-based vectors for transfection controls. However, this effect does not significantly alter the interpretation of the experiment. Mutation of the putative Cdx2-binding sites did significantly diminish the Cdx2 responsiveness of the chimeric promoter. In addition, the repressor region of the DSC2 promoter inhibited not only basal promoter activity but the Cdx2 responsiveness of the pGL2-Promoter construct (Fig. 7B). These chimeric promoter studies serve to support the observations made using DSC2 promoter truncations by showing that elements from the DSC2 promoter provide Cdx2 responsiveness and transcriptional repression when placed in a heterologous promoter.

To better localize repressor activity, additional DSC2 promoter truncations were generated in the pGL2-Basic vector as before, G1 (−923/−9), G2 (−718/−9), G3 (−629/−9), and G4 (−603/−9; Fig. 7C). Transient transfection of these reporter constructs along with a Cdx2 expression vector suggested that there might be more than one repressor site within this region of the promoter between −1,119 and −525 (Fig. 7C). There was a significant increase in luciferase activity between the pMDMGI and G1 constructs, and a further significant increase as the promoter was truncated to G2. Thereafter, we did not see any additional increases, and the G4 construct remained significantly diminished compared with pMDM18. The results of these studies imply that there may be as many as three different repressor regions operating within the DSC2 promoter between −1,119 and −525. We conclude that the DSC2 promoter is negatively regulated by a potent transcriptional repressor in Colo 205 cells. Binding by Cdx1 or Cdx2 to the distal DC1 and DC2 promoter elements enhances DSC2 gene expression by disrupting the actions of this repressor complex.

Discussion

The epithelium lining the intestine and colon is quite remarkable. It has evolved in such a way as to maximize absorptive capacity while still serving as a complete barrier to invasive organisms and noxious external environments. Critical to this function are cell to cell adhesive junctions that bind the single layer of epithelial cells together (29). These junctions enhance the structural integrity of the epithelium and prohibit the extracellular passage of microorganisms and large macromolecules. Three types of cell-cell adhesive junctions are thought to be critical for the integrity of intestinal epithelium: adherens junctions, tight junctions, and desmosomes. The mechanisms regulating the development of these junctions in enterocytes and colonocytes are imperfectly understood. In particular, the role of intestine-specific factors in promoting cell-cell adhesion is poorly defined. In the present study, we
showed that the intestine-specific transcription factors Cdx1 and Cdx2 directly regulate DSC2 gene expression. Enhanced DSC2 protein levels may contribute to the increased desmosome formation observed after the expression of Cdx1 or Cdx2 in some cell lines (17, 18, 20, 21).

Cdx Expression Regulates Desmosome Formation and DSC2 Gene Expression in Colon Cancer Cells

In our previous studies with Colo 205 cells, we had noted the induction of desmosomes when Cdx1 or Cdx2 expression was restored (Fig. 1; refs. 17, 18). We therefore confirmed
Cdx-mediated induction of DSC2 mRNA and protein and extended this relationship to other colon cancer cell lines. An analysis of the DSC2 gene sequence identified two consensus Cdx-binding sites in the distal promoter region, suggesting that the DSC2 gene might be a transcriptional target for Cdx1 and Cdx2.

Using novel methodologies, we determined that these distal promoter elements, DC1 and DC2, are bound by Cdx1 and Cdx2 in vitro EMSA assays. Moreover, a ChIP analysis of the DSC2 promoter confirmed that Cdx2 binds this region of the promoter in vivo. Studies with DSC2 promoter-reporter constructs further supported this novel conclusion. The full-length promoter was induced by Cdx2; however, a truncation in which the distal DC1 and DC2 sites were deleted was no longer Cdx2 responsive. Lastly, when this distal region was subcloned into a heterologous promoter, the pGL2-Promoter vector, Cdx2 responsiveness was reduced when the DC1 and DC2 sites were mutated. Taken together, these findings indicate that DSC2 gene expression in colonocytes is regulated in part by the intestine-specific transcription factors Cdx1 and Cdx2. They seem to bind to distal enhancer elements of the gene promoter and thereby induce DSC2 gene expression. Although other cell-cell adhesion proteins are recognized as Cdx transcriptional targets (11, 12), this would be the first report to tie Cdx1 and Cdx2 to the regulation of intestinal cell desmosomes and DSC2 gene expression. Moreover, it is the first report to our knowledge exploring the molecular regulation of the DSC2 promoter and DSC2 gene expression since a description of the gene was first published (4, 25).

Several questions remain unresolved. The role of proximal promoter elements in the Cdx-mediated induction of DSC2 gene expression is still an open question. Our sequence analysis and transient transfection studies seemed to suggest that cryptic but highly responsive Cdx elements are located between nucleotides −525 and −332 (Fig. 5B). However, our ChIP study suggested that this region is not highly occupied by Cdx2 in vivo. Moreover, these proximal elements are apparently unavailable or ineffective when the putative repressive region is retained but the distal Cdx-binding sites DC1 and DC2 are removed, as in the pMDM16 reporter (Fig. 5). We therefore suspect that these cryptic elements may contribute to DSC2 gene expression but are not as critical as the distal DC1 and DC2 sites.

Another question to be answered is how Cdx binding to the DC1 and DC2 sites induces DSC2 gene expression. Is it by activating transcription or disrupting a repressor complex? Typically, Cdx1 and Cdx2 act as activators of transcription, binding proximal promoter regions to enhance promoter activity (10). However, the Cdx2-responsive elements in the DSC2 promoter are quite distant from the transcription initiation site of the gene. Moreover, a potent transcriptional repressor region is located in the promoter between the Cdx binding and transcription initiation sites. Deletion of these distant Cdx-binding sites results in a promoter that not only lacks Cdx responsiveness but has significantly reduced basal level of activity as well. Thus, it is not clear at the present time if Cdx1 and Cdx2 (a) act as transcriptional activators or (b) disrupt a repressor complex or (c) some combination thereof. Progress in understanding this question awaits the identification of the DSC2 repressor mechanism.

**A Transcriptional Repressor Negatively Regulates DSC2 Gene Expression in Colo 205 Cells**

In the absence of Cdx1 or Cdx2, DSC2 mRNA and protein levels are significantly diminished in Colo 205 cells. As suggested above, one explanation for this could be the presence of transcriptional repressors that suppress DSC2 gene expression in Colo 205 and other cells. This would not be unprecedented. The expression of Cdx1 and Cdx2, another cell adhesion gene related to DSC2, is regulated in part by transcriptional repressors (9). Our studies with DSC2 promoter truncations strongly suggest that a repressor protein or repressor complex acts to inhibit DSC2 gene expression in the absence of Cdx1 or Cdx2. This transcriptional repressor seems to bind the region between −1,119 and −525 because when this region was deleted, there was a significant increase in transcriptional activity in response to Cdx2. Moreover, when this region was subcloned into the pGL2-Promoter construct, it effectively silenced both basal and Cdx2-responsive transcriptional activity (Fig. 7B). Additional promoter truncations suggest that there may be as many as three different repressor regions within that 600-nucleotide stretch.

The identity of the DSC2 repressor or repressors remains unknown. E-cadherin gene transcription is inhibited by several different DNA-binding proteins that bind specific E-box elements within the proximal E-cadherin promoter (9). However, for DSC2, we tend to believe that there are several repressor proteins involved in a repressor complex rather than a single inhibitory factor. In support of this, E-cadherin expression is maintained in Colo 205 cells, suggesting that the known E-cadherin repressors are not present or not active.

**FIGURE 7.** The putative Cdx2 distal responsive sites and the repressor element function in heterologous promoters. A, Site-directed mutagenesis was applied to the pMDM16 DSC2 reporter to mutate the Cdx2-responsive elements DC1 and DC2. These elements were mutated separately or together. The resulting promoters were fully sequenced and then subcloned into the pGL2-Basic reporter using the same restriction sites as the original pMDM16 construct. Reporter assays with the mutant DSC2 promoter constructs were done as before and compared with the truncation mutants for responsiveness to Cdx2. Activity of the human DSC2 reporter constructs was normalized as before. Gray columns, transfected with the control pRC/CMV empty construct; black columns, transfected with the pRC/CMV-Cdx2 expression vector. Assays were done in triplicate. Columns, mean values of luciferase activity (n = 3); bars, SD. B, Fragments of the DSC2 promoter containing either both consensus Cdx2-responsive sites (wild-type and mutated) or the region containing repressor activity were subcloned into the pGL2-Promoter construct to determine if these DNA elements can affect a heterologous promoter. These chimeric promoter constructs were transfected into Colo 205 cells as before, along with a Cdx2 expression vector. Gray and black columns are as indicated above. Activity of the human DSC2 reporter constructs was normalized as before. Assays were done in triplicate. Columns, mean values of luciferase activity (n = 4); bars, SD. Statistical analysis was done using ANOVA followed by a multiple comparison test.
Moreover, as we pointed out above, mutation of DC1 and DC2 seemed to disrupt the actions of the repressor, although deletion of these sequences did not. This suggests that multiple proteins covering a broad length of the promoter might be involved. Lastly, we have generated smaller deletions of the repressor region of the DSC2 promoter. We have observed that repressor activity is diminished stepwise as more of the region is deleted rather than a complete loss of all repressor activity with a single deletion. This suggests to us multiple proteins binding across much of the inhibitory region we defined. Identifying these repressors remains a focus of our future efforts.

**The Regulation of the DSC2 Gene May Provide Insights into Desmocollin Switching and EMT**

The identification of a repressor regulating DSC2 gene expression is reminiscent of E-cadherin gene regulation and the role played by repressors such as Twist, Snail, and Slug in EMT during epithelial carcinogenesis (8, 30). Inhibition of E-cadherin gene expression is necessary for EMT to occur. In colon and breast cancer, the E-cadherin expression is commonly suppressed by aberrant expression of developmental regulators such as Snail and Twist. These factors recognize E-box sequences in the proximal promoter and act to repress E-cadherin gene expression. Coincident with loss of E-cadherin expression is the induction of N-cadherin, which some studies suggest may promote cell migration and an invasive phenotype (31). This is sometimes known as cadherin switching.

Thus, it is of interest to consider the role played by the DSC2 gene repressors in EMT and desmocollin switching. We know that in the majority of colon cancers, DSC2 gene expression is lost, with the concomitant induction of DSC1 and DSC3 expression (7). We can speculate that the switch to DSC1 and DSC3 may foster EMT and an invasive phenotype. It has been suggested that disruption of desmosomes may promote Wnt/β-catenin signaling. Plakoglobin, a component of desmosomes, is an armadillo protein that can replace β-catenin in cadherins junctions. This frees up β-catenin to migrate to the nucleus and alter gene expression patterns and foster transformation. Alternatively, there is evidence to suggest that desmosomal assembly can reverse EMT and promoter mesenchymal to epithelial transition even in the absence of changes in E-cadherin levels (6, 32). In summary, the transcriptional repression of DSC2 gene expression in Colo 205 cells seems to be analogous to the regulation of E-cadherin during EMT.

An improved understanding of this process and its contribution to EMT awaits the identification of the negative repressors. However, based on reports from the literature and our observations here, a model is suggested. Cdx2 levels are believed to be diminished during colon carcinogenesis, particularly at tumor borders where the most invasive cells reside. Moreover, Cdx2 levels seem to be inversely related to Snail expression, suggesting that Cdx2 expression may be negatively regulated by Snail (33). We would predict that the loss of Cdx2 expression in colon cancer cells would lead to loss of DSC2 expression and ultimately disruption of the cell-cell desmosomal junctions.

In the work presented here, we showed that the DSC2 gene is a direct transcriptional target of the intestine-specific transcription factors Cdx1 and Cdx2. We expect that this contributes to the Cdx-mediated induction of desmosomes that we and others have observed. The Cdx transcription factors bind to distal enhancer elements and promote gene expression by disrupting the activity of potent transcriptional repressors binding nearby. This frees up proximal promoter elements to initiate gene transcription. Loss of DSC2 gene expression is common in human colon cancers and may contribute to the EMT that is critical for cancer metastasis. We conclude that Cdx expression may function to suppress EMT by promoting strong cell-cell binding through the induction of adherens, tight, and desmosomal junctions in intestinal epithelial cells.

**Materials and Methods**

**Cell Culture**

Colo 205, DLD1, HT29, SW480, LoVo, and T84 colon cancer cells were obtained from the American Type Culture Collection or the Cell Center (University of Pennsylvania). Cells were all maintained as recommended by the American Type Culture Collection. MIGR1-infected, MIGR-Cdx1–infected, and MIGR-Cdx2–infected Colo 205 cells and MIGR-Flag-Cdx2 Colo 205 cells were generated from Colo 205 cells as described (17, 18).

**Western Blots**

Whole-cell extracts were prepared as described (22, 23, 34). Briefly, cells were washed twice in PBS, removed with rubber cell scraper, pelleted, and then resuspended in 80 μL of buffer A (1 × PBS with 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, 1 mmol/L NaVO4). Two volumes of 2 × lysis buffer were added [250 mmol/L Tris-Cl (pH 7.4), 10% SDS, 20% glycerol, 1 mmol/L DTT], and the cells were heated to 100°C for 5 min. The cells were placed on ice briefly, then sonicated for 15 s, and stored at −70°C. Protein concentration was determined by the bicinchoninic acid method (Pierce). Whole-cell lysates (25 μg) were analyzed by SDS-PAGE and electroblotted. The antibodies for DSC2 (RDI Research Diagnostics, Inc.) and Cdx2-88 (BioGenex Laboratories) were used for Western blotting. Cdx1 (CPSP) polyclonal antibody was previously described (35). For a Western blot loading control, we used anti-actin A-4700 (GE Healthcare) according to the manufacturer’s recommendations and exposed to X-ray film (Denville Scientific, Inc.).

**Quantitative Real-time PCR**

Total RNA was isolated from MIGR1-infected, MIGR-Cdx1–infected, and MIGR-Cdx2–infected Colo 205 cells using RNaseasy (Qiagen). The First-Strand cDNA Synthesis kit (Invitrogen) was used for cDNA synthesis. Primer sequence and PCR concentrations are listed in Table 1. For the reverse transcription-PCR, cDNA and primers were mixed with SYBR Green Reverse Transcription-PCR Master Mix (Applied Biosystems) and then assayed in an ABI Prism 7000 sequence detection system as directed by the manufacturer. A ribosomal phosphoprotein, 36B4, was used as the normalization control.
Production of shRNA Vector

The shRNA vector targeting pSUPER.Retro.neo.Cdx2 was generated by annealing complementary oligonucleotides (sense primer sequence, AACTTTCGTCCGTGTGTTCCACC-TGTTC; antisense, AAGTGAAGACCGAGGAAAGCCTGTC) encoding a hairpin shRNA. The annealed oligonucleotides were then cloned into pSUPER.Retro.neo vector (OligoEngine). Retroviral supernatants were made and collected as above, and retrovirally infected DLD1 cells were isolated by neomycin selection.

Promoter-Reporter Studies

The DSC2 promoter region deletion constructs pMDM16 (−1,697/−9), pMDM18 (−525/−9), pMDM19 (−332/−9), and pMDM20 (−134/−9) were kindly provided by Dr. Roger S. Buxton (4). Additional deletion constructs of DSC2 promoter region pMDM16 (−1,697/−9) were generated by PCR. Specific length fragments of the 5′-flanking region of the human DSC2 gene were amplified by PCR and subcloned into the KpnI and Nhel sites of the pGL2-Basic vector. KpnI and Nhel restriction sequences were included in the forward and reverse PCR primers, respectively. Primer sequences are listed in Table 1. Additionally, several fragments of the 5′-flanking region of the human DSC2 gene between pMDM16 and pMDM18 were subcloned into pGL2-promoter vector. PCR-amplified DSC2 promoter fragments with HindIII restriction sites included in the primers were subcloned in the HindIII site of the pGL2-promoter vector. Primer sequences are indicated in Table 1. All new promoter constructs generated by PCR were fully sequenced to confirm both orientation and sequence fidelity to the template promoter.

Site-directed mutagenesis of the putative Cdx2-binding motifs in the pMDM16 was carried out with the QuikChange Site-Directed Mutagenesis kit (Stratagene) using complementary oligonucleotides with the mutated codon and Pfu DNA polymerase. Oligonucleotide sequences are indicated in Table 1. The DNA sequence of each mutant construct was verified by sequencing. To confirm enhancer and repressor activities, DSC2 promoter fragments were also amplified by PCR and then subcloned into the pGL2-Promoter vector rather than the pGL2-Basic vector.

Colo 205 cells were transfected using the Nucleofection method (Amaxa Biosystems) following the company’s guidelines. Colo 205 cells (5 × 10⁶) were transfected for each experiment. For reporter assays, a DNA transfection mixture was prepared consisting of 0.1 to 5 μg of the reporter construct and 50 ng of pRL-CMV Renilla (Promega), pRC/CMV-Cdx1 or pRC/CMV-Cdx2 expression vectors were used to transiently express Cdx1 or Cdx2 and have been previously described (22, 23, 34). pcR2.1 vector (Invitrogen) was used to equalize the amount of transfected DNA. The pRL-CMV vector served as a transfection control. Briefly, cells were resuspended in 100 μL of Nucleofector Solution V (Amaxa Biosystems), mixed with cDNA, and pulsed using the program A-24. Immediately after, cells were gently transferred into prewarmed fresh medium. Transfection efficiency was ~40% in analysis of green fluorescent protein–positive cells (data not shown).

Luciferase activity was determined 24 h after transfection using a Dual-Luciferase Reporter Assay System (Promega). Each transfection was measured in duplicate. Transfection efficiencies were normalized to Renilla luciferase activity and protein concentration, and the results are expressed as the mean relative luciferase activity SD of at least three independent experiments.

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared as described (36). Protein concentration was determined using the bicinchoninic acid kit (Pierce). Sense and antisense oligonucleotides corresponding to the Cdx-binding sites or their mutants were annealed and labeled with ³²p-labeled dCTP or dATP by Klenow (New England Biolabs). Sequences of these oligonucleotides are listed in Table 1. EMSAs were done as described previously (22, 34). To do supershift assay, the binding mixtures were incubated for 10 min at room temperature in the presence of 1 μL of Cdx1 (CPSP) or Cdx2 (Cdx2-88) antibodies before electrophoresis.

ChIP Assay

Nucleosome ChIP was done according to the methods of Kouskouti and Talimadis (37). After cross-linking of the cells with 1% formaldehyde for 10 min at room temperature, glycine (final concentration of 125 mmol/L) was added to stop the cross-linking. The cells were washed with ice-cold PBS/0.5 mmol/L phenylmethylsulfonyl fluoride and then scraped off the plate. The nuclei were prepared by resuspending the cells in sucrose buffer A, containing 0.32 mol/L sucrose, 15 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 0.5% bovine serum albumin, 0.5 mmol/L spermine, 0.15 mmol/L spermidine, and 0.5 mmol/L DTT, followed by Dounce homogenization. The nuclear suspension was layered over an equal volume of sucrose buffer B, containing 30% sucrose, 15 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 0.5 mmol/L spermidine, 0.15 mmol/L spermine, and 0.5 mmol/L DTT. After centrifugation at 3,000 rpm for 15 min, purified nuclei were resuspended in buffer N, containing 0.34 mmol/L sucrose, 15 mmol/L HEPES (pH 7.5), 60 mmol/L KCl, 15 mmol/L NaCl, 0.5 mmol/L spermidine, 0.15 mmol/L spermine, and 0.15 mmol/L β-mercaptoethanol. CaCl₂ was added to the samples to a final concentration of 3 mmol/L. Then, 30 units of micrococcal nuclease (U.S. Biochemical) were added and the mixture was incubated for 5 min at 37°C. The reactions were stopped by the addition of an equal volume of 2× sonication buffer [90 mmol/L HEPES (pH 7.9), 220 mmol/L NaCl, 10 mmol/L EDTA, 2% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 0.5 mmol/L phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, protease inhibitor cocktail from Sigma]. Insoluble precipitates were pelleted by centrifugation at 14,000 rpm for 15 min. Then, the soluble chromatin present in the supernatant was collected. The supernatant lysate was preclarified by rotating with 40 μL Protein G-Sepharose 4 Fast Flow (GE Healthcare) for 2 h at 4°C. The lysates were then incubated with 10 μg monoclonal Cdx2-88 antibody, anti-Flag M2 antibody (Sigma), or anti-actin antibody (Sigma), respectively. Protein G-Sepharose 4 Fast Flow (40 μL) was then added and precipitation was done overnight with agitation at 4°C. The precipitated material was de-cross-linked and purified and then analyzed by PCR using the indicated primer sets.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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