The Tumor Suppressor Adenomatous Polyposis Coli and Caudal Related Homeodomain Protein Regulate Expression of Retinol Dehydrogenase L*

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Development of normal colon epithelial cells proceeds through a systematic differentiation of cells that emerge from stem cells within the base of colon crypts. Genetic mutations in the adenomatous polyposis coli (APC) gene are thought to cause colon adenoma and carcinoma formation by enhancing colonocyte proliferation and impairing differentiation. We currently have a limited understanding of the cellular mechanisms that promote colonocyte differentiation. Herein, we present evidence supporting a lack of retinoic acid biosynthesis as a mechanism contributing to the development of colon adenomas and carcinomas. Microarray and reverse transcriptase-PCR analyses revealed reduced expression of two retinoid biosynthesis genes: retinol dehydrogenase 5 (RDH5) and retinol dehydrogenase L (RDHL) in colon adenomas and carcinomas as compared with normal colon. Consistent with the adenoma and carcinomas samples, seven colon carcinoma cell lines also lacked expression of RDH5 and RDHL. Assessment of RDH enzymatic activity within these seven cell lines showed poor conversion of retinol into retinoic acid when compared with normal cells such as normal human mammary epithelial cells. Reintroduction of wild-type APC into an APC-deficient colon carcinoma cell line (HT29) resulted in increased expression of RDHL without affecting RDH5. APC-mediated induction of RDHL was paralleled by increased production of retinoic acid. Investigations into the mechanism responsible for APC induction of RDHL indicated that β-catenin fails to repress RDHL. The colon-specific transcription factor CDX2, however, activated an RDHL promoter construct and induced endogenous RDHL. Finally, the induction of RDHL by APC appears dependent on the presence of CDX2. We propose a novel role for APC and CDX2 in controlling retinoic acid biosynthesis and in promoting a retinoid-induced program of colonocyte differentiation.

Colon cancer arises from distinct genetic events that initiate and promote tumor formation. An inherited colon cancer predisposition, familial adenomatous polyposis, results from mutations in a single gene known as adenomatous polyposis coli (APC).1 This syndrome is characterized by the appearance of hundreds to thousands of colon adenomas in affected individuals. Recent investigations have generated a model describing downstream events controlled by APC. In the current model, APC regulates the activity of a transcriptional pathway that may control colonocyte proliferation (for a review, see Refs. 1 and 2). It does so by regulating the levels of β-catenin, a protein found initially to function as a link between extracellular adhesion molecules and the cytoskeleton. It appears, however, that β-catenin also regulates transcription through a partnership with TCF/LEF transcription factors. In cells expressing functional APC, APC acts to repress β-catenin levels through regulation of ubiquitin-mediated proteolysis. Low levels of β-catenin prevent activation of TCF/LEF. In cells harboring mutated APC, β-catenin accumulates. This accumulation allows assembly of β-catenin-TCF/LEF complexes and activation of the transcriptional capabilities of TCF/LEF (1, 2). β-catenin-TCF/LEF-dependent transcriptional activation of specific cell cycle regulatory genes, like c-myc (3) and cyclin D1 (4), may underlie the development of colon adenomas and colon carcinomas (1–3, 5–8). In addition to sustained cell proliferation, colonocytes within adenomas and carcinomas display differentiation defects (9–13). For example, crypts from colon adenomas are deficient in mucin-producing goblet cells (14), one of the three predominant, terminally differentiated cell types seen within normal colon crypts. Although APC/β-catenin pathway target genes such as c-myc and cyclin D1 offer mechanistic insights into disregulation of colonocyte proliferation, few of the current APC pathway target genes have easily identifiable roles in cellular differentiation.

Retinoids are a class of small lipid mediators derived from vitamin A that have important roles in vision, cell growth, and embryonic development (15, 16). A number of studies implicate retinoids in normal colonocyte function and in the development of colon neoplasms. For example, vitamin A-deficient animals display abnormalities in many epithelial tissues, including colon (17–23). These abnormalities include decreased mucus production, expansion of proliferation zones within the crypt, and ion flux alterations (17–23). At a molecular level, vitamin A-deficient rats showed diminished expression of TGF-β2 in certain epithelial tissues, including intestinal mucosa (24). Systemic administration of retinoic acid (RA) induced the
expression of TGF-β2 and TGF-β3 in these animals. In other studies, retinoid analogs have proven effective in preventing 5-azoxymethane-induced colon carcinoma formation in rats (17, 25-28). Retinoids also induce markers of differentiation, inhibit cell growth, increase cell adhesion, reduce colony formation, block anchorage-independent growth, and suppress invasiveness in colon cancer cells (29-32). Finally, recent studies have demonstrated that RA can not only inhibit the transcriptional activity of β-catenin (33) but can also independently promote the translocation of β-catenin from the cytoplasm to the membrane (34). These effects of RA on β-catenin function are thought to inhibit proliferation and promote differentiation, respectively (34). Although these studies offer evidence supporting a role for retinoids in colonocyte function, the specific cellular controls governing retinoid response pathways remain undefined in colon tissues.

In addition to the nuclear hormone receptors, retinoid responsiveness within cells is governed by retinoid availability (35, 36). For the most part, cells acquire retinoids in the form of retinol, an inactive precursor. Tissues must, therefore, convert retinol into RA in order to activate the network of nuclear receptors required to evoke retinoid transcriptional responses. The enzymes that catalyze these conversions fall into three distinct classes that include the alcohol dehydrogenases, the short-chain dehydrogenases/reductases, and the aldehyde dehydrogenases. Alcohol dehydrogenases and short-chain dehydrogenase/reductase enzymes convert retinol into the aldehyde, retinal. Further conversion of retinol into RA in RA is carried out by the aldehyde dehydrogenase enzyme family. Enzymes in each class have broad substrate specificities and can oxidize or reduce many physiologically important alcohols or aldehydes including ethanol, steroids, and retinoids. The actions of RA, in turn, can be limited by catabolism via cytochrome P450 enzymes (35, 36). Although the biochemistry of these retinoid biosynthetic and metabolic enzymes is emerging, little is known about the regulation of these enzymes within tissues or specific cell types.

In order to define the molecular pathways that may govern APC-dependent differentiation of colonocytes, we have analyzed gene expression profiles in colon adenomas and carcinomas compared with normal colon. Our analyses revealed that colon adenomas and carcinomas show consistent down-regulation of the RA biosynthetic enzymes retinol dehydrogenase 5 (RDH5) and retinol dehydrogenase-like (RDHL). Given that loss of RA biosynthetic genes may contribute to the lack of differentiation observed in colon adenomas and carcinomas, we investigated the regulatory mechanisms that control the expression of RDH5 and RDHL. We found in a survey of normal human tissues that, whereas both RDHL and RDH5 were expressed in the colon, RDH5 expression appears relatively restricted to the colorectal adenoma and carcinoma tissues. We have characterized the introduction of APC into APC-deficient colon cancer cells induced RDH5 expression. Furthermore, the intestinal specific transcription factor, CDX2, targets RDH5 and acts synergistically with APC in the induction of RDH5. Since RA holds known differentiation factor, CDX2, targets RDH5 and acts synergistically with RDHL expression. Furthermore, the intestinal specific transcription factor, CDX2, targets RDH5 and acts synergistically with APC in the induction of RDH5. Since RA holds known differentiation factor, CDX2, targets RDH5 and acts synergistically with RDHL expression.}
translational start site) of the RDH5 promoter and −1637 to +83 of the RDH5 promoter were PCR-amplified from normal human genomic DNA (Clontech) using specific primers (RDH5, forward (5′-GAAGATACACTTGGGTAGAG-3′) and reverse (5′-ACACCATGTCCTTGTACGTTAC-3′); RDH5, forward (5′-GCTGGTCTCCAGTGTACGTTAC-3′) and reverse (5′-TTATAGCTTGTGCCCCAAGGAC-3′)). PCR products were then inserted upstream of the firefly luciferase gene in the pGL3 basic vector (Promega) to create RDH5LUC and RDH5LUC, respectively. 

PRL::Luc contains −36 to +36 of the prolactin gene driving expression of the luciferase gene and was kindly provided by Dr. Andrew Thorburn (Wake Forest University, Winston-Salem, NC). The CDX1 and CDX2 expression vectors were constructed by RT-PCR from normal colon RNA using specific primers (CDX1, forward (5′-GGCGGAGATCATGATGGTATGCTGGTACGTTAC-3′); reverse (5′-GGCGGAGATCATGATGGTACGTTACGAGTACCTC-3′); CDX2, forward (5′-GGCGGAGATCATGATGGTACGTTACGAGTACCTC-3′) and reverse (5′-GGCGGAGATCATGATGGTACGTTACGAGTACCTC-3′)). The RT-PCR products were then cloned into a pCDNA3.1 His C vector (Invitrogen). The β-catenin S73A and DN-LEF expression vectors were kindly provided by Dr. Donald Ayer (University of Utah, Salt Lake City, UT). For luciferase assays, RDH5LUC or RDH5LUC reporters were co-transfected with a Rous sarcoma virus (RSV)-Renilla luciferase reporter plasmid that was used to normalize transfection efficiencies.

**Electrophoretic Mobility Shift Assays—** 22-Mer oligonucleotides representing −338 to −359 in the RDH5 promoter containing putative CDX2 sites were annealed and 5′-end-labeled with [α-32P]ATP using T4 kinase (MBI Fermentas). The labeled oligonucleotides were then purified over a micro Bio-spin 6 column (Bio-Rad) to remove unincorporated nucleotides. For binding reactions, 6 μg of extract from 293 cells overexpressing CDX2 were incubated with 1 μl (50,000–200,000 cpm) of [32P]-labeled probe in a 10-μl final volume of 1× binding buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 0.25 mg/ml poly[d(dC)-dC]-poly[d(dC)-dC]). For competition and supershift assays, a 10- or 50-fold molar excess of unlabeled oligonucleotide or 2 μg of anti-His tag antibody or anti-CDX2 antibody were added 15 min prior to the addition of labeled oligonucleotide. Complexes were separated on a 6% acrylamide gel in 1× TBE at 250 V for 3 h. The gel was then dried and exposed to a phosphor screen and visualized on a PhosphorImager (Amersham Biosciences).

**Northern Blotting—** Total RNA was isolated using Trizol (Invitrogen) followed by poly(A) RNA selection using a Poly(A) Tract mRNA Isolation Kit (Promega). Poly(A) RNA was fractionated through formaldehyde-containing agarose gels and transferred onto nylon membranes (Amersham Biosciences). Probes were generated using the Rediprime II random prime labeling system (Amersham Biosciences) supplemented with [γ-32P]ATP. Hybridizations with [32P]-labeled probes were carried out using ULTRAbuf buffer (Ambion) as recommended by the manufacturer.

**RA Extraction and HPLC Analysis—** Cells were treated with 100 nmol ATROIL at 80–90% confluence for 12 h. Medium was removed, and cells were scraped into PBS for protein quantification. After the addition of 100 mlm of internal standard TTNPB, the medium was acidified with 6 N HCl (0.03X volume) and extracted with an equal volume of hexane containing 0.1 mg/ml butylated hydroxytoluene. The resulting solution was mixed vigorously and spun down at 11,500 rpm for 20 min. Following centrifugation, the organic phase was transferred to a glass vial, dried under nitrogen, and reconstituted in 100 μl of 1:1 MeSO/MeOH for HPLC analysis. Extracted RA is expressed as pmol of RA/mg of protein/mml of TTNPB.

Retinol dehydrogenases are down-regulated in colon adenomas and carcinomas. In order to identify signaling pathway alterations in neoplastic colon, we performed microarray expression analyses on colon adenomas and carcinomas in comparison with a pool of normal colon tissue. A striking feature of our colon tumor progression data was that 80% of the differentially expressed genes were down-regulated in adenoma and carcinoma tissues as compared with normal (data not shown). Two genes in particular showing down-regulation in colon adenomas and carcinomas caught our attention. The first gene encoded RDH5, an enzyme that catalyzes the conversion of retinol into retinal. The second gene encoded RDH5, a recently described, novel retinol dehydrogenase (described by Soref et al. (38), but referred to as hRDH-TBE). Each of these genes was down-regulated at least 2-fold in ~70% of both adenoma and carcinoma tissues relative to normal (Fig. 1A).

Due to small tissue sizes and correspondingly low yields of mRNA samples, we obtained limited data on RDH5 by microarray (see "Experimental Procedures"). We thus used quantitative RT-PCR to assess the expression levels of RDH5 and RDH5 in an additional 10 patient-matched normal and carcinoma colon tissues. Fig. 1B shows that expression of RDH5 was decreased at least 2-fold in 9 of 10 carcinoma samples in comparison with the matched, normal appearing colon tissue, whereas RDH5 was decreased in 6 of 10 samples examined. As a positive marker for distinguishing colon carcinoma from normal, cyclin D1 expression levels were increased in each of the carcinoma samples relative to normal (data not shown) (39, 40). We also noted the loss of tags corresponding to RDH5 in two
Colon Tumor Cells Lack RDHL and RDH5 and Fail to Convert Retinol to Retinoic Acid—Given the absences of RDH5 and RDHL in colon carcinomas, we examined their expression levels in a panel of commonly studied colon carcinoma cell lines. Among the cell lines examined were cells known to harbor mutations in APC or β-catenin (Caco-2, Colo 205, DLD-1, HT29, SW480, and HCT116). Only RKO cells contain both wild type APC and β-catenin. In agreement with the data from human tumors, each of seven colon cancer cell lines expressed low or undetectable levels of RDHL and RDH5 in comparison with normal colon (Fig. 2B). To assess the capacity of these cell lines to produce retinoic acid, we quantified retinoic acid production following incubation of each cell type with 100 nmol of all-trans-retinol for 24 h. Retinoic acid was extracted from tissue culture medium using acidified hexane and retinoic acid levels determined by HPLC in comparison with the internal extraction standard TTPNB. Due to the lack of a method for culturing primary colon epithelial cells, we chose to compare the results from the colon cancer cell lines to normal human mammary epithelial cells, which express RDH5 at levels similar to that of normal colon (data not shown). Fig. 2C shows that each of the colon cancer cell lines converted retinol into retinoic acid poorly.

APC Regulates RDHL Expression—Since RDH5 and RDHL expression was reduced in most colon adenomas and carcinomas as well as colon carcinoma cell lines with APC pathway defects, we began considering mechanisms that would account for their lack of expression. We gave particular attention to the possibility that the regulation of RDH5 and RDHL was connected to the APC pathway. To address this, we utilized the HT29 cell line containing a ZnCl2-inducible APC gene constructed and described by Morin et al. (42). In the absence of ZnCl2, the APC-inducible cells only express mutant forms of APC. Upon the addition of ZnCl2, wild-type APC expression is induced. An HT29 cell line containing a ZnCl2-inducible LacZ gene served as a negative control. We treated each cell line with 100 μM ZnCl2 for 24 h and analyzed endogenous gene expression by Northern analysis. Fig. 3A demonstrates that induction of APC led to a 3.2-fold induction of RDHL without any detectable increase in RDH5 expression. LacZ-inducible cells showed no induction of RDHL or RDH5. The induction of RDHL following restoration of wild-type APC was also time-dependent. Quantitative RT-PCR analysis detected induction of RDHL as early as 8 h following the addition of 100 μM ZnCl2 (Fig. 3B). Induction of RDHL persisted for 12 and 24 h but declined by 48 h when cell viability became limiting. The temporal induction of RDHL was consistent with the temporal induction of APC.

Given that APC induced the expression of RDHL (Fig. 3, A and B), we expected to see an increase in retinol dehydrogenase activity following induction of APC. To accomplish this, cells were again induced to express APC and RDHL. Cells were then provided 50 nM retinol for 4 h. Following incubation, RA was extracted and quantified as above. Consistent with the induction of RDHL, we observed a statistically significant increase in the ability of HT29 cells to convert retinol into RA after induction of APC. We saw no increase in RA production in the LacZ-inducible cells.

APC Regulation of RDHL Is Independent of β-Catenin—APC induction of RDHL (Fig. 3, A and B) focused our attention on understanding the transcriptional mechanisms that control RDHL expression downstream of APC. The most obvious possibility was that elevated levels of β-catenin served to repress RDHL expression. We thus examined the first 1000 base pairs of the RDHL promoter as well as the RDH5 promoter for putative TCF/LEF consensus sequences ((A/T)^3(A/T)CAAAAG).
bars and cells were treated with 100 

sites fused to a luciferase reporter gene. Fig. 4 we used TOP-FLASH, which contains multimerized TCF/LEF activity of 

/H9252 C FLASH, but with mutated TCF/LEF sites) served as negative

and FOP-FLASH (the equivalent reporter vector to TOP-

that whereas 

used to identify 

press luciferase expression in 293 cells, a cell line that is often

APC-inducible and LacZ-inducible cells were treated for 24 h with 100 

A increases conversion of retinol to RA in HT29 cells.

then exposed to 50 mM retinol for 4 h. Medium was extracted, and RA

levels were determined by reversed phase HPLC. TTNPB was utilized

to identify 

catenin using a dominant-negative LEF1 construct (DN-

catenin, RDHL:LUC is not induced. Based on this

result, we discarded the model wherein β-catenin directly regu-

lates RDHL.

RDHL Is Regulated by CDX Transcription Factors—Since 

β-catenin and DN-LEF did not appear to regulate RDHL expression, we considered the possibility that RDHL is controlled by APC independently of β-catenin. To identify new candidates for transcriptional regulation of RDHL, we examined the RDHL promoter for additional, canonical transcription factor binding sites. In this analysis, we found that the first 1000 base

pairs of the RDHL promoter contained seven TTTAT motifs
(Fig. 4A) that have been shown to bind to the caudal related homeodomain proteins, CDX1 and CDX2 (43). By comparison, the same region of the RDH5 promoter contained only one of these motifs (Fig. 4A). CDX1 and CDX2 have important roles in regulating gastrointestinal development in vertebrates (44–47), and they, like RA, have antiproliferative, prodifferentiative effects in colon cells (48–51). Their expression is highly specific to the intestines, and they are down-regulated in certain human colon tumors (52–54). Finally, CDX2 is mutated in RKO cells, the only cell line used in Fig. 2, B and C, that harbors wild type APC and β-catenin. We, therefore, hypothesized that the CDX transcription factors could control RDHL expression. To test this hypothesis, we asked whether CDX1 and CDX2 could activate RDHL:LUC. Fig. 5A shows the induction of RDHL:LUC activity in HCT116 colon cancer cells co-transfected with CDX1 or CDX2. Both CDX1 and CDX2 induced RDHL:LUC but failed to induce RDH5:LUC.

To confirm the binding of CDX2 to putative binding elements within the RDHL promoter, we designed an oligonucleotide corresponding to nucleotides -359 to -338 within the RDHL promoter and used this in an electrophoretic mobility gel shift assay. Nuclear protein extracts from 293 cells transfected with His-tagged CDX2 showed two complexes that were effectively competed by the addition of a cold, unlabeled competitor oligonucleotide. Antibodies to either the His tag or CDX2 protein supershifted the complexes, thus confirming binding by CDX2. Finally, we asked whether the expression of CDX2 in a CDX2-deficient cell line caused induction of endogenous RDHL. Fig. 5C shows that expression CDX2 in RKO cells causes the induction of the endogenous levels of RDHL as measured by Northern analysis.

RDHL:LUC spans -2228 to +1071 of the RDHL promoter and contains 18 putative CDX2 response elements. Note that RDHL:LUC contains 10 putative TCF/LEF sites (Fig. 6A) but does not appear to be regulated by β-catenin (Fig. 4, B and C). In an attempt to pinpoint the region of the RDHL promoter that is responsive to CDX2, we made a series of promoter deletions (Fig. 6A), fused each upstream of the luciferase gene, and assessed the ability of CDX2 to induce each reporter construct. Fig. 6B shows a gradual decrease in CDX2 induction as putative CDX2 sites are eliminated from the promoter. Outside of the RDHL TATA-box, no single mutation eliminated CDX2 induction of RDHL (data not shown).

**APC and CDX2 Regulate RDHL Synergistically**—The above data suggested the possibility that APC and CDX2 regulate RDHL. Two pieces of evidence suggested that APC and CDX2 may work in a dependent manner to regulate RDHL. First, RDHL is down-regulated in tumors and cell lines harboring wild type APC. For example, sequence analysis of the APC mutation cluster region in the nine carcinomas showing reduced RDHL expression in Fig. 1B revealed only six with mutations (data not shown). This suggests intermediates between APC and RDHL. Consistent with this, RKO cells contain wild type APC and β-catenin but lack CDX2. To examine the relationship between APC and CDX2 in the regulation of RDHL, we examined whether APC can induce RDHL:LUC in a colon carcinoma cell line, SW480, that contains very low levels of CDX2 (data not shown). Fig. 7 shows that APC alone was incapable of inducing RDHL:LUC in SW480 cells. In contrast, CDX2 alone activated RDHL:LUC ~2-fold. Induction of RDHL:LUC was dramatically enhanced by co-transfection of APC with wild-type CDX2, suggesting interaction of the two in regulating RDHL. RDH5:LUC was not induced by any of the treatments.

**DISCUSSION**

The existence of biosynthetic and metabolic pathways for retinoids implies that the control of cellular responses to retinoic acid must, at one level, reside in the control of RA biosynthesis and metabolism. In the present study, we provide data that support the loss of RA biosynthetic enzymes as a downstream consequence of APC mutation, but not necessarily a consequence of β-catenin dis regulation. Our results are consistent with a model wherein APC and the intestine-specific tran-
scription factor, CDX2, control the RA biosynthetic capacity in colon cells. Evidence for this model includes the following. (i) Expression levels of RDH5 and RDHL were depressed relative to normal in over 70% of the neoplastic tissues that we examined; (ii) wild type APC induced only RDHL in an APC-deficient colon carcinoma cell line; (iii) APC induction of RDHL paralleled increased RA production in HT29 cells; (iv) the intestine-specific transcription factor CDX2 activated an RDHL promoter construct as well as endogenous RDHL expression; and (v) APC induction of RDHL was enhanced by the presence of wild-type CDX2.

Studies in model organisms highlight the importance of RA biosynthesis and metabolism in development and differentiation. For example, deletion of the retinoid-metabolizing P450 enzyme CYP26 in mice disrupted anteroposterior axis development, normal hind brain patterning, vertebral identity, and development of posterior structures (55–57). Similarly, mice with a targeted disruption of the retinaldehyde dehydrogenase gene Raldh2 die in midgestation, display shortening along the anteroposterior axis, and fail to form limb buds (58). It is clear from these studies that the biosynthesis and metabolism of RA plays an important role in development and differentiation. Loss of regulatory control of RA biosynthetic genes could, therefore, alter cell growth and differentiation in the colon, thus contributing to the development of colon adenomas and carcinomas. Support for the loss of retinoid biosynthesis in contributing to tumor formation comes from recent studies showing that certain breast cancer cell lines failed to synthesize RA (59). Moreover, reintroduction of ALDH6, a retinaldehyde dehydrogenase, restored the ability of MCF-7 breast cancer cells to synthesize RA (60). Finally, retSDR1 and LRAT, two genes involved in retinol storage, were found to be lost in neuroblastoma (61) and prostate cancer (62), respectively.

Few studies have revealed the regulatory mechanisms that control expression of RA biosynthetic genes in any tissues. The loss of RDH5 and RDHL expression in colon cancer emphasizes the importance of understanding these regulatory mechanisms. We found that reintroduction of the tumor suppressor APC induced RDHL in colon cancer cell lines. Our findings imply that the loss of APC in most colon cancers could account for the similar loss of RDHL that we observed by microarray and RT-PCR (Fig. 1, A and B). Although mutations in APC appear to predict loss of RDHL, loss of RDHL does not appear to predict the presence of an APC mutation. Indeed, a number...
of tumors and at least two cell lines failed to express RDHL despite harboring wild type APC. This suggests a defective, intermediate step between APC and RDHL.

In explaining this, we focused our attention on the possibility that RDHL was repressed by β-catenin. This, however, appears not to be the case for two reasons. First, overexpression of β-catenin and a dominant-negative LEF1 construct failed to regulate an RDHL promoter construct either positively or negatively (Fig. 4, B and C). Second, RKO cells lack RDHL expression but express wild type APC and β-catenin. This lack of regulation by β-catenin implies a function for APC that is independent of its known role in regulating levels of β-catenin. A number of studies suggest a β-catenin-independent function of APC in cellular differentiation. Mariadason et al. (63) showed that neither inhibition of β-catenin transcriptional activity nor translocation of β-catenin to the membrane was sufficient to induce two of four differentiation markers examined in a Caco-2 cell model of intestinal differentiation. In addition, Dang et al. (64) have demonstrated β-catenin-independent regulation of gut Kruppel-like factor 4 by APC and CDX2. It is therefore likely that β-catenin independent pathways can promote differentiation in colonocytes.

In considering β-catenin-independent regulation of RDHL, our attention was drawn to the CDX transcription factors. CDX1 and CDX2 were likely candidates for regulation of RDHL for a number of reasons. First, like RDHL, they are highly expressed in the colon (52, 53) and lost in certain colon adenocarcinomas (52–54). Second, they have been shown to cause growth arrest and to promote differentiation upon re-introduction into colon cancer cell lines (48, 51). Finally, CDX2 is mutated in RKO cells. Consistent with these findings, the RDHL promoter contains several consensus CDX binding elements (Fig. 6A) that are recognized by CDX2 in vitro (Fig. 5B) and that appear required for CDX2 activation of an RDHL promoter construct (Fig. 6B). In addition, CDX2 induced endogenous RDHL in RKO cells (Fig. 5C). Altogether, these data support a model whereby CDX2 activates endogenous RDHL expression in colonocytes.

We found that APC may require CDX2 to induce RDHL (Fig. 7). It is presently unclear how APC and CDX2 may interact in regulating RDHL. We were unable to detect changes in expression of CDX2 following APC induction in HT29 cells (data not shown), despite previous reports that CDX2 is inducible by APC in HT29 cells (65). The idea that APC may not regulate transcription of the CDX2 gene is suggested by the finding that familial adenomatous polyposis tissues express normal levels of CDX2 protein (54). However, a role for APC in post-translational regulation of CDX2 cannot be dismissed, since there does appear to be a relationship between APC and CDX2. First, familial adenomatous polyposis tissues lack expression of the reported CDX2 target gene, Kruppel-like factor 4 (64). Furthermore, Aoki et al. (66) have recently reported that Apc+/−Cd2−/−mice showed increased colonic polyp numbers in comparison with Apc+/−Cd2+/+mice. This suggests a requirement for inactivation of both APC and CDX2 in colon polyp formation and raises the possibility that CDX2 protein is not functional in familial adenomatous polyposis colon polyps despite its normal levels of expression (54).

Our finding that APC and CDX2 control RDHL fits with recent studies suggesting that RA can inhibit the oncogenic effects of β-catenin. Easwaran et al. (33) demonstrated that RA-bound RA receptor α can bind to and sequester β-catenin, leading to down-regulation of β-catenin transcriptional activity. The same group later showed that the differentiation-associated morphological changes induced by RA in a breast cancer cell line are probably mediated through cadherin-dependent recruitment of β-catenin to the cellular membrane (34). Interestingly, the ability of RA to induce β-catenin translocation was independent of its ability to inhibit β-catenin-mediated transcription (34), suggesting that RA has separable roles in inhibiting proliferation and promoting differentiation. Still, the finding that a number of genes can be synergistically activated by Wnt-1 and RA (67) warrants additional investigations to clarify the relationship between Wnt signaling and retinoids in colon tumor development.

Two separate studies have characterized the enzymatic activity of RDHL. Soref et al. (38) demonstrated that RDHL (referred to as 1021 in their publication) increased the ability of tracheobronchial epithelial cells to convert retinol to RA, whereas Chetyrkin et al. (68) determined that RDHL (referred to as 3α-HSD in their publication) prefers different substrates in vitro. Specifically, Chetyrkin et al. (68) found that RDHL was 100 times more efficient as a 3α-hydroxysteroid dehydrogenase than as a retinol dehydrogenase. Their in vitro studies demonstrated that RDHL can catalyze the conversion of 3α-tetrahydroprogesterone (allopregnanolone) to dihydroprogesterone and 3α-androstenediol to the potent androgen, dihydrotestosterone. Presently, the prevalent enzymatic activity of RDHL in colonocytes remains uncertain. We found that induction of APC was paralleled by an increase in RDHL but not RDH5. This was also accompanied by an increased ability of the cells to convert retinol into RA (Fig. 3C) and supports findings that RDHL may convert retinol into retinaldehyde in cells.

Although the roles for retinoids in intestinal epithelial cell function remain unclear, studies in vitamin A-deficient rats predict an important function for retinoids in intestinal mucosa. Glick et al. (24) found that vitamin A-deficient rats have decreased levels of TGF-β2 in their intestinal mucosa. Administration of RA to these vitamin A-deficient animals restored TGF-β2 levels. This work emphasizes the importance of retinoids in controlling expression of these ligands in vivo and illustrates the responsiveness of intestinal mucosa to retinoids. In other studies, vitamin A-deficient animals displayed gastrointestinal abnormalities that included decreased mucus production, expansion of proliferation zones within colon crypts, and ion flux alterations (17–23). In addition, retinoid analogs have proven effective in preventing 5-azoxymethane-induced colon carcinoma formation in rats (17, 25–28). Our findings suggest that the RA response pathway is a target for inactivation in colon cancer. We provide data that support the silencing of RA biosynthesis as a downstream consequence of APC mutation but not necessarily a consequence of β-catenin deregulation. We offer a new model explaining the potential relationship between APC, CDX2, retinoid biosynthesis, and differentiation. Specifically, APC and CDX2 may control intracellular levels of RA and, ultimately, an RA-mediated program of differentiation. Although several target genes for APC/β-catenin/TCF/LEF have been described, there have been no reports of specific, prodifferentiation signaling pathways, like retinoids, that are under the direct control of APC. Ultimately, this work could lead directly to a testable clinical hypothesis aimed at pharmacological restoration of retinoid activity.

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