CdX1 is a homeodomain transcription factor that regulates intestine-specific gene expression. Experimental evidence suggests that CdX1 may be involved in cell cycle regulation, but its role is ill defined and the mechanisms have not been explored. We used stable transfection of inducible constructs and transient expression with a replication-deficient adenovirus to induce CdX1 expression in rat IEC6 cells, a non-transformed intestinal epithelial cell line that does not express CdX1 protein. Expression of CdX1 markedly reduced proliferation of IEC6 cells with accumulation of cells in the G0/G1 phase of the cell cycle. Cell cycle arrest was accompanied by an increase in the hypophosphorylated forms of the retinoblastoma protein (pRb) and the pRb-related p130 protein. Protein levels of multiple cyclin-dependent kinase inhibitors were either unchanged (p16, p18, p21, p27, and p57) or were not detected (p15 and p19). Most significantly, levels of cyclins D1 and D2 were markedly diminished with CdX1 expression, but not cyclins D3, E, or the G1 kinases. Additionally, cyclin-dependent kinase-4 activity was decreased in association with decreased cyclin D protein. We conclude that CdX1 regulates intestinal epithelial cell proliferation by inhibiting progression through G0/G1, most likely via modulation of cyclin D1 and D2 protein levels.

Homeobox genes encode for transcription factors that have been implicated as critical regulatory proteins for a variety of developmental and differentiation processes in organisms throughout the phylogenetic tree (reviewed in Refs. 1–4). Moreover, there is evidence that members of this family of transcriptional proteins are involved in the control of cellular proliferation and oncogenesis (5). The CdX1 gene family includes mammalian homeobox genes that are related to the Drosophila melanogaster gene caudal (6). Three mouse CdX genes have been identified, CdX1 (7), CdX2 (8–10), and CdX4 (11); two genes have been identified thus far in humans, CDX1 (12) and CDX2 (13). CdX1 and CdX2 are expressed in many tissues early in development, but are restricted to the intestinal and colonic epithelium later in fetal life and in the adult (7, 11, 14–17).

Several lines of evidence suggest that CdX1 and CdX2 are important nuclear proteins involved in the regulation of the phenotype of intestinal epithelial cells (18–22). CdX1 (23) and CdX2 (10, 23–28) interact with DNA elements that have an AT-rich consensus sequence (TTTAT), similar to other homeodomain proteins. Binding of CdX1 (23) and CdX2 (10, 23) to intestinal gene promoters leads to transcriptional activation that is mediated through an activation domain localized to the amino-terminal region of the protein (29, 30). Most studies have shown that CdX2 activates transcription of various genes (10, 23, 27, 28, 31), whereas fewer studies have examined CdX1 as an activator of gene transcription (17, 23). The role of these homeobox genes in regulation of proliferation is of particular interest, given the tightly controlled patterns of proliferation in the intestinal epithelium. CdX2 is able to inhibit cellular growth as shown by studies in transfected cell lines (18), but the effect of CdX1 on cellular proliferation is not as well defined. The genes that are known to be regulated by either CdX1 or CdX2 are generally ones that define functional phenotype (for example, surcace-ismaltase (Ref. 10), glucagon (Refs. 27 and 28), intestinal phospholipaseA/lysophospholipase (Ref. 23), and carbonic anhydrase (Ref. 32)) and are not known regulators of cellular proliferation.

Several studies have examined the role of CdX1 on proliferation in intestinal cell lines. CdX1 was shown to transform NIH-3T3 cells in a focus-forming assay in a study that tested many homeobox genes for transforming properties (5). In another study, a colon cancer cell line that expresses small amounts of endogenous CdX1 and CdX2 was transfected with CdX1 sense and antisense expression constructs (19). These investigators found that overexpression of CdX1 with the sense construct had no effect on growth, but that the CdX1 antisense-expressing cells had a significant reduction in their growth rate. Based on these findings, the authors hypothesized that CdX1 acted as a growth promoter, although CdX protein levels were not examined to determine whether there were indeed changes in CdX1 levels. Recently, another human colon cancer cell line, HT29, was transfected with CdX1, CdX2, or both CdX1 and CdX2 expression constructs (21). HT29 clones expressing CdX2 or both CdX1 and CdX2 had significantly reduced growth rates compared with controls. CdX1 expression alone had no effect on the growth of these cells, but neither CdX1 nor CdX2 protein levels were measured in this study.
Significantly, only CDX1 and CDX2 expressed together could reduce the tumor growth rate of the HT29 clones in nude mice. Two important weaknesses of these studies include the use of cells that constitutively express CDX genes and that levels of the expressed proteins were not measured. This experimental design might lead to a selection of cells that were able to grow despite CDX gene expression, or for cells that expressed very low levels of CDX1.

The expression of CDx genes has been investigated in human colorectal tumors. Expression of CDX2 mRNA (33) and protein (13) is decreased in a large percentage of colorectal cancers. Expression of CDX1 mRNA (33) and protein (34) is also decreased in the majority of colorectal cancers. In studies using immunohistochemistry to detect CDX1, there appears to be a graded decrease in immunoreactivity between normal, adenomatous, and cancer cells (34). From these data, there appears to be a marked decrease in the expression of both CDX1 and CDX2 in colorectal cancers.

Therefore, it is currently unclear how Cdx1 is involved in the control of cellular proliferation. The purpose of our study was to examine the effects of Cdx1 on growth regulation of a non-transformed intestinal cell line. We found that Cdx1 inhibits cellular proliferation by blocking progression through the cell cycle in G1. This G1 arrest was accompanied by decreased expression of the D-cyclins, inhibition of cyclin-dependent kinase activity, and accumulation of the hypophosphorylated forms of the retinoblastoma protein (pRb) and the pRb-related p130. These findings have important implications for understanding the functional role of Cdx1 in the regulation of intestinal epithelial cell proliferation.

MATERIALS AND METHODS

Construction of Expression Vectors—An inducible Cdx1 expression vector, pMT-Cdx1, was constructed by subcloning the coding sequence of Cdx1, released by digestion of pEVRF1Cdx1 (16) with KpnI and XbaI, into pMTCB6, which contains the promoter of the sheep metallothionein I gene (35).

Production and Characterization of Adenovirus Vectors—Mouse Cdxi cDNA was subcloned into the adenoviral transfer vector, pAdCMVlink, and used to produce the expression vector Adeno-Cdxi. pAdCMVlink without a cDNA insert was used to generate the control Adeno-NULL virus. The linearized adenoviral transfer plasmid was cotransfected with adenoviral DNA into 293 cells to allow for homologous recombination. Viral plaques were then picked and expanded on Adeno-NULL virus. The linearized adenoviral transfer plasmid was CMVlink without a cDNA insert was used to generate the control of Cdx1, released by digestion of pEVRF1Cdx1 (16) with KpnI and XbaI, into pMTCB6, which contains the promoter of the sheep metallothionein I gene (35).

Regulation of Proliferation by Cdx1

The focus assay, 7 × 10^5 cells from each stable line (3T3MT1, 3T3Cdx1MT1, and 3T3Cdx2MT17) were mixed with 9.3 × 10^4 NIH3T3 cells and plated in six-well plates. The culture medium was changed twice weekly for 4 weeks. At 2 and 4 weeks, cells were fixed with Bouin’s solution for 1 h, rinsed with PBS, and stained with 4% Giemsa reagents for 24 h. Dishes were rinsed briefly with distilled water and then scored for the number of foci.

Cell Proliferation and Flow Cytometry Analysis—Brdu incorporation was measured using the 5-bromo-2′-deoxyuridine labeling and detection kit I (Roche Molecular Biochemicals). BrdUrd labeling of IEC6 cells was performed over 1 h, and then the cells were processed according to the instructions provided with the kit, except that in the final step, Hoechst dye and biotinylated streptavidin was added to the wash reagent (final concentration = 2 μg/ml) to stain all nuclei. At least 200 cells from each well were counted using a fluorescent microscope, and those incorporating the BrdUrd label were expressed as a percentage of all cells counted. These percentages were transformed using the arcsine transformation prior to statistical analysis with analysis of variance and Tukey key tests.

Flow cytometry. IEC6 cells were infected and cultured as described. DNA content was measured using a rapid propidium iodide staining method. Approximately 250,000 cells (one 100-mm dish) were trypsinized, washed once in PBS, and then resuspended in 0.3 ml of STAIN solution (3% polyethyleneglycol 8000, 50 μg/ml propidium iodide, 360 units/ml RNase A, 0.1% Triton X-100, 3.6 mM citric acid buffer, pH 7.2). The cells were incubated for 30 min at 37 °C, then 0.35 ml of SALT solution (3% polyethyleneglycol 8000, 50 μg/ml propidium iodide, 0.1% Triton X-100, 0.4 μM NaCl, pH 7.2) was added, and the solution incubated in the dark at 4 °C for at least 1 h prior to flow cytometry and DNA quantitation using a FACSScan (Becton Dickinson).

Immunoblot Analysis—Protein extracts of IEC6 cells were made from trypsinized cells resuspended in 80 μl of buffer A (1X PBS with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.2 mM PMSF, 1 mM NaF, and 1 mM NaVO₄). Two volumes of 2X lysis buffer was added (250 mM Tris-Cl, pH 7.4, 10%, SDS, 20% glycerol, and 2% β-mercaptoethanol), and the cells 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 BCA protein assay (Fierce). 50 μg of whole cell extract was analyzed by a 12% SDS-PAGE electrophoresis and electroblotting to nitrocellulose membranes. The membranes were incubated for several hours at room temperature with PBS/milk, and then incubated overnight at 4 °C with the primary antibody. The following antibodies were used: for retinoblastoma protein (pRb), G3-245 (PharMingen); for cyclin D1, O16-137 (Upstate Biotechnology, Inc.) and SC-246 (Santa Cruz); for p130, SC-317; for p107, SC-250; for cyclin A, SC-751; for cyclin B1, SC-245; for cyclin D2, SC-452; for cyclin D3, SC-182; and for cyclin
Regulation of Proliferation by Cdx1

E, SC-481; for Cdk2, SC-163; for Cdk4, SC-601; for Cdk6, SC-177; for p15, SC-613; for p16, SC-1661; for p18, SC-865; for p19, SC-1063; for p21, SC-397; for p27, SC-1641; for p57, SC-1037 (Santa Cruz); for actin, A-4700 (Sigma); and anti-Cdx1 antibody (34). After washing membranes three times for 10 min with PBS/Tween, blots were incubated with the secondary antibody (anti-rabbit HRP, anti-goat HRP, or anti-mouse HRP) at room temperature. Finally, blots were washed three times and developed with the ECL-Plus Western blotting kit by Amersham Pharmacia Biotech.

Northern Blot and Ribonuclease Protection Analysis—Total RNA was isolated by the guanidinium thiocyanate/CsCl method from several 100-mm plates infected and cultured as described (18). 50 μg of RNA was run on a 8.0% agarose gel with 2× MOPS buffer. The RNA was transferred to a nitrocellulose membrane, the filter was air-dried, and the RNA was UV cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene). The RNA was stained with 0.005% methylene blue after blotting to evaluate for equal transfer.

RESULTS

Inhibition of Intestinal Cell Proliferation by Cdx1 in Stably Transfected IEC6 Cells—IEC6 cells are non-transformed intestinal epithelial cells that were originally isolated from neonatal rat small intestine (37). They have some characteristics of intestinal crypt cells, but are morphologically undifferentiated and do not express genes that are associated with a differentiated phenotype (37). Since they lack expression of both Cdx1 and Cdx2, we have used IEC6 cells to evaluate the functional role of Cdx genes in intestinal epithelial differentiation (18). The pattern of IEC6 cell accumulation in tissue culture was markedly affected by the expression of Cdx2 (18). For 2–3 days following induced expression of Cdx2 in stably transfected IEC6 cells, there was a marked decrease in the accumulation of cells in culture (18). This period was followed by exponential growth of cells, reaching confluence at a higher density than cells that were not induced to express high levels of Cdx2 (18).

To determine the effect of Cdx1 on IEC6 cell proliferation, stable cell lines were made using an inducible expression construct for Cdx1. Immunoblots for Cdx1 proteins showed that stable cell lines containing the Cdx1 expression vector expressed low constitutive amounts of protein, which was induced following addition of zinc sulfate to the culture medium (Fig. 1A). The expressed Cdx1 protein was competent to bind to a known DNA binding element, the SIF1 element in the sucrase-isomaltase gene promoter (10) (Fig. 1B). Since the SIF1 element has been shown to have two adjacent Cdx binding sites (23), the gel shift has two specific complexes labeled A and B, which represent the monomer and dimer complexes, respectively. The cell lines transfected with the Cdx1 construct grew more slowly than cell lines transfected with the vector alone (Fig. 2). Induction of Cdx1 resulted in slower accumulation of cells in culture when compared with uninduced cells. However, the effect of induction was not great since the rate of growth of the uninduced cells was already diminished. It is possible that the decreased growth of uninduced cells is due to the small amount of expressed Cdx1 in the absence of inducing media (Fig. 1A).

Cyclin-dependent Kinase Activity Analysis—Several 100-mm plates of IEC6 cells were infected and cultured as described. At 48 h, they were briefly trypsinized, washed twice in PBS, then resuspended in IP buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween 20, 1 mM NaF, 0.1 mM Na3VO4, 1 mM PMSF, 1 mM dithiothreitol, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 150 μg/ml benzamidine, and 10 μg/ml trypsin inhibitor). Cells were briefly sonicated, then incubated in IP buffer at 4 °C with rotation for 30 min. Cellular debris was pelleted by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatant was saved and was further cleared by repeat centrifugation as before. The protein concentration of the supernatant was measured with the Bio-Rad protein assay (Biorad). Duplicate 100 μg samples of protein extract was used in each kinase reaction. The lysates were first precleared with prewashed Protein G beads (Life Technologies, Inc.), then with prewashed Protein A beads (Life Technologies, Inc.). These cleared lysates were then subjected to immunoprecipitation with 1 μg of either anti-Cdk4 (sc-260, Santa Cruz) or 1 μg of rabbit pre-immune IgG (Santa Cruz) for 2 h at 4 °C with rotation. Prewashed Protein A beads were added, and the incubation continued for an additional 1 h. The IP products were pelleted by centrifugation at 4000 rpm for 5 min at 4 °C, and washed twice with IP buffer, then three times with PBS/Tween (Amersham Pharmacia Biotech), anti-mouse/HPR, or anti-goat/HPR (Santa Cruz) for 45 min at room temperature. Finally, blots were washed three times and developed with the ECL-Plus Western blotting kit by Amersham Pharmacia Biotech.

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vector consistently grew to a lower density than the cells transfected with the vector alone. This finding suggested that even low level expression of Cdx1 in uninduced transfected cells may inhibit IEC6 cell growth. Therefore, we used an adenoviral vector to express Cdx1 in IEC6 cells. Adenovirus expressing Cdx1 was made as described under “Materials and Methods” and shown to express authentic Cdx1 protein by both Western blot (Fig. 1A) and EMSA (Fig. 1C). Comparison of Cdx1 protein levels on the immunoblot (Fig. 1A) shows that Cdx1 levels were much greater in the adenovirus-infected cells than in the stable lines.

The effects of Adeno-Cdx1 was compared with the same adenovirus without a cDNA insert, Adeno-NULL. The number of infectious particles for both adenovirus were determined by dilutional infections in 293 cells. The multiplicity of infection (m.o.i.) was varied in a series of IEC6 cell infections to determine the effect on cell viability and efficacy of infection and protein expression. It was determined by immunofluorescence for Cdx1 protein that an m.o.i. of 50 resulted in expression of Cdx1 in greater than 90% of cells in the culture dish (data not shown). Thus, an m.o.i. of 50 was used for all experiments.

The accumulation of IEC6 cells infected with Adeno-Cdx1 was markedly inhibited when compared with uninfected control cells or those infected with Adeno-Null (Fig. 3A). Additionally, there was a marked decrease in the percentage of cells incorporating BrdUrd into DNA (Fig. 3B). Thus, expression of Cdx1 inhibits proliferation of IEC6 cells, as suggested by the experiments with stably transfected cells.

Cdx1 Gene Expression Blocks Progression of the Cell Cycle in G₁—Flow cytometry was used to evaluate cell cycle progression of IEC6 cells. Cells infected with Adeno-Cdx1 showed a marked increase in the percentage of cells in G₁ with a decrease in cells in S phase and G₂ in comparison to uninfected cells and cell infected with Adeno-Null (Table I). Of note, the flow cytometry profiles did not show a subdiploid peak of DNA, suggesting that apoptosis did not result from expression of Cdx1 (data not shown). Therefore, Cdx1 expression in IEC6 cells inhibits proliferation by blocking cell cycle progression in G₁.

Cell Cycle Protein Changes Associated with Cdx1 Expression—To explore the mechanism of the G₁ block, we first examined the expression of the retinoblastoma family of proteins, which are key regulators of the G₁/S transition. In cells expressing Cdx1, there was a shift in pRB to the hypophosphorylated form, there was a marked increase in hypophosphorylated p107, and a decrease in p107 (Fig. 4). These changes in Rb proteins are known to alter transcriptional events that inhibit the transition from G₁ to S phase.

We next examined cell cycle proteins that directly or indirectly affect the phosphorylation of Rb proteins. Cyclins D1 and D2 are both markedly decreased in cells expressing Cdx1 with only a slight decrease in cyclin D3 (Fig. 5). Cdkks 4 and 6, which both associate with D cyclins, are only slightly decreased in comparison to cells infected with null virus. Cyclin A and B1 were both decreased, as occurs in any event that blocks the G₁/S transition. There was no change in cyclin E. Since an important mechanism for inhibition of cyclin-dependent kinase activity is induction of inhibitor proteins, we examined the expression of multiple known cyclin-dependent kinase inhibitors. There was no change in expression of p16, p18, p21, p27, or p57 in cells expressing Cdx1 (Fig. 5). Immunoblot were also performed for p15 and p19, but there was no detectable protein (data not shown).

Cyclin-dependent Kinase Activity Is Decreased by Expression of Cdx1—The decrease in cyclin D1 and D2 protein levels represents a potential mechanism by which phosphorylation of Rb proteins is decreased, resulting in inhibition of the cell
cycle. However, the maintained cyclin D3 could potentially support enough cyclin D-dependent kinase activity to allow progression through G1 and the G1/S transition. To test this question, we measured cyclin-dependent kinase 4 (Cdk4) activity in cellular extracts. Cdk4 kinase activity was decreased 60% of full activity in cells expressing Cdx1 (Fig. 6). Since Cdk4 protein is not significantly altered in cells expressing Cdx1 (Fig. 6), the decreased kinase activity is likely due to low levels of D cyclins. These data establish the link between decreased cyclin D protein levels and hypophosphorylation of Rb proteins.

Cdx1 Expression Decreases Cyclin D1 mRNA—The analysis of the expression of cell cycle proteins indicated that the likely cause of G1 arrest was a decrease in the levels of D cyclins. Therefore, we examined the level of expression of cyclin D1 mRNA following induction of Cdx1. There was a dramatic reduction of cyclin D1 mRNA upon expression of Cdx1 in IEC6 cells with no change noted in control-infected cells (Fig. 7A). We next examined the expression of mRNAs simultaneously for cyclin D1, D2, and D3 using a ribonuclease protection assay (Fig. 7B). This experiment showed that the mRNA levels of the three D cyclins decreased, as would be predicted from the protein levels with cyclin D1 and D2 mRNAs being markedly reduced, whereas there was little change in mRNA for cyclin D3 (Fig. 7B). Therefore, expression of Cdx1 in IEC6 cells causes a reduction in cyclin D1 and D2 mRNA and protein, with little change in cyclin D3.

**DISCUSSION**

Cdx transcription factors appear to have multiple functions in the establishment and maintenance of the intestinal epithelium. One critical feature of the organization of the intestinal epithelium is the compartmentation of proliferating cells in crypts and the continual renewal of differentiated cells on the villus from this proliferating compartment. The control of the proliferative state of crypt cells is important for maintaining...
the cellular equilibrium in the normal epithelium and for adapting to pathophysiologic stimuli. Taken together, the data presented in this report shows that Cdx1 is able to inhibit growth of intestinal epithelial cells by blocking progression of the cell cycle in G1. Moreover, inhibition of cell cycle progression appears to be due to decreased cyclin D1 and D2 and the decreased levels of protein correlate with decreased mRNA.

A recent report came to a different conclusion regarding the effect of CDX1 on proliferation (21). These investigators constitutively expressed human CDX1 in human adenocarcinoma cell line HT-29 and examined the effect on proliferation and differentiation. They found that stable expression of CDX1 did not affect the proliferation of HT-29 cells, whereas co-expression of CDX1 with CDX2 augmented the growth inhibitory effect of CDX2. There are a number of explanations for the differences in our results in IEC6 cells. First, we used the mouse Cdx1 cDNA and these investigators used the human cDNA. It is doubtful that this is the reason for the differences since these two homologous proteins are highly similar. Second, the cell lines used are very different. Our studies were performed in a non-transformed rat intestinal cell line that does not form tumors in nude mice, whereas HT-29 cells are human adenocarcinoma cells. Cellular growth and signaling pathways may be very different between these two lines, which may affect the ability of Cdx1 to regulate proliferation. Third and most importantly, it is likely that there were very different levels of expression in the two studies. The expression of Cdx1 in the HT-29 cells was only evaluated using a luciferase reporter construct that is activated by Cdx proteins, and there was no direct evidence of protein expression. The luciferase reporter assay is very sensitive and suggests that the actual levels of Cdx1 protein may have been very low in the stably transfected lines. Thus, when the different experimental designs are considered, these two apparently contradictory results are potentially explained by differences in cell lines and levels of Cdx1 expression.

It is unclear whether the cell cycle inhibitory effect of Cdx1 is important for regulation of intestinal epithelial growth in vivo. In the adult small intestinal epithelium, Cdx1 protein is found in greatest amount in the nuclei of crypt cells with decreasing levels in villus cells as they move toward the villus tip (34). Similarly, the highest levels of expression in the colonic epithelium are found in crypt cells with less in the surface epithelial cells (34). However, it is important to note that Cdx1 is expressed throughout the villus as well as the crypt, albeit at lower levels. In Cdx1 null mice, no gross abnormalities were found in the epithelium, but a careful histological analysis has not yet been reported (17). Since Cdx1 is also important for expression of enterocyte genes, there may be other changes in Cdx1 null mouse epithelium that alters proliferation in addition to loss of Cdx1 alone. Additionally, changes in Cdx2 expression in the Cdx1 null mice may mitigate the effect of loss of Cdx1. Thus, the available in vivo data are compatible with multiple possibilities including a pro-proliferative effect, an anti-proliferative effect, or no effect on proliferation. Our data suggest that Cdx1 may inhibit the cell cycle and thereby serve as a break on proliferation in crypt cells. In fact, the cell cycle inhibitory effect is evident over a wide range of levels of Cdx1 protein (compare IEC6 stable lines to adenovirus-infected cells; Fig. 1A). However, it is currently unclear how these protein levels in cell lines compare with the protein expression in colonocytes in the intact mucosa. Moreover, all conclusions of function in cell lines must be tempered by the fact that the microenvironment of the intact epithelium in which the enterocyte resides is very different from cultured cells.

Cdx1 has also been suggested to play a role in neoplasia. A previous study suggested that Cdx1 was able to transform NIH-3T3 cells (5). We were unable to confirm these findings since we found no increase in transformed foci or soft agar colonies in 3T3 cells expressing Cdx1. In contrast to the previous study, we confirmed the expression of Cdx1 in the NIH-3T3 clones. Thus, we conclude that Cdx1 does not transform NIH-3T3 cells. The second association with neoplasia is a change in expression in human colonic adenocarcinomas. We (34) and others (33) have shown that the majority of colorectal cancers have decreased levels of Cdx1 in comparison to normal colonic mucosa. Preliminary data suggest that expression of Cdx1 in some colon cancer cell lines that do not express Cdx1 results in inhibition of cell growth (38). Since HT-29 cells are not affected by Cdx1 expression (21), the role in growth control of cancer cells will require additional studies.

There are many targets for regulation of the mammalian cell cycle. The balance between activators and inhibitors of specific kinases determines progression through the G1 phase and entry into S phase. The Rb proteins are important gatekeepers of the G1-S transition by regulating the activity of the E2F family of transcriptional activators. Our data are consistent with Rb family members as the ultimate targets of Cdx1 protein expression since there was an increase in the inactive hypophosphorylated form of RB and p130, and loss of p107. Phosphorylation of Rb proteins may be affected by alterations in many proteins and their multiprotein complexes. Expression of Cdx1 does not change levels of the cyclin-dependent kinases or their inhibitors. However, there is a marked decrease in expression of cyclins D1 and D2, which serve to activate the kinase activity of CDK4 and CDK6. As a result, Cdk4-dependent kinase activity is decreased in cells expressing Cdx1. Thus, it is likely that depletion of these D-type cyclins results in decreased Rb family phosphorylation by activated CDKs 4 and 6 and inhibition of progression of the cell cycle. Since cyclin D1 and D2 mRNAs are decreased in parallel with the proteins, it appears that Cdx1 affects either transcription of the cyclin D genes or degradation of the mRNAs, or both. Unraveling this mechanism will require
Taken together, our results suggest a role for Cdx1 in the regulation of intestinal epithelial cell proliferation as an inhibitor of cell cycle progression through a decrease in D-type cyclins. We hypothesize that the potency of the Cdx1 inhibitory effect is dependent on other cellular signaling pathways and the state of cellular differentiation. An understanding of the mechanism by which Cdx1 affects the cell cycle will provide insight into maintenance of the delicate balance of proliferation and differentiation in the intestinal epithelium and the pathogenesis of colonic neoplasia.
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