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Expression of Id Helix-Loop-Helix Proteins in Colorectal Adenocarcinoma Correlates with p53 Expression and Mitotic Index[1]

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ABSTRACT

Id helix-loop-helix (HLH) proteins function as regulators of cell growth and differentiation and when overexpressed can induce malignant transformation. In a series of 34 cases of primary human colorectal adenocarcinoma, immunoreactivity for Id1, Id2, and Id3 was found to be significantly elevated in tumor compared with normal mucosa ($P = 0.001$ for Id1 and Id2; $P = 0.002$ for Id3). No elevation of Id expression was observed in 17 cases of adenoma. Expression of Id1 and to a lesser extent of Id2 was correlated with mitotic index ($P = 0.005$ for Id1; $P = 0.042$ for Id2) in human adenocarcinomas, and expression of all three Id proteins was correlated with p53 immunoreactivity (a marker of mutational 'inactivation' of p53 function; $P = 0.002$ for Id1; $P = 0.006$ for Id2; $P = 0.016$ for Id3). In normal intestinal mucosa of p53-null mice and in spontaneous tumors arising in *Min*+/− mice, expression of all three Id proteins was also found to be up-regulated. Antisense oligonucleotide blockade of Id protein expression inhibited the proliferation of human adenocarcinoma cells. Enforced, ectopic expression of the E47 basic HLH (bHLH) protein in human adenocarcinoma cell lines efficiently sequestered endogenous Id proteins as Id-bHLH heterodimers, as shown by coimmunoprecipitation and subcellular colocalization studies. This led to growth arrest of the cells. Enforced overexpression of a mutant E47 protein, deficient in transactivation and DNA binding function, also partially inhibited cell growth. Taken together, these data imply that deregulated expression of Id proteins in colorectal adenocarcinoma arises at least in part as a consequence of loss of p53 function and contributes to the uncontrolled proliferation of tumor cells in colorectal cancer.

INTRODUCTION

Members of the Id family of HLH[3] proteins play a pivotal role in the regulation of cell lineage commitment, growth, and differentiation in most, if not all, mammalian cell lineages (reviewed in Refs. 1–3). There are four Id family members in mammals (Id1–4). They function by binding to and antagonizing the activities of several classes of transcriptional regulator-bHLH proteins (4, 5), Rb "pocket" proteins (6–8), Ets domain proteins (9), and Pax homeodomain-containing proteins (10). Of these, heterodimeric interactions with members of the bHLH protein family (mediated via the HLH domain) are established as being functionally the most important (1, 2, 4, 5). Accumulating evidence supports the view that the maintenance of a critical balance between a cell's complement of bHLH and Id proteins is important for cell fate decisions of growth and differentiation; expression of Id proteins is typically high in actively proliferating cells and is down-regulated as a prerequisite for exit from the cell cycle and terminal differentiation (1–3).

Enforced expression of *Id* genes in a variety of cell types promotes proliferation (1) and, under appropriate physiological conditions, also drives apoptosis (11–13). In addition, Id function is required for the G_1 to S-phase transition of the cell cycle (14–16). In common with several other positive regulators of G_1 to S-phase transition, Id genes function as cooperating oncogenes in the immortalization of primary cells (11, 17) and will also induce disordered cell growth in fibroblast and epithelial cell lines (18, 19). More recent data have shown that Id function is required for vascularization and invasiveness of tumor growth *in vivo* (Ref. 20; reviewed in Ref. 2). Moreover, targeted expression of Id genes in transgenic mice, which typically results in a differentiation block, has also been shown to cause lymphomas (21, 22) and tumors of the intestinal epithelium (23).

Deregulated expression of *Id* genes has been described in tumor cell lines from lung, colon, pancreas, and in both neuronal and astrocytic tumor lines of the nervous system (reviewed in Ref. 3). A similar deregulation of Id expression has been reported in some primary human tumors such as seminomas (24), pancreatic adenocarcinomas (25, 26), squamous cell carcinomas (27), and mammary tumors (28). Significantly, high level expression of Id1 has been reported to be more frequently associated with infiltrating, more aggressive mammary epithelial tumors than with ductal tumors, which suggests a link between Id expression and disease progression (28). We report here on a systematic analysis of Id protein expression in primary human and mouse intestinal tumors. We have found that, compared with most normal intestinal mucosa, tumor tissues (but not premalignant adenomas) express high levels of the Id1–3 proteins. Id expression is correlated with mitotic index and with p53 expression level (a marker of mutational modulation of p53 function) in human colorectal adenocarcinoma. Normal mucosa from *p53*-null mice displayed increased expression of Id1–3, and abrogation of Id function in colorectal adenocarcinoma cell lines by antisense oligonucleotide blockade or by enforced expression of bHLH protein led to growth arrest. These data imply that deregulated expression of Id proteins in colorectal cancer arises in part as a consequence of loss of p53 function and contributes to the uncontrolled proliferation of this tumor type.

MATERIALS AND METHODS

Patient Samples. Samples were obtained with informed consent from 34 patients undergoing radical surgery for colorectal cancer. All of the patients were classified according to the Jass (29) and Tumor-Node-Metastasis (TNM) classifications (30). Tumor ploidy was assessed by flow cytometric determination of DNA content as described previously (31). Other data for the tumor series have been reported previously (31).

The human colonic adenocarcinoma cell lines, HCLO (32) and LS174T (33), were obtained from the American Type Culture Collection and grown in RPMI 1640 medium supplemented with 10% FCS.

Mouse Tissues. *p53*-null mice (*p53*−/−; Ref. 34) and *Min*/+ mice (35) were maintained and genotyped as described previously (36). For immunohistochemical analysis, guts were excised, flushed with ice-cold PBS and fixed in 4% formaldehyde in PBS (pH 7.4) overnight at 4°C. Samples were washed

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[3] The abbreviations used are: HLH, helix-loop-helix; LacZ, β-galactosidase; bHLH, basic HLH; aHLH, antagonist HLH; TRITC, tetramethylrhodamine isothiocyanate; RIPA, radioimmunoprecipitation assay (buffer); HB, homogenization buffer; BrdUrd, bromodeoxyuridine; DAPI, 4,6-diamidino-2-phenylindole.

three times in 70% ethanol before dehydration through graded alcohols and xylene and were finally embedded in paraffin wax. Sections were cut at 3-μm thickness, dried onto 3-aminopropyl triethoxysilane-coated slides, and stored at 4°C until use (31).

Antibodies. Mouse anti-p53 IgG (DOH7, M7001) was obtained from Dako (High Wycombe, United Kingdom). Rabbit anti-p53 IgG (CM5) was a kind gift from Prof. D. P. Lane (University of Dundee, Dundee, United Kingdom). Rabbit anti-Id IgG antibodies (Id1, sc-488; Id2, cs-489; and Id3, sc-490) and anti-E47 rabbit antibody were purchased from Santa Cruz Biotechnology (Autogen Bioclear, Wilts, United Kingdom). In some experiments, a rabbit anti-Id3 antibody preparation, RD7 (37), was used. Rabbit anti-LacZ antibody was purchased from 5 Prime to 3 Prime Inc. Antirabbit secondary antibodies (biotinylated, FITC-conjugated, or TRITC-conjugated) were obtained from Dako. Antibody specific to the Myc-epitope tag (9E10) was purchased from Sigma Life Sciences (Poole, Dorset, United Kingdom).

Immunohistochemistry. Immunohistochemistry was carried out as described previously (31). Anti-Id3 IgG was used at 50 ng/ml; anti-Id1 and anti-Id2 were used at 100 ng/ml and anti-Id4 was used at 200 ng/ml. Mouse anti-p53 IgG was used at 100 ng/ml, CM5 rabbit anti-p53 IgG was used at a dilution of 1:20,000. Biotinylated secondary antibody was used at 1200 dilution. Vector ABC reagents were used in accordance with the manufacturer's instructions to amplify immunoreactivity. Final detection used 3–3′diaminobenzidine as the chromogen. Slides were counterstained with thionine and mounted with XAM permanent mountant. Immunostaining specificity was rigorously established form trial experiments using homologous and heterologous blocking peptides as described previously (11, 31).

The staining of sections was quantitated as described previously (31) by assigning a score out of three for intensity of immunoreactivity (INT) and for the proportion of (epithelial) cells stained (PROP). The product of these two values was taken to give the overall total score (TS). Tumor sections were viewed and evaluated by two independent observers without prior knowledge of histopathology data (31). Statistical analysis was carried out by using SPSS for Windows (version 7.5.1; SPSS Inc.). Data were compared using a nonparametric Kruskal-Wallis test for ANOVA, and a Mann-Whitney test was used to assess the difference between individual data sets. The level of significance was determined at $P \leq 0.05$. Correlations between immunostaining data and other variables were assessed by calculating Spearman's correlation coefficients.

Immunoprecipitation and Western Blotting. Intestinal tissues were surgically removed and either cryopreserved or lysed directly in RIPA buffer (50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS). For experiments with cell lines, cells were also lysed in RIPA buffer for direct immunoprecipitation-Western analysis. However, for coimmunoprecipitation analysis, cells were lysed in "HB" buffer [0.3 M sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP40]. Both lysis buffers contained a mixture of protease inhibitors; 0.1 mM phenylmethylsulfonyl fluoride; leupeptin, bestatin, and aprotinin (100 μg/ml each); and 2 mM levamisole. For some experiments, intestinal epithelial cells were prepared by the Weiser method (38). Before Western blotting, Id proteins were initially subjected to immunoprecipitation from RIPA or HB lysates. This and subsequent Western analysis was performed as described previously (24, 37).

Antisense Oligonucleotide Blockade of Id Protein Expression. Unmodified antisense oligonucleotides for each of the *Id* mRNAs, *Id*1–3, were essentially as described in previous studies (14, 16, 25). The sequences were: *Id1*, GCGACTTTCATGATTGG; *Id2*, AGGCTTTCATGCTGACCGC; and *Id3*, CAGCGCCTTCATGCTGGGGAG. Oligonucleotides of the corresponding sense sequences were used as controls. After dissolving in 10 mM Tris/0.1 mM EDTA (pH 7.4), the oligonucleotides were added as an equimolar mixture at various concentrations to subconfluent cultures of cells in 8-well chamber slides under standard growth conditions. After 17 h, the cells were pulsed for 2 h with BrdUrd and immunostained for BrdUrd by using a commercial kit (Boehringer) essentially as described by the supplier. A minimum of 300 cells were scored for each concentration of oligonucleotide used.

Transfection and Immunofluorescence Analysis of Cell Lines. Expression vectors encoding Id proteins (pcDNA-Id3, pcDNA-Id2, pcDNA-Id1), E47 (pcDNA3E47), and LacZ (pEQ176) have been described previously (11). A mutant version of E47 (aHLH), defective in both transactivation and DNA-binding functions (see Fig. 8*A* in "Results") was constructed by PCR mutagenesis and inserted into the vector, pcDNA3, by standard recombinant DNA techniques. The presence of mutations in the basic, DNA-binding region that have been shown previously to abolish DNA binding (39), were confirmed by DNA sequencing. Subconfluent cultures of cells in 60-mm dishes were transfected with a total of 8 μg of DNA by using the standard calcium phosphate procedure. Next day, cells were either lysed in HB buffer for immunoprecipitation analysis or else reseeded onto slides for immunofluorescence analysis. After attachment, cells were fixed and stained for either Id3 (using FITC-conjugated secondary antibody) or E47 (using TRITC-conjugated secondary antibody) and counterstained with DAPI, as described previously (11, 37). In separate experiments, cells on slides were pulsed for 2 h with BrdUrd and then fixed and immunostained for LacZ; BrdUrd was then incorporated by using a commercial kit (Boehringer) according to the manufacturer's instructions. Slides were finally mounted in Vectashield mountant containing DAPI and evaluated by fluorescence microscopy. For quantitative analysis, a minimum of 200 LacZ-positive cells were evaluated for each transfection.

RESULTS

Expression of Id Proteins in Normal and Tumor Tissue from Human Colonic Epithelium. Immunostaining of normal human colonic epithelium revealed low but detectable expression of Id1, Id2, and Id3 protein as illustrated in Fig. 1*A*. No significant staining for Id4 was detected (data not shown). The specificity of immunoreactivity for each Id antibody was established in control experiments using homologous/heterologous peptide competition and preimmune sera, as reported previously (Ref. 9; data not shown). The pattern of staining for each Id protein in normal human colonic mucosa (Fig. 1*A*) was broadly similar to that previously reported for the small intestine in mouse (23). Immunoreactivity was observed primarily in the upper third of the colonic crypts and in the table region between the tops of the crypts. The staining for Id1 and Id2 was mostly cytoplasmic, whereas that for Id3 was predominantly nuclear.

A panel of 34 well-characterized human colorectal adenocarcinomas was screened by immunohistochemistry with each of the four Id antibodies. With the exception of Id4, which, as in normal intestinal epithelium, was not significantly expressed in tumor tissue, all of the Id proteins were expressed at higher levels in tumor *versus* normal

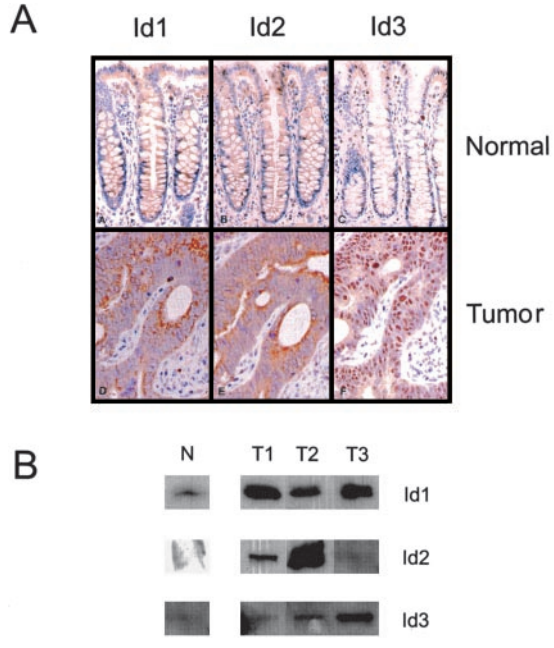

Fig. 1. Expression of Id proteins in human adenocarcinoma. *A*, immunostaining of representative sections from normal and tumor (*T1*, *T2*) colonic epithelia. *B*, Western analysis of immunoprecipitates of Id protein from three primary colonic adenocarcinomas (*T1–T3*) compared with normal colonic epithelium (*N*).

tissue. Fig. 1*A* illustrates typical staining for a representative tumor compared with normal mucosa from the same patient; and in Fig. 1*B*, three tumors are compared with normal mucosal epithelium by Western analysis. Fig. 2 summarizes the quantitative data comparing immunoreactivity of tumor and normal mucosa for all 34 patients. The elevated expression of Id1, Id2 and Id3 in tumor compared with normal tissue was statistically significant ($P < 0.001$ for Id1 and Id2; $P = 0.002$ for Id3; Wilcoxon signed rank test) for the patient series as a whole (Fig. 2). Despite the overall increase in tumor-associated Id immunoreactivity, there was considerable heterogeneity both in the proportion of tumor cells staining, the intensity of staining, and, particularly for Id3, the nuclear-cytoplasmic distribution of Id staining (Fig. 2). By contrast, mucosal samples displayed a narrower range of immunoreactivity. Interestingly, three mucosal specimens (*A*, *B*, and *C* in Fig. 2) were exceptional and gave consistently high immunoreactivity scores for different Ids. When the data for the patient series was further evaluated, we found no relationship between the profile of Id protein staining and tumor histotype, node involvement, ploidy, sex, or expression of the Bcl-2 family proteins, Bcl-w and Bcl-2 (data not shown). However, as shown in Table 1, there was a significant association between tumor stage and Id2 staining, with Jass stage IV tumors displaying significantly greater Id2 expression than either stage I ($P = 0.027$) or stage III tumors ($P = 0.033$). In addition, compared with either right-sided or rectal tumors, nuclear Id3 staining was significantly higher in left-sided tumors ($P = 0.049$; $P = 0.007$; Table 1). We noted no significant increase in staining of any of the three Id proteins in 17 colorectal adenomas (data not shown).

Table 2 *Spearman correlation coefficients for relationships between Id expression, p53 status, and mitotic index in the human colorectal adenocarcinomas*[a]

	p53 PROP[b]	p53 TS	Mitotic index
Id1	**0.457**	**0.517**	**0.59**
PROP	($P = 0.007$)	($P = 0.002$)	($P = 0.005$)
TS	0.173	0.248	**0.556**
			($P = 0.008$)
Id2	**0.41**	**0.462**	**0.444**
PROP	($P = 0.016$)	($P = 0.006$)	($P = 0.044$)
TS	0.331	**0.4**	**0.447**
		($P = 0.019$)	($P = 0.042$)
Id3-C	**0.366**	**0.411**	0.341
PROP	($P = 0.033$)	($P = 0.016$)	
TS	0.251	0.317	0.312

[a] Bold figures indicate a significant correlation, at $P \leq 0.05$.
[b] PROP, proportion of cells stained; TS, total score; C, cytoplasmic immunoreactivity.

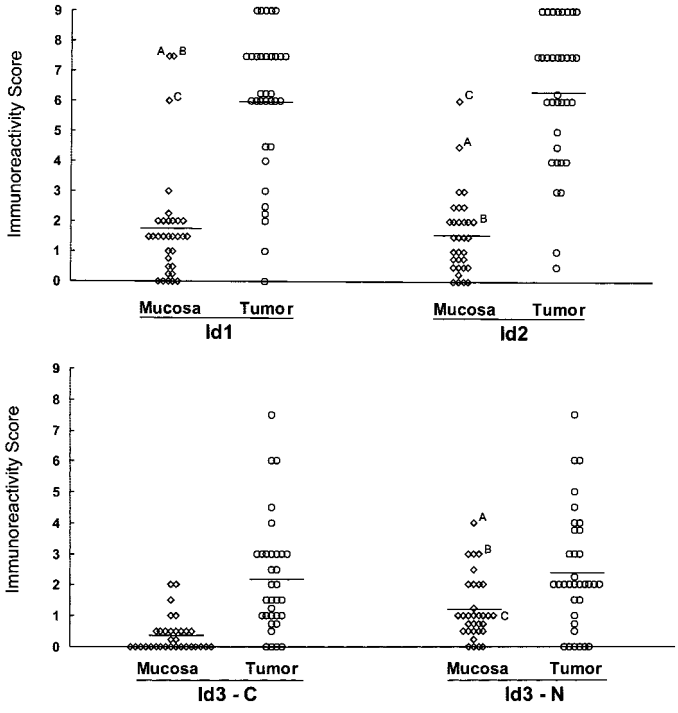

Fig. 2. Id protein expression levels in normal mucosa and tumor tissue from patients with colonic adenocarcinoma. Scatter plots are shown for immunoreactivity of mucosa and tumor specimens for the patient series. For Id3, the data for cytoplasmic (*Id3-C*) and nuclear staining (*Id3-N*) are shown separately. The immunoreactivity score represents the total score (TS) which was taken as the product of the intensity score (INT) and the score for the proportion (PROP) of immunoreactive cells within the specimen. *Solid horizontal lines*, mean values. Three mucosal specimens (*A*, *B*, and *C*) displayed consistently elevated levels of Id immunoreactivity.

Id protein Immunoreactivity Correlates with p53 Immunoreactivity and Mitotic Index in Colorectal Tumors. Using the DOH7 anti-p53 antibody, we found that increased staining is a reliable marker of mutant p53 protein that possesses a higher stability than wild type (40). Mutational loss of p53 function is a common event in colorectal tumorigenesis (40). As shown in Table 2, we found a statistically significant correlation between Id and p53 immunoreactivities. For Id1, Id2, and Id3, the proportion of tumor cells that were positive for Id protein correlated with both the proportion of p53-positive cells and with the total p53 staining. In the case of Id2, this correlation extended to the total staining pattern for this Id protein. Both the proportion of Id-positive cells and the total Id staining was correlated with mitotic index for Id1 and to a lesser extent for Id2, but not for Id3 (Table 2).

Because the correlation between loss of p53 function, as reflected by increased immunoreactivity, and Id expression was so striking, we determined whether loss of p53 function in isolation might influence Id protein expression levels by an analysis of intestinal epithelium in *p53*−/− mice. As shown in Fig. 3, intestinal crypts from *p53*−/− mice displayed a higher level of expression of all three Id proteins

Table 1 *Id staining in human colorectal adenocarcinomas*

Values represent the mean ± SE of TS values for Ids 1, 2, and 3 for tumors grouped according to Jass stage and site of lesion.

	Id1		Id2		Id3-nuclear		Id3-cytoplasmic	
	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal	Tumor
Jass stage								
I ($n = 11$)	1.6 ± 0.6	5.5 ± 0.8	1.4 ± 0.4	5.4 ± 0.7	1.2 ± 0.4	1.7 ± 0.6	0.2 ± 0.1	2.1 ± 0.6
II ($n = 6$)	1.7 ± 0.5	6.7 ± 1.2	1.3 ± 0.3	7.4 ± 1.3	0.8 ± 0.2	2.8 ± 0.4	0.2 ± 0.1	3.1 ± 0.8
III ($n = 10$)	2.0 ± 0.7	5.7 ± 0.7	1.6 ± 0.3	5.7 ± 0.6	1.5 ± 0.4	2.5 ± 0.6	0.8 ± 0.3	2.0 ± 0.6
IV[a] ($n = 7$)	1.7 ± 0.7	6.6 ± 0.3	4.8 ± 3.0	7.7 ± 0.4[b]	1.1 ± 0.2	2.9 ± 0.9	0.3 ± 0.1	1.8 ± 0.3
Site								
Right ($n = 9$)	1.2 ± 0.3	4.6 ± 0.9	1.4 ± 0.3	5.3 ± 0.9	1.4 ± 0.3	2.0 ± 0.7	0.6 ± 0.2	1.3 ± 0.3
Left ($n = 12$)	1.8 ± 0.6	7.0 ± 0.4	3.4 ± 1.9	7.4 ± 0.5	1.6 ± 0.4	3.5 ± 0.6[b]	0.4 ± 0.2	2.6 ± 0.5
Rectum ($n = 13$)	2.0 ± 0.6	5.9 ± 0.7	1.6 ± 0.5	6.0 ± 0.7	0.8 ± 0.2	1.6 ± 0.4	0.2 ± 0.1	2.4 ± 0.6

[a] All of the Jass stage 4 tumors were $T_4N_{2-4}M_0$ (TNM staging), Dukes stage C1.
[b] Subgroups with significantly elevated Id expression relative to other subgroups. These are: (*a*) Id2 TS for Jass IV > Id2 TS Jass I and III at $P = 0.027$ and $P = 0.033$, respectively; and (*b*) Id3-nuclear TS for left-sided tumors > Id3-nuclear TS for both right-sided and rectal tumors, at $P = 0.049$ and $P = 0.007$, respectively (Mann-Whitney test).

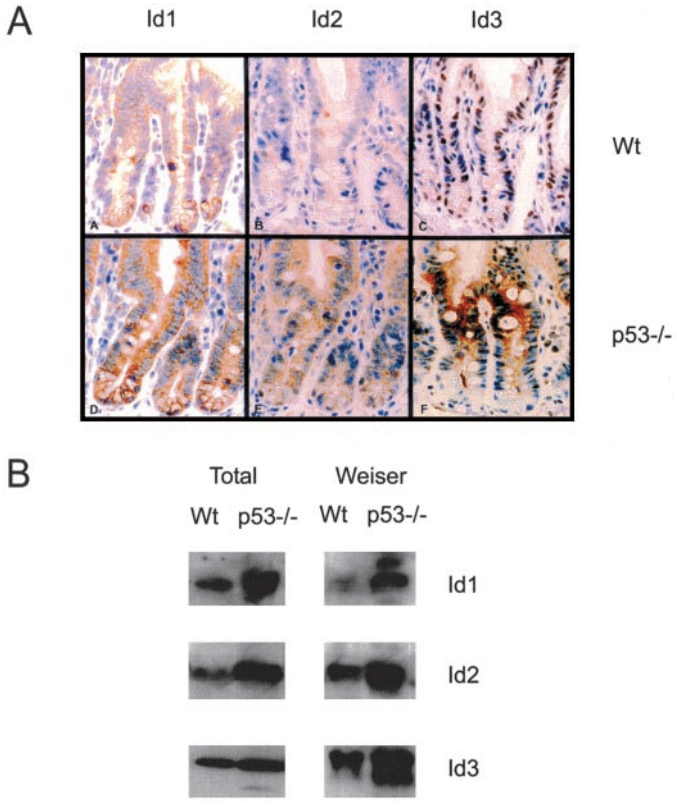

Fig. 3. Expression of Id proteins in intestinal epithelia from wild-type and *p53*−/− mice. *A*, immunostaining of Id protein in sections from wild-type (*Wt*) or p53-null (*p53*−/−) mice. *B*, Western analysis of immunoprecipitates of Id protein from intestinal epithelia of Wt and *p53*−/− mice. Cell lysates were obtained from intestinal epithelial cell preparations processed by the Weiser method (see "Materials and Methods").

when compared with wild type. Intriguingly, for Id3, there was also a marked change in subcellular distribution associated with *p53*−/− crypts, with strong cytoplasmic immunoreactivity in cells at the top of the crypt and at the crypt-villus junction. No significant elevation of Id protein expression was observed in *p53*+/− mice (data not shown).

Id Protein Expression Is Deregulated in Mouse Adenocarcinomas. To investigate the generality of elevated Id protein expression as a marker of intestinal epithelial tumorigenesis, we extended the analysis to the *Min*+/− mouse model of tumorigenesis. This mouse strain develops spontaneous tumors of the intestinal epithelium (35). Tumor material and matched normal tissue was investigated in six mice. As shown in Fig. 4, increased expression of Id1–3 was evident in both small cystic adenomas and in larger tumor masses. No expression of Id4 was detectable in either normal or malignant mouse intestinal epithelium (data not shown). The pattern of Id staining was essentially invariant among different mouse tumors.

Suppression of Adenocarcinoma Cell Proliferation by Antisense Id Oligonucleotides. We next investigated the requirement of Id protein expression for the proliferation of epithelial adenocarcinoma cells. As a model, we used two well-characterized human adenocarcinoma cell lines, HCLO (32) and LS147T (33). As with primary tumors, both of these cell lines expressed abundant Id1, Id2, and Id3 protein but were negative for Id4 (Fig. 5). Fig. 6*A* shows that the incubation of both of the cell lines with antisense oligonucleotides specific for *Id1*, *Id2*, and *Id3* resulted in a dose-dependent suppression of cell growth as assessed by the percentage of cells in S phase of the cell cycle. Neither control, sense oligonucleotide, or carrier buffer had any discernible affect on cell growth (Fig. 6*A*). As shown in Fig. 6*B*, antisense inhibition of cell growth was accompanied by a modest reduction of expression of one or more of the Id proteins in each cell line. These observations are consistent with previous studies using antisense Id oligonucleotides in other cell types (14, 16, 25) and suggest that cell growth can be attenuated by only a modest reduction in the level of the cellular Id protein level.

Suppression of Adenocarcinoma Cell Proliferation by bHLH Protein. The HCLO and LS147T cell lines were used to investigate the importance of Id-bHLH protein interactions in supporting the proliferation of adenocarcinoma cells. Of the known bHLH E proteins (the preferred heterodimerization partners for Id proteins) only the E2A-encoded proteins, E47/E12 are known to be abundantly expressed in epithelial cell lineages (Refs. 19, 23; data not shown). As shown in Fig. 7*A*, only ~50% of the intracellular Id protein is associated with E2A protein in coimmunoprecipitates prepared from exponentially growing cultures of the two tumor lines (compare Western analysis of immune supernatant and immunoprecipitate lanes from cells transfected with control, pcDNA3 vector, in Fig. 7*A*). When cells were transfected with vector expressing E47, a greater proportion of each Id protein could be immunoprecipitated with the anti-E2A antibody (Fig. 7*A*). However, only ~5–10% of the cells in these transiently transfected populations expressed the exogenous E47 protein (data not shown). Therefore, to show more definitively that the pool of endogenous Id proteins is associated with the transfected E47 protein, we exploited the fact that Id proteins lack a nuclear localization signal and are found predominantly in the cytoplasm/perinuclear region of many cell types, whereas their bHLH partners such as E47 possess a strong nuclear localization signal and are usually localized exclusively to the nucleus (37). As shown in Fig. 7*B*, whereas cells not expressing exogenous E47 protein displayed a diffuse, cytoplasmic staining of Id3, those cells expressing exogenous E47 exhibited strong nuclear staining of Id3. Similar results were obtained for Id1 and Id2

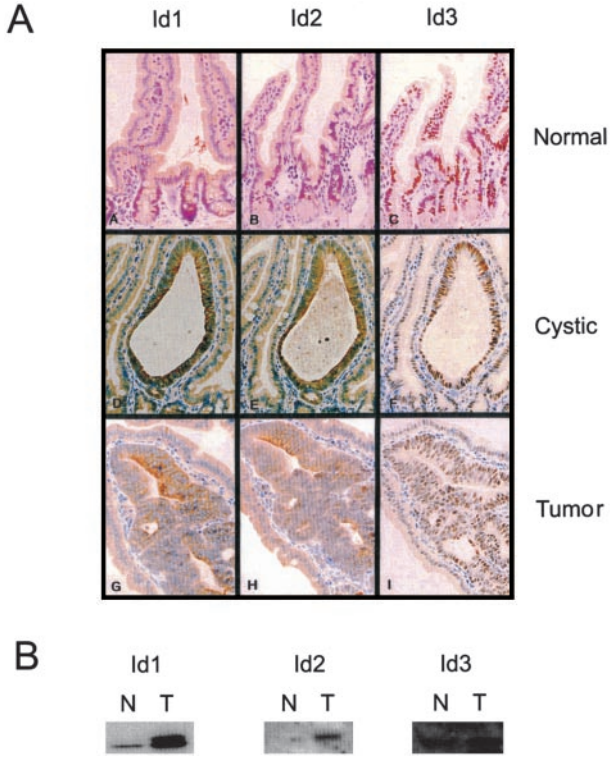

Fig. 4. Expression of Id proteins in normal and tumor tissue from *Min*/+ intestinal epithelium. In *A*, immunostaining of "normal" epithelium, cystic tumors, and adenomas is shown for comparison. In *B*, Western analysis of immunoprecipitates of Id proteins from normal mouse intestinal epithelium (*N*) are compared with a representative mouse adenoma (*T*) that was excised from the epithelium.

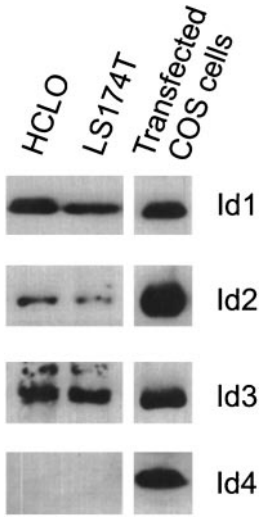

Fig. 5. Id protein expression in human colonic adenocarcinoma cell lines. Western analysis for each of the four Id proteins was performed on immunoprecipitates of Id proteins prepared from the two cell lines, HCLO and LS174T. As positive controls, lysates were prepared and analyzed in parallel from Cos7 cells transiently transfected with pcDNA3 expression vectors encoding each Id protein.

(data not shown). This colocalization of E47 and Id proteins in the nuclei of E47-transfected cells is consistent with sequestration of cellular pools of Id protein as an Id-E47 heterodimer. To determine whether such cells expressing a functional excess of bHLH E protein over Id protein are affected in their proliferative capacity, we assessed BrdUrd incorporation in transiently transfected cells by using the *LacZ* gene as a marker. As shown in Fig. 7*C*, the ability of E47 overexpressing cells to progress into the S phase of the cell cycle was suppressed by 80–90% in both cell lines.

These experiments suggest that the proliferative capacity of intestinal adenocarcinoma cell lines can be modulated by perturbing the balance of cellular pools of Id and bHLH proteins; an excess of Id protein supports proliferation, whereas an excess of bHLH protein, with accompanying loss of "free" Id protein, results in cell cycle arrest. However, it could be argued that the E47-induced growth arrest observed in these experiments is as much a consequence of the high levels of exogenous E47 protein as the sequestration of cellular pools of Id protein into a heterodimer state in transfected cells. To overcome this caveat, we designed an attenuated mutant of E47 in which the region imparting transcriptional transactivation function (NH_2-terminal to the HLH domain) is deleted and the basic DNA binding domain is mutated to abolish DNA binding ability as described previously by Chakraborty *et al.* (39). The structure of this mutant (antagonist, aHLH protein), which also possesses a myc epitope tag at the NH_2 terminus, is depicted in Fig. 8*A*. The HLH domain of the aHLH protein is preserved so that it should still be able to form heterodimers. However, because bHLH-bHLH dimerization is stabilized by DNA binding, the mutant should preferentially dimerize with Id protein [the stabilization of which does not require DNA binding (see Ref. 1)]. As shown in Fig. 8*B*, the aHLH protein could be detected in transfected cells by Western blotting using E47 antiserum after immunoprecipitation with 9E10 antibody, specific for the myc epitope tag present on the aHLH protein. However, as shown in Fig. 8*C*, when a construct encoding wild-type E47 was cotransfected with aHLH vector, none of the E47 protein was coimmunoprecipitated with the aHLH protein; essentially all of the E47 protein remained in the immune supernatant (Fig. 8*C*). Cells were then transfected with the aHLH construct, and the extent of association with either endogenous or exogenous (co-transfected) Id protein was evaluated by immunoprecipitation with anti-myc tag antibody followed by Western blotting with antisera for each of the three Id proteins (Fig. 8*D*). Somewhat surprisingly, for both the endogenous and exogenous Id proteins, only Id1 and not Id2 nor Id3 was found to associate with the aHLH protein. Despite this altered dimerization potential of the aHLH protein (which presumably arises as a consequence of removal of the NH_2-terminal region and or mutation of the basic, DNA-binding domain), adenocarcinoma cells transiently transfected with this construct still displayed an appreciable suppression in proliferative capacity (3- to 4-fold) as assessed from the percentage of cells in S phase of the cell cycle (Fig. 8*E*). This observation is consistent with data obtained by antisense oligonucleotide blockade (Fig. 6) in showing that a partial reduction of the cell's complement of Id protein impairs proliferation. Taken together, the data strongly imply that proliferation of adenocarcinoma cells is dependent on the elevated expression levels of Id protein observed in these tumor cells.

DISCUSSION

A substantial body of evidence from experimental model systems now supports a role for Id proteins in tumorigenesis mechanisms (reviewed in Refs. 2, 3). However, studies on primary human tumors have shown that deregulated expression of Id genes is not apparently

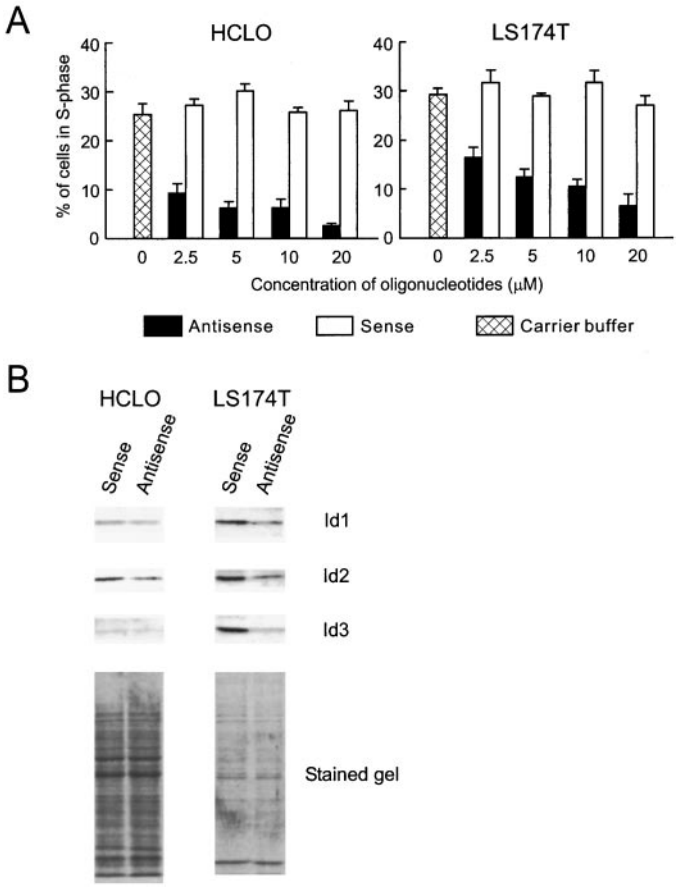

Fig. 6. Inhibition of adenocarcinoma cell proliferation by Id antisense oligonucleotides. In *A*, cells were seeded in chamber slides and treated with the indicated concentrations of an equimolar mixture of either sense or antisense Id1, Id2, and Id3 oligonucleotide. An additional control of treatment of cells with carrier buffer was also included. After 17 h, cells were pulsed with BrdUrd for 2 h. The percentage of cells in S phase was determined by scoring BrdUrd-positive cells detected by immunostaining. The data shown are from two experiments, each performed in triplicate. *B*, lysates from cells treated with a 10-μM mixture of either sense or antisense oligonucleotides for Id1, Id2, and Id3 were analyzed by Western blotting for Id protein expression. *Bottom panel*, a representative Coomassie Blue-stained gel to show equivalence of protein loading.

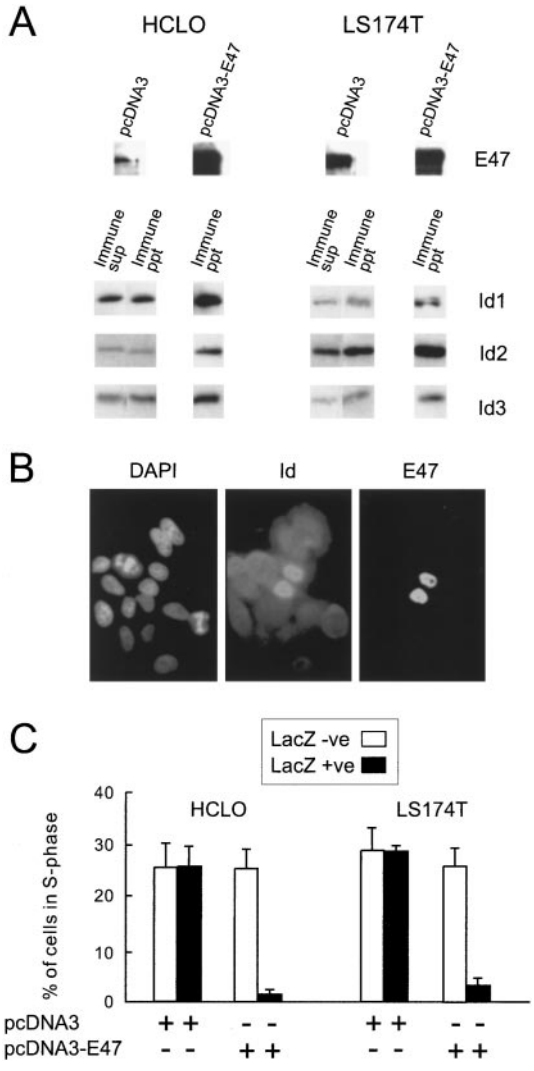

Fig. 7. Suppression of epithelial tumor cell proliferation by E2A protein. In *A*, both human colorectal cell lines, HCLO and LS174T, were transiently transfected either with control (empty vector, pcDNA3) or with vector expressing the E2A bHLH protein, E47 (pcDNA3-E47). Lysates were prepared and subjected to immunoprecipitation with anti-E47 antibody followed by Western analysis with anti-E47 antibody (*top panel*). For pcDNA3 transfected cells, the immune supernatant (containing Id protein not associated with endogenous E protein) was subjected to immunoprecipitation with each of the Id antibodies (*immune sup*). An equivalent proportion of E47 immunoprecipitate (containing Id protein associated with endogenous E protein) was redissolved and similarly immunoprecipitated with each Id antibody (*immune ppt*) for comparison as shown in the *bottom panel*. For pcDNA3 transfected cells, E47 immunoprecipitates were redissolved and subjected to immunoprecipitation with anti-Id antibodies to show the association with endogenous Id protein. Immunoprecipitates were subjected to Western analysis with each of the anti-Id antibodies indicated. Additional control samples from Cos7 cells, transiently transfected with Id (or E47) constructs were used to confirm the identities of bands as indicated (see Fig. 5). In *B*, HCLO cells, transiently transfected with the E47 expression vector, were fixed and immunostained for Id3 and E47 proteins, then counterstained with DAPI. The representative field of view shows colocalization of Id3 and E47 proteins in the nucleus in cells expressing the exogenous E47 protein. In *C*, the HCLO and LS174T cell lines were transiently cotransfected with LacZ vector, together with either empty control vector, pcDNA3, or with vector expressing the E2A bHLH protein, E47 (pcDNA3-E47) as indicated. Twenty-four h later, the cells were pulsed for 2 h with BrdUrd, fixed, and then immunostained for codetection of LacZ and incorporated BrdUrd. The percentage of cells in S phase was evaluated by scoring a minimum of 200 cells from both LacZ-positive and LacZ-negative populations in each transfection. The data were taken from two experiments.

a universal feature of malignancy; most primary leukemias, for example, do not display abnormalities in Id expression (41, 42). Among solid tumor types, the published data are still relatively limited (Refs. 24–28; reviewed in Refs. 2, 3). The present study represents the first systematic survey of Id protein expression in colorectal cancer and also addresses the relationship with histopathological and other features of disease. Our studies revealed that deregulated expression of Id1–3, but not of Id4, is a consistent feature of both human and mouse

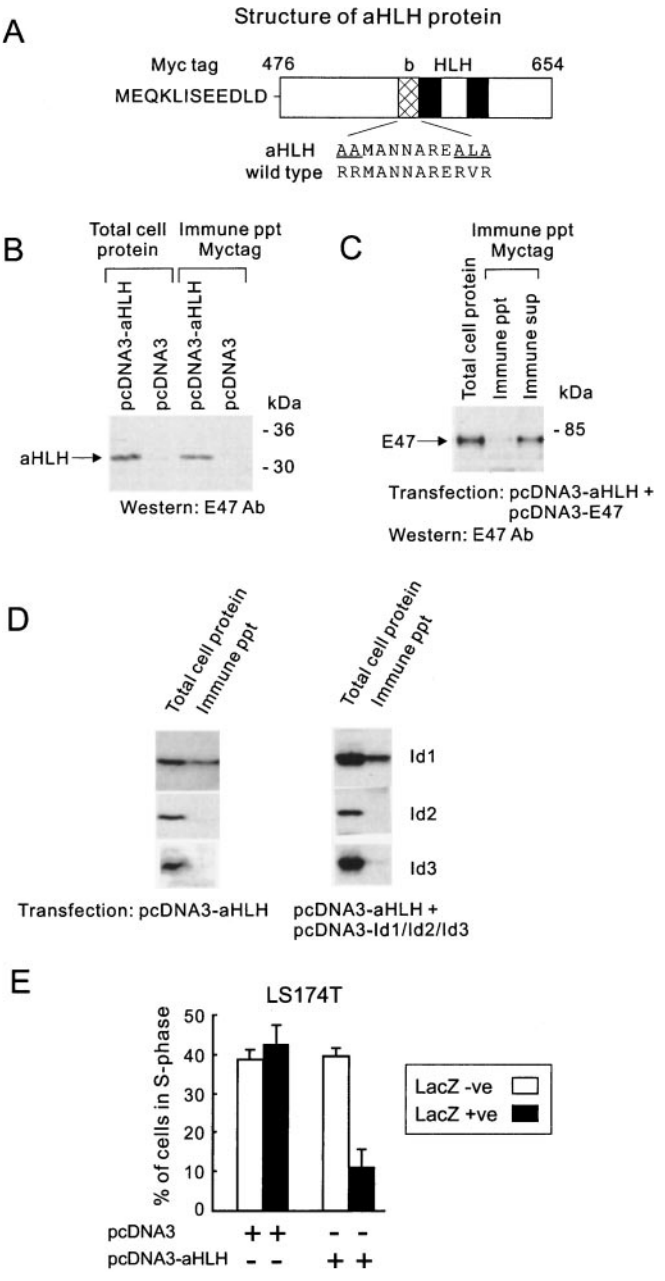

Fig. 8. Suppression of epithelial tumor cell proliferation by the aHLH protein. *A*, structure of the aHLH mutant construct derived from E47. In *B*, cells were transfected with either empty vector, pcDNA3, or with pcDNA3-aHLH; and lysates were subjected to immunoprecipitation with 9E10 antibody, specific for the myc-epitope tag. Immunoprecipitates (*Immune ppt*) were analyzed by Western blotting using E47 antibody. Samples of total cell protein from an equivalent number of transfected cells were analyzed as a control. *kDa*, M_r in thousands. In *C*, cells were cotransfected with the aHLH expression construct and pcDNA3-E47, and lysates were analyzed by immunoprecipitation for the myc epitope tag [immunoprecipitate (*Immune ppt*) and immune supernatant (*Immune sup*) are compared]. E47 antibody was used in Western blot analysis. In *D*, cells were transfected either with pcDNA3-aHLH or with pcDNA3-aHLH plus constructs expressing each of the Id proteins as indicated. Equivalent cell numbers were analyzed by Western blotting for each Id protein, using either whole cell protein or cell lysate that had been immunoprecipitated with 9E10 antibody, specific for the myc tag on the aHLH protein. In *E*, cells were cotransfected with LacZ vector, together either with control empty vector, pcDNA3, or with pcDNA3-aHLH, as indicated. Twenty-four h later, the cells were pulsed for 2 h with BrdUrd, fixed, and then immunostained for detection of LacZ and incorporated BrdUrd. The percentage of cells in S phase was evaluated by scoring a minimum of 200 cells from both LacZ-positive and LacZ-negative populations in each transfection. The data were taken from two experiments.

intestinal epithelial tumors. Interestingly, no significant elevation of Id expression was seen in human colorectal adenomas, which implies that up-regulation of Id expression levels occurs as a relatively late event during tumorigenesis of intestinal epithelium, at least in humans.

In most patients, we found that Id protein levels in normal mucosa were low and fell within a narrow range. However, in three patients, who also displayed particularly high Id expression levels in tumor tissue, the mucosal expression levels were also consistently high. Evaluation of histopathological and other data revealed no obvious distinguishing features in these patients. However, because expression of Id genes is well documented to be responsive to a plethora of growth factor signals (1), it is possible that paracrine mechanisms involving factors secreted by tumor tissue might explain the elevated Id expression in the normal mucosa of these patients. This possibility may have relevance to tumorigenesis mechanisms because Id proteins are involved in vascularization and tumor invasiveness (19, 20).

Evaluation of the patient series as a whole revealed several significant associations with histopathological and other features of tumors. Of these, the correlation with mitotic index and p53 expression levels was highly significant. The correlation between mitotic index and expression of Id1, and to a lesser extent with expression of Id2, may well reflect the ability of Id proteins to enhance cellular proliferation when experimentally overexpressed in a number of cell types (1), including cells of epithelial lineage (Ref. 19).[4] We were able to show that the correlation between expression of all three Id proteins and p53 expression (as a marker of mutational modulation of p53 function) can be explained by a direct causal relationship, because loss of p53 function in *p53*-null mice led to up-regulation of Id protein levels in otherwise normal mucosa. It seems likely, therefore, that loss of p53 function represents an important mechanism through which Id protein expression is deregulated in colonic adenocarcinoma cells.

Expression of Id proteins is known to be regulated through mechanisms, as yet poorly understood, that operate at both the transcriptional and translational levels (1). The precise determinants of p53-dependent (and p53-independent) up-regulation of Id expression in tumor cells are, therefore, likely to be complex. However, in common with p53, Id proteins are rapidly up-regulated in response to genotoxic stress stimuli, and at least one gene target for p53, $p21^{WAF-1/CIP1}$, is also reportedly regulated by Id proteins (43). Thus, some cross-talk between the p53 and Id signaling pathways might well be expected.

A major pathway of cell-fate determination is regulated by a critical balance between the opposing functions of bHLH and Id proteins (1–3). Several studies have shown that abrogation of Id function by antisense oligonucleotide blockade or by microinjection of antibodies (resulting in a functional excess of the cell's compliment of bHLH protein) results in an arrest in the G_1-to-S phase transition of the cell cycle and to the inhibition of cellular proliferation (14–16). Antisense Id oligonucleotide blockade similarly led to the suppression of proliferation of adenocarcinoma cells in the present study. By enforced expression of the bHLH protein, E47, which is known to form stable heterodimers with Id proteins (5), we showed by both coimmunoprecipitation and immunofluorescence colocalization studies that essentially all of the cellular pools of Id protein become associated with the E47 protein in transfected cells. Under these conditions of experimentally manipulated bHLH protein excess, cellular proliferation was significantly suppressed. Moreover, we found that the sequestration of cellular pools of Id1 by using an antagonist HLH protein that was defective in DNA binding and transactivation function also resulted in the suppression of adenocarcinoma cell proliferation. Thus, we infer that a functional excess of Id protein is necessary to support the proliferation of these tumor cells. Given the known oncogenic properties of Id proteins established in various experimental models (2, 11, 17–22), and the observation that targeted transgene expression of Id1 in murine intestinal epithelium is associated with tumor induction (23), it is likely that the up-regulated expression of Id proteins seen in primary human colorectal tumors is a major determinant of tumorigenesis of the colonic epithelium.

Mutations in a number of onco/tumor suppressor genes such as *APC*, *DCC*, *K-ras*, and *p53* are involved in tumorigenesis of colonic epithelium (44). Perturbations in the expression of several important regulators of the cell cycle such as cyclin D3 (45), Cdk2 (46), $p21^{WAF-1/CIP1}$ (47) and $p27^{Kip1}$ (48) have also been described in this tumor type. However, the mechanisms through which primary genetic lesions ultimately modulate the expression of these cell cycle regulators and the role of the latter in colorectal tumorigenesis is not known. Our data show that deregulated expression of Id protein family members is likely to be an important determinant in colorectal tumorigenesis and arises at least in part through a loss of p53 function.

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