Menin, the product of the tumor suppressor gene MEN1, is widely expressed in mammalian endocrine and non-endocrine tissues, including intestine. Its known abundant expression in several types of cells with high proliferative capacity led us to investigate the physiological function of the protein menin in intestinal epithelium, one of the most rapidly growing epithelia. Here we showed that the Men1 gene is mainly expressed in the crypt compartment of the proximal small intestine and that its expression was increased during fasting in vivo, both suggesting a role of menin in the control of cell growth. Indeed, specific reduction of menin expression by transfected antisense cDNA in the rat duodenal crypt-like cell line, IEC-17, increased cell proliferation. The latter is correlated to a loss of cell-cycle arrest in G1 phase by resting cells and an overexpression of cyclin D1 and cyclin-dependent kinase (Cdk)-4. Furthermore, these cells lost the inhibition of proliferation induced by transforming growth factor-β1, associated with a decrease of transforming growth factor-β type II receptor expression. As a result of deregulated proliferation, antisense menin transfected IEC-17 cells became tumorigenic as shown in vitro as well as in vivo in immunosuppressed animals. These results indicate that menin contributes to proliferation control in intestinal epithelial cells. The present study reveals an unknown physiological function for menin in intestine that may be important in the regulation of epithelial homeostasis.

Menin is a 610 amino acid protein encoded by the tumor suppressor gene MEN1 (1). Germline mutations are responsible for multiple endocrine neoplasia type 1 (MEN1), an autosomal-dominant cancer syndrome featuring parathyroid cell hyperplasia and tumors of the pituitary and duodeno-pancreatic endocrine tissues. Biallelic inactivation of the Men1 gene in mice results in embryonic lethality at mid-gestation (2, 3). Heterozygote Men1 mutant mice develop the similar range of endocrine tumors as seen in the human MEN1 syndrome (4).

Menin is highly conserved in humans and rodents (5, 6). The protein sequence does not include consensus motives from which its putative function could be deduced. Menin has been shown to interact directly with an ever increasing list of molecular partners such as JunD, Smad3, nm23, NF-κB (for a review, see Ref. 7). It is predominantly located in the nucleus, with two independent nuclear localization signals (1).

Despite the endocrine specificity of the MEN1 syndrome tumors, menin RNA and protein are widely expressed in endocrine and non-endocrine rodent and human tissues (6, 8–10), suggesting a large panel of physiological functions for the protein. In the intestinal tract of mouse and rat, menin RNA was detected by Northern blotting in small and large intestine (6, 8). Interestingly, in adult human tissues, the expression of the MEN1 gene detected by in situ hybridization was predominant in actively proliferating cells such as the proliferative phase of endodermium and parabasal cells of the esophageal mucosa, but faint in the secretory phase of the endodermium (10).

The aim of the present study was to investigate the physiological function of menin in the intestinal epithelium, which is among the most rapidly renewing tissues of the mammalian organism. First, we provide direct evidence that the Men1 gene is mainly expressed in the crypt compartment of the small intestine. We then demonstrate that menin inactivation increases cell proliferation and promotes tumorigenesis both in vitro and in vivo in intestinal epithelial cells.

**EXPERIMENTAL PROCEDURES**

**In Situ Hybridization—**Deparaffinized mouse proximal small intestine sections were treated for 10 min with pepsin (0.4%) in 0.2 N HCl, followed by ethanol treatment and air drying. Hybridization with digoxigenin-labeled riboprobes was done at 65 °C overnight in 50% formamide, 1× salt, 1× Denhardt’s solution, 10% dextran sulfate, and 250 μg/ml yeast RNA. The unhybridized probe was removed in 2× SSC and 50% formamide at 65 °C for 30 min, followed by rinses with decreasing concentrations of SSC. Nonspecific sites were blocked with 2% blocking reagent (Roche Applied Science) in 0.1 mol/liter maleic acid, 0.15 mol/liter NaCl, pH 7.5, 0.1% Tween 20, and 2% goat serum for 1 h at room temperature. Slides were incubated overnight at room temperature with alkaline phosphatase-conjugated polyclonal sheep anti-digoxigenin antibody (Roche Applied Science). The reaction product was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Roche Applied Science). Sense and antisense digoxigenin-labeled riboprobes were generated from linearized templates by in vitro transcription with T3 or T7 RNA polymerase in the presence of digoxigenin-UTP (Roche Applied Science). A plasmid (IMAGE clone ID.402210, Research Genetics) containing the 900-bp 3′-end of the Men1 cDNA was used as a template. The plasmid was linearized either with EcoRI and tran-
scribed with T3 RNA polymerase, or with NotI and transcribed with T7 RNA polymerase, respectively, for antisense and sense probe. The sections, first used for in situ hybridization as described above, were subjected next to fluorescent indirect immunohistochemistry with an anti-proliferating cell nuclear antigen antibody (1:150, Santa Cruz Biotechnology).

Cell Culture and Stable Transfection—The rat duodenal cell line IEC-17 (11) was routinely maintained in DMEM, 10% FCS, 25 units/ml insulin, 2 mM glutamine, 100 units/ml penicillin, and 50 mM streptomycin.

The expression plasmid encoding the human menin cDNA cloned in an antisense orientation into the EcoRI site of pcDNA3.1 (+) was a gift from Dr. Hendy (McGill University, Montreal, Canada) (12). The empty pcDNA3.1 vector (V) and the pcDNA3.1/AS menin plasmid (AS) were stably transfected into IEC-17 cells using the Exgen reagent as described by the supplier (Euromedex). To select for stable expression, the transfected cells were grown in standard culture medium containing G418 (700 µg/ml) starting 2 days after transfection. Except during experiments, G418 was maintained in culture medium.

Proliferation Assays—Cells were plated at 10,000 cells per well in 24-well plates. The cell number was estimated at 24-h intervals by counting with a hemacytometer the number of cells detached after trypsin treatment.

DNA synthesis was determined through [3H]thymidine incorporation (1 mCi/ml) during a 4-h period as described previously (13).

Cell-cycle Analysis—Cells in growth phase were synchronized by exposing the culture to FCS-deprived DMEM supplemented with 0.1% bovine serum albumin for 48 h. Quiescent cells were stimulated by the addition of 10% FCS for 24 h. Cells were harvested, fixed, and stained with propidium iodide using the BD Cycle TEST™PLUS Reagent Kit according to the manufacturer’s instructions (BD Biosciences). Flow cytometry was then performed by using a Galaxy apparatus (DAKO).

Luciferase Assay—Cells were seeded at 60,000 cells per well in 12-well plates. Twenty-four hours later, cells were transfected with 2 µg of the reporter plasmid (p3TP-Lux or TOPFlash) and the pRL-TK plasmid expressing Renilla (0.1 µg) using Exgen reagent. The reporter system p3TP-Lux contained the TGFβ-responsive PAI-1 promoter driving luciferase expression (14). The reporter construct TOPFlash contained three copies of a mutant T cell factor-4 binding site cloned into the luciferase pGL3-basic plasmid (15). Cells were harvested 48 h later. Luciferase and Renilla activities were measured by using the dual-luciferase reporter assay system (Promega). Luciferase activity was normalized to the relative Renilla activity in each experiment.

RNA Isolation and Reverse Transcription (RT) - PCR—RNA from cells or tumor samples was prepared with TRIzol reagent (Euromedex, France). 2 µm RNA samples were reverse-transcribed for 1 h at 42 °C by avian myeloblastosis virus reverse transcriptase (Finnzymes) using a specific antisense primer. The PCR was carried out on 3 µl of the cDNA generated from the RT reaction, 50 pmol of each oligonucleotide pair, 0.4 mM each of 2'-deoxynucleoside 5'-triphosphate and 1.5 units of TaqDNA polymerase in 10× buffer as described by the manufacturer (Invitrogen) with the following conditions: 94 °C for 30s; 55 °C for 45s; 72 °C for 45s; for 30 cycles (menin) or 20 cycles (TGFβ type II receptor (TβRII) and cyclinophilin A5). Primers used were as follows: pcDNA3.1 polyclonal site primer, sense, 5'-GAA CCC ACT GCT TAC TGG CTT ATC GAA-3'; human menin, antisense, 5'-GCC AGC GCA AAG GCC TCT GAA CTA CTG-3'; TβRII, sense, 5'-TCA CTA GGC ACC GTC TCA GCS-3'; TβRII, antisense, 5'-AGG ACA ACC CGA ATG CAC AC-3';
cyclophilin A, sense, 5′-CTT GTC CAT GGC AAA TGC TG-3′; cyclophilin A, antisense, 5′-GTG ATC TTC TTG CTC TTG-3′. PCR products were separated by electrophoresis on 2% agarose gels.

**Protein Preparation and Western Analysis**—Cells were lysed in cold solubilization buffer containing 1% Triton X-100, 50 mM Hepes (pH 7.5), 150 mM NaCl, 2 mM Na3VO4, 10 mM NaF, 100 units/ml aprotinin, 20 μg/ml leupeptin, and 0.2 mg/ml phenylmethylsulfonylfluoride. Cell extracts were clarified (14,000 × g for 15 min at 4°C). Cytosolic and membrane fractions were prepared as described (16). After electrophoresis, proteins were transferred onto nitrocellulose membranes (polyvinylidene difluoride membranes for menin expression). Western blotting was performed as described previously (13). The polyclonal antibodies used were menin (1:1500), cyclin D1 (1:200), cyclin D3 (1:1000), Cdk4 (1:200), TβRII (1:500) (Santa Cruz Biotechnology), and cyclophilin A (Upstate Biotechnology, 1:3000). The mouse monoclonal antibodies used were β-catenin (1:200, Santa Cruz Biotechnology), α-tubulin (1:25,000, Sigma), and actin (1:5000, ICN).

**Soft Agar Assays**—IEC-17/AS and IEC-17/V cells were plated at 50,000 cells/well in 0.4 ml of DMEM supplemented with 5% FCS and 0.2% low-melting-temperature agarose (Sigma) in 12-well plates coated with a 0.75-ml layer of DMEM supplemented with 5% FCS and 0.6% low-melting-temperature agarose. Dishes were kept at 37°C and 5% CO2 and monitored after 2 weeks for colony formation. As a positive control, we used the colonic adenocarcinoma HT-29 cell line (12.5 × 104/well) in the same conditions. Cells were photographed after 2 weeks. All experiments were performed in triplicate.

**Animal Studies**—Tumorigenicity was assayed by subcutaneous injection of 1 × 106 IEC-17/V or IEC-17/AS cells resuspended in 50 μl of cold phosphate-buffered saline mixed with 50 μl of matrigel (BD Biosciences) in the abdominal region of Wistar newborn rats. Rats were subsequently immunodepressed as described previously (13). Animals were sacrificed 3 weeks after cell inoculation and tumors were removed, fixed in 10% formalin, and then embedded in paraffin for histological analysis or frozen directly to liquid nitrogen for RNA analysis. Tumor volumes were calculated by the formula π/6 × length × width × thickness. Furthermore, subcutaneous dorsal injections of IEC-17/V or IEC-17/AS cells (3 × 106 cells with matrigel) were also given to 4–6 week-old athymic (Balb/c nu/nu) female mice. They were sacrificed at 6 or 12 weeks after injection.

The fasting and refeeding study comprised three groups of male Swiss CD1 mice (Charles River Laboratories) as described by Nian et al. (17). Briefly, the first group of animals was fed ad libitum, the second group was deprived of food for 48 h, the third group was fasted for 48 h, followed by unrestricted access to food for 24 h. A 10-cm segment from duodeno-jejunal was harvested in the euthanized animals and rinsed in phosphate-buffered saline. Duodeno-jejunal mucosa was scraped from the underlying seromuscular layers and homogenized in buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, NaF 100 mM, EDTA 10 mM, 10 mM Na3VO4, 1% Nonidet P-40, and complete, EDTA-free protease inhibitor mixture (Roche Applied Science). Protein extracts were submitted to Western blot analysis as described above.

To analyze cell proliferation in vivo, wild type and Men1 heterozygous mice (4) were injected intraperitoneally with 1.5 mg of 5-bromodeoxyuridine (BrdUrd, Roche Applied Science). Tissues were collected on the next day, fixed, and paraffin embedded. BrdUrd incorporation was evaluated by immunocytochemistry.

**Histochemistry and Immunostaining**—Sections of the paraffin-embedded tissue samples were subjected to standard hematoxylin and eosin-staining. Detection of cytokeratin was performed by using a mouse monoclonal antibody (DAKO). Antigen-antibody complexes were revealed by the streptavidin-biotin technique (DAKO), using diaminobenzidine as chromogen. For immunocytochemical study, cells were grown in 8-well chamber slides, fixed with cold methanol, incubated for 1 h with the anti-β-catenin antibody (1:100), and followed by the appropriate fluorescent secondary antibody.

**RESULTS**

**Men1 Transcript Is Expressed in Intestinal Crypts and Its Expression Increased During Fasting**—The expression of the Men1 transcript was examined by in situ hybridization in proximal small intestine (duodeno-jejunal segment) of mice using a riboprobe containing an antisense Men1 cDNA sequence. As depicted in Fig. 1A, Men1 transcript was strongly expressed in the crypts, whereas weak expression was seen in dispersed cells of the villi. No hybridization was detected in the muscular layers or when the sense probe was used (Fig. 1B). After hybridization, immunostaining of the sections with an anti-proliferating cell nuclear antigen antibody revealed that expression of proliferating cell nuclear antigen, a marker of proliferative activity, correlated with the strongest Men1 expression in crypts (Fig. 1C).

To connect the in vitro results to the physiological state, we used the fasting-refeeding model which decreases cell crypt proliferation (17, 18). In the Western blot analysis, menin expression was stronger in the mucosa of fasted and fasted-
refed animals compared with that in ad libitum-fed mice (Fig. 2), suggesting an adaptive role of menin facing altered epithelial growth.

The expression of menin was also compared between Men1 heterozygous and wild-type mice of about 5 months by using Western blotting and in situ hybridization. As shown in Fig. 3, A and B, no clear difference in the expression of the protein or the Men1 transcript was evidenced. Furthermore, the BrdUrd incorporation was not increased in intestinal epithelium of heterozygous mice (Fig. 3 C).

Use of Antisense Menin in IEC-17 Reduces Menin Expression—To study the biological effects of menin inactivation in intestinal cells, IEC-17 cells were subjected to transfection with different constructs. Twelve and seven clones were obtained after transfection of the empty vector (IEC-17/V) and the pcDNA3.1/AS menin plasmid (IEC-17/AS), respectively. One empty vector (V403) and two antisense menin (AS303 and AS313) transfectants were used throughout the study. Specific RT-PCR was performed to control the expression of human menin antisense transcripts: a strong signal was detected in the AS303 and AS313 clones but not in the control V403 clone (Fig. 4 A). Western blot analysis showed a strong reduction of the rat (endogenous) menin protein expression in antisense menin-transfected AS303 and AS313 clones, as compared with menin expression in empty vector-transfected cells (V403) (Fig. 4 B).

Antisense Menin Expression Stimulates Proliferation and Prevents G1 Arrest of Quiescent Cells—Cell proliferation was studied by performing a growth curve on a 5-day period. As shown in Fig. 5 A, antisense menin-transfected cells grew faster than empty vector-transfected control cells. DNA synthesis was also assessed after [3H]thymidine incorporation. As shown in Fig. 5 B, [3H]thymidine incorporation was increased 2.4- and 4-fold in AS303 and AS313 clones, respectively, as compared with the V403 clone.

After a 48-h exposure to serum-deprived medium to achieve quiescence, 92% of V403 cells were arrested in G1 phase, whereas 48 and 51%, respectively, of AS303 and AS313 cells were in G1 phase, and about 22% of cells of each antisense clone entered S phase (Fig. 6 A). A 24-h serum stimulation allowed V403 cells to progress in S phase (22%), whereas the percentage of AS303 and AS313 cells in S phase increased only slightly (Fig. 6 B). The data evidenced disruption of the cell-cycle control in antisense menin-transfected cells.

Increased Proliferation Is Associated with Increased Expression of Proteins Involved in G1 to S Progression—D-type cyclins are major positive regulators expressed during the G1 phase of the cell cycle and form complexes with their catalytic partner cyclin-dependent kinase 4 (Cdk4). As D1- and D3-type cyclin expression has been shown in cells within the crypt compartment (19) and in a rat intestinal cell line (20), their expression, as well as that of Cdk4, was investigated in the three clones. For this purpose, cells were grown in complete culture medium for 24 h and then serum-deprived for 24 h. The immunoblotting data in Fig. 7 showed that cyclin D1, cyclin D3, and Cdk4
expression in serum-starved, quiescent IEC-17 clones was upregulated in antisense menin-transfected clones.

Overexpression of Cyclin D1 Does Not Involve the β-Catenin Pathway—Because cyclin D1 expression is regulated by β-catenin through transcriptional activation of the cyclin D1 gene (21), we investigated whether an increase in the cytoplasmic level of β-catenin in the antisense menin-transfected clones could be responsible for the abnormally high cyclin D1 expression. Protein extracts were prepared from cytoplasmic and membrane fractions and analyzed by Western blotting. The comparative level of β-catenin in the cytoplasmic versus the membrane compartment was similar in antisense menin-transfected clones and in the empty vector-transfected clone (Fig. 8A). Furthermore, a strong membranous expression of β-catenin was seen in V403, AS 303, and AS 313 cells by immunostaining (Fig. 8B). Finally, the TOPFlash reporter gene activity was compared in the three clones as well as in the colorectal cancer cell line Isreco-1 used as control (because of its mutation in the APC gene; Ref. 22). As shown in Fig. 8C, TOPFlash activity was very low in V403, AS303, and AS 313 cells, whereas in Isreco-1 cells, TOPFlash activity was more than 300-fold higher. These data suggest that the overexpression of cyclin D1 was not induced by a deregulation of the β-catenin pathway.

Menin Inactivation Antagonizes TGFβ1-mediated Inhibition of Cell Proliferation and Down regulates the Expression of TβRII—Because the proliferation of IEC-17-expressing antisense menin cDNA was increased, sensibility to TGFβ1, a growth factor known to inhibit intestinal epithelial cell proliferation (23), was assessed. As expected, [3H]thymidine incorporation in V403 cells was inhibited by 55% when exposed to 10 ng/ml of TGFβ1. Conversely, AS303 and AS313 cells showed only a small, non-significant decrease of [3H]thymidine incorporation (6 and 13%, respectively) (Fig. 9A).

The expression of TβRII was studied by Western analysis using a specific antisemur. A decrease of TβRII expression was observed in the two antisense menin-expressing clones (Fig. 9B). Furthermore, RT-PCR demonstrated a decrease of the TβRII transcript (Fig. 9C).

Finally, we examined whether the TGFβ1 signaling pathway was also affected. To address this question, we selected the validated TGFβ1-responsive PAI-1 promoter (14). V403, AS303, and AS313 cells were transiently transfected with the 3TP-Lux construct. As expected, the transcriptional activity of the PAI-1 promoter was increased by TGFβ1 in V403 cells. This effect was maintained in AS303 and only slightly reduced in clone AS313 (Fig. 9D).

Reduced Menin Expression Is Associated with Tumorigenic Transformation of IEC-17 Cells—V403, AS303, and AS313 clones were tested for their anchorage-independent growth in a colony-forming soft agar assay. The colon carcinoma HT-29 cell line was used as positive control, forming large colonies under the given experimental conditions. V403 cells did not form any colonies in soft agar, whereas AS303 and AS313 cells grew numerous small colonies (Fig. 10A).

To test the ability of these cells to form tumors in vivo, cells were injected subcutaneously into immuno-suppressed newborn rats using matrigel as adjuvant. Matrigel was previously described as providing strong mitogenic support for the rapid growth of non-aggressive cells (24). At sacrifice 21 days after injection, the V403 clone did not induce any tumors in the 23 rats used for the study. On the contrary, injections of AS303 and AS313 clones promoted the formation of small tumors in 30/30 rats and 27/29 rats, respectively. Injections without the addition of matrigel were also performed with the V403 and AS303 clones. Only the later cell line gave tiny tumors (data not shown).

To confirm that the tumors obtained did indeed result from the growth of antisense menin-transfected cells, the presence of antisense menin transcripts (derived from the antisense cDNA) was checked by specific RT-PCR performed with total RNA prepared from tumor samples. The two tumors used in this experiment expressed the same antisense menin transcript as the parental AS303 clone grown in culture (Fig. 10B). V403 clone in culture was used as a negative control.

Tumors obtained after subcutaneous injection of IEC-17/AS clones in immuno-suppressed newborn rats ranged from 47 to 293 mm3 (mean ± S.D. = 141 ± 21 mm3) for AS303 clone and 17 to 167 mm3 (59 ± 12 mm3) for AS313 clone. They were formed by large lobules of medium-sized, monomorphic cells, characterized by a high nucleo-cytoplasmic ratio, a large, often nucleilated nucleus, and scarce or limited cytoplasm (Fig. 2C, left panel). The epithelial nature of IEC-17 cells was demonstrated by their constant expression of cytokeratins, as detected using a pan-cytokeratin antibody (Fig. 10C, right panel). The tumors did not express chromogranin A, a specific marker
of endocrine cells (data not shown). Tumor lobules were associated with an abundant fibrous stroma. They were located mainly in the subcutaneous tissue, but sheets of tumor cells constantly invaded the adjacent dermis and muscle layers (Fig. 10C, left panel). Similar tumors were obtained after subcutaneous injection of V403, AS303, and AS313 clones into nude mice (data not shown).

DISCUSSION

The development of endocrine tumors in patients carrying germline mutations of the MEN1 gene and the frequently observed LOH in MEN1 tumor led to the assumption that menin is a tumor-suppressor (2, 25). The broad expression of menin, however, does not fit an exclusive role in endocrine structures. In fact, menin mRNA is especially abundant in proliferative cell types, such as endometrium or parabasal cells of the esophagus (10), suggesting a possible role in the control of cell growth outside the endocrine glands or cells. In support of this hypothesis, stable transfection of Ras-transformed NIH3T3 fibroblasts with menin decreased cell proliferation rate (26). In gut, menin is expressed from duodenum to distal colon. The amplitude of mRNA signal suggests that expression is not restricted to endocrine cells, because these account for only about 1% of the epithelial cells. The present study demonstrated that the Men1 gene is mainly expressed in the crypts of the mouse proximal small bowel. Next, we attempted to evaluate the physiological relevance of the link between menin and proliferation in intestinal cells using a fasting-refeeding approach. Menin expression in the intestinal mucosa increased during the fasting period and remained so within the 24-h test period after refeeding. These data suggest an adaptive role of menin when intestinal proliferation is endogenously altered and strengthen the concept that menin may be involved in the control of intestinal cell proliferation.

Because crypts are the proliferative compartment of intestinal epithelium, we went on to test the effects of a decreased cell menin level on the proliferation rate of the rat, non-transformed, crypt-like duodenal cell line IEC-17 (11). IEC-17 cells were found to express clearly detectable levels of menin mRNA and protein. Using a strategy formerly applied to the pituitary GH4Cl cell line (12), we were able to strongly and stably reduce menin expression. In vitro, this menin reduction resulted in a significant increase of cell proliferation rate that was consistent between the two tested clones. Cell-cycle analysis demonstrated that antisense menin-transfected clones continued to grow in resting culture conditions, without arrest in G1 phase, suggesting that these cells were relaxed for growth factor requirement. The increased apoptosis rate could have been triggered by reduction of the cell menin level. However, neither Hoechst 33258 staining nor caspase 3 activity indicated an increased resistance to apoptosis in the antisense transfected clones (data not shown). We then turned to possible alterations of two pathways that are known to play important roles in the regulation of proliferation of intestinal cells, namely, D cyclins and the TGFβ1 pathway.

Cyclin D1 is a critical target of many proliferative signals to intestinal epithelial cells. In the mouse small intestine, the expression of cyclin D1 and Cdk4 has been shown to be limited to cells within the crypt compartment (19, 26, 27). An overexpression of cyclin D1 has been described in the IEC-6 cell line upon transformation by activated Ras (28). More recently, cyclin D3 was also reported to be involved in the control of intestinal cell proliferation (18). In the present study, we demonstrated that reduced menin expression was associated with significant overexpression of cyclin D1, cyclin D3, and Cdk4 in IEC-17 cells. Cyclin D1 overexpression could have reflected the fact that the cyclin D1 gene is a target of the β-catenin/LEF-1 pathway (21). In a recent study, Clavel et al. (29) described a frequent nuclear redistribution of β-catenin in a panel of lung neuroendocrine tumors. This possibility was studied in the IEC-17 clones, but the data from immunocytochemistry, Western blotting, and TOPFlash reporter plasmid transfection clearly suggested that cyclin D1 overexpression in the present system did not result from the deregulation of the β-catenin pathway.

An alternative explanation is up-regulation of the cyclin D1 gene at the transcriptional level by other transactivators. It is known that NF-κB regulates cyclin D1 gene transcription through specific binding sites in the promoter (30). Menin interacts with NF-κB in the nucleus and is a potent inhibitor of NF-κB-mediated transcriptional activation (31). Thus, the possibility remains that antisense menin expression suppressed a negative control of endogenous menin on the NF-κB transcriptional effects upon cyclin D1. Further experiments are in progress to prove this hypothesis.
TGFβ1 is a potent negative growth regulator in intestinal epithelium (20, 23). Furthermore, inhibition of cyclin D1 expression is recognized as an integral component of TGFβ1-mediated growth inhibition in intestinal epithelial cells (32). In the present study, we found that the antisense menin-expressing clones were resistant to TGFβ1-induced growth inhibition. This result is relevant, because IEC17 cells express both TGFβ1 and TβRII, so that autocrine or paracrine effects are likely to occur in vitro and in vivo. Down-regulation or mutation of TβRII is one of the mechanisms by which tumor cells become resistant to growth inhibitory actions of TGFβ1. For example, transformation of rat intestinal epithelial cells (IEC-6 and RIE-1) by activated H-Ras results in down-regulation of TβRII along with resistance to TGFβ1 growth inhibitory effects (33, 34). We observed a decreased expression of the TβRII in the antisense menin-expressing clones. These results are reminiscent of those described in the SV40-transformed human esophageal epithelial cell line HET-1A. In the latter, the overexpression of cyclin D1 resulted in a decreased expression of the TβRII accompanied by the loss of growth inhibition induced by TGFβ1 (35).

Prior data suggested a link between menin and TGFβ1 pathway (12). Kaji et al. showed that inactivation of menin by antisense strategy blocks TGFβ1 signaling by inhibiting smad3/4-DNA binding at specific transcriptional regulatory sites in the pituitary GH4C1 cell line. By using the validated TGFβ-sensitive 3TP-lux plasmid, we demonstrated that the transcriptional response remains functional in the antisense menin-expressing clones despite the reduction of TβRII expression, suggesting that the residual levels of TβRII were sufficient to activate some pathways of TGFβ1 signaling. Similar results were recently reported by Fujimoto et al. (36) after activation of Ras in the RIE cell line. Furthermore, cyclin D1 overexpression in human hepatocarcinoma cell lines induced the loss of TGFβ1-mediated growth inhibition, whereas the transcriptional response of PAI-1 remained functional (37). Our data are consistent with the hypothesis that the loss of TGFβ1-induced growth inhibition in IEC-17 cells with low menin levels resulted from decreased TβRII expression rather than from blockade of TGFβ1 signaling.

The above observations led us to test whether the inactivation of endogenous menin in the IEC-17 cell line might induce a neoplastic transformation of these cells. Indeed, expression of antisense menin RNA in the non-transformed intestinal epithelial IEC-17 cells induced two hallmarks of neoplastic cells: anchorage-independent growth in soft agar, and tumor formation in immuno-suppressed newborn rats or nude mice. An underlying challenge was then to explore whether these tumors would show endocrine differentiation. It may be stressed that nothing is known of the stage at which intestinal cells undergo neoplastic change with an endocrine pattern, i.e. whether they are progenitor cells with the potential of further endocrine differentiation, or cells already engaged in the endocrine lineage. The possibility thus remained that reduction of
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