Up-Regulation of Fibroblast Growth Factor-Binding Protein, by β-Catenin during Colon Carcinogenesis

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Abstract

Fibroblast growth factor-binding protein (FGF-BP) releases immobilized FGFs from the extracellular matrix and can function as an angiogenic switch molecule in cancer. Here we show that FGF-BP is up-regulated in early dysplastic lesions of the human colon that are typically associated with a loss of adenomatous polyposis coli and up-regulation of β-catenin. In addition, FGF-BP expression is induced in dysplastic lesions in ApcMin/+ mice in parallel with the up-regulation of β-catenin. Also, in cell culture studies FGF-BP is induced by β-catenin through direct activation of the FGF-BP gene promoter. We conclude that FGF-BP is a target gene of β-catenin.

Introduction

We have shown previously that the secreted binding protein FGF-BP can act as a chaperone for locally stored FGFs and enhance their angiogenic activity, thus allowing FGF-BP to serve as an angiogenic switch molecule in cancer (1). Consistent with a role for FGF-BP in cancer, ribozyme-targeted depletion of FGF-BP from human colon cancer or squamous cell carcinoma cells showed a rate-limiting role for FGF-BP in tumor growth and angiogenesis (1). Hence, we proposed that this molecule is one of the “angiogenic switch” mechanisms required for malignant progression (1–3).

We found that FGF-BP is expressed at high levels in the murine gut during embryonic development, down-regulated in the adult (4, 5), but expressed at high levels in some colon cancer tissues and cell lines (1). To evaluate regulation of FGF-BP during colon carcinogenesis we initiated a series of studies with normal and pathological colon biopsies to determine at what stage of transformation the gene is up-regulated. Here we report that FGF-BP expression is highly up-regulated in dysplastic lesions, i.e., early on during colon carcinogenesis. These early lesions are associated with mutations in β-catenin, and/or a loss of function of the APC tumor suppressor gene has been identified in >80% of sporadic colon carcinomas (6). To assess the possible contribution of the loss of APC to FGF-BP up-regulation, we used a well-defined murine model, the B6 ApcMin/+ mouse, which carries one mutant APC allele and develops polyposis on loss of the residual wild-type APC allele (7, 8). In this model we found that FGF-BP and β-catenin expression was induced in polyposis, as well as in a rare ACF, the earliest discernible stages of transformation (9). Furthermore, cell culture studies show that increases in endogenous β-catenin by treatment with LiCl result in a significant increase in FGF-BP mRNA levels, and cotransfection assays demonstrate transcriptional activation of the FGF-BP gene promoter by β-catenin through T-cell factor (TCF) sites. We conclude that FGF-BP is a novel target gene of the Wnt/β-catenin pathway.

Materials and Methods

Tissue Samples, Immunohistochemistry, and in Situ Hybridization. Paraffin-embedded archival tissues were provided by the tumor tissue core facility of the Lombardi Cancer Center with patient identifiers removed. Dr. Moser (University of Wisconsin) and Drs. Herfarth and Schoelmerich (University of Regensburg) provided samples from mouse models. H&E stains of archival tissues were reviewed by a pathologist to verify the diagnosis. The categorization followed Dukes’ classification (10). Serial sections of 4 μm were used for FGF-BP protein staining or FGF-BP mRNA detection by in situ hybridization. The in situ hybridization protocol was described earlier using human and mouse FGF-BP riboprobes (4, 11) that were digoxigenin-labeled using the DIG RNA labeling mix (Roche). For the immunohistochemistry a rabbit polyclonal anti-FGF-BP antibody (diluted 1:150 in 2% BSA/PBS) was used. As described earlier, this antibody recognizes murine, rat, and human FGF-BP in archival tissue sections (5, 11). A β-catenin monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Stained cells were divided into three grade levels: grade 0, negative (absence of color); grade 1, moderately stained with an obvious brown color; and grade 2, vividly stained dark brown. A tissue section was considered as negative when <30% of the same morphological structure (normal mucosa, dysplasia, or cancer) showed any color (grade 0; Ref. 12). All of the others were scored as positive in the analysis. In a subset of samples angiogenesis was assessed after CD31 staining using light microscopy at ×200 in areas containing the highest number of capillaries or hot spots as described earlier (13).

Cell Culture, Transfections, and Reporter Assays. The cell lines CaCo-2 (colon cancer), SKBr3, and MDA-MB468 (breast cancer) were from the American Type Culture Collection (Springfield, VA). The HCT-116 cell line with somatic cell knockout of the activated β-catenin allele was provided by Dr. Todd Waldman (Georgetown University; Ref. 14). The cells were maintained in DMEM with 10% FBS at 37°C and 5% CO2. Twenty-four h before transfection, cells were seeded in 12-well plates at a density of 1 × 105 cells/well in DMEM +10% FBS. With CaCo-2 cells, for each well 0.75 μg of DNA constructs and 10 μl of LipofectAMINE reagent were combined with 200 μl of OPTI-MEM1 (Life Technologies, Inc.) and incubated for 30 min at room temperature. Appropriate amounts of OPTI-MEM1 were added to the solution to bring the volume to 1 ml, and the mixture was placed on cells for 3 h at 37°C. The cells were then washed twice with Iscove’s Modified Medium (IMEM, Life Technologies, Inc.) and incubated in DMEM +10% FBS for 18 h. For SKBr3 cells, DNA constructs were mixed in a 1:2 ratio with FuGENE (Roche) reagent in serum-free IMEM and incubated at room temperature for 30 min. The FuGENE/DNA solution was then added to the cells with DMEM +10% FBS medium and incubated at 37°C for 18 h. Transfection efficiency was determined by cotransfection with 4.0 ng of the Renilla luciferase reporter vector pRL-CMV (Promega). After the 18-h incubation, cells were lysed in 100 μl of Passive Lysis Buffer (Promega). Ten μl of the cell extract was assayed for firefly and Renilla luciferase activity with the Dual-Luciferase Reporter assay system (Promega). FGF-BP promoter constructs (−1060/+62, −118/+62, −93/+62, and −77/+62) were cloned into the pGL3 vector as described previously (15). Two consensus TCF sites (S-A/T77/ S-A/T62) are present in the −1060/+62 fragment, and one is present in the −118/+62 fragment. The −93/+62 and −77/+62 fragments lack these sites. Transfections were performed using FuGENE (Roche) reagent in serum-free IMEM and incubated at room temperature for 30 min. The FuGENE/DNA solution was then added to the cells and incubated for 18 h. Cell lysates were assayed for Renilla and luciferase activity using the Dual-Luciferase Reporter System (Promega) as above.

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The abbreviations used are: FGF-BP, fibroblast growth factor binding protein; APC, adenomatous polyposis coli; FBS, fetal bovine serum; ACF, aberrant crypt focus; VEGF, vascular endothelial growth factor; TPA, 12-0-tetradecanoylphorbol-13-acetate.
A/T CAAAG-3') located at −1030 and at −545 were deleted by PCR-based mutagenesis using the following primers: forward primers 1030-del-F: 5'-TACTG CAATG TCT GGT TAA ACA GAC CC-3' and 545-del-F: 5'-CATG CACCC ATT CAT TTA TTG AGA GTG G-3'; reverse primers 1030-del-R: 5'-GGG TCT TAA GTT GTA TAA ACA GAC ATT TG-3' and 545-del-R 5-CCA CTC TCA ATA AAT GAA TGG GTG ACT G-3'. All of the constructs were sequenced to confirm mutations. SKBR3 cells were transfected with 100 ng of luciferase cDNA, 0.1 ng Renilla, and 300 ng of pCDNA3 or 300 ng pCDNA3-β-catenin using Fugene 6 transfection reagent (Roche). Experiments were typically performed in duplicate and repeated as indicated in the legends to the figures. β-Catenin, E-cadherin, and TopFlash expression constructs were described previously (16). The pCDNA3 cloning vector was purchased from Invitrogen.

Northern Analysis. MDA-MB468 cells were plated in 10-cm dishes and grown to 70% confluence in IMEM +10% FBS 24 h before treatment. The cells were treated with LiCl (30 mM) + insolit or with NaCl (30 mM) + insolit dissolved in 10 ml of IMEM +10% FBS. Sixteen h after initiation of treatment total RNA was isolated using the RNA STAT-60 protocol (RNA STAT-60, Tel-Test, Friendswood, TX). Thirty μg of total RNA were run on a 1.2% formaldehyde-agarose gel. Blotting and hybridization with the human FGF-BP probe were performed as described previously (15).

Data Analysis. The Prism/GraphPad program was used for data analysis. P < 0.05 were considered significant.

Results

FGF-BP Is Up-Regulated during the Initiation of Human Colon Carcinogenesis. We had observed previously that FGF-BP is highly expressed during murine gut development and is down-regulated in the adult mouse (4). FGF-BP is also highly expressed in some human colon cancer cell lines and tissues (1). The stages of malignant progression toward colon cancer have been well delineated, and we hypothesized that analysis of colon biopsies of different pathological stages could indicate possible genetic alterations associated with the up-regulation of FGF-BP. A survey of human colon biopsy material (Fig. 1) showed that FGF-BP expression was detectable in 12 of 76 histologically normal appearing samples. In contrast with this, a high portion of the samples with moderate to severe dysplasia expressed FGF-BP (62 of 85; \( P < 0.0001 \) normal versus dysplastic mucosal; \( x^2 \) test). Interestingly, even individual dysplastic crypts (closed arrow in Fig. 1A) located within otherwise normal mucosa (open arrow in Fig. 1) show expression of FGF-BP. It is of note that the increased expression of FGF-BP in dysplastic lesions coincides with a significant increase in blood vessel density from 80 ± 7 to 154 ± 9 vessels/field as measured by CD31 staining in the lamina propria (normal mucosa versus severe dysplasia; \( P < 0.001, t \) test).

To assess whether FGF-BP would be up-regulated by all of the pathological alterations in the gut, we studied a series of biopsy samples from different stages of inflammatory bowel disease. Only 3 of 26 samples from patients with ulcerative colitis (n = 18) or Crohn’s disease without apparent dysplasia (n = 8) showed detectable FGF-BP expression. This frequency of expression was not different from the normal mucosa controls (\( P > 0.05, x^2 \)). Because inflammatory bowel disease does not induce FGF-BP expression, we hypothesized that the up-regulation of FGF-BP at the onset of colon epithelial malignant transformation may be due to an early genetic event, such as loss of the APC tumor suppressor function associated with the initiation of dysplasia. To address this question, we used the APC heterozygous B6 ApcMin/+ mouse (17) as an animal model system.

FGF-BP Expression in B6 ApcMin/+ Mouse Adenoma Coincides with Cellular Relocation and Increase in β-Catenin Protein. As a first step, we compared FGF-BP expression in the intestines of wild-type C57BL/6J mice relative to that in B6 ApcMin/+ mice. We found no differences in baseline expression of FGF-BP (data not shown). This suggests that the loss of function of one allele in the B6 ApcMin/+ mice is not sufficient to alter the signal toward FGF-BP expression. In the normal epithelium β-catenin is sequestered at the membrane and is rarely found in the cytoplasm or nucleus (Ref. 18; see Fig. 2J). When APC becomes defective in intestinal crypt cells of B6 ApcMin/+ mice, regulation of β-catenin is lost (17), and the epithelium progresses to early stages of malignancy. We used the accumulation of cytoplasmic and nuclear β-catenin in microscopic sections as a read-out for the loss of APC function, and probed serial sections of normal and adenoma tissues for both FGF-BP and β-catenin expression.

Tissues were harvested from animals between 91 and 132 days of age, and analyzed for β-catenin protein by immunohistochemistry and for FGF-BP mRNA by in situ hybridization. Dysplastic lesions within normal mucosal tissue (Fig. 2, A and E, darker H&E staining) showed an elevation of the β-catenin protein (Fig. 2, B and F, brown stain) and of FGF-BP mRNA (Fig. 2, C and G, dark blue stain). Fig. 2, D and H, show negative controls for FGF-BP detection. In a survey of tissues we found that 21 of 27 adenomas were strongly positive for FGF-BP (>30% of the adenoma surface area; Fig. 2, C and G). Adjacent normal intestine was also examined for FGF-BP expression, and only 5 of 19 samples showed any expression of FGF-BP (\( P < 0.001 \), normal versus adenomas, \( x^2 \), Fig. 2K). On careful inspection of serial sections, we found a striking coincidence of expression of FGF-BP mRNA (Fig. 2, C and G) and of β-catenin protein (Fig. 2, B and F) in
the same tissue areas. We also searched tissues for the earliest microscopically discernible stage of dysplasia, ACF, that is observed at a rate of 0.14 per mouse (19). Although we observed only a single ACF, there was coincidence of expression of the two genes in cells that formed this ACF in the midst of normal mucosal areas (Fig. 2, I and J).

In addition to the tissues derived from the ApcMin/+ model, we also examined sections from intestinal polyps in a dextran sulfate-induced model of inflammatory colon disease (20). No increase in FGF-BP expression was observed in this model (data not shown). This finding corroborates the lack of expression of FGF-BP in human clinical inflammatory bowel disease of different stages (see above). We concluded from these studies that FGF-BP expression is induced during the initiation of malignancy, and we hypothesized that this could occur as a result of the activation of the Wnt/β-catenin pathway.

**Lithium Induces Endogenous FGF-BP mRNA Expression.** To determine whether β-catenin is directly involved in the regulation of the FGF-BP gene, we examined whether lithium-induced β-catenin stabilization affects the levels of endogenous FGF-BP mRNA. Lithium inhibits glycogen synthase kinase-3β, a negative regulator of β-catenin (21). For the experiments we used MDA-MB468 breast cancer cells because they express detectable FGF-BP and show intact β-catenin regulation. The MDA-MB468 cells were treated for 16 h with LiCl and inositol, which prevents inositol 1,4,5-triphosphate depletion by LiCl (21). Cells treated with LiCl and inositol increased β-catenin protein levels and showed a 3-fold induction of FGF-BP mRNA as compared with control treatment (NaCl+inositol; Fig. 3). The NaCl+inositol control showed no significant effect on basal FGF-BP mRNA expression (data not shown). Thus, increasing the level of free β-catenin coincides with induction of endogenous FGF-BP mRNA, lending additional support to the notion of β-catenin as a regulator of FGF-BP expression.

**β-Catenin Regulates FGF-BP Promoter Activity.** To investigate whether the FGF-BP gene is a transcriptionally regulated target of β-catenin, we cotransfected a wild-type β-catenin expression vector with an FGF-BP expression vector containing the −1060/+62 fragment of the FGF-BP promoter upstream of a luciferase reporter. This 1060-bp FGF-BP promoter fragment contains numerous consensus transcription factor-binding sites that are necessary for the transcriptional activity of the promoter (15). In the CaCo-2 colon cancer cell line, which harbors an APC mutation and expresses endogenous FGF-BP, we found that β-catenin expression induces FGF-BP promoter activity up to 3-fold over basal levels (Fig. 4A). This increase in promoter activity in CaCo-2 cells is comparable with β-catenin induction of the TopFlash promoter, a known β-catenin sensitive promoter containing multimerized TCF sites (data not shown).

Because CaCo-2 cells harbor an APC mutation that results in high levels of endogenous β-catenin, we chose the SKBR3 breast cancer cell line as a model for a next series of studies, because these cells have no known mutations in the APC/β-catenin signaling pathway and express low basal levels of β-catenin. Thus, these cells provide a more sensitive system to observe the effects of β-catenin overexpression and dissect the pathway of induction. In these cells, the FGF-BP promoter activity is induced by expression of exogenous β-catenin (Fig. 4B). To determine whether this induction is a β-catenin-specific effect, we cotransfected E-cadherin, an adhesion molecule that binds to the internal armadillo repeats of β-catenin and functions as a dominant-negative regulator of β-catenin by preventing its translocation to the nucleus. Cotransfection with E-cadherin reversed β-catenin induction of the FGF-BP promoter (Fig. 4B). We also found that FGF-BP promoter activity of the full-length promoter −1060/+62 was reduced in isogenic HCT-116 colon cancer cells with somatic cell
knockout of the activated β-catenin allele (14) as compared with promoter activity in wild-type HCT-116 cells (Fig. 4D). Taken together with the results of the β-catenin effects on endogenous levels of FGF-BP mRNA, these data indicate that β-catenin is a transcriptional regulator of the FGF-BP gene.

**β-Catenin Regulatory Region in the FGF-BP Promoter.** To identify the regions necessary for regulation of the FGF-BP promoter by β-catenin, we transfected SKBR3 cells with 5′ deletion constructs of the FGF-BP promoter/reporter constructs (15). β-Catenin had a minimal background effect (<2-fold) on luciferase activity of the pGL3-basic empty vector (Fig. 4B), similar to nonspecific background effects that we observed previously with this vector (15). Deletion from −1060 to −118 reduced the β-catenin induced promoter activity by >70%. An additional deletion to −93 had no effect on the induction of the promoter by β-catenin, but deletion to −77 negated all of the β-catenin induction of the promoter to background levels of the pG33 vector (Fig. 4B). The experiments with the FGF-BP promoter/reporter constructs in the HCT-116 knockout cells, which have their activated β-catenin allele deleted (14), showed a significant reduction of constitutive promoter activity of the full-length construct. Constitutive activity of the −118/+62 construct, however, was not altered by the deletion of the activated β-catenin allele (Fig. 4D). This finding complements the different indutability of the activity of these constructs by transfection of exogenous β-catenin in the SKBR3 cells (see Fig. 4B).

We found that the FGF-BP promoter contains two TCF consensus binding sites at −545 and −1030, and deleted these sites by PCR. Interestingly, only deletion of the distal TCF site at −1030 reduced β-catenin induction of promoter activity, whereas deletion of the proximal site at −545 had no significant effect (Fig. 4C). We conclude from these results that β-catenin induction of FGF-BP promoter activity involves regulatory regions in the distal promoter.

**Discussion**

In this study, we demonstrate that FGF-BP expression is up-regulated during early stages of human colon epithelial malignant progression, *i.e.*, during the earliest dysplastic stages associated with a loss of the APC tumor suppressor gene, and is induced in the intestinal adenomas of the B6 ApcMin/+ mouse. We show that FGF-BP expression coincides with the expression of β-catenin in early lesions, *i.e.*, adenomas and already an ACF in the B6 ApcMin/+ mouse, suggesting that FGF-BP lies downstream of the β-catenin signaling cascade. Finally, the studies in cultured cells show that β-catenin activates transcription from the FGF-BP promoter, thus providing evidence that this gene is a target of β-catenin.

Our previous analysis of FGF-BP expression in the developing mouse gut had shown that epithelial cells positioned at the bottom of the crypts express FGF-BP and that this expression is lost in cells maturing along the crypt/villus axis (4). More recently, positioning of epithelial cells along the crypt/villus axis and imposition of a crypt precursor phenotype was found associated with a gradient of β-catenin/TCF activity that shows its maximum at the bottom of the crypts and is reduced as cells differentiate during their migration up the crypt (22, 23). It is likely that the FGF-BP expression that we observed in histologically normal tissues represents staining of sections of the lower third of crypts and, hence, the region with high β-catenin activity. Also, FGF-BP expression may indicate a very early stage in the transition to dysplasia that is not yet manifest from the H&E staining. Fig. 1, A and B, shows such an example of dysplastic lesions with surrounding normal mucosa. Interestingly, the histologically normal crypts that do not express FGF-BP show some staining for the protein in sections that transverse the bottom of the crypt as indicated by the narrow opening of the crypt (compare the two crypts indicated by open arrows in Fig. 1A). With respect to distinct pathological alterations, the lack of FGF-BP expression in inflammatory human bowel disease and in the rodent animal model equivalent (20) suggest that inflammatory pathways in the colon do not lead to an up-regulation of FGF-BP either on their own or through cross-talk with the β-catenin signaling.

FGF-BP is an activator of growth factors in the FGF-family, and our studies lend support to the idea that the FGF family plays a role in the development of the early angiogenic phenotype in colon cancer. The induction of the angiogenic phenotype in colon cancer is a multifaceted process requiring the cooperation of numerous factors during the different steps of malignant progression. In a study of levels of FGF-2 and VEGF in serum samples from colon cancer patients, it was suggested that FGF-2 may act as an early inducer of the angiogenic phenotype (24). FGF-BP up-regulation in intestinal adenomas may indeed trigger this by providing the chaperone that can release the immobilized FGF-2. In support of this notion, we found increased angiogenesis coincident with FGF-BP expression in human colon dysplastic lesions. Other factors, such as VEGF, probably cooperate with FGF-2 to maintain the process of angiogenesis throughout the stages of tumor formation. VEGF expression is found in adenomas of the colon; however, unlike our findings with FGF-BP, increased VEGF expression levels are correlated with later stages of the disease, and VEGF expression is increased in carcinomas as compared with adenomas as well as in metastatic versus nonmetastatic colon cancer (25).

Not only does FGF-BP appear to be a novel proangiogenic target of β-catenin that is up-regulated at an early stage of premalignant lesions, it seems that its regulation is through areas of the FGF-BP
two proteins were also found overexpressed in intestinal adenomas of the 1-kb promoter region between −1060 and −118 in the β-catenin regulation of FGF-BP. This also indicates that β-catenin is not activating the promoter via indirect activation of the mitogen-activated protein kinase signaling pathways. Examination of the 1-kb promoter region between −1060 and −118 for possible transcription factor recognition sites using Transfac analysis revealed two potential TCF/lymphoid enhancer factor (LEF) sites, which are known to be involved in β-catenin regulated gene transcription. Deletion of these sites showed that only the −1030 site contributes to β-catenin-induced promoter activation, whereas the site at −545 does not. Interestingly, there is also a perfect consensus site for REL/ nuclear factor κB present in this region, which is of interest because β-catenin can interact directly with nuclear factor κB and might, thus, contribute in addition to regulation of the gene promoter (27). The second surprise of the FGF-BP gene promoter analysis was that the region between −93 and −77 was also required for full β-catenin induction of the promoter. We have demonstrated previously that this region harbors an SP1 binding site and can bind SP1 specifically. However, this site is not required for growth factor or TPA-regulated gene transcription (15). It remains to be determined whether this site acts cooperatively with upstream regulatory factors in the β-catenin induction of FGF-BP gene transcription. The Sp1 and Krueppel-like factors that bind to GC boxes are known to play a role in cell growth and tumor progression. However, their role in early events in colon carcinogenesis has not been defined.

In conclusion, β-catenin, one of the most significant oncogenic proteins in colon cancer, has been implicated in several key steps of the path to malignancy. The cell cycle regulatory genes, c-Myc and cyclin-D1, have both been identified as targets of β-catenin. These two proteins were also found overexpressed in intestinal adenomas of the B6 ApcMin/+ mouse. Furthermore, β-catenin activates matrix metalloproteinase 7, an enzyme that plays a role in invasion and metastasis. Additionally, APC and E-cadherin, two proteins that are closely tied to β-catenin function, are important for induction of apoptosis and cell-cell adhesion, respectively. Our identification of FGF-BP as a direct target of β-catenin transcriptional activation suggests that β-catenin can also play a role in promoting the switch to the angiogenic phenotype observed early in the malignant progression of colon cancer.

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References


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