Identification of BMP and Activin Membrane-bound Inhibitor (BAMBI), an Inhibitor of Transforming Growth Factor-β Signaling, as a Target of the β-Catenin Pathway in Colorectal Tumor Cells*

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The Wnt signaling pathway is activated in most human colorectal tumors. Mutational inactivation in the tumor suppressor adenomatous polyposis coli (APC), as well as activation of β-catenin, causes the accumulation of β-catenin, which in turn associates with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and activates transcription of their target genes. Here we show that β-catenin activates transcription of the BMP and activin membrane-bound inhibitor (BAMBI)/NMA gene. The expression level of BAMBI was found to be aberrantly elevated in most colorectal and hepatocellular carcinomas relative to the corresponding non-cancerous tissues. Expression of BAMBI in colorectal tumor cell lines was repressed by a dominant-negative mutant of TCF-4 or by an inhibitor of β-catenin-TCF interaction, suggesting that β-catenin is responsible for the aberrant expression of BAMBI in colorectal tumor cells. Furthermore, overexpression of BAMBI inhibited the response of tumor cells to transforming growth factor-β signaling. These results suggest that β-catenin interferes with transforming growth factor-β-mediated growth arrest by inducing the expression of BAMBI, and this may contribute to colorectal and hepatocellular tumorigenesis.

The conversion of an intestinal epithelial cell into a fully transformed, metastatic cancer cell requires mutations in multiple proto-oncogenes and tumor suppressor genes (1, 2). Mutations in the tumor suppressor adenomatous polyposis coli (APC)1 and β-catenin occur during the early stages of this process. The product of the APC gene interacts with various proteins including β-catenin, Axin, Ras-specific guanine nucleotide exchanger Asef, kinesin superfamily associated protein 3, EB1, microtubules, and the human homolog of the Drosophila Discs large (hDLG) (3–11). Through interacting with these molecules, APC functions in multiple signaling pathways, thereby regulating Wnt signaling, actin and microtubule cytoskeletal networks, and cell morphology and migration (8, 12–18).

β-catenin is an essential component of the Wnt signaling pathway and plays important roles in development and tumorigenesis (19–22). Wnt signaling promotes the stabilization and accumulation of β-catenin, which, in turn, interacts with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and activates transcription of downstream genes such as c-Myc, cyclin D1, and Axin2 (23–27). APC interacts with β-catenin, glycogen synthase kinase-3β, casein kinase 1α, and Axin or the closely related factor conductin/Axil (3–7, 28–30). By recruiting β-catenin into this multi-protein complex, APC promotes its proteasome-mediated degradation, hence mutations in APC or β-catenin result in the accumulation of β-catenin in colorectal tumor cells (31, 32). Mutations in β-catenin and Axin have also been identified in many other types of tumors, including hepatocellular carcinoma, ovarian cancer, and endometrial cancer (33–35). Thus, constitutive activation of β-catenin-TCF-mediated transcription is believed to be a critical step in the tumorigenesis of various types of tumors.

The transforming growth factor-β (TGF-β) pathway inhibits the growth of multiple epithelial cell types, and loss of this negative regulation is believed to contribute to tumor development, including colorectal tumorigenesis. Indeed, inactivating mutations in the type II receptor (TβRII), SMAD2, and SMAD4 have been reported to occur during colorectal tumorigenesis. In addition, it has been reported that the TGF-β receptor is often down-regulated in tumor cells, or otherwise unavailable at the cell surface, thus allowing tumor cells to escape the growth inhibitory activities of TGF-β (36, 37).

TGF-β signals through a heteromeric cell-surface complex of two types of transmembrane serine/threonine kinases, the type I (TβRI) and type II receptors. TβRII activates TβRI, which, in growth factor-β, TβRI/TβRII, type I/II receptors; ICAT, inhibitor of β-catenin-TCF, BAMBI, BMP and activin membrane-bound inhibitor; Ad-ICAT, adenovirus encoding ICAT; GFP, green fluorescent protein.

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sequence similarity to the extracellular domain of TβRI and inhibits the proliferation of colorectal tumor cells containing mutations in APC or β-catenin. All colorectal carcinoma cells containing mutations in Axin (42–45). In this study, we examined the effects of ICAT on gene expression in the human colorectal tumor cell line SW48, in which β-catenin-TCP4-mediated transcription is aberrantly activated because of a mutation in β-catenin. We found that ICAT suppresses expression of the BAMBI (BMP and activin membrane-bound inhibitor). Furthermore, we found that β-catenin activates transcription of BAMBI and that BAMBI expression is aberrantly elevated in most colorectal and hepatocellular carcinomas, compared with the corresponding non-cancerous tissues. The product of the BAMBI gene is a transmembrane protein that lacks an intracellular kinase domain but has sequence similarity to the extracellular domain of TβRI and thereby inhibits TGF-β signaling by forming a heterodimer with TβRII (46). Our data suggest that β-catenin-mediated overexpression of BAMBI may be one of the strategies by which tumor cells escape the growth inhibitory activities of TGF-β.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids and Recombinant Viruses**—For construction of the BAMBI-luciferase reporter plasmids, a human bacterial artificial chromosome clone RP13–43N24, which contains the entire promoter region and several exons and introns, was purchased from BACPAC Resources. The longest construct (7.1 kb) and deletion constructs were generated by cloning each sequence into the appropriate enzyme site in the pGL3-basic vector (Promega). Site-directed mutagenesis of each TCF/LEF binding element was created by standard PCR procedures. In brief, sections were deparaffinized in xylene, and then rehydrated with a graded ethanol series before antigen retrieval, performed according to standard procedures. In brief, sections were deparaffinized in xylene, and then rehydrated with a graded ethanol series before antigen retrieval, performed according to standard procedures. In brief, sections were deparaffinized in xylene, and then rehydrated with a graded ethanol series before antigen retrieval, performed according to standard procedures. In brief, sections were deparaffinized in xylene, and then rehydrated with a graded ethanol series before antigen retrieval, performed according to standard procedures.
Ad-ICAT revealed that BAMBI protein expression was also repressed by ICAT (Fig. 1B).

These results suggest that the expression of BAMBI is regulated by β-catenin. Therefore, we examined the effect of an active mutant of β-catenin, β-catenin-S33Y, on the expression of BAMBI. β-catenin-S33Y is a mutant that was initially identified in human colorectal tumors, and contains a tyrosine in place of the normal serine at residue 33 (S33Y), a change that is targeted in human colorectal tumors, and contains a tyrosine in place of the normal serine at residue 33 (S33Y), a change that renders the protein resistant to APC-mediated degradation (34). Northern blot analysis of RNA isolated from COS-1 cells infected with Ad-LacZ, Ad-ICAT, or Ad-FLAG-β-catenin-S33Y, with anti-BAMBI antibody or anti-α-tubulin antibody. Cells were infected with adenoviruses at a multiplicity of infection of 100. B, immunoblotting analysis of lysates from SW48 cells infected with Ad-LacZ, Ad-ICAT, or Ad-TCF-4-ΔN, or from COS-1 cells infected with Ad-LacZ or Ad-FLAG-β-catenin-S33Y, with anti-BAMBI antibody or anti-α-tubulin antibody. Cells were infected with adenoviruses at a multiplicity of infection of 100. C, time course of BAMBI up-regulation after Ad-FLAG-β-catenin-S33Y infection of COS-1 cells. Total RNA was isolated at the indicated times and analyzed by Northern blotting. Two different blots were hybridized with the BAMBI and G3PDH probes, with EtBr staining serving as a loading control. Lysates prepared at the indicated times were subjected to immunoblotting analysis with FLAG antibody or anti-α-tubulin antibody. Cells were infected with adenoviruses at a multiplicity of infection of 20.

Next we investigated whether transcription of BAMBI is directly regulated by β-catenin. We isolated a BlnI(−3841)–NcoI(+3866) genomic fragment encompassing the upstream and downstream regions of the transcription start site (Fig. 2A) and examined whether the BAMBI promoter is contained within this fragment. When a XhoI(−586)–NheI(+82) fragment was inserted upstream of a luciferase reporter gene in the sense and antisense orientations and transfected into COS-1 cells, the sense construct exhibited about 170-fold higher luciferase activity than the antisense construct or empty vector (Fig. 2B). Thus, the BAMBI promoter is presumably contained within this fragment. However, the activity of the reporter containing this BAMBI promoter region (Fig. 2A, −586(+82)+luc) was not enhanced by cotransfection with β-catenin-S33Y (Fig. 2A). Similarly, β-catenin-S33Y did not stimulate the activity of a reporter construct that contains the further upstream regions containing at least three consensus sites for TCF-binding (Fig. 2A, −3384(+82)+luc). Therefore, we examined the β-catenin responsiveness of the region downstream of the BAMBI promoter (SacI(+421)–SacI(+3806) fragment). We inserted this fragment upstream of the SV40 minimal promoter in a luciferase reporter vector (Fig. 2A, Int1-luc) and examined the construct for responsiveness to β-catenin. When trans-
Fig. 2. β-catenin-TCF-mediated transactivation of the BAMBI promoter. A, schematic representation of the BAMBI promoter region. O, consensus TCF-binding sites (CTTTGA/TA/T); Δ, potential TCF-binding sites, which differ from the consensus TCF-binding sites by one base; X, mutated TCF-binding sites (three nucleotides in O and two nucleotides in Δ were mutated). The nucleotide sequence of this region is available from the GenBank™/EBI Data Bank (accession no. AL390996). The transcription initiation site was adopted from the DataBase of human Transcriptional Start Sites (DBTSS; Ref. 54). The fragments indicated in the figure were inserted directly upstream of the luciferase gene in pGL3 (-3384 + 82-luc and -586 + 82-luc) or upstream of the SV40 minimal promoter in a luciferase reporter vector (Int1-luc, Int1-mut1-5-luc, and Int1-mut-All-luc), respectively. COS-1 cells were transfected with β-catenin-S33Y (0.8 μg) along with the indicated promoter construct (0.4 μg); luciferase activity was measured. pTOP-tk- and pFOP-tk-luciferase (0.4 μg) were used as positive and negative controls, respectively. Bars, standard deviation of triplicate assays. B, a fragment containing nucleotides -586 to +82 was inserted in the sense or antisense orientation into pGL3 (-586 + 82-luc(S) or -586 + 82-luc(AS), respectively). COS-1 cells were transfected with these reporter constructs, and luciferase activity was measured. C, effects of a dominant-negative mutant of TCF-4 on the activity of the BAMBI promoter in colorectal tumor cells. SW480, HCT116, and SW48 cells were transfected with a luciferase reporter plasmid (0.4 μg, pTOP-tk-luc, or Int1-luc) and increasing amounts of TCF-4-ΔC (0, 0.1, 0.4, and 1.2 μg), and luciferase activity was measured. D, left, effects of dominant-negative mutants of TCF-4 and ICAT on β-catenin-mediated activation of the BAMBI promoter. COS-1 cells were transfected with a luciferase reporter plasmid (0.3 μg, pTOP-tk-luc or Int1-luc) along with indicated plasmids (0.6 μg for β-cat S33Y, 1.2 μg for others), and luciferase activity was measured. In all of the luciferase-reporter assays in this figure, the total amount of plasmid DNA was adjusted with pcDNA3.1(−) empty plasmid. The pRL-tk Renilla luciferase reporter (0.07 μg) was co-transfected to normalize transfection efficiency. Right, upper panel, detection of exogenously expressed FLAG-tagged TCF4-ΔC, TCF4-ΔN, and ICAT. Lysates prepared from COS-1 cells transfected with the indicated plasmids were subjected to immunoblotting with anti-FLAG antibody. Right, lower panels, nuclear localization of TCF4-ΔC. COS-1 cells were transfected with FLAG-tagged TCF4-ΔC and stained with anti-FLAG antibody (left panel). The nucleus was stained with TOTO-3 (right panel).
fected into COS-1 cells, activity of this reporter was found to be enhanced significantly by \( \beta \)-catenin-S33Y (Fig. 2A). These results suggest that this region, which encompasses intron 1, is responsible for \( \beta \)-catenin-mediated transactivation. Intron 1 contains at least five consensus sites for TCF-binding, and synthetic oligonucleotides containing each of these sites demonstrated specific binding to the DNA-binding region of TCF-4, fused to glutathione \( S \)-transferase, in an electrophoretic mobility-shift assay (data not shown).

Next we examined whether transcription of BAMBI is activated by the endogenous \( \beta \)-catenin-TCF complexes using the colorectal tumor cell lines SW480, HCT116, and SW48. SW480 contains mutated APC and wild-type \( \beta \)-catenin, whereas HCT116 and SW48 possess wild-type APC and mutated \( \beta \)-catenin. The activity of Int1-luc but not \( \beta \)-catenin-S33N was repressed in a dose-dependent manner in SW480, HCT116, and SW48 cells by a dominant-negative mutant of TCF-4, TCF-4-C, which contains the amino-terminal \( \beta \)-catenin-binding domain but lacks the carboxyl-terminal DNA-binding domain (Fig. 2C). This mutant was constructed as a fusion to the nuclear localization signal of SV40 and was found to localize efficiently to the nucleus (Fig. 2D). Under these experimental conditions, TCF-\( \Delta \)C also repressed the activity of pTOP-tk-luciferase, which contains optimal TCF-binding sites upstream of a luciferase reporter gene, in these cell lines.

These results imply that BAMBI is a direct target for \( \beta \)-catenin-TCF-mediated transactivation. However, we noticed that even when all five consensus TCF-binding sites in Int1-luc were mutated, some \( \beta \)-catenin responsiveness remained (Fig. 2A, Int1-mut1-luc). In addition to these five sites, we also mutated four sites in Int1-luc, which diverge one nucleotide from the TCF-binding consensus sequence. This construct (Fig. 2A, Int1-mut-All-luc) exhibited slightly lower activity than Int1-mut-5-luc, suggesting that these sites contribute only a little to \( \beta \)-catenin-mediated transactivation. Furthermore, the \( \beta \)-catenin-mediated transactivation of Int1-luc was only weakly inhibited by TCF-4-\( \Delta \)N, whereas it was significantly inhibited by ICAT as well as TCF-4-C (Fig. 2D). The weak effect of TCF-4-\( \Delta \)N is not due to low levels of expression, because immunoblotting analysis revealed that TCF-4-\( \Delta \)N and TCF-4-C were expressed at similar levels in these experiments. On the other hand, TCF-4-\( \Delta \)N as well as TCF-4-\( \Delta \)C and ICAT exhibited significant inhibitory effects on the \( \beta \)-catenin-mediated transactivation of pTOP-tk-luciferase. TCF-4-\( \Delta \)N differs from TCF-4-C in that the former blocks TCF-binding to DNA, whereas TCF-4-\( \Delta \)C is able to inhibit the interaction of a number of proteins, including \( \beta \)-catenin. These results suggest that BAMBI may be transcriptionally regulated not only by \( \beta \)-catenin-TCF complexes, but also by \( \beta \)-catenin complexed to other proteins. This finding is consistent with recent reports showing...
that complexes of β-catenin with partners other than TCF can also transactivate target genes (47–50).

Northern blotting analysis revealed that BAMBI is expressed at very low levels in human colon, small intestine, thymus, and peripheral bloods, at moderate levels in brain, skeletal muscle, liver, and lung, and at high levels in heart, spleen, kidney, and placenta (data not shown). Because β-catenin accumulates in most human colorectal tumors due to mutations in APC or β-catenin, we expected BAMBI expression to be up-regulated in colorectal tumors. We examined BAMBI expression in 18 paired colorectal tumors and adjacent non-cancerous tissues by semi-quantitative RT-PCR analysis. In 13 of 18 (72%) cases, the expression level of BAMBI was very high in colorectal tumors but was very low in the corresponding non-cancerous tissues (Fig. 3A). In the other five cases, BAMBI expression was almost equal to or lower than that of the non-cancerous tissues. Immunostaining experiments also demonstrated that BAMBI is expressed at high levels in colorectal tumors but at very low levels in non-cancerous tissues (Fig. 3B). Furthermore, we found that BAMBI expression is higher in hepatocellular carcinomas than in adjacent normal tissues in three of five cases, consistent with the fact that AXIN1 as well as β-catenin is mutated in a certain subset of hepatocellular carcinomas (35, 51). Consistent with the fact that expression of AXIN2 is activated by β-catenin and up-regulated in colorectal tumors (25, 27), we observed that the expression patterns of BAMBI and AXIN2 were similar in most tumors examined.

Because BAMBI has been shown to act as an antagonist of TGF-β signaling (46, 52), we examined the effect of overexpression of BAMBI on the growth of tumor cells in the presence of TGF-β. In these experiments, we used the prostate tumor cell line DU145, because it responds to TGF-β and does not exhibit aberrant β-catenin-mediated transactivation (data not shown), suggesting that it does not possess mutations in APC, β-catenin, or AXIN. We transfected BAMBI into DU145 cells and performed a colony formation assay in the presence or absence of TGF-β. TGF-β strongly inhibited colony formation among DU145 cells that were transfected with GFP or mutant BAMBI-GFP lacking the amino-terminal extracellular domain (BAMBLAN-GFP). In contrast, BAMBI-GFP-transfected cells were resistant to the growth inhibitory effects of TGF-β (Fig. 4A). Immunoblotting analysis of BAMBI-GFP-transfected cells propagated from TGF-β-resistant colonies revealed that BAMBI-GFP is indeed expressed in these cells (Fig. 4B). We also examined the effect of BAMBI-GFP on TGF-β-mediated transactivation in DU145 cells using the reporter p3TP-lux, which contains a TGF-β-responsive element from the PAI-1 promoter placed upstream of a luciferase reporter gene. Luciferase assay revealed that TGF-β-mediated transactivation is repressed by exogenously expressed BAMBI-GFP (Fig. 4C). These results imply that BAMBI has the potential to inhibit TGF-β-mediated transactivation and cell growth inhibition.

Loss of TGF-β responsiveness provides an advantage for developing tumors (36, 37). Most colorectal tumors possess mutations in a component of the TGF-β signaling pathway such as TβRII, SMAD2, or SMAD4. Our results show that, in addition, an inhibitor of TGF-β signaling, BAMBI, is induced by β-catenin and overexpressed in colorectal and hepatocellular carcinoma cells. In the multistep progression into cancer, mutations in the Wnt signaling pathway generally precede those in other tumor suppressor genes and oncogenes (1, 20). Therefore, it is interesting to speculate that overexpression of BAMBI may allow adenomas and tumor cells to escape the growth inhibitory activities of TGF-β until a component of TGF-β signaling is mutated. BAMBI also interferes with signaling pathways other than that mediated by the SMADs; thus, its overexpression may be more advantageous to oncogenic transformation than mutation of the SMADs alone. It is also possible that β-catenin-induced BAMBI expression plays a role in embryogenesis. These possibilities could be examined using Bambi knockout mice, as well as Apc and Bambi double-knockout mice. Furthermore, our findings suggest that BAMBI may have the potential as a target for therapy using monoclonal antibodies (53). Development of monoclonal antibodies to BAMBI as anti-tumor reagents is currently under way in our laboratory.

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