The PDZ Protein Tax-interacting Protein-1 Inhibits β-Catenin Transcriptional Activity and Growth of Colorectal Cancer Cells*

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Wnt signaling is essential during development while deregulation of this pathway frequently leads to the formation of various tumors including colorectal carcinomas. A key component of the pathway is β-catenin that, in association with TCF-4, directly regulates the expression of Wnt-responsive genes. To identify novel binding partners of β-catenin that may control its transcriptional activity, we performed a mammalian two-hybrid screen and isolated the Tax-interacting protein (TIP-1). The in vitro complex formation between β-catenin and TIP-1 was verified by coimmunoprecipitation, and a direct physical association was revealed by glutathione S-transferase pull-down experiments in vitro. By using a panel of deletion mutants of both proteins, we demonstrate that the interaction is mediated by the PDZ (PSD-95/DLG/ZO-1 homology) domain of TIP-1 and requires primarily the last four amino acids of β-catenin. TIP-1 overexpression resulted in a dose-dependent decrease in the transcriptional activity of β-catenin when tested on the TOP/FOPFLASH reporter system. Conversely, siRNA-mediated knock-down of endogenous TIP-1 slightly increased endogenous β-catenin transcriptional function. Moreover, we show that overexpression of TIP-1 reduced the proliferation and anchorage-independent growth of colorectal cancer cells. These data suggest that TIP-1 may represent a novel regulatory element in the Wnt/β-catenin signaling pathway.

β-catenin for ubiquitin-mediated proteasomal degradation. Stabilized β-catenin enters the nucleus where it binds members of the TCF family of transcription factors, thereby activating the expression of Wnt-target genes including c-myc, PPARγ, matrilslyn, and cyclin D1.

Periodical renewal of the colorectal mucosa is strictly governed by the progressive decrease of Wnt signals as cells move from the bottom of the crypt to the surface of the lumen (4). Mutations that inactivate APC or render β-catenin immune to phosphorylation by GSK3β cause signal-independent accumulation of β-catenin (5). This results in the formation of adenomatous polyps, which may acquire additional genetic changes and transit into fully metastatic carcinomas (4). Therefore, the control of levels, localization, and transcriptional activity of β-catenin provides a potential intervention point in the treatment of colorectal cancer. β-Catenin is also a structural component of mammalian cells, linking the adhesion molecule cadherin to cytoskeletal actin filaments (6). During epithelial-mesenchymal transition, invasive tumor cells show profound alterations in their cytoskeletal structure and adhesion properties (7). Recent observations suggest that the equilibrium between cytoplasmic and membrane-associated pools of β-catenin is regulated by APC, a lack of which may result in changes of cell adhesion and migration during colorectal carcinogenesis (8).

PDZ1 (PSD-95/DLG/ZO-1 homology) domains are small protein-protein recognition modules that bind well defined C-terminal residues in a sequence-specific manner (9). Several PDZ domain-containing proteins localize to sites of cell-cell contacts and are involved in the assembly of diverse signaling complexes (10). An increasing number of PDZ proteins have been shown to shuttle between these signaling complexes and the nucleus, thus exerting transcription regulatory functions (11–13). β-Catenin is target of at least two PDZ proteins, LIN-7 (14) and MAGI-1 (15).

Here we provide evidence that the PDZ protein TIP-1 binds β-catenin and inhibits its transcriptional activity. We show that overexpression of TIP-1 results in reduced proliferative capacity of colorectal cancer cells, suggesting an important role for this protein in the control of Wnt/β-catenin signaling.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Constructs, and Transfections—Cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum. Transfections were performed using FuGENE 6 according to the manufacturer’s instructions (Roche Applied Science). Expression plasmids were subcloned into the expression vector pCMV-Tag2b (Stratagene) or pCMV-Tag2a (Clontech), which contain an upstream cytomegalovirus (CMV) promoter and a downstream polyadenylation signal. The expression vectors were transfected into cells using LipofectAMINE 2000 (Invitrogen). Cell morphology was analyzed using light microscopy.

Cell viability was determined by trypan blue exclusion and flow cytometry. Annexin V binding and FLICA staining were performed as described (16). Western blot analyses were performed as described (17). Immunoprecipitation was performed using protein G-Sepharose 4B and rabbit polyclonal antibodies against β-catenin (BD Biosciences) or anti-human PDZ domains (9M2). Immunoprecipitates were subjected to SDS-PAGE followed by western blot analysis. The expression levels of the transfected proteins were determined by densitometric analysis using Image J software (National Institutes of Health). The results were analyzed using Student’s t test.
with 10% fetal bovine serum, penicillin, and streptomycin in a humidified incubator with 5% CO₂ at 37°C.

The protein coding sequence of murine TIP-1 was subcloned into the following expression vectors: pCMV-Myc, pEGFP-C1 (Clontech), pGEX4T-1 (Amersham Biosciences), and pcDNA3 (Invitrogen) in fusion with an N-terminal Myc tag. The coding sequence of murine β-catenin was subcloned into pCMV-HA (Clontech) and pcDNA3. β-Catenin (-1–777) was generated by PCR and subcloned into pcDNA3. The various deletion mutants of β-catenin and TIP-1 used for interaction mapping were generated by PCR. The coding sequence of human T-cell lymphotropic virus-1 Tax (kindly provided by Dr. M. Giacca) and mouse rhotekin (amino acids 38–551) was subcloned in pcDNA3. TOFLASH and FOPFLASH reporters contain wild type (WT) and mutant TCF-4 consensus binding sites, respectively, and were described previously (16). pG5Luc contains five tandem repeats of Gal4 binding sites and was purchased from Promega. Transfections were performed with LipofectAMINE 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science) reagents according to the manufacturers’ recommendations.

**Antibodies, Immunofluorescence Analysis, and Western Blotting—** Anti-TIP-1 polyclonal antisera were obtained from rabbits immunized with GST-TIP-1. The following monoclonal antibodies were used: anti-Myc (9E10, Santa Cruz Biotechnology; 9B11, Signal Transduction Laboratories), anti-HA (12CA5, Roche Applied Science), and anti-β-catenin (Signal Transduction Laboratories). Polyclonal antibodies specific for actin were purchased from Sigma. Fluorescein isothiocyanate- and rhodamine isothiocyanate-conjugated secondary antibodies for immunofluorescence and horseradish peroxidase-conjugated secondary antibodies for Western blotting were purchased from Sigma. Indirect immunofluorescence analysis was performed as described previously (17), and images were taken with a Leica DMLB microscope and a Photometrics Coolscan camera. Western blotting was performed according to standard protocols, and bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

**Immunoprecipitation and GST Pull-down Assay—** Immunoprecipitation was carried out as described previously (17, 18). Purification of GST and GST-TIP-1, in vitro translation (TNT kit, Promega), and pull-down assays were performed as described previously (17).

**Mammalian Two-hybrid and Reporter Assays—** The applied two-hybrid method has been published previously (19). For TOP/FOPFLASH reporter assays, cells were transfected in 24-well plates at 2 × 10⁵ cells/well/dish with 100 ng of luciferase reporter was cotransfected with expression vectors as indicated in each experiment for a total of 250 ng of DNA/well. pRL-CMV (Promega) was cotransfected (5–10 ng/well) to normalize samples for transfection efficiency (Dual luciferase kit, Promega). Cells were harvested 24 h later, and luciferase activity was determined with a Turner Designs luminometer.

**RNA Interference—** Small interfering RNA duplexes (siTIP-1, 5′-AAGCUCCGUCAAGAGUAC-3′ and siControl (20)) were purchased from Ambion. Cells grown on 24-well plates were transfected with siRNAs using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s suggestions. 24 h later, cells were transfected with the indicated reporter constructs using FuGENE 6 reagent and luciferase activity was measured 1 day later.

**Colony Formation and Soft Agar Assays—** Subconfluent cells grown on 6-mm dishes were transfected with 1 μg of pcDNA3 or pcDNA3/Myc-TIP-1. 24 h later, cells were replated on three 10-cm dishes in the presence of 1 mg/ml G418 (Sigma). After 2 weeks of selection, surviving colonies were fixed with 3% paraformaldehyde stained with Giemsa and counted.

**Cell Lines with Stable Expression of Myc-TIP-1 Were Obtained as Described (21)—** For anchorage-independent growth assays, 5 × 10⁴ cells were plated in 2 ml of soft agar (0.5% agarose in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics) on the surface of 4 ml of hard agar (1%) in 6-cm dishes. After 3 weeks, large colonies were counted.

**RESULTS**

**β-Catenin Interacts with TIP-1—** In search for new interacting partners of β-catenin, we employed a modified mammalian two-hybrid (M2H) assay described recently (19). β-Catenin in fusion with the VP16 transactivation domain was expressed in CHO-K1 cells together with ~15,000 mouse full-length cDNAs fused in-frame with the DNA binding domain of Gal4, and interaction was monitored with the pG5Luc reporter. Several known binding partners of β-catenin were identified among the expressed BD fusion proteins including TCF-4, APC, GSK3β, cadherin, and inhibitor of β-catenin and TCF-4 (ICAT) (data not shown). In addition to these molecules, we screened out the murine homologue of the PDZ domain protein, TIP-1, as a novel binding partner of β-catenin (Fig. 1A).

TIP-1 has been first identified in a yeast two-hybrid screen as a Tax-interacting protein (22), and recent work has shown that it can also bind glutaminase L and rhotekin (23, 24). Human and mouse TIP-1 are highly similar small proteins (14 kDa) that seem to be involved in the Rho signaling pathway (24).

To verify the interaction between β-catenin and TIP-1, coimmunoprecipitation experiments were performed on lysates from 293 cells expressing Myc-tagged TIP-1 and HA-tagged β-catenin. As shown in Fig. 1B, β-catenin was readily observed in the anti-Myc immunocomplex. Similar results were obtained by using U2OS cells (data not shown).

β-Catenin is a component of the adherens junctions but exerts its transcriptional activity in the nucleus, whereas TIP-1 has been shown to be a cytoplasmic protein (24). To correlate the interaction data with subcellular localization, we performed immunofluorescence analysis of ectopically expressed proteins in U2OS cells. As expected, HA-β-catenin showed mainly nuclear localization (Fig. 1C), whereas Myc-TIP-1 localized both to the cytoplasm and to the nucleus (Fig. 1C). The subcellular localization of overexpressed TIP-1 was confirmed with two different monoclonal antibodies against Myc tag and with a rabbit polyclonal antiserum against TIP-1. Similar results were obtained by using 293, NIH3T3, and HCT-116 cells or when TIP-1 was expressed in fusion with the green fluorescent protein (data not shown). These results suggest that β-catenin and TIP-1 interact in vivo and that complex formation probably takes place in the nucleus.

To test whether the association between TIP-1 and β-catenin is direct, we performed pull-down assays with purified GST-TIP-1 in the presence of in vitro translated β-catenin. As positive controls, we used in vitro translated human T-cell lymphotropic virus-1 Tax and rhotekin, two previously described binding partners of TIP-1 (22, 24). As shown in Fig. 1D, a strong and specific interaction was detected between TIP-1 and β-catenin, suggesting that β-catenin physically associates with TIP-1.

**TIP-1 Binds β-Catenin via Its PDZ Domain—** TIP-1 belongs to the class I of PDZ proteins that are known to recognize the X(S/T)(V/I/L)-COOH motif in their targets C terminus (25), a sequence that is also possessed by β-catenin (DTDL-COOH). In fact, two class I PDZ proteins, MAGI-1 and LIN-7, have been shown to interact with β-catenin through these residues (14, 15).

First, we wanted to confirm that the association with β-catenin is mediated by the PDZ domain of TIP-1. The single PDZ domain of TIP-1 is located centrally and flanked by short N- and C-terminal sequences (22, 23). Various deletion mutants of TIP-1 were tested in an in vivo interaction assay with coexpressed β-catenin, and the obtained results clearly demonstrated the requirement of the intact PDZ domain for complex formation (Fig. 2A). Interestingly, although the removal of the first 12 amino acids of TIP-1 had no significant effect on the interaction with β-catenin, deletion of the last eight residues resulted in a 3-fold reduction of binding efficiency, suggesting a possible role for this protein region in the formation or stability of the complex.

We next wanted to dissect the target sequence in β-catenin that is required for the interaction with TIP-1. For this purpose, we performed in vitro pull-down assays with GST-TIP-1 by using either the WT or a mutant form of β-catenin that lacks
the PDZ-recognition motif from its C terminus (β-catenin-(1–777)). As reported in Fig. 2B, the readily observed interaction between β-catenin and TIP-1 was almost completely abolished by the removal of the DTDL sequence from the C terminus of β-catenin. To our surprise, however, β-catenin-(1–777) was able to bind TIP-1 in vivo although significantly weaker than the full-length protein (Fig. 2C). This result suggests the existence of either an in vitro non-accessible second binding site or, alternatively, a protein that may act as a molecular bridge to mediate the interaction between TIP-1 and β-catenin.

To clarify this point, we repeated the in vivo interaction assay with several deletion mutants of β-catenin and mapped the region spanning between amino acids 173 and 483 responsible for the reduced complex formation with TIP-1 in the absence of the C-terminal DTDL sequence (Fig. 2D). Although it is known that PDZ proteins can recognize internal sequences as well (26), the size and the three-dimensional structure of the identified segment makes the presence of such a motif rather unlikely. On the other hand, this region comprises Armadillo repeats 2–8 that is the binding surface for several known β-catenin-interacting proteins (27). Taken together, these results clearly demonstrate a direct physical association between the extreme C terminus of β-catenin and the PDZ domain of TIP-1 and reveal an alternative mechanism for complex formation in vivo.

TIP-1 Inhibits the Transcriptional Activity of β-Catenin—Because β-catenin contains several transactivating elements in its C terminus (28), we wished to analyze the biological significance of the interaction by measuring the transcriptional activity of β-catenin upon coexpression of TIP-1. As a first approach, β-catenin-(683–781) in fusion with the DNA binding domain of Gal4 was coexpressed with increasing amounts of Myc-TIP-1 in CHO-K1 cells and transcriptional activity was measured with the pG5luc reporter. As shown in Fig. 3A, TIP-1...
FIG. 2. Mapping the interaction domains of β-catenin and TIP-1. A, VP16-β-catenin and full-length or deletion mutants of TIP-1 fused to the Gal4 DNA binding domain were expressed in CHO-K1 cells as indicated, and protein-protein interaction was determined with the pG5Luc reporter. B, [35S]labeled in vitro translated WT-(1-781) or mutant-(1-777) β-catenin was incubated with Sepharose-bound GST or GST-TIP-1 (TIP-1) as shown, and complexes were precipitated. After SDS-PAGE, the labeled proteins were visualized by autoradiography. 25% of the in vitro translated proteins used in each reaction were loaded as Input. C–D, Gal4-TIP-1 was expressed in CHO-K1 cells together with WT or mutant forms of β-catenin in fusion with the VP16 transactivation domain as indicated, and protein-protein interaction was determined with the pG5Luc reporter.
repressed the transcriptional activity of the BD-β-catenin chimeric in a dose-dependent manner.

To test the effect of TIP-1 on the transactivation function of β-catenin under more physiological conditions, we applied the TOPFLASH and FOPFLASH reporters that contain a series of either WT or mutant TCF-4 binding sites, respectively, upstream of the luciferase gene (16). 293 cells were cotransfected with fixed amounts of β-catenin (either WT or 1–777 mutant) and luciferase reporters together with increasing amounts of TIP-1. As shown in Fig. 3B, TIP-1 inhibited β-catenin transcriptional activity in a dose-dependent manner. The transcriptional activity of β-catenin-(1–777) was also decreased by TIP-1, but significantly higher amounts of TIP-1 were required to the same inhibitory effect with respect to the WT (Fig. 3B). This observation is in line with the in vivo binding data (Fig. 2, C and D) and confirms the existence of a secondary interaction in a functional assay. TOPFLASH and FOPFLASH reporters had the same basal activity in these cells in the absence of exogenous β-catenin, and overexpression of TIP-1 had no effect on any of the reporters under this condition (data not shown).

We next addressed whether TIP-1 could repress the transcriptional activity of endogenous β-catenin in colorectal cancer cells harboring either truncated APC (HT-29) or mutations in the GSK3β phosphorylation sites of β-catenin (HCT-116 and SW48). Overexpression of TIP-1 decreased TOPFLASH reporter activity in a dose-dependent fashion in all of the three cell lines (Fig. 3, C and D, and data not shown), suggesting that TIP-1 could act as a potent inhibitor of Wnt/β-catenin signaling.

**Endogenous TIP-1 Affects β-Catenin Function**—To understand the role of TIP-1 in the β-catenin pathway, we first assessed whether complex formation occurs between endogenous proteins. As shown in Fig. 4A, β-catenin was efficiently coimmunoprecipitated with TIP-1 from HCT-116 cells, thus confirming that the interaction takes place under physiological conditions. We next measured the transcriptional activity of β-catenin in HCT-116 cells where expression of endogenous TIP-1 was down-regulated using small interfering RNAs specific for human TIP-1 (siTIP-1). As shown in Fig. 4B, we observed a modest but reproducible increase in TOPFLASH reporter activity in cells transfected with siTIP-1 as compared with the ones transfected with control siRNA (siCONT).
TIP-1 Reduces the Growth Rate of Colorectal Cancer Cells

Overexpression of β-catenin results in increased proliferation and enhanced anchorage-independent growth of normal epithelial cells, underlying its oncogenic function (29). Overexpression of ICAT, a reported inhibitor of β-catenin transactivation function (30), reduces the growth and colony-forming ability of tumor cells in soft agar (31).

Therefore, we performed colony formation assays in HCT-116 and HT-29 cells. Equal amounts of empty or TIP-1-encoding expression vectors conferring resistance to neomycin were transfected, and surviving colonies were counted after 2 weeks of selection in Geneticin-containing medium. As shown in Fig. 5A, a 50% reduction in colony numbers was observed in the TIP-1-transfected cells. This decrease in the number of resistant colonies could be attributed to either the death or the reduced growth rate of TIP-1-expressing cells. Transient transfection of TIP-1 did not cause cell death, although we observed a significant inhibition (30–50%) of S phase entry in those cells by bromodeoxyuridine incorporation assay (data not shown).

Cell lines of HCT-116 and HT-29 expressing Myc-TIP-1 were established next to assess their colony formation ability in soft agar. As shown in Fig. 5B, the anchorage-independent proliferative capacity of the Myc-TIP-1-expressing cells was significantly reduced. Altogether these results demonstrate that ectopically expressed TIP-1 inhibits the growth properties of colorectal cancer cells harboring deregulated β-catenin activity.
DISCUSSION

In this work, we have shown that the PDZ protein TIP-1 physically interacts with β-catenin and inhibits its transcriptional activity. TIP-1 binds primarily to the PDZ recognition sequence located at the extreme C terminus of β-catenin. Deletion of these residues, however, did not completely abolish association between the two proteins in vivo, and we have defined a secondary interaction region comprising Armadillo repeats 2–8 by deletion mapping. The size, structure, and location of this segment suggest the existence of a protein, which binds β-catenin and TIP-1 simultaneously, hence mediating their complex formation. Further research is required to identify this hypothetical protein. In addition, we have also identified eight amino acids of TIP-1, flanking its PDZ domain C-terminally, which seem to be required for efficient interaction with β-catenin. Future works may address whether these residues confer specificity for substrate recognition.

The evidence that overexpression of TIP-1 inhibits the transcriptional activity of β-catenin while down-regulation of endogenous TIP-1 expression has a positive effect on it strongly suggests that TIP-1 is a critical component of the β-catenin regulatory network. TIP-1 may exhibit its action by affecting the stability or subcellular localization of β-catenin, or alternatively, it may influence complex formation between β-catenin and TCF-4 or components of the transcriptional machinery. Ectopic expression or knock-down of TIP-1 had no detectable effect on the protein levels of β-catenin as judged by Western blot analysis (data not shown).

TIP-1 has been shown to be exclusively cytoplasmic (24), although we observed both nuclear and cytoplasmic localization of the overexpressed protein (Fig. 1C). Because of its small size, it is plausible that TIP-1 freely shuttles between the cytosol and the nucleus. Notably, human T-cell lymphotropic virus-1 Tax shuttles between the cytoplasm and the nucleus. However, we were not able to detect visible changes in the localization of β-catenin upon overexpression of TIP-1 (Fig. 1C and data not shown).

The intrinsic transactivating capability of β-catenin relies on the presence of transactivating elements located both N- and C-terminally that are able to recruit general transcription factors like TATA-binding protein for target gene promoters (28). Moreover, the more potent C-terminal transactivation domain directly associates with the transcriptional coactivators p300/CPB (34–36) and the chromatin remodeling factor Brg-1 (37). Displacement of these proteins or TCF-4 from β-catenin by competitive binding is seen as a prospective therapeutic approach for the treatment of colorectal cancers. Indeed, ICAT binds Armadillo repeats 5–12 and inhibits β-catenin transcriptional activity by displacing both TCF-4 and p300 (38, 39). It is important to note that the primary and secondary binding sites of TIP-1 on β-catenin partially overlap with the interaction region of p300 and TCF-4, respectively. Experiments aimed to investigate whether the inhibitory effect of TIP-1 is based on competitive binding are in progress in our laboratories.

The evidence that TIP-1 overexpression reduces both colony formation and anchorage-independent growth of colorectal can-

cer cells raises the possibility that it may function as a tumor suppressor. Interestingly, the TIP-1 gene is located at the short arm of human chromosome 17 that is frequently lost in colon carcinomas (40). It will be important to see the presence and/or level of TIP-1 transcript in the various stages of colorectal tumorigenesis. Information regarding the regulation of the gene and identification of novel targets of its product may help to better understand the biological function and mechanism of action of TIP-1.

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