Traf2- and Nck-Interacting Kinase Is Essential for Wnt Signaling and Colorectal Cancer Growth

Miki Shitashige, Reiko Satow, Takaumi Jigami, Kazunori Aoki, Kazufumi Honda, Tatsuhiro Shibata, Masaya Ono, Setsuo Hirohashi, and Tesshi Yamada

Abstract

T-cell factor-4 (TCF4) is a transcription factor essential for maintaining the undifferentiated status and self-renewal of intestinal epithelial cells. It has therefore been considered that constitutive activation of TCF4 by aberrant Wnt signaling is a major force driving colorectal carcinogenesis. We previously identified Traf2- and Nck-interacting kinase (TNIK) as one of the proteins that interact with TCF4 in colorectal cancer cells, but its functional significance has not been elucidated. Here, we report that TNIK is an activating kinase for TCF4 and essential for colorectal cancer growth. TNIK, but not its catalytically inactive mutant, phosphorylated the conserved serine 154 residue of TCF4. Small interfering RNA targeting TNIK inhibited the proliferation of colorectal cancer cells and the growth of tumors produced by injecting colorectal cancer cells s.c. into immunodeficient mice. The growth inhibition was abolished by restoring the catalytic domain of TNIK, thus confirming that its enzyme activity is essential for the maintenance of colorectal cancer growth. Several ATP-competitive kinase inhibitors have been applied to cancer treatment and have shown significant activity. Our findings suggest TNIK as a feasible target for pharmacologic intervention to ablate aberrant Wnt signaling in colorectal cancer.

Introduction

The majority of colorectal cancers have somatic mutations in one of two genes involved in the canonical Wnt signaling pathway: the adenomatous polyposis coli (APC) and β-catenin (CTNNB1) genes. More than 80% of colorectal cancers show mutation of the APC gene (1), and half of the remainder have CTNNB1 gene mutation (2, 3). Mutation of either gene causes failure of β-catenin degradation and accumulation of β-catenin protein (4–6). The accumulated β-catenin protein is translocated into the nucleus, where it forms complexes with T-cell factor (TCF)/lymphoid enhancer factor (LEF) family DNA-binding proteins and transactivates their target genes (7–9).

TCF4 is a member of the TCF/LEF family of transcription factors, which comprises LEF1 (LEF1), TCF1 (TCF7), TCF3 (TCF7L1), and TCF4 (TCF7L2; ref. 10). Among these factors, the aberrant activation of TCF4 by accumulated β-catenin has been implicated in colorectal carcinogenesis (11). TCF4 is commonly expressed in colorectal cancer cells (12). Physiologically, TCF4 is essential for maintaining the undifferentiated status of intestinal crypt epithelial cells. Mice lacking Tcf7l2 show no proliferative compartment in the crypt regions (13). Induction of dominant-negative TCF4 restores epithelial cell polarity and converts colorectal cancer to a single layer of columnar epithelium (14), indicating that constitutive activation of TCF4 is necessary for maintaining the malignant phenotype.

Wnt signaling is a major force driving colorectal carcinogenesis, but not all the molecules mediating the signal can be drug targets. Restoration of the loss of function resulting from mutation of the APC gene is not realistic with current medical technology. We have therefore been searching for drug target molecules downstream of APC, especially in the nucleus (15). We previously identified 70 proteins present in the immunoprecipitate with anti-TCF4 antibody using highly tuned liquid chromatography and mass spectrometry (LC-MS; ref. 16). Among those proteins, Traf2- and Nck-interacting kinase (TNK; ref. 17) attracted our interest because various small-molecule kinase inhibitors have been applied successfully to cancer treatment (18–20). Here, we report that colorectal cancer cells are highly dependent on the expression and kinase activity of TNK for proliferation in vitro and in vivo. During the preparation of this article, Mahmoudi and colleagues also identified Tnk as a protein interacting with Tcf4 in the mouse intestinal crypt (21). Their data overlapped partly with ours: TNK is an activating kinase for the TCF4 and β-catenin transcriptional complex. TNK is a feasible target of pharmacologic intervention for manipulation of the aberrant Wnt signaling pathway.
Materials and Methods

Cell lines and antibodies

The human embryonic kidney cell line HEK293 and the human colorectal cancer cell line DLD1 were obtained from the Health Science Research Resources Bank. The cervical cancer cell line HeLa was obtained from the Riken Cell Bank. The human colorectal cancer cell lines HCT-116 and WiDr were purchased from the American Type Culture Collection. The numbers of living cells were counted by trypan blue dye exclusion using a hemocytometer. Antibodies used in this study and their suppliers are listed in Supplementary Table S1.

Immunoprecipitation

Total cell lysates were prepared as described previously (16). The lysates were incubated at 4°C overnight with the indicated antibody or relevant control IgG and precipitated with Dynabeads protein G (Dynal Biotech).

Immunoblot analysis

Protein samples were fractionated by SDS-PAGE and blotted onto Immobilon-P membranes (Millipore). After incubation with the primary antibodies at 4°C overnight, the blots were detected with the relevant horseradish peroxidase–conjugated antimouse or antirabbit IgG antibody and enhanced chemiluminescence Western blotting detection reagents (GE Healthcare; ref. 22). Nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce; ref. 22).

Immunohistochemistry

Fifty cases of sporadic colorectal cancer were selected from the pathology archive panel of the National Cancer Center Hospital (Tokyo, Japan). Immunoperoxidase staining was done using the avidin-biotin complex method (23).

Liquid chromatography and mass spectrometry

Protein bands in SDS-PAGE gels were visualized by Coomassie blue staining and digested using modified trypsin (Promega) as described previously (24). The tryptic peptides were then fractionated with a 0% to 80% acetonitrile gradient (200 nL/min for 1 hour) using a 150-μm i.d. × 5 cm C18W-3 separation column (KYA Technologies). The peptides were then analyzed with a Q-Star liquid chromatography and mass spectrometer equipped with one of the luciferase reporters and phRL-TK transcriptional activity. Cells were transiently transfected in triplicate with pBind, pAct, and pGLuc (Promega) plasmids using the Lipofectamine 2000 reagent (Invitrogen; refs. 24, 26).

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Recombinant protein production

Glutathione S-transferase (GST) fusion proteins were synthesized using the ENDEXT Wheat Germ Expression Kit (CellFree Sciences). Hemagglutinin (HA)-tagged recombinant TNIK proteins were synthesized using rabbit reticulocyte lysate (TnT T7 Quick Coupled Transcription/Translation System, Promega).

RNA interference

Four siRNAs (CTNNB1-9, CTNNB1-11, TNIK-J-004542-12, and TNIK-J-004542-13) were synthesized and annealed by Dharmaco. Three nontargeting control RNAs (X, IX, and VIII) were purchased from Dharmaco. The SureSilencing short hairpin RNA (shRNA) plasmids for human TNIK (pGeneClip-TNIK1, pGeneClip-TNIK2, and pGeneClip-TNIK3) and a negative control were purchased from SuperArray Bioscience.

Luciferase reporter assay

A pair of luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate), was used to evaluate TCF/LEF transcriptional activity. Cells were transiently transfected in triplicate with one of the luciferase reporters and phRL-TK (Promega; ref. 23). Luciferase activity was measured with the Dual-luciferase Reporter Assay System (Promega) and normalized using Renilla reniformis luciferase activity as an internal control (28). Data are presented as the ratio of TOP-FLASH to FOP-FLASH (TOP/FOP ratio).

Colony formation assay

Twenty-four hours after transfection, 400, 300, and 1,000 μg/mL G418 (Geneticin, Invitrogen) were added to the culture media of HeLa, DLD1, and HCT-116 cells, respectively. Cells were stained with Giemsa solution (Wako) after selection for 8 days (16).
Real-time reverse transcription-PCR

Total RNA was prepared with an RNeasy Mini Kit (Qiagen). DNase-I-treated RNA was random primed and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). The TaqMan Universal PCR master mix and predesignated TaqMan Gene Expression probe and primer sets were purchased from Applied Biosystems. Amplification data measured as an increase in reporter fluorescence were collected using the PRISM 7000 Sequence Detection system (Applied Biosystems). mRNA expression level relative to the internal control [β-actin (ACTB)] was calculated by the comparative threshold cycle (C_T) method (26).

Adenovirus vectors

The replication-incompetent recombinant adenovirus vectors were prepared by Cre/loxP-mediated recombination of a S360 adenoviral cosmid, which is an Ad 5 derivative designed with deletion of E1 and partial deletion of E3 (29), and the pAdCMVloxP or pAdCMV-TNIKΔC plasmid. The generated adenovirus vectors were designated AdCMV-Control and AdCMV-TNIKΔC, respectively. Cesium chloride–purified viruses were desalted by sterile Bio-Gel p-6 DG chromatography (Econopac DG 10, Bio-Rad) and diluted for storage in 13% glycerol/PBS solution. All viral preparations were confirmed to be free of E1+ adenovirus by PCR assay (30).

Mouse experiments

HCT-116, DLD1, WiDr, or HeLa cells (5 × 10^6) suspended in 0.1 mL of PBS were s.c. inoculated into the flanks of 5-week-old female BALB/c nu/nu nude mice (SLC). One week later, the developed tumors were treated with siRNA together with atelocollagen (AteloGene, KOKEN). The final concentration of siRNA was 30 μmol/L and that of atelocollagen was 0.5%. A 0.2-mL volume of siRNA solution was injected directly into each tumor. siRNA remains intact in vivo for at least 1 week with the support of atelocollagen (31, 32). In some experiments, 5 × 10^4 pfu recombinant adenovirus vectors were coinjected. Tumor volume was determined as V = ABπ/6, where A and B represent the largest and smallest dimensions, respectively (33). Mouse experiments were carried out according to the guidelines of the National Cancer Center Research Institute (Tokyo, Japan), which meet all the ethical requirements stipulated by Japanese law. The experimental protocols were reviewed and approved by the institutional ethics and recombination safety committees.

Results

TNIK is a component of the TCF4 and β-catenin complex

We analyzed the composition of proteins immunoprecipitated with anti-TCF4 antibody in two colorectal cancer cell lines (DLD1 and HCT-116) and identified TNIK (ref. 16; Supplementary Fig. S1). DLD1 has a truncating mutation in the APC gene and lacks the other allele, and HCT-116 has a missense mutation in the CTNNB1 gene (2). TNIK was detected by immunoblotting of immunoprecipitates with anti-TCF4 or anti-β-catenin antibody, but not with control IgG. Conversely, β-catenin and TCF4 proteins were immunoprecipitated with anti-TNIK antibody (Fig. 1A). These results indicate that TCF4, β-catenin, and TNINK proteins form a complex in colorectal cancer cells. Two-hybrid assay revealed that TNINK interacted with TCF4 through amino acids 1–289, including the kinase domain (Fig. 1B). Amino acids 100–216 of TCF4 were necessary for interaction with TNINK (Fig. 1C).

Phosphorylation of TCF4 by TNINK

TCF4 protein was phosphorylated by TNINK (WT, wild-type; Fig. 2A–C), but not by the catalytically inactive mutant of TNINK with substitution (K54R) of the conserved lysine 54 residue in the ATP-binding pocket of the kinase domain (17). Tandem mass spectrometry (MS/MS) revealed that the serine 154 residue of TCF4 was phosphorylated by TNINK (WT) (Supplementary Fig. S2). Consistently, substitution of the serine 154 residue by alanine (S154A) abolished the phosphorylation of TCF4 by TNINK (Fig. 2C). The serine 154 residue of human TCF4 (TCF7L2) is conserved among species (Supplementary Fig. S3A) and shared with TCF3 (Supplementary Fig. S3B). TCF4 was phosphorylated on transfection of DLD1 cells with TNINK (WT), but not with TNINK (K54R) (Fig. 2D).

Nuclear translocation of TNINK

The catalytic activity of TNINK seems to be necessary for its nuclear translocation and interaction with TCF4 (Supplementary Fig. S4). DLD1 cells were transfected with HA-tagged TNINK (WT) or catalytically inactive TNINK (K54R) and analyzed by immunofluorescence microscopy (Supplementary Fig. S4A) and immunoprecipitation (Supplementary Fig. S4B). The wild-type TNINK was incorporated into the nuclei, whereas the K54R substitution significantly inhibited the nuclear translocation of TNINK (Supplementary Fig. S4A) and reduced the amount of TNINK interacting with TCF4 (Supplementary Fig. S4B).

The serine 764 (S764) residue of TNINK had been identified as a phosphorylation site by LC-MS/MS–based random sequencing of protein kinases (34), but its functional significance had remained unexplored. TNINK protein was distributed along the filamentous cytoskeleton (Supplementary Fig. S4C, anti-TNIK), whereas phosphorylated TNINK (anti-TNIKpS764) was detected mainly in the nuclei and co-localized with TCF4 (anti-TCF4).

Activation of TNINK by Wnt signaling

The phosphorylation and nuclear translocation of TNINK seem to be mediated, at least partly, through Wnt/β-catenin signaling (Fig. 3). TNINKpS764 was detected in colorectal cancer cells, but not in untransformed HEK293 cells (Fig. 3A). Transient transfection of HEK293 cells with β-catenin stabilized by deletion of the NH2-terminal glycogen synthase kinase 3β phosphorylation site (β-cateninΔN134; ref. 26) induced the S764 phosphorylation and nuclear translocation of TNINK (Fig. 3B). Knockdown of β-catenin (CTNNB1) by siRNA (constructs 9 and 11) abolished the phosphorylation
and nuclear translocation of TNIK in colorectal cancer cells (Fig. 3C).

The expression of TNIK was examined in clinical specimens of colorectal cancer (Supplementary Fig. S5). Although the overall expression level of TNIK protein did not differ significantly between cancer and normal mucosa (Supplementary Fig. S5A and B), the expression of phosphorylated TNIK (pS764) was increased in cancer cells compared with neighboring normal intestinal epithelial cells (Supplementary Fig. S5C and D). Nuclear TNIKpS764 was detected most predominantly in the invasive front of colorectal cancer (Supplementary Fig. S5E), where β-catenin was accumulated in the nucleus and cytoplasm (Supplementary Fig. S5F).
Regulation of β-catenin–evoked transcriptional activity by TNIK

We then investigated the effects of TNIK on the transcriptional activity of the TCF4 and β-catenin complex (Supplementary Fig. S6). HeLa cells have wild-type APC and CTNNB1 genes, and their TCF/LEF transcriptional activity is kept repressed (2, 35). Cotransfection with HA-tagged wild-type TNIK (WT), but not with TNIK (K54R), enhanced the β-cateninΔN134–evoked TCF/LEF transcriptional activity (Supplementary Fig. S6A). TNIK did not significantly affect transcriptional activity in the absence of β-cateninΔN134, indicating that the effects of TNIK are dependent on activation of Wnt signaling. Conversely, knockdown of TNIK by siRNA against TNIK (constructs 12 and 13) abolished the β-cateninΔN134–evoked TCF/LEF transcriptional activity (Supplementary Fig. S6B), which was also attenuated by an established inhibitor of Wnt signaling, dominant-negative TCF4 (TCF4DN; ref. 14). Knockdown of TNIK by shRNA against TNIK (constructs 1, 2, and 3) abolished the β-cateninΔN134–evoked colony formation, which was also inhibited by TCF4DN, but did not significantly affect the proliferation of HeLa cells that were not cotransfected with β-cateninΔN134 (Supplementary Fig. S6C).

TNIK is essential for colorectal cancer cell proliferation

Transient transfection with TNIK (WT), but not with TNIK (K54R), into DLD1 and HCT-116 cells also enhanced their TCF/LEF transcriptional activity (Supplementary Fig. S7A).
The effects were less remarkable, probably because endogenous TCF4 had already been activated by TNIK in these colorectal cancer cells. Then, substitution of the serine 154 residue of TCF4 by alanine (S154A) abolished the TNIK (WT)–induced TCF/LEF transcriptional activity (Supplementary Fig. S7B). Knockdown of TNIK suppressed the TCF/LEF transcriptional activity (Fig. 4A), proliferation (Fig. 4B), and colony formation (Fig. 4C) of DLD1 and HCT-116 cells. The expression of known target genes of the β-catenin and TCF/LEF complexes (36–40), such as axis inhibitor-2 (AXIN2), c-myc (MYC), c-jun (JUN), and matrixsin (MMP7), except for cyclin D1 (CCND1), was significantly reduced by transient transfection with siRNA against TNIK (Fig. 5). The siRNA-mediated reduction of transcriptional activity and cell proliferation was rescued by cotransfection with the kinase domain (amino acids 1–289) of TNIK (TNIKΔC), which lacks the sequences targeted by siRNAs (Supplementary Fig. S8).

Regression of established tumors after injection of siRNA against TNIK

We next examined the effects of TNIK on the growth of human colon cancer cells in vivo (Fig. 6). HCT-116 cells were implanted in the flanks of immunodeficient mice. One week after the inoculation, siRNA against TNIK (12 or 13) mixed with atelocollagen (31) was injected into the tumors (224.5 ± 8.9 mm³). Three days after the siRNA injection, some tumors were excised, and the silencing of TNIK was confirmed by real-time PCR (Fig. 6A). The volume of xenografts was monitored for 18 days after siRNA injection (Fig. 6B). We found that the tumors regressed almost completely after an injection of siRNA against TNIK. Figure 6C and D shows the appearance of representative mice and excised tumors. Tumors treated with siRNA against TNIK (12 or 13) were significantly smaller than those that were untreated (No treat), treated with only atelocollagen (Ateo only), or treated with control RNA (X or IX; Fig. 6D). We observed similar regression of established tumors after an injection of siRNA against TNIK in two other colorectal cancer cell lines, DLD1 and WiDr (Supplementary Figs. S9 and S10), but not in Wnt-inactive HeLa cells (Supplementary Fig. S11). The siRNA-mediated reduction of tumor growth was rescued by coinjection of an adenovirus vector encoding the kinase domain of TNIK (TNIKΔC; Supplementary Fig. S12). The detection of cleaved poly(ADP-ribose) polymerase 1 (Supplementary Fig. S12B) indicated the induction of apoptosis by silencing of TNIK.

Discussion

The Wnt signaling pathway plays an important role in the maintenance of intestinal epithelial stem cell reservoirs. In fact, undifferentiated cells at the bottom of the intestinal crypts accumulate nuclear β-catenin (41). Mahmoudi and colleagues showed that TNIK interacted with and phosphorylated β-catenin as well as TCF4 (21). We further showed that TNIK phosphorylated the S154 residue of TCF4 (Fig. 2; Supplementary Fig. S2). The recombinant TCF4 protein was directly phosphorylated by the in vitro translation product of TNIK (Fig. 2B and C), and the effects of TNIK were compromised by substitution of the S154 residue of TCF4 (Fig. 2C). Substitution of the serine 154 residue of TCF4 by alanine (S154A) abolished the TNIK (WT)–induced TCF/LEF transcriptional activity of colon cancer cells (Supplementary Fig. S7B), indicating that the TNIK-mediated phosphorylation of the residue is essential for the transcriptional activity of TCF4. The serine residue of TCF4 is conserved from human to zebrafish and also in TCF3 (Supplementary Fig. S3), indicating the biological importance of its phosphorylation.
Constitutive activation of the Wnt signaling pathway seems to be necessary for maintenance of the undifferentiated status and self-renewal of colorectal cancer cells (14, 42). TNIK was essential for the transcriptional activity of the TCF4 and β-catenin complex in colorectal cancer cells (Fig. 4; ref. 21).

We newly observed that TNIK was essential for the continuation of colorectal cancer cell proliferation. Knockdown of TNIK by different siRNA constructs induced marked suppression of the TCF/LEF transcriptional activity and colorectal cancer cell proliferation (Fig. 4). To exclude the off-target
effects of siRNA, we performed rescue experiments. Because the target sequences of both the TNIK siRNAs (12 and 13) are located in the COOH-terminal half of TNIK, we designed plasmid and adenovirus vectors encoding only the NH2-terminal catalytic domain (amino acids 1–289) of TNIK (i.e., TNIKΔC), which mediates the interaction with TCF4 (Fig. 1B; ref. 21). These constructs successfully rescued the activity of TNIK that had been downregulated by siRNA in vitro (Supplementary Fig. S8) and in vivo (Supplementary Fig. S12).

Knockdown of TNIK did not significantly affect the growth of Wnt-inactive cells [Supplementary Figs. S11 and S6C, β-cateninΔN134(–)], indicating that the suppression of colorectal cancer cell growth is mediated, at least partly, by blockage of Wnt signaling.

We also found that β-catenin overexpression induced TNIK phosphorylation in Wnt-inactive cells (Fig. 3B) and the suppression of β-catenin by siRNA decreased TNIK phosphorylation in colorectal cancer cells (Fig. 3C). These observations indicate that the activation of TNIK is mediated by Wnt signaling. The immunohistochemistry data obtained from clinical samples (Supplementary Fig. S5) support this notion. Activated TNIK is then translocated into the nucleus and augments the transcriptional activity of TCF4. This positive feedback circuit seems to be essential for the continuation of colorectal cancer cell renewal. Elucidation of the molecular pathway that connects the Wnt signal to TNIK, however, will be an issue for future studies.

TNIK was originally identified as a novel member of the germinal center kinase family that interacts with tumor necrosis factor receptor–associated factor-2 (Traf2) and Nck (17). In addition to activation of the c-jun NH2-terminal kinase pathway, TNIK induces disruption of the filamentous actin structure, thereby inhibiting cell spreading (25). Therefore, the significant suppression of colorectal cancer growth by TNIK siRNA (Fig. 6; Supplementary Figs. S9 and S10) may not be solely attributable to the inhibition of Wnt signaling. In fact, we (Fig. 4A) and others (21) have observed that siRNA targeting TNIK induced only a few-fold decrease of TCF/LEF transcriptional activity in colorectal cancer cells. A full explanation of the molecular mechanisms behind the marked cell growth–suppressive effect will require further detailed studies.

The Wnt signaling pathway is an attractive target for anticancer therapy, but only a few “druggable” targets have
been found in this pathway (43, 44). In the present study, we have clearly shown that TNIK is essential for the continual growth of colorectal cancer. Several synthetic small compounds that bind to the ATP-binding pockets of protein kinases competitively with ATP have been incorporated successfully into oncological practice (18–20). For example, imatinib, which blocks the Bcr-Abl fusion kinase of chronic myeloid leukemia (CML), is currently the first-line therapeutic drug for CML. A new drug targeting TNIK might be effective for the treatment of patients with colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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