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Direct interaction of menin leads to ubiquitin-proteasomal degradation of β-catenin

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ABSTRACT

Menin, encoded by the multiple endocrine neoplasia type 1 (*MEN1*) gene, is a tumor suppressor and transcription regulator. Menin interacts with various proteins as a scaffold protein and is proposed to play important roles in multiple physiological and pathological processes by controlling gene expression, proliferation, and apoptosis. The mechanisms underlying menin's suppression of tumorigenesis are largely elusive. In this study, we showed that menin was essential for the regulation of canonical Wnt/β-catenin signaling in cultured cells. The C-terminal domain of menin was able to directly interact with and promote ubiquitin-mediated degradation of β-catenin. We further revealed that overexpression of menin down-regulated the transcriptional activity of β-catenin and target gene expression. Moreover, menin efficiently inhibited β-catenin protein levels, transcriptional activity, and proliferation of human renal carcinoma cells with an activated β-catenin pathway. Taken together, our results provide novel molecular insights into the tumor suppressor activity of menin, which is partly mediated by proteasomal degradation of β-catenin and inhibition of Wnt/β-catenin signaling.

1. Introduction

The human *MEN1* (multiple endocrine neoplasia type 1) gene, identified by positional cloning in 1997, encodes menin, a 610-amino acid protein, which is predominantly found in the nucleus [1,2]. The loss of function of menin due to missense or nonsense mutations along the entire *MEN1* gene is associated with *MEN1* syndrome; this syndrome is characterized by the development of tumors in multiple endocrine organs such as the parathyroid, anterior pituitary, and pancreatic islets [1]. Homozygous *MEN1* knockout mice die *in utero* (E11.5–13.5) with multiple developmental defects, suggesting that menin has an essential role in early development [3]. Heterozygous knockout mice grow normally, but the adult mice eventually develop endocrine tumors, similar to *MEN1* patients [4].

The mechanism by which the loss of menin leads to MEN1 is still unclear. Menin interacts with diverse partners. By acting as a scaffold protein, menin is engaged in a complex network of interactions and plays a critical role in the control of gene expression and intracellular signaling [5]. In the nucleus, menin can associate with chromatin remodeling factors such as histone methyltransferases, MLL1 and MLL2, or histone deacetylases (HDACs) to regulate target genes [6–8]. Its protein partners further include various transcription factors including β-catenin, JunD, activating protein-1 (AP-1), nuclear factor-κB (NF-κB), SMAD proteins [9–13]. Furthermore, menin regulates *GBX2* and *IL6* transcription through direct interaction with SUV39H1 [14,15]. This complex list of interacting partners implicates menin in multiple biological pathways including cell proliferation, apoptosis, and genome integrity [16].

The canonical Wnt/β-catenin signaling pathway plays an important role both in embryonic development and carcinogenesis. It regulates many biological processes, such as cell fate determination, cell proliferation, apoptosis, migration, and stem cell maintenance [17]. Dysregulation of those pathways is involved in the development of a variety of carcinomas, such as colorectal cancer, hepatocellular carcinoma, ovarian cancer, melanoma, medulloblastoma, and renal cell carcinoma (RCC) [18,19].

In this report, to elucidate the role of menin in the Wnt/β-

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catenin pathway, we investigated the effect of menin on β-catenin stability, localization, and nuclear function. We showed direct interaction of menin with β-catenin by determining the interaction surfaces on both proteins. Menin promoted β-catenin degradation via an ubiquitin-mediated proteasomal degradation pathway. Furthermore, menin inhibited human RCC cells through repression of β-catenin without changing the mRNA level of β-catenin. We therefore propose new molecular insights for the tumor suppressor function of menin that targets β-catenin.

2. Materials & methods

2.1. Cell cultures and transfection

HEK293T cells were obtained from the American Type Culture Collection (Manassas, USA). The renal cell carcinoma cell line RCC4 was obtained from the European Collection of Cell Cultures (London). HEK293T and RCC4 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 50 units/ml penicillin/streptomycin (HyClone, USA) in a humidified incubator at 37 °C and 5% CO_2. Transfection was carried out using jetPEI DNA transfection reagent (Polyplus transfection, USA) and FuGENE HD transfection reagent (Promega, USA).

2.2. Plasmids and antibodies

Plasmids used in this study, pcDNA3-Myc/His menin (full length), pcDNA3-Myc/His menin 1-150 (150×), 145–610 (ΔN145), 330–610 (ΔN330), 360–610 (ΔN360), 420–610 (ΔN420), and 485–610 (ΔN485), were previously described [14,15]. Plasmids such as pcDNA3 Flag-β-catenin, pDEST HA-β-catenin WT, S45A, S33A, pGEX5T1-β-catenin, pGEX4T1-Arm (141–664), pGEX4T1-β-catenin (1–133), pcDNA3 HA-Dvl2-ER, pcDNA Myc β-TrCP, and pCR3-HA-VHL were kindly donated as described in Acknowledgements. Plasmid M50 Super 8× TOPFLASH was obtained from Addgene (#12456). The anti-menin antibodies were purchased from Bethyl (A300-105A, USA) or Thermo Fisher Scientific (PA5-19584, USA). Anti-β-catenin antibody (610154) was from BD (USA). Anti-Myc (M4489), anti-HA (H3663), anti-FLAG (F1804), and anti-β-Actin (A1978) antibodies were from Sigma Aldrich (USA). Anti-ubiquitin antibody (sc-1833) was from Santa Cruz (USA). Anti-α-tubulin (ab15246) and anti-Lamin B1 (ab8982) antibodies were from Abcam (UK).

2.3. Quantitative real time PCR (qRT-PCR)

Total RNA was purified with a RNeasy plus mini kit (Qiagen, USA) per the manufacturer's instructions. One microgram (1 μg) of purified RNA was used to synthesize cDNA using a reverse transcriptase reaction mixture containing oligo dT or random hexamers and gene-specific primers (Thermo Fisher Scientific). Quantitative real-time PCR was performed using the CFX96™ Real Time System (Bio-Rad, USA). PCR amplification was carried out using the KAPA SYBR FAST Master Mix (KAPA Biosystem, USA) and relative quantification was performed with the $2^{-\Delta\Delta CT}$ (Livak) program method. The mRNA level was normalized to the values of GAPDH for each reaction. Primers used for qRT-PCR are described in Supplementary Table 1.

2.4. Western blot analysis & immunoprecipitation

Cells were washed twice with cold PBS. Cell lysates were prepared using lysis buffer [25 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.5% NP40]. After incubating for 30 min on ice, cell lysates were centrifuged (13,000 rpm at 4 °C) for 10 min. For immunoprecipitation (IP), HEK293T cells were harvested 24 h after DNA transfection and lysed in a lysis buffer containing protease inhibitors. Lysates were immunoprecipitated with appropriate antibodies along with protein-A/G magnetic beads (Thermo Fisher Scientific). Western blots and immunoprecipitates were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to immunoblotting with appropriate antibodies.

2.5. GST pull-down assay

Recombinant proteins were expressed as fusion proteins with glutathione S-transferase (GST) in DH5α. When the cell optical density reached 0.6–0.9 OD, cells were harvested and lysed in PBS containing Triton X-100, protease inhibitors, and phosphatase inhibitors. GST fusion proteins were combined with GST-beads (20% slurry) for 30 min in a 4 °C chamber. GST-beads were prepared by washing with PBS buffer three times. HEK293T total lysates (25 μg) containing Myc/His menin, were added to the GST-beads and incubated overnight in a 4 °C chamber. Beads were washed with PBS buffer three times, resolved by SDS-PAGE, and subjected to immunoblotting and Coomassie staining.

2.6. Luciferase assay

HEK293T cells were transiently transfected in triplicate with M50 Super 8× TOPFLASH luciferase reporter and various combinations of expression vectors using the jetPEI transfection method. After 24 h, luciferase activity (duplicate for each group) was measured using the Bright-Glo luciferase Assay (Promega) and a FB12 luminometer (Berthold technologies, Germany).

2.7. Cell proliferation assay

RCC4 cells were seeded into 6-well plates and transfected with the pcDNA3 Myc/His menin vector using the FUGENE HD method. Cells were cultured in the optional media and cell numbers were counted 72 h after transfection. The number of cells was counted with a Tali™ Image Based Cytometer (Invitrogen, USA).

3. Results

3.1. Tumor suppressor menin reduces the protein stability of β-catenin

To investigate the role of menin in the regulation of β-catenin, we examined its effect on β-catenin levels in HEK293T cells that had been co-transfected with Myc/His menin and Flag β-catenin. Interestingly, expression of menin significantly inhibited β-catenin protein levels in a dose-dependent manner (Fig. 1A). Next, we examined whether menin affected cellular localization of β-catenin by analyzing the cytoplasmic and the nuclear fractions. As shown in Fig. 1B, menin and β-catenin were detected in both the cytoplasm and the nucleus and an increasing amount of menin efficiently led to a decrease of β-catenin in both fractions; this indicated that menin reduced the levels of β-catenin without changing its localization. Overexpression of menin was sufficient to reduce nuclear and cytoplasmic β-catenin. Furthermore, we confirmed that endogenous β-catenin was also decreased due to menin expression (Fig. 1C), however, the mRNA level of β-catenin was unaltered (Fig. 1D), indicating that menin effectively decreased β-catenin without affecting its transcription. Next, we examined whether menin affected the protein stability of β-catenin. We found that menin-mediated down-regulation of β-catenin was efficiently blocked by MG132 (Fig. 1E), suggesting that menin promotes β-catenin degradation at the protein level without affecting gene