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Homeodomain-interacting protein kinase 2 (HIPK2) targets β-catenin for phosphorylation and proteasomal degradation

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ABSTRACT

The regulation of intracellular β-catenin levels is central in the Wnt/β-catenin signaling cascade and the activation of the Wnt target genes. Here, we show that homeodomain-interacting protein kinase 2 (HIPK2) acts as a negative regulator of the Wnt/β-catenin pathway. Knock-down of endogenous HIPK2 increases the stability of β-catenin and results in the accumulation of β-catenin in the nucleus, consequently enhancing the expression of Wnt target genes and cell proliferation both *in vivo* and in cultured cells. HIPK2 inhibits TCF/LEF-mediated target gene activation via degradation of β-catenin. HIPK2 phosphorylates β-catenin at its Ser33 and Ser37 residues without the aid of a priming kinase. Substitutions of Ser33 and Ser37 for alanines abolished the degradation of β-catenin associated with HIPK2. In *ex vivo* mouse model, HIPK2 knock-down resulted in accumulation of β-catenin, thereby potentiated β-catenin-mediated cell proliferation and tumor formation. Furthermore, the axis duplication induced by the ectopic expression of β-catenin was blocked by co-injection of HIPK2 mRNAs into *Xenopus* embryos. Taken together, HIPK2 appears to function as a novel negative regulator of β-catenin through its phosphorylation and proteasomal degradation.

1. Introduction

The Wnt/β-catenin signaling pathway plays important roles in animal development [1]. The aberrant regulation of Wnt signaling cascade component is associated with human disease, and mutations of APC, Axin and β-catenin have been studied extensively in colon and liver cancers [2]. Although many signaling molecules participate in the transduction of signals from the Wnt receptor to downstream components, β-catenin plays a pivotal role in the signaling pathway. In the absence of the Wnt signal, cytosolic β-catenin is constitutively degraded via phosphorylation-dependent ubiquitination and subsequent proteasomal clearance. The Wnt ligand/receptor-induced signaling cascade results in stabilization of β-catenin and an increase in the levels of β-catenin in the nucleus where it functions as a coregulator of TCF/LEF transcription factors for Wnt target gene activation [3,4]. The canonical mechanism of β-catenin regulation involves a destruction complex where β-catenin is phosphorylated by priming kinases at the Ser45 site and subsequently by glycogen synthase kinase 3β (GSK3β) at the Thr41, Ser37 and Ser33 sites [5,6]. Phosphorylated β-catenin is recognized by β-TrCP, a component of the SCF E3 ligase complex, and is degraded by the ubiquitin-mediated proteasome system [7,8].

HIPK2 has been shown to act as a coregulator of homeodomain transcription factors [9–12] and as a tumor suppressor through the phosphorylation of cellular target proteins, including p53, CtBP, AML and p300/CBP [13,14]. HIPK2 appears to exert multiple functions depending on the binding partner or on the phosphorylation of the downstream target proteins in different signaling pathways. Although there is evidence supporting that HIPK2 may be involved in the Wnt signaling pathway [15,16,22], it was not well known how HIPK2 directly regulates intracellular β-catenin levels. In this study, we report that HIPK2 can bind directly and phosphorylate β-catenin, and consequently degrades β-catenin. The knock-down of endogenous HIPK2 augments the stability of β-catenin and the expression of β-catenin target genes. A stable tumor cell line in which HIPK2 was silenced using an HIPK2 shRNA displays accelerated proliferation. In addition, HIPK2 expression blocked the axis duplication induced by the injection of β-catenin mRNA into *Xenopus* embryos. These results strongly indicate that HIPK2 is a novel negative regulator of Wnt signaling operating via the direct phosphorylation and degradation of β-catenin, a key component of the Wnt signaling pathway.

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2. Materials and methods

2.1. Cell transfection and luciferase reporter assays

Cell cultures, transient transfection and luciferase assays were performed as described previously [17,18]. The HEK293 and RKO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco). Cells were grown on six-well plates and transiently transfected with reporter plasmids and various expression plasmids using Fugene6 reagent (Roche Molecular Biochemicals). For the transcription assays, cells were transfected with 0.4 μg of TOP-FLASH reporter plasmids, 0.6 μg of β-catenin plasmid, 2 μg of HIPK2/K221R plasmids and 0.1 μg of pCMV-β-gal plasmid encoding β-galactosidase was included in all transfections to normalize the transfection efficiency in combination as indicated in figures. Thirty-six hours after transfection, the luciferase activities were measured from duplicate plates using the Luciferase Reporter Assay System (Promega) and a Genios luminometer (TECAN). All experiments were repeated at least three times.

2.2. In vitro phosphorylation of β-catenin

In vitro β-catenin phosphorylation was performed as described previously [9]. In brief, affinity-purified GST-β-catenin was mixed with GST-HIPK2 (aa 1–629) in 30 μl of kinase buffer (50 mM HEPES pH 7.0, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 0.15 M NaCl, 5 mM $MgCl_2$) and incubated for 30 min at 30 °C. The samples were resolved by SDS–PAGE, and the amounts of phosphorylation were evaluated by Western blotting with Ser33, Ser37-specific anti-phospho-β-catenin antibody (Cell signaling).

See Materials and methods section of Supplementary data for detailed experimental procedures for plasmid constructions, mRNA injection into *Xenopus* embryo, reverse transcription-PCR, GST pull-down assays, Western blot and immunoprecipitation, Establishment of shRNA-expressing cell lines and subcutaneous injection of CT-26 cells into BALB/c mice.

3. Results

3.1. Knock-down of HIPK2 increases β-catenin stability

In a yeast-two hybrid screen using the C-terminus (aa 503–1189) of HIPK2 as bait, we identified β-catenin as a HIPK2-interacting protein. The association of endogenous HIPK2 with β-catenin was also verified via co-immunoprecipitation with anti-β-catenin antibody followed by Western blotting using anti-HIPK2 antibody (Fig. S1). In the course of co-immunoprecipitation experiments, we have seen that the level of β-catenin was decreased by forced expression of HIPK2. Therefore, we assessed the effects of HIPK2 silencing on the level of β-catenin and the β-catenin-mediated transcriptional activation. The knock-down of endogenous HIPK2 enhanced the level of β-catenin (Fig. 1A) and β-catenin-mediated transactivation of the *cyclin D* promoter as well as the TOP-FLASH reporter gene (Fig. 1B and C). As a positive control, the expression plasmid for shRNA against GSK3β was utilized in parallel. Since β-catenin is expressed at a relatively low level and is not associated with plasma membrane in RKO cells [19], RKO cell was chosen for establishment of HIPK2 knock-down cell line to study the effect of HIPK2 on β-catenin stability. The stability of β-catenin was remarkably enhanced in RKO-shHIPK2 cells when compared with that observed in RKO cells (Fig. 1D). These results indicate that the downregulation of HIPK2 increases the stability of β-catenin. We further evaluated the effects of HIPK2 silencing on cellular functions of β-catenin in terms of its localization and activation of target genes. The immunocytochemistry of endogenous β-catenin showed that β-catenin in the RKO-shHIPK2 cell line was detected in both the nucleus and the cytoplasm, while β-catenin in RKO cells was detected in the cytoplasm. Additionally, the quantity of β-catenin, which was determined by intensities of fluorescence, was increased to a greater degree in RKO-shHIPK2 cells than in RKO cells (Fig. 1E). Consistently, the expressions of *c-myc*, *cyclin D* and *DKK1*, typical target genes of β-catenin, were increased to a higher degree in RKO-shHIPK2 cells than in RKO cells (Fig. 1F and G). Taken together, HIPK2 knock-down resulted in the stabilization and concomitant translocalization of β-catenin to the nucleus, and transactivation of β-catenin target genes.

3.2. HIPK2 down-regulates Wnt signaling-mediated transactivation through β-catenin degradation

In order to characterize function of HIPK2 on the regulation of β-catenin, a series of experiments was carried out using transient expression of wild-type and catalytically inactive HIPK2 mutant. Transcription assays were conducted via the transfection of Wnt3a or β-catenin expression plasmids in combination with wild-type or catalytically inactive HIPK2. As shown in Fig. 2A, HIPK2 repressed the Wnt3a- and Wnt1-mediated transactivation of reporter gene. HIPK2 also inhibited β-catenin-mediated transactivation of the reporter gene in a catalytic activity-dependent manner (Fig. 2B). In addition, transcription from the promoter of Wnt target gene, such as *cyclin D*, was repressed by HIPK2 (Fig. 2C). These results indicate that HIPK2 inhibits Wnt/β-catenin-mediated transactivation of target genes.

Because HIPK2 knock-down increased β-catenin stability, we wondered whether β-catenin is degraded by HIPK2. The Western blot revealed that HIPK2 remarkably reduced β-catenin stability, which was blocked by administration of cells with MG132, thereby indicating that HIPK2 induced the proteasomal degradation of β-catenin as GSK3β (Fig. 2D). Consistently, β-catenin stability was dramatically reduced upon HIPK2 expression (Fig. 2E, HIPK2 panel). The inhibitory effect of HIPK2 was compromised by the inactivation of its catalytic activity (Fig. 2E, HIPK2 KR panel). The expression of the HIPK2 N-terminus with an active catalytic domain (HIPK2 1–629) resulted in suppression of β-catenin-mediated transactivation (Fig. 2B, lane 5) and β-catenin degradation (Fig. 2F), in contrast to the catalytically inactive mutant HIPK2 (HIPK2 1–629 K221R) (Fig. 2B and F). Taken together, these results suggest that HIPK2 represses transactivation of Wnt target genes through β-catenin degradation.

3.3. HIPK2 targets β-catenin for phosphorylation and proteasomal degradation

Phosphorylation-dependent β-catenin regulation by HIPK2 led us to test whether HIPK2 can directly phosphorylate β-catenin. The Western blot analysis revealed that the level of β-cat ΔN was not influenced by HIPK2 expression while wild-type β-catenin was decreased by HIPK2 in a manner depending on catalytic activity of HIPK2 (Fig. 3A). These results indicate that degradation of β-catenin by HIPK2 depend on the N-terminal phosphorylation sites of β-catenin in a HIPK2's catalytic activity-dependent manner, suggesting that N-terminal region of β-catenin might be a phosphorylation target of HIPK2. In cultured HEK293 cells, the coexpression of HIPK2 and β-catenin resulted in β-catenin phosphorylation, which was determined by Western blot using the S33- and S37-specific phospho-β-catenin antibody (Fig. 3B). The affinity-purified wild-type β-catenin was phosphorylated by HIPK2 *in vitro*, but substitutions of Ser33 and Ser37 to alanines abolished its phosphory-