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Terminal regions of β-catenin are critical for regulating its adhesion and transcription functions

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ABSTRACT

β-Catenin, the central molecule of canonical *Wnt* signaling pathway, has multiple binding partners and performs many roles in the cell. Apart from being a transcriptional activator, β-catenin acts as a crucial effector component of cadherin/catenin complex to physically interact with actin cytoskeleton along with α-catenin and E-cadherin for regulating cell-cell adhesion. Here, we have generated a library of β-catenin point and deletion mutants to delineate regions within β-catenin that are important for α-catenin-β-catenin interaction, nuclear localization, and transcriptional activity of β-catenin. We observed a unique mechanism for nuclear localization of β-catenin and its mutants and show that N-terminal exon-3 region and C-terminal domain of β-catenin are critical for this activity of β-catenin. Furthermore, we show HepG2 cells have high β-catenin mediated transcriptional activity due to the presence of an interstitial deletion at the N-terminal region of β-catenin. Due to this deletion mutant (hereupon called TM), GSK3β and HDAC inhibitors failed to show any impact whereas curcumin significantly inhibited β-catenin mediated transcriptional activity reiterating that TM is primarily responsible for the high transcriptional activity of HepG2 cells. Moreover, we show the recombinant TM does not physically interact with α-catenin, localizes predominantly in the nucleus, and has nearly two-fold higher transcriptional activity than the wildtype β-catenin.

1. Introduction

Wnt/β-catenin signaling plays critical roles in many biological processes, including stem cell maintenance, embryonic development, and cell destination [1,2]. There are two pools of β-catenin within the cell which are required for two principal roles that include its involvement in cell-cell adhesion junction at the membrane and its transcriptional functions in nucleus [3–6]. As a component of adhesion junction, β-catenin interacts with E-cadherin and α-catenin [7,8]. This trimeric complex then associates with cytoskeleton [9,10]. Cytoplasmic pool of β-catenin is usually kept low *via* destruction complex (APC, Axin, GSK3β and CK1) [11,12], wherein β-catenin is phosphorylated and then degraded through ubiquitination-proteosomal pathway [13]. However, upon Wnt signaling stabilized β-catenin translocates to the nucleus and associates with the TCF/LEF transcriptional factors to regulate gene expression [4].

As a proto-oncogene, β-catenin is implicated in cancer development [14–16]. Aberrant activation of Wnt/β-catenin pathway, due to APC and axin mutations, β-catenin exon-3 missense mutations, N-terminal interstitial deletions [17–20], is commonly found in a variety of cancers [21–24]. Moreover, α-catenin deletions, that promote hyper-proliferation of epidermal cells [25–27], and mutations are seen in many cancer cell-lines as well as primary cancers [28]. Effect of α-catenin regulation on tumor development occurs primarily through β-catenin [29]. Overexpression of α-catenin has been shown to decrease the β-catenin transcriptional activity while the down-regulation of α-catenin has the opposite effect [25–27]. Nevertheless, the mechanism underlying this α-catenin-dependent regulation of β-catenin remains elusive.

β-catenin is composed of three domains: N-terminal domain (NTD) (~150 residues), central arm-repeat domain (~500 residues) and a C-terminal domains (CTD) (~100 residues) [30,31]. These NTD and

Abbreviations: TCF, T-cell factor; TOP, TCF4 optimal promoter; GFP, green fluorescent protein; GFP-WT, GFP recombinant wildtype beta-catenin protein; TM, truncation mutant of beta-catenin; ΔN, deletion from N-terminal; ΔC, deletion from C-terminal; 24AA, 24 amino acids; GSK-3β, glycogen synthase kinase 3β; NPC, nuclear pore complex; SAHA, suberoylanilide hydroxamic acid; HDAC, histone deacetylase.

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CTD are mutually interregulated because they influence the binding of each other to the arm-repeats, as well as the binding of other factors to the arm-repeat and the terminal regions [32–34]. The unstructured nature of terminal domains compounds the β-catenin structural studies as well as inhibitor identification [35,36]. Thus exact role of these terminal regions in β-catenin stabilization, adhesive and transcriptional activities needs further investigation.

Multiple studies have been carried out to understand the role of exon-3 point mutants in cancer and cell signaling, conversely deletions mutations, more specifically in-frame deletions [20,37], seen in many cancers have received less attention. N-terminal interstitial deletion have been observed in many cancers [38–40] and cancer cell lines like HepG2 [41], HSC39/40 [42] and hepatoblastomas [43]. HepG2, a liver specific cell line, has two forms of β-catenin, a wildtype protein and a truncated protein that is shorter than wildtype [41].

In this study, we generated a library of β-catenin variants and carried out comprehensive and comparative analysis of these proteins using multiple assays in order to ascertain their role in cell-cell adhesion and transcription. Furthermore, we identified translocation of β-catenin and its variants into the nucleus in a unique fashion that is determined by sequences in its terminal regions.

2. Material and methods

2.1. Cell lines and transfections

HEK293 and Hep3B cells were obtained from American Type Culture Collection (ATCC), while as HepG2 cells were obtained from Sigma. All transfections were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.2. Plasmids

TM was cloned using total RNA extracted from HepG2 cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was used as the template for RT-PCR performed with gene specific primers to amplify the truncated version of β-catenin. GFP-WT β-catenin construct was produced by PCR amplification using 6×-Flag wildtype β-catenin as template. The PCR products were subcloned into the pEGFP-N3 vector (Clontech, Palo Alto, *CA*) between *Nhe*I and *Bam*HI restriction sites. All the deletion constructs were generated using similar strategy. β-Catenin site-specific mutants were generated by QuickChange Site-Directed Mutagenesis kit (Stratagene) using GFP-WT or GFP-TM as template. GST-tagged α-catenin was generated using flag-tagged α-catenin plasmid (kindly gifted by Lu Z, M.D. Anderson Cancer Center, Houston, USA) as template and inserted into the pGEX4T1 vector (Amersham Pharmacia Biotech) between *Eco*RI site and *Not*I restriction sites. His-WT and His-TM constructs were created using GFP-WT and GFP-TM as templates and subcloned into the pET28a Vector (Novagen) between the *Nco*I and *Sal*I restriction. All constructs were sequenced to verify the integrity of each clone. The primers used for PCR amplification to create these constructs are listed in Supplementary Table 1.

2.3. Antibodies

Anti-β-catenin (SC-7963), *anti*-cyclinD1, *anti*-GFP, *anti*-GAPDH, HRP-conjugated secondary antibodies; *anti*-mouse IgG and *anti*-rabbit IgG were from Santa Cruz Biotechnology.The *anti*-β-actin (CST) and *anti*-α-catenin (Cat No-610,193, BD Biosciences) were used.

2.4. Western blot analysis

For Western blot analyses, cells were trypsinized and washed in PBS, and then resuspended in RIPA buffer-A (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mg/ml leupeptin, 5 mg/ml aprotinin, 1 mM PMSF) and incubated for 40 min on ice with a vortex of 10 s after every 10 min. The lysates were cleared by centrifugation at 14,000 rpm for 20 min at 4 °C, and the protein concentration of the lysates was determined using a Bradford assay. Equal quantity of proteins were resolved on 8 to 12% SDS-PAGE and blotted onto PVDF membrane (Immobilon-P Transfer Membrane Millipore) at 110 V for 2 h. The blots were blocked with 4% BSA in TBST(20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween20) for 1 h and then incubated with primary antibody to β-catenin(1:1000), cyclin D1(1:250), GFP mAb (1:500), GAPDH mAb (1:500), α-catenin (1:200) and β-actin (1:2000) for 1–3 h at room temperature. Blots were then washed with TBST for 3 times after the interval of 5 min. The secondary antibodies used were: *anti*-mouse IgG (1:500) and goat anti-rabbit IgG(1:2000) and after incubations for 1–2 h, blots were detected using enhanced chemiluminescent HRP substrate (Millipore).

2.5. Immunoprecipitation

HEK293 cells were transfected either with GFP-WT or GFP-TM constructs of β-catenin. After 24 h post transfection, cell lysates 700 μl (700 μg protein) were made in RIPA buffer-B (0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mg/ml leupeptin, 5 mg/ml aprotinin, 1 mM PMSF) and precleared by incubation with 0.5 μg of goat *anti*-mouse IgG antibody together with 20 μl of resuspended volume of agarose beads (Protein A/G PLUS-Agarose) for 30 min at 4 °C with continuous mixing. After centrifugation at 2500 rpm for 5 min, the supernatant was incubated with 1 μg *anti*-GFP mAb for 2 h at 4 °C. To these immunocomplexes 30 μl of agarose beads (Protein A/G PLUS-Agarose) were added and further incubated for 2 h at 4 °C. Pellet was collected by centrifugation at 3000 rpm (approximately 1000 ×*g*) for 5 min at 4 °C and washed 3–4 times with RIPA buffer-B prior to being denatured. The immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with *anti*-α-catenin antibody. 10% of total cell lysate, used as input, were also subjected for SDS-PAGE and immunoblotting.

2.6. α-Catenin knockdown

Hep3B cells were transiently transfected with 100 nM control or α-catenin siRNA for 72 h and then lysed and processed for immunoblotting with *anti*-α-catenin antibody, immune detection by GAPDH antibody was used for confirming the equal loading of lysates. For Luciferase assays, after 48 h post transfection with control or α-catenin siRNA, Hep3B cells were transfected with TOP or FOP FLASH DNA and Renilla plasmids by Lipofectamine 2000, and grown further for 24 h. TOP-FLASH readings were taken as described above. Small interfering α-catenin RNA (α-catenin siRNA) was from Santa Cruz Biotechnology.

2.7. Recombinant protein purification and pull-down assays

β-Catenin-pET28a (His-WT and His-TM) as well as α-catenin-pGEX4T1 full length protein were expressed in *Escherichia coli*. Briefly, recombinant plasmids were transformed in *E. coli* BL21 (DE3) expression system, and induced with 0.6 mM IPTG (Sigma) at 0.6 O.D, and grown further for 4–6 h at 37 °C. Bacteria were harvested at 6000 rpm, and the pellet was kept at −80 °C.

Purification of His-tagged β-catenin proteins (His-WT and His-TM) were performed using Ni-NTA beads (QIAGEN). Cleared lysates of bacterial pellets were incubated with Ni-NTA beads at 4 °C with continuous mixing for 2 h. The incubated beads were loaded on a polypropylene coloumn (QIAGEN) and washed with 20 mM and 50 mM imidazole followed by elution of protein with 200 mM imidazole. Purification of GST-tagged α-catenin protein was performed using Glutathione HiCap matrix QIAGEN). The protein was eluted with 15 mM L-Glutathione reduced (Sigma). All fractions were checked on SDS-PAGE, and the