Biochemical and Biophysical Research Communications 436 (2013) 571–577

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tubulin polymerization promoting protein 1 (TPPP1) increases β-catenin expression through inhibition of HDAC6 activity in U2OS osteosarcoma cells

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ARTICLE INFO

Article history:
Received 15 May 2013
Available online 29 May 2013

Keywords:
β-Catenin
TPPP1
ROCK1
Microtubule
Deacetylation
Phosphorylation
Mitogen

ABSTRACT

The Rho-associated coiled-coil kinase (ROCK) family of proteins, including ROCK1 and ROCK2, are key regulators of actin and intermediate filament morphology. The newly discovered ROCK substrate Tubulin polymerization promoting protein 1 (TPPP1) promotes microtubule polymerization and inhibits the activity of Histone deacetylase 6 (HDAC6). The effect of TPPP1 on HDAC6 activity is inhibited by ROCK signaling. Moreover, it was recently demonstrated that ROCK activity increases the cellular expression of the oncogene β-catenin, which is a HDAC6 substrate. In this study, we investigated the interplay between ROCK-TPPP1-HDAC6 signaling and β-catenin expression. We demonstrate that β-catenin expression is increased with ROCK signaling activation and is reduced with increased TPPP1 expression in U2OS cells. Further investigation revealed that ROCK-mediated TPPP1 phosphorylation, which prevents its binding to HDAC6, negates TPPP1-mediated reduction in β-catenin expression. We also show that increased HDAC6 activity resulting from ROCK signaling activation reduced β-catenin acetylation at Lys-49, which was also accompanied by its decreased phosphorylation by Caesin kinase 1 (CK1) and Glycogen synthase kinase 3β (GSK3β), thus preventing its proteasomal degradation. Overall, our results suggest that ROCK regulates β-catenin stability in cells via preventing TPPP1-mediated inhibition of HDAC6 activity, to reduce its acetylation and degradation via phosphorylation by CK1 and GSK3β.

1. Introduction

Rho-associated coiled-coil kinase (ROCK) signaling is a key regulator of several downstream targets, most notably a large array of actin regulatory and intermediate filament proteins. More recently, it was demonstrated that ROCK regulates cellular β-catenin expression, via an as yet undefined mechanism. Enhanced ROCK signaling increases β-catenin expression [1] and its transcriptional target cyclin D1 [2], whereas down-regulation of ROCK signaling reduces β-catenin and cyclin D1 levels [3]. These findings suggest that ROCK-mediated increases in cell proliferation may be partially due to increased β-catenin expression.

β-catenin is an oncogene that plays a crucial role in the canonical Wnt signaling pathway. Binding of Wnt ligands to the Frizzled (Fz)/LRP5/6 (Low Density Lipoprotein receptor protein 5 or 6) co-receptor complex activates the Wnt signaling cascade to increase β-catenin levels, through inhibition of its degradation by the "destruction complex", a multi-protein assembly consisting of the Adenomatous polyposis coli (APC), axin, Casein kinase 1 (CK1) and Glycogen synthase kinase 3β (GSK3β) proteins. Elevated β-catenin levels are accompanied by its cytoplasmic-nuclear translocation, where it binds to the Lymphoid enhancer factor/T-cell factor (LEF/TCF) family of DNA-binding proteins to displace their transcriptional repressor Groucho and promote the transcription of Wnt target proto-oncogenes including c-myc and cyclin D1 [4,5]. In the absence of Wnt signaling, β-catenin is tethered to the assembled "destruction complex", at which point it is fated for destruction via systematic phosphorylation at Ser45 by CK1 [6] and at Ser-31, Ser-37 and Thr-41 by GSK3β [7]. These modifications enable its binding to β-transducin repeat-containing protein (β-TrCP), a component of the ubiquitin E3 ligase, promoting β-catenin ubiquitination and proteasomal degradation [8–10]. In addition to these post-translational modifications, β-catenin is also acetylated on Lys-49, a site frequently mutated in tumors [11]. Acetylation is catalyzed by the CREB-binding protein (CBP)/p300 acetyltransferase, whereas its deacetylation is performed by Histone deacetylase 6 (HDAC6) [12]. Mutation of the Lys-49 to Arg,

Abbreviations: CK1, caesin kinase 1; GSK3β, glycogen synthase kinase 3β; HDAC6, histone deacetylase 6; ROCK, rho-associated coiled-coil kinase; TPPP1, tubulin polymerization promoting protein 1; β-TrCP, β-transducin repeat-containing protein.

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http://dx.doi.org/10.1016/j.bbrc.2013.05.076

an amino acid that cannot be acetylated, increases β-catenin-mediated transcriptional activation of c-myc compared to its wild-type counterpart [11], suggesting that β-catenin acetylation regulates its stability, cytoplasmic-nuclear shuttling and/or gene promoter affinity.

Tubulin polymerization promoting protein 1 (TPPP1) is a Microtubule (MT) regulatory protein that drives MT polymerization and stabilization [13]. It stabilizes MTs through binding and inhibition of HDAC6 activity, resulting in increased levels of acetylated MT [14]. We recently demonstrated that the ROCK-TPPP1 signaling pathway prevents TPPP1-mediated inhibition of HDAC6 activity [15] resulting in decreased MT acetylation. Moreover, we established that TPPP1 overexpression decreases osteosarcoma (U2OS) cell proliferation while its knockdown increases it [16].

The aim of this study was to investigate a mechanism that contributes to ROCK-mediated regulation of β-catenin levels. We show here that ROCK signaling regulates β-catenin levels via preventing the TPPP1/HDAC6 interaction to increase β-catenin deacetylation and expression.

2. Materials and methods

2.1. Plasmid constructs

pcDNA3-Flag-ROCK1Δ4 and pcDNA-Flag-ROCK1Δ4-KD constructs were a generous gift of Dr. S. Narumiya (Kyoto University, Japan) [17]. pBABE-Flag-TPPP1 [18], pBABE-Flag-TPPP1-S32A/S107A/S159A (TPPP1^{3Ala}) and pBABE-Flag-TPPP1-S32E/S107E/S159E (TPPP1^{3Glu}) plasmids were generated as previously described [15].

2.2. Mammalian cell culture

U2OS cells were cultured in DMEM supplemented with 10% FBS and maintained in a 5% CO_2 atmosphere at 37 °C. RNAi experiments were performed with hTPPP1 ON TARGETplus SMARTpool siRNA (Dharmacon). Cells were transfected with 10 nM of siRNA using the Lipofectamine™ 2000 (Life Technologies) transfection reagent in Opti-MEM® according to manufacturer's recommendations. Cells expressing ROCK constructs were transiently transfected with the appropriate constructs using the FuGene® 6 transfection reagent according manufacturer's instruction. U2OS cell lines stably expressing TPPP1 were generated by infection with amphotropic retroviruses as previously described [15].

2.3. Immunofluorescence microscopy

Cells grown on uncoated glass coverslips were fixed with ice-cold 100% methanol for 5 min followed by blocking in 10% FBS for 1 h at room temperature. Blocked cells were incubated with the following primary antibodies (Ab) overnight at 4 °C: Anti-Flag IgG (1:100) [19], Anti-β-Catenin IgG (1:1000) [Sigma; Cat #C7082] and Anti-phospho-MLC IgG S18/T19 [Cell Signalling; Cat #3674]. Incubation with anti-TPPP1 IgG (1:100) [18] was performed at room temperature for 1 h. Secondary Ab incubations were performed for 45 min at room temperature with the following Abs: Anti-Mouse IgG Alexa Fluor 488 (1:400) [Molecular Probes; Cat #A-11034], Anti-Rat IgG Alexa Fluor 594 (1:200) [Molecular Probes; Cat #A-21209] or Anti-Rabbit IgG Alexa Fluor 594 (1:400) [Molecular Probes; Cat #A-21207] together with Hoechst [1:10,000]. Coverslips were mounted on fluorescent mounting medium and stored at 4 °C. Images were captured on a Nikon C1 confocal microscope using the NIS software with a PLAPO VC 60X02PH NA1.4 objective lens. Confocal images were saved as grayscale TIFF files. Brightness adjustments and pseudo-coloring was performed with Adobe Photoshop v11.0.2 and applied to all images in a comparative group.

2.4. Immunoblotting

Immunoblotting was performed as previously described [15]. The following Abs were used: Anti-acetyl-α-Tubulin Lys40 (1:5000) [Sigma; Cat #T7451], Anti-acetyl-β-catenin Lys49 (1:500) [Cell Signalling; Cat #5934], Anti-c-myc (1:1000) [Life Technologies; Cat #AHO0062], Anti-Flag 9H1 clone (1:3000) [19], Anti-GAPDH HRP (1:3000) [Cell Signalling; Cat #3683], Anti-TPPP1 (1:1000) [18], Anti-α-tubulin (1:5000) [Sigma; Cat #T5168], Anti-β-catenin (1:3000) [Sigma; Cat #C7082], Anti-phospho-β-catenin Ser45 (1:1000) [Cell Signalling; Cat #5964], Anti-phospho-β-catenin Ser31/37/Thr41 (1:1000) [Cell Signalling; Cat #5961] and Anti-phospho-MLC Ser18/Thr19 (1:1000) [Cell Signalling; Cat #3647]. Protein band densitometry was performed using the ImageQuant 7 software (Molecular Dynamics (CA, USA)).

3. Results

3.1. ROCK regulates β-catenin levels in U2OS cells

Previous studies described that ROCK signaling increases β-catenin expression in various cell lines. To confirm that this is also the case in U2OS cells, we transiently expressed constitutively active Flag-ROCK1Δ4 (Flag-ROCK1), its K105G kinase dead mutant (Flag-ROCK1-KD) or vector control. Analysis of total β-catenin levels by immunoblotting (Fig. 1A) and immunofluorescence microscopy (Fig. 1B) showed that overexpression of Flag-ROCK1, but not that of Flag-ROCK1-KD, resulted in increased β-catenin levels as well as an increase in the level of its transcriptional target c-myc. Furthermore, inhibition of ROCK activity, by treatment of U2OS cells with the small-molecule ROCK inhibitor Y-27632, decreased β-catenin levels when analyzed by immunoblotting (Fig. 1C) and immunofluorescence microscopy (Fig. 1D). Therefore, we clearly show that ROCK signaling regulates β-catenin expression in U2OS cells.

3.2. ROCK-TPPP1 signaling regulates β-catenin levels

Our recent study demonstrated that TPPP1 knockdown increases cell proliferation [16]. Since β-catenin is a well known mitogen that is regulated by HDAC6 activity [20], we investigated the possibility that TPPP1, through inhibition of HDAC6 activity, may reduce β-catenin levels. We previously established that ROCK-TPPP1 signaling negates TPPP1-mediated inhibition of HDAC6 activity [15], therefore we analyzed β-catenin expression in stable U2OS cells expressing wild-type TPPP1, TPPP1^{3Ala} (ROCK phospho-inhibitory mutant), TPPP1^{3Glu} (ROCK phospho-mimetic mutant) or vector by immunoblotting (Fig. 2A) and immunofluorescence microscopy (Fig. 2B). Our analysis revealed that overexpression of wild-type TPPP1 or TPPP1^{3Ala} significantly decreased β-catenin levels, with TPPP1^{3Ala} exhibiting a greater reduction in β-catenin levels. In contrast, overexpression of TPPP1^{3Glu}, a mutant that mimics its ROCK phosphorylated form, had no affect on β-catenin expression. Moreover, down-regulation of endogenous TPPP1 levels with a TPPP1-specific siRNA significantly increased β-catenin levels and its transcriptional target c-myc compared to the non-targeting (NT) control as demonstrated by immunoblotting (Fig. 2C) and immunofluorescence microscopy (Fig. 2D). These results suggest that ROCK and TPPP1-mediated regulation of β-catenin expression is dependent on their modulation of HDAC6 activity. This is supported by the differences in β-catenin levels in the TPPP1 and TPPP1^{3Ala} expressing cells that is likely due to partial phosphorylation of the wild-type TPPP1.