Biochemical and Biophysical Research Communications 441 (2013) 555–559

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Ubiquitin E3 ligase SCF$^{\beta\text{-TRCP}}$ regulates TRIB2 stability in liver cancer cells

Yongxia Qiao [a,1], Yue Zhang [b,*,1], Jiayi Wang [c,*]

[a] School of Public Health, Shanghai Jiaotong University, Shanghai 200025, China
[b] Department of Central Laboratory, Shanghai Tenth People's Hospital of Tongji University, Shanghai 200072, China
[c] Department of Laboratory Medicine, Shanghai Tenth People's Hospital of Tongji University, Shanghai 200072, China

ARTICLE INFO

Article history:
Received 9 October 2013
Available online 5 November 2013

Keywords:
Cullin1
Hepatocellular carcinoma (HCC)
Ubiquitination
Protein stability

ABSTRACT

Tribbles homolog 2 (TRIB2) is functionally important for liver cancer cell survival and transformation. Our previous study demonstrates TRIB2 is stable in liver cancer cells due to the impaired ubiquitination by Smurf1. However, overexpression of Smurf1 alone cannot completely abolish TRIB2 protein expression, whether other potential factors involved in the degradation of TRIB2 still remains unclear. In the present study, we reveal that the stability and ubiquitination of TRIB2 can also be controlled by ubiquitin E3 ligase SCF$^{\beta\text{-TRCP}}$. Depletion of either Cullin1 or β-TRCP up-regulates TRIB2 protein expression. Moreover, knockdown of β-TRCP extends the half-life, whereas reduces ubiquitylation of TRIB2. Similar to Smurf1, β-TRCP exerts its role through the TRIB2 Degradation Domain (TDD) at the N-terminus of the TRIB2 protein. Hence, we add TRIB2 to the substrate list of SCF$^{\beta\text{-TRCP}}$ and the finding may be helpful in the treatment of TRIB2 dependent liver cancer.

1. Introduction

TRIB2 is a member of the tribbles family pseudokinase proteins originally identified by their roles in Drosophila morphogenesis [1]. Emerging evidence suggests a potential role of tribbles proteins in both solid and non-solid malignancies [2–5]. By using a combination of genomic and cellular approaches, we recently identified TRIB2 as a critical downstream effecter of Wnt signaling in liver cancer cells [6]. Moreover, we found TRIB2 is also functionally important for liver cancer cell survival and transformation, and acts as a crucial signaling nexus to couple the Hippo/YAP and C/EBPα pathways to Wnt-induced liver tumorigenesis [6]. Given that TRIB2 modulates many cellular functions, it can be hypothesized that aberrant TRIB2 expression may be responsible for the development of human cancer and could represent a novel therapeutic target.

Our and other studies suggest that TRIB2 functions as a protein that interacts with ubiquitin E3 ligases, and thereby modulates the protein stability of downstream effecters [3–7]. However, the stability control of TRIB2 per se is still largely unclear. Protein ubiquitination mediated by the ubiquitin proteasome system (UPS) plays an essential regulatory role in critical cellular processes. In this system, E3 ubiquitin ligases play an indispensable role by recognizing specific substrates, and facilitating or directly catalyzing ubiquitin transfer to the respective molecular targets [8]. The relevance of the E3s in several biological processes is emphasized in vivo and often accompanied by the occurrence of cancer [9,10]. Most recently, we described ubiquitin E3 ligase Smurf1 is involved in the ubiquitination and proteasomal degradation of TRIB2. Phosphorylation of TRIB2 by P70S6K was also found to promote Smurf1 induced degradation. Impaired ubiquitination and phosphorylation by Smurf1 and P70S6K in liver cancer cells contributes to the relative higher expression and more stability of TRIB2 compared to that in the normal hepatic cells [11]. However, overexpression of either Smurf1 or P70S6K cannot completely abolish TRIB2 protein expression in liver cancer cells. Therefore it is important to reveal other potential related factors involved in the degradation of TRIB2.

The SCF is a four-subunit RING-type E3 ligase consisting of the RING domain protein Rbx1, two scaffold proteins (Culin1 and SKP1), and one of the many F-box proteins [12]. The F-box proteins determine substrate specificity [12]. β-Transducin Repeat-Containing Proteins (β-TRCP) is a member of the F-box protein family. β-TRCP was reported that plays redundant roles in the ubiquitination of various of substrates, including β-catenin [13], IkB [14], and YAP [24]. In our previous study, we demonstrated that TRIB2

Abbreviations: TRIB2, tribbles homolog 2; HCC, hepatocellular carcinoma; IF, immunofluorescence; CHX, cycloheximide; β-TRCP, beta-transducin repeat containing E3 ubiquitin protein ligase; SCF complex, SKP1–cullin–F-box complex; TDD domain, TRIB2 degradation domain.

* Corresponding authors. Address: No. 301 Middle Yanchang Rd, Shanghai Tenth People's Hospital of Tongji University, Shanghai 200072, China. Fax: +86 21 66313245 (Y. Zhang), +86 21 66303643 (J. Wang).

E-mail addresses: zhangyue0147@126.com (Y. Zhang), karajan2@163.com (J. Wang).

[1] These two authors contribute equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.123

interacts with β-TRCP at C-terminus [6], however; there is no direct evidence showing that β-TRCP regulates TRIB2 stability.

In this study, we reveal that the stability and ubiquitination of TRIB2 can be also controlled by Ubiquitin E3 ligase $SCF^{\beta\text{-}TRCP}$. Similar to Smurf1, β-TRCP exerts its role through the TRIB2 Degradation Domain (TDD) at the N-terminus of the TRIB2 protein. Hence, we add TRIB2 to the substrate list of $SCF^{\beta\text{-}TRCP}$ and the finding may be helpful in the treatment of TRIB2 dependent liver cancer.

2. Materials and methods

2.1. Cell culture and vectors

Liver cancer cell lines Bel-7402 and SMMC-7721 cells were cultured in DMEM. Cells were treated by 50 μg/ml protein synthesis inhibitor, cycloheximide (CHX, Sigma), or 25 μM proteasome inhibitor, MG132 (Cayman) 5 h before harvest. Constructions of the expression plasmids for β-TRCP, Cullin1 and TRIB2 as well as the lentiviral ShRNA against β-TRCP were described in our previous study [6,9,11]. Lentiviral shRNA against Cullin1 (TRCN0000003392) was purchased from Open Biosystem. Dominant negative forms of Cullin2, 3, 4A, 4B, and 5 were constructed according to the previous study [15].

2.2. Immunofluorescence (IF), and western blotting (WB)

For IF, cells were fixed by 4% paraformaldehyde for 15 min, washed with PBS and blocking buffer (3% FBS + 1% HISS + 0.1% Triton X-100), and then incubated overnight at 4 °C in primary antibodies against TRIB2 (Abnova, #H00028951-M04) and Cullin1 (Epitomics, #2436). Alexa-Fluor-488 or -555 fluorescent conjugated secondary antibodies (life technologies) were used for detection.

For WB, proteins were resolved on SDS–PAGE gels followed by standard WB. Primary antibodies used were: β-TRCP (Cell Signaling Technology (CST), #4394), HA (CST, #3724 or #2367), Flag (Sigma, #F3165 or CST, #2368), Myc (CST, #2278 or #2276), Ub (CST, #3933), TRIB2 (Abcam, ab84683), GFP (Epitomics, #1533) and GAPDH (CST, #5174).

2.3. Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described [6,9,11]. qPCR primers used for detection TRIB2 were listed as follows: forward: GGTGTGCAAGGTGTTTGATATCAG, and reward: GAAGGAATGCATGTCCCCATAG.

2.4. Immunoprecipitation

Cells were washed with PBS and subsequently lysed in Western/IP lysis buffer (Beyotime). Protein lysates were centrifugated at 14,000×*g* for 10 min to pellet debris. After preclearing for 1 h with 50 μl of protein A/G-Sepharose (Novex), the supernatants were incubated at 4 °C overnight with 3 μg antibodies as indicated crosslinked to protein A/G-Sepharose beads. Beads were washed five times with lysis buffer, resuspended in SDS loading buffer, and analyzed by WB analysis with antibodies as indicated.

2.5. In vivo and in vitro ubiquitination Assays

For the in vivo ubiquitination assay, cells were treated with MG132 (25 μM) for 5 h before harvest to avoid the proteasome-mediated degradation. The cell lysate was prepared in HEPES lysis buffer supplemented with protease inhibitors (CST), and proteins were immunoprecipitated with the indicated antibody and detected by WB with antibody as indicated.

For the in vitro ubiquitination assay, active E1 and E2 (Boston Biochemistry), HA-Ub (a gift from Jiabin Sun from Shanghai Jiaotong University), $SCF^{\beta\text{-}TRCP}$ complex (GST-BTRC, GST-Cullin1, GST-Skp1 and GST-Rbx1) (Abnova), and TRIB2-Flag (expressed in HEK293T cells and purified by immunoprecipitation with an anti-Flag antibody) were incubated at 30 °C for 2 h, and the assay was terminated with protein loading buffer as described by Guo, et al. [16].

3. Results

3.1. Cullin1 interacts and controls TRIB2 expression

As multisubunit Cullin–Ring complexes comprise the largest known family of E3 ligases [17], we first tested whether a specific Cullin–Ring complex was involved in regulating TRIB2 degradation. Because both TRIB2-Flag and Cullin1-Myc were cloned into the pcDNA3 vector, and the expression of the two proteins were all driven by the CMV promoter, limited cellular context needed for the activation of the CMV promoter may be shared by these two plasmids when they co-transfected. Thus, increasing concentration of pcDNA-Cullin1-Myc may reduce the CMV promoter efficiency from the co-transfected constant amount of pcDNA-TRIB2-Flag plasmid, and inevitably leading to the does dependent down-regulation of the TRIB2-Flag without demonstrating whether Cullin1-Myc protein has a genuine negative effect on TRIB2-Flag protein expression. To avoid this, increasing concentration of pcDNA-TRIB2-Flag (0.25 μg for lane 1, 0.5 μg for lane 2, 0.75 μg for lane 3, and 1 μg for lane 4) expression plasmid was co-transfected with increasing concentration of pcDNA-Cullin-Myc expression plasmid (0 μg for lane 1, 0.1 μg for lane 2, 0.15 μg for lane 3, and 0.25 μg for lane 4) into Bel-7402 cells, and we found that even increasing expression of TRIB2-Flag could not prevent its down-regulation by overexpression of Cullin1-Myc in a doses dependent manner (Fig. 1A). Furthermore, ectopic expression of other dominant-negative forms of Cullins had no notably effect on TRIB2 expression in Bel-7402 cells (Fig. 1B), indicating a specific role of Cullin1 on TRIB2 stability.

To further support the role of Cullin1 on TRIB2 expression, we infected Bel-7402 cells with shRNA against Cullin1, and found endogenous TRIB2 could be up-regulated by knockdown of Cullin1 (Fig. 1C). Moreover, overexpression of Cullin1 was sufficient to reverse Cullin1 shRNA induced TRIB2 expression (Fig. 1D), suggesting the specificity of the Cullin1 shRNA.

To confirm the interaction between TRIB2 and Cullin1, we performed microscopy analysis and observed co-localization of endogenous TRIB2 and Cullin1 in both Bel-7402 and SMMC-7721 cells (Fig. 1E). By co-imunoprecipitation (co-IP) experiments, we also found that TRIB2-HA could be readily pulled down by Cullin1-Myc (Fig. 1F), further support a physiological role for Cullin1 in the control of TRIB2 stability. Take together; the above data strongly suggests the involvement of the SCF type of E3 ligase complex in the regulation of TRIB2 stability.

3.2. β-TRCP is the specific F-box protein that controls TRIB2 expression

Although Cullin1 controls TRIB2 expression has been described in the Section 3.1, the identity of the specific F-box protein that binds with Cullin1 to form a functional SCF type of E3 ligase complex remains unknown. In our previous study, we reported an F-box factor, β-TRCP has a direct interaction with TRIB2 [6]. However, whether β-TRCP acts as a regulator to TRIB2 stability is still unclear. In support of β-TRCP capable of regulating TRIB2