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Ubiquitin ligase Cbl-b acts as a negative regulator in discoidin domain receptor 2 signaling via modulation of its stability

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ABSTRACT

Discoidin domain receptor 2 (DDR2), a collagen receptor tyrosine kinase, initiates signal transduction upon collagen binding, but little is known as to how DDR2 signaling is negatively regulated. Herein we demonstrate that Cbl family member Cbl-b predominantly promotes the ubiquitination of DDR2 upon collagen II stimulation. Cbl-b-mediated ubiquitination accelerates the degradation of activated DDR2. Finally, the production of MMP-13, a downstream target of DDR2, is enhanced in Cbl-b-knocked down MC3T3-E1 cells and Cbl-b-deficient mouse primary synovial fibroblasts. Thus, Cbl-b, by promoting the ubiquitination and degradation of DDR2, functions as a negative regulator in the DDR2 signaling pathway.

Structured summary of protein interactions:
DDR2 physically interacts with **Cbl-b** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

Collagen proteins, major components of extracellular matrix (ECM), play important roles in the regulation of cell function and behavior aside from the maintenance of tissue structure and integrity. The signals from extracellular collagens to the cells are transduced by specific cell-surface receptors, including integrins and discoidin domain receptors (DDRs) [1,2].

DDRs, consisting of DDR1 and DDR2, belong to the receptor tyrosine kinase (RTK) subfamily and exhibit a slow but sustained phosphorylation kinetics in response to collagen binding [3]. DDR2 expression is mainly detected in mesenchymal-derived cells [4,5]. Growing biological evidences have demonstrated that DDR2 can regulate cell proliferation, migration, differentiation, as well as extracellular matrix remodeling and epithelial-mesenchymal transition (EMT) [3,6–12]. Upregulation of DDR2 signaling was reported to be associated with diverse human diseases, such as arthritis and cancer [13]. Elevated expression of matrix metalloproteinases (MMPs) that primarily mediate the degradation of ECM represents an important cell response to DDR2 activation [8,14,15]. However, up to date little is known as to how the DDR2 signaling is negatively regulated or attenuated.

Covalent modification of activated RTKs with ubiquitin, which is carried out by a cascade of enzymatic reactions involving E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase), has been well established as a major mechanism for negative regulation of RTK signaling [16]. Cbl family, which consists of three homologues known as Cbl (or c-Cbl), Cbl-b and Cbl-c (or Cbl-3) in mammals, belongs to RING-type E3 ubiquitin ligases [17]. Cbl proteins-mediated ubiquitination can target activated tyrosine kinases for degradation, either by facilitating their endocytic sorting into lysosomes or by promoting their proteasomal degradation [18]. In contrast to the wide expression pattern of Cbl and Cbl-b, Cbl-c expression was reported to be limited in epithelial cells [19], in which DDR2 is always not expressed [4,5]. Thus, in this study, we aimed to investigate whether DDR2 can be negatively regulated by Cbl or Cbl-b.

2. Material and methods

2.1. Materials

The detailed information for the regents and antibodies were described in Supplementary data. The sense sequence of Cbl-b small interfering RNA (siRNA) is: 5′-GCUUCUCUUGUUAGUUUAA

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TT-3′. Xpress-tagged wild-type Cbl, Cbl-b or Cbl-b mutant (Trp to Ala mutation, Cbl-b-W400A) as well as HA-tagged ubiquitin plasmids were used as described previously [20]. Wild type DDR2 or its mutants were tagged with Myc and expressed using pcDNA3.1(-) vectors [21].

2.2. Cell culture and treatment protocols

Cell culture and transfection of HEK 293T and MC3T3-E1 cells were described in Supplementary data. Primary mouse synovial fibroblasts were established by cultivating type B synoviocytes from the synovial tissues of Cbl-b$^{+/+}$ or Cbl-b$^{-/-}$ mice on the B6 background as previously described [21]. The animal protocol was approved by the Committee on the Ethics of Animal Experiments of The La Jolla Institute for Allergy and Immunology (USA). Synovial fibroblasts were used between the fourth to eighth passages. For collagen stimulation, collagen II was dissolved in 0.2% acetic acid at the concentration of 2 mg/mL and coated onto culture plates (6 μg/cm^2). To analyze MMP-13 production in synovial fibroblasts, the cells were treated with collagen II in serum-free media for 48 h. Cell lysates and the culture medium were both collected and the culture medium was concentrated 20-fold before detection.

2.3. In vivo ubiquitination assay, co-immunoprecipitation and immunoblot

For the detection of ubiquitinated DDR2, cells were lysed in Nonidet P-40 (NP-40) lysis buffer [1% NP-40, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM sodium pyrophosphate, 2 mM orthovanadate and 10 μg/ml of both aprotinin and leupeptin]. The cell lysates were heated at 95 °C for 20 min in the presence of 1% SDS to disrupt non-specific protein interactions. The heated cell lysates were then diluted to make the final concentration of SDS to 0.1% for further immunoprecipitation (IP) with antibodies against Myc or DDR2. Anti-Myc or anti-DDR2 immunoprecipitates were separated by SDS–PAGE and immunoblot was performed using antibodies recognizing HA or Ub. To detect the association of DDR2 with Cbl-b, the transfected cells were lysed in Nonidet P-40 (NP-40) buffer and then directly subjected to co-immunoprecipitation.

2.4. Pulse-chase assays

HEK 293T cells were transfected with Myc-DDR2 together with pEF or Cbl-b. 16 h after transfection, the cells were cultured on collagen II-coated plates for another 2 h and then treated with methionine-free DMEM containing 5% dialysed FCS for 1 h, followed by labeling with 100 μCi [35S] methionine (ICN Biomedicals, Inc., Costa Mesa, CA) for 1 h. Cells were then washed three times and cultured for various durations in complete DMEM medium. At each time point, the cell lysates were prepared and then immunoprecipitated with anti-Myc. After separation of the immunoprecipitates, the gel was dried and autoradiographied at −70 °C overnight. The radio-labeled protein bands were quantified using NIH Image J software.

2.5. Real time PCR

Cbl-b primer sequences were 5′-ACGGGTTCACCAAAGTCTTC-3′ (forward) and 5′-ATTGGTGGAGGTCTTTCAGG (reverse). MMP-13 and GAPDH primer sequences were the same as described previously [9,21]. The procedures of RNA isolation and real time PCR were described in Supplementary data.

2.6. Statistical analysis

All experiments were performed independently in triplicate. The results are expressed as the mean ± S.D. of triplicate independent samples. A student's t test was used to examine the differences between the two groups of data. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cbl-b predominantly promotes DDR2 ubiquitination

To investigate whether Cbl or Cbl-b is involved in the ubiquitination of DDR2, HEK 293T cells were transiently co-transfected with Myc-DDR2, HA-Ub and Xpress-Cbl or Xpress-Cbl-b, and then plated onto native collagen II-coated surface to stimulate DDR2 phosphorylation. It was shown that collagen II stimulation led to the formation of a high molecular weight smear that is indicative of ubiquitinated DDR2 protein. The intensity of this smear was enhanced sharply by the ectopic expression of Cbl-b, but only slightly by Cbl (Fig. 1A, *upper panel*). Cbl-b-caused increase in the ubiquitination level of DDR2 was consistent with the decrease in the amount of phosphorylated but non-ubiquitinated form of DDR2 (Fig. 1A, *middle panel*). To further confirm that the ubiquitination of DDR2 directly correlates with its phosphorylation status, we measured the ubiquitination ability of the indicated point mutants of DDR2 (Fig. 1B). As previously reported [22], Y736F and Y741F DDR2 represent phosphorylation-null mutants, while Y740F mutant exhibits enhanced phosphorylation. We found that Y740F DDR2 had a higher ubiquitination level than wild-type DDR2. In contrast, Y736F and Y741F mutants were resistant to induction of ubiquitination (Fig. 1B). These data collectively suggest that Cbl-b may serve as a major regulator of collagen-induced DDR2 ubiquitination.

We then examined whether DDR2 interacts with Cbl-b. HEK 293T cells were co-transfected with Myc-DDR2 and Xpress-Cbl-b in the presence or absence of type II collagen and the cell lysates were then subjected to co-immunoprecipitation assay. As shown in Fig. 1C, Cbl-b could be detected in anti-Myc immunoprecipitates from collagen II-treated cells but not in those from un-stimulated cells. Thus, upon activation, DDR2 can form complex with Cbl-b.

Considering that the E3 ligase activity of Cbl-b can be abolished by a Trp to Ala mutation (W400A) in its RING finger domain [20], we examined the capability of the RING-defective Cbl-b WA to regulate DDR2 ubiquitination. As shown in Fig. 1D, Cbl-b WA mutant failed to mediate the conjugation of ubiquitin to DDR2, suggesting that the functional RING finger domain in Cbl-b is indispensible for its modulation of DDR2 ubiquitination.

3.2. Cbl-b overexpression decreases the protein stability of DDR2

Because ubiquitin-modified tyrosine kinases can be degraded in either lysosome or proteasome [18], we then examined which pathway is linked to DDR2 ubiquitination. HEK293T cells co-expressing Myc-DDR2, HA-Ub and Xpress-Cbl-b were stimulated with collagen II for 2 h, and then treated with proteasome or lysosome inhibitor for 30 min. MG132, a potent proteasome inhibitor, resulted in a dramatic accumulation of ubiquitinated DDR2. In contrast, lysosomal inhibition with NH_4Cl showed no obvious effects (Fig. 2A). This indicates that proteasome pathway might participate in further processing of ubiquitinated DDR2 protein.

We then examined whether the protein stability of DDR2 was affected by Cbl-b using pulse-chase experiment. As shown in Fig. 2B, in both control and Cbl-b-expressing cells, the newly synthesized DDR2 protein (lower bands) were converted into