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E3 ubiquitin ligase CHIP interacts with C-type lectin-like receptor CLEC-2 and promotes its ubiquitin-proteasome degradation

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ABSTRACT

C-type lectin-like receptor 2 (CLEC-2) was originally identified as a member of non-classical C-type lectin-like receptors in platelets and immune cells. Activation of CLEC-2 is involved in thrombus formation, lymphatic/blood vessel separation, platelet-mediated tumor metastasis and immune response. Nevertheless, the regulation of CLEC-2 expression is little understood. In this study, we identified that the C terminus of Hsc70-interacting protein (CHIP) interacted with CLEC-2 by mass spectrometry analysis, and CHIP decreased the protein expression of CLEC-2 through lysine-48-linked ubiquitination and proteasomal degradation. Deleted and point mutation also revealed that CHIP controlled CLEC-2 protein expression via both tetratricopeptide repeats (TPR) domain and Ubox domain in a HSP70/90-independent manner. Moreover, reduced CHIP expression was associated with decreased CLEC-2 polyubiquitination and increased CLEC-2 protein levels in PMA-induced differentiation of THP-1 monocytes into macrophages. These results indicate that CLEC-2 is the target substrate of E3 ubiquitin ligase CHIP, and suggest that the CHIP/CLEC-2 axis may play an important role in the modulation of immune response.

1. Introduction

The ubiquitin-proteasome system plays vital roles in a broad array of cell biological functions including damaged protein degradation, gene transcriptional modulation, cell cycle, cell signaling and immune response [1–7]. The process is dependent on polyubiquitin chain conjugation to target proteins through three enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-protein ligase. E3 ubiquitin-protein ligases mediate a critical step in substrate recognition and are believed to be essential in ubiquitination reactions [8].

C-terminus of Hsc70-interacting protein (CHIP) is identified as a E3 ubiquitin ligase involved in ubiquitination processes of several proteins, such as c-myc, Smad, Runx2 and steroid receptor co-activator 3 (SRC-3) [9–12]. CHIP contains TPR (tetratricopepdide repeat) domain in the N-terminal as well as a Ubox domain in the C-terminal, which has the activity of ubiquitin ligase [13]. CHIP could mediate the degradation of target proteins via the ubiquitin-proteasome system in cooperation with heat shock proteins [13,14]. Therefore, CHIP participates in the quality control of proteins and is critical to determine cell fate [15,16]. Accumulating evidences reveal that CHIP is involved in various physiological processes as well as diverse pathological conditions including neurodegenerative diseases, cardiovascular diseases and tumors [17–21].

C-type lectin-like receptor 2 (CLEC-2) is a transmembrane receptor highly expressed in platelets and to some extent in immune cells [22–24]. With the engagement of its endogenous ligand podoplanin and exogenous ligand snake toxin rhodocytin, CLEC-2 could elicit downstream signals via the single YxxL immunoreceptor tyrosine-based activation motif (ITAM) within its cytoplasmic tail by coupling with the spleen tyrosine kinase (SYK), and functions in lymphatic/blood vessel separation, platelet aggregation and immune response [25,26]. CLEC2-deficient mice also die at the embryonic and neonatal stages, suggesting a crucial role of CLEC-2 in development [27]. Nevertheless, little is understood about the regulation of CLEC-2 expression so far. In our study, we screened the interacting partners of CLEC-2 using immunoprecipitation and mass spectrometry analysis, and found that CHIP functioned as an E3 ubiquitin ligase of CLEC-2.

Abbreviations: CLEC-2, C-type lectin-like receptor 2; CHIP, C terminus of Hsc70-interacting protein; PMA, phorbol myristate acetate; CHX, cycloheximide; HEK293T, human emborynic kidney 293T; MS, mass spectrometry; FACS, fluorescence-activated cell sorting; ITAM, immunoreceptor tyrosine-based activation motif; SYK, spleen tyrosine kinase; TPR, tetratricopeptide repeats; PBS, phosphate-buffered saline.

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2. Materials and methods

2.1. Antibodies and reagents

Anti-Myc antibody was purchased from Merck Millipore. Rabbit anti-CHIP antibody, rabbit anti-ubiquitin antibody and mouse β-actin antibody were purchased from Cell Signaling Technology. Mouse anti-HA antibody, normal mouse IgG, normal rabbit IgG, normal goat IgG and CHIP siRNA (h) were from Santa Cruz Biotechnology. Mouse anti-human CLEC-2 antibody was from R&D Systems. MG132, chloroquine, cycloheximide (CHX) and PMA (phorbol myristate acetate) were from Sigma Aldrich.

2.2. Cell culture

All cell lines were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Human embryonic kidney 293T (HEK293T) and HeLa cells were cultured in DMEM (Sigma Aldrich) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. THP-1 monocytes were cultured in RPMI-1640 media (Gibco) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO2.

2.3. Plasmid construction

Human CLEC-2 cDNA was a generous gift from Dr. Yukio Ozaki (Yamanashi Medical University, Japan). The coding sequence of CLEC-2 was in frame sub-cloned into pcDNA3.1-Myc/His vector (Invitrogen) to generate Myc/His-tagged constructs. The complete coding region of human CHIP (NM_005861) was amplified from cDNA library of Hela cells. The coding sequence of CHIP was in frame sub-cloned into pRK7-N-FLAG vector to generate Flag-tagged wild-type CHIP. Two CHIP deletion mutants comprising amino acids 1–197 (CHIP ΔUbox) and 143–303 (CHIP ΔTPR) were generated by amplifying the coding sequence from Flag-tagged wild-type CHIP. Point mutations of CHIP (CHIP K30A and CHIP H260Q) were generated by amplifying the coding sequence from Flag-tagged wild-type CHIP with overlap extension polymerase chain reaction. The primers used for plasmid construction are listed in Table 1. All constructs were verified by sequencing.

2.4. Transfections and stable cell line construction

The plasmids were transiently transfected into HEK293T or Hela cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. THP-1 cells were incubated with scramble or CHIP shRNA lentiviral particles following the manufacturer's protocols (Santa Cruz Biotechnology). Stably transfected THP-1 cells were selected with 0.1 μg/mL puromycin.

Table 1
Lists of all primers used for cloning and mutants of CHIP.

CHIP	Primers: 5′-3′
WT	F-CAAAAGCTTATGAAGGGCAAGGAGGAG
	R-AAAGAATTCTCAGTAGTCCTCCACCCAGCC
ΔTPR	F-ACAAAGCTTAAGAAGAAGCGCTGGAAC
	R-AAAGAATTCTCAGTAGTCCTCCACCCAGCC
ΔUbox	F-CAAAAGCTTATGAAGGGCAAGGAGGAG
	R-AACGAATTCTCACTGCTGGGCCCGGACGTG
K30A	F1-CAAAAGCTTATGAAGGGCAAGGAGGAG
	R1-GCGCGCAGGAGCTCGCGGAGCAGGGCAATC
	F2-GATTGCCCTGCTCCGCGAGCTCCTGCGCGC
	R2-AAAGAATTCTCAGTAGTCCTCCACCCAGCC
H260Q	F1-CAAAAGCTTATGAAGGGCAAGGAGGAG
	R1-ACATCGAGGAGCAGCTGCAGCGTGTGG
	F2-CCACACGCTGCAGCTGCTCCTCGATGT
	R2-AAAGAATTCTCAGTAGTCCTCCACCCAGCC

2.5. Co-immunoprecipitation

HEK293T cells were grown to 50–60% density in 100 mm dishes and transfected with indicated plasmids. Transfected cells were collected and washed with PBS. All cells were solubilized with IP lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 15 mM $MgCl_2$, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM PMSF), and co-immunoprecipitation was performed as before [28].

2.6. Mass spectrometer analysis

HEK293T cells were transfected with Myc-tagged CLEC-2 plasmids, and immunoprecipitation was performed using normal mouse IgG or anti-Myc tag antibody. Immunoprecipitates were separated in SDS-PAGE gel and visualized by silver staining. Specific bands co-immunoprecipitated with anti-Myc antibody were cut and subjected to mass spectrometry analysis. The experiments were performed on a Nano Aquity UPLC system (Waters Corporation, Milford, MA) connected to a quadrupole-Orbitrap mass spectrometer (Q-Exactive) (Thermo Fisher Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source.

2.7. Western blotting

Briefly, the proteins from cell lysates or immunoprecipitates were separated by standard 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were washed, blocked, and incubated with primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The reactions were detected by enhanced chemiluminescence assay.

2.8. Confocal microscopy

Hela cells were seeded on glass coverslips and transfected with indicated plasmids. After 24–36 h, the transfected cells were washed twice with PBS, fixed with 4% paraformaldehyde at room temperature, and permeabilized. Then cells were incubated with primary antibodies for 1 h, followed by incubation with fluorescent secondary antibodies (Jackson). Nuclei were visualized with DAPI. The cells were visualized with laser confocal scanning microscope (Leica Microsystems Heidelberg GmbH, Germany).

THP-1 cells were harvested on Culture Slides (Falcon, USA) with PBS by centifugation at RCF of 500*g* for 10 min. Then, the adherent cells were fixed, permeabilized, stained, and visualized under the laser scanning confocal microscope as above.

2.9. Flow cytometry analysis

HEK293T cells were transfected with indicated plasmids, and harvested 36 h later. Cells were incubated with mouse anti-Myc primary antibody for 1 h at 4 °C, washed three times with ice-cold PBS, and subsequently incubated with Alexa Fluor 488-labeled secondary antibody for another 30 min at 4 °C. Then cells were washed, suspended in PBS and subjected to fluorescence-activated cell sorting (FACS) analysis by a BD FACSCalibur (BD Biosciences). Data were analyzed by FlowJo software (Tree Star, USA).

3. Results

3.1. E3 ubiquitin ligase CHIP interacts with CLEC-2

To explore the potential interacting partners regulating the expression of CLEC-2, we performed co-immunoprecipitation and NanoUPLC-nano-