

学位論文内容の要旨

博士の専攻分野の名称 博士 (医 学) 氏名 鈴木 正宣

学位論文題名

TRIM39 negatively regulates NFκB signals through stabilization of Cactin
(TRIM39 は Cactin の安定化を介して、NFκB シグナルを負に制御する。)

Background and Objectives

NFκB functions as an important regulator for cell survival, immunity, inflammation, carcinogenesis and organogenesis. In this NFκB signaling pathway, ubiquitination plays important roles in regulation of the activation of NFκB. Skp1-Cul1-F-box protein complex (SCF)-type ubiquitin ligase, SCF^{β-TrCP} interacts with and ubiquitinates IκB protein phosphorylated by IKK. Furthermore, the activation of IKK requires K63-linked polyubiquitin [2,6]. It has also been reported that linear ubiquitin chains are necessary for NFκB activation [7]. These findings indicate that activation of the NFκB signaling pathway greatly depends on several steps of ubiquitination.

Ubiquitination is an important posttranslational modification used by eukaryotic cells. The conjugation of ubiquitin to the target protein is catalyzed by several components, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3).

E3 ubiquitin ligases are the most directly responsible for recognition of the substrate in the ubiquitin conjugation system. E3 ubiquitin ligases have been classified into three groups. Tripartite motif-containing (TRIM) proteins are defined as proteins that consist of a RING domain, one or two B-boxes, a coiled-coil motif, and carboxyl-terminal unique domains. TRIM family proteins are involved in various biological processes, and their alterations therefore often lead to diverse clinical disorders, such as developmental disorders, neurodegenerative diseases, vulnerability to viral infection, and carcinogenesis. It has been reported that approximately half of the TRIM proteins are involved in inflammatory signaling pathways.

TRIM39 is a one of the TRIM family proteins and has an E3 activity, leading to the degradation of some proteins, while TRIM39 has been reported to stabilize some proteins. The *TRIM39* gene is located in the MHC region within chromosome 6p21–23, in which there are six other *TRIM* genes (*TRIM10*, *TRIM15*, *TRIM26*, *TRIM27*, *TRIM31* and *TRIM38*) and many genes that are related to immune response. It has been reported that TRIM39 is involved in the regulation of type I interferon response. In addition, a single nucleotide polymorphism (SNP) on exon 9 of *TRIM39* has been identified as one of the genetic factors in Behcet's disease by multiple logistic regression analysis. These findings suggest that TRIM39 is related to inflammatory signaling pathways. However, the role of TRIM39 in inflammatory signaling pathways has not been fully elucidated.

In this study, with the aim of elucidating the molecular function of TRIM39 in inflammatory signaling pathways, we performed yeast two-hybrid screening using TRIM39 as bait, and examined how TRIM39 affects the identified protein and functions in inflammatory signaling pathways.

Materials and Methods

1. Cloning of cDNAs and plasmid construction

Human *TRIM39* and *Cactin* cDNAs were amplified from a human B cell cDNA library by polymerase chain reaction (PCR). The amplified fragments were subcloned into expression vectors for eukaryotic cells, for a yeast two-hybrid system, and for a baculovirus protein expression system.

2. Yeast two-hybrid screening

To screen for proteins that interact with TRIM39, the yeast strain L40 was transformed with a B-cell Matchmaker cDNA library using the lithium acetate method.

3. Transfection, immunoprecipitation and immunoblot analysis

HEK293T cells were transfected by the calcium phosphate method. The cell lysates were centrifuged and the resulting supernatant was incubated with an antibody. After addition of protein A-Sepharose, each of the mixtures rotated. The resin was washed, and then boiled in SDS sample buffer. Immunoblot analysis was performed to detect immune complexes.

4. Generation of Recombinant Protein.

His₆-tagged TRIM39 and Cactin were expressed in the Sf9 insect cell line by a baculovirus protein expression system. The recombinant His₆-tagged proteins were purified by using ProBond metal affinity beads.

5. *In vitro* binding assay

The mixtures of purified recombinant proteins were incubated with the antibody. After addition of protein A-Sepharose, each of the mixtures rotated. The resin was washed and then boiled in SDS sample buffer. Immunoblot analysis was performed to detect immune complexes.

6. RNA interference

In some experiments, siRNA were transfected into HEK293T cells or HeLa cells with Lipofectamine RNAiMAX.

7. Protein stability assay with cycloheximide.

HEK293T cells were transfected with siRNA specific for human TRIM39 (siTRIM39). Forty-eight hr after transfection, the cells were cultured in the presence of cycloheximide for the indicated times. Cell lysates were subjected to immunoblot analysis.

8. Real-time PCR

Total RNA was isolated from HEK293T or HeLa cells, followed by reverse transcription. The resulting cDNA was subjected to real-time PCR. The average threshold cycle (Ct) was determined from independent experiments.

9. Dual-luciferase assay

Expression vectors, reporter plasmids and the internal control plasmid coding Renilla luciferase were transfected into the cells. Forty-eight hr after transfection, cells were incubated with TNF α or with PolyIC for 6 or 12 hr, and assayed by the Dual-Luciferase Reporter Assay System.

Result

To clarify the function of TRIM39, we isolated TRIM39-interacting proteins from a human B cell cDNA library by yeast two-hybrid screens. Using TRIM39 as a bait, we obtained human Cactin as candidate. To examine whether TRIM39 interacts with Cactin in mammalian cells, we performed an *in vivo* binding assay, which showed that TRIM39 specifically bound to Cactin in mammalian cells. Next, to examine whether TRIM39 directly binds to Cactin, we performed an *in vitro* binding assay between TRIM39 and Cactin. The result showed there was direct interaction between TRIM39 and Cactin.

We speculated that TRIM39 also affected the stability of Cactin. Protein stability analysis showed that knockdown of TRIM39 caused a decrease of endogenous Cactin, suggesting that TRIM39 upregulates the stability of Cactin protein.

To confirm that Cactin regulates NF κ B mediated transcription, we performed a luciferase reporter assay, which showed that Cactin significantly suppressed NF κ B-mediated transcriptional activity. We also found that the mRNA expression level and protein level of Cactin was significantly increased by TNF α treatment. These findings indicated that Cactin also provides a negative feedback loop in the NF κ B pathway.

Finally, we examined whether TRIM39 acts as a regulator of transcription including the NF κ B and Toll-like receptor (TLR). Luciferase reporter assays revealed that knockdown of TRIM39 significantly promoted NF κ B- and ISRE-mediated transcriptional activity induced by TNF α and PolyIC. Quantitative real-time PCR revealed that the mRNA expression levels of IL-6 and IL-8 were significantly increased in HeLa cells in which TRIM39 had been knocked down. These findings suggest that TRIM39 negatively regulated NF κ B- and TLR-mediated transcriptional activity. Furthermore, we also showed by luciferase assay that overexpression of Cactin suppressed the activity of NF κ B even in cells in which TRIM39 had been knocked down, which indicated that the stabilization of Cactin by TRIM39 negatively regulates NF κ B activity.

Discussion

In this study, we identified Cactin as TRIM39-interacting proteins. Cactin was identified as a novel protein interacting with Cactus, the *Drosophila* ortholog of I κ B α , which is important for negative regulation of the NF κ B signaling pathway. Human Cactin has been reported to interact with I κ BL (Inhibitor- κ B-like), which is one of negative regulators of the NF κ B signaling pathway. The human *I κ BL* is localized in the MHC class III region of chromosome 6, in which the *TRIM39* gene is also localized.

According to a previous report, the SNP on exon 9 of *TRIM39* is involved in Behcet's disease. Furthermore, the polymorphism of the *I κ BL* gene locus, which is known to interact with Cactin, is associated with several inflammatory diseases such as, rheumatoid arthritis, Takayasu's arteritis, ulcerative colitis, systemic lupus erythematosus, Sjogren's syndrome, type 1 diabetes, and multiple sclerosis. These findings imply that the dysfunction of TRIM39, Cactin, and I κ BL may be involved in the etiology of inflammatory diseases.

Conclusion

In conclusion, we revealed that TRIM39 regulated NF κ B- and TLR-mediated transcriptional activity through stabilization of Cactin, which provided the negative feedback loop in the NF κ B pathway. However, it is still unclear how TRIM39 regulates NF κ B activity with Cactin. Further study on these proteins will lead not only to the elucidation of their molecular function but also to therapeutic benefits for these inflammatory diseases.