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Thesis Outline

MKRN2 is a novel ubiquitin E3 ligase for the p65 subunit of NF-κB and negatively regulates inflammatory responses

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Introduction

For protection from pathogens, nuclear factor κB (NF-κB) is a pivotal transcription factor for expression of cytokines (IL-6, IL-12, TNF-α), chemokines, growth factors (G-CSF), and effector enzymes to activate innate immunity and subsequent acquired immunity mediated by T and B cells. PDLIM2 was identified as a nuclear ubiquitin E3 ligase targeting the p65 subunit of NF-κB, thereby terminating NF-κB activation in the nucleus. To further investigate how NF-κB activation is regulated, I applied a yeast two-hybrid screening strategy to identify molecules that can interact with PDLIM2 and suppress NF-κB signaling. Isolated MKRN2 was demonstrated to be a protein that can bind to PDLIM2 and negatively regulate NF-κB signaling.

Results and Discussion

1. Identification of MKRN2 by yeast two-hybrid screening

I used a full length murine PDLIM2 as the bait protein for use in a yeast two-hybrid screening for PDLIM2-interacting proteins. A cDNA library prepared from Mouse Embryo 17-day was screened and nineteen clones encoding proteins that specifically interacted with the PDLIM2 bait were isolated. One of these clones encoded MKRN2 (makorin ring finger protein 2), a member of the MKRN protein gene family.

2. MKRN2 is a RING finger domain-containing protein that can bind to PDLIM2

MKRN family consists of four proteins that all have three C3H-type zinc finger domains, which contain three conserved Cys plus one His, at the N-terminus, followed by an unusual Cys-His motif, a C3HC4-type RING finger domain, and a fourth C3H-zinc finger domain at the C-terminus. I predicted that MKRN2 would also possess ubiquitin E3 ligase activity with RING finger domain and investigated whether MKRN2 regulates NF-κB-mediated signaling similar to PDLIM2. Whole cell extracts were prepared from HEK293T cells transfected with His-tagged PDLIM2 together with c-Myc-tagged MKRN2 and subjected to immunoprecipitation with a c-Myc antibody and then

analyzed by Western blotting with a His antibody. As a result, MKRN2 was coimmunoprecipitated with PDLIM2.

3. MKRN2 binds the p65 subunit of NF-κB and negatively regulates NF-κB signaling

I next tested the effect of MKRN2 on NF-κB-mediated gene activation in a reporter assay and found that MKRN2 markedly inhibited p65-mediated transactivation in a dose-dependent manner. I then examined if MKRN2 bound to p65 by a coimmunoprecipitation experiment in 293T cells and demonstrated that MKRN2 was immunoprecipitated with p65. Moreover, I visually confirmed this interaction in HeLa cells transfected with expression plasmids encoding p65 fused with EGFP (enhanced green fluorescent protein) and c-Myc-tagged MKRN2 and imaged by indirect immunofluorescence using epi-fluorescence microscopy. These data suggest that MKRN2 binds to the p65 subunit of NF-κB and inhibits p65-mediated NF-κB activation.

4. MKRN2 promotes p65 ubiquitination and degradation through its RING finger domain

RING finger domains are well known for possessing ubiquitin E3 ligase activity, therefore I next determined whether MKRN2 could ubiquitinate p65 protein and found that MKRN2 enhanced p65 polyubiquitination in a RING finger domain-dependent manner. Moreover, MKRN2 overexpression decreased p65 in the soluble nuclear fraction but increased it in the insoluble nuclear fraction. Treatment of NIH3T3 cells with MG132, an inhibitor of proteasomal degradation, resulted in accumulation of p65 in the insoluble nuclear fraction. These data suggest that MKRN2 shuttles p65 from soluble to insoluble nuclear compartments, where p65 is ultimately degraded by the proteasome. These data indicate that MKRN2 promotes polyubiquitination and degradation of p65 through its RING finger domain.

5. MKRN2 and PDLIM2 synergistically promote p65 ubiquitination and degradation

The effect of MKRN2 on p65 ubiquitination and degradation was quite similar to that of PDLIM2. I therefore examined whether MKRN2 and PDLIM2 work cooperatively to regulate p65 activation. Knockdown of MKRN2 by siRNA impaired PDLIM2-mediated polyubiquitination and degradation of p65, indicating that MKRN2 is required for the activity of PDLIM2 to suppress NF-κB activation. Moreover, coexpression of PDLIM2 and MKRN2 resulted in a marked induction of polyubiquitination and degradation of p65, compared to the expression of either PDLIM2 or MKRN2. These data suggest that MKRN2 and PDLIM2 synergistically promote polyubiquitination and subsequent degradation of p65.

6. Enhanced p65-mediated inflammatory responses accompany MKRN2 deficiency

I knocked down MKRN2 in bone marrow-derived dendritic cells by using siRNA, then stimulated the cells with LPS and analyzed p65 by immunoblot with the p65 mAb. Specific knockdown of MKRN2 resulted in a substantial increase in soluble nuclear p65 protein compared to control cells, but the amounts of cytoplasmic p65 were unaffected by MKRN2 deficiency. Next, I examined TLR-induced proinflammatory cytokine production in MKRN2 knock down dendritic cells and observed a consistent two- to fivefold increase in IL-6, IL-12p40, TNF-α and G-CSF transcripts in response to LPS compared to control cells. These data suggest that MKRN2 inhibits NF-κB-mediated inflammatory responses by controlling nuclear p65 protein levels.

Conclusions

In this study, MKRN2 was isolated based on its ability to associate with PDLIM2 using yeast two-hybrid screening and identified as a novel ubiquitin E3 ligase for p65.

I demonstrated that MKRN2 bound to p65 and promoted polyubiquitination and proteasome-dependent degradation of p65 through its RING finger domain, negatively regulating inflammatory responses in dendritic cells.

Moreover, I also showed that MKRN2 and PDLIM2 synergistically control p65 polyubiquitination and degradation.

Finally, I demonstrated that MKRN2 knock down in dendritic cells resulted in enhanced expression of proinflammatory cytokines, indicating that MKRN2 negatively regulates NF-κB-mediated inflammatory responses.

As the constitutive activation of NF-κB at sites of inflammation is observed in human diseases such as rheumatoid arthritis and bronchial asthma, the MKRN2-mediated pathway to inhibit p65 activation could be a useful new molecular target for the treatment of autoimmune and inflammatory diseases.

Publication

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