論文内容の要旨

論文題目 : Identification and characterization of a novel IκB protein

(新規 IκB タンパク質の同定および解析)

氏名　山内　俊平

All living organisms are constantly exposed to various microbes, such as virus and bacteria, and vertebrates have developed sophisticated defense system against them, i.e. innate immunity and acquired immunity. Innate immunity is the first line of defense against infectious pathogens, mediated by antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs). Acquired immunity is the late response process mediated by highly diverse antigen receptors on T cells or B cells, and antibodies produced by B cells. The diversity of antigen receptors and antibodies is generated by gene rearrangements and allows responses to a wide variety of potential antigens. Innate immune responses play key roles in the regulation of acquired immune responses by producing a battery of cytokines.

Innate immune responses are initiated by APCs that recognize specific structure of a invading pathogens

by patter recognition receptors, Toll-like receptors (TLRs), which recognize a molecular pattern of pathogens, e.g. nucleic acids or components of bacterial cell walls. Binding of such pathogens to TLRs leads to activation of the transcription factors, such as nuclear factor kappa B (NF-κB) and interferon regulator factors (IRFs). They induce the production of pro-inflammatory cytokines and chemokines, which activate innate and adaptive immune responses against invading pathogens. Therefore, it is important to understand the regulatory mechanisms of cytokine expressions from APCs via TLRs.

NF-κB plays a central role in regulating the induction of pro-inflammatory cytokines by TLRs. NF-κB is composed of homo- or hetero-dimer of NF-κB/Rel family proteins, including p65 and p50. The activity of NF-κB is strictly regulated by a member of inhibitor of NF-κB (IκB) proteins. All IκB proteins are characterized by their multiple ankyrin repeat domains and IκBα is a prototypical member of this family. In unstimulated cells, IκBα forms a complex with NF-κB and inhibits the nuclear translocation of NF-κB, keeping it as an inert complex in the cytoplasm. Stimulation of TLRs induces phosphorylation and ubiquitin-dependent degradation of IκBα, leading to release of NF-κB. Free NF-κB then translates into the nucleus and initiates transcription of target genes, including pro-inflammatory cytokines and chemokines, by binding to κB sites in their promoters or enhancers.

In addition to such canonical IκB proteins that are present in the cytoplasm and inhibit the NF-κB activity, a distinct class of IκB proteins has been recently found. Those atypical IκB proteins, including BCL-3, IκBNS and IκBζ, are generally not expressed in unstimulated cells. As they are expressed in the nucleus by TLR stimulation and regulate the NF-κB-mediated transcriptional activity, they are also called as nuclear IκB proteins. Studies on those nuclear IκBs, especially using knockout mice, clearly show that these molecules play important roles in the expression of pro-inflammatory cytokines in the innate immune responses.

In our laboratory, microarray analysis of bone marrow derived DCs (BMDCs) was performed to find

novel molecules involved in the regulation of inflammation. Among many genes expressed in BMDCs, I found one novel gene encoding ankyrin-repeats. This gene had been registered as Ankrd42 in databases, but its function had not been described. Since ankyrin-repeats were known to be important for the interaction with NF-κB, I decided to characterize this protein in detail. The protein encoded by the gene consists of 516 amino acid residues and has 8 ankyrin repeats in the NH_2-terminal region. As it was structurally similar to IκB proteins, I named it IκBη and further analyzed its expression and functions.

IκBη mRNA was rather ubiquitously expressed in all the tissues examined and highly expressed in the brain, lung, testis and ovary, and expressed not only in DCs but also macrophages, T cells and B cells. As described above, IκB proteins are categorized into two groups, cytoplasmic IκBs and nuclear IκBs. Through the immunofluorescence staining and biochemical fractionation, I found that IκBη was predominantly expressed in the nucleus, indicating that IκBη is a nuclear IκB protein. In contrast to the previously characterized nuclear IκB proteins that are highly inducible by LPS in macrophages, IκBη was only slightly induced by stimuli and rather constitutively expressed in macrophage cell line Raw264.7 cells. These results indicate that IκBη is a unique nuclear IκB that is constitutively and ubiquitously expressed in various tissues and blood cells.

One key feature of IκB proteins is their interaction with NF-κB components, and nuclear IκB proteins are known to interact with p50 rather than p65. Co-immunopreciptation assay revealed that IκBη was co-immunoprecipitated with p50 subunit of NF-κB, but not with p65. This result indicates that IκBη associates with a p50 homodimer or a heterodimer of p50 with an NF-κB subunit. Since it is known that nuclear IκB proteins prefer to interact with the p50 subunit of NF-κB, rather than p65, these results suggest that IκBη is characteristically similar to the nuclear IκB proteins, rather than typical cytoplasmic IκBs. I also analyzed the interaction between IκBη and DNA by using avidin-biotion-conjugated DNA-binding (ABCD) assay. IκBη interacted with the IL-6 promoter and also interacted with NF-κB subunits in response to LPS, suggesting that IκBη forms a transcriptional complex on the IL-6 promoter to act as

transcriptional cofactor.

To address the transcriptional activity of IκBη, I employed luciferase reporter gene assay using the NF-κB binding site and found that IκBη enhanced the NF-κB-mediated transcription, suggesting that IκBη positively regulates the NF-κB's activity. To reveal the physiological role of IκBη as a regulator of NF-κB, I knocked down the expression of IκBη in Raw264.7 cells by using siRNA. Knockdown of IκBη suppressed the production of various pro-inflammatory cytokine mRNA, including TNFα, IL-6, IL-1β, G-CSF, GM-CSF, and CXCL-2, indicating that IκBη regulates the NF-κB's transcriptional activity. It is known that there are at least two types of NF-κB-mediated gene expression in response to TLR signaling, i.e. primary and secondary response genes. Primary response genes, such as TNFα and IL-1β, are expressed immediately in response to TLR stimulation in the absence of protein synthesis, while the expression of secondary response genes, such as IL-6, G-CSF and GM-CSF, requires newly synthesized mediators of NF-κB and occurs after the primary responses. Knockdown experiments indicate that IκBη regulates both primary and secondary response genes. Because IκBη is constitutively expressed in the nucleus, it may regulate the primary response genes without protein synthesis.

In contrast to IκBη, IκBζ, another nuclear IκB, is known to be rapidly induced by TLR signaling without protein synthesis, and newly synthesized IκBζ, is essential for the induction of a subset of TLR-dependent secondary response genes. Since knockdown of IκBη showed little or no effects on the expression of IκBNS and IκBζ, IκBη may affect secondary response genes by a mechanism independent of such nuclear IκB proteins, and both IκBη and IκBζ seem to be required for the expression of secondary response genes.

In conclusion, IκBη is a new nuclear IκB protein that contributes to NF-κB-mediated transcription as a nuclear cofactor, and plays an important regulatory role in innate immune responses by regulating the expression of inflammatory genes. Although this study has focused on the innate immune responses of macrophages to TLR signaling, ubiquitous expression of IκBη suggests that it may also play an important role for regulation of NF-κB signaling in non-immune cells.