論文内容の要旨

論文演題

Comparison of the structure and functional analysis of the AF-1 transactivation region of the insect ecdysone receptor

(昆虫の脱皮ホルモン受容体 AF-1 転写活性化領域の構造比較と機能解析)

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Morphogenetic events during insect development are triggered by ecdysteroids. The major insect ecdysteroid, 20-hydroxyecdysone (20E), binds directly to a heterodimeric transcription factor comprising two nuclear receptors, the ecdysone receptor (EcR) and the ultraspiracle (USP), and regulates various cellular processes including cell proliferation, cell differentiation, and cell death. The EcR/USP heterodimer binds to ecdysone response elements in the promoter of various ecdysteroid-responsive genes, and activates a complex transcriptional cascade. The EcR gene was identified in several non-insect arthropods (crustaceans, arachnids, and scorpions) as well as insects, and the molt-regulating function of the ecdysteroid/EcR system is conserved in the major subgroups of Arthropoda.

The ecdysteroid/EcR system has been studied extensively in the selected holometabolous insects such as a fruit fly *Drosophila melanogaster* and a silkmoth *Bombyx mori*. In holometabolous insects, there are two or three EcR isoforms (A and B1 isoforms; *Drosophila* has an additional B2 isoform) that are produced from a single genetic locus by differential promoter usage and alternative splicing. EcR isoforms have a common C-

terminal region that includes the DNA binding and ligand binding domains (the C-F domains), but also have isoform-specific regions in the N-terminal A/B domain (Fig. 1A). In holometabolous insects, different EcR isoforms govern distinct ecdysteroid-stimulated responses during metamorphosis. In *Drosophila*, the EcR-A isoform is predominantly expressed in the proliferative tissues during metamorphosis and is required for adult-specific developmental processes. In contrast, EcR-B isoforms (B1 and B2 isoforms) are expressed in larva-specific tissues and are involved in 20E-triggered larval tissue remodeling during metamorphosis. Because only the A isoform has been identified in basal direct-developing (ametabolous and hemimetabolous) insects, it is not known whether direct-developing insects have multiple EcR isoforms with distinct physiologic functions.

The key to understand the mechanism of the isoform-specific responses to 20E is the isoform-specific transcription activation functions. Generally, the nuclear receptors have two transcriptional activation functions (AF-1 and AF-2), and both AF regions are involved in recruitment of co-regulatory proteins (Fig. 1B). The EcR isoforms have the isoform-specific region in the A/B domain, which contains the ligand-independent activation function (AF)-1 region. Several studies of the AF-1 region in *Drosophila* EcR isoforms have revealed that the A/B domain of *Drosophila* EcR-B isoforms have strong transactivation activity, whereas that of the A isoform has weaker transactivation activity. The AF-1 of *Drosophila* EcR-B1 mainly locates in the N-terminal region (amino acid residues 1-53), whose sequence is considerably conserved among higher holometabolous insects such as flies, mosquitoes, and moths. However, the structural basis and molecular mechanisms underlying the isoform-specific AF-1 functions remain obscure.

The ecdysteroid/EcR system is conserved among arthropods, and the EcR-A and -B1 isoforms were found in several non-insect arthropods as well as insects. Thus, I hypothesized that the mechanisms of the isoform-specific AF-1 region-mediated transcriptional regulation are essentially conserved across arthropod species. If so, even if the isoform-specific AF-1 region of each EcR isoform varies in length and sequence across species, the essential structural basis for transcriptional regulation might be conserved. In search for the essential structural basis of the isoform-specific AF-1 activation function of each EcR isoform, I performed a comprehensive structural comparison of the isoform-specific regions of insect EcR-A and -B1 isoforms. The EcR isoforms were newly identified from 51 species of insects and non-insect arthropods, including direct-developing ametabolous and hemimetabolous insects. The comprehensive structural comparison revealed that the isoform-specific region of each EcR isoform contained evolutionally conserved microdomain structures and insect subgroup-specific structural modifications (Fig. 2): The A isoform-specific region generally contained four conserved microdomains, the SUMOylation motif, the nuclear localization signal, the (D/E)(D/

E)W motif, and the A-box (Fig. 2A). On the other hand, the B1 isoform-specific region contained three conserved microdomains, the S-rich motif, the SP residues, and the DL-rich motif. In addition, the EcR-B1 isoform of holometabolous insects had a novel (K/R)RRW motif at the N-terminal end (Fig. 2B).

To evaluate the functional roles of the conserved microdomains in transcriptional regulation, I performed the luciferase reporter assay. The isoform-specific regions of the *Drosophila* EcR-A and -B1 isoforms were C-terminally fused to the DNA binding domain of the GAL4 transcription factor, and were expressed in the *Drosophila* S2 cells. The reporter assay revealed that the isoform-specific region of EcR-A and -B1 isoforms have a weak transactivation activity and a strong transactivation activity, respectively, in the S2 cells. In addition, the reporter assay using the microdomain-deletion mutants revealed that the microdomain-mediated transcriptional regulations: In the A isoform-specific region, the SUMOylation motif and the A-box were involved in transcriptional regulation. In the B1 isoform-specific region, the (K/R)RRW motif and the DL-rich motif were involved in transcription activation activity (Fig. 3). Given that the nuclear receptor AF-1 is involved in cofactor recruitment, the microdomain structures identified in the isoform-specific region of each EcR isoform might function as signature motifs and/or as targets for cofactor proteins. To test this hypothesis, I examined the microdomain-protein(s) interaction in the isoform-specific region of the *Drosophila* EcR-B1 isoform by using the fluorescence correlation spectroscopy (FCS) and the pull-down assay. The FCS assay using the motif-deletion mutants of the EGFP-fused B1 isoform-specific region revealed that the (K/R)RRW motif is involved in protein-protein interaction in the nucleus of the *Drosophila* S2 cells. Moreover, I obtained a ~50 kDa (K/R)RRW-motif-interacting protein from the *Drosophila* S2 cells by pull-down assay. These data suggest that the holometabolous insect EcR-B1 isoform acquired additional co-regulatory protein and transcriptional regulation mechanisms, which are mediated by the novel (K/R)RRW motif.

This is the first study to determine the structural basis of the nuclear receptor AF-1 activation function based on the comprehensive structural comparison and the molecular evolutionary analysis. This study provides crucial insights into the isoform-specific transcriptional regulation mechanism of the insect EcR isoforms, as well as the structure-function relationships within the EcR isoform-specific AF-1 regions. Moreover, this is the first report on the identification of the EcR-B1 isoform in direct-developing insects. Further comparative studies on the developmental functions of the each EcR isoform in the holometabolous and direct-developing insects will shed light on the molecular mechanisms underlying the evolution of the complete metamorphosis.

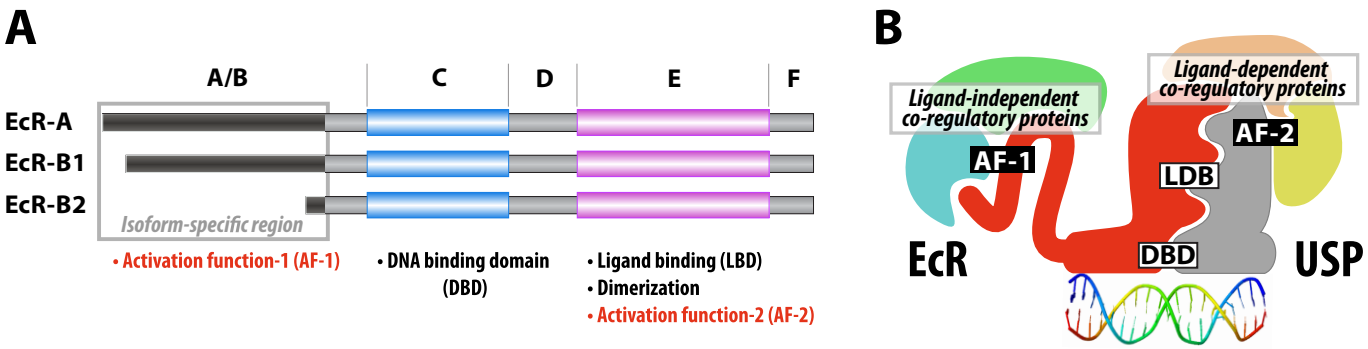

Fig. 1. A) Domain structure of insect EcR isoforms. EcR isoforms have the isoform-specific region in the N-terminal A/B domain, which contains the ligand-independent activation function (AF-1). EcR-B2 isoform was only found in *Drosophila*. **B) Schematic representation of the functional EcR transcriptional complex.** EcR/USP heterodimer bind to the EcRE and recruit co-regulatory proteins on the AF-1 and AF-2. The EcR isoform-specific AF-1 results in the differential recruitment of regulatory proteins.

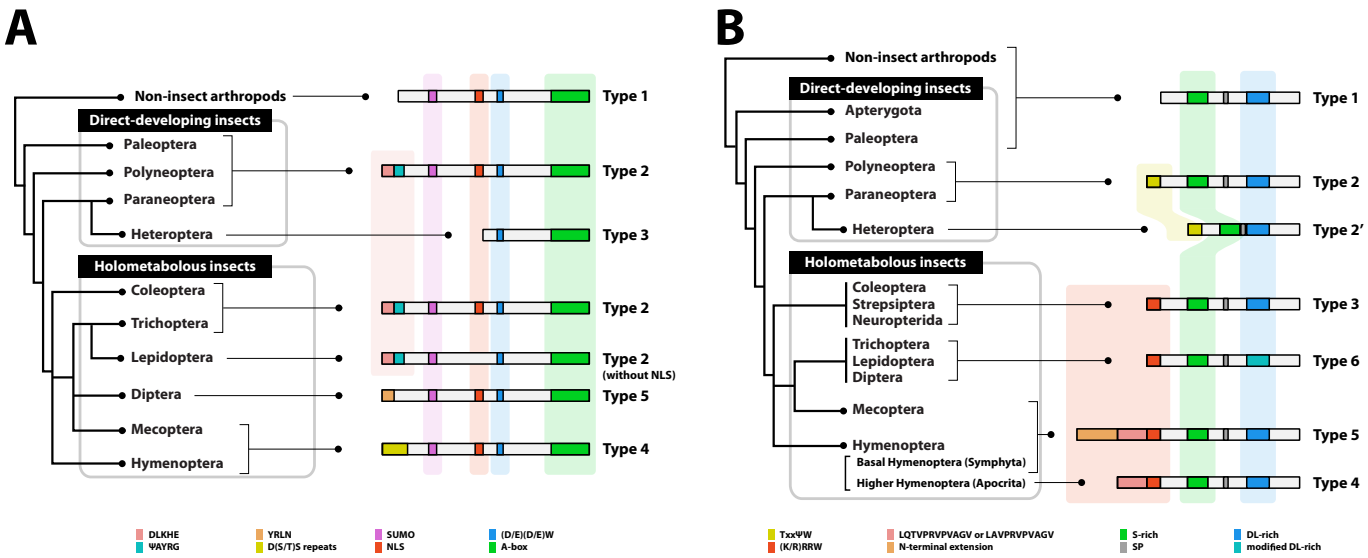

Fig. 2. Structural diversities of the EcR-A isoform-specific AF-1 region in insects. A) Evolution model for the structural modification in the A isoform-specific AF-1 region in insects. B) Evolution model for the structural modification in the B1 isoform-specific AF-1 region in insects. Schematic representation shows the phylogenetic relationship of insect subgroups with the structural types of the A isoform-specific region. Conserved structures are indicated by colored boxes.

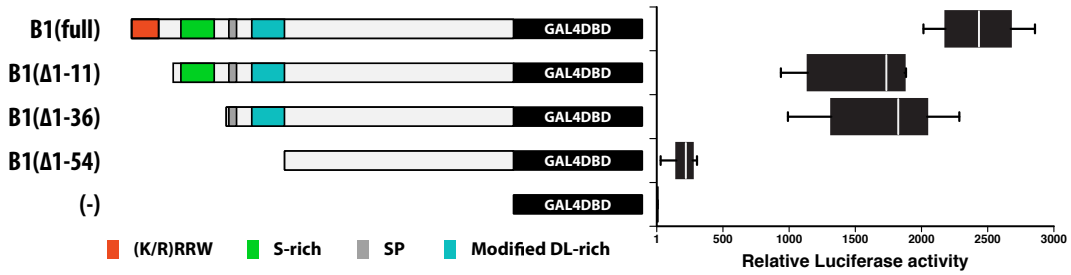

Fig. 3. Luciferase assay to evaluate the AF-1 functions of the *Drosophila* EcR-B1 isoform-specific regions. The isoform-specific region of EcR-A and B1 isoforms were C-terminally fused to the GAL4 DNA-binding domain (GAL4DBD). The isoform-specific region of the *Drosophila* EcR-B1 isoform showed strong transactivation activity in *Drosophila* S2 cells. The deletion of the (K/R)RRW motif and the DL-rich motif resulted in decreased transactivation activity.