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A novel Rel protein and shortened isoform that differentially regulate antibacterial peptide genes in the silkworm *Bombyx mori*☆

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Abstract

Two cDNAs encoding novel Rel proteins were cloned from the silkworm, *Bombyx mori*. These cDNA clones (*BmRelA* and *BmRelB*) showed identical nucleotide sequences except for the 5′-region. *BmRelB* cDNA derived probably from an alternatively spliced mRNA lacked 241 bp nucleotides at the 5′-region of the *BmRelA* cDNA, resulting in a loss of the first 52 amino acids. Expression of antibacterial peptide genes was strongly inhibited upon infection with *Micrococcus luteus* in transgenic silkworms in which *BmRel* gene expression was knocked down, suggesting that these two Rel proteins are involved in activation of antibacterial peptide genes. Co-transfection experiments indicated that BmRelB activated the *Attacin* gene strongly and other genes to a lesser extent, whereas BmRelA activated *Lebocin 4* gene strongly and *Attacin* and *Lebocin 3* genes very weakly. The Rel homology domain of BmRelA and BmRelB was shown to bind specifically to κB sites of antibacterial peptide genes. Proline-rich domains of the BmRels were necessary for activation of antibacterial peptide genes. These results illustrate that a minor structural change in Rel proteins can provoke a dramatic differential activation of antibacterial peptide genes, suggesting a novel regulatory mechanism for insect antibacterial peptide gene expression.

Keywords: Rel protein; Insect immunity; Antibacterial peptide; Transgenic silkworm; Gene knockdown; Differential transcriptional regulation

1. Introduction

The innate immune system in insects and mammals shares structurally and functionally related factors for elimination of invading microbes. Toll homologues and the components involved in signal transduction of immune-related protein gene expression, such as TAK, FADD and Myd88, are known to have significant conservation between *Drosophila melanogaster* and mammals [1]. Rel/NFκB molecules, transcription factors that bind to κB sites and control immune related genes, are also remarkably conserved in *Drosophila* and mammals [2]. A common feature of Rel/NFκB molecules is that all proteins have a well-conserved domain, so-called Rel homology domain (RHD), involved in DNA binding, dimerization and interaction with Inhibitor κB (IκB) [3]. In *Drosophila*, three mammalian Rel/NF-κB homologues that control antibacterial and antifungal peptide genes have been identified. Two Rel/NF-κB proteins, Dorsal [4] and Dorsal-related immunity factor (Dif) [5], are activated by the Toll pathway in response to infection with fungi and Gram-positive bacteria. These factors are localized in the cytoplasm and interact with the mammalian IκB homologue Cactus in unstimulated cells. In response to an infection, Dif and Dorsal translocate

☆ The nucleotide sequences reported in this paper have been deposited in the GSDB/DDBJ/EMBL/NCBI nucleotide sequence databases (accession no. AB096087 for BmRelA and AB096088 for BmRelB).

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into the nucleus by dissociating from Cactus, and activate antifungal peptide genes such as *drosomycin* [6]. On the other hand, another Rel/NF-κB protein, Relish, is activated by the IMD pathway in response to infection with Gram-negative bacteria [7,8]. Relish comprises the N-terminal Rel homology domain (RHD) and C-terminal ankyrin repeat domain. N-terminal fragment including RHD of Relish is released by endoproteolytic cleavage in response to bacterial infection, and translocates from the cytoplasm to the nucleus activating antibacterial peptide genes such as *diptericin* [9]. Recently, several Rel/NF-κB homologues from other insects have been cloned and characterized. According to structural features, insect Rel/NF-κB protein can be categorized into two types, Dif-Dorsal type or Relish type. Gambif1 from *Anopheles gambiae* [10] and A.d.RelA from *Allomyrina dichotoma* [11] belong to the Dif-Dorsal type because these proteins do not have ankyrin repeat domains and their RHDs have much higher homology with Dorsal than Relish. On the contrary, Relish from *Aedes aegypti* [12] and SRAM, a 59-kDa κB-binding protein from *Sarcophaga peregrina* [13], are identified as Relish-type proteins because of the C-terminal ankyrin-repeat domain.

In spite of the detailed functional analysis of Rel proteins in the activation of antibacterial and antifungal peptide genes in *D. melanogaster* [14], their physiological role in other insects still remains obscure. Several antimicrobial peptide genes such as *CecropinA* [15], *CecropinB* [16], *Attacin* [17], *Lebocin* [18] and *Moricin* [19] have been identified from *Bombyx mori*. These genes contain conserved κB motifs in the 5′-upstream region and nuclear factors that bind to κB motif were found by electrophoresis mobility shift assay (EMSA) [20], suggesting the Rel/NF-κB homologues also control antimicrobial peptide genes in *B. mori*. However, the silkworm immune system has not been analyzed well because the lack of transgenic lines with knockout or knockdown-immune genes has been a serious limitation for studying silkworm immunity. We have recently developed a novel method to establish transgenic silkworms, *B. mori*, using *Autographa californica* nucleopolyhedrosis virus and *piggyBac* transposable elements [21]. On the other hand, gene silencing caused by RNA interference has been successfully achieved in *B. mori* [22]. Taking advantage of the combination of these techniques, we attempted *Rel* gene silencing in transgenic *B. mori* to analyze the role of Rel proteins in the activation of antibacterial peptide genes.

In this paper, cloning and functional analysis of two novel Rel protein cDNAs from the silkworm, *B. mori*, are reported. The Rel protein and a shortened isoform likely produced as a result of alternative splicing are structurally identical except for the N-terminal region. The results of functional analysis of these Rel proteins showed that gene expression of some antibacterial peptides were suppressed in *BmRel*-knockdown transgenic silkworms infected with *Micrococcus luteus*. The results also indicated that a minor structural difference in the Rel proteins can cause a significant functional difference in regulation of antibacterial peptide gene expression. This suggests a novel regulatory mechanism for insect immune gene expression.

2. Materials and methods

2.1. Insects

B. mori (C602 strain) was reared on an artificial diet (Nihonnosanko) in a rearing room at 25 °C under a controlled environment (11 h light and 13 h dark). Fifth instar larvae were used in the experiments.

2.2. cDNA cloning and nucleotide sequencing

Total RNA was prepared from the fat body of *B. mori* larvae and first strand cDNA synthesized as described previously [11]. Forward and reverse degenerate primers to clone *B. mori* Rel proteins by reverse transcriptase-polymerase chain reactions (RT-PCR) were designed on the basis of conserved RHD sequences described by Barillas-Mury et al. [10]; 5′-GITT(A/C)GITA(T/C)GA(A/G)TG(T/C)GA(A/G)GG(A/G/C/T)(A/C)G-3′ (forward primer) and 5′-AT(A/G)TCTTC(T/C)TTI(T/G)(G/C)IA(T/C)(T/C)TTITC(A/G)CA-3′ (reverse primer), where I denotes inosine. A 584 base pair (bp) fragment was obtained. 3′-rapid amplification of cDNA ends (3′-RACE) was performed using the RT-PCR fragment as a template, a forward primer, 5′-GACAAGAAGGCCATGAGCGA-3′ (F1) and the *Not*I-(dT18) primer (Amersham Pharmacia Biotech)(reverse primer). An 1177 bp PCR product was subcloned. 5′-RACE was conducted using a 5′-RACE System for Rapid Amplification of cDNA End Reagent Assembly, Ver. 2 (Gibco BRL) with the following primers: 5′-ATCCTTGTTGACGCAGGACA-3′, 5′-GATAGAGCATATCTTGATTG-3′ and 5′-TGTAGGATAAGTTTTGTTCT-3′. Two kinds of 5′ cDNA fragments and a 3′ cDNA fragment were isolated.

2.3. Detection of BmRelA and BmRelB mRNA

The two 5′ cDNA fragments were confirmed to have the same 3′ nucleotide sequences by RT-PCR using a combination of a forward primer 5′-CATCGAGGATTTACGCACAC-3′ (corresponding to positions 22 to 41) and a reverse primer 5′-TACTGTCGGCCTCAACGTTGAGGT-3′ (corresponding to positions 2277 to 2300) or a combination of a forward primer 5′-TCGGACAAGCGAGAGCGAAGAGA-3′ (corresponding to positions 278 to 300) and a reverse primer 5′-TACTGTCGGCCTCAACGTTGAGGT-3′ (corresponding to positions 2277 to 2300) under the following conditions. The reaction mixture including 10 pmol of forward and reverse primers and first strand cDNA corresponding to 0.1 μl of total RNA was kept at 95 °C for 1 min, then 40 cycles of PCR (95 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min) were done. Amplified fragments