Homeobox gene *Caudal* functions as an immuno-responsive transcription factor for Rel/NF-κB-dependent and independent antifungal response in *Drosophila* innate immunity

Thesis by
Ji-Hwan Ryu

Department of Medical Science

The Graduate School of

Yonsei University

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Ji-Hwan Ryu is approved.

윤 주 헌

Thesis Supervisor

이 원 재

Thesis Committee

최 인 흠

Thesis Committee

용 태 순

Thesis Committee

박 각 현

Thesis Committee

The Graduate School
Yonsei University

December, 2002

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지금까지 끝없는 보살핌과 기도로 후원해 주신 아버님, 어머님, 항상 따듯한 관심으로 동생을 지켜봐 주신 형, 누나, 매형 에게도 감사의 마음을 전합니다. 어려운 상황에서 우리 내외를 따뜻하게 맞아주시고, 격려 해주신 장인 어른, 장모님께 진심으로 감사 드립니다. 저를 위해 많은 조언을 아끼지 않으신 형님 내외분들께도 감사의 마음을 전합니다. 끝으로 넓은 이해와 사랑으로 공부하는 남편을 믿고 따라준 나의 사랑스러운 아내 지영과 앞으로 태어날 우리 아기에게 이 논문을 바칩니다.

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LIST OF ABBREVIATIONS

PGRP : peptidoglycan recognition protein

DLAK : drosophila LPS-activated kinase

DIF : dorsal like immune factor

Cad-LacZ : caudal promoter-lacZ

Dro-LacZ : drosomycin promoter-lacZ

Dro-GFP : drosomycin promoter-green fluorescence protein

Cec (Wt)-GFP : intact cecropinA1 promoter-green fluorescence protein

Cec (Mut)-GFP : point mutated cecropinA1 promoter-green fluorescence protein

hs-GAL4 : heat shock-gal4

da-GAL4 : daughterless-gal4

Cad-IR : caudal-inverted repeat

CecA1 : cecropinA1

hBD : human beta defensin

ABSTRACT

Homeobox gene *Caudal* functions as an immuno-responsive transcription factor for Rel/NF-κB-dependent and independent antifungal response in *Drosophila* innate immunity

Ji-Hwan Ryu

Department of Medical Science

The Graduate School of Yonsei University

Directed by Professor Joo-Heon Yoon

The hallmark of the innate immunity in *Drosophila* is the rapid and signal-dependent induction of Rel/NF-κB-dependent antimicrobial peptide genes. Although the κB-motif, found in all known antimicrobial peptide genes, plays a pivotal role in the initial induction of innate immune response, the possible involvement of transcription factors other than Rel/NF-κB has been proposed for the infection-induced cooperative regulation and the constitutive expression of the immune genes. However, the molecular identities and exact mechanisms of such transcription factors remain obscure. Here, we present evidence showing that the *Drosophila* homeobox gene product Caudal is both necessary for the full activation of the Rel/NF-κB-dependent antifungal Drosomycin gene and the constitutive expression of the Rel/NF-κB-independent antifungal Drosomycin gene, and antibacterial CecropinA1 gene in a tissue specific manner. The ectopic expression of Caudal drastically enhances the basal Drosomycin reporter activity *in vivo*, and *in vitro*. Moreover we found that Caudal co-immunoprecipitates with Dif/NF-κB and functionally cooperates in the

induction of the Drosomycin gene expression in *Drosophila* immune cells. Furthermore, using transgenic fly that interfere the endogenous caudal expression and activation, we confirm that caudal functions as activator for Drosomycin gene expression in Rel/NF-κB-dependent or Rel/NF-κB-independent way in a tissue specific manner, and we also showed that Caudal functions as repressor in specific region of whole intestine. As a result, we suggest that Caudal, transcription factor other than Rel/NF-κB, functions as a regulator of *Drosophila* innate immune signaling pathway, and give the insight to studying the immune response in epithelia of local immune tissues.

Key words: innate immunity, homeobox, caudal, antifungal drosomycin

I . INTRODUCTION

The innate immune system is a first line of host defense aimed at neutralizing microbial infections, and this system also plays an instructive role in the induction of adaptive immunity.[1] *Drosophila*, although devoid of adaptive immunity, still possess an efficient immune system against invading microorganisms.[2-5] The presence of microbial cell wall components in the body rapidly induces the *de novo* synthesis of innate immune genes, such as antimicrobial peptide genes.[2-5] At present, seven distinct immune-inducible antimicrobial peptides have been discovered. They include the antibacterial peptides Cecropin, [6] Attacin, [7] Defensin, [8] Drosocin, [9] and Diptericin, [10] the antifungal peptide Drosomycin, [11] and the antibacterial/antifungal peptide Metchnikowin.[12] These peptides act in concert to suppress bacterial and fungal propagation. Flies carrying mutations of molecules involved in antimicrobial signaling, are severely affected in terms of their viability during infection, demonstrating the central role of innate immunity in the host's defenses.[13-27]

The early step of innate immunity signaling involves the recognition of non-self molecules, by soluble and/or membrane anchored "non-clonal pattern recognition receptors". [1,28] In *Drosophila*, Gram-negative bacteria binding protein plays an important role in the recognition of common non-self motifs (*e.g.* lipopolysaccharide (LPS)) and in the subsequent immune signal transmission required for antimicrobial peptide synthesis.[29,30] In humans, such pathogen motifs are recognized by distinct membrane-bound Toll-like receptors, which subsequently initiate the innate immune signaling pathway.[31,32] Although it is an essential signaling mediator for the antifungal response, *Drosophila* Toll does not function as a pattern recognition receptor.[33] An active form of spaetzle generated by the proteolytic cascade is thought to be the extracellular ligand of Toll.[13,33] Recently, several groups identify two peptidoglycan recognition proteins (PGRPs) in the fruit fly that are probable pattern recognition receptors for the insect innate immune response.[23, 25]

Analysis of the promoter sequences of various antimicrobial peptide genes revealed that most of them contain multiple κB sites, which have striking similarities with the NF-κB sites found in many inducible innate immune genes in mammals.[34] In

Drosophila, immune signal-induced activation of Rel/NF-κB factor is an essential requirement for the transcriptional initiation of antimicrobial peptide genes.[14, 15, 35, 36, 37] Moreover, LPS-induced Rel/NF-κB signaling is mediated by *Drosophila* LPS-activated kinase (DLAK), a *Drosophila* homologue of mammalian IKKβ,[19, 20, 38] and *Drosophila* IKKγ.[17] Three Rel/NF-κB factors, Dorsal, Dorsal-like immune factor (Dif) and Relish, are involved in *Drosophila* antimicrobial response. Genetic evidence demonstrates that Dif/NF-κB is mainly involved in Toll-mediated antifungal Drosomycin expression, whereas Relish/NF-κB is more closely implicated in the expression of Gram-negative bacteria (or LPS)-induced antibacterial peptide genes (e.g. Cecropin, Attacin, Diptericin, etc.)[15-19] Nevertheless, both pathways synergistically activate different antimicrobial peptide gene expressions, suggesting that different homo- and hetero-dimers formed by Dif/NF-κB and Relish/NF-κB may regulate different target gene regulation preferences.[39]

The production of antimicrobial peptides is also an important aspect of host defense in vertebrates, Similar to insects, mammals express multiple peptide antibiotics, such as the antibacterial defensins and cathelicidins and the antifungal histatins.[40, 41] Among them, two human β–defensins have been identified, which are expressed in many epithelia, either constitutively or in response to infection.[42-45] This situation contrasts with the systemic release of antimicrobial peptides into the hemolymph of *Drosophila*. Recently, it is reported that many *Drosophila* antimicrobial peptides are expressed at barrier epithelia in local tissue such as respiratory, digestive, and reproductive tract in the inducible or constitutive manner.[46, 47]

Apart from the Rel/NF-κB family of transcription factors, little is known about other transcription factors involved in the induction of antimicrobial peptide genes. Recently, Serpent, a developmental transcription factor, was found to regulate antibacterial Cecropin gene via a GATA motif proximal to a κB site.[48, 49] Two mammalian *cis*-elements, such as interferon-responsive element and novel LPS response element, were found in the promoter regions of the fruit fly Diptericin gene and the silkworm Cecropin gene, respectively.[50, 51] Although, the existence and

importance of the nuclear factors responsible for the binding of these elements have been biochemically demonstrated, the molecular nature of these factors is presently unknown.

Caudal is a DNA binding nuclear factor via its homeodomain, a conserved helix-turn-helix domain rich in basic amino acids.[52] Moreover, a high level of Caudal is needed in posterior structures to activate the segmentation gene *fushi tarazu*, and *spalt* which is involved in terminal specification.[53, 54] Caudal expression in post-fetal life is restricted to organs that display cell renewal or remodeling, such as the intestine.[52] Homologues of Caudal have been identified in many mammals and their primary roles in intestine development and colon tumorigenesis are well-documented.[55, 56, 57]

In our study, we report that Caudal is an immuno-responsive cotranscription factor together with Dif/NF-κB in *Drosophila*. We also show that caudal is a critical regulator for local immune response of antimicrobial Drosomycin and CecropinA genes in *Drosophila* independent of Rel/NF-κB activation.

Ⅱ. MATERIALS AND METHODS

1. Cell culture

Drosophila immunocompetent Schneider cells (ATCC CRL-1963) were maintained exactly as described previously,[30] and stably transformed cells expressing Caudal were maintained in the presence of 300 μg/ml of hygromycin.

2. Antibody production

The activation domain of Caudal (amino acids 1-272) was used to produce glutathione S-transferase fusion recombinant protein, according to the manufacturer's instruction (Pharmacia). Mouse antiserum was produced using recombinant protein, and specific anti-Caudal antibody was purified by affinity as previously described.[58]

3. *In vivo* localization of Caudal promoter-LacZ transgenic fly

Flies were maintained at 25 ℃ on standard food. Oregon^R flies were used as the standard wild-type strain. The transgenic fly were dissected in Ringer's solution. Fixation was carried out in 0.5 % glutaraldehyde in PBS for 20 min and tissues were stained in 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside. Histochemical analysis of β-galactosidase expression and quantitative measurements of enzyme activity were performed, as has been described previously.[59]

4. Construction of plasmids

An open reading frame encoding Caudal was amplified by PCR and cloned into the *Drosophila* transfection vector, pPacPL, which contains the *actin 5C* promoter.[37] to generate pPacPL-Caudal. The activation domain (amino acids 1-272) and DNA binding domain (amino acids 273-427) of Caudal were PCR-amplified and subsequently cloned into pPacPL to generate pPac-Caudal-AD and pPacPL-Caudal-BD, respectively. All Caudal expression constructs (Caudal, Caudal-AD and Caudal-BD) were hemagglutinin (HA)-tagged in front of the stop codon. To create the Caudal repressor construct (pPacPL-CaudalEn-R).[60] *Drosophila engrailed* repressor domain (amino acids 1-296 of the *Drosophila engrailed* protein) was amplified and inserted

in front of pPacPL-Caudal-BD to allow the production of an in-frame N-terminal fusion of *engrailed* repressor domain to the BD of Caudal. Full-length Dif/NF-κB (a gift from Dr. Engström, Stockholm University, Sweden) was V5 epitope-tagged and subsequently subcloned into pPacPL to generate pPacPL-Dif-V5. A 2.4 kB upstream fragment of the Drosomycin promoter was amplified by PCR from pP[CaSpeR] plasmid (a gift from Dr. Lemaitre, CNRS, France).[46] and sub-cloned into the pGL3 luciferase reporter gene (Promega) to generate pGL3-Droso-Luc. The pPacPL-LacZ was constructed as previously described.[38]

5. Reporter gene assay

In the case of immunocompetent *Drosophila* cells, transient transfections were carried out using the calcium phosphate method.[61] All transfections contained 100 ng of pPacPL-LacZ as an internal standard to allow the determination of transfection efficiency and 100 ng of the luciferase reporter constructs (pGL3-Droso-Luc or pGL3-Cec-Luc). In addition, various amounts of pPacPL-Caudal, alone or together with Caudal mutant constructs, were co-transfected. At 48 hr after transfection, luciferase activity was measured according to the manufacturer's instructions (Promega). In the case of LPS stimulation, cells were incubated with LPS (10 μg/ml) for 6 hr. Toll signaling was activated by co-transfecting 1 μg of pJL195-Toll$^{\Delta LRR}$. Luciferase activity was normalized to β-galactosidase activity to correct for variations in the transfection efficiency. In the case of NIH-3T3 cells, semi-confluent cells were co-transfected with 100 ng of pGL3-Droso-Luc or pGL3-Cec-Luc, and pcDNA3-Caudal. pcDNA3-Caudal-BD was also used as a dominant-negative construct for Caudal. SuperFect™ (Qiagen) was used for the transfection according to the manufacturer's instruction.

6. Overexpression of Caudal *in vivo*

The ORF of the Caudal cDNA was subcloned into the pPUAST vector,[62] and used to generate transgenic animals for Caudal under the control of UAS promoter by P-element mediated transformation.[63] One transgenic strain with UAS-Caudal on the

second chromosome was obtained. To overexpress Caudal, transgenic flies carrying two copies of the UAS-Caudal were crossed with hs-GAL4$^{89\text{-}2\text{-}1}$, the heat shock driver stock (generated by A. Brand). Heat shock was carried out at 37 ℃ for 1 hr and fly were allowed to recover for 17 hr. Flies were subsequently infected for 3 hr by pricking with a thin needle previously dipped into a heat-killed mixture of overnight cultures of *Escherichia coli*, *Micrococcus luteus*, and *Saccharomyces cerevisiae*.

7. Co-immunoprecipitation and Western blot analysis

Total protein extracts (approximately 500 μg) from cells transiently transfected with Caudal and V5 epitope-tagged Dif/NF-κB were immunoprecipitated with anti-Caudal antiserum (5 μl) or pre-immune serum (5 μl) for 2 hr at 4 ℃. Protein G-agarose beads were then added to the reaction mixture and incubation continued for another hour at 4 ℃. The beads were pelleted and washed five times with lysis buffer (100 mM NaCl, 50 mM HEPES, pH 7.5, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM PMSF and protease inhibitor mixture; Roche Molecular Biochemicals). Immunoprecipitated proteins were then boiled in SDS-PAGE sample buffer and subjected to Western blot analysis.[64] To detect Dif/NF-κB-V5, a monoclonal anti-V5 antibody (Invitrogen) was used for 2 hr at a dilution of 1:1000 in TBST (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, and 0.02 % Tween 20) in the presence of 5% bovine serum albumin. Following primary antibody, the blot was washed and incubated for 1 hr with goat anti-mouse IgG conjugated to horseradish peroxidase diluted at 1:2000. Detection was carried out using an ECL Western blot detection kit (Amersham Pharmacia Biotech).

8. GFP-reporter genes

GFP-expressing *D rosophila* a re a nalyzed d irectly u nder a s tereomicroscope (e.g. Leica MZFLI II) equipped with epefluorescent illumination (excitation filter 480/40 nm; dichroic filter 505 nm; emission filter 510 nm).

9. Construct of caudal RNAi transgenic fly lines

To construct the caudal-IR, two 510 bp-long caudal plasmid DNA (nucleotide position 70-579 of the coding sequence) was amplified by PCR and was inserted as an inverted repeat (IR) into pUAST, a transformation vector, and two 510 bp-long inverted repeats (IR) of the construct were separated by an unrelated 180 bp DNA sequence that acts as a spacer, to make a hairpin-loop–shaped RNA. Transformation of *Drosophila* embryos was carried out in the w^{1118} fly stock. Each experiment was repeated using at least two independent UAS-RNAi insertions for each construct tested. In this study, we used fly adults carrying one copy of the UAS-RNAi construct combined with one copy of the GAL4 driver such as hs-GAL4, da-GAL4, and c729-GAL4.

Ⅲ. RESULTS

1. In the absence of immune stimulation for Rel/NF-κB activation, Caudal can trans-activate the basal level of antifungal Drosomycin gene.

We investigated whether Caudal is involved in the regulation of innate immune genes, such as antimicrobial peptide genes. For this purpose, we used immunocompetent *Drosophila* Schneider cells that do not normally possess any basal Caudal expression (data not shown). This cellular system permitted us to identify the role of Caudal in immunity, in the absence of any effects due to endogenous Caudal. We first transiently transfected the *Drosophila Caudal* gene under the control of *actin 5C* promoter (pPacPL-Caudal) and either antifungal Drosomycin reporter (pGL3-Droso-Luc). The ectopic expression of Caudal strongly induced basal Drosomycin reporter activity (more than 30-fold activation in the presence of 3 μg of pPacPL-Caudal) in a concentration-dependent manner in *Drosophila* immunocompetent Schneider cells (Sn cell) (Fig 1A). To determine the specificity of this induction, we overexpressed Caudal-BD (deletion mutant containing only a DNA binding homeodomain) in order to saturate the putative Caudal-binding site(s). It was found that the overexpression of Caudal-BD specifically inhibited Caudal-induced Drosomycin expression, whereas the overexpression of Caudal-AD (deletion mutant containing only *trans*-activating domain) failed to inhibit reporter activity (Fig 1B). These results suggest that Caudal transcription factor specifically activates Drosomycin gene expression and that Caudal-induced reporter activity is Caudal site-specific. To determine whether Caudal activity is important in the innate immune signaling under more physiological condition, we generated transgenic flies that used the GAL4/UAS system to overexpress Caudal. To induce Caudal overexpression, adult fly carrying hs-GAL4/UAS-Caudal and Dro-GFP (2.2kb Drosomycin promoter fused with GFP containing vector) were subjected to heat shock treatment (37 °C for 1 hr). The same fly without heat treatment or fly carrying only Dro-GFP with heat treatment were used as controls. Analysis of Dro-GFP intensity showed that Caudal overexpression was observed only in fly carrying hs-GAL4/UAS-Caudal and Dro-

GFP after heat shock treatment (Fig 2). These findings are good agreement with our *in vitro* results presented in the previous sections, and show that Caudal activity is indeed important for the full induction of antifungal immune genes.

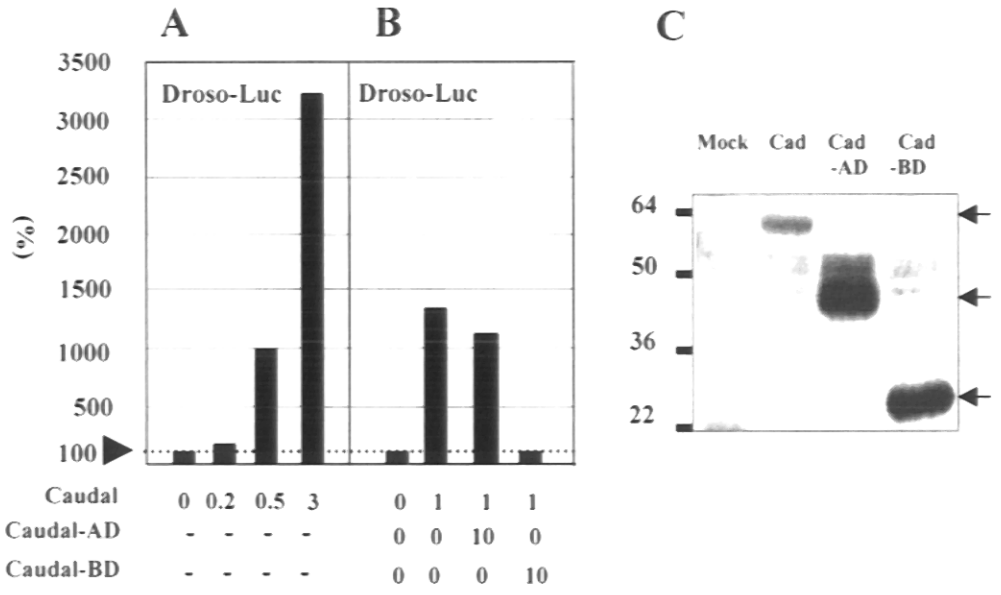

Figure 1. Effect of Caudal overexpression on basal Drosomycin reporter activity in *Drosophila* immunocompetent cells. (A) Increasing amount of pPacPL-Caudal was co-transfected with 100 ng of Droso-luciferase construct together with 100 ng of β-galactosidase construct in immunocompetent Schneider cells. At 48 hr after transfection relative luciferase activity was calculated by dividing the luciferase activity by the corresponding cotransfected β-galactosidase activity. Normalized luciferase activity in the absence of Caudal expression was taken arbitrarily as 100, and the results are presented as relative expressions. (B) The inhibition of Caudal-induced Drosomycin reporter activity by the overexpression of the Caudal-BD mutant. A fixed amount of pPacPL-Caudal (1μg) was co-transfected with either 10 μg of Caudal-AD mutant construct or 10 μg of Caudal-BD mutant construct together with 100 ng Droso-luciferase and 100 ng of β-galactosidase construct. Relative luciferase activity was calculated as described above. (C) The level of expressed Caudal, Caudal-BD or Caudal-AD in each lot of transfected cells in (B) was analyzed by immunoblot analysis using a monoclonal anti-HA antibody. In all experiments, the reporter assay was repeated at least three times, and the results obtained were found to be highly reproducible.

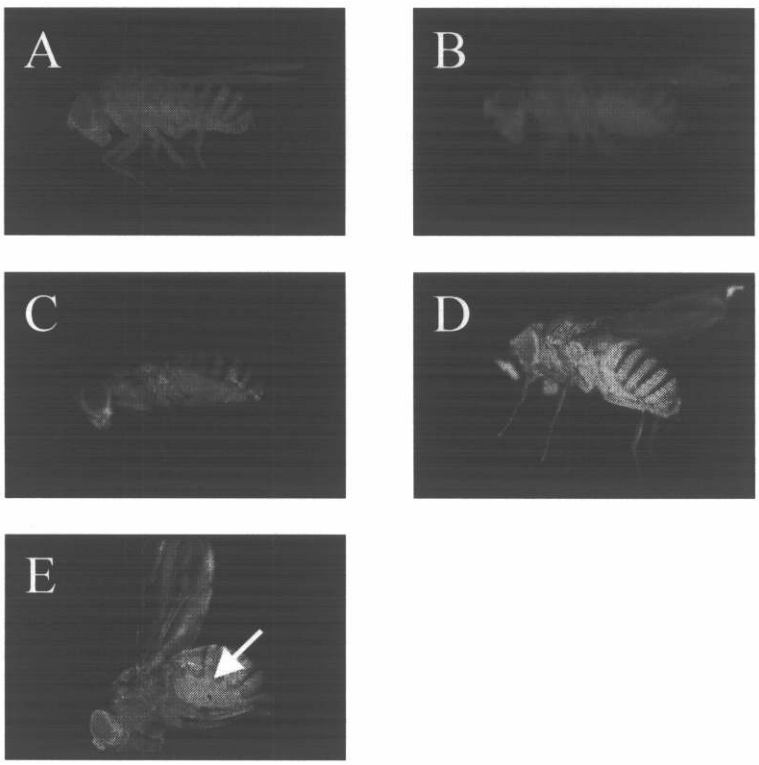

Figure 2. Effect of Caudal overexpression on basal Drosomycin reporter activity in transgenic fly. GFP illumination of whole body from transgenic fly carrying the Dro-GFP construct (A, B) or Dro-GFP, hs-GAL4, and UAS-Cadal construct (C, D) in the absence of immune challenging. Transgenic fly carrying the Dro-GFP, hs-GAL4, and UAS-Cadal construct with heat shock treatment (37 °C for 45 min) increased the GFP intensity compared with transgenic fly carrying the Dro-GFP, hs-GAL4, and UAS-Cadal construct without heat shock treatment (37 °C for 1 hr) (C, D). Following heat shock treatment, flies were allowed to recover for 17 hr. (E) Transgenic flies carrying only Dro-GFP were immunized for 48 hr (melanized scar at the abdomen, arrow), strongly express fluorescent illumination in fat body.

2. Caudal is required for full activation of Drosomycin gene through the caudal binding element in Drosomycin gene promoter.

To identify regions of the caudal binding element in Drosomycin gene promoter, fragments of the 5'-flanking region of the gene were cloned upstream of the firefly luciferase cDNA in the reporter plasmid pGL3 and transfected into Sn cell (Fig 3A). In the presence of caudal, The –2.2 kb flanking region reporter construct directed the maximal transcriptional activity with 11-fold higher relative luciferase expression compared with the –2.2 kb flanking region reporter construct with the absence of caudal. The transcriptional activity driven by the –1.1 kb flanking region fragments was the same value compared with the –2.2 kb flanking region reporter construct with the presence of caudal. The transcriptional activity driven by the –1.0 kb flanking region fragments was reduced the same level compared with the –2.2 kb flanking region reporter construct with the absence of caudal. Therefore, we inspected the promoter regions of the Drosomycin between –1.1 kb and –1.0 kb to find out correct caudal binding element and identified three elements with two pairs (Fig. 3A). The identification of putative *cis*-acting elements was refined further by analyzing the activities of a final series of point mutation constructs. Among the point mutation constructs, activity of the construct which has four point mutated cis elements (s1, s2, s3, s4) was completely reduced the same level compare with the –1.1 kb flanking region report construct. To examine direct binding of caudal protein to the four binding elements, EMSA was performed using caudal binding domain fused with GST protein and caudal overexpressed *Drosophila* larvae nuclear extract (Fig. 3B). Supershift experiments with specific anti-caudal immune serum showed that four elements are interacted specifically with caudal protein. Thus Caudal is required for full activation of Drosomycin gene through the caudal binding element in Drosomycin gene promoter.

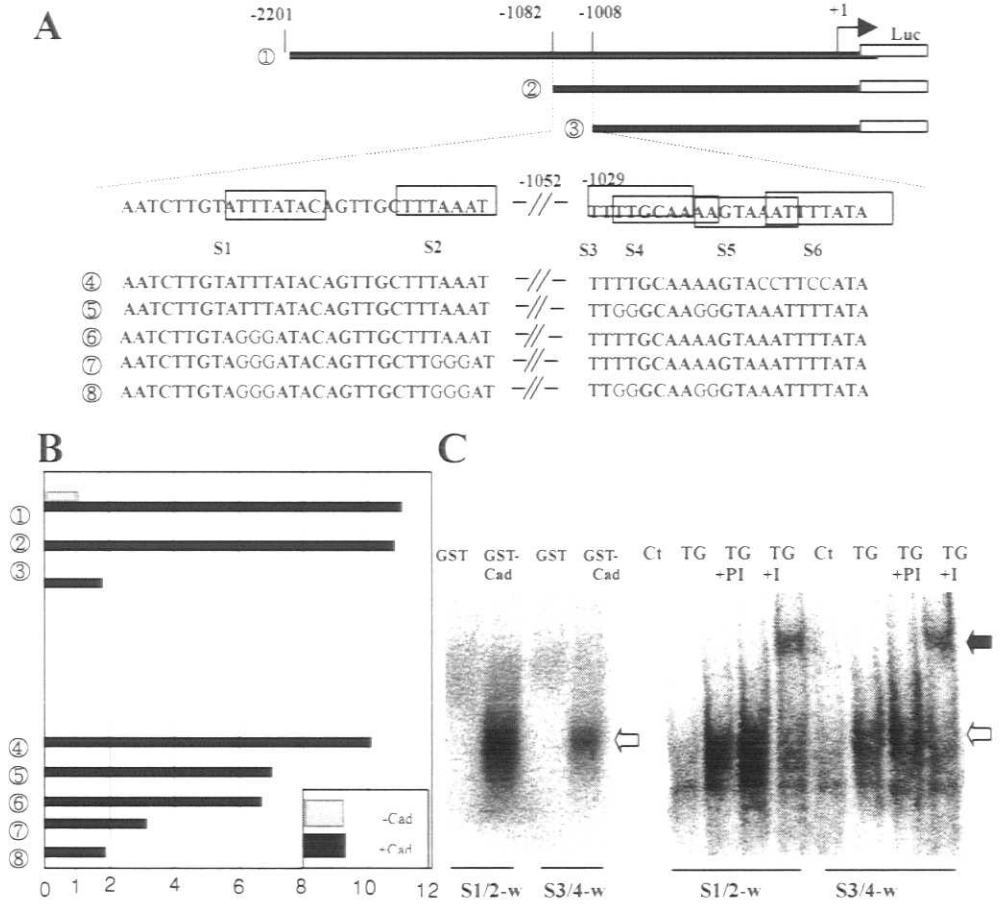

Figure 3. Caudal is required for full activation of Drosomycin gene through the caudal binding element in Drosomycin gene promoter. (A) Schematic representation of the deleted, or point mutated Drosomycin promoter constructs designated as number 1-8 used for reporter gene assay. (B) Transient expression levels of luciferase activity under the control of different lengths of wild-type (①), or deleted (②, ③), or point mutated (④ - ⑧) promoters of Drosomycin are represented by gray (without caudal) and black (with caudal) bars. (C) EMSAs were done using the ^{32} P-labeled two putative caudal binding elements, s1/2wt and s3/4wt probe, and using recombinant protein which caudal binding domain was fused with GST protein (left panel) and caudal overexpressed *Drosophila* larva nuclear extract (right panel). White arrow indicate specific binding of caudal protein, and preimmune or immune anti-caudal antibody were used for supershift experiments demonstrating the presence of the Caudal protein in the complex. Black arrow indicates the complex that supershifted with addition of anti-Caudal antibody.

3. Following immune stimulation causing Rel/NF-κB activation, Caudal can enhance the signal-dependent inducibility of Drosomycin and functional cooperation and physical interaction between Caudal and Dif/NF-κB

As the amount of Caudal expression is directly proportional to the basal reporter activity of Drosomycin, we further attempted to analyze whether Caudal can enhance Drosomycin reporter activity following immune signals. Genetic evidence demonstrates that the antifungal Drosomycin gene is mainly up-regulated via Toll signaling and is also weakly turned on in response to LPS by the *imd* pathway, which is independent of any Toll input. Therefore, we used ligand independent constitutively active mutant Toll construct ($Toll^{\Delta LRR}$) and bacterial LPS to initiate Toll and *imd* signaling, respectively. Under our experimental conditions, we routinely obtained a 50-fold activation of the Toll-induced Drosomycin promoter (Fig 4A), which is comparable to that reported previously. When increasing amounts of the expression plasmid pPacPL-Caudal were introduced with a constant amount of pGL3-Droso-Luc reporter construct, Toll-inducibility of Drosomycin was drastically enhanced in a dose-dependent manner, i.e., more than a 400-fold induction in the presence of 3μg of pPacPL-Caudal (Fig. 4A). Moreover, -2.2 kbΔ that was discard the 80 bp flanking region fragments between –1.0 kb and –1.1 kb directed the almost same transcriptional activity with the –2.2 kb flanking region reporter construct with the absence of caudal. In the case of LPS stimulation, we also observed the enhanced LPS-inducibility of the Drosomycin reporter in the presence of Caudal expression, specifically, more than 10-fold higher inducibility than in control cells without Caudal expression (Fig 4B). These results suggest that Caudal activity is necessary for the full inducibility of the Drosomycin gene in response to immune signals. Previous studies have shown that Dif/NF-κB is involved in the induction of Drosomycin gene expression following microbial infection because we found that Caudal regulates Drosomycin gene expression *in vivo* and *in vitro*, we further investigated whether Caudal and Dif/NF-κB can function cooperatively to activate Drosomycin promoter. Drosomycin reporter construct was transfected into *Drosophila* immunocompetent cells with Caudal- or Dif/NF-κB- expression plasmids, individually or together. As

shown in Fig. 5A, the co-transfection of reporter gene with Caudal and Dif/NF-κB expression plasmid maximally increased reporter activity, indicating a transcriptional synergy between Caudal and Dif/NF-κB. To understand the underlying molecular basis behind this observed synergistic activation, we investigated whether Caudal and Dif/NF-κB physically interact with each other. Cell lysate from Schneider cells overexpressing Caudal and V5 epitope-tagged Dif/NF-κB were immunoprecipitated with anti-Caudal antiserum and analyzed by immunoblot using monoclonal anti-V5 epitope antibody. Fig. 5B shows that Dif/NF-κB was co-immunoprecipitated with Caudal, whereas no Dif/NF-κB was observed when pre-immune serum was used in the immunoprecipitation reaction. These results indicate that Caudal can form a protein-protein complex, directly or indirectly, with Dif/NF-κB and that their physical interaction may elicit transcriptional synergy.

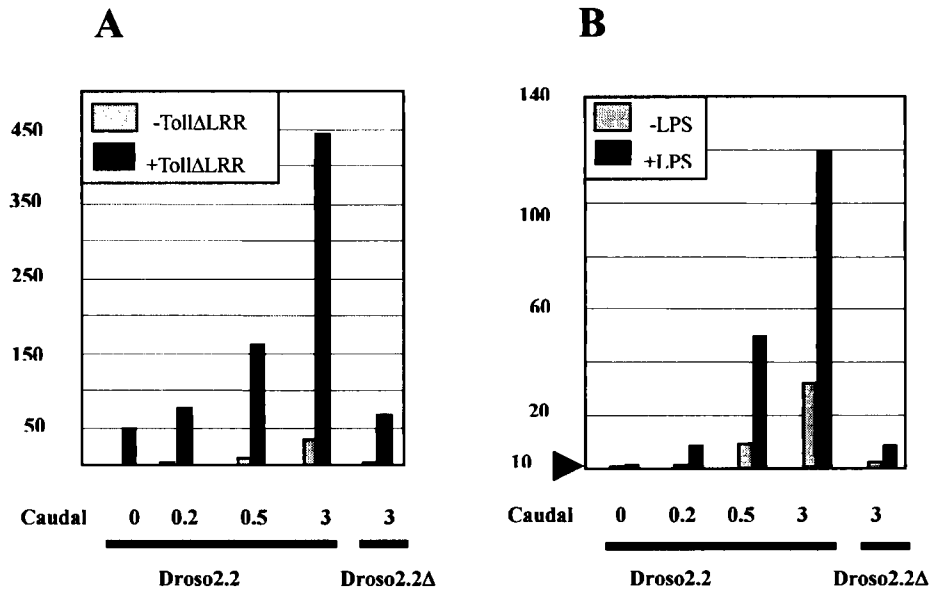

Figure 4. Caudal can enhance the signal-dependent inducibility of Drosomycin. (A) Increasing amounts of pPacPL-Caudal were co-transfected without (gray bar) or with (black bar) a constitutively active version of Toll construct (Toll$^{\Delta LRR}$) (1 μg) and 100 ng of Droso-luciferase and 100 ng of β-galactosidase construct in immunocompetent Schneider cells. (B) Increasing amounts of pPacPL-Caudal were co-transfected with 100 ng of Droso-luciferase together with 100 ng of β-galactosidase construct in immunocompetent Schneider cells. At 48 hr after transfection, the cells were incubated without (gray bar) or with (black bar) LPS (10 μg/ml) for 7 hr. In all experiments, the normalized luciferase activity in the absence of Caudal expression and without immune stimulation (Toll activation or LPS treatment) was taken arbitrarily to be 100, and results are presented as the corresponding relative expressions. Relative luciferase activity calculations were performed as described in Figure 1. Each bar represents the average of at least three independent experiments.

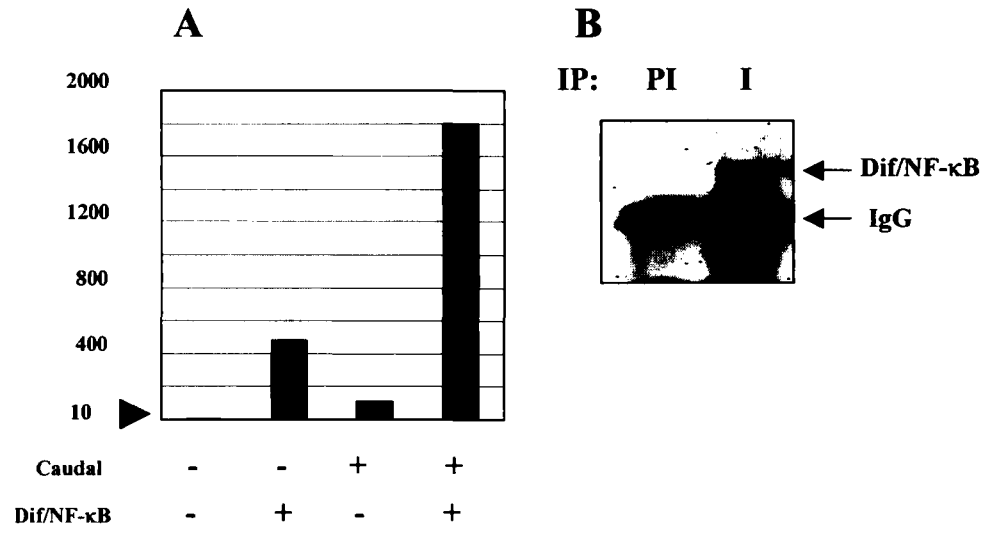

Figure 5. Functional cooperation and physical interaction between Caudal and Dif/NF-κB. (A) The Drosomycin reporter construct was co-transfected into *Drosophila* immunocompetent Schneider cells with Caudal (1 μg) and Dif/NF-κB (1 μg) expression constructs, individually or in combination. Results were normalized to the activities of those cells transfected with the reporter gene only, which were assigned a value of 100. Each bar represents the average of at least three independent experiments. (B) Caudal can form a protein-protein complex with Dif/NF-κB. *Drosophila* immunocompetent Schneider cells were transfected with Caudal and V5 epitope-tagged Dif-NF-κB simultaneously. Total cell extracts (500 μg) were prepared and immunoprecipitated (IP) with preimmune and immune anti-Caudal serum respectively. Equal amounts of the same extract were also immunoprecipitated with pre-immune serum. Immunoprecipitated proteins were analyzed by Western blot analysis using anti V5 antibody to detect co-immunoprecipitated Dif/NF-κB.

4. Caudal-RNAi a nd Caudal-repressor transgenic fly blocks infection-induced antifungal Drosomycin gene expression

To further confirm the role of Caudal as a transcriptional activator of the Drosomycin gene, we have generated transgenic flies carrying either UAS-CadEn-R or UAS-Cad-IR. UAS-CadEn-R construct was made that the transcriptional activation domain of Caudal was removed and replaced with the strong transcriptional repressor domain of the *Drosophila engrailed* protein. This chimeric construct was cloned under the control of *Drosophila* UAS promoter. In addition, UAS-Cad-IR construct consists of two 510 bp-long inverted repeats (IR) of the gene separated by an unrelated 180 bp DNA sequence that acts as a spacer, to make a hairpin-loop–shaped RNA. The schematic diagram of these construct is shown in Fig 6A. In order to see whether this construct can repress induction of Drosomycin expression by caudal, we performed a co-transfection assay using Droso-Luc in the absence or presence of UAS-CadEn-R and UAS-Cad-IR (Fig. 6A). The assay showed that Drosomycin promoter-Luciferase reporter activity is decreased more than 4 times in the presence of UAS-CadEn-R or UAS-Cad-IR.

This result confirms that these caudal repressor constructs are efficient for inhibiting the Drosomycin gene expressions in Sn cell (Fig. 6B). Figure 6C show that the expression of UAS-CadEn-R or UAS-Cad-IR transgenic fly induced by hs-GAL4, which directs expression of GAL4 ubiquitously after heat shocks downregulate the immune challenged Drosomycin gene expressions. These *in vitro* and *in vivo* results suggest that Caudal is the specific transcriptional activator for antifungal Drosomycin gene.

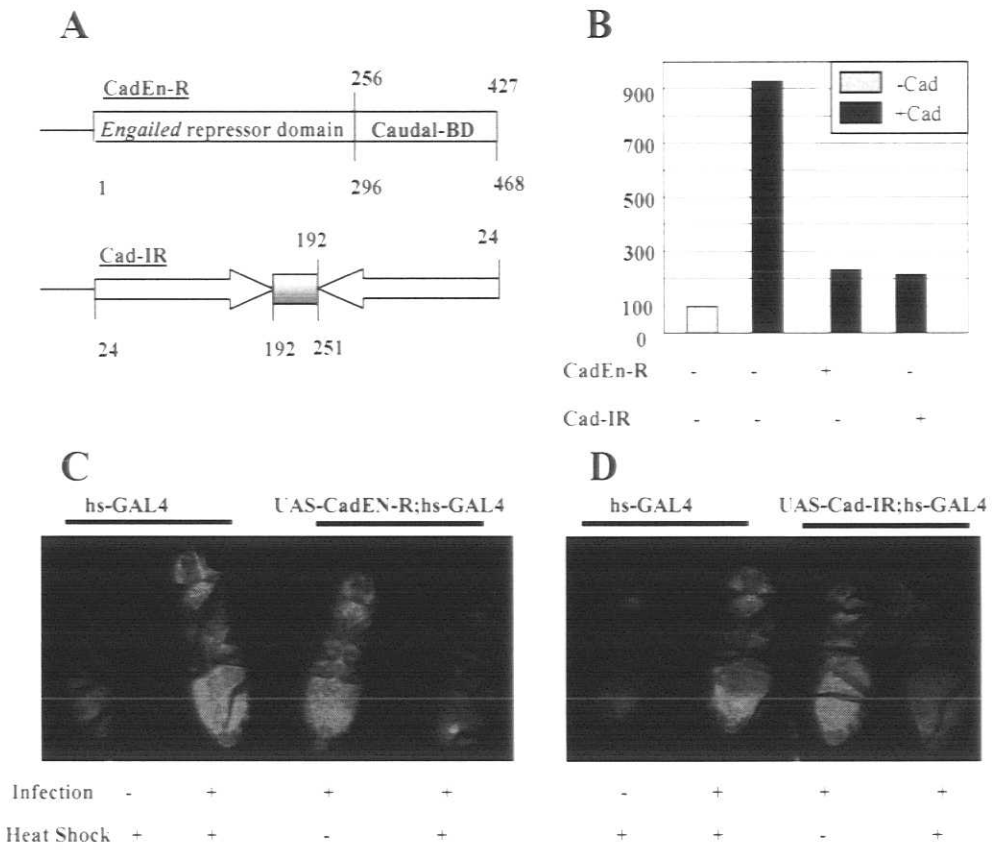

Figure 6. Inhibition of infection induced Drosomycin reporter activity by the overexpression of Caudal repressor construct (CadEn-R) and Caudal RNAi (Cad-IR) *in vitro* and *in vivo*. (A) A schematic diagram of the Caudal-repressor construct and Cad-IR used in this study. In case of CadEn-R, numbers on the top denote amino acid sequence numbers derived from *Drosophila* Caudal. The numbers on the bottom represent positions within fusion protein. In case of Cad-IR, numbers on the top and bottom amino acid sequence numbers derived from *Drosophila* Caudal (B) Fixed amounts (2 μg) of Caudal were co-transfected with PpacPL-CadEn-R and PpacPL-RNAi and Droso-luciferase and, 100 ng of β-galactosidase construct in immunocompetent Schneider cells. Results are presented as relative expressions. Each bar represents the average of at least three independent experiments. (C) Inducible expression of CadEn-R and Cad IR with a hs-GAL4 decrease the infection induced Dro-GFP activity in adult fly. Flies were incubated for 1 hr at 37 °C and 1day recovery after were infected with a mixture of Gram-negative (*E. coli*) and Gram-positive (M. luteus) bacteria, and 2days after intensity of Dro-GFP was observed.

5. Localized expression of the Caudal-LacZ reporter gene and the Drosmycin-GFP reporter gene in fly in the absence of experimental immune challenge

To identify the constitutive expression organs of caudal and drosomycin gene, we have generated transgenic flies carrying caudal promoter-LacZ (Cad-LacZ), and drosomycin promoter-GFP (Dro-GFP) reporter gene. All transgenic adults carrying the Cad-Lacz reporter displayed strong colorization in full salivary glands and female sexual organs such as spermatheca and seminal receptacle and male sexual organs such as ejaculatory duct, but all transgenic adults carrying the Cad-LacZ reporter showed weak colorization in fat body (Fig. 7). Transgenic adult flies carrying the Dro-GFP reporter displayed strong fluorescence in the same organs, where LacZ staining value in Cad-LacZ transgenic fly was strong, such as salivary gland, spermatheca and seminal receptacle and partial ejaculatory duct, but no fluorescence was detected in fat body in the absence of immune challenge. There is strong fluorescence in fat body in the presence of immune challenge. These results suggest the strong probability that there are close relationships between Caudal and Drosomycin in view of constitutive local innate immunity.

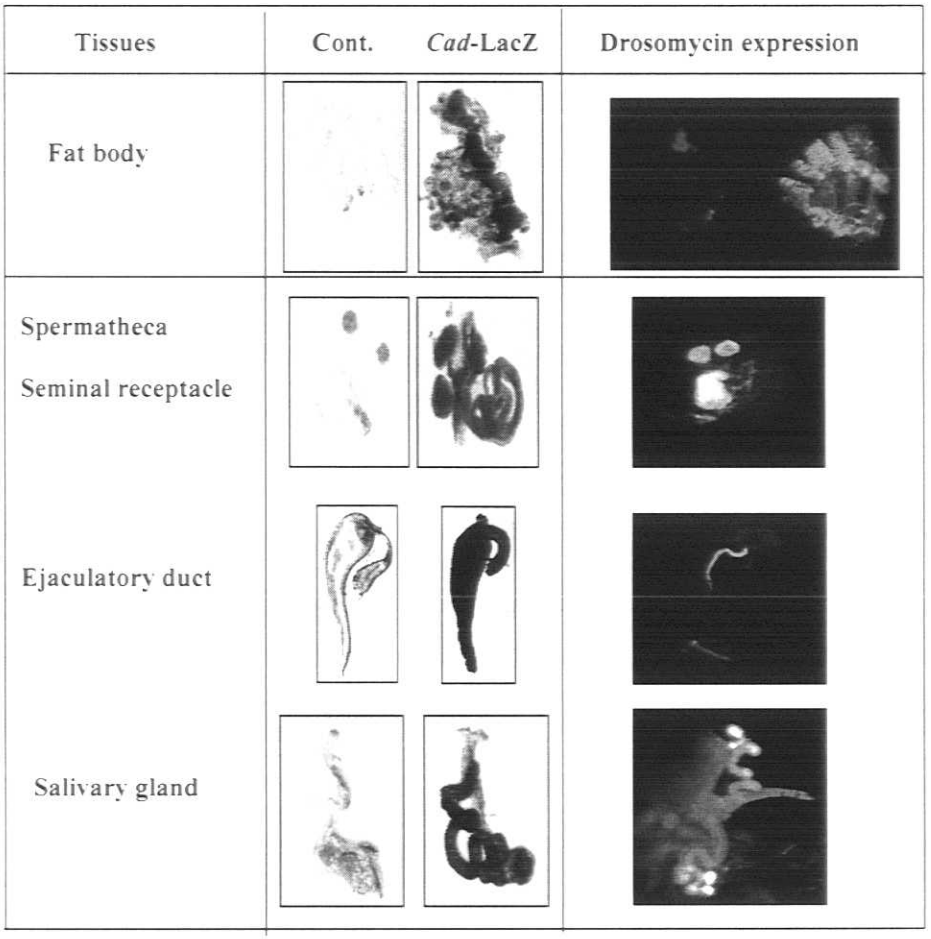

Figure 7. Constitutive expression patterns of the Cad-LacZ reporter gene and Dro-GFP reporter gene in various adult tissues in the absence of experimental immune challenge. (A) Cad-LacZ transgenic adult express the reporter gene in systemic immune related tissue (fat body) and local immune related tissue; reproductive tissue such as spermatheca, seminal receptacle, and ejaculatory duct, secretory tissue such as salivary gland (B) Dro-GFP transgenic adult constitutively express the reporter gene at the same region that Cad-LacZ reporter gene was constitutively expressed.

6. Caudal RNAi transgenic fly blocks the Drosomycin gene expression in constitutive local immune tissue in the absence of experimental immune challenge

To address Caudal's role as a transcriptional activator of the Drosomycin gene in local immune tissue in the absence of immune challenge *in vivo*, we expressed the UAS-caudal-IR transgene using the c729-GAL4 insertion which was known to be expressed in salivary gland specifically. Figure 8 shows that the expression of UAS-Cad-IR induced by c729-GAL4 in transgenic fly downregulate the Drosomycin gene expression in the salivary gland, important local immune tissue. These results suggest that Caudal is the critical transcriptional activator for antifungal Drosomycin gene expression in local immune tissue.

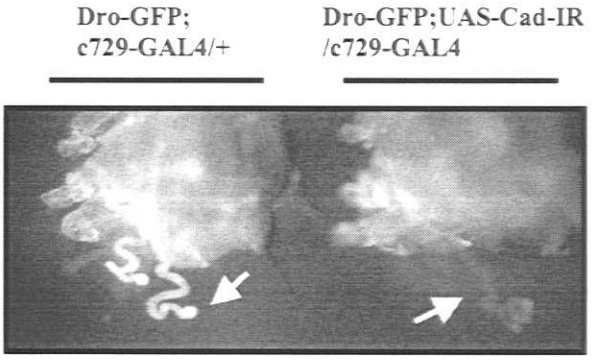

Figure 8. Caudal RNAi transgenic fly blocks the Drosomycin gene expression in salivary gland. Inducible expression of Caudal RNAi with the c729-GAL4 driver (right pannel), without the c729-GAL4 driver (left pannel) decreased Dro-GFP activity in salivary gland. Arrows indicate salivary gland.

7. Caudal fuctions as transcriptional activator on CecropinA1 gene expression in reproductive organs in the absence of experimental immune challenge, *in vivo*

The two caudal binding motifs in CecA1 gene promoter has been demonstrated to be necessary for expression of CecA1 reporter constructs in Sn cell in our previous study. To investigate the function of the caudal binding motif for tissue-specific expression *in vivo,* we generated seven transgenic flies carrying the Cec (Mut)-GFP reporter construct, in which the caudal core sequence TTTA was altered CCCA. Transgenic animals carrying the Cec (Mut)-GFP or the unmodified Cec (Wt)-GFP reporter construct (Fig. 9A) were not challenged and the reporter expression was analysed as GFP illumination. Fly carrying the Cec (Mut)-GFP reporter construct showed strong decrease of the reporter gene in the ejaculatory duct and spermatheca, male and female reproductive tracts respectively (Fig. 9B). Moreover, we expressed the UAS-caudal-IR transgene using the daughterless-GAL4 (da-GAL4) insertion which expresses GAL4 strongly and ubiquitously. Figure 9C show that the expression of UAS-Cad-IR in transgenic fly downregulate the CecA1 gene expression in the ejaculatory duct and spermatheca. We conclude that the caudal binding motif is necessary for constitutive expression of the CecA1 gene in the reproductive tracts, and that caudal is also the important transcriptional regulator for CecA1 gene expression in local immune tissue.

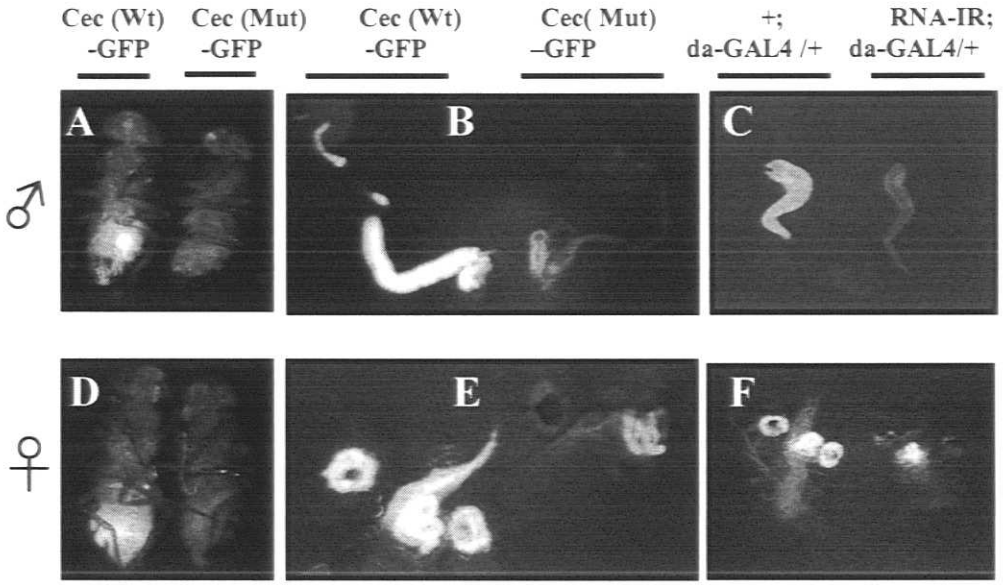

Figure 9. Caudal's functions as transcriptional activator on CecropinA1 gene expression in reproductive organs. GFP illumination of tissues, or whole body from transgenic fly carrying the Cec (Wt)-GFP construct (left pannel of A,B,D,E) or the Cec (Mut)-GFP construct (right panel of A,B,D,E) in the absence of immune challenging. Transgenic fly carrying the Cec (Mut)-GFP reporter decreased the GFP intensity compared with transgenic fly carrying the Cec (Wt)-GFP reporter in the male (A) and female (D) whole body, respectively, and in the ejaculatory duct (B), spermatheca and seminal receptacle (E). Inducible expression of Caudal RNAi with the Daughterless-GAL4 driver (right panel), without the daughterless-GAL4 driver (left panel) decreased Cec (Wt)-GFP activity in ejaculatory duct (C), Inducible expression of Caudal RNAi with the daughterless-GAL4 driver (right panel), without the dauterless-GAL4 driver (left panel) decreased Cec (Wt)-GFP activity in spermatheca and seminal receptacle (F).

8. CecropinA1 is constitutively expressed in the ejaculatory duct in an IMD/Relish-independent manner

To address whether the regulation of CecA1 gene expression in localized immune tissues is dependent of Rel/NF-κB activation or not, we analyzed expression of the Cec (Wt)-GFP transgene in the ejaculatory duct of Relish^{E20} , Relish knock-out mutant fly. As shown in Figure 10, the constitutive expression of Cec (Wt)-GFP in the male ejaculatory duct was not affected in Rel/NF-κB inactivated Relish^{E20} mutant fly. Altogether, these results indicate that Caudal regulates the CecA1 gene expression as activator in the ejaculatory duct in an IMD/Relish-independent manner.

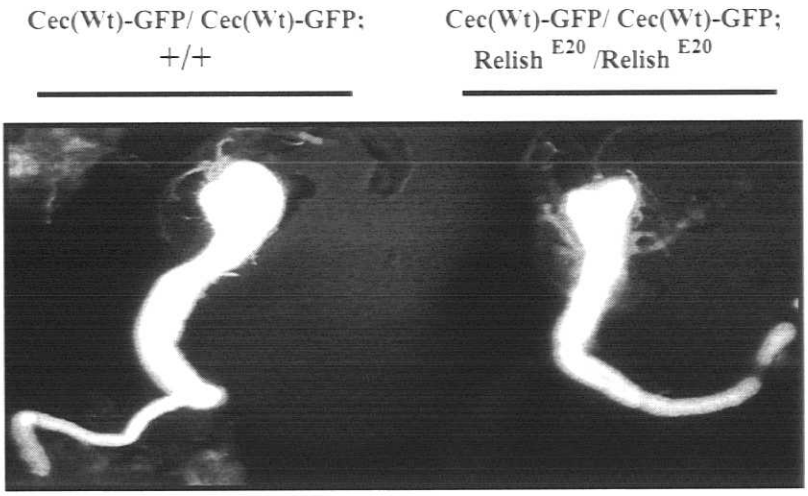

Figure 10. CecropinA1 is constitutively expressed in the ejaculatory duct in an IMD/Relish-independent manner. In transgenic fly carrying the Cec (Wt)-GFP reporter with Relish^{E20}, Relish knock mutant background (right panel), or without Relish^{E20}, Relish knock mutant background (left panel), there is no difference between GFP intensity of these two transgenic fly in the ejaculatory duct.

9. Caudal functions as repressor in specific regions of the whole intestine, *in vivo*

Upon examination of a large number of unchallenged transgenic larvae and adults carrying Cec (Mut)-GFP reporter gene construct, we detect a localized expression of GFP in restricted areas of the midgut epithelium, and we first monitored β-galactosidase activity in the whole digestive tract from transgenic fly carrying Cad-LacZ transgene. As shown in Figure 11A, the transgenic larvae and adults carrying the Cad-LacZ reporter displayed strong colorization in the whole digestive tracts. Moreover, we confirmed that in all seven transgenic fly lines carrying the Cec (Mut)-GFP reporter construct, strong green fluorescence was observed in restricted areas of the midgut epithelium such as proventriculus and posterior midgut compared with those of Cec (Wt)-GFP (Fig. 11B). These results suggest that caudal functions as repressor of CecA1 gene expression through binding to caudal binding motif in the limited regions of midgut epithelium.

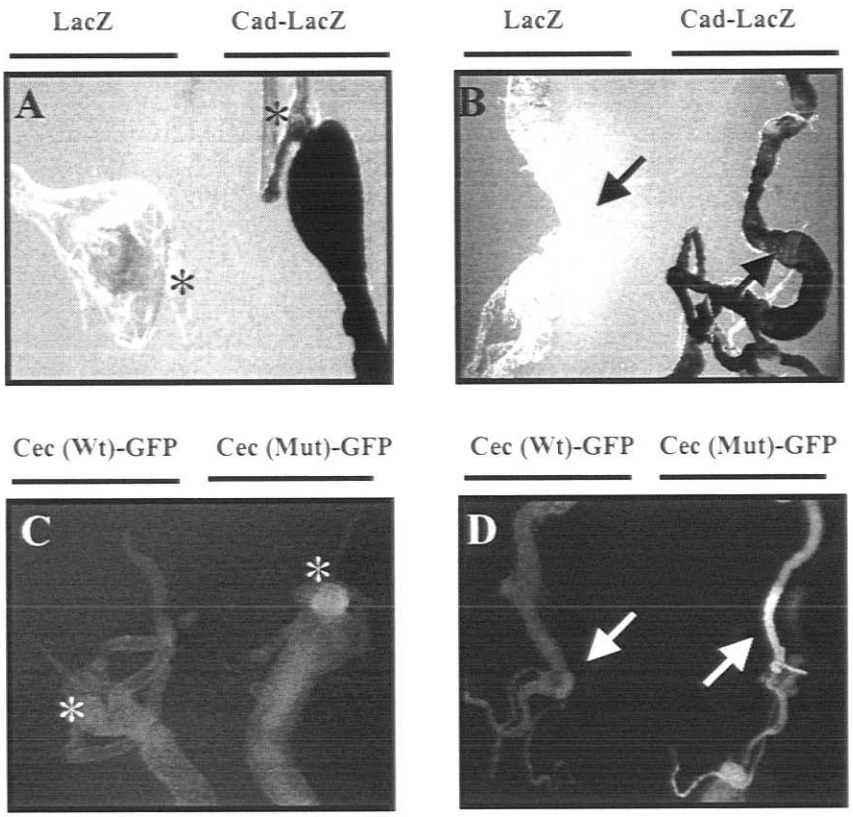

Figure 11. Caudal's function as repressor in specific regions of the whole intestine. X-gal staining of tissues from transgenic fly carrying the Cad-LacZ construct (right panel of A, B) or the only LacZ construct (right panel of A, B) in the whole intestine. All tissues were stained for two hours. Caudal strongly express in the whole intestine including preventriculus (asterisk), and posterior midgut (arrow) (A, B). GFP illumination of tissues from transgenic fly carrying the Cec (Wt)-GFP construct (left pannel of C,D) or the Cec (Mut)-GFP construct (right pannel of C,D) in the absence of immune challenging. Transgenic fly carrying the Cec (Mut)-GFP reporter increased the GFP intensity compared with transgenic fly carrying the Cec (Wt)-GFP reporter in preventriculus (C), and posterior midgut (D).

Ⅳ. DISCUSSION

The homeobox transcription factor Caudal is known to regulate the antero-posterior body axis of *Drosophila*.[52, 65] In mammals, these genes are known to participate in early embryogenesis, the development of the intestine and colon tumorigenesis.[55, 56, 57, 66, 67] However, apart from their developmental roles, the other physiological functions of the Caudal/Cdx homeobox genes are substantially unknown. Genetic evidence demonstrates that at least two distinct immune signaling pathways (the *imd* and the *Toll* pathways) are employed to induce antimicrobial peptide genes in *Drosophila*. The *Imd* pathway, is directly initiated by bacterial components, such as LPS, and is responsible for the induction of various antibacterial peptides.[15, 17, 19, 20, 22, 30, 38, 68] On the other hand, the *Toll* pathway, is initiated by a specific ligand, spaetzle, and mainly controls antifungal Drosomycin gene induction.[13, 16, 18, 19, 28] In the present study, Toll- and LPS- induced Drosomycin reporter activities are greatly enhanced in the presence of Caudal (Fig 4A, and B), suggesting that Caudal mediates both the Toll- and LPS- signals required for Drosomycin expression. It is possible that "immune signal-activated" Dif/NF-κB, efficiently activates the antifungal Drosomycin gene in association with "immune signal-induced" Caudal. Such transcriptional cooperation may lead to a coordinate assembly of transcriptional machinery, which contributes to the highly inducible nature of the Drosomycin promoter. Caudal co-immunoprecipitates and functionally cooperates with Dif/NF-κB in immunocompetent Schneider cells (Fig. 5A, and B), partly supporting this hypothesis.

The κB-motif, found in all known antimicrobial peptide genes, is considered to be the most important *cis*-element, which facilitates instantaneous *de novo* synthesis of these genes following infection signal.[4] Mutation analysis of other putative *cis*-elements in the proximity of the κB motif has revealed that the κB motif alone is insufficient to induce the full activation of these genes.[50, 51] These putative *cis*-elements are homologous to those of the mammalian acute phase genes: the IL-6 responsive elements and the interferon responsive element in

Drosophila Diptericin promoter.[50] , and the novel LPS-responsive element in silkworm Cecropin promoter.[51] However, the biochemical nature of the molecules responsible for the binding to such motifs is presently unknown. Only the GATA motif has been characterized at the molecular level. GATA motif and its binding factor serpent, are required for the correct LPS-inducibility of the Cecropin gene *in vivo* and *in vitro*.[48, 49] Such factor(s) may act in cooperation with immune-activated Rel/NF-κB factors to fully induce distinct antimicrobial peptide genes. Upon infection, the transcripts of all three known *Drosophila* Rel/NF-κB factors are rapidly upregulated. Apart from the Rel/NF-κB family, Caudal is the first immune-responsive transcription factor identified to date in *Drosophila*. Our *in vitro* (Fig. 1) and *in vivo* study (Fig. 2) shows that Caudal activity is necessary for the full induction of the Dif/NF-κB-controlled antifungal gene. In mammals, Rel/NF-κB-dependent target gene expression often necessitates functional cooperation between Rel/NF-κB and a wide range of transcriptional regulators (DNA binding transcription factors including AP-1,[69] C/EBP,[70] SRF,[71] SEF,[72] Stat6,[73] Smads,[74] the architectural protein HMG I (Y),[75] and co-activators, such as, CREB-binding protein,[76, 77] members of the p160 family of co-activators,[78] and CBP-associated factor.[79] Moreover, the functional cooperation between Rel/NF-κB and one of these factors greatly influences the ability of Rel/NF-κB to regulate distinct target genes in a selective manner. The potential role of Caudal as an essential positive regulator of κB-dependent Drosomycin gene implies that the level of Caudal in a specific cell in a given environmental condition may be a critical determinant of *Drosophila* antifungal response.

It was reported that in *Drosophila*, antimicrobial peptide genes are expressed in several epithelia, which are potentially in contact with the environment. These include the respiratory tract, the oral region and the digestive tract, the malphigian tubules, and the male and female reproductive tracts.[47] These tissues are sites of major physiological functions, such as gas exchange, nutrient absorption, water conservation, and reproduction, which necessitate host-environment interaction, and where cells are likely to encounter microorganisms. Interestingly, in most tissues that

antimicrobial peptide genes are expressed, homeobox transcription factor Caudal is strongly expressed in absence of immune challenging in our study, and the constitutive expression of Dro-GFP in the female spermathecae and seminal receptacle and salivary glands was not affected in *imd* and *Toll* loss-of-function which inhibit Rel/NF-κB activation.[47] Moreover, we also showed that Relish^{E20}, Relish knock-out mutant fly could not downregulate the constitutive expression of the CecA1-GFP in the ejaculatory duct, indication that activation of Drosomycin and CecropinA1 in these tissues is not dependent on *imd* and Toll pathway. Altogether, these results suggest that Caudal plays a critical role in the control of antimicrobial promoter inducibility in surface epithelia of some local immune responsive tissues.

In mammals, many antimicrobial peptides present at epithelial surfaces are derived either from synthesis by resident epithelial cells or from storage granules in the cytoplasm of specialized white blood cells, such as neutrophils localized in the vicinity of the epithelium. Among them, three β-defensins, human β-defensins hBD-1, hBD-2, and hBD-3, are expressed in various organs, either constitutively or in response to infection.[42-45] In vitro, primary human bronchial epithelial cells cultured without immunostimulants expressed HBD-1 mRNA ; however, HBD-2 and –3 were expressed only after stimulation with heat-killed bacteria (*P. aereginosa, S. pheumoniae*), phorbol myristate acetate of a cytokine 'cocktail'. [80] Evidence to date suggests that hBD-2 is up-regulated via the NF-κB transcription factor pathway in intestinal epithelial,[81] and tracheal epithelial cells.[82] This signaling pathway is critical for many cellular responses to inflammatory stimuli,[83] and the hBD-2 gene (DEFB2) promoter region contains three potential NF-κB binding sequences.[84] Nevertheless, the role of NF-κB in β-defensins regulation is not fully understood, although it is known to activate transcription of several genes involved in inflammation and immune responses. Moreover, it has been shown that NF-κB is neither critical nor sufficient for hBD-2 regulation by *Fusobacterium nucleatum* in human gingival epithelial cell.[85] In *Drosophila*, most of antimicrobial peptide gene expression is regulated by imd pathway in the presence of local immune challenging,[47] but unexpectedly, we showed that fly lines carrying the Cec (Mut)-GFP that was point

mutated at Caudal binding element expressed strong green fluorescence in restricted areas of the midgut epithelium such as preventriculus and posterior midgut compared with those of Cec (Wt)-GFP in the absence of local immune challenging (Fig. 10B) ; therefore, there are at least two key questions : first, how does Caudal function as repressor at the specific regions in whole intestine epithelia, second, what is the role of Rel/NF-κB in intestine epithelia in the presence of local immune challenging or not The role of Caudal as repressor could be thus explained by the presence of corepressor, rather than inactivation of caudal itself by upstream signal pathway, in that caudal is highly expressed in whole intestine epithelia. Therefore, corepressor could be existed by region specific manner, and repress the CecropinA1 gene expression through the interaction of caudal in specific part of whole intestine epithelia. The refractory nature of intestine epithelial cells to naturally infected pathogen could be thus explained by the repression of Rel/NF-κB activation. In local immune challenging heavier than natural infection, Rel/NF-κB could be activated and upregulate the CecropinA1 gene expression. As a result, we suggest that Caudal is the critical regulator in *Drosophila* innate immune signaling by the manner of dependent of Rel/NF-κB, or independent of Rel/NF-κB.

V. CONCLUSION

In this study, we showed that Caudal can trans-activate the basal level of antifungal Drosomycin gene in the absence of immune stimulation for Rel/NF-κB activation *in vivo*, and *in vitro*, and these regulations were mediated by direct Caudal's binding to caudal binding element in Drosomycin gene promoter. We also showed that following immune stimulation causing Rel/NF-κB activation, Caudal can enhance the signal-dependent inducibility of Drosomycin and functional cooperation and physical interaction between Caudal and Dif/NF-κB. Using Caudal-RNAi and Caudal-repressor transgenic fly, we demonstrated that infection-induced antifungal Drosomycin gene expression was blocked by inducible interference of endogeneous caudal expression and inhibition of caudal transcriptional activity. Moreover, we demonstrated that Caudal RNAi transgenic fly blocks the Drosomycin gene expression in constitutive local immune tissue in the absence of experimental immune challenge, and using Cec (Mut)-GFP transgenic fly, we showed that Caudal regulates the CecA1 gene expression as activator or repressor in a tissue specific manner independent of Rel/NF-κB activation. As a result, we suggest that Caudal is the critical regulator in *Drosophila* innate immune signaling by the manner of dependent of Rel/NF-κB, or independent of Rel/NF-κB.

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국문요약

*Drosophila*의 선천성 면역에 있어서의 homeobox gene인 *Caudal*의 역할 및 조절기전에 대한 규명

연세대학교 대학원 의과학과

류지환

지도교수 윤주헌

대부분의 생물체는 초기 병원균에 대항하는 선천성 면역체계를 가지고 있고, 이러한 초기 면역반응에 있어서, 항균 펩타이드가 매우 중요한 역할을 수행한다고 알려져 있다. 초파리 내에서도, 많은 종류의 항균 펩타이드가 존재하고, 침입균을 제거하는데 있어 중요한 기능을 한다고 알려져 있다. 또한 모든 초파리 항균 펩타이드 유전자의 promoter regione에는 κB-motif가 존재하고 있고, 이러한 κB-motif가 항균 펩타이드 유전자의 발현유도에 중요한 역할을 한다고 알려져 있다, 그러나, 최근에 항균 펩타이드 유전자의 발현유도에 대한 많은 연구를 통하여, Rel/NF-κB 이외의 다른 전사인자가 존재하고, 이러한 전사인자가 초파리 면역유전자의 발현조절에 중요 할 수 있다는 가능성을 제시하게 되었다. 따라서 본 연구에서는, 세포수준에서, 초파리 homeobox 유전자로 알려진 caudal이 병원균 감염시 Rel/NF-κB와의 상호작용에 의해 초파리 항균 펩타이드인 Drosomycin의 발현조절에 중요한 전사인자임을 밝혔고, 또한,

초파리 내에서의 Caudal의 발현과 활성을 억제하는 형질전환 초파리를 이용하여, 위의 사실을 확인하였다. 한편, 또 다른 항균 펩타이드인 CecropinA1 promoter region내에 Caudal 전사인자가 결합하는 특정 위치를 찾았으며, 이러한 결과를 토대로, 형질전환 초파리를 만들었다. 이러한 형질전환 초파리를 이용하여, Caudal이 Rel/NF-κB 전사인자에 의존 없이 조직 특이적인 방법으로 CecropinA1의 발현조절에도 중요한 역할을 한다는 것을 밝혔다. 결과적으로, 본 연구는 초파리의 면역 신호전달에 있어서, Caudal이 면역반응에 관계하는 다양한 조직에서, 기존의 Rel/NF-κB와는 다른 전사인자로서 기능함을 초파리 모델시스템을 이용하여 제시하였고, 이러한 결과를 토대로, 인간의 선천성 면역체계를 조직 특이적인 측면으로 이해함에 있어서 중요한 연구가 되리라 기대된다.

핵심되는 말 : innate immunity, homeobox, caudal, antifungal drosomycin