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Induction Patterns of Suppressor of Cytokine Signaling (SOCS) by Immune Elicitors in *Anopheles sinensis*

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Suppressor of cytokine signaling (SOCS) is known to be as a negative feedback regulator in Janus kinase signal transducer and activator of transcription signaling. Highly conserved SOCS box domain was cloned from a Korean malaria vector, *Anopheles sinensis*. Sequence analysis indicates that it has identity to *Anopheles gambiae* (96%), *Aedes aegypti* (94%), *Drosophila melanogaster* (78%), *Mus musculus* (72%) and *Homo sapiens* (72%), respectively. Tissue specificity RT-PCR demonstrated that the expression level of *AsSOCS* transcript was high at abdomen, midgut, and ovary, whereas developmental expression patterns showed that the level of *AsSOCS* was high at egg, early pupae, and adult female. On the other hand, RT-PCR analysis after bacterial challenge showed that *SOCS* mRNA was strongly induced in larvae. In addition, it was also induced by various immune elicitors such as lipoteicoic acid, CpG-DNA, and laminarin. It seems that *AsSOCS*, repressor of JAK-STAT pathway, is highly conserved in mosquito, and may play an important role in mosquito innate immune response.

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Introduction

JAK pathway is pivotal to many developmental processes and immune responses in vertebrates and invertebrates, and also regulation of JAK signaling is equally important. In order to limit JAK activity in cells, there are several conserved protein families that regulate JAK activation. These include phosphatases, Protein Inhibitors of Activated STATs (PIAS), and the suppressors of cytokine signaling (SOCS) family (Alexander *et al.*, 2004). In insect, the JAK cascade has been investigated in several experimental models such as *Drosophila melanogaster* (Yan *et al.*, 1996), *Anopheles gambiae* (Barillas-Mury *et al.*, 1999), *Aedes aegypti* (Lin *et al.*, 2004), *Culex tritaeniorhynchus* (Lin *et al.*, 2004). In human malaria vector, *A. gambiae*, a new insect member of the STAT family of transcription factors (Ag-STAT) has been cloned from the human malaria vector *Anopheles gambiae* (Barillas-Mury *et al.*, 1999). Bacterial challenge results in nuclear translocation of Ag-STAT protein in fat body cells and induction of DNA-binding activity that recognizes a STAT target site. In vitro treatment with pervanadate (vanadate and H_2O_2) translocates Ag-STAT to the nucleus in midgut epithelial cells. In addition, time bomb model has been

suggested on the basis of the interactions between anopheline midgut epithelial cells and *Plasmodium berghei* (Pb) ookinetes during invasion of the mosquito by the parasite. The time bomb model showed that *P. berghei* ookinetes invade polarized columnar epithelial cell, and induce nitric oxide synthease (NOS) expression (Barillas-Mury *et al.*, 2000; Han *et al.*, 2000; Han and Barillas-Mury, 2002). The effector molecules mediating immune response, such as nitric oxide can be potentially toxic to the host, specifically to midgut cells. Under these harmful conditions in the NOS-overexpressed cells, it can be hypothesized that SOCS must be up-regulated to tightly regulate the JAK-STAT signal pathway and the level of NOS potentially produced from JAK-STAT pathway in the Pb-invaded cells.

Based on the concepts described above, the long term goal of the current study is to elucidate the precise role of SOCS in the Pb-invaded midgut cells. As part of our effort to address this possibility, we have been attempting to clone the SOCS box fragment from a Korean malaria vector, *Anopheles sinensis*, and also see if mosquito SOCS is induced by various immune elicitors such as bacteria, lipoteichoic acid, CpG-DNA and laminarin.

Materials and Methods

Insects

Females of *Anopheles sinensis* were collected at Jangdeung-dong, Gwangju, Jeollanam-do where is non-epidemic areas of malaria in Korea. Mosquitoes were collected by Black Hole (Bio-Trap, Korea). Collections were started at 19:00 and continued until 07:00 the next morning. Larvae were reared in declorinated tap water, and fed on fish food (TetraMin Baby) (Shin *et al.*, 2003). Adult mosquitoes were fed on cotton containing 10% sugar solution, blood-fed on anaesthetized mouse (Balb/c), and reared in a 12 : 12 h light : dark cycle at 26 ± 2°C and 70% relative humidity.

Cloning of *AsSOCS* box domain

AsSOCS gene was isolated using PCR method with degenerate primers based on two protein regions well conserved between *Anopheles gambiae* and *Drosophila SOCS*. *A. sinensis* genomic DNA was used as template. *SOCS box* (540 bp) obtained from PCR reaction was cloned into TA cloning vector and the sequence data was confirmed using BLAST search.

RNA sample collection for tissue specificity and developmental stages

For the tissue specificity experiments, adult mosquitoes were dissected under stereo microscope to collect thoraces, abdomens, midguts, and ovaries. In addition, eggs, 4th instar larvae, early pupae (white pupae), late pupae (black pupae) and adult female were used for developmental expression profile of *AsSOCS* mRNA. All the collected samples were stored in RNAlater solution (Qiagen) at −80°C till use.

Immunization and sample collection for RNA isolation

Bacteria used were *Escherichia coli*. Mosquito larvae were pierced in the thoracic segments using a needle which was dipped in bacteria. RNA from the following times was examined: 0 and 4 hrs after inoculation. 1 μg of LTA, laminarin and CpG-DNA per mosquito adult female were injected into the thoracic segments using a microcapillary needle. RNA from the following times was examined: 0.6 hrs and 12 hrs post injection.

RNA isolation, cDNA synthesis and RT-PCR

Total RNA was isolated from *A. sinensis* whole body using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was also isolated from the samples stored at −70°C, and first-strand cDNA was synthesized from 2 μg of total RNA using *AccuPower* RT PreMix (Bioneer, Korea) and an oligo $(dT)_{12-18}$ primer as described in the manufacturer's protocol. The ribosomal protein S7 gene sequence and actin was used as positive internal control. PCR cycle numbers were constant for a particular sequence in the multiple samples analyzed in a given experiment and chosen empirically to attain comparable band intensities for the different markers in each experiment while avoiding saturation. The primers used were as follows: *A. sinensis actin* forward, 5'-TGTACGCTTCCGGTCGT-3' *A. sinensis actin* reverse, 5'-TCGCACTTCATGATGGA-3'. *A. sinensis SOCS* foward, 5'-ATCGAGCAGTTCAATCACAA-3' *A. sinensis SOCS* reverse, 5'-GGTACGCCTTCAGAGA CTTT-3'.

Results and Discussion

Cloning of *AsSOCS box* and multiple alignment

PCR-based cloning approach was used to clone a highly conserved SOCS box domain using degenerate primer, and 540-bp partial fragment was cloned into TA vector. Sequence analysis indicates that it has high identity (72~96%) to SOCS box motif from various organisms such as *A. gambiae, Ae. aegypti, D. melanogaster, Mus musculus*, and *Homo sapiens* (Fig. 1A). This strongly indicates that SOCS box motif is highly conserved in diverse organisms, ranging from mosquitoes to human. In addition,

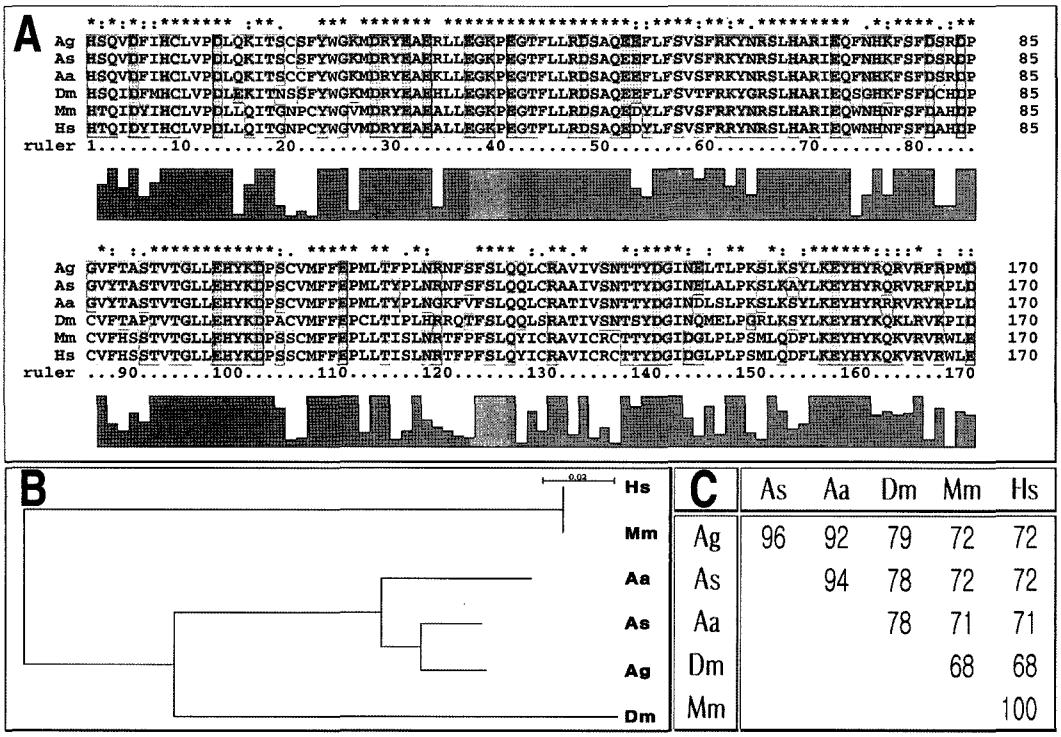

Fig. 1. Relationship of *AsSOCS* (As) to other SOCS (A) Aligned sequences of the conserved domains of *AsSOCS* (As) and five other SOCS. (B) Dendrogram based on the alignment of the SOCS sequence, constructed using CLUSTAL X (Thompson *et al.*, 1997). (C) Matrix of percentage amino acid identities in the aligned central region between SOCS. *Anopheles gambiae* (Ag), *Drosophila Melanogastar* (Dm), *Mus musculus* (Mm) and *Homo sapiens* (Hs).

phylogenetic tree using CLUSTAL X (Thompson *et al.*, 1997) showed a similar data (Fig. 1B). Finally, matrix of percentage amino acid identities in the aligned central region between SOCS showed that *AsSOCS* box has highest homology to AgSOCS (Fig. 1C).

It has been reported that there are three different types of homologues of mammalian SOCS in *Drosophila* (Rawlings *et al.*, 2004). It includes *SOCS36E* (Karsten *et al.*, 2002; Rawlings *et al.*, 2004), *SOCS44A*, and *SOCS16A*. Considering the facts that SOCS gene family is highly conserved, it is highly likely to have similar types of SOCS homologues in mosquito. Until now, no study has been made to clarify the roles of SOCS during immune responses in fruit fly and mosquitoes. In this context, it would be interesting to know which type of SOCS is preferentially induced depending on the type of pathogens or immune elicitors.

SOCS box motif has been known to interact with elongin-B and -C (Kamura *et al.*, 1998; Zhang *et al.*, 1999; Elliott *et al.*, 2004). We have generated a high quality of *AsSOCS* antibody. Thus we are planning to conduct immunoprecipitation experiment to see if *As-SOCS* box motif interacts with mosquito elongin-B and -C in the Pb-invaded midgut cells. In addition, another experiment will be conduced to test *AsSOCS* may interact with JAK molecules under the similar conditions.

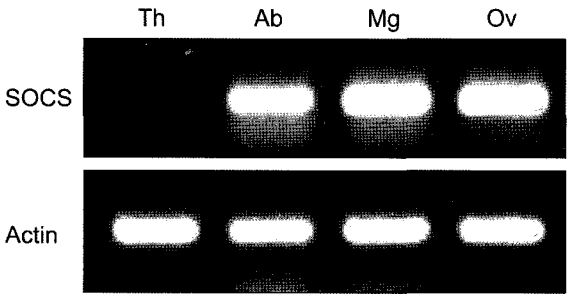

Fig. 2. Tissue-specific expression pattern of *As-SOCS* box. The loading of equal amounts of RNA per lane was verified by actin marker. Th, Thorax; Ab, abdomen; MG, midgut; Ov, ovary.

Spatial and developmental expression of *AsSOCS* in various tissues

RT-PCR analysis for tissue specificity indicated that the highest expression was detected at abdomen, midgut and egg (Fig. 2). However, it was not possible to detect the signal from head tissue. In addition, developmental expression patterns showed that the level of *AsSOCS* was

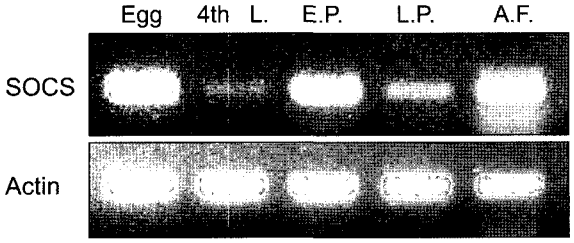

Fig. 3. Developmental expression profile of AsSOC mRNA. The loading of equal amounts of RNA per lane was verified by actin marker. 4th L, 4th instar larvae; E. P., early pupae; L. P., late pupae; A. F., adult female whole body.

high at egg, early pupae and adult female. It has been reported that drosophila *SOCS36E* mRNA was also developmentally expressed, with maximal levels between 2 and 12 hrs of embryonic stage. The *SOCS36E* mRNA level rapidly declines at the end of embryogenesis and remains low during larval stages with a modest increase during pupal development (Callus *et al.*, 2002). In contrast, the level of *AsSOCS* mRNA in a Korean malaria vector, *A. sinensis* was very high at early pupal stage (white pupae) although the precise role of the *AsSOCS* increased at this stage remains to be further elucidated.

Up-regulation of *AsSOCS* mRNA by various immune elicitors

To see if SOCS, negative feedback regulator of JAK-STAT signaling cascade, is induced by various immune elicitors, we have conducted infection experiments under the various conditions. RT-PCR analyses showed that SOCS mRNA was strongly induced in larvae in response to bacteria containing LPS (Fig. 4A). Furthermore, laminarin, CpG (Bacterial DNA) and LTA (gram positive cell wall component) initially, for unknown reasons, decrease the level of SOCS transcripts at 6 hrs but enhanced its level in 12 hrs post-injection.

It has been well known that during initial interaction of pathogens with the host's immune system, pathogen-associated molecular pattern (PAMP) triggers cells of the innate immune system. Conserved bacterial components such as LPS, peptidoglycan, lipoteichoic acid, or bacterial CpG-DNA have been characterized as structural correlates of PAMP. Indeed, it appears that PAMPs injected into mosquito thorax could be involved in activating JAK-STAT signaling pathway present in mosquito innate immune system, and furthermore the SOCS repressor of JAK-STAT pathway is elevated.

We have cloned *AsSOCS* box motif from a Korean malaria vector, *A. sinensis*. As expected, *AsSOCS* was strongly induced in response to various immune elicitors such as bacteria, LTA, CpG-DNA and laminarin in both

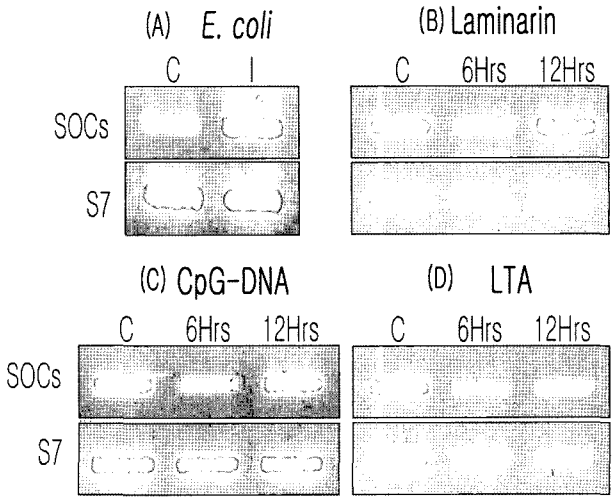

Fig. 4. Induction pattern of SOCS by various immune elicitors. (A) *AsSOCS* mRNA in larvae was strongly induced in response to bacteria (*E. coli*). (B, C and D) *AsSOCS* was also induced in adult mosquitoes by various immune elicitors such as laminarin, CpG-DNA and LTA.

larvae and adult mosquitoes. However, it should be tested to see if *Plasmodium berghei* induces the transcript of *AsSOCS* from the Plasmodium-invaded midgut cells. The previous report showed that *P. berghei* ookinetes invade polarized columnar epithelial cell, protrude towards the midgut lumen, and induce nitric oxide synthease (NOS) expression. The effector molecules mediating immune response, such as nitric oxide can be potentially toxic to the host. One of the signaling pathways known to be directly linked to the generation of NOS is JAK-STAT signal transduction pathway. In this context, we will focus on the followings: (1) RT-PCR will be conducted to see if Plasmodium induces *AsSOCS* in Plasmodium-invaded midgut epithelium, (2) Full length cDNA of *AsSOCS* will be screened cDNA from As-cDNA library we generated, (3) The precise role of SOCS in the midgut cells will be elucidated in the context of the interactions between mosquito midgut epithelial cells and *Plasmodium berghei* ookinetes. For example, knowledge of how *AsSOCS* proteins are regulated and/or degraded might provide insights into how SOCS repressor can be used by Plasmodium. For instance, Plasmodium has been reported to be killed in midgut by NOS/NO molecules (Luckhart *et al.*, 1998). Thus, if Plasmodium in the midgut cells develops one way to reduce the level of NOS/NO, the rate of Plasmodium survival will be greatly increased. To test this possibility, we are also planning to conduct RNAi experiment and evaluate the potential effects of *AsSOCS* knockdown on the Plasmodium survival rate by counting ookinete and oocyst numbers in midgut.

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