Characterization of Two Mosquito STATs, AaSTAT and CtSTAT

Differential Regulation of Tyrosine Phosphorylation and DNA Binding Activity by Lipopolysaccharide Treatment and by Japanese Encephalitis Virus Infection

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Two mosquito STATs, AaSTAT and CtSTAT, have been cloned from Aedes albopictus and Culex tritaeniorhynchus mosquitoes, respectively. These two STATs are more similar to those of Drosophila, Anopheles, and mammalian STAT5 in the DNA binding and Src homology 2 domains. The mRNA transcripts are expressed at all developmental stages, and the proteins are present predominantly at the pupal and adult stages in both mosquitoes. Stimulation with lipopolysaccharide resulted in an increase of tyrosine phosphorylation and DNA binding activity of AaSTAT and CtSTAT as well as an increase of luciferase activity of a reporter gene containing Drosophila STAT binding motif in mosquito C6/36 cells. After being infected with Japanese encephalitis virus, nuclear extracts of C6/36 cells revealed a decrease of tyrosine phosphorylation and DNA binding activity of AaSTAT which could be restored by sodium orthovanadate treatment. Taking all of the data together, this is the first report to clone and characterize two mosquito STATs with 81% identity and to demonstrate a different response of tyrosine phosphorylation and DNA binding of these two STATs by lipopolysaccharide treatment and by Japanese encephalitis virus infection.

Mosquitoes, like other insects, have efficient humoral and cellular defense system(s) that exhibit prominent similarity to the innate immunity in vertebrates (1, 2). The innate immunity is the first line defense of insects and mammals to combat microbes. This ancestrally common defense system in Drosophila and mammals depends on conserved intracellular signaling pathways that can respond rapidly to infection by transcriptional regulation (1, 3). Two distinct pathways are involved in the regulation of antimicrobial peptides in the fat body. First is the Toll/Dif pathway, which is activated mainly by fungi and Gram-negative bacteria, and the second is the Imd/Rel pathway, which is activated mainly by Gram-positive bacteria. These two conserved pathways mediate differential expression of antimicrobial peptides via distinct nuclear factor-κB-like transcription factors (4–6).

In addition to the Toll/Dif and Imd/Rel pathways, the Drosophila JAK/STAT pathway, which is involved in several developmental events, has also been shown to regulate the cellular immune response (7). Recently, it has been demonstrated that a complement-like humoral protein TEP1 was produced via JAK/STAT pathway in Drosophila after bacterial challenge (8). Moreover, the Anopheles STAT could translocate into nuclei of the fat body cells after bacterial challenge (9). The activation of c-Jun N-terminal kinase and JAK/STAT pathways after LPS stimulation in addition to Toll/Dif and Imd/Rel pathways was also detected by a genomewide approach in Drosophila (10). All data suggested that there is a tight connection among the pathways of Toll/Dif, Imd/Rel, and JAK/STAT in the innate immune responses in the insect. In this scenario, the JAK/STAT pathway is most likely to be activated to produce complement-like humoral factors after the activation of the Toll/Dif or Imd/Rel pathway for secretion of antimicrobial peptides to accelerate bacterial clearance. However, the molecular mechanism linking these two pathways remains to be characterized.

In mammals, the interferon (IFN) signaling through JAK/STAT is an essential pathway to defend viral infection (11, 12). The well known IFN signaling pathway begins with the binding of IFN-α/β or IFN-γ to its cognate receptors on the surface of cells, followed by the activation of JAK/STAT and the translocation of activated STAT dimer to the nucleus for binding to interferon-stimulated response element or γ-interferon-activated sites on target genes and activates the transcription of interferon-stimulated genes that encode proteins with potent antiviral, antiproliferative, and anti-inflammatory effect (13–15). Although these IFN immune responses through the JAK/STAT pathway to combat viral infection are evolutionally conserved in mammals, there is no evidence to support whether equivalent homologs of IFN signal transduction pathways are present in mosquitoes to combat virus.

Mosquitoes can transmit a variety of arboviruses that cause different diseases in mammals (16). Aedes albopictus (Skuse) and Culex tritaeniorhynchus Giles mosquitoes are known to transmit flaviviruses such as Dengue virus and Japanese encephalitis virus (JEV), which cause severe diseases in human...
For their survival, arboviruses must have developed strategies to replicate in both vertebrate and invertebrate to avoid the efficient immunity produced by these two different hosts (18), although mosquito does not have adaptive immunity. We are interested in the regulation of the JAK/STAT pathway in the immune reactions of these two mosquitoes in response to LPS stimulation and JEV infection. To our knowledge, this laboratory has sequenced the genome of one A. albopictus and Aedes aegypti strain and random hexamers in a 50-μl reaction mixture containing 5 mg of template DNA, 2 μl of 10× SYBR Green Master Mix, 1.5 mM MgCl₂, and 100 ng of actin primers (a-3F5, a-3F6, and a-actin-R, 5′-TCGCCATCGGATCATCGGTC-3′; and a-3end, 5′-CGAC-3′, and c-actin-F, 5′-GAACGACCCGACCATCACGTGGTCC-3′) or C3F2 (5′-GGTACCTTGTGATCATCTCTCCG-3′) for a-actin-R, 5′-CGATCAGCAGTC-3′). These two primers and first strand cDNAs of the two mosquitoes, PCR was performed to isolate STAT homologs cDNA fragment. The resulting PCR product of 700 bp was cloned into pGEM-T easy vector (Promega) and sequenced.

Rapid Amplification of cDNA Ends (RACE)—The 5′- and 3′-ends of AaSTAT and CβSTAT mRNAs were obtained by the RACE technique using the Marathon-Ready cDNA Amplification kit (CLONTECH, Palo Alto, CA) according to the supplier’s instructions. The first PCR was performed with a 27-mer sense primer (AP1) specific for the adaptor and an AaSTAT-specific antisense primer A5F1 (5′-ATCCATGATCCTCCGCACCGACATGTC-3′) or a CβSTAT-specific antisense primer CS5F1 (5′-TCTGATATCTCCGCCAGATGTCACCT-3′), then the second round of PCR was carried out with a nested 22-mer sense primer (AP2) and a nested-gene-specific antisense primer A5F2 (5′-TTGGACCTGCTGCTG-3′) or CS2F2 (5′-TTGAGGCTGTTCCCGGATGCGG-3′). Similarly, 3′-RACE was performed with two rounds of PCR, first with the AP1 primer and an AaSTAT-specific primer A5F1 (5′-GAAGCTGATCCGTCCGCGATGC-3′) or a CβSTAT-specific primer CS1F1 (5′-TGGCCAGAAGGCTGCTGCT-3′) or CS2F2 (5′-TTGAGCCTGCTGCGCAGCG-3′). Sequences in the SH2 domain and 3′-untranslated region respectively), were performed in a 50-μl reaction mixture containing 25 ng of the specific actin primers from A. albopictus (a-actin-F, 5′-TCCGATCCAAGGCTGTGTC-3′; and a-actin-R, 5′-GAGTGTGATACAGGCTGTCG-3′) or AaSTAT-specific primers (a-3F5, 5′-CCAGACACCGGCAGCGA-3′; and a-3end, 5′-CCTCTTACCCGCTCCAGATTAGC-3′, corresponding to sequences in the activation domain and 3′-untranslated region, respectively). Other PCR were performed in a 50-μl reaction mixture with the specific actin primers from C. triaenohydrinus (a-actin-F, 5′-TCCGTATCCCGGCTGTGTC-3′; and c-actin-R, 5′-GAGTGTGATACAGGCTGTCG-3′) or CβSTAT-specific primers (c-3F3, 5′-CAGATCTGGACATCAGGCGTACCC-3′; and c-3end, 5′-ACAGTGGCCACCCCCACCTGATGACGC-3′, corresponding to sequences in the SH2 domain and 3′-untranslated region respectively), 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.5 unit of ExTaq (TaKaRa Shuzo, Shiga, Japan). The conditions for amplification were denaturation at 96 °C for 2 min, then 35 cycles of 96 °C for 1 min, 50 °C for 30 s, and 72 °C for 30 s, and final extension at 72 °C for 5 min. A negative control was performed in the absence of RNA. The products were resolved on a 1.2% agarose gel and stained with ethidium bromide.

Quantitative Real Time PCR Analysis—Gene expression of AaSTAT was also determined by quantitative real time PCR analysis on the DNA Engine Opticon 2 System (MJ Research Inc., Reno, NV) with the FastStart DNA master SYBR Green I (Roche Applied Science) as described previously (23). The reaction mixture contained 6 μl of template cDNA, 2 μl of 10× SYBR Green Master Mix, 1.5 mM MgCl₂, and 100 ng of primers (a-3F5, a-3F6, and a-actin-F, a-actin-R for AaSTAT gene and actin gene, respectively) at a final volume of 20 μl. The reactions were denatured at 95 °C for 10 min and cycled 50 times under the following parameters: 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s. At the end of the PCR, the temperature was increased from 65 to 95 °C at a rate of 1 °C/min, and the fluorescence was measured every 15 s to construct the melting curve. A nontemplate control was run with every assay, and all determinations were performed at least in duplicate to achieve reproducible results. The actin gene was used as the reference gene. The threshold cycles (Ct) were recorded for all samples for both the AaSTAT gene and the reference gene. The relative gene copy number was calculated from the Ct of the AaSTAT gene and the reference gene. By subtracting the Ct of the reference gene from the Ct of the AaSTAT gene, the ΔCt value for the AaSTAT gene was determined.

Plasmid Construction—The expression vector, pcAaSTAT-HA and pcCβSTAT-HA were constructed by inserting the open reading frame of AaSTAT and CβSTAT before a HA tag and between the Hind III and Kpn sites of pHA-YUN, which is derived from pcDNA3. The pHA-YUN plasmid was kindly provided by Dr. H. J. Kung (University of California at Davis Cancer Center, Sacramento). The JH1 DNA of C. triaenohydrinus (235 amino acids) was used as the sequence for the HA tag. A negative control of the expression number AY278117 was generated by PCR with primers containing EcoRV and NotI restriction sites on both ends and cloned into the C terminus of pcAaSTAT-HA and pcCβSTAT-HA to generate pcAaSTAT-HA-cJH1 and pcCβSTAT-HA-cJH1, respectively. The resulting clones were confirmed by DNA sequencing.

The reporter plasmid 2XDrai was kindly given by Dr. M. Yamaguchi (Laboratory of Cell Biology, Aichi Cancer Center Research Institute, Nagoya, Japan). This reporter plasmid contains the Drosophila raf gene promoter with two recognition consensus sequences (TTCGGCGGAA) for Drosophila STAT and a luciferase reporter gene (27).

In Vivo Transcription and Translation—The TNT-coupled transcription/translation system (Promega) was used to synthesize AaSTAT-HA, CβSTAT-HA, AaSTAT-HA-cJH1, and CβSTAT-HA-cJH1 proteins in vitro according to the manufacturer’s instructions. Briefly, 0.2–1 μg of DNA plasmids and 1–20 μl of [35S]methionine were added to the master mixture and incubated for 90 min at 30 °C. The synthesized polypeptides were analyzed by SDS-PAGE and visualized by autoradiography. For subsequent bandsheet analysis and Western blotting, [35S]methionine was replaced by cold methionine (final concentration, 1 mM).

Cell Cultures—Mosquito C6/36 cells (25, 26) were grown in RPMI 1640 medium supplemented with 2% fetal bovine serum, 50 units/ml penicillin G, 50 μg/ml streptomycin, 50 μg/ml gentamicin, 25 mM HEPES in a humidified atmosphere of 5% CO₂ at 28 °C. All the culture regents were purchased from Hyclone Laboratories (Logan, UT). To infect with JEV, an ~80% confluent monolayer of C6/36 cells grown in 10-mm dish was first absorbed with JEV at multiplicity of infection 0.2 for 1 h at
28 °C. After adsorption the unbound viruses were removed by gentle washing with serum-free culture medium, and fresh medium containing 2% fetal calf serum was added to the dish for further incubation at 28 °C. Alternatively, after infection with JEV for 1 day, C6/36 cells were treated with 50 µM sodium orthovanadate (Sigma) and harvested on day 3 postinfection.

**Virus and Virus Infection—**JEV strain NT113, isolated from mosquitoes in Taiwan in 1985 (27), was inoculated onto a confluent monolayer of C6/36 cells in RPMI medium containing 2% fetal calf serum. Cells were maintained for 5 days at 28 °C, and then supernatant fluids were harvested and stored at −70 °C. This virus titer was 1 × 10^6 plaque-forming units/ml as determined by plaque assay on BHK21 cells. This virus stock, from the Institute of Preventive Medicine, Taipei, Taiwan, was used as a positive control for C6/36 cells for future assay.

**Mosquitoes—**The colonized mosquitoes used in the study were *A. albopictus* (Skuse) (Chungsho strain) and *C. tritaeniorhynchus* Giles (Peitou strain), which were maintained on 10% sucrose solution in a humidified (80%) insectary at 26−28 °C with 14/10-h light/dark cycles. Larvae were reared in water pans and fed on commercial Tetramin fish food and yeast powder. These mosquitoes were kindly provided by S. Hung (Center for Disease Control, Department of Health, Taipei, Taiwan). Between 5 and 7 days after emergence mosquitoes were inoculated intrathoracically (28, 29) with LPS (Escherichia coli serotype 0127:B8, Sigma). The injection volume was 98 nl containing 9.8 ng of LPS.

**Preparation of Whole Cell Lysates and Nuclear Extracts—**Whole cell lysates were prepared as described previously (30). In brief, cells were washed twice with phosphate-buffered saline and lysed in the lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) containing 0.2 mM Na3VO4 and 0.2 mM phenylmethylsulfonyl fluoride. The mosquito embryos, larvae, pupae, and adults were also homogenized with the same lysis buffer. Extracts were centrifuged at 4 °C for 10 min at 13,000 × g, and the resulting supernatants were used for subsequent Western blotting. Nuclear extracts were prepared according to the procedures described previously (31). Briefly, cells were washed twice with phosphate-buffered saline and solubilized with buffer A (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM Na3VO4, 0.1 mM NaF, 20 mM Na3P04, 20 mM Na3P04, 10% glycerol, 1 µg/ml of aprotinin, pepstatin, and leupeptin, pH 7.9) on ice for 30 min and then vortexed vigorously and centrifuged at 12,000 × g at 4 °C for 1 min. The supernatant was the cytoplasmic part at this stage. The pellets were then extracted with buffer B (20 mM HEPES, 350 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na3VO4, 0.2% Nonidet P-40, 10% glycerol, 1 µg/ml aprotinin, pepstatin, and leupeptin, pH 7.9), rotated at 4 °C for 30 min. The extracts were centrifuged at 13,000 × g at 4 °C for 5 min, and these supernatants, nuclear extracts, were quickly frozen and stored at −70 °C for subsequent use by electrophoretic mobility shift assay (EMSA) or Western blotting.

**cDNA cloning of adult female mosquitoes for EMSA was carried out by submerging the mosquitoes in liquid nitrogen for 5 min and then homogenized in buffer A. All the protein concentrations were determined by the Bradford method (Bio-Rad).

**Antibodies and Western Blotting—**Monoclonal Abs against α-tubulin was purchased from Sigma and Oncogene (Cambridge, CA). Monoclonal Ab against γ-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The remaining Abs were purchased from Santa Cruz Biotechnology (CA), and their specificities were determined by the manufacturer’s instructions.

**EMSA—**EMSA was performed as described previously (35). Briefly, reactions were performed by the addition of nuclear extracts, adult mosquito extracts, or products of *in vitro* transcription and translation in the presence of 32P-labeled double-stranded oligonucleotide probes (10,000 cpm/μg) to the binding buffer: 20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10 mM MgCl2, 0.5 mM ZnCl2, 0.02% Nonidet P-40, 10 µg/ml bovine serum albumin, 10% glycerol, and 2 µg of poly(dI-dC) in a 25-µl reaction volume. An excess of unlabeled specific or mutant oligonucleotides was added as competitor. 1 µl of antiserum (1:2 dilution in phosphate-buffered saline) was also added as indicated. After incubation at room temperature for 15 min, the reaction mixture was loaded on 5% polyacrylamide gel in 0.5 × TBE and run at 200 V for 2 h at room temperature. The oligonucleotides probes used in this assay were commercially available, corresponding to the DNA binding motifs for mammalian STATs (Santa Cruz). The sequences of the upper strand of the normal and the corresponding mutant oligonucleotides are listed in Table 3 of Ref. 30. The probes were prepared by annealing the upper and lower strands of oligonucleotides, one of which was end labeled with γ-32P-ATP by using T4 polynucleotide kinase (Promega).

**Transactivation Assay—**The plasmids used for transfections were purified by Qiagen plasmid purification kit (Qiagen). For transactivation assay, plasmid transfections were conducted by using the CELL- FECTIN kit (Invitrogen) in 6-well plates. For investigation of the endogenous AsaSTAT activation by LPS, approximately 80% confluent C6/36 cells were transfected with 0.2 µg of 2XdraSTATwt for 24 h; subsequently 10 µg/ml LPS was added into the culture medium. After stimulation with LPS for 30, 60, and 90 min, cells were harvested and extracted for luciferase activity assay. 2XdraSTATwt contains the raf gene promoter of Dro sophila with two recognition consensus sequences for Dro sophila STAT and a luciferase gene (24). The C6/36 cells were harvested at 24, 25, and 25.5 h post-transfection and assayed for luciferase activity using luciferase assay kit Firelite according to the manufacturer’s instructions (Packard). Final luciferase activity was obtained after normalizing protein concentration of each sample.

**RESULTS**

**Isolation and Characterization of AnSTAT and CisSTAT Genes—**Two STAT homologs were isolated from mosquitoes *A. albopictus* and *C. tritaeniorhynchus* by RT-PCR amplification using degenerate primers containing sequences corresponding to two stretches of amino acid residues, KQPPQV(M/I)K and TFEWEFL(Y/F)T/A, which are highly conserved between mammalian and insect STATs (underlines in Fig. 1). The first strand cDNA from larvae of these two mosquitos was used as template. Only one PCR product of 700 bp was obtained, the sequences from which were used to design the primers A5F1, A3F1, C5F1, and C3F1 for further 5'- and 3'-RACE to obtain full-length cDNA of the AnSTAT and CisSTAT. The full-length AnSTAT cDNA encompasses 3579 bp including a 2724-bp coding region, which encodes a protein of 907 amino acid residues, as well as a 524-bp 5'-untranslated region and 331-bp 3'-untranslated region (Fig. 1). The full-length CisSTAT cDNA consists of 4014 bp including a 2643-bp coding region, which encodes a protein of 880 amino acid residues, plus 843 bp upstream and 528 bp downstream of the open reading frame. These two sequences were deposited in GenBank with accession numbers AY299686 and AY299687, respectively.

The overall organization and the deduced amino acid residues of these two mosquito STATs were compared with known STATs of *Anopheles, Drosophila*, and human are shown in Fig. 1. Sequence alignment shows that AnSTAT and CisSTAT have the same functional domain organization as the known STATs and highly conserved sequences between the DNA binding domain and SH2 domain and lower homology in the N-terminal protein interaction domain, coiled-coil domain, and C-terminal transactivation domain. The conserved amino acid residues are also annotated. For example, a tyrosine at residue 685 to be phosphorylated by JAKs during activation (20) in the C-terminal transactivation domain of both AnSTAT and CisSTAT is conserved. An arginine at residue 31 in the N terminus whose
Comparison of deduced amino acid sequences of *Aa*STAT and *Ct*STAT with those from *Ag*STAT, *Dm*STAT, and human STAT5a. The encoded amino acid sequences were aligned using the PILEUP program (Genetic Computer Group). Gaps are introduced to optimize alignment and are shown as dashes. Arrowed brackets indicate the boundaries of functional domains of the STAT polypeptide. The putative phosphorylated tyrosine residue is indicated by a black star. Black underlines indicate the two conserved regions from which the degenerate oligonucleotides were designed and used to clone STAT homologs from cDNA of *A. albopictus* and *C. tritaeniorhynchus*. Black shadows indicate identity in amino acid residues. Accession numbers for the five sequences are the same as in Fig. 2.
Interestingly, the length of C-terminal activation domain of STATs is conserved through all the STATs (36), suggesting that methylation was reported affecting STAT dephosphorylation, is conserved through all the STATs. Based on the amino acid sequence comparison in all STATs, phylogenetic analysis was performed in the central conserved region from the DNA binding domain to the SH2 domain. As illustrated in Fig. 2, AoSTAT, CtSTAT, and Anopheles AgSTAT, Drosophila DmSTAT, and human STATs5 and STAT6 are more closely related to each other with about 50% identity (data not shown) and constitute an ancient class of the STAT family. In contrast, human STAT1, STAT2, STAT3, and STAT4 form another diverged group. These analyses raised the possibility that an ancestral STAT gene had been duplicated in the split between insects and vertebrates to create these two classes of STAT family.

The mRNA and Protein Expression Profiles of AoSTAT and CtSTAT Are in the Ancient Class of the STAT Family—Based on the amino acid sequence comparison in all protein sequences, AoSTAT and CtSTAT are 81% identical to each other and show 35 and 28% identity to those of Anopheles and Drosophila. Moreover, AoSTAT and CtSTAT displayed 30 and 27% identity to human STATs5 and STAT6, respectively, but with only 18–20% identity to another class of human STAT family (Table I). To investigate further the evolutionary relationship between AoSTAT, CtSTAT, and other STAT family members, phylogenetic analysis was performed in the central conserved region from the DNA binding domain to the SH2 domain. As illustrated in Fig. 2, AoSTAT, CtSTAT, Anopheles AgSTAT, Drosophila DmSTAT, and human STATs5 and STAT6 are more closely related to each other with about 50% identity (data not shown) and constitute an ancient class of the STAT family. In contrast, human STAT1, STAT2, STAT3, and STAT4 form another diverged group. These analyses raised the possibility that an ancestral STAT gene had been duplicated in the split between insects and vertebrates to create these two classes of STAT family.

The mRNA and Protein Expression Profiles of AoSTAT and CtSTAT at Different Developmental Stages—The mRNA expression level of AoSTAT and CtSTAT at the embryonic, larval, pupal, adult stages, and C6/36 cells was analyzed by RT-PCR. As shown in Fig. 3A, a 305-bp DNA fragment could be amplified from all stages examined in A. albopictus. The quantitative real-time PCR analysis (Fig. 3B) also revealed that equivalent mRNA expression of AoSTAT was found at all stages except pupal and male mosquito, in which their AoSTAT mRNA levels were higher. In Fig. 3C, an 819-bp DNA fragment was also amplified at all stages examined but lower at embryonic stage in C. tritaeniorhynchus. A similar conclusion was obtained by quantitative real-time PCR (data not shown). These results showed that AoSTAT and CtSTAT were ubiquitously transcribed at all developmental stages, comparable with the expression patterns of Anopheles STAT gene (9). When the protein expression of AoSTAT was analyzed by Western blotting using anti-AoSTAT polyclonal Abs, a major band of about 102 kDa was shown in C6/36 cells, pupae as well as adults of A. albopictus, but not found in embryos and larvae (Fig. 4A, upper). Similarly, anti-CtSTAT Abs detected a protein band of about 100 kDa in pupae and adults of C. tritaeniorhynchus, lower amount in larvae, but not in embryos (Fig. 4C). The detectable bands were not observed when the membranes were reprobed with the preimmune sera at the same dilution (data not shown). The above membranes were reprobed with mAb against α-tubulin, a band about 55 kDa and 58 kDa was observed in equal amounts in C6/36 cells and adult stages of A. albopictus (Fig. 4A, lower) and adult stages of C. tritaeniorhynchus (data not shown), respectively. The antibody could not detect the corresponding proteins in the protein samples from embryo, larva, and pupa possibly because of the presence of other isoforms of α-tubulin. The integrity of all protein samples was shown by Coomassie Blue staining (Fig. 4B). These data demonstrated that AoSTAT and CtSTAT proteins were expressed with detectable amount at pupal and adult stages, suggesting that they may play some roles during developmental stages guarding against environmental stress.

In Vitro Translated AoSTAT-HA-cJH1 and CtSTAT-HA-cJH1 Fusion Proteins Are Tyrosine-phosphorylated and Bound
to Mammalian STAT5-responsive Element—To investigate the biochemical properties of the AaSTAT and CtSTAT proteins, we adopted the strategy developed by Berchtold et al. (37) to generate constitutively active AaSTAT and CtSTAT variants and to characterize their DNA binding properties in a cell-free system. AaSTAT-HA-cJH1 and CtSTAT-HA-cJH1 fusion proteins were generated by fusing the JH1 domain of the JAK from C. tritaeniorhynchus mosquito into the C-terminal end of AaSTAT and CtSTAT proteins, respectively, as described previously (30). In vitro transcription and translation products of AaSTAT-HA, AaSTAT-HA-cJH1, CtSTAT-HA, and CtSTAT-HA-cJH1 were all recognized by mAb against the HA tag, but only AaSTAT-HA-cJH1 and CtSTAT-HA-cJH1 were recognized by anti-pY99 mAb (Fig. 5A), demonstrating that the JH1 domain of JAK from C. tritaeniorhynchus can autophosphorylate or transphosphorylate AaSTAT-HA-cJH1 and CtSTAT-HA-cJH1 in vitro, respectively.

We next investigated whether AaSTAT, CtSTAT, AaSTAT-HA-cJH1, and CtSTAT-HA-cJH1 bind DNA in vitro. As shown in Fig. 5B, AaSTAT-HA-cJH1 and CtSTAT-HA-cJH1 formed complexes with the STAT5 binding motif (Fig. 5B, lanes 2 and 6). In contrast, AaSTAT and CtSTAT, without cJH1 domain, did not bind to the STAT5 binding motif (Fig. 5B, lanes 1 and 5). The shifted bands were not seen in the presence of a 50-fold excess of cold probe (Fig. 5B, lanes 3 and 7) but sustained in the presence of mutant competition (Fig. 5B, lanes 4 and 8). These results indicate that tyrosine phosphorylation of AaSTAT and CtSTAT by JAK is required for the DNA binding of the AaSTAT and CtSTAT. Several commercial available mammalian DNA binding motifs for mammal STATs (sis-inducible elements, STAT1, 3, 4, 5, 6) were applied in similar EMSA experiments. In agreement with the phylogenetic analysis in Fig. 2, among the STAT response elements tested, AaSTAT-HA-cJH1 and CtSTAT-HA-cJH1 displayed specific and strong binding to the STAT5 binding motif, weak binding to STAT3, 4, 6 elements, and not at all to either sis-inducible elements or STAT1 binding motif (data not shown). Therefore, the STAT5 binding motif was used further to test the DNA binding activity of the endogenous STAT proteins in adult mosquito or its derived C6/36 cells in EMSA.

AaSTAT and CtSTAT Were Activated in Response to LPS Stimulation—C6/36 cells transfected with 2XDrafSTATWT reporter plasmid for 24 h and then were treated with LPS for 30, 60, and 90 min before being harvested. There was a 2-3-fold

Fig. 2. The phylogenetic tree analysis of AaSTAT, CtSTAT, and other STAT family members. The amino acid sequences between DNA binding, linker domain, and SH2 domains of AaSTAT and CtSTAT were aligned with those of 10 known STAT proteins. The phylogenetic tree was constructed by using the NEIGHBOR-JOINING program together with bootstrap analysis using 1,000 replicates provided by ClustalX. Branch lengths are proportional to sequence divergence. Branch labels record the stability of the branches more than 1,000 bootstrap replicates. GenBank accession numbers of the sequences used are as follows: Caenorhabditis elegans (CeSTAT), Z70754; Drosophila melanogaster (DmSTAT), Q24151; human h-STAT1 (HsSTAT1), P42224; h-STAT2 (HsSTAT2), P52830; h-STAT3 (HsSTAT3), P40763; h-STAT4 (HsSTAT4), Q14765; h-STAT5a (HsSTAT5a), P42229; h-STAT5b (HsSTAT5b), NP036580; h-STAT6 (HsSTAT6), P42226; Anopheles gambiae (AgSTAT), AJ010299; A. albopictus (AaSTAT), AA99686; C. tritaeniorhynchus (CtSTAT), AA99687.
increase in luciferase activity after 30-min LPS stimulation and declined gradually (Fig. 6A). In Fig. 6B we show that anti-AaSTAT and anti-pY99 detected equivalent signals in C6/36 cells under LPS treatment, suggesting a high basal level of tyrosine phosphorylation in the culture cells. However, LPS treatment can still enhance the transcriptional activity of the AaSTAT. These results were in agreement with the previous report that the Gram-negative bacterial cell wall (LPS) induced the activation of endogenous STAT in Drosophila cells (10).

To examine further the activation of AaSTAT and CtSTAT by LPS in vivo, the adult females of these two mosquitoes were injected intrathoracically with LPS for 3 h, and the whole cell extracts were prepared for EMSA analysis. As showed in Fig. 7A, the DNA-protein complexes were formed in the LPS-treated mosquitoes, but not in the control (lanes 1 and 6 versus lanes 5 and 10). These complexes were competed completely in the presence of a 50-fold excess of unlabeled probes (lanes 2 and 7) but not with an excess of mutant probes (lanes 3 and 8). Supershift of the DNA-protein complexes was observed in the presence of anti-AaSTAT or anti-CtSTAT Ab, respectively (lanes 4 and 9). The same extracts from LPS-treated or control mosquitoes were inspected by Western blotting using anti-AaSTAT or anti-CtSTAT polyclonal Abs and anti-pY99 mAb. As shown in Fig. 7B, the AaSTAT and CtSTAT proteins were detected with equal amounts, and they were tyrosine phosphorylated mainly in the group of LPS treatment. Taken together, the intrathoracic injection of LPS induces AaSTAT and Ct-STAT proteins in vivo along with an increase of their specific DNA binding activity and tyrosine phosphorylation.

JEV Infection Inhibits the Activation of AaSTAT, and Sodium Orthovanadate Treatment Reverses This Effect in C6/36 Cells—To investigate whether virus infection would influence the activity of AaSTAT, the nuclear extracts of JEV-infected C6/36 cells were examined by EMSA. In normal C6/36 cells culture, there was basal activity of endogenous AaSTAT in the absence of LPS and Ct-STAT-HA-cJH1 (sixth and eighth lanes) were detected by Western blot analysis using anti-HA mAb (first, second, fifth, and sixth lanes), or anti-pY99 mAb (third, fourth, seventh, and eighth lanes). In B, the same fusion proteins were incubated with labeled STAT5 binding motif and followed by EMSA. In vitro transcription and translation products of AaSTAT-HA and CtSTAT-HA were used as control (lanes 1 and 5). The binding of fusion protein to STAT5 binding motif was completely abolished by the addition of a 50-fold molar excess of cold competitor (lanes 3 and 7) but remained unchanged when mutant oligonucleotide was added at a 50-fold molar excess (lanes 4 and 8).

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infection in C6/36 cells compromises tyrosine phosphorylation of AaSTAT protein and thus suppressed its activation as decreased DNA binding ability. To study further whether the reduced tyrosine phosphorylation of AaSTAT involves phosphatase activities, a tyrosine phosphatase inhibitor, sodium orthovanadate, was added into C6/36 cells infected with JEV (Fig. 9, lane 3), AaSTAT tyrosine phosphorylation was restored by such a treatment.

DISCUSSION

In this study, we cloned two STAT homologs from A. albopictus and C. tritaeniorhynchus mosquitoes and investigated their functional responses to LPS stimulation and JEV infection from the prospect of DNA binding activity and protein tyrosine phosphorylation status. Approximately 50% of sequence identity of these two STATs compared with those of Anopheles, Drosophila, and human STAT5 were found in the conserved regions from DNA binding domain to SH2 domain. They are much more similar to each other with 81% identity in the overall protein sequences (Table I). Their C-terminal transactivation domains, however, are unusually long and significantly more divergent (Fig. 1). Although two STAT genes were annotated in the complete genome sequence of A. gambiae (38), only one STAT gene has been isolated in each mosquito in this study. Similarly, there is only one STAT gene in the analogous insect Drosophila (19, 39). The possibility of having another STAT gene requires further investigation.

Recently, a number of constitutively activated mammalian STATs have been identified and constructed (40, 41). A fusion protein consisting of STAT5 and the kinase domain of JAK2 or JAK1 was also reported to be constitutively active (30, 37). In this study, the JH1 domain of JAK from mosquito C. tritaeniorhynchus was fused to the C terminus of AaSTAT and CtSTAT proteins to generate their constitutive active forms, namely AaSTAT-HA-cJH1 and CtSTAT-HA-cJH1. Similarly, each fusion protein showed tyrosine kinase activity and was phosphorylated on tyrosine (Fig. 5A). They also exhibited specific DNA binding activity to mammalian STAT5 binding motif (Fig. 5B). The core sequence of STAT5 binding motif is TTCTAGGA, which resembles the binding motif of human STAT5 (TTCCTNA/ GGAA) (42) and Drosophila DmSTAT (TTCNNNGAA) (19). These results indicate that the biochemical properties of these mosquito STAT proteins are more akin to mammalian STAT5 and Drosophila STAT, consistent with the higher homology between the DNA binding domains of these proteins (Fig. 2).

So far, only two insect STAT genes have been isolated and characterized from Drosophila and malaria mosquito, Anopheles gambiae. In Drosophila, a single JAK homolog, hopscotch (hop) (43) and a STAT protein have been identified (19, 39). Thus, the existence of an invertebrate JAK/STAT system with one JAK and one STAT has been established. The receptor of JAK/STAT signal transduction pathway in Drosophila has been identified recently (44, 45). The JAK/STAT pathway in Drosophila is not only involved in larval hematopoiesis but also involved in a number of developmental events (7, 46, 47). Moreover, the Drosophila STAT protein plays important roles in regulating the mitogen-activated protein kinase cascade through D-raf gene activation in the immune response. A STAT

**FIG. 6.** Endogenous AaSTAT was activated in response to LPS stimulation in C6/36 Cells. C6/36 cells were treated with 10 g/ml LPS for 30, 60, and 90 min before harvesting. The reporter plasmid (0.2 of Aa or blank control were probed with anti-STAT Ab (B). The nuclear extracts of C6/36 cells treated with LPS/H9262 reduced tyrosine phosphorylation of STAT and increased DNA binding ability. To study further whether the STAT tyrosine phosphorylation was restored by such a treatment.

**FIG. 7.** AaSTAT and CtSTAT were activated in response to LPS stimulation in vivo. Adult females extracts of A. albopictus and C. tritaeniorhynchus mosquitoes inoculated with LPS for 3 h were used for EMSA (A). Approximately 10 g of extracts was incubated with STAT5 probe. Binding complexes were completely abolished by unlabeled probes in a 50-fold molar excess, but not by an excess of mutant probes. Anti-AaSTAT and anti-CtSTAT polyclonal Abs partially de-probed with anti-pY99 mAb (B). Identical protein extracts were probed with anti-AaSTAT or anti-CtSTAT Abs to show equivalent amounts of protein (B, upper) and reprobed with anti-pY99 mAb (B, lower) to monitor the phosphorylation of STAT.
binding motif was identified in the promoter region of Drosophila Raf gene (24). The second member of insect STAT family (AaSTAT) with a possible role in immune response has been cloned from the human malaria vector A. gambiae. The AaSTAT was shown to translocate into the nuclei of the fat body cells after bacterial challenge (9). Also, microarray analysis of transcription pattern of Drosophila SL2 cells after LPS treatment revealed sequential activation of c-Jun N-terminal kinase and JAK/STAT pathways in addition to Toll/Dif and Imd/Rel pathways, which might be transmitted through the Drosophila Tak1 (10). Moreover, the gene encoding the complement-like TEP1, which might bind to the surface of bacteria and promote phagocytosis by hemocytes, had been identified as a target of the JAK/STAT pathway through the Toll cascade (8). All above observations provide direct evidences that the STAT pathway may participate in the immune response and contribute to antimicrobial infection. Our results are that 1) the AaSTAT and CtSTAT are activated at the level of protein tyrosine phosphorylation and DNA binding after LPS treatment (Fig. 7A); and 2) the D-raf reporter activity was elevated in C6/36 cells after LPS treatment (Fig. 6). These results are consistent with the previous observations mentioned above. Taken together, LPS stimulation in vivo and in vitro could activate the transcriptional activity of AaSTAT and CtSTAT, which are involved in immunity.

The mosquito cell line C6/36 was established from A. albopictus (25, 26) and used to culture many flaviviruses (48–50). JEV, a mosquito-borne flavivirus, infects more than 35,000 fatal cases annually, causes permanent neurological sequelae in survival patients (17), and infects a broad range of vertebrate species (51). JEV must develop unique strategies to replicate in both mosquitoes and many vertebrate hosts that have efficient immune system against the virus. In previous studies (52, 53), JEV appeared to be lifelong and was presented in most organs of mosquito while infected, indicating that JEV had escaped barriers and the innate immune responses in this mosquito. Although A. albopictus is not the major vector in contrast to C. tritaeniorhynchus for JEV, the JEV (54, 55) and West Nile virus, an encephalitic flavivirus (56), had indeed been isolated from A. albopictus. We then investigated the immune response of mosquito to the virus in JEV-infected C6/36 cells to study the regulation of AaSTAT pathway. A diminished DNA binding activity as well as decreased tyrosine phosphorylation of AaSTAT were observed in nuclear extracts of JEV-infected cells (Fig. 8), suggesting that JEV infection may interfere with the tyrosine phosphorylation of AaSTAT, probably through the induction of cellular phosphatase(s) or the inactivation of JAK or other tyrosine kinase(s) by viral products. Our data also showed that the addition of sodium orthovanadate, a tyrosine phosphatase inhibitor, to the JEV-infected cells could restore the tyrosine phosphorylation of AaSTAT protein (Fig. 9A), suggesting that at least the induction of cellular phosphatase(s) may be in part the cause of decreasing AaSTAT tyrosine phosphorylation. These results were comparable with a previous report that human cells infected with Sendai virus showed blocking of interferon signaling by inhibiting the tyrosine phosphorylation of Tyk2, which resulted in the subsequent failure in tyrosine phosphorylation of the STATs (57). However, it is unclear whether an analogous IFN is present in mosquitoes or whether a parallel pathway is present which could be activated by JEV through mosquito AaSTAT/CtSTAT signaling to induce antiviral effects. In summary, two mosquito STATs were cloned and their biochemical properties, such as tyrosine phosphorylation, DNA binding, and transactivation activity, were characterized by LPS treatment and by JEV infection.

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Characterization of Two Mosquito STATs, AaSTAT and CjSTAT: DIFFERENTIAL REGULATION OF TYROSINE PHOSPHORYLATION AND DNA BINDING ACTIVITY BY LIPOPOLYSACCHARIDE TREATMENT AND BY JAPANESE ENCEPHALITIS VIRUS INFECTION

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