Identification of Immune-Related Protein Kinases from Mosquitoes (Aedes aegypti)

Key Words
Mosquito
Aedes aegypti
Tyrosine kinase
Serine/threonine kinase
Insect immunity

Abstract
Protein kinases are known to be involved in signal transduction for numerous physiological events. However, little is known about the roles of protein kinases in insect immunity. A fragment around 150 bp was amplified by polymerase chain reaction using cDNA templates from bacterial inoculated mosquitoes and primers corresponding to the conserved domain of protein kinases. Based on sequence analysis, 11 groups of protein kinases were characterized including 3 nonreceptor tyrosine kinases, 3 receptor tyrosine kinases, 3 serine/threonine kinases, and 2 novel protein kinases. The most abundant kinase obtained in this study reveals a high degree of similarity to human cholinesterase-related cell division controller (CHED) protein kinase. The expression of this mosquito CHED-like kinase is not detectable in normal female mosquitoes, but induced only after bacterial inoculation and trauma. A mosquito protein kinase was demonstrated to share homology with a plant Tousled gene, but has not yet been characterized in the animal system. In addition, analysis of the sequences of several protein kinases cloned from mosquitoes suggests that they might be involved in the regulation of cellular or humoral immunity.

Introduction
Mosquitoes are of medical importance because they are vectors for many parasitic and viral diseases of humans and animals, such as malaria, lymphatic filariasis, dengue fever and yellow fever. A characteristic attribute of these diseases is that infectivity is acquired through a specific sequence of development, differentiation and migration within the vector insect. In mosquitoes, these pathogens must evade or overcome various protective barriers, such as the peritrophic membrane, digestive proteases, and immune responses. To understand the mechanisms involved in this interaction and the means by which parasites avoid the host’s defensive responses is critical for designing future strategies for controlling mosquito-borne diseases.

Insects are equipped with both cellular and humoral defense systems [18, 19, 26]. To date, except for the melanization which is an effective defense reaction of mosquitoes, the biochemical nature of the factors involved in mosquito immunity to parasites is largely unknown [26]. However, numerous antibacterial peptides
have been isolated and characterized in many insects. These immune proteins are induced after a bacterial challenge and reveal a broad spectrum of activity against Gram-positive and/or Gram-negative bacteria [8, 18]. In the mosquito *Aedes aegypti*, an important antibacterial peptide, defensin, was purified and sequenced [3, 23], the cDNA corresponding to defensin A (AaDefA) was cloned and characterized, revealing that the basic characteristics of AaDefA are similar to those of *Drosophila* defensin [6, 11]. Recently, a mosquito defensin gene was isolated and characterized as well [7]. Preliminary data of defensin gene reveal that several mammalian immune response regulatory elements, such as nuclear factor κB binding site, nuclear factor interleukin 6 binding site, and interferon consensus response element binding site were found in the upstream and downstream regions. This implies that the regulation of the mosquito defense pathway may use some similar enzymes or factors as mammals do.

Protein kinases are the largest superfamily of enzymes, many of which mediate the response of eukaryotic cells to external stimuli. Although their structures, regulation modes, and substrate specificities are diverse, the catalytic domain of protein kinases consists of 12 conserved subdomains folding into a common catalytic core structure [15]. All protein kinases in this superfamily characterized with regard to substrate specificity are classified into protein serine/threonine kinases and protein tyrosine kinases. A physiological function of protein kinases is to participate in protein phosphorylation cascades. Three major classes of input which are known to modulate protein phosphorylation cascades are extracellular signals, cell cycle checkpoints and environmental or nutritional stresses [15]. In addition, protein kinases are demonstrated to be involved in signal transduction of hemocytes in the insect, *Ceratitis capitata*, in response to lipopolysaccharide (LPS) stimulation [5]. For instance, Sluss et al. [29] isolated a *Drosophila* MAP kinase, DJNK, which was activated by LPS. This study reports the identification and cloning of several mosquito protein kinases which may be related to immune responses or cell proliferation.

### Materials and Methods

**Materials**

Modified T7 DNA polymerase, Sequenase, was supplied by United States Biochemical Co. Restriction enzymes were purchased from Boehringer Mannheim Co. Perkin Elmer Cetus was the source of reagents for the polymerase chain reaction (PCR). Primers were obtained from Genosys Biotechnologies Inc. The Wizard Minipreps DNA Purification system was from Promega Co. Amersham Life Science was the source of Hybond™-N+ membranes. [α<sup>32</sup>P]dATP (3,000 Ci/mmol) for labeling, and [35S]dATP (1,000–1,500 Ci/mmol) for sequencing, were obtained from New England Nuclear Chemical Co. The Ultraspec-II RNA isolation kit was purchased from Biotex Laboratories Co. Bacterial culture media were supplied by E. Merck. All other reagents were of analytical grade from Sigma Chemical Co.

**Animals**

Mosquitoes, *A. aegypti*, were reared as described by Cho et al. [6]. Antibacterial peptides were induced in adult females three to five days after eclosion. Mosquitoes were infected by intrathoracic injections of 0.25 μl of *Escherichia coli* LE392 suspended in mosquito saline (150 mM NaCl, 4 mM KCl, 3 mM CaCl<sub>2</sub>, 1.8 mM NaHCO<sub>3</sub>, 0.6 mM MgCl<sub>2</sub>, and 25 mM Hapes, pH 7.0 at 27°C). *E. coli* cultured in LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) were pelleted by centrifugation, then resuspended in mosquito saline to an OD<sub>600</sub> of 1. The bacterial suspension was heated at 100°C for 10 min before injection.

**Cloning and Sequencing of Mosquito Protein Kinases**

Female mosquitoes were collected 2, 6, 11, 18, and 24 h post intrathoracic injections of *E. coli* and were combined for total RNA isolation using the Ultraspec-II RNA isolation kit. PolyA+ RNA was prepared with the PolyA Tract mRNA Isolation System III (*Promega*). Complementary DNA was reverse transcribed from the purified mRNA and used as templates for PCR. A sense primer (mpk-p1), 5'-agtctagaaa(g/a)(g/a)tn(a/g)/tn(a/g/k)ang(t/c)tt(t/c)gg-3', and an antisense primer (mpk-p2), 5'-atctgcagnc(a/g)(a/g)(a/g/k)ang(c/a)canaca(a/g)tc-3', which include an XbaI and PstI digestion site at their 5' ends, respectively, were designed based on two conserved domains, K(V/I)(S/A/T)DFG and DVW(S/A)(F/Y)G. PCR amplification was performed as follows: 94°C for 10 min; 1 min at 94°C, 2 min at 48°C, and 2 rain at 72°C for 5 cycles; 40 s at 94°C, 2 rain at 55°C, and 2 rain at 72°C for 25 cycles; extension at 72°C for 5 min. The amplified product was precipitated and subcloned into pGEM vectors. Positive clones were identified with PCR using mpk-p1 and mpk-p2 primers, then sequenced from both directions.

**Analysis of Nucleotide and Amino Acid Sequences**

Putative mosquito protein kinases were analyzed with PC/Gene (Dominique Garin, Geneva, Switzerland) and GCG (University of Wisconsin Genetic Computer Group) softwares.

**Results**

**Cloning of Mosquito Protein Kinase Partial cDNA Fragments Using PCR**

To obtain protein kinases related to mosquito immune response or hemocyte proliferation, mRNA was prepared from bacterial inoculated mosquitoes. In the conserved catalytic domain, subdomain VII to IX was used for primer design. Primers mpk-p1 and mpk-p2 were degenerated from K(V/I)(S/A/T)DFG and DVW(S/A)(F/Y)G, respectively. A predominant band (150 bp) was detected after PCR (fig. 1). To study the types of immune-related pro-