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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Identification of a new type of haematopoietic progenitor kinase-interacting protein (HIP-55) in *Aedes aegypti* mosquito haemocytes and its involvement in immunity-like functions in mosquito: a molecular study

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Abstract
In this study, we characterize the HIP-55 protein in the mosquito *Aedes aegypti* for the first time. HIP-55 is a 55-kDa HPK1-interacting protein that is also called SH3P7. HIP-55 constitutively binds HPK1 'via' an HPK1 proline-rich motif 2(PR2) through its C-terminal SH3 domain. HIP-55 critically interacts with ZAP-70, and this interaction was induced by TCR signalling. ZAP-70 phosphorylated HIP-55 at Tyr-334 and Tyr-344 in vitro and in vivo. In our previous findings, AaZAP gene expression strongly proved that AaZAP-70 was involved in immunity-like functions in mosquito. Northern blot analysis of HIP-55 mRNA expression confirmed that it is only expressed in the abdomen and haemocyte tissues; this prediction correlates 100% and a polyclonal antibody also confirmed its localization in haemocytes and the abdomen. We prepared extracts to show the cytoplasmic expression (CE) of this protein. Previous results had proven that this protein is secreted from the cytoplasm; thus, we confirmed here that the protein is a cytoplasmic adaptor protein in mosquitoes and mammalian systems. Furthermore, our polyclonal antibody against HIP-55 also demonstrated that this protein is found in haemocytes and abdomen tissues, which assumes that the protein may be involved in phagocytic-like functions. RNAi (siRNA) silencing studies were used to degrade mosquito HIP-55; however, silencing only slightly affected the HIP-55 sequence and the gene transcriptional level. To characterize this protein, we cloned 609 bp from the 1.6-kb full-length cDNA using a pET28 vector for polyclonal antibody production.

Keywords HIP-55 · HPK1 · ZAP-70 · *Aedes aegypti* · An actin-binding adaptor protein · Haemocytes · SH3 domain

Introduction

Blocking pathogens in mosquitoes to reduce their transmission is a new avenue for controlling mosquito-borne diseases. The absence of T cells in insects characterizes the non-existence of an acquired immune system. The consistent humoural immunity in insects plays various roles including immune protein production against antigens and promoting signalling pathways to control haemolymph clotting and melanin management (Lavine and Strand 2001; Lavine and

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Strand 2003). Insect humoural immunity is also involved in cellular activities such as encapsulation, phagocytosis and nodulation (Lavine and Strand 2002). Many types of haemocytes circulate in haemolymph, which emerges from stem cells from the mesoderm that differentiated into particular lineages such as granulocytes and plasmatocytes. Haemocyte levels decrease during infection; thus, to stabilize the immunity, haematopoietic tissues will continuously engage in haemocyte production.

In mammals, T cells play a critical role in defending against pathogens. Zeta-chain-associated protein kinase 70 (ZAP-70) was verified as a 'zeta' chain-binding protein of the T cell receptor (TCR). It plays a critical role in mediating T cell activation, which triggers complicated signalling cascades, leading to transcriptional enhancements, cell proliferation and differentiation. In T cells, it has been demonstrated that HIP-55 is associated with the upstream kinase ZAP-70. As an actin-binding adaptor, HIP-55 interacts with and binds to tyrosine-phosphorylated ZAP-70 upon TCR stimulation. HIP-55 inducibly interacts with and is phosphorylated by the upstream kinase ZAP-70 in TCR signalling (Han et al. 2003). It is also possible that HIP-55 may function in regulating ZAP-70 activation by modulating the recruitment of ZAP-70 to the TCR complex through cytoskeleton reorganization in TCR signalling (Kane et al. 2000). HPK1 binds to several adaptor proteins in the TCR signalling pathway. The phosphorylation of ITAMs in the TCR/CD3 complex induces ZAP-70 binding, which is in turn phosphorylated by LcK ZAP-70, which phosphorylates numerous proteins including LAT, SLP-76, Vav, HIP-55 and HPK1. ZAP-70 binds to and phosphorylates all these signalling molecules to mediate protein-protein interactions. ZAP-70 is an SH2 domain-containing protein that phosphorylates HIP-55 once. This interaction mediates the signal to HIP-55 SH2 'via' the SH3 domain to activate the protein and promote its participation with downstream signalling effector molecules (Boomer et al. 2005). Based on Han et al. (2005) and our previously unpublished data on AaZAP-70, we aimed to determine the exact functions of these two proteins. Thus, we recently focused on the protein tyrosine kinase ZAP-70-associated downstream signalling molecule HIP-55, which contains an SH3 domain and is an actin-binding adaptor protein in mosquitoes. HIP-55 (haematopoietic progenitor kinase 1 (HPK1)-interacting protein of 55 kDa, also called SH3P7) is a novel SH3 domain-containing protein that was proposed to be involved in TCR signalling (Ensenat et al. 1999). HIP-55-interacting HPK1 is involved in many cellular signalling cascades including MAPK, antigen receptor, apoptosis, growth factor and cytokine signalling (Boomer et al. 2005). HPK1 tyrosine phosphorylation and kinase activation depend on the presence of adaptor protein interaction and tyrosine phosphorylation by ZAP-70, which is a crucial proximal protein tyrosine kinase for TCR signalling that is conserved between humans and mice and is a member of the drebrin/Abp1 class of actin-binding proteins (Boomer et al. 2005; Ensenat et al. 1999). The structure of HIP-55 contains one ADF, two consensus Y-sites and a C-terminal SH3 domain and has no actin polymerization activity (Kessels et al. 2000; Ensenat et al. 1999). HPK1 interacts with many adaptor proteins that are capable of modulating many intracellular signalling pathways. The diversity of HPK1 functions (both positive and negative) may be explained by the ability of HPK1 to bind or phosphorylate various adaptor proteins during T cell activation. HPK1is a family kinase involved in a variety of cellular signalling events, such as antigen receptor signalling, adaptor protein binding signalling and HPK1 activation, and whose functions depends on the presence of an adaptor protein. Upon phosphorylation, HPK is responsible for binding to other signalling molecules. Similar to haematopoietic lineage cell-specific protein 1 (HS1), drebrin/Abp1 and the cortactin family, which bind F-actin but not actin monomers, HPK1 is involved in signalling events in multiple organisms and cells. The drebrin/Abp1 family is conserved from yeast to mammals and is characterized by the presence of a homologous N-terminal actin-depolymerizing factor homology (ADF-H) domain (Ensenat et al. 1999; Drublin et al. 1990; Lappalainen et al. 1998; Han et al. 2003). This family includes SH3P7 in mouse (Kessels et al. 2000; Larbolette et al. 1999) and Abp1 in yeast (Drublin et al. 1990). The ADF-H domain is also found in proteins that regulate the disassembly of actin filaments such as cofilins and twin filings. It has many known binding partners and has been studied in several cell types, though most extensively in T cells (Pasquet et al. 1999; Gibbins et al. 1998). HIP-55 interacts with HPK1 (Ensenat et al. 1999), a serine/threonine protein kinase involved in TCR signalling (Hu et al. 1996; Ling et al. 2001; Ling et al. 1999). Haematopoietic progenitor kinase 1 (HPK1) is a haematopoietic-specific mammalian Ste20-like protein serine/threonine kinase (Hu et al. 1996; Kiefer et al. 1996) and a member of the MAPK kinase family of kinases, which was cloned from mouse haematopoietic progenitor cells using a PCR-based strategy (Hu et al. 1996; Kiefer et al. 1996). HPK1 is a 97-kDa kinase with restricted expression in haematopoietic organs and cells. Haematopoietic progenitor is an important proximal mediator of T cell receptor-induced NF-kB activation. Upon TCR stimulation, both HPK1 and HIP-55 translocate to the T cell/APC contact site and glycolipid-enriched microdomains (Ling et al. 2001; Han et al. 2003; Le Bras et al. 2004). HIP-55 contains an actin-binding domain at its N-terminus and an SH3 domain and its C terminus (Ensenat et al. 1999) and binds to filamentous actin and co-localizes with actin filaments (Kessels et al. 2000; Larbolette et al. 1999). Mosquito AaHIP-55-like cDNA was first isolated from our laboratory. Thus, investigation of the involvement of AaHIP-55 in immune signalling and host defence is an important issue when examining

immune regulation in mosquitoes. In this study, we successfully cloned a novel HIP-55 gene from adult-stage mosquito cDNA. In mosquitoes, the N-terminal region of *Aedes aegypti* HPK1 interacts with an approximately 40.5-kDa HIP-55 protein via ExPASy ID VIRT20787 prediction. To date, there have been several reports of HIP-55 genes in mammals; however, the gene encoding this protein has not been examined in insects such as mosquitoes.

The function of HIP-55 in TCR signalling is not well understood, though it has been shown thatHIP-55 interacts with HPK1 (Ensenat et al. 1999) through a serine/threonine to associate with TCR signalling (Ling et al. 2001; Liou et al. 2000). HIP-55 also intensifies HPK1 kinase activity and JNK in an overexpression system (Ensenat et al. 1999). Src and Syk/ZAP-70 family tyrosine kinases activate HIP-55 and TCR or BCR stimulate the HIP-55 tyrosine for phosphorylation (Larbolette et al. 1999; Nagata et al. 1999; Han et al. 2003). Early events in TCR signalling are initiated by tyrosine kinases, namely ZAP-70-Lck and ZAP-70, which interact with HIP-55 (Kane et al. 2000). TCR signalling and immune synapse formations are also affected by HIP-55, as it is an actin-binding adaptor protein that controls cytoskeleton reorganization. Antigen-presenting cell (APC)-regulated T cell activation was performed with various actin-binding proteins. The mAbp1 cell invasion inhibitory effect on the mAbp1 SH3 domain is essential (Boateng et al. 2016), and the same process was executed with the actin-related protein Arp2/3 in the complex formed by the Wiskott-Aldrich syndrome protein (WASP) (Mullins 2000). Actin nucleation is controlled by the WASP-Arp2/3 complex and promotes actin filament elongation and branching. HIP-55 interacts with TCR signalling upstream kinase ZAP-70 to promote its phosphorylation (Han et al. 2003). HIP-55 may control ZAP-70 activation by modifying the need for ZAP-70 in TCR complex-mediated cytoskeleton reorganization during TCR signalling, as ZAP-70 is critical for TCR signalling and T cell function (Elder 1998; Williams et al. 1998). In mosquitoes, our previous finding suggests that AaZAP-70 is involved in T lymphocyte receptor-like signalling (unpublished data). Here, we found the adaptor protein may have a T lymphocyte-like function. For example, it is well-known that after adaptor protein binding, TCR signalling plays a vital role in mediating several functions, such as participation in new signalling events. Cloning and functional analysis of the AaHIP-55 gene from mosquitoes is important for determining immune-like signalling functions in mosquitoes. To study the function of this gene, to knockdown RNA interference, HIP-55 in Jurkat T cells in mice was used by Han et al. (2003). As a result, the signalling events induced by TCR stimulation, such as HPK1 and JNK activation (JNK upstream signalling molecule), were defective, but not the Erk kinase activity after TCR stimulation (Han et al. 2003), suggesting that HIP-55 may be an important regulator of TCR signalling (Fig. 1).

In this study, we show that the HIP-55 protein accumulates in mosquito haemocytes and in the abdomen. Mosquito haemocytes have been characterized as differentially responsive to invading pathogens. They have been recently described in several medically important mosquito species, as these cells rapidly and effectively respond to pathogen invasion through phagocytosis and by mediating humoural immune responses (Hernandez et al. 1999; Da Silva et al. 2000; Hernandez-Martinez et al. 2002; Hillyer and Christensen 2002; Hillyer et al. 2003a, b). As it has been well-studied, we conclude that this newly identified HIP-55 protein from mosquito haemocytes could be involved in the phagocytic functional activity. Mosquito haemocytes not only represent diverse cellular processes but also include numerous gene products related to immunity, many of which parallel those employed in vertebrate defence responses.

Materials and methods

Mosquito maintenance

The yellow fever (Liver pool) strain of the mosquito *A. aegypti* was cultured in a 26 °C room at a humid temperature. The room specialized for mosquito culture was set up with an incubator suitable for mosquito rearing. The healthy mosquitoes used in the present studies emerged from pupa raised in the incubator and specialized room. The mosquitoes were routinely fed 5% sugar (sucrose) solutions.

Sample collection

The thorax and gut regions were collected separately from 10 mosquito abdomens, and 20–25 haemocytes were collected from dissected mosquitoes with the help of a microscope using sterilized needles. Aag_2 cells were collected from the mosquito cell culture facility room containing CO_2-free air. Haemocytes, abdomen, gut and thorax were washed once with mosquito PBS solution.

RNA isolation and cDNA synthesis

For RNA isolation, total RNA from 10 mosquitoes was isolated using the Trizol reagent (Invitrogen, Waltham, MA, USA). Several isolation steps were followed after the RNA was subject to quantification using a NanoDrop (NanoDrop, USA). Finally, the RNA was resuspended in RNase-free DEPC water with a final volume of 25 μL and stored at − 70 °C until further use. First strand synthesis of cDNA from the RNA was performed in a reaction containing 2 μg of total RNA, 1 μl of 0.1 μg/μl AR-17 oligo dt tailing primer, 2 μl of 10 mM dNTPs (10 mM dATP, 10 mM dCTP, 10 mM dGTP and 10 mM dTTP), 5 μl of 5× buffer (250 mM Tris-HCl, pH

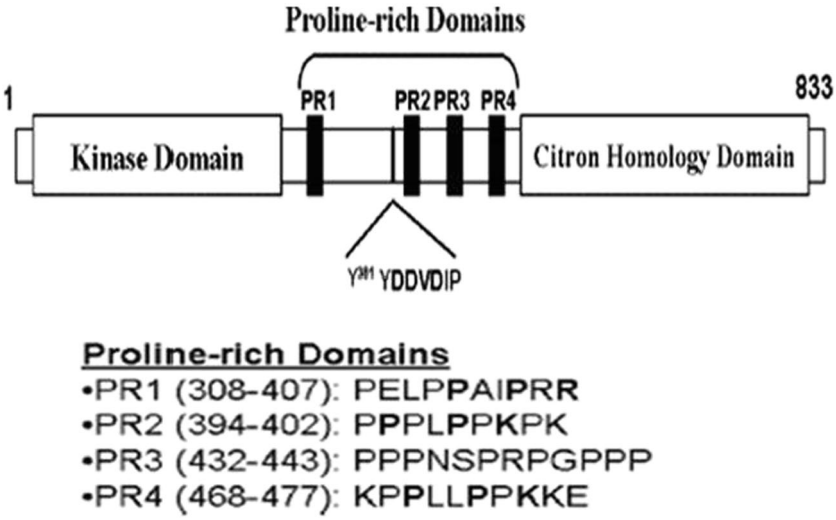


Fig. 1 HPK1 structure. HPK1 contains an N-terminal serine/threonine kinase domain, a C-terminal citron homology domain (CHD), four proline-rich motifs (PR1, PR2, PR3 and PR4), a caspase cleavage site (DDVD) and a tyrosine residue at 381, which when phosphorylated is responsible for HPK1 binding to SLP-76. The four proline-rich domain sequences are also listed. Picture courtesy of Boomer et al. (2005)[7]

8.3, at 25 °C with 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT), 1 μl of RNase inhibitor, and 1 μl M-MLV Reverse transcriptase and water to a total volume of 25 μl according to the manufacturer's protocol (Promega kit, Madison, USA). The tube containing the reaction was gently flicked and incubated in a water bath for 1 h at 42 °C. The reaction was heated to deactivate the RNase and then stored at − 70 °C until further PCR amplification.

Plasmid construction and transformation

A 0.6-kb (609 bp) HIP-55 fragment was partially cloned or amplified from the 1.6-kb full-length sequence via PCR (Perkin Elmer, USA) with a set of gene-specific forward (P1) and reverse (P2) primers (the primers synthesized by Mission Biotech, Nangang, Taiwan). To clone the TA II vector (NEB, UK), the PCR products were ligated with T4 DNA ligase (NEB, UK) with the correct vector insert ratio (1:3 M) and the 20-μl ligation reaction was performed overnight in a 42 °C water bath (Thermo Biotech, Taiwan). After transforming *E. coli* DH5 α competent cells with 20 μl of the ligation product, the samples were placed in a water bath for heat shock at 42 °C for 90 s and then immediately cooled on ice. The cells were allowed to grow 1 h at 37 °C in a bacteriological shaker and then the pellet was collected by mild centrifugation at 4000*g* for 4 min. Finally, the pellet was plated with a Luria broth Petri plate containing ampicillin. Upon selection of a successful clone, the positive TA clone HIP-55 plasmid was sequenced and then further used for protein production. For construction of the AaHIP-55 protein, the AaHIP-55 gene was constructed to include BamH1 and EcoR1 cutting sites. The BamH1 and EcoR1 products were sub-cloned into the pET vector 28 (+) expression vector and selected from the LB + AP Petri plate. We checked for clone insertion using the pET vector series T7 forward and HIP-55 gene-specific reverse primers for the correct insert size. Afterwards, this clone was further subject to AaHIP-55 recombinant protein production. For the positive clone identification, the clone was sequenced at the Yang Ming University Sequence Resource Center in Taipei.

Recombinant protein isolation from bacterial *E. coli*

We transformed the DH5-α-transformed plasmid containing 609 bp of AaHIP-55 with BamH1 and EcoR1 cut sites into M15 phage cells for recombinant AaHIP-55 protein production using a 1-mM IPTG inducible system with the selectable resistant marker AP/KM. A 1-ml overnight culture was transformed into 1 l of Luria broth and grown at 37 °C until reaching an OD of 0.5, after which 100 μl IPTG/1 l was added to the culture. The culture was harvested at different intervals, such as 2, 4 and 6 h, and the bacterial crude culture was subjected to centrifugation at 5000 rpm for 5 min. The pellet was harvested, completely dissolved in lysis buffer (8 M urea and 50 mM Tris, pH 8.0) and lysed using a French Press sonicator (Electron Corporation sonicator MA, USA) to break the bacterial cell wall. The lysed bacterial culture was centrifuged at 15,000 rpm for15 min (Beckman Coulter LE-80 K ultracentrifuge, USA). To confirm the induction of the recombinant AaHIP-55 protein, the lysed supernatant was examined by 10% SDS-PAGE with control samples at zero hours (non-IPTG) as well as samples with IPTG after 2, 4 and 6 h.

AaHIP-55 recombinant protein purification using column affinity chromatography

The Ni-resin binding assay (column affinity chromatography) column was washed two times with 0.5 M EDTA. For protein binding, 10 ml of 50 mM $NiSO_4$ was transferred to the column and incubated for 5–10 min because the recombinant protein 6× HIS-tag needs to bind to the fusion protein (nickel-binding protein). The sonicated bacterial lysate was transferred into

the column affinity chromatography, and protein and Ni-resin were allowed to equilibrate for a few minutes to promote binding. The column was then washed with low stringent lysis buffer containing 25 mM imidazole and eluted with high stringent buffer containing 250 mM imidazole. The eluted buffer contained only purified recombinant AaHIP-55 protein. The eluted protein was confirmed and checked with 10% SDS-PAGE and by Western blot.

Rabbit immunization

We produced recombinant AaHIP-55 extracellular domain (ECD) for polyclonal antibody production with the pET-28 protein expression system purchased from QIAGEN Co., MD, USA. The recombinant histidine-tag fusion protein was purified by using metal-affinity chromatography. Before animal immunization, the purified recombinant protein was passed through a protein eluter (Bio-Rad, Protein Eluter, USA) to elute the entire protein with the help of an elution buffer (1MTris, 1% SDS and 14mmglycine, pH 8.2) for 4 h at 0.25 Amps (Bio-Rad, Powerpac, USA) at 4 °C. We used a two-step pure AaHIP-55 ECD for rabbit antisera production. Before injection with 1 μl purified antigen, gel electrophoresis was performed to check the antigen/protein concentration. We used 2 μg of antigen for subcutaneous injection into New Zealand rabbits with the help of emulsified complete Freund's adjuvant (Sigma, USA). Two weeks after the first immunization, the rabbit was reinjected (booster injection)

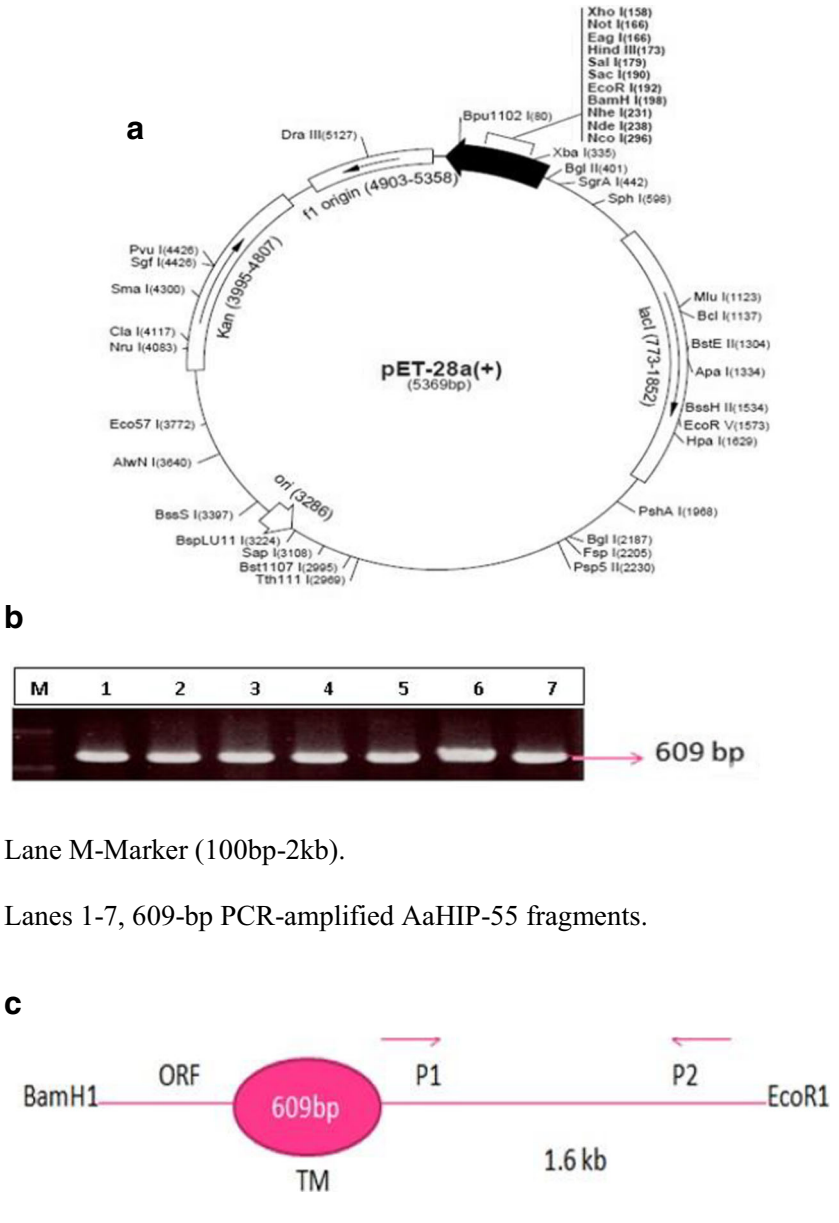


Fig. 2 **a** pET-28a(+) vector map site. **b** PCR-amplified fragment from the1.6-kb full-length AaHIP-55cDNA. **c** Aa HIP-55 cloning region with the cutting site

Lane M-Marker (100bp-2kb).

Lanes 1-7, 609-bp PCR-amplified AaHIP-55 fragments.

with the same concentration of antigen. In total, three injections were carried out for antiserum production (Fig. 2).

Polyclonal antibody production

Prior to animal immunization, 5 ml of pre-immune serum was collected as a control. The serum was collected 2 weeks after the first immunization and maintained at room temperature for 30 min for serum separation from crude blood cells. After clear serum was viewed as the upper phase, it was carefully collected from the upper phase for further antibody purification and spun down at 10,000 rpm for 20 min at 4 °C. Several spins with a refrigerated centrifuge (Eppendorf, Germany) were executed. The supernatant was discarded and the pellet was dissolved in dialysis buffer (1 M Na_2HPO_4 and 20 mM $NaHPO_4$). Finally, the dissolved buffer was transferred to a dialysis membrane for salt removal. During this process, the dialysis chamber was kept in a 4 °C refrigerator for 12 h and then extended for another 12 h after changing the dialysis buffer. The buffer from the dialysis membrane was collected and spun down 10,000 rpm for 10 min at 4 °C. The supernatant was carefully saved in long-term storage or stocked after the dialysis process. The sera were mixed with 50% glycerol and stored − 80 °C until use.

SDS-PAGE and Western blotting analysis

Ten *A. aegypti* mosquito lysates were used in this study. The entire mosquito was homogenized using a Teflon homogenizer for 1–2 min with lysis buffer PBS (pH 8.0) until the mosquito was completely lysed. Then, the clear lysate was spundown in an Eppendorf tube (Eppendorf, Germany, 4 °C cooling centrifuge) at 14,000 rpm for 10 min at 4 °C. The upper layer (clear lysate) was collected without disturbing the sediments (200 μl). Next, protein estimation was performed according to the Bradford et al. (1976) method; the Bradford reagent (Bio-Rad, USA) contained 1 ml consisting of 200 μl of reagent and 800 μl of ddH_2O. One microliter of protein sample was used to determine the protein concentration at an OD of 595 nm; the samples were mixed thoroughly before the OD measurements. Finally, the total mosquito protein (75 μg) was resolved by 10% SDS-PAGE with the appropriate current voltage for 3 h, before the gel containing the cellular proteins was subject to Western blot analysis. The gels were transferred to polyvinylidene difluoride membrane (St. Louis, MO, USA) at 4 °C for 1 h 10 min at 60 mA for complete protein transfer. The correct orientation of the transferred protein was carefully noted and the membrane was incubated with the primary anti-HIP-55 antibody (diluted 1:5000) overnight at 4 °C. Then, the membrane was carefully removed and blocked non-specifically with 5% non-fat milk powder for 1 h at room temperature. The membrane was then washed three times with PBS-saline buffer before secondary antibody (HRP conjugate) hybridization. The membrane was hybridized with anti-rabbit (HRP) IgG (1:500) for 30 min at room temperature. Finally, the membrane was dried using an air dryer and the background was enhanced with chemiluminescence ECL solution (Bio-Rad, USA) that was spread on the membrane for a few seconds. The membrane was carefully placed in the X-ray cassette to expose the membrane onto the X-ray film.

Northern blotting and isotope labelling

Multi-tissue poly (A) + blots were run with 2 μg RNA from haemocyte and abdomen tissues collected from mosquitoes. The probe was approximately 1.3 kb full-length HIP-55 cDNA obtained by restriction digestion of the AaHIP-55 plasmid with BamH1 and HindIII. The cDNA probe was radiolabelled with 2.5 μL α-^{32}p dCTP (25 μci $mMol^{-1}$; Radiation and Isotope Tech. Helsinki, Poland) and amplified by PCR with 15 cycles of denaturation at 94 °C for 5 min, annealing at 55 °C for 25 s, extension at 72 °C for 10 min and a final hold at 4 °C. The hybridization and washing conditions used were those described in the manual provided with the ExpressHyb hybridization solution (Amersham Biotech, UK). This process was performed for 12 to 18 h at 68 °C in a hybridization oven (Hybrid, Perkin Elmer, Germany). The day after hybridization, the blots were washed three times with SSC solution at different temperatures. The first was in 20× sodium saline citrate buffer (SSC) with 0.5% SDS buffer for 30 min at 68 °C, followed by two or three washes with 0.1× SSC with 0.1% SDS buffer solution at 22 °C in a hybrid oven (Thomas Scientific, NJ, USA) with gentle washing. Finally, the membrane was covered with a wrapper sheet, placed into a cassette (Amersham, UK) and exposed to an X-ray film (Konica Rx-X-ray film) in a Kodak darkroom image station. For exposure, the membrane was kept at − 80 °C (Nuarie, USA); the film was developed 5 days later (Kodak, film developer, Japan) to visualize the spots.

Cytoplasmic extract preparation

We prepared cytoplasmic extracts from the mosquito AP61 cell line. To determine the cytoplasmic expression of the protein, we used 1 ml of AP61 cells from aculture flask (BD, Bioscience) and a sterilized culture scoop to scratch the cells and collected them (contained approximately 4×10^7cells). The cells are washed with PBS buffer and collected by centrifugation at 1000 rpm for 10 min. The pellet was resuspended in 5 pellet volumes of cytoplasmic extract (CE) buffer (1× solution containing 10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (*v*/*v*) NP40, 1 mM DTT and 1 mM PMSF) with the pH adjusted to 7.6. Convenient concentrated stocks of these reagents were prepared in 10 ml volumes of 1× CE buffer that could be incubated on ice for 3 min. The preparation was spun using a microcentrifuge (Eppendorf, Germany)

at 1000–1500 rpm for 5 min at 4 °C and then the cytoplasmic extracts from the pellet were removed for the preparation. The obtained pellet was resuspended in 1× CE buffer. The protein cytoplasmic extracts were subjected to 10% SDS-PAGE and the expression of the cytoplasmic protein was confirmed by Western blot analysis using an antibody against AaHIP-55.

dsRNA preparation and RNAi silencing

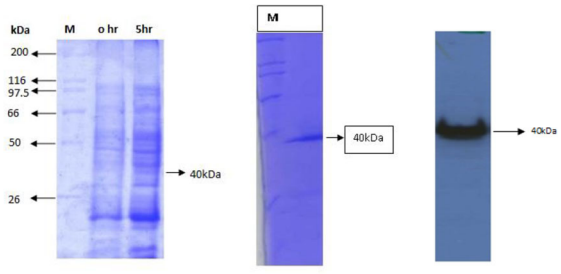
We maintained two sets of mosquitoes in the AaHIP-55 RNAi silencing study; one set was a template for another set containing dsGFP as a marker. In this section, we synthesized 20 μl of dsRNA and dsGFP at two concentrations (200 and 350 ng) (two tubes) separately. Then, 1 μl of dsRNA was removed from the stock solution with a microsyringe attached to a microscope. We carefully injected the dsRNA into the mosquito thorax region, preventing mosquito injury, and collected the RNA from the experimental mosquito at varying intervals, such as 24, 48 and 72 h, for first strand (cDNA) synthesis. For each interval, we used five mosquitoes to collect RNA for cDNA synthesis with PCR confirmation. In another set of mosquitoes, we simultaneously injected 1 μl of dsGFP at the abovementioned template concentration. The purpose of dsGFP was to verify RNAi silencing in the template samples. We collected dsRNA and dsGFP from both sets of mosquitoes and created cDNA using a PCR reaction with 30 cycles and specific primers to check whether the products displayed silencing. Additionally, 10 μl of dsRNA and dsGFP PCR product template was simultaneously loaded and analysed using a 1% TAE agarose gel.

Results

Novel mosquito AaHIP-55 protein studies using standardizing partial cloning and functional analysis techniques were performed to generate polyclonal antibodies from the N-terminal sequence. From the NCBI database, 1621 nucleotides encoding 540 amino acids of a 60-kDa protein were predicted for AaHIP-55. The 609-bp from MSID to VEYLI, which was approximately *60 kDa using the right reading front, was cloned for polyclonal antiserum production. We produced the N-terminal recombinant HIP-55 protein from *A. aegypti* with a size of 40 kDa as predicted by ExPASy. AaHIP-55 positively sequenced clones were subjected to BLAST for multiple sequence alignment and overlapping fragments analysis. The results identified that the sequence contains an initiation codon (ATG) followed by a stop codon (UCA). With the help of PCR, we synthesized this short fragment for further antibody production, RNAi silencing studies and localization studies in the abdomen and gut haemocytes. The localization of the 60-kDa mosquito HIP-55 protein was confirmed to be in the haemocyte and abdomen based on its interaction with the synthesized AaHIP-55 polyclonal antibody (Fig. 3).

Next, we predicted our deduced mosquito HIP-55 amino acid sequence and compared it with the HIP-55 N-terminal region from other related available sequences by multiple sequence alignment. Interestingly, we found that the N-terminal region of mosquito HIP-55 aligns with the AaHIP-55 SH3 domain, which consists of a short peptide putative actin-binding domain; it also showed 70–80% similarity and aligned with human HIP-55, dreprin, coactosin and Abp1 (Fig. 4a). Dreprin and coactosin are SH3 domain-containing actin-binding proteins that are involved in actin cytoskeleton reorganization during immune synapse signalling. These results suggest that mosquito HIP-55 could have the same role during actin cytoskeleton and immune synapse signalling formation.

The hybridization of the total mosquito components, including Aag_2, C636, abdomen, thorax, gut, fat tissues and haemocytes, with the two-step purified AaHIP-55 polyclonal antibody and the SDS-PAGE data suggested that all the mosquito samples tested contain the HIP-55 protein (Fig. 5b). We confirm this finding by Western blot to determine whether these proteins are HIP-55 or related proteins. Surprisingly, the Western blot showed that three of the extracts contain AaHIP-55 compared to other haemocyte samples having more HIP protein. We found few contrasting results from the Western blot, as the samples containing abdomen and whole mosquitoes showed a small amount of pre-sized protein in the gel compared with other bands. To confirm the exact size of the protein, we used the Rf value to measure its size; it was estimated to be 60 kDa from the data shown in Fig. 5a.We assumed that this protein was *Aedes* HIP-55-associated protein. Our polyclonal sera had suggested this protein was HIP protein. The Northern blot observation of AaHIP-55 mRNA expression is similar to the data obtained from the Western blot. Additionally, our other data related to the cytoplasmic expression of AaHIP-55 is similar to that of tested human HIP-55 cytoplasmic expression. This is because HIP-55 is a cytoplasmic adaptor protein in both vertebrate and invertebrate systems; thus, we proved here for the first time the cytoplasmic expression of this adaptor protein in invertebrates, which was already reported in vertebrates by Deckert et al. (2004). This protein is a cytoplasm-specific actin-binding SH3 domain-containing adaptor protein. Boomer et al. (2005) demonstrated that HIP-55 shows tissue-specific restricted expression and is only expressed in haematopoietic tissues; however, we found that the protein is also expressed in invertebrates such as in mosquitoes. Hence, we conclude this protein is expressed not only in vertebrate haematopoietic tissue but also in invertebrate tissues such as insect haemocytes, abdomen and whole mosquito tissue extracts. We found that the HIP-55 SH3 domains in the mosquito N-terminal



HIP-55 sequence similarity between *Homo sapiens* and mosquito *Aedes aegypti* is approximately 70-80%.

d Amino acid sequence in *Aedes aegypti* compared to HIP-55 N-terminal region in another related human sequence.

ID:VIRT20787/exPASY.

ATG → 6X His-Tag →

MRNCSHHHHHHIVNDFKVKDYSQANDVQKPV
GTNYSRVIPTKEINPAERDEFWRKEEEEEKHR
LQLEYDRKLQETVKLEEERRRREEREFEKREAAL
EAPQSPDKNYARQTSDSSKTERDREIKQIVEASG
KVVSSAKARFLNSALSPIHIQQTVAPELIMSPDS
SNEETSLINELKAEVEAEQNGGQNGTTAEPVVQ
EFFDPNATIDLSDEDNSRIKARALYDYQAADESE
ISFDPGDIITHIDQIDEGWWQGLAPDGTYGLFP
ANYVELI* stop.

e The deduced amino acid sequence of Human HIP55 is shown. The putative SH3 domain is underlined.

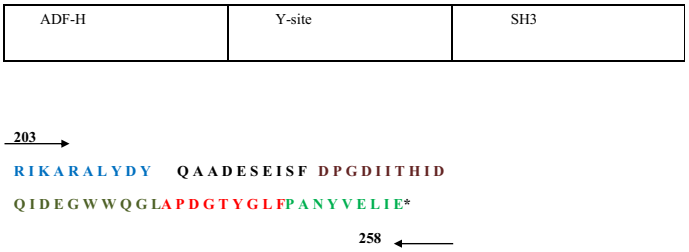
ATG →

MAANLSRNGPALQEAYVRVVTEKSPTDWALFTYEGNSNDIRVAGTGEGGLEEMVEELNSGKV
MYAFCRVKDPNSGLPKEVLINWTGEGVNDVRKGACASHVSTMASFLKGAHVTINARAEEDVEP
ECIMEKVAKASGANYSFHKESGRFQDVGPQAPVGSVYLKTNAVSEIKRVGKDSFWAKAEKEEE
NRRLEEKRRAEEAQRQLEQERREREIREAARREQRYQEQGGEASPQRTWEQQQEVVSRNRNEQ
ESAVHPREIFKQKERAMSTTSISSPQPGKLRSPFLQKQLTQPETHFGREPAAAISRPRADLPAEEPA
PSTPPCLVQAEEEAVYEEPPEQETFYEQPPLVQQQGAGSEHIDHHIQGQGLSGQGLCARALYDYQ
AADDTEISFDFENLITGIEVIDECWWRGYGPDGHFGMFPANYVELIE*stop.

(Courtesy of Ensenat et al.,1999).

f

Predicted *Aedes aegypti* mosquito N-terminal HIP-55 structure. The amino acids are mosquito SH3 domain-containing putative amino acids.



(Extracted from cloned N-terminal AaHIP-55 sequence**)**

Fig. 3 Expression of recombinant AaHIP-55 protein cloned from the pET28(+) vector in *E. coli* cells separated by 10% SDS-PAGE. **a** Ni-NTA affinity column-purified AaHIP-55 His-Tag fusion protein. **b** Bacterial lysate was subjected to 10% SDS-PAGE and the expression of recombinant protein was detected with an anti-His-tag antibody. **c** Amino acid sequence of *Aedes aegypti* compared with the HIP-55 N-terminal region from a related human sequence. **d** Amino acid sequence of human HIP-55. **e** The deduced amino acid sequence of AaHIP-55 is shown; the putative SH3 domain is underlined. **f** Diagram of the AaHIP-55 protein showing the ADF homologous domain, two consensus tyrosine site and SH3 domain

sequence consisting of amino acids 203 to 258 were present and have putative functional domains in the expressed fusion protein. Therefore, we predicted that the expressed product would show phagocytic activity functions.

We interpreted our data from AaHIP-55 mRNA expression by successfully targeting HIP mRNA expression with Northern blot analysis, which showed a single 1.6-kb transcript in the abdomen tissue and haemocyte studies. This indicates that HIP-55 mRNA is ubiquitously expressed in these two tissues (Fig. 5b); moreover, HIP-55 mRNA expression is in the same tissues as we expected, which was confirmed by Northern blotting (Fig. 5b). The obtained 1.6-kb mRNA transcript product is similar to Western blot results, meaning that we can conclude that endogenous protein expression from protein lysates and HIP mRNA expression are only in the abdomen and haemocytes. Thus, the intracellular localization of the *A. aegypti* HIP-55 protein was detectable using the N-terminal anti-AaHIP-55 antibody and mRNA expression was detected using a radiolabelled isotope method. As it was detectable, we found that the levels of the N-terminal mRNA product in haemocytes and abdomen were similar to the Western blot analysis results. The synthesized polyclonal antibodies recognized AaHIP-55 protein, providing further information regarding their possible roles in haemocyte functions and defence systems in this organism. Mosquito haemocytes have been characterized and immunocytochemical and cytochemical assays have demonstrated that these cells are differentially responsive to invading pathogen; moreover, haemocytes function has also been analysed by measuring phagocytosis. As haemocytes are invertebrate phagocytes, our new findings may suggest that AaHIP-55 participates in haemocytes during encapsulation. We also assume that haemocytes containing the AaHIP-55 protein could be involved in encapsulation because encapsulation refers to haemocyte binding to larger targets such as parasite and protozoa.

Mosquito haemocytes are not only involved in diverse cellular processes but also include numerous gene products related to immunity, many of which parallel those employed in vertebrate defence responses.

Mosquito cytoplasmic expression of the AaHIP-55 protein was similar with that of mammalian HIP-55, but how?

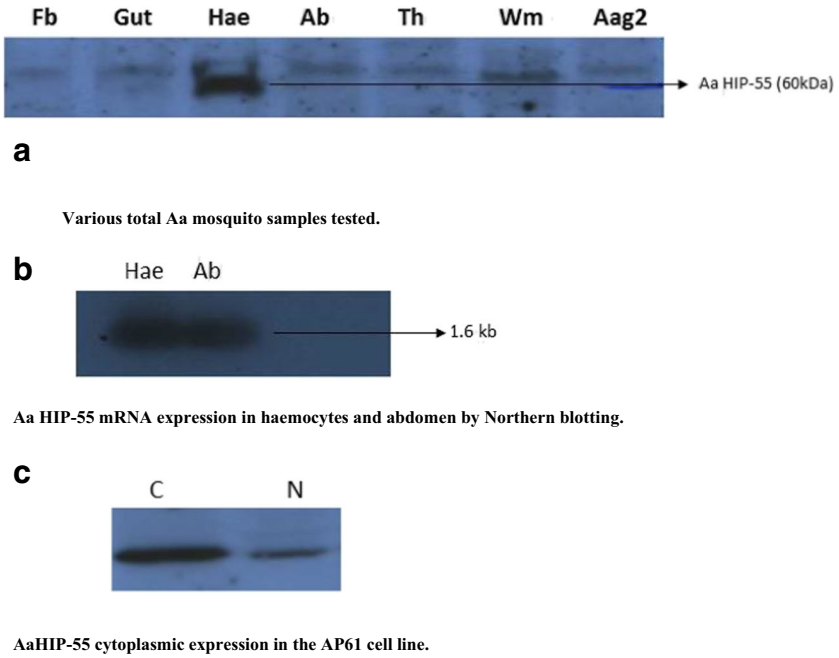


Fig. 4 The purified anti-HIP-55 polyclonal antibody was used in a Western blot to detect endogenous protein in various cell lines and tissues: Lanes 1–7: lane1, fat body; lane2, gut; lane3, haemocytes; lane4, abdomen; lane5, thorax; lane6, whole mosquito and lane7, Aag2. Total protein from *A. aegypti* (7 μg) and various tissues and cell lines were resolved by 10% SDS-PAGE and then transferred to a PVDF membrane for AaHIP-55 antibody hybridization for Western blot studies. **a** Northern blots probed with HIP-55 cDNA show a 1.6-kb transcript in haemocytes and the abdomen. **b** AaHIP-55 expression detected in the cytoplasm (C) and nucleus (N). **c** The protein extracts were subjected into Western blot analysis using antibodies against AaHIP-55

Using mammalian Jurkat T cells, the expression of this protein in the cytoplasm is already well-documented in vertebrates. Interestingly, we observed the same results when examining the mosquito AP61 cell line. Our data correlate with the mammalian system; thus, we conclude that this is a cytoplasmic adaptor protein that shows cytoplasm-specific expression in both vertebrates and invertebrates. This is the first intensive study to clone and sequence the mosquito cytoplasmic adaptor protein AaHIP-55 localized to the membrane.

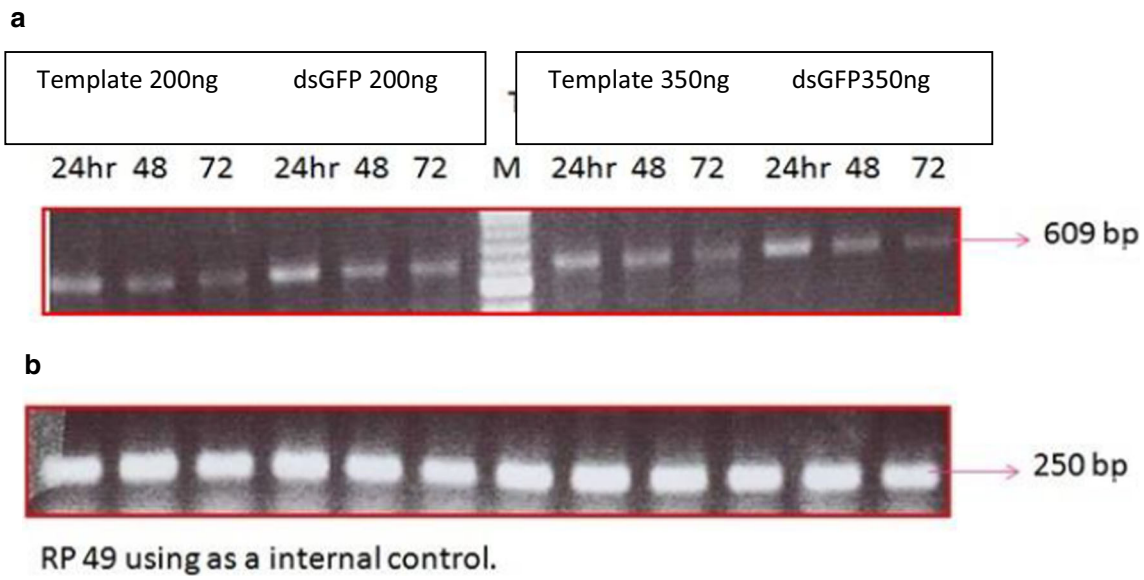


Fig. 5 RNAi silencing with the AaHIP-55 sequence. **a** Bands after 24, 48 and 72 h with the template (200 ng of dsRNA) and after 24, 48 and 72 h with 200 ng of the dsGFP positive control marker. **b** cDNA quality check for both the template and dsGFP siRNAs using ribosomal gene 49 (RP49) as an internal control

To investigate the protein, we prepared the cytoplasm and nuclear extracts from an Ap61 cell line by Western blot, which showed low AaHIP-55 signal in the nuclear protein extract and strong detection in the cytoplasm (Fig. 5c). We cannot currently explain the low HIP-55 protein signal in the nucleus, as its function is still unknown. We assume this indication may be full-length AaHIP-55 containing an active nuclear localization signal (NLS). The AaHIP-55 NLS function may require in vitro testing. The localization of the AaHIP-55 cDNA NLS will further be examined by linking the signal to red fluorescent protein in a reporter gene assay. In the future, we may perform a reporter gene transfection to study the function of active NLS contained in the HIP-55 cDNA. In our data, the endogenous expression of mosquito protein lysates analysed by SDS-PAGE and subsequently by Western blot showed an expected molecular mass of AaHIP in mosquito of 60 kDa; therefore, we designated the novel molecule as HIP-55, a 60-kDa HPK1-interacting protein in mosquitoes. This was confirmed by Western blot predictions from *A. aegypti*, haemocytes and abdomen, and whole mosquito extracts (Fig. 5a) and by the pI theoretical/average through ExPASy (Fig. 3e). Our conclusion on the size of HIP-55 in mosquitoes suggests that it varies slightly between vertebrates and invertebrates; however, we strongly believe that the protein is AaHIP-55 because it has been proven using polyclonal antibodies.

In the AaHIP-55 RNAi silencing studies, we maintained two sets of mosquitoes, one set as a template and the other, with dsGFP, as a marker. In this part, we synthesized 20 μl of dsRNA and dsGFP at two concentrations (200 and 350 ng) (two tubes) separately. We selected different siRNA target sites along the full-length gene sequence. The results showed that the input dsRNA processed and degraded the HIP-55 sequence for 72 h at both the 200 ng and 350 ng template preparations, leading to limited silencing, and the dsGFP marker 200 and 350 ng showed dsRNA template binding. Based on template dsRNA verification with the dsGFP marker, AaHIP-55-mediated silencing is not effective, but small amounts of silencing occurred at the siRNA target, indicating that RNAi silencing can affect the mosquito HIP sequence. We observed silencing with the template at 200 and 350 ng after 72 h, but it is not effective; however, this small change may affect the protein transcriptional level. We assumed that gene silencing will affect the transcriptional levels (data not shown) and diverted the TCR-like function. Our experimentally predicted data using RNAi silencing and overexpression experiments showed that HIP-55 is regulated and the complex is found in the mosquito. Thus, further experiments will need to be performed to explore the functions of this gene and protein.

The mosquito HIP-55 protein was expressed in all the samples and in the cell line, and the expression levels appear to be high in haemocytes and the abdomen and showed little signal in the cell lines. Thus, our evidence suggests that HIP-55 is an important adaptor protein involved in mosquito immunity-like functions. Addition investigations focused on cloning and the functional analysis of AaZAP-70 and downstream-encoded AaHIP-55 from mosquitoes may be important for determining mosquito immune signalling (T lymphocyte)-like functions. It will be worthwhile studying the function of HIP-55 in the mosquito system. In this study, we have increased the current knowledge on the regulators and signalling pathways required for insect haemocyte immune responses. We have uncovered essential components involved in haemocyte-mediated immunity. Much work is still needed to understand how haemocytes recognize invaders 'via' transmembrane receptors and how the signals produced are transmitted to their targets.

Discussion

The aim of the present work was to characterize the AaHIP-55 protein in the *A. aegypti* mosquito using different molecular approaches such as dsRNA, Western blot and both mRNA and cytoplasm expression analysis. Actin-binding HIP-55 may also potentially affect immune synapse formation and TCR signalling by regulating cytoskeleton reorganization. Several actin-binding proteins have been involved in the formation of immune synapse signalling (IS) and T cell activation by mediating or stimulating antigen-presenting cells (APCs) (Holsinger et al. 1998; Fuller et al. 2003). Our aim was to explore the activity of the HIP-55 protein, which acts as an adaptor protein that combines with MAP4K1 in mosquitoes (Zhang and Zhang 2015). Haemocytes play roles in various insect defence mechanisms with distinct immune system references to the expression of genes and their products. These immune responses contribute many common elements that serve to eradicate pathogens in the haemolymph. Granulocytes are phagocyte cells present in *A. aegypti* and *Armigeres subalbatus* that can consume numerous bacteria and are important for cytoskeletal reorganization (Greenberg and Grinstein 2002; Hillyer et al. 2003a). Our purified antibody was hybridized with proteins in extracts from total mosquito, Aag_2, C636, abdomen, thorax, gut, fat tissues and haemocytes. Protein lysates from these samples were subjected to SDS-PAGE to check whether the mosquito has this same protein. Western blot confirmed that all these extracts contained the AaHIP-55 protein; specifically, our prepared haemocyte extracts displayed more signal than the other samples. This is because haemocytes are phagocytes and function in phagocytosis. We identified haemocytes containing AaHIP-55, suggesting that haemocytes are essential for the insect immunity or immune signalling such as TCR function; even though insects have no T cells, haemocytes may function similar to T cells in TCR. It was also reported by Le Bras et al. (2003) that the HIP-55 actin cytoskeleton plays an essential role in T cell activation. HIP-55 targeted to the APC contact

zone by the formation of T cell-APC conjugates involves the Rac/Cdc42-WASP pathway, leading to Arp2/3 activation as well as actin polymerization at the T cell-APC contact site (Snapper et al. 1998; Krause et al. 2000).

Our earlier findings proved that mosquito ZAP-70 has a T cell-like function (data not published). Investigation of the involvement of AaZAP-70 in immune signalling is important to determine immune regulation in mosquitoes; therefore, we performed some experiments, such as examining AaZAP-70 induction after bacterial challenge and AaZAP cellular localization immunofluorescence assay in the mosquito cell line AP61. After LPS challenge, ZAP was induced and membrane localization was observed using confocal microscopy. An active NLS signal was found in the AaZAP sequence and Western blot of nuclear protein extracts from AP61 cells revealed a strong AaZAP signal. All these experiments were conducted to confirm the immunity of the interested protein; this was the first intensive study of AaZAP involvement in mosquito immunity. ZAP-70-interacting HIP-55 is an actin-binding adaptor protein that interacts with and is tyrosine-phosphorylated by ZAP-70, which is a crucial proximal protein tyrosine kinase for TCR signalling. It is well-known that ZAP-70 is a T cell receptor zeta chain-binding protein and plays a critical role in mediating T cell activation. Here, we noticed for the first time that the N-terminal AaHIP-55 sequence has active tyrosine residue sites (PTK), which is similar to our earlier identification of AaZAP-70 tyrosine sites. These tyrosine sites are potential response sites and capable of further activation of several signalling molecules to transmit TCR-induced proximal signals to downstream effectors. For their activation and further roles, all immune signalling proteins need active tyrosine residue sites or residue sites that phosphorylate immune receptor tyrosines-based activation motif in the CD3 zeta tail. Phosphorylated tyrosine-based activation motifs recruit ZAP-70 through the SH2 domain and further activate several immune signalling molecules such as Grb2. Thus, we concluded here that the tyrosine residues are important for activating additional signalling molecules and that active AaZAP-70 interacts with AaHIP-55 through tyrosine sites (Fig. 4a). While we correlated our data with Vassilis and Lampropoulou (2009), our Western blot results on the haemocyte contents of this AaHIP-55 protein showed a reduction in haemocytes reduction during infection time. Compared with the Vassilis and Lampropoulou (2009) report, haemocytes are produced from haematopoietic tissues, while HIP-55 is an HPK1-interacting protein. Thus, it is possible that haemocytes are produced from haematopoietic tissues during infection, while the HIP-55 protein is derived from haematopoietic tissues to balance the lost haemocytes. Hence, our data correlate well with the findings of Vassilis and Lampropoulou (2009).

Interestingly, our Western blot results explain that the signal of this protein is found in the abdomen because the abdomen is part of the foregut, one location of the pathogen invasion site. Thus, perhaps the HIP-55 protein provides immunity to defend against pathogens during pathogen invasion in the gut. Subsequently, it is very clearly proven from our Western blot studies that mosquito haemocytes contain the newly identified HIP-55 protein (Fig. 3). Based on the NCBI database prediction, the full-length mosquito HIP-55 protein is approximately 60 kDa. Thus, for antibody production and overexpression studies, we cloned a 609-bp partial sequence from full-length AaHIP cDNA that was cut with specific primers, yielding an approximately 40-kDa protein. The RNAi silencing affects HIP-55 gene expression by gradually reducing the activity of the gene due to changes in its transcriptional level. T cell activation is crucially engaged in actin cytoskeleton activity, while its role in TCR endocytosis remains unknown, though it may regulate HIP-55 in non-immune cells for endocytic processes (Kessels et al. 2000; Mise-omata et al. 2003). Crucial mosquito haemocytes function as originators and mediators in mosquito innate immunity. The reason behind the lack of resources in mosquito haemocytes is that it is ambitious to collect, culture and store these cells for certain periodsin vitro. Periosteal haemocytes assemble on the exterior part of the heart due to distinct antigenic induction, while haemocytes and immunogenicity already present at sites are undergoing with rapid haemolymph movement (Sigle and Hillyer 2016). Haemocytes phagocytose and melanise numerous microbes and malaria parasites (Da Silva et al. 2000; Hernandez et al. 1999; Hernandez-Martinez et al. 2002; Hillyer et al. 2003a, b). There are many subpopulations among mosquito haemocytes that have been confirmed by immunocytochemical and cytochemical assays as accountable for the occupation of pathogenicity (Hernandez et al. 1999; Hillyer and Christensen 2002). Mosquito haemocyte cells are differentiated to have many roles, including infrastructural aspects, effects on phagocytosis or play a role in the existence and action of enzymes employed in the melanin synthesis pathway (Hernandez et al. 1999; Hillyer et al. 2003a, b; Johnson et al. 2003). It is believed that haemocytes are involved in pattern recognition for the continuing synthesis of immune proteins from fats (Beerntsen et al. 2000). Phagocytosis plays a role in primary innate immunity and mediates consecutive adaptive immune responses. Similar to vertebrates, insects are also subpopulated by phagocytic cells, which are also considered to have macrophage-like characteristics against antigens. These phagocytic characteristics were shown through numerous signalling molecules, reorganization of the cytoskeleton and apoptotic components (Greenberg and Grinstein 2002).

Finally, we conclude that the newly identified and characterized mosquito HIP-55 protein is of prime importance in mosquito immunity by engaging in phagocyte-like functions. Additional studies are needed to determine the function of this protein. Our preliminary data will help to provide a platform for further possible research.

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Compliance with ethical standards

Mosquitoes are important vectors for viral infection which makes it a massive issue to eradicate. Our research tends to open a new avenue of immune-like function studies in the mosquito to control it as a viral parasite. This study is of use for the society affected with heavy losses due to mosquito-mediated viral infection all over the world. Our contribution and the importance of our works make it important to include our manuscript to this journal. As our Yang ming medical school ethical committee had not considered our study for ethical clearance regarding mosquitoes as a model animal, we declare that due to the importance of the research regarding mosquito and its efficiency as parasite, our manuscript should be published. Hence, we selected the journal to expose our research which mainly deals in the immune-like function protein which may tend to eradicate the viral vector by preventing millions of death in tropical countries like India and around the world.

Conflict of interest The authors declare that they have no conflict of interest.

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