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***Aea*/RACK1 expression and localization in response to stress in C6/36 HT mosquito cells**

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Abstract

The Receptor for Activated C Kinase 1 (RACK1), a scaffold protein member of the tryptophanaspate (WD) repeat family, folds in a seven-bladed β -propeller structure that permits the association of proteins to form active complexes. Mosquitoes of the genus *Aedes* sp., are vectors of virus producing important diseases such as: dengue, chikungunya and yellow fever. Based on the highly conserved gene sequence of *Aeae*RACK1 of the mosquito *Aedes aegypti* we characterized the mRNA and protein of the homologous *Aea*RACK1 from the *Ae. albopictus*-derived cell line C6/36 HT. Two protein species differing in MW/pI values were observed at 35 kDa/8.0 and 36 kDa/6.5. The behavior of *Aea*RACK1 was studied inducing stress with serum deprivation and the glucocorticoid dexamethasone. Both stressors induced increase of the expression of *Aea*RACK1 mRNA and proteins. In serum-deprived cells *Aea*RACK1 protein was located cortically near the plasma membrane in contrast to dexamethasone-treated cells where the protein formed a dotted pattern in the cytoplasm. In addition, 33 protein partners were identified by immunoprecipitation and mass spectrometry. Most of the identified proteins were ribosomal, involved in signaling pathways and stress responses. Our results suggest that *Aea*RACK1 in C6/36 HT cells respond to stress increasing its synthesis and producing phosphorylated activated form.

Keywords

C6/36 HT cells; Receptor for Activated C; Kinase (RACK1); Scaffold protein; Stress conditions; *Aedes* mosquito

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1. Introduction

The receptor for activated C kinase 1 (RACK1) is a highly conserved protein [1–5] that contains seven tryptophan-aspartate repeats (WD40). RACK1 is the best characterized member of the WD multidomain scaffold protein family and coordinates a variety of important cell activities such as signal transduction, adhesion, migration, development and immune and stress response among others [6–9]. RACK1 binds specifically to other proteins as the active form of protein kinase C (PKC β) [10,11], tyrosine kinase Src [12], integrin β subunit [13], β isoform of the thromboxane A2 receptor (TP β) a G protein-coupled receptor (GPCR) [14], and the G $\beta\gamma$ subunits of heterotrimeric G proteins [15].

Dexamethasone, a synthetic glucocorticoid, has been used to induce stress in several systems and it has been observed that it produces pleiotropic effects, depending of dose, affecting multiple signaling pathways [16,17]. In T cells, it has been observed that overexpression of RACK1 protects from apoptosis induced by high dose of dexamethasone and leads to translocation of PKC β , suggesting that the PKC β activation or changes in interactions with other molecules could be the mechanisms for the antiapoptotic effects of RACK1 [18]. Other functions of RACK1 include modulation of protein synthesis as a component of the 40S ribosomal subunit and regulate miRNA biogenesis and function [19–22]. Under stress conditions, RACK1 is incorporated into stress granules probably as part of 40S ribosome subunit, which in this condition stalls in a preinitiation complex preventing the synthesis/accumulation of misfolded proteins [8,23,24].

RACK1 is a conserved molecule and in insects there are studies suggesting its participation in signaling pathways regulating diverse cell functions [25–29]. In *Drosophila melanogaster*, RACK1 is ubiquitously expressed and it has a crucial role in the 20-hydroxyecdysone (20E) regulation, which modulates multiple steps of development [26,27]; RACK1 is also involved in the autophagy response to starvation, potentially acting as a scaffold protein during the formation of autophagosomes [28], and in the novel p38/MAPK/RACK1 pathway regulating proteostasis in aging and stressed muscle [29].

Mosquito-borne diseases are the most widespread infections in humans and they are serious global health challenges [30]. *Aedes sp.* mosquitoes are important vectors of dengue, chikungunya and yellow fever [31], and a model predicts a further spread of *Aedes albopictus*, particularly under climate change conditions [32]. Given the importance of this mosquito, their study is significant in order to understand its biology and vector competence [33]. C6/36 HT cell line was derived from *Ae. albopictus* mosquito, which is accepted as an *in vitro* model for studying insect vectors of human diseases because it can be easily infected with several viruses [34,35]. Several studies have implicated RACK1 in mediating distinct types of cell stress responses but the role of RACK1 in mosquitoes has not been explored. In this study, we report the identification of RACK1 in the *Ae. albopictus* cell line C6/36 HT (*Aea*/RACK1), which shares a high degree of sequence identity with those of other species. Also, we analyzed the expression and protein interaction of RACK1 under stress conditions of serum deprivation and exposure to the glucocorticoid dexamethasone. The results suggest the involvement of RACK1 in the possible activation of several cell signaling pathways.

2. Materials and methods

2.1. Bioinformatic analysis

Alignment of RACK1 sequences of several organisms was carried out using the ClustalW multiple alignment program available at Biology Workbench 3.2 (San Diego Supercomputer Center). The GenBank accession numbers of the sequences used in the alignment are *Aeae*RACK1 (*Aedes aegypti*, gi|157168005 [XP001663282.1] [AAEL013069]), *Dm*RACK1 (*D. melanogaster*, NP477269), *Cq*RACK1 (*Culex quinquefasciatus*, XP001863303.1), *Ag*RACK1 (*Anopheles gambiae*, XP319347.2), *Bm*RACK1 (*Bombyx mori*, NP001041703.1), *Ce*RACK1 (*Caenorhabditis elegans*, NP501859.1), human-RACK1 (*Homo sapiens*, NP006089.1) and *Sc*RACK1 (*Saccharomyces cerevisiae*, P38011.4).

The protein modeling tool Swiss-Model (<http://swissmodel.expasy.org/workspace/>) was used to generate a model for the structure of *Aeae*RACK1 [36]. *Aeae*RACK1 was also analyzed for putative functional association networks according to the STRING 9.0 Server (string-db.org) [37].

2.2. *Ae. albopictus* cell line C6/36 HT

C6/36 HT cells from *Ae. albopictus* [38,39], adapted to grow at 34 °C, were cultured in Minimum Essential Medium (MEM; Gibco, Life Technology), supplemented with 7% fetal calf serum, vitamins (Gibco, Life Technologies), 0.370 g/l sodium bicarbonate and 50 U/ml of penicillin and 50 µg/ml of streptomycin [40].

2.3. Cell viability

For viability assay 3.4×10^6 cells in exponential phase growth in 96 wells plates were gently washed with serum-free MEM. The cells were then switched to 100 µl of fresh serum-free MEM medium alone (serum deprivation) or dexamethasone [DEX, (11β, 16α)-9-fluoro 11, 17, 21-trihydroxy-16-1-methylpregna-1, 4-diene-3, 20-dione, (Sigma-Aldrich Corporation)], was added at the specified concentrations (8, 13, 25, 38 and 51 µM) and the cells were incubated for 30, 60 and 120 min. Cells without treatment were used as control. Cell viability was measured using the CellTiter 96® AQueous One Solution Reagent (Promega Corporation, Woods Hollow Road, Madison, WI, USA) following manufacturer's protocol. The amount of soluble formazan produced by cellular reduction of MTS substrate was recorded by absorbance at 490 nm using a 96-well plate reader (Corning, NY, USA). The experiments were performed in triplicate. Statistical analysis of data was performed by one-way ANOVA where the significant difference was considered $p < 0.05$.

2.4. Apoptosis assay

For apoptosis assays, Annexin-V-Fluos Staining Kit (Roche Diagnostics GmbH, Mannheim Germany) was used following manufacturer's protocol. 1×10^6 C6/36 HT cells were gently washed as before and then switched to serum-deprived or dexamethasone containing medium (13 and 51 µM) and incubated at 34 °C for 2 h. Then, the cells were washed with PBS, pH 7.2 and centrifuged at $200 \times g$ for 5 min. The cell pellet was resuspended in 100 µl of Annexin-V-Fluos/Propidium iodide labeling solution, and incubated at 25 °C for 10 min. After labeling, cells were analyzed in a flow cytometer (CyAn ADP Analyzer, Beckman

Coulter). In each experiment 10,000 cells were examined and Summit Version 5.1 software (Beckman Coulter Inc, CA, USA) was used for data acquisition and analysis. The experiments were conducted in triplicate.

2.5. Cell treatments

To investigate the effect of serum deprivation and exposure to dexamethasone on RACK1, 7.5×10^6 C6/36 HT cells in exponential growth phase were gently washed with serum-free MEM and then cultured in 10 ml of serum-free MEM (serum deprivation) or treated with 13 μ M dexamethasone for 120 min. Cells without treatment were used as control. These experiments were conducted in three separate trials. After treatments, the C6/36 HT cells were washed twice with PBS and centrifuged at $800 \times g$. Cells were lysed in sample buffer 2X (0.5 M Tris-HCl, 10% glycerol, 5% SDS), with protease (Complete, Roche Diagnostics, Mannheim Germany) and phosphatase (PhosSTOP, Roche Diagnostics, Mannheim Germany) inhibitors. Cell lysates were centrifuged at $16,000 \times g$ at 4 °C for 15 min, supernatants recovered and used immediately or stored at -80 °C until use.

2.6. 2-DE

Analysis of proteins by 2-DE was carried out using immobilized pH gradient (IPG) strips for the first dimension [41]. After treatments, C6/36 HT cells were resuspended in 200 μ l sample buffer [7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG Buffer (GE Healthcare Bio Sciences AB, Sweden), 40 mM DTT] with protease and phosphatase inhibitors, and homogenized. Samples were centrifuged at $16,000 \times g$ at 4 °C for 15 min, supernatants were precipitated with acetone and the pellets were solubilized in 200 μ l of rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.75% IPG Buffer, 20 mM DTT, and 1% bromophenol blue) supplemented with protease and phosphatase inhibitors. Protein concentration was determined using 2-D Quant kit (GE Healthcare), and 200 μ g of protein were applied to Immobiline™ Drystrips pH 3–10 NL, 7 cm (GE Healthcare) and rehydrated overnight at room temperature. IPG strips were electrofocused (IEF) in an Ettan IPGphor 3 system (GE Healthcare) following the manufacturer's instructions. Focused IPG strips were used immediately or stored at -80 °C until use. For second dimension, IPG strips were treated for 15 min in equilibration buffer (6 M urea, 2% SDS, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol) plus 65 mM DTT followed for 15 min in the same buffer plus 67.5 mM iodoacetamide and, equilibrated strips were placed on top of 12% SDS-PAGE. Gels were stained with Bio-Safe colloidal Coomassie Blue Stain (Bio-Rad Laboratories, Inc. CA, USA) and images recorded using the ImageQuant LAS 4000 System (GE Healthcare).

2.7. Western blot

For one-dimension (1D) Western blot, 40 μ g of protein per sample was resolved in 12% SDS-PAGE. Gels were stained with Coomassie blue and replica gels were electrotransferred onto nitrocellulose (NC) membranes and blocked with 3% BSA in TBS-T. Membranes were incubated with a rabbit polyclonal to human RACK1 (anti-RACK1) (ab62735, AbCam, Cambridge, MA, USA; dilution 1:10,000) overnight at 4 °C, followed by a secondary antibody goat anti-IgG Rabbit-HRP conjugate (65–6120, Invitrogen, Flynn Rd, Camarillo CA, USA; dilution 1:40,000). Signals were detected using the Supersignal West Pico Chemiluminescent Kit (Thermo Scientific, Rockford, USA) in an ImageQuant LAS 4000

System (GE Healthcare). The NC membrane was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA) following manufacturer's instructions and reacted with an anti- β actin monoclonal antibody as loading control (ab8224, Abcam Cambridge, MA, USA; dilution 1: 40,000), followed by a goat anti-Mouse IgG, (H + L) HRP conjugate (AP308P, Millipore, dilution 1:40,000). A densitometric analysis was carried out using ImageQuant TL LAS 4000 software. Non-stimulated A431 cell lysate (human carcinoma cell line; 12–301, Millipore Temecula CA, USA) was used as a positive control for RACK1. Assays were performed in duplicate.

For 2-DE blots, a replica 2-DE gel per experimental condition (control, serum deprivation and dexamethasone treatment), was electrotransferred onto NC membrane, blocked and reacted with anti-RACK1 (ab62735), followed by a goat anti-IgG Rabbit-HRP conjugate, as stated before. Signal detection was performed as before. A control using only the secondary antibody was performed. Assays were performed in duplicate. The protein spots that reacted with anti-RACK1 were excised from the replica Coomassie stained 2-DE gels and analyzed by mass spectrometry (ESI-LC-MS/MS).

2.8. Data analysis

For 1D WB quantitation, digital images were obtained as before and densitometric analysis of the band intensities was performed using ImageQuant LAS 4000 software (GE Healthcare). Results were statistically analyzed by Student's *t* test for unpaired samples with a $p < 0.001$ considered significant. For quantification of protein spot differences in 2-DE Coomassie stained gels and 2-DE WB, digital images were recorded using the ImageQuant LAS 4000 system and spot intensities were analyzed using the ImageMaster 2D Platinum 7.0 (GE Healthcare) software. Spots from 2-DE WB were detected, quantified and matched automatically. The amount of protein at each spot was expressed as volume percentage in order to correct for gel to gel variations in the intensity of the protein spots. The resulting sets of averaged spot relative volumes were analyzed by ANOVA with a $p < 0.05$.

2.9. Immunoprecipitation assay

C6/36 HT cells were serum-deprived and lysed in a nondenaturing lysis buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA) with protease and phosphatase inhibitors. Cell lysates were centrifuged at 4 °C, supernatants were collected and protein concentration was determined using BCA protein assay (Thermo Scientific, Rockford IL, USA). Samples of 2 mg of protein from total extract were pre-cleared with IgG/rProtein G-coupled agarose beads (Invitrogen) in immunoprecipitation non-denaturing lysis buffer with protease and phosphatase inhibitors. The beads were centrifuged and supernatant recovered. The pre-cleared protein extracts were incubated with anti-RACK1 antibody overnight at 4 °C on a rotary shaker. Control sample was incubated with a non-specific rabbit IgG antibody (negative control). The IgG/rProtein G-coupled agarose beads blocked with albumin were added to the mixtures, which were then incubated for 2 h at 4 °C on a rotatory platform. Subsequently, beads with immune complexes were washed three times with washing buffer (150 mM NaCl, 10 mM Tris–HCl pH 7.5, 1% NP-40, 5 mM EDTA). Proteins were extracted by incubation in loading buffer (5% SDS, 10% glycerol, 0.5 M Tris–HCl, pH 8.0) boiled for 4 min and subjected to 12% SDS-PAGE. Proteins were transferred

onto NC membranes, blocked and incubated with specific anti-RACK1 (ab62735). The experiments were performed in duplicate. Protein bands were excised from a replica Coomassie blue stained gel and analyzed by LC-MS/MS.

2.10. Mass spectrometry analysis by LC-MS/MS

Analysis and identification of proteins by ESI-LC-MS/MS was performed at the Protein Core Facility of the Columbia University Medical Center (New York, NY, USA). Selected protein spots or bands were manually excised from Coomassie blue stained gels, destained, digested and the resulting peptides were extracted and reduced/desalted. To obtain partial sequences of peptides ESI-LC-MS/MS was performed on a Micromass hybrid quadrupole/time of flight mass spectrometer (Micromass QTOF I, Waters, USA) equipped with an LC Packings nanoflow and operating in positive ion mode electrospray or in an hybrid quadrupole time of flight mass spectrometer (QSTAR XL Hybrid LC-MS/MS system; Applied Biosystems, Foster City, CA) in positive ion mode, at room temperature, with an IonSpray source for the API 150EX, API 3000, and QSTAR systems (Applied Biosystems) in the Protein Chemistry Section, Research Technology Branch, NIAID, NIH (Rockville, MD, 20852, USA). Raw data files were processed using the MassLynx ProteinLynx software and MASCOT engine (<http://www.matrixscience.com/>) using the NCBI nr database. Mascot search parameters of the tryptic peptides were adjusted considering possible oxidation of methionine residues, and carbamidomethylation at cysteine residues and, monoisotopic mass values, as variable modifications. The acquired MS and MS/MS data were integrated and searched (Mascot v. 2.1, Matrix Science) against the UniProt Knowledgebase (UniProtKB/Swiss-Prot) for *Ae. aegypti* species plus contaminant protein databases.

2.11. Real-time RT-PCR analysis

For the analysis of RACK1 expression under the different experimental conditions, quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was conducted. In brief, total RNA was isolated from C6/36 HT cells using Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA was treated with TurboDNase (Fermentas, Thermo Scientific). The quality of the RNA was tested with UV spectrophotometer and gel electrophoresis. The first strand cDNA was synthesized from 500 ng of total RNA using oligo (dT) primers, and SuperScript II reverse transcriptase (Invitrogen, Life Technologies, CA, USA) following the manufacturer's protocol.

To confirm the presence of RACK1 in C6/36 cells, a fragment of 369 bp was obtained using specific primers corresponding to *AeaeRACK1* (AAEL013069) sequence by RT-PCR. Forward primer: 5'-CCG TGT CGC CCG ATG GTT CC-3' (positions 596 to 615) and Reverse primer 5'-GAG CCG AGA CCG ACA CCT GC-3' (positions 931 to 912) were used. The fragment was cloned in PCRTM2.1-TOPO vector (Invitrogen) and sequenced (3500xL Genetic Analyzer, Applied Biosystems, Life Technologies). Match of *AeaeRACK1* fragment showed 100% identity with the sequence of *AeaeRACK1* (data not shown).

To quantify the *AeaeRACK1* expression by qRT-PCR, all reactions were performed with SYBR Green PCR Master Mix (Invitrogen) on the 7500 Fast Real-Time PCR System (Applied Biosystems). The primers were designed from the *Ae. aegypti* RACK1

(AAEL013069) sequence with assistance of REAL-TIME PCR program available at <http://www.idtdna.com/scitools/Applications/RealTimePCR/>: forward 5'-CAT CAG TGA CGT TGT CCT CTC-3' (positions 195–216), and reverse 5'-GGA CAT CCT TGG TAT GGT CTT C-3' (positions 304–326), to obtain a fragment of 113 bp corresponding to an internal region (forward exon 2 and reverse exon 3). In addition, primers: forward 5'-GAG ATC GAG TTC AAC AGC AAG A-3' (positions 145–167) and reverse 5'-GAG AAC TTC TTC TCC AGC TCA C-3' (positions 236–258) to obtain a fragment of 113 bp of 40S ribosomal S7 of *Ae. aegypti* [46] were used as reference for the amplifications. Relative changes in mRNA amounts were calculated using the 2^{-CT} method [42].

2.12. Immunofluorescence assays

For immunofluorescence assays, C6/36 HT cells grown at 60% confluence on coverslips in supplemented MEM, serum-deprived MEM and MEM with 13 μ M dexamethasone for 120 min, were fixed with 1% paraformaldehyde for 30 min at room temperature. Cells were permeabilized with ice-cold acetone for 3 min and treated with a blocking solution (PBS, pH 7.2, 10% FCS, 3% BSA, 10 mM glycine) for 60 min at 37 °C. Samples were incubated with anti-RACK1 (1:50) rabbit polyclonal antibody (ab62735) and goat anti-IgG Rabbit Alexa 488 conjugate as secondary antibody (A-11034, Invitrogen, Paisley, UK). DAPI (H-1200, VECTASHIELD, Vector Laboratories, Inc., Burlingame, USA; dilution 1:400) was used to stain nuclei. Fluorescence was visualized directly under a Leica LSM-SPC-5 Mo inverted confocal microscope fitted with HCX PL apo lambda blue 63 $\times$ 1.4 oil immersion lens. Images were recorded and processed using the LAS AF software (Leica TCS-SPE, USA). Untreated C6/36 HT cells preparations were used as control.

3. Results

3.1. Sequence analysis of AeaeRACK1

A full-length RACK1 protein and mRNA sequences were obtained from the *Ae. aegypti* genome database (<https://www.vectorbase.org/>), given that the *Ae. albopictus* genome is not sequenced. The amino acid sequence of AeaeRACK1 is 100% identical to *Cx. quinquefasciatus* and *An. gambiae*, it has 87% of identity and 93% similarity to RACK1 of *B. mori* and *D. melanogaster*, 74% identity and 84% similarity to *H. sapiens*, 66% identity and 79% similarity to *C. elegans*, and 50% identity and 67 % similarity to *S. cerevisiae* (Fig. S1). AeaeRACK1 is predicted as a protein of 311 amino acids with a molecular weight of 34.89 kDa and pI 7.97. Multiple amino acid sequence alignment indicated that AeaeRACK1 contains seven conserved Gly-His (GH) and WD (or their equivalents) canonical elements similar to human RACK1 [43]. The constructed model of AeaeRACK1 showed a seven-bladed (repeats) β -propeller structure with blades arranged radially around a central axis (Fig. 1A), similar to RACK1 of human and other species [1,43]. WD-repeats are conserved domains of approximately 40–60 amino acids that possess a GH dipeptide in the N-side and have a WD or equivalent dipeptide at the C-terminus. Repeats I, II, IV and V contain (GH) dipeptides, while repeat III showed Asp-His (DH), repeat VI contained Glu-His (EH) and repeat VII has Asp-Pro (DP) dipeptides at the N-terminal ends. Repeats II, V and VI had WD dipeptides at the C-terminal ends, while repeat I had Trp-Lys (WK), repeats III and IV contain Trp-Asn (WN), and repeat VII had Trp-Gln (WQ) (Fig. 1B).

3.2. Effect of dexamethasone on C6/36 HT cells survival

To investigate the effects of dexamethasone on viability of C6/36 HT cells doses from 8, 13, 25, 38 and 51 μM were tested for 30, 60 and 120 min. Dexamethasone doses of 38 and 51 μM diminished the viability under 80% at 60 min and reached less than 75% at 120 min. In comparison, doses of 8, 13, and 25 μM conserved viability above 80% for 120 min (Fig. S2).

3.3. Effect of serum deprivation and dexamethasone on C6/36 HT cells apoptosis

Serum deprivation and dexamethasone induced C6/36 HT cells apoptosis was evaluated by Annexin V/Propidium Iodide double staining followed by flow cytometry after 2 h of treatment. In the control group the apoptotic cells were $4.72 \pm 0.16\%$ (Fig. S3A), in the serum deprivation group were $9.83 \pm 0.15\%$ (S3B). In dexamethasone treatments apoptotic cells numbers were concentration-dependent with $7.31 \pm 0.09\%$ at (13 μM) and $18.43 \pm 0.19\%$ ($p < 0.05$) in 51 μM (Fig. S3 C and D). In subsequent experiments, 13 μM dexamethasone treatment was used because cells maintain their viability above 80%. The serum-deprived cells presented, compared with control cells, a moderated but significant increase in apoptosis ($p < 0.05$). Besides, the necrotic population of cells, detected by the Propidium iodide stain, remained at non-significant levels in all tested conditions.

3.4. Effect of stress on AeaRACK1 protein expression

A commercial polyclonal anti-RACK1 detected suitably the *Aea*RACK1 from C6/36 HT cells lysate in the Western blot assay and, it was used to determine abundance of *Aea*RACK1 in C6/36 HT cells under serum deprivation and a non-toxic dose of dexamethasone (13 μM) treatments. In basal conditions a very faint band of 35 kDa was detected; in contrast, the levels of *Aea*RACK1 significantly increased ($p < 0.05$) in response to serum deprivation condition and dexamethasone treatment, in a 62% and 45%, respectively (Fig. 2A and B). To confirm the identity and look for possible post-translational modifications (PTM) of the molecule recognized by the anti-RACK1 antibody, a 2-DE Western blot assay was conducted with C6/36 HT cells incubated in serum deprivation condition using the same antibody (Fig. 2C). Interestingly, the anti-RACK1 antibody recognized two spots of 35 kDa with pI 8.0 and 36 kDa with pI 6.5. In addition, LC-MS/MS analysis confirmed that both proteins were RACK1 (Table 1).

3.5. Differential abundance of AeaRACK1 species

After the observation that *Aea*RACK1 increase significantly under stress conditions and that there are two species, it was interesting to study the proportion of each moiety under these conditions. Cells in basal conditions had similar proportions of both *Aea*RACK1 species (Fig. 3A and B). Under serum deprivation, the spot 1 (35 kDa/pI 8.0) increased 2.94-fold in respect to control and spot 2 (36 kDa/pI 6.5) increased 5.35-fold (Fig. 3C and D). Comparatively, after exposure to dexamethasone spot 1 increase 4.91-fold and spot 2 increased 7.55-fold (Fig. 3E and F). The ratio spot 2/spot 1 was 1.82-fold in serum deprivation and 1.54-fold with dexamethasone (Fig. 3G).

3.6. AeaRACK1 functional associations

Since RACK1 is a scaffolding protein with many known binding partners and functions, we investigated the associations between *Aea*RACK1 with other proteins by an immunoprecipitation assay using anti-RACK1. C6/36 HT cells were serum-deprived and dexamethasone treated, cell lysate immunoprecipitated and proteins resolved by SDS-PAGE (Fig. 4A). The presence of *Aea*RACK1 was confirmed in lysates and immunoprecipitates by WB using anti-RACK1 antibody (Fig. 4B and C). Seven major protein bands were observed in both stress conditions, those of serum-deprived samples were excised from the stained gel and identified by LC-MS/MS (Fig. 4A and Tables 2 and 3). *Ae. albopictus* proteins that interacted with *Aea*RACK1 protein could be classified in eleven categories according to Gene Ontology annotations of biological processes at the VectorBase DB (Fig. 5). The most abundant groups corresponded to proteins involved in translation/ribosomal structure, proteins with functions of signaling and proteins with WD-repeat both implicated in a wide variety of crucial functions. Others proteins were clustered into categories of those participating in protein folding and assembly, response to oxidative stress and transmembrane and intracellular transport, among others (Table 3).

The associations observed for *Aea*RACK1 were in good agreement with those described for RACK1 from other species available in previous reports and with an *Aeae*RACK1 interactome generated using the STRING 9.05 database that contains information about known and predicted protein-protein interactions. This analysis revealed that *Aeae*RACK1 AAEL013069 (GI:157168005; XP_001663282.1) could interact with protein kinase c (AAEL000810), 40S ribosomal protein S12 (AAEL010299), ribosomal protein S3 (AAEL008192), 40S ribosomal protein S7 (S7), 40S ribosomal S20 (AAEL009506), 40S ribosomal protein S10 (AAEL002047), 40S ribosomal protein S26 (AAEL002832), 40S ribosomal protein S2 (AAEL010168), 40S ribosomal protein S9 (40sRpS9) and 40S ribosomal protein S14 (RpS14) (Fig. S4).

3.7. Effect of stress on AeaRACK1 gene expression

The expression of *Aea*RACK1 mRNA in C6/36 HT cells was studied in serum deprivation and dexamethasone treatment by qRT-PCR. The transcription level of *Aea*RACK1 was about 2-fold higher in cells under serum deprivation for 2 and 4 h, and 1.5-fold higher in cells treated with dexamethasone for 2 h, than in control C6/36 HT cells (Fig. 6).

3.8. Localization of AeaRACK1 protein

Considering that in different systems RACK1 translocated as part of the response to environmental stimuli [42], we study the location of *Aea*RACK1 in serum deprivation and dexamethasone-treated C6/36 cells. In untreated cells *Aea*RACK1 was scarce and located in very small dots dispersed in the cytoplasm. In cells incubated in serum-free medium, an increase in the signal of *Aea*RACK1 was observed, and the molecule was located mainly near the plasma membrane and in a smaller proportion in dots in the cytoplasm. In cells treated with dexamethasone the *Aea*RACK1 signal was more intense and uniformly distributed in numerous dots in the cytoplasm (Fig. 7).

4. Discussion

RACK1 proteins are conserved in all eukaryotes and have multiple physiological roles as adaptor proteins, regulating cellular events including translation and signaling. We identified the sequence of the scaffold protein RACK1 in the *Ae. aegypti* genome [44] and confirmed the *AealRACK1* expression in the *Ae. albopictus* C6/36 HT cell line with 100% identity. *AeaeRACK1* gene codes for a predicted protein identical to those of *Cx. quinquefasciatus* and *An. gambiae* mosquitoes and exhibits high homology with those of *B. mori* and *D. melanogaster*, *H. sapiens*, *C. elegans*, and *S. cerevisiae*, indicating that *AeaeRACK1* is evolutionary conserved (Fig. S1).

The *AeaeRACK1* predicted configuration has a β -propeller structure with seven blades formed by WD domains arranged radially around a central axis (Fig. 1A) similar to that of humans [1,43] and *Arabidopsis thaliana* [45] suggesting that *AeaeRACK1* may interact with other proteins, forming regulatory complexes [1,14,45,46]. In fact, using an elegant strategy by means of synthetic peptides as competitors, it has been demonstrated the region of $G\beta\gamma$, another WD40 family molecule, critical for the interaction with RACK1 and through this contact RACK1 can regulate the function of a subclass of $G\beta\gamma$ effectors [47]. RACK1 can be found in monomer and dimer forms [5,45,48] and it has been reported that Ser-146 in WD-repeat IV is required for human RACK1 dimerization [5,46]. However, the *AeaeRACK1* sequence does not contain Ser-146, but contain other Ser/Thr residues in this region which could be potential targets mediating *AeaeRACK1* dimerization after phosphorylation as has been observed in *S. cerevisiae* and *Dictyostelium discoideum* [5,47]. We used the NetPhos software [46] to predict phosphorylation sites within the WD-IV dimerization domain of *AeaeRACK1*. Significant scores (>0.5) were obtained for Serine-157 and Thr-173 (0.94 and 0.62, respectively) (Fig. S5), and could be potential targets for phosphorylation. In addition, in *AeaeRACK1* sequence there is a His-147 residue, which is equivalent in position to those of *S. cerevisiae* RACK1 (yRACK1) where it acts as switch between monomeric and dimeric forms [5].

In several systems has been reported that RACK1 expression and localization is modified by environmental conditions and their activities are important in stress responses [49]. In order to study the effects of stress on C6/36 cells we used two known stimuli, serum deprivation and the glucocorticoid dexamethasone. To study the effect of dexamethasone in a non-toxic condition that allows us to study the reaction of *AeaeRACK1* in stressed C6/36 HT cells, we selected a dose from a viability dose response curve. Dexamethasone at 51 μ M concentration during 2 h significantly reduced the viability of cells and induced apoptosis as reported (Figs. S2 and S3) [50–52]. At 13 μ M dexamethasone during 2 h C6/36 HT cells viability was over 80% and apoptosis lower than 10% and was selected for subsequent experiments as a non-toxic stress dose.

To detect *AeaeRACK1* we used a polyclonal antibody prepared against a region near the N-terminal (positions 2–16) of human RACK1 (TEQMTLRGTLKGHNG). In spite of this peptide is 60% identical and 73% similar to those of *AeaeRACK1* (TETLQLRGQLVGHS) the antibody recognized suitably the insect sequence, as it was confirmed by mass spectrometry of spots recognized in 2-DE WB (Table 1).

WB detection of *Aea*RACK1 in C6/36 HT showed that untreated cells have low protein level; in previous studies in invertebrates (*Penaeus monodon*, *Pinctada martensii* *Choristoneura fumiferana* and *Crassostrea angulate*) it was documented that RACK1 expression levels vary in tissues according to environmental, metabolic and developmental conditions of the cells [9,25,53,54]. Recent studies also showed that RACK1 expression in *Drosophila* L3 larva cells is negligible [27]. Accordingly, it is possible that the low levels of both, *Aea*RACK1 mRNA and protein observed in untreated C6/36 HT cells may reflect their larval origin.

In our study, both serum deprivation and dexamethasone treatments induced in C6/36 HT cells a RACK1 protein increase. Interestingly, we found two *Aea*RACK1 protein species by 2-DE and LC-MS/MS from spots visualized by Western blot. The first species corresponded to the predicted molecular weight and pI of *Aea*RACK1 (34.89/7.97) from translation of the coding sequence, the second species, slightly heavier and more acidic pI (36/6.5), was consistent with post-translational modifications (PTMs) as glycosylation, which in RACK1 from different mammalian cells during cellular stress may cause a small increase in molecular weight [55–57] and phosphorylation that add negative charges which is consistent with human RACK1 reported species [46]. RACK1 phosphorylation is required for various functions as its interaction with Src (pTyr 228/246 in WD-repeat VI) [12], binding of PP2A and β 1 integrin (Tyr-302 in WD7) [6] and FAK (Tyr-52) [58] among others. The ratio in the intensity between the two *Aea*RACK1 species detected suggests that in the control condition there are similar quantities of a basal molecule and another with one phosphorylated residue. In comparison, in both stress conditions the total protein abundance, and the ratio between them changes, suggesting that total *Aea*RACK1 protein increase and the proportion of the phosphorylated species is higher under stress conditions, which is consistent with an activation of RACK1.

The expression of both, protein and mRNA *Aea*RACK1 increments in C6/36 HT cells under serum deprivation and dexamethasone treatment similarly to those reported in *Drosophila* larva fat body during starvation, where deletion of RACK1 impaired both basal and starvation-induced autophagy. Under the effect of other stress inductors, RACK1 also increase, in *P. monodon* RACK1 expression levels rose when animals were treated with hydrogen peroxide and a non-enzymatic antioxidant function has been suggested [9]. Similarly, RACK1 was upregulated in *A. thaliana* under salt stress [59]. We observed that at 4 h of treatment the *Aea*RACK1 mRNA significantly increased more by effect of serum deprivation than for dexamethasone treatment. It has been observed that stressors produce different responses on RACK1 expression in the organisms studied by activation of specific signaling routes [60]. However at this moment we cannot explain yet this difference which could be an effect of the low dose of dexamethasone and this issue should be studied further.

Dexamethasone, a drug with diverse effects on cells and considered as xenobiotic [61], induced an increase in *Aea*RACK1 expression. In other systems an increase in RACK1 expression resulted in resistance to apoptosis as was observed in T cells, treated with Dexamethasone or Goniothalamine [18,62]. However this role is controversial because there are some reports where RACK1 expression correlates with apoptosis in tumoral cells [63].

Proteins interacting with *Aea*RACK1 under serum deprivation and dexamethasone treated were immunoprecipitated and those recovered from serum-deprived cell extracts were identified by mass spectrometry. In this condition *Aea*RACK1 associated with 33 proteins, which were grouped into eleven groups of functional associations (Fig. 5, Table 3). Most of the proteins identified in this work have been independently reported as partners of RACK1 in different systems, suggesting that our results are *bona fide*. Beside this, we identified HSP 90, endoplasmic (a HSP 90 variant) and HSP 70 which have not been reported before as directly associated to RACK1, there are reports that RACK1 and HSP 90 compete for Hypoxia-Inducible Factor 1a (HIF-1a) a HLH transcription factor regulating the fundamental maintenance of oxygen homeostasis in metazoans [64]. Other identified proteins associated to *Aea*RACK1 were two uncharacterized oxidoreductases which could be involved in stress signaling pathways [60]. Most of the detected proteins are part of ribosomal structure and/or participate in protein synthesis (25%), consistent with the reported localization and function of RACK1 in the ribosome to regulate translation [23,65,66], and WD-repeat/signaling proteins (31%), as observed in other systems [1,67]. In a search of protein interactions in the STRING 9.05 server, we detected *Aea*RACK1 interactions similar to those observed for human RACK1 [1,68], mainly association with ribosomal proteins (Fig. S4). Taken together, these data suggest that both *Aea*RACK1 and *Aeae*RACK1 are constituents of ribosome and may be involved in the regulation of protein translation [65,66,69–71], in response to multiple stimuli, such as stress and drug treatments [49,62].

In different physiological conditions, RACK1 is translocated to various cellular compartments (cytosol, endoplasmic reticulum and the nucleus), depending on its association with protein partners [49]. Under serum deprivation the location of *Aea*RACK1 was cortical in a pattern similar to those described for neurons, where under effect of dioxin associates with PKC β II forming a complex and migrating to a membrane location [72]. In NIH 3T3 fibroblasts treated with PMA, RACK1 was translocated from the perinuclear region to the plasma membrane, where it associated with Src [12,73]. Also, the translocation of RACK1 from the cytosol to the plasma membrane in mammalian cells was documented as a mechanism for the regulation of the protein G β γ transduction signal [15].

In dexamethasone-treated cells the translocation of *Aea*RACK1 generated a dotted pattern similar to those observed when RACK1 is incorporated into stress granules after exposure to hypoxia, heat shock or sodium arsenite, where it prevents the activation of a stress-responsive MAPK pathway, which in turn lead to cell survival instead of cell apoptosis [8,18]. These observations indicate a differential response depending of stressor and directing a specific and effective survival response (Fig. 7). However, it is currently unknown if the numerous dots observed in the cytoplasm of C6/36 HT cells upon dexamethasone treatment, correspond to sequestration of *Aea*RACK1 to stress granules through its binding to 40S ribosome subunit [8]. The possible association of *Aea*RACK1 to other molecules in these locations and the resulting effects await investigation.

In a previous report, the regulatory upstream region of gene GNB2L1, a human homolog of RACK1 was analyzed, several known and evolutionary conserved transcriptional elements were found and their activity was confirmed by deletion analysis [74,75]. Using

bioinformatics tools we analyzed the upstream region of *AeaeRACK1* gene and transcription factor binding sites were detected (Fig. S6), including a consensus sequence for activating protein 1 (AP-1) (–109 position), which is a general activator element. Interestingly other elements for the binding of transcription factors activated by stress are present such as CCAT/enhancer binding protein (C/EBP) (–116) which is a mediator of the endoplasmic reticulum stress signaling pathway characterized by the stress granules formation; a glucocorticoid response element (GRE) at the position –303 involved in the response to dexamethasone, and peroxisome proliferator-activated receptor alpha (PPAR α) (–819) responding to other hormonal stress related factors as prostaglandins. It is important to point that glucocorticoids have multilevel transcription regulation [76,77]. Two consensus sequence for Specificity protein 1 (Sp1) (–349 and –369), a cAMP Response Element Binding protein (CREB) (–496), the insect hormone Ecdysone response element (EcRE) (–636), and E-twenty six family (c-Ets-1) (–904) [78] were present too. The conformation of this set of elements is very suggestive to explain the RACK1 response under the stress conditions used in this work, supporting the activation of transcription in both stress conditions, but coordinating a different outcome due to the action of other genes that could share elements responding to drug or starvation differentially and a transcription inactivation conducted by glucocorticoid receptors (transrepression) [77]. Future research should investigate the RACK1 behavior in other physiological conditions as embryonic development and metamorphosis and other stress inductors as xenobiotics, including insecticides.

5. Concluding remarks

In this work we use the sequence of *AeaeRACK1* gene from the mosquito *Ae. aegypti* available in the VectorBase database to do predictions about the sequence and behavior of the molecule in a larval cell line C6/36 HT from *Ae. albopictus*. The results confirmed that this is a highly conserved molecule both, in structure and cellular behavior. C6/36 HT cells submitted to serum deprivation and the presence of dexamethasone increased the expression of *AeaRACK1* both in mRNA and protein, generating two protein species differing probably in phosphorylation and glycosylation, favoring the increment of the modified species. In addition, the localization of the protein, was different in these conditions suggesting a differential response to these specific stress conditions. As a scaffold protein *AeaRACK1* associates with numerous partners and in this work a preliminary description of the molecules associated, mainly ribosomal and signaling proteins, is presented, supporting its role as a ribosomal component probably modulating translation and regulatory functions.

The results obtained in this work indicate the potential use of our experimental model to study the physiological effects of *AeaRACK1* in diverse signaling pathways, and in the expression of regulatory molecules in insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

RACK1	Receptor for activated C kinase
AP-1	activating protein 1
GRE	glucocorticoid response element
CREB	cAMP Response Element Binding protein

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Biological significance

Insect cells adapt to numerous environmental stressors, including chemicals and invasion of pathogenic microorganisms among others, coordinating cellular and organismal responses. Individual cells sense the environment using receptors that trigger signaling pathways that regulate expression of specific effector proteins and/or cellular responses as movement or secretion. In the coordination of responses to stress, scaffold proteins are pivotal molecules that recruit other proteins forming active complexes. The Receptor for Activated C Kinase 1 (RACK1) is the best studied member of the conserved tryptophan-aspartate (WD) repeat family. RACK1 folds in a seven-bladed β -propeller structure and it could be activated during stress, participating in different signaling pathways. The presence and activities of RACK1 in mosquitoes had not been documented before, in this work the molecule is demonstrated in an *Aedes albopictus*-derived cell line and its reaction to stress is observed under the effect of serum deprivation and the presence of glucocorticoid analog dexamethasone, a chemical used to cause stress *in vitro*.

Fig. 1 –.
Model of *Aea*RACK1. (A) The predicted protein sequence of *Aea*RACK1 was used to construct a three-dimensional model using the human RACK1 structure as template (PDB ID: 2zkqa). Each blade of the propeller is made up of four antiparallel β -strands and forms a WD domain, indicated by Roman numerals I to VII. (B) Sequence alignment of the seven WD domains of *Aea*RACK1, which are flanked GH or equivalents dipeptides (enclosed in a dashed line box), WD or equivalent dipeptides (enclosed in a continuous line box).

Fig. 2 –.

Expression of *Aea/RACK1* in C6/36 HT cells in serum deprivation or dexamethasone treatment. The C6/36 HT cells were serum-deprived (SERUMDEPRIV) or dexamethasone(DEX, 13 μ M) treated. (A) Cell lysates were resolved in 1D electrophoresis and Western blot was performed using anti-RACK1 polyclonal antibody followed by an anti-IgG-rabbit polyclonal antibody coupled to peroxidase. Lanes: A431-Human carcinoma cell line used as positive control; lane CTL—C6/36 HT cells untreated-control; lane SERUM DEPRIV—cells incubated with serum-free MEM; lane DEX—dexamethasone treatment. Same blot was tested with an anti β -actin antibody as loading control. (B) RACK1 presence was determined by densitometric quantification of the unsaturated images of Western blot. Relative expression was calculated using the corresponding value of β -actin. Experiments were conducted in duplicate (* $p = 0.01$; ** $p < 0.05$). (C) Total proteins of C6/36 HT cells under serum deprivation treatment were resolved in the first dimension on an IPG strip pH 3–10 NL and in the second dimension on a 12% SDS-PAGE. Gel was stained with Bio-Safe Coomassie blue. (D) Identification of *Aea/RACK1* by 2-DEWB. A replica 2-DE gel was blotted onto NC membrane and incubated with a specific anti-RACK1 antibody followed by an anti-IgG-rabbit polyclonal antibody coupled to peroxidase. Two

protein spots (indicated by arrows) recognized by anti-RACK1 were detected. Both proteins spots were identified as RACK1 by MS (Table 1).

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Fig. 3 –.

Abundance of two *Aea/RACK1* species in C6/36 HT cells in serum deprivation and dexamethasone treatment. The images of the two *Aea/RACK1* protein species detected by 2-DE Western blot under different treatments (A, C, E) were analyzed using the ImageMaster 2D Platinum 7.0 software and 3D graphics of the density of the spots were generated (B, D, F). (A and B) Cells without treatment; (C and D) cells serum-deprived; (E and F) cells with dexamethasone (13 μ M). Circles and arrows show protein spots 1 (35 kDa/pI 8.0) and 2 (36 kDa/pI 6.5). Spot 2 showed higher molecular weight and intensity and a lower pI. (G) Quantification of protein abundance of two protein spots.

Fig. 4 –.

Proteins associated with *Aea*/RACK1 during serum deprivation or dexamethasone treatment. Protein extracts of C6/36 HT cells were immunoprecipitated with an anti-RACK1 antibody. (A) Coomassie blue stained proteins resolved by SDS-PAGE. Input: total extracts; IP:IgG, reaction with a non-related antibody. IP: RACK1, immunoprecipitation of cells lysates using an anti-RACK1 antibody. Lanes 1 and 6—C6/36 control cells extract; lanes 2, 4 and 7—serum deprivation; lanes 3, 5 and 8—cells treated with dexamethasone (13 μ M). Major protein bands were excised from the gel and identified by MS (arrows 1–7). The presence of RACK1 in total extracts (INPUT) and in immunoprecipitates (IP: RACK1) was verified by immunoblot (IB). (B) Input and IP: RACK1 in the absence (–) or presence (+) of dexamethasone. Panel C: Input and IP:RACK1 in control (–) and serum-deprived cells (+). Lanes IgG—immunoprecipitation negative control conducted with a non-related IgG antibody. MCF7—total extracts of human mammary cancer cells used as positive control.

Fig. 5 –.
Classification of the identified *Ae. albopictus* proteins in biological processes. Categories were obtained from the gene ontology/annotations of biological processes at VectorBase DB.

Fig. 6 –.

Expression of *Aea/RACK1* in C6/36 HT cells serum-deprived and dexamethasone treated. A time course of the expression of *Aea/RACK1* mRNA in C6/36 HT cells serum-deprived and treated with dexamethasone (13 μ M) were determined by qRT-PCR, using the comparative 2^{-CT} method, comparing with the expression of control cells and normalizing with *S7* mRNA as an internal control. Results are represented as mean $\pm$ SE (N = 3).

Fig. 7 –.

Localization of *Aea/RACK1* in C6/36 cells under serum deprivation and dexamethasone treated. C6/36 HT cells untreated (A–C), serum-deprived (D–F) or treated with dexamethasone (13 μ M) (G–I), were fixed with 1% paraformaldehyde and permeabilized with ice-cold acetone. The *Aea/RACK1* protein was localized using anti-RACK1 polyclonal antibody and with Alexa Fluor 488-conjugated secondary antibody (green color) (A, D and G). Nuclei were DAPI stained (blue color) (B, E and H). Merge images (C, F and I). Cells were examined using confocal microscopy.

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Table 1 –
Identification of *Aea*RACK1 protein from C6/36 HT cell line by 2-DE/WB and LC-MS/MS.

Spot num	Protein	NCBI Acc num	Theor Mr/pI ^a	Exp Mr/pI ^b	Pept Ident ^c	SC % ^d	Mascot score
1	Receptor for activated protein kinase (RACK1) <i>Aedes aegypti</i>	gi 157168005	34,891/7.97	35,000/8.0	9	33	385
2	Receptor for activated protein kinase (RACK1) <i>Aedes aegypti</i>	gi 157168005	34,891/7.97	36,000/6.5	6	20	261

Spots 2-DE resolved were identified by LC-MS/MS on a Micromass QToF1 (Waters, USA).

Mascot scores > 60 and $p < 0.05$ from Mascot search on MS/MS spectra data.

^aTheoretical mass (Mr) and pI reported in UniProtKB (www.uniprot.org).

^bExperimental mass (Mr) and pI.

^cNumber of peptides matched by LC-MS/MS for each identified proteins by Mascot program.

^dSequence coverage: percentage of amino acids of matched peptides in relation to the full sequence of identified proteins.

Table 2 –

Proteins associated to *AeeRACK1* in serum-deprived C6/36 HT cells identified by LC/MS/MS.

Band num	Sequence Id	Protein name	Function	VectorBase Acc Num ^a	Theor/Exp Mr ^b	Theor pI ^c	Pep Ident ^d	Score	SC % ^e
1	Q16KZ2	Endoplasmic	Protein folding, response to stress, ATP binding	AAEL012827	91.0/100	4.66	14	882	30
1	Q0IFN2	Eukaryotic translation elongation factor	GTPase activity, catalyzes the elongation of peptide chains in protein biosynthesis	AAEL004500	94.0/100	5.96	11	586	12
1	Q175R3	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	SERCA is a transmembrane ion transporter key to the regulation of Ca ²⁺ (Calcium pump)	AAEL006582	112.0/100	5.27	7	352	10
1	Q16IM0	Clathrin heavy chain	Intracellular protein transport, vesicle-mediated transport	AAEL013614	191.0/100	5.50	6	407	6
1	Q177S9	Coatomer WD associated region (fragment)	Intracellular protein transport, vesicle-mediated transport	AAEL006040	106.0/100	4.95	5	327	9
2	Q16FA5	Heat shock protein 90	Protein folding, response to stress	AAEL010585	81.5/80	4.79	9	492	15
2	Q16SH1	Spermatogenesis associated factor	Belongs to the AAA ATPase family	AAEL010585	88.7/80	5.17	8	451	12
3	Q17LF3	Coronin	WD-repeat protein, actin cytoskeleton regulator	AAEL001360	61.4/65	6.02	4	183	13
3	J9HSH6	Heat shock protein 70	Protein folding and assembly	AAEL017315	90.3/65	4.91	5	418	10
3	Q175F8	Putative oxidoreductase	Oxidation-reduction process	AAEL006684	60.8/65	7.83	4	281	11
4	Q16S95	Quinone oxidoreductase	Oxidoreductase activity	AAEL010668	50.5/45	5.47	7	393	19
4	Q17LW2	Phosphoinositide-binding protein putative	WD-repeat protein, it participates in signaling	AAEL001212	46.1/45	7.06	6	380	16
4	Q16P63	Actin	Major component of the cytoskeleton	AAEL011750	44.7/45	5.88	4	250	16
4	Q17K03	Cop9 complex subunit	Component of the 26S proteasome	AAEL001874	46.7/45	5.44	4	228	13
4	Q17J37	GTP-binding protein	Proteins involved in signal transduction	AAEL002160	44.7/45	6.62	3	240	11
5	Q1HQK6	Serine-threonine kinase receptor-associated protein (strap)	Protein G subunit β , WD40 repeat. It participates in signaling	AAEL007382	36.0/37	5.99	8	550	33
5	Q1HR05	Serine/threonine-protein phosphatase S	Catalyzes the dephosphorylation of pS and pT residues	AAEL009275	37.3/37	5.89	4	264	17
5	Q17FQ8	CRAL/TRIO domain- containing protein	Lipid transport and metabolism, membrane trafficking in TGN, signal transduction	AAEL003347	36.8/37	6.62	3	148	12
5	Q16QF1	Annexin	Calcium-dependent phospholipid binding	AAEL011302	35.7/37	4.55	9	525	29
5	Q17MH7	Guanine nucleotide-binding protein beta 3 (G protein beta3)	WD-repeat protein is implicated in signal transduction	AAEL001041	37.0/37	5.97	2	121	8

Band num	Sequence Id	Protein name	Function	VectorBase Acc Num ^a	Theor/Exp Mr ^b	Theor pI ^c	Pep Ident ^d	Score	SC % ^e
6	Q1HQU2	60S ribosomal protein L5	5S rRNA binding. Structural constituent of ribosome. Translation	AAEL004325	34.1/33	10.25	11	623	35
6	Q1HRQ2	Activated protein kinase C receptor	Rack1, WD-repeat protein is implicated in signal transduction and others	AAEL013069	34.9/33	7.78	8	432	29
6	Q8MQT0*	Ribosomal protein P0	Ribosome biogenesis	AAM97779*	33.8/33	5.33	4	186	17
6	Q1HRU0	ADP/ATP translocase	Mitochondrial transmembrane transport	AAEL004855	32.9/33	10.01	4	265	16
6	Q1HQT4	Translation initiation factor 2B alpha subunit	IF2B, translation initiation factor activity	AAEL010251	34.1/33	5.28	6	347	25
7	Q16ZR8	40S ribosomal protein SA	Structural constituent of ribosome. Translation	AAEL008083	31.5/30	4.75	3	165	14
7	Q1HR57	Mitochondrial porin	Transmembrane transport. Voltage-gated anion channel activity	AAEL001872	30.7/30	8.89	13	939	49
7	Q1HRS6	40S ribosomal protein S4	Translation, ribosomal structure and biogenesis	AAV69397	29.6/30	10.71	7	379	28
7	Q1HRR3	40S ribosomal protein S3a	Translation, ribosomal structure and biogenesis	AAEL005901	30.1/30	9.89	7	433	26
7	Q4PKD7	Proliferating cell nuclear antigen	DNA replication and repair	AAEL012545	29.0/30	4.47	3	175	21
7	Q1HR32	60S ribosomal protein L2/L8	Structural constituent of ribosome. Translation	AAEL000987	28.6/30	11.46	5	299	15
7	Q1HR36	14-3-3 protein zeta	Scaffold protein implicated in the regulation of a large spectrum of both general and specialized signaling pathway	AAEL006885	28.2/30	4.63	3	273	18
7	Q1HR13	Prohibitin	Scaffold protein that modulates many signaling pathway, cell survival, apoptosis, metabolism and others	AAEL009345	29.9/30	5.23	4	237	26

SDS-PAGE resolved bands were identified by LC-MS/MS in a QSTAR XL Hybrid System. Applied Biosystems. Analysis by Mascot v.2.1 program.

* Sequence of Aedes albopictus [European Nucleotide Archive (www.ebi.ac.uk)].

^a VectorBase Accession number (www.vectorbase.org).

^b Theoretical/Experimental mass (Mr).

^c Theoretical pI reported in UniProtKB (www.uniprot.org).

^d Number of peptides matched by LC-MS/MS for each identified proteins by Mascot program.

^e Sequence coverage: percentage of amino acids of matched peptides in relation to the full sequence for each identified proteins.

Table 3 –

Functional classification of all 33 identified proteins by immunoprecipitation and MS.

Categories ^a	Protein
Protein folding Endoplasmic and assembly (3)	Endoplasmic Heat shock protein 90 Heat shock protein 70
Response to oxidative stress (2)	Putative oxidoreductase Quinone oxidoreductase
DNA synthesis (1)	Proliferating cell nuclear antigen
Cytoskeleton proteins (1)	Actin
Translation, ribosomal structure (8)	60S ribosomal protein L5 60S ribosomal protein L2/L8 40S ribosomal protein SA 40S ribosomal protein S3a 40S ribosomal protein S4 Ribosomal protein P0 Eukaryotic translation elongation factor Translation initiation factor 2B alpha subunit
WD-repeat proteins (6)	Activated protein kinase C receptor Coatomer WD associated region (fragment) Coronin Guanine nucleotide-binding protein beta3 (G protein beta3) Phosphoinositide-binding protein putative Serine-threonine kinase receptor-associated protein (strap)
Signaling proteins (6)	Activated protein kinase C receptor CRAL/TRIO domain-containing protein GTP-binding protein Guanine nucleotide-binding protein beta3 Prohibitin 14-3-3 protein zeta
Transmembrane transport (3)	ADP/ATP translocase Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (SERCA) Mitochondrial porin
Intracellular transport (2)	Clathrin heavy chain Coatomer WD associated region (fragment)
ATP-binding proteins (2)	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type Spermatogenesis associated factor
Calcium ion binding (1)	Annexin

^aCategories were taken from Gene Ontology annotations of biological process at VectorBase DB.