

GP35 ANOAL, an abundant acidic glycoprotein of female *Anopheles albimanus* saliva

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Abstract

Salivary glands of female mosquitoes produce proteins, not completely described yet, that participate in carbohydrate and blood feeding. Here, we report an acidic glycoprotein of 35 kDa (GP35 ANOAL) secreted in the saliva of the malaria vector mosquito *Anopheles albimanus*. GP35 ANOAL is produced exclusively in the distal lateral lobes of adult female salivary glands, it has a pI of 4.45 and is negatively stained by regular silver stain. An 888 bp cDNA clone encoding a predicted product of 240 amino acids has a signal peptide, potential post-translational modification sites, and a disintegrin signature RGD. The GP35 ANOAL sequence depicts high similarities with the 30 kDa saliva allergen of *Aedes aegypti*, 30 kDa allergen-like hypothetical proteins, and GE-rich proteins present in several *Anopheles* species, as well as in *Ae. albopictus* and *Culex pipiens quinquefasciatus*. The function of this protein family is still unknown.

Keywords: *Anopheles albimanus*, salivary glands, sex-specific, glycoprotein, GP35, mosquito.

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Introduction

Blood-sucking insects have developed special salivary glands fitted for sugar intake from plants and blood from vertebrates. Female mosquito salivary glands are composed of two lateral lobes, with distinct proximal and distal portions, and a medial lobe (Wright, 1969). Molecules related to blood feeding are synthesized by the medial and the distal portion of lateral lobes (James *et al.*, 1991; Smartt *et al.*, 1995). The male glands consist of a single small and narrow lobe with similar morphology and function to the female proximal regions of the lateral lobes that produce enzymes involved in sugar feeding (James, 1994; Moreira-Ferro *et al.*, 1999).

Transmission of malaria to vertebrate hosts depends upon the ability of *Plasmodium* sporozoites to invade the salivary glands of female vector mosquitoes. Salivary gland invasion is limited to the medial and distal segments of the lateral lobes (Sterling *et al.*, 1973; Rossignol *et al.*, 1984), a pattern corresponding to the lobes specialized in the secretion of blood feeding compounds, which contain different proteins and bear specific carbohydrates on their surface (Perrone *et al.*, 1986; Stark & James, 1996; Moreira-Ferro *et al.*, 1999).

Mosquito saliva contains a variety of bioactive compounds that are injected into the host's skin during blood feeding, and counteract the host's immune and haemostatic responses, including blood coagulation, platelet aggregation, and vasoconstriction (Ribeiro, 1987, 1995; Law *et al.*, 1992; Stark & James, 1996; Isawa *et al.*, 2002). Using high throughput genomic strategies, databases have been made available on gene sequences of *Aedes aegypti* (Valenzuela *et al.*, 2002b), *Anopheles gambiae* (Arcà *et al.*, 1999a,b, 2005; Dana *et al.*, 2005; Kalume *et al.*, 2005a,b; Francischetti *et al.*, 2002; Lanfrancotti *et al.*, 2002), *An. darlingi* (Calvo *et al.*, 2004), and *An. stephensi* (Valenzuela *et al.*, 2003). Some of these are grouped in gene families, common in all mosquitoes so far studied, that match with proteins of known function (e.g. mucins, apyrases, peroxidases, maltases, antithrombins, Factor XII inhibitors, antimicrobial peptides), but the identity and function of many others await identification. Unravelling the complex composition and function of saliva in vector mosquitoes is

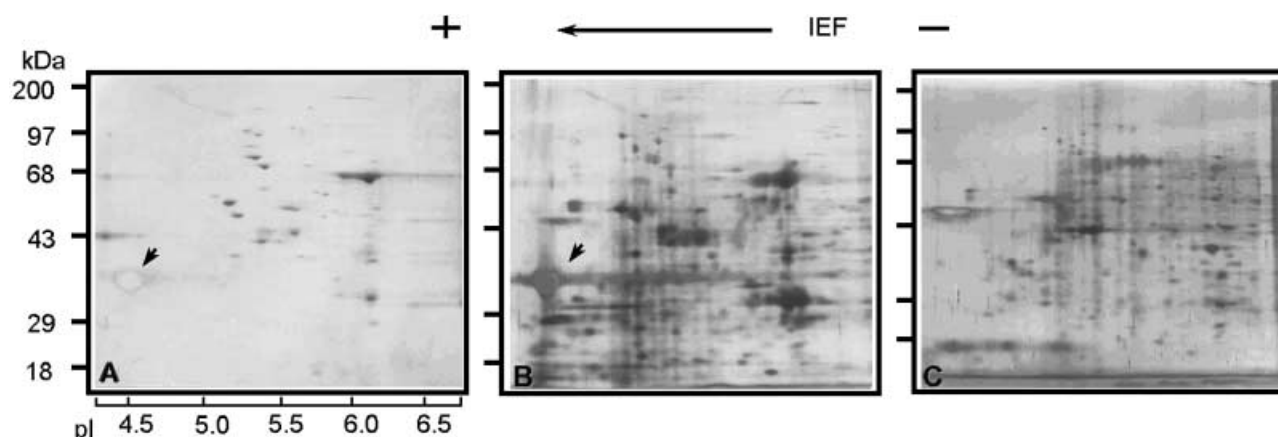


Figure 1. Proteins of *An. albimanus* salivary glands analysed by 2D electrophoresis. Protein extracts of female and male salivary glands were analysed by 2D (ampholytes mix 5–7/3–10, 10% SDS–PAGE) and stained with mono- and polychromatic silver methods. (A) Proteins from 200 pairs of female salivary glands stained with monochromatic silver, the 35 kDa protein band is negatively stained. (B) Proteins from 200 pairs of female salivary glands stained with polychromatic silver. (C) Proteins from 400 pairs of male salivary glands stained with polychromatic silver. GP35 is shown with an arrow.

essential for the understanding of the blood feeding process and its interaction with malaria parasites leading to vertebrate host infection (Rodríguez & Hernández-Hernández, 2004).

Among the extensive number of proteins of unknown function, a family of acidic 30–36 kDa proteins was first identified as an allergen in *Ae. aegypti* (GI:14423642; Peng and Simons, 1997; Brummer-Korvenkontio *et al.*, 1997; Simons & Peng, 2001), and later in all anopheline and culicine mosquitoes studied so far (GI:31979116; GI:37201974; Francischetti *et al.*, 2002; Valenzuela *et al.*, 2002a,b, 2003; Ribeiro *et al.*, 2004; Calvo *et al.*, 2004). A member of this family is the second most abundantly expressed gene in salivary glands of female *An. gambiae* (Arcà *et al.*, 2005), but no information on the anatomical site of production and its relation to the bloodmeal process is available. In this work, we describe the site, time of production and secretion, as well as the molecular structure of a new member of this family, a glycoprotein of 35 kDa (GP35 ANOAL) produced in the salivary glands of female *An. albimanus*, one of the main vectors of malaria in Mexico and Central America (Rodríguez, 2005).

Results

An acidic protein of 35 kDa of salivary glands from An. albimanus female mosquitoes

To study the total proteins of female *An. albimanus* salivary glands, whole glands were dissected and analysed by two-dimensional electrophoresis (2D). Approximately 100 protein spots were visualized in gels treated with monochromatic silver stain, but a very abundant spot of 35 kDa, with a pI of 4.45, was very conspicuous as it was 'negatively' stained and appeared as a white spot surrounded by a dark

circle (Fig. 1A). In contrast, more than 370 proteins were observed with polychromatic silver staining, including the 35 kDa protein that was brown stained (Fig. 1B, arrow). Whole male salivary glands resolved by 2D and polychromatic silver stained presented a different pattern and although several spots coincided with those observed in female salivary gland preparations, the 35 kDa spot was absent (Fig. 1C). The 35 kDa protein was not present in female midguts, ovaries and haemolymph preparations analysed with the same methods (Cázares-Raga *et al.*, 1998, 2000). These results indicate that the 35 kDa protein is exclusive of female salivary glands.

The 35 kDa protein is expressed near mosquito emergence

To investigate the expression of GP35 during the maturation process after adult mosquito emergence (AE), proteins of whole female salivary glands from 0, 12, 24, 48, 72, 96 and 120 h AE were run by 1D and 2D electrophoresis and silver stained using mono- and polychromatic procedures (Fig. 2). The 35 kDa protein was easily detected as a clear band in 1D gel and, as a spot in 2D gels. The negatively stained band was observed at 0 h AE and as a constant broad band at later times (Fig. 2A). This protein was visualized in 2D gels as a small spot at 0 h AE, and increased in size and density up to 24 h AE, remaining constant up to 120 h (5 days) (Fig. 2B,C).

The 35 kDa protein is a saliva glycoprotein (GP35) produced in the distal lateral lobes of salivary glands

To investigate which regions of the salivary gland express the 35 kDa molecule, protein extracts of distal and proximal lateral and medial lobes were separated by 1D electrophoresis and subjected to Western blot analysis using a

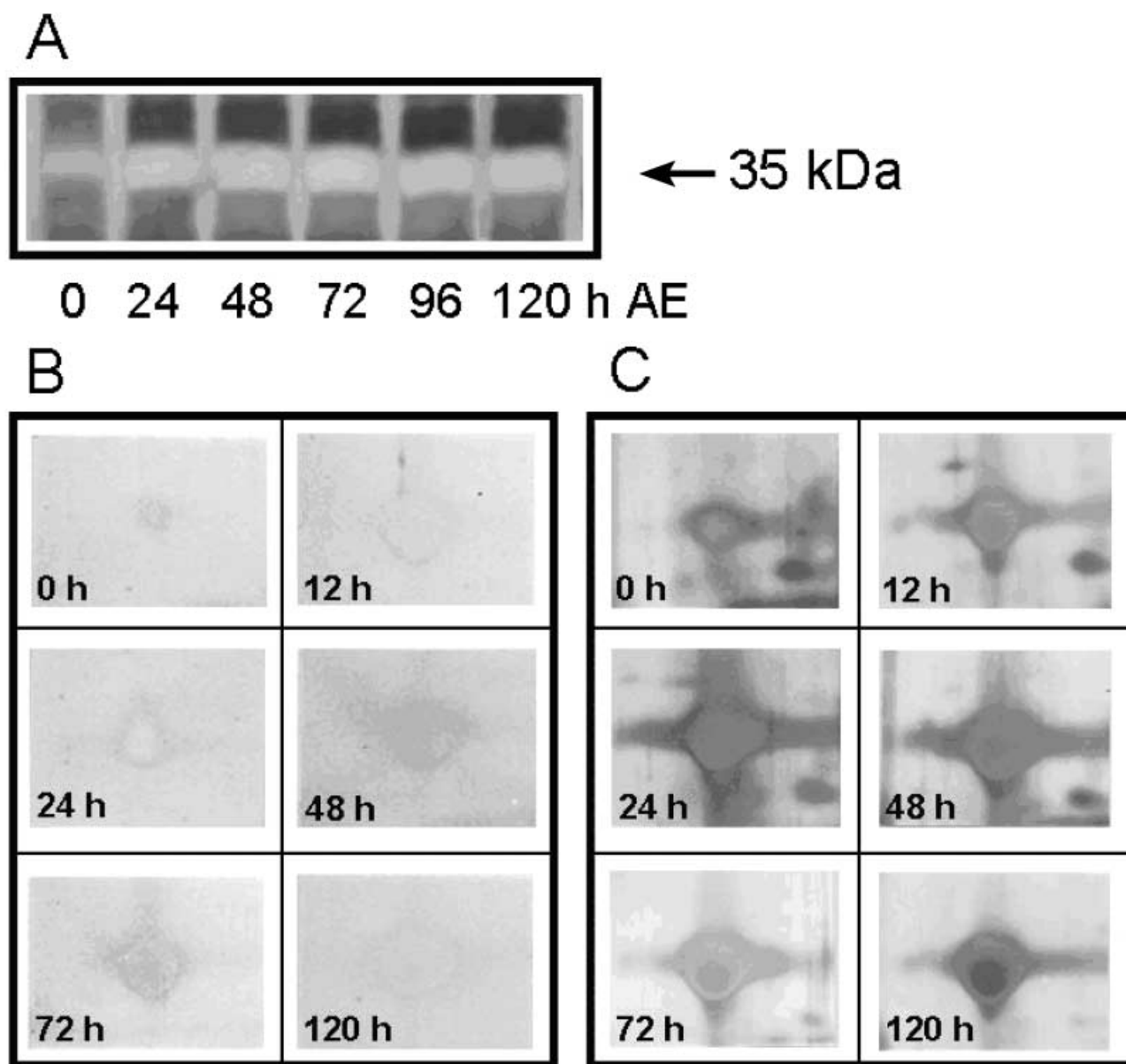


Figure 2. Expression of GP35 in *An. albimanus* female salivary glands after mosquito emergence. Whole salivary glands were obtained at times between 0 and 120 h after adult emergence (AE), and their protein extracts were electrophoresed in 12% SDS–PAGE and 2D (ampholytes mix pH 5–7/3–10, 12% SDS–PAGE). (A) Unidimensional resolution of GP35 ANOAL protein of 10 pairs of salivary glands per lane, monochromatic silver stained. GP35 ANOAL proteins of salivary glands (200 pairs) were resolved in two-dimensional gels and monochromatic silver stained (B) or polychromatic silver stained (C). Only the region corresponding to the 35 kDa protein is presented.

mouse monoclonal antibody (mAb 1B11:IgG1) that recognizes a 35 kDa band in whole salivary gland preparations. The protein was observed only in whole salivary glands and distal lateral lobes preparations (Fig. 3A,B). In order to investigate the presence of carbohydrate moieties in the 35 kDa protein, a specific carbohydrate oxidation reaction was conducted indicating the glycoprotein character of the molecule (Fig. 3C,D) that was consistent with a positive labelling with biotinylated concanavalin A (data not shown). To investigate whether GP35 is secreted in the saliva, a spit

blot assay was performed. mAb 1B11 produced abundant and intense reaction with mosquito saliva drops deposited on a nitrocellulose membrane (NCM) indicating the presence of the 35 kDa protein (Fig. 3E). No reaction was observed using preimmune sera (Fig. 3F).

Molecular analysis of GP35

Based on the N-terminus sequence of GP35 spot (NED-TAEE), a primer was designed (AAC GAG GAT ACN GCN GAG GAG) and a fragment of 772 bp generated by

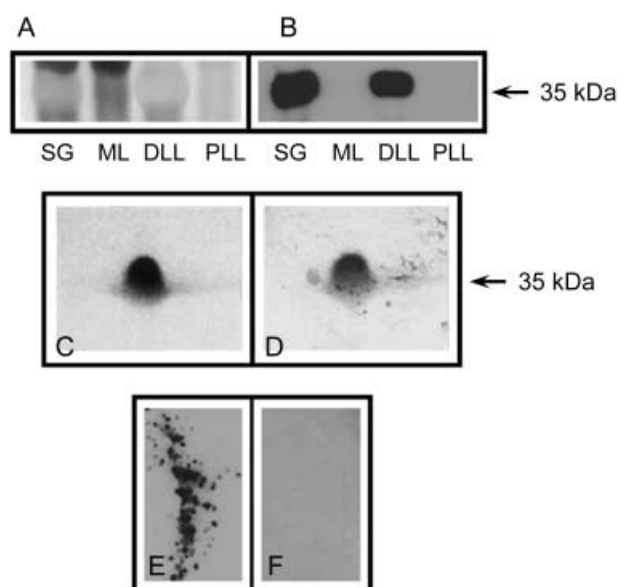


Figure 3. GP35 is a saliva glycoprotein exclusively expressed in the distal lateral lobe of *An. albimanus* female salivary glands. Complete female salivary gland proteins and equivalents of separated lobes were electrophoresed by 1D (12% SDS–PAGE) or 2D. (A) Proteins of 10 whole salivary glands and equivalents of separated lobes analysed by 1D and silver stained and (B) detected by Western blot, using mAb 1B11 against mosquito saliva, and revealed with a second antibody goat antimouse–HRP, and enhanced through chemiluminescence. SG: whole salivary glands, ML: medial lobes, DLL: distal lateral lobes, PLL: proximal lateral lobes. Three hundred whole salivary glands were separated by 2D electrophoresis, transferred on to NCM and treated with sodium periodate, labelled with biotin, and subsequently detected using streptavidin–alkaline phosphatase (SAP) and colour development. The 35 kDa protein stained with Coomassie blue in a control gel (C). Labelling indicates the presence of carbohydrate residues in the molecule (D). (E) Saliva from *An. albimanus* females was obtained by allowing mosquitoes to bite on NCM. The membranes were incubated with mAb 1B11 revealed as before or with a preimmune mouse serum (F). Each black spot corresponds to the reaction of antibodies with proteins deposited during mosquito bites on NCM.

3'RACE. The sequence of this cDNA fragment showed 32% identity with a 30 kDa salivary gland allergen of *Ae. aegypti* (GI:14423642). To obtain the complete sequence of GP35 mRNA, this fragment was used as a probe for screening an *An. albimanus* female salivary gland cDNA library, and 10 clones (GP35-631.1–10 ANOAL) with unique cDNA sequences of 888 nucleotides (nt) encoding uninterrupted open reading frames were selected from tertiary screening. The sequence of the 3'RACE fragment was exactly contained in the cDNA clones. The *An. albimanus* GP35-631.3 ANOAL cDNA clone (GP35ANOAL, accession number: DQ115976) consisted of 888 nt, 240 deduced amino acids, had an expected molecular weight of 25 957.98 Da and a theoretical pI of 4.27. A putative signal peptide was identified at the 5'-terminus of the sequence plus 61 nt of the 5'UTR; a stop codon, plus 250 nt of the 3'UTR, a polyadenylation signal, AATAAA, was also identified at the 3' end, indicating that the carboxy-terminal region of the deduced

protein was complete (Fig. 4). GP35 ANOAL contains several potential post-translational modifications predicted in its amino acids sequence (Fig. 4). Among them, two potential N-glycosylation and nine potential O-glycosylation sites, three protein kinase C (PKC), nine casein kinase II (CK2), and one tyrosine kinase (TYR) phosphorylation site. A possible cell attachment signature RGD was identified in the amino acid sequence of the protein (Barja-Fidalgo *et al.*, 2005). GP35ANOAL is rich in DEGSK (13.3/13.3/9.2/9.2/8.8%, respectively) with a net negative charge (–35): total number of negatively charged residues (D + E) is 64 and positively charged residues (R + K) are 29.

Sequence databases searching revealed that the GP35 sequence has similarities with an allergen of *Ae. aegypti* (Valenzuela *et al.*, 2002b; Peng & Simons, 1997), *Ae. albopictus* (GI:56417505), and several sequences named 30 kDa allergens and GE-rich salivary gland proteins from *An. gambiae* (Francischetti *et al.*, 2002; Arcà *et al.*, 2005), *An. stephensi* (Valenzuela *et al.*, 2003), *An. darlingi* (Calvo *et al.*, 2004), and *An. dirus* (GI:31979116), and *Cx. pipiens quinquefasciatus* (Peng & Simons, 1997; Ribeiro *et al.*, 2004), which form the 30 kDa allergen GE-rich protein family (Fig. 5A,B). All these proteins have similar molecular weight and pI, forming an acidic protein family (Table 1). Although all these proteins have in common a 30 kDa allergen domain toward their carboxy terminus (ProDom PD605055), a GE-rich module is present in the N-terminal half of only some of them (ProDom PD715399 and PD548579), suggesting different functions among the members of this family (Fig. 5C). Phylogenetic analysis (Clustal W program) of anopheline and aedine protein members grouped aedines in a distinct set; anopheline molecules were grouped in setting GP35 ANOAL very close with its homologue of *An. darlingi* (*Nyssorhynchus* subgenus). On the other hand, *An. dirus* and *An. gambiae* (*Cellia* subgenus) were very closely placed in the tree, but *An. stephensi*, another *Cellia* member, was placed in a different branch (Fig. 5A,B).

The complete sequence of a different independent GP35 clone (accession number DQ115977) showed minute differences: 886 nt in length differing only in five nucleotides (nt 87: G for T, nt 129: G for A, nt 867: A for G; nt 887 and 888 were absent). These differences in the sequence changed two amino acid residues in positions 29: G for C and 43: A for T, into the mature sequence, that did not modify the protein characteristics. These differences in the sequence could correspond to gene polymorphism among individual mosquitoes in the sample.

GP35 mRNA is expressed in salivary glands of adult females

The expression of GP35 mRNA was studied by reverse transcription–polymerase chain reaction (RT–PCR) using oligonucleotide primers corresponding to an internal region

Figure 4. Sequence of *An. albimanus* GP35 cDNA and its predicted translation product. The sequence of the cDNA encoding GP35 from *An. albimanus* is presented along with the corresponding predicted translation product. The sequence shows a signal peptide (cursive capital letters), cleavage site (vertical arrow), N-terminal sequence (bold capital letters), RGD domain (square), primer for 3'RACE (cursive small letters), forward and reverse primers for RT-PCR (underlined with arrow), possible N-glycosylation (broken underline), O-glycosylation (dotted underline), CK2 (underline), PKC (double underline), Tyr (bold underline) phosphorylation and polyadenylation (bold cursive minor letters) sites. Asterisks correspond to stop codons and initiation methionine is a cursive and bold capital 'M'.

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H E V K N E F S T R F Q L T A V T M K L 20
3  caccgaggtcaagaacgaattcagcacgagattccagctcaccggttaacaatgaagct
Y L L L V S T L G L A L I V A ↓ R P N E 40
63  taccttcttttagtaagcactctcgccctcgcatattgtggcgcccgaccacaaacgag
D T A E E T S A L G P E D G D A S D A P 60
123 gataccggccgaggaaacatccgcttttaggaccagaagacggagatgcgagtgatgctccg
R G D T P E D T T D E G D A D T S E E H 80
183 aggggagataccctgaggatactactgacgaaggagatgaggacacctctgaggagcat
D M L T S D D E V E D H G K E S E N G D 100
243 gacatgctaatacctcgatgatgaagtagaagatcatggcaaggaatccgagaatggcgat
D T A D G T D E A E S T D G E S D G S A 120
303 gatactcgccgatggaacggacgaggcagagtgcaaccgatggcgaagtgatggtatctgct
E D G A G G S G E G G K G G K E S A R N 140
363 gaagacggagcaggaggtcttgagaggggtggaagggtggcaaggaatcgccgaggaat
T Y R Q V H K L L K K V M K V D V K D K 160
423 acgtatcgccaggtgcacaagctcttgaaaggtgatgaaggtggacgttaaggacaag
Y L K S F I V A R L Q E R L M N P T I D 180
483 tacctcaagtcgttcgttgcctacaggagcgaactcatgaacctacgatcgat
L V G N I E K Y S K I K E C F S S V E K 200
543 ctggtcgccgaacatcgaaaagtactcgaagatcaaggaatgcttcagttcggtcgagaaa
D T K D L V K E S E K A Y K E C S E D K 220
603 gataccaaggacctagtcaaggagtcggaaggcttcacaagaatgctcgaggacaag
S N T S C G S E G T R E L D D G L I D L 240
663 agcaacacaagctcggttcgaaggtactagagagctcgacgacggactcatcgatttg
E Q E L S D C I V E K R D A D S E * V P 260
723 gaggcaggaaatctcggtatcggttgagaagcgtgacgccgactctgagtaggtgccc
T S V H A C Q K S * Q * R I K * N N K I 280
783 acaagcgtccatgcttgcagaagagttgacagtgaggatcaaatgaacaaataaaatc
R S A G I A N L K K K K K K K K 295
843 cggagtgccggtattgcaaatctaaaaaataaaaaaataaaaaa 888

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Table 1. Hypothetical proteins of salivary glands with identity to GP35 ANOAL

UniProt	Specie	Source (name of protein)	pl	MW Da	Score/E-value	Identity %	GenBank GI	Ref
Q45TQ1*	<i>An. albimanus</i>	GP35 salivary gland protein	4.27	25 958	-----	-----	71389018	
-----	<i>An. darlingi</i>	30K allergen	4.11	25 659	177 2e-42	75	-----	1
Q5TNZ6	<i>An. gambiae</i>	ENSANGP00000028522	4.13	26 904	670 3e-62	58	57912048	2
Q7YT44	<i>An. dirus B</i>	GE rich salivary gland protein	4.18	27 383	623 2e-57	54	31979116	3
Q7YT37	<i>An. stephensi</i>	GE rich salivary gland protein, anti-platelet aggregation protein precursor	4.05	28 504	601 5e-55	49	83016745	4
Q86M95	<i>An. stephensi</i>	GE rich salivary gland protein	4.01	28 459	585 3e-53	48	29501379	5
O01949	<i>Ae. aegypti</i>	ALL3 AEDAE, 30 kDa salivary gland allergen Aed a3 precursor	4.25	27 146	285 1e-21	31	14423642	6
Q5MIT8	<i>Ae. albopictus</i>	GE rich salivary gland protein 30k-4	4.05	27 874	275 1e-20	29	56417505	7
Q8T9T8	<i>Ae. aegypti</i>	Putative 30 kDa allergen-like protein	4.57	23 584	251 4e-18	27	18568321	8
Q6TRY5	<i>Cx. pipiens</i>	Putative salivary protein	4.61	24 369	235 2e-16	27	38350684	9
	<i>quinquefasciatus</i>							

*Accession DQ115976 and DQ115977 (GI:71389018 and GI:71389020). The computation was performed at the Swiss Institute of Bioinformatics (SIB) using the BLAST network service, Program NCBI-BLAST2 and the database UniProt were used. pl and MW were obtained by ExPASy-Compute pl/Mw tool, <http://www.expasy.org>. References and GenBank: 1. Calvo *et al.*, 2004, http://www.ncbi.nlm.nih.gov/projects/Mosquito/A_darlingi_sialome; 2. *Anopheles gambiae* str. PEST <http://www.ncbi.nlm.nih.gov>; 3. Jariyapan *et al.*, 2003, <http://www.ncbi.nlm.nih.gov>; 4. Yoshida *et al.*, 2003, <http://www.ncbi.nlm.nih.gov>; 5. Valenzuela *et al.*, 2003; 6. Xu *et al.*, 1997; 7. Arcà and Ribeiro, 2004; 8. Valenzuela *et al.*, 2002b; 9. Ribeiro *et al.*, 2003.

of the GP35 coding sequence and total RNA from eggs, first to fourth larval stages, pupae, and salivary glands of adult male, sugar-fed female, and females from 1 to 168 h (7 days) after blood feeding. A fragment of the expected size (571 bp) was observed only in adult female salivary glands (Fig. 6B,C), with an almost constant expression level even after blood intake (Fig. 6D,E). No signal was detected in larval, pupal and male preparations (Fig. 6B). A 571 bp probe was also used in a Northern blot experiment to investigate the mRNA size of GP35 in total RNA of adult female salivary glands, obtaining an mRNA of $\approx$ 900 bp (Fig. 6A).

Discussion

We describe here the complete sequence of GP35 ANOAL, an acidic glycoprotein secreted within the saliva of female *An. albimanus*. Albeit the relevance of this anopheline mosquito as a malaria vector in the Americas (Rodriguez, 2005), only few proteins have been described from the salivary glands of this mosquito, including a peroxidase/catechol oxidase (Ribeiro & Valenzuela, 1999), an anti-thrombin peptide, named anophelin (Valenzuela *et al.*, 1999; Francischetti *et al.*, 1999), and a glycoprotein of unknown function, GP65-1 (Montero-Solís *et al.*, 2004).

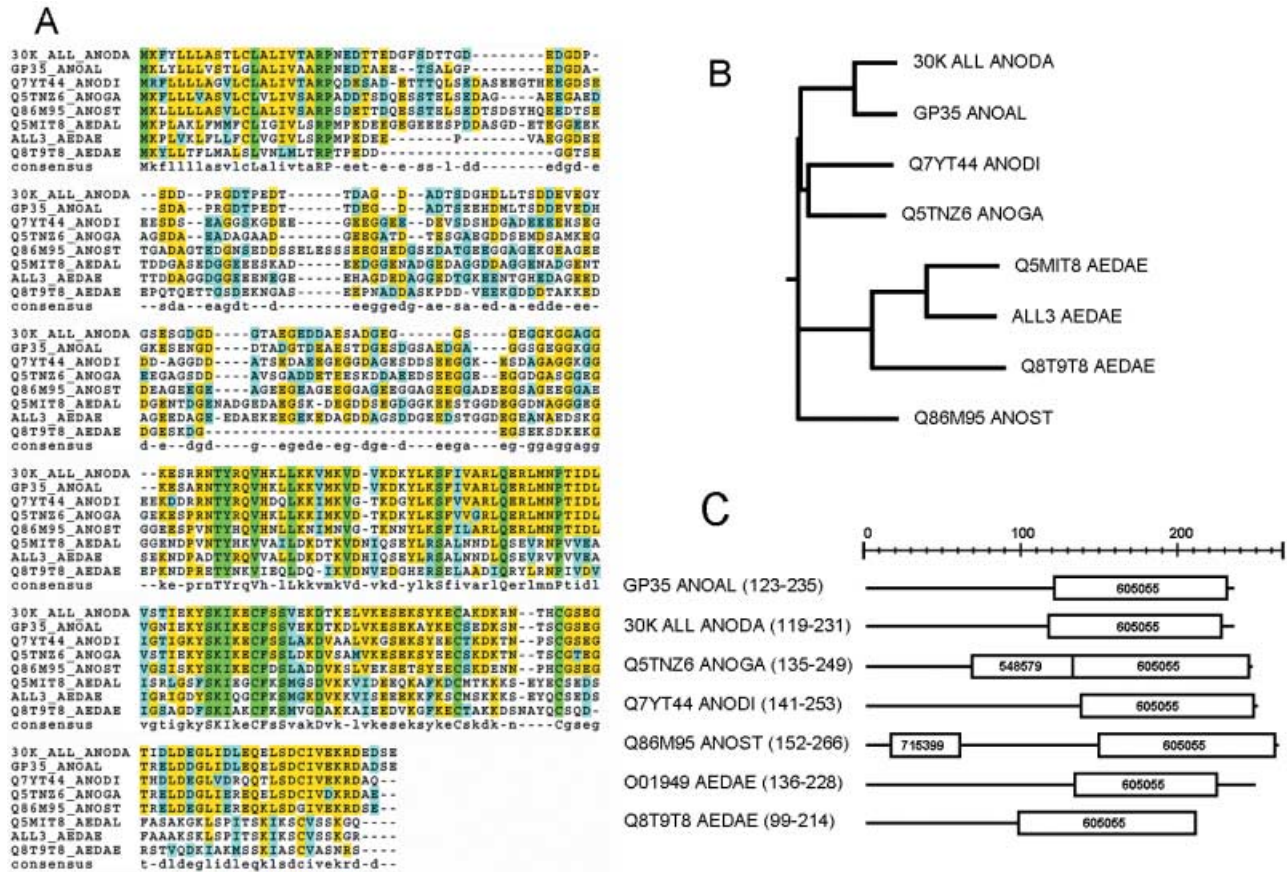


Figure 5. Comparison of GP35 ANOAL with other salivary gland molecules of culicines. (A) Deduced amino acid sequences of salivary molecules of different mosquitoes were BLAST searched and recovered sequences were aligned using ClustalW program and a relation tree was constructed (B). Sequences identification numbers correspond to those described in Table 1. (C) Sequences were analysed for ProDom database registered domains. 605055 correspond to a 30 kDa allergen domain and 715399 and 548579 to GE-rich domains. Amino acid residues shaded yellow correspond to identical residues, green to completely conserved residues, and cyan to similar residues.

Recent studies on the transcriptome and proteome of salivary glands of several mosquito species, including *An. gambiae* (Francischetti *et al.*, 2002; Arcà *et al.*, 2005), *An. stephensi* (Valenzuela *et al.*, 2003), *An. darlingi* (Calvo *et al.*, 2004), *Ae. aegypti* (Valenzuela *et al.*, 2002b), *Ae. albopictus* (GI:56417505), and *Cx. pipiens quinquefasciatus* (Ribeiro *et al.*, 2004), led to the primary sequence of many putatively secreted salivary proteins. These studies have predicted about 70 proteins that are putatively secreted in the saliva, but the function of less than 10 of these proteins has been characterized at the protein level (Ribeiro & Valenzuela, 2003; Arcà *et al.*, 2005).

The cDNA GP35 ANOAL predicted a protein of 240 amino acids. The full coding sequence showed the presence of a signal peptide, which agrees with our experimental observation that GP35 ANOAL is a secretion protein. The protein depicts a pI (4.45) and an apparent M_r ($\approx 35\,000$ Da) that differ from those predicted through cDNA sequence analysis (pI of 4.27 and M_r of 25957.98), suggesting post-translational modifications. It has been demonstrated that the acidic

character adds stability, solubility and protease resistance to proteins (Li *et al.*, 1999). These GP35 ANOAL characteristics (amino acid sequence and acidic characteristics) are common to the 30 kDa salivary allergen and GE-rich protein groups that probably form a single family. Both groups bear in the C-terminal half a 30 kDa salivary gland allergen motif (ProDom database PD605055) but GE-rich proteins have, in addition, a long region of low complexity, consisting mainly of G and E residues towards the N-terminal half (ProDom PD715399 and PD548579). The first member of this family was described as an allergen in *Ae. aegypti* (Brummer-Korvenkontio *et al.*, 1997; Simons & Peng, 2001) and later found in all other mosquito sialotranscriptomes so far constructed, including four *Anopheles* species (Francischetti *et al.*, 2002; Valenzuela *et al.*, 2002a,b, 2003; Calvo *et al.*, 2004; Arcà *et al.*, 2005; data available at <http://www.ncbi.nlm.nih.gov/projects/omes/#Ad1>) and *Cx. pipiens quinquefasciatus* (Ribeiro *et al.*, 2004). In addition, we have identified a protein with similar molecular weight, pI, and stain characteristics in *An. pseudopunctipennis*

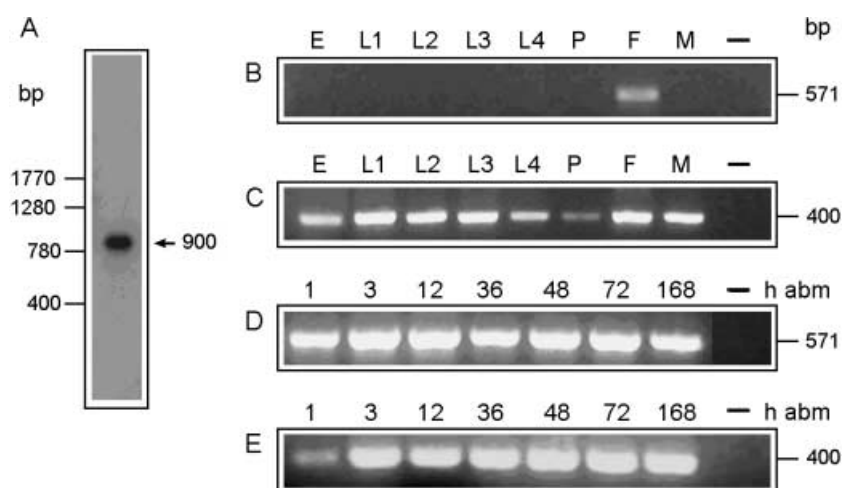


Figure 6. Size of mRNA and expression profile of GP35 during mosquito life cycle. A Northern blot hybridization was carried out using radiolabelled α -[^{32} P]dATP-GP35 specific probe on complete adult female salivary gland total RNA, and RT-PCR using total mRNA from larval to adult stages salivary glands and specific oligonucleotide primers conducted. (A) Northern blot hybridization, a band of approximately 900 bp is detected. (B–E), RT-PCR expression analysis of: (B) GP35 during mosquito development, a band of approximately 571 bp is shown. (D) GP35 at different hours after blood meal (h abm), the same band of 571 bp is shown. (C,E) A band of 400 bp corresponding to an actin fragment was used as constant expression gene during life cycle stages and after blood ingestion, respectively. (E) Eggs; L1–L4 indicate larva instars salivary glands; P, pupa salivary glands; (F) adult female salivary glands; M, adult male salivary glands; –, control without RNA.

(data not shown). These data taken together indicate that this family is possibly common to the culicidae group.

Mosquito salivary proteins have been proposed as robust markers for closely related species, as they seem to be rapidly evolving, compared with housekeeping proteins (Valenzuela *et al.*, 2003). The molecular structure of the GE-rich family and 30 kDa proteins has two distinct regions, one more conserved in their C-terminus region among all family members, as compared with N-terminus region that presents lesser similarity mainly due to differences in the contents of Gly (G) and Glu (E) residues. Accordingly, the C-terminal region could reflect the phylogenetic relationship among member proteins, which is more evident in related species and groups, as happens for GP35 ANOAL with its homologue in *An. darlingi* (Calvo *et al.*, 2004) both belonging to the *Nyssorynchus* subgenus. However, members of *Cellia* subgenus, *An. gambiae* (GI:57912048) and *An. dirus* (Valenzuela *et al.*, 2003) were grouped together, and *An. stephensi* was placed in a different branch reflecting the high complexity of this subgenus, and indicating that the value of this molecule as a taxonomic marker should be investigated further.

The 30 kDa salivary gland protein of *Ae. aegypti* (GI:14423642; PD605055) is an active inducer of allergic response (Simons & Peng, 2001; Peng & Simons, 2004), and shares high similitude with GP35 ANOAL. Although the precise participation of the 30 kDa allergen domain is currently unknown, molecules of this family induce IgE production in subjects allergic to mosquito bites (Peng & Simons, 1997; Peng *et al.*, 2001; Palosuo *et al.*, 1997; Brummer-Korvenkontio *et al.*, 1997; Valenzuela *et al.*,

2003). These allergens contain several possible phosphorylation and N-glycosylation sites, which may play important roles in the induction of allergic responses (Wal, 2001; Malandain, 2005).

The GP35 ANOAL is glycosylated and binds to concanavalin A, indicating the presence of N-glycans with mannose type residues. Putative O-glycosylation sites occur in all identified members of the 30 kDa allergen family; most of these sites are located on the amino half of the molecules (predictions of glycosylation sites were conducted using: NetOGlyc 3.1 server, Julenius *et al.*, 2005; and NetNglyc 1.0 server, Gupta *et al.*, 2004, <http://www.cbs.dtu.dk/services>) (data not shown). Aside from the N-glycosylation sites documented here for GP35 ANOAL, only the *An. gambiae* homologue has a putative one; these sites are located on the carboxy terminus region in both molecules. These differences are in line with a diversification of these molecules, in which the C-terminus structure is conserved, and a more diverse N-terminus may confer different functions (Francischetti *et al.*, 2002).

Like the homologue produced by *An. gambiae* females (Arcà *et al.*, 2005), GP35 ANOAL is one of the most abundant proteins produced in salivary glands of *An. albimanus*, its production in other mosquitoes has not been assessed, but, at least in anophelines, it is likely to be similar.

The *An. gambiae* homologue is selectively produced by female salivary glands, but its final destination in the saliva was only speculated, based on its signal peptide sequences (Arcà *et al.*, 2005). We documented here that GP35 ANOAL is secreted in *An. albimanus* saliva, and that it is produced only in the distal lateral lobes, specialized

gland regions of the adult female mosquito salivary glands that are associated with blood feeding (Stark & James, 1996; Moreira-Ferro *et al.*, 1999).

Protein production begins around the time of mosquito emergence; although during the pupal stage neither the messenger for GP35 ANOAL nor the protein (not shown) were detected, but in recently emerged females small amounts of the protein were observed, indicating that production begins closely before emergence, increased during mosquito maturation, before the first blood feeding, and then remained unchanged during the whole adult life. This is similar to the production of other salivary proteins of *An. albimanus*, such as GP65-1 (Montero-Solís *et al.*, 2004) and GP55 (Pérez-Bonilla *et al.* in preparation), that are injected with the saliva during blood ingestion. The production of GP35 ANOAL is constant as has been described for proteins of salivary glands of *An. stephensi* (Poehling, 1979) and *An. darlingi* (Moreira *et al.*, 2001), as well as for the synthesis of messengers of apyrase of *An. darlingi* (Moreira *et al.*, 2001) and apyrase and D7 of *An. gambiae* (Nirmala *et al.*, 2005), which remain unchanged after blood feeding.

An RGD signature was identified by sequence analysis of GP35 ANOAL, which could confer the ability to bind to integrins (Williams *et al.*, 1990) and inhibit molecular interactions dependent on the adhesive functions of these proteins on cell surfaces (Marcinkiewicz *et al.*, 1999; Zhou *et al.*, 2000), including inhibition of platelet aggregation (Gasmi *et al.*, 2001). Nevertheless, disintegrin signature based on RGD tripeptide could be unspecific and a platelet inhibitor function for GP35 ANOAL remains a hypothesis. Supporting the importance of RGD or other potential integrin-binding motifs (Barja-Fidalgo *et al.*, 2005) is the fact that they are also observed in homologous proteins: RGD in *An. darlingi* (30 kDa allergen ANODA) and KGD in *An. dirus* (GI:31979116), and VGD in *Ae. aegypti* (GI:18568321) and *Ae. albopictus* (GI:56417505).

Another argument supporting the possible antiplatelet role of GP35 ANOAL is its similarity with a molecule proposed as the anopheline antiplatelet protein (AAPP), another possible member of the 30 kDa-allergen family, and a possible platelet inhibitor from the saliva of *An. stephensi* (GenBank accession number BAE53440; Yoshida & Watanabe, 2006): our current hypothesis is that GP35 ANOAL could have an antiplatelet function.

The diversity in the N-terminus section among the proteins of the family indicates that they could have different functions, including those already attributed to other molecules in mosquito saliva: inhibitors of haemostasis or antibacterial molecules (Korayem *et al.*, 2004). These and other possible interactions of these abundant molecules with *Plasmodium* parasites within the vector-mosquito's salivary gland contents and in the wound, produced during blood feeding, warrant further research.

Experimental procedures

Mosquitoes

White stripe strain *Anopheles albimanus* mosquitoes (Chan *et al.*, 1994) were reared at the insectary of the Centro de Investigación de Paludismo, INSP, Tapachula, Chiapas at 27 °C and 87% relative humidity. Adult mosquitoes were routinely maintained on a 10% sucrose solution and, when required, blood fed on rabbits.

Salivary gland dissection

Mosquitoes were cold anaesthetized, and their salivary glands were dissected in phosphate-buffered saline, pH 7.2 (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) as described (Andrews *et al.*, 1997; Montero-Solís *et al.*, 2004). To separate the different salivary gland regions, the medial lobe was cut at its junction with the lateral lobes and removed. The lateral lobes were cut at the intermediate section to separate the proximal and distal regions. Immediately after dissection, whole salivary glands and the separated lobes were dissolved in loading buffer for either one- or 2D electrophoresis.

Uni-dimensional and two-dimensional gel electrophoresis

Complete salivary glands or separated lobes were dissected in PBS, pH 7.2, supplemented with a protease inhibitors cocktail [100 µM tosyl lysyl chloromethyl ketone (TLCK), 100 µM tosyl phenylalanyl chloromethyl ketone (TPCK), 100 µM leupeptine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 µM pepstatin, 10 mM ortho-phenanthroline] (North, 1989) and immediately placed in sample buffer. Twelve per cent sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE, 1D) was performed using 10 salivary gland pairs and the equivalent of distal and proximal lateral and medial lobes. The samples were placed in SDS sample buffer (0.125 M Tris base, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) (Laemmli, 1970). Two-dimensional electrophoresis was performed according to O'Farrell (1975). Immediately after dissection, 200 (or as indicated) salivary gland pairs were placed in lysis buffer [9.5 M ultrapure urea, 2% NP-40 (v/v), 2% ampholytes (v/v) pH 5–7/3–10 (2 : 1 relation) (Bio-lyte Ampholyte, Bio-Rad, Hercules, CA, USA), 5% 2-mercaptoethanol (v/v), in H₂O], and stored at –75 °C until used. Salivary gland samples were centrifuged at 18 000 × g for 10 min at 4 °C, and the supernatant used to load first dimension gels. The second dimension was run in 10% SDS–PAGE. Gels were mono- or polychromatic silver (Wray *et al.*, 1981; Nielsen & Brown, 1984) or Coomassie Brilliant Blue (CB) stained, or transferred on to NCM (Hybond ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA) for immunoblot analysis (Towbin *et al.*, 1979).

Antibody production

A mAb 1B11:lgG1 was selected from a panel of mAbs produced against an extract of female *An. albimanus* salivary glands using a mouse hybridoma line SP2/O (González-Cerón, unpublished).

Western blot analysis

Ten female *An. albimanus* salivary gland pairs and the equivalent of distal and proximal lateral and medial lobes were separated by SDS–PAGE. Gel was silver-stained and a replica gel was electro-transferred onto NCM and reacted with mAb 1B11:lgG1 diluted

1:2000 during 3 h at room temperature (Towbin *et al.*, 1979), followed by a goat antimouse, peroxidase conjugated antibody (Pierce, Rockford, IL, USA). The blots were developed by enhanced chemiluminescence peroxidase following manufacturer's protocol (ECL Western Blotting Analysis System, Amersham, Piscataway, NJ, USA) (Rosewell & White, 1978) and exposed to Kodak XK1 X-ray films.

Saliva from female *An. albimanus* was also obtained by allowing mosquitoes to bite on an NCM previously wet with 10 mM sodium bicarbonate (Billingsley *et al.*, 1991). The membranes were blocked with 5% non-fat dried milk-PBS for 60 min at room temperature, and incubated with the mAb 1B1:lgG1 for 3 h at room temperature. Blots were incubated with goat antimouse, peroxidase conjugated and developed as above.

Glycosylation analysis

Protein extracts of 300 female *An. albimanus* salivary gland pairs were resolved by 2D electrophoresis, transferred onto NCM, and subjected to carbohydrate oxidation to label them with biotin, and subsequent detection using streptavidin-alkaline phosphatase and colour development reagents with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate dipotassium/nitrotetrazolium blue chloride). This procedure was carried out using an Immun-Blot Kit for Glycoprotein Detection (Bio-Rad), following the supplier's instructions.

N-terminal sequencing

To sequence the N-end of the negatively stained 35 kDa spot, 300 female *An. albimanus* salivary gland pairs were run in a 2D gel, and transferred onto polyvinylidene difluoride membrane (PVDF) in CAPS (3-[cyclohexylamine]-1-propane-sulphonic acid) buffer (Matsudaira, 1997). The 35 kDa spot was visualized on the PVDF membrane stained with Ponceau red, cut, and N-end sequenced by Edman degradation procedure in a Beckman LF3000 protein sequencer. Based on N-terminal sequence of GP35 (NEDTAE) and the codon usage for *An. albimanus* (San Diego Super Computer, SDSC, <http://www.sdsc.edu>), a specific primer was designed (5'-AACGAGGATACNGCNGAGGAG-3'; GP35 intern specific primer) that was used to anchor a specific RT-PCR amplification following the rapid amplification from cDNA end 3' (3'RACE), as described below.

Amplification and cloning of GP35 cDNA

Approximately 600 ng of total RNA was isolated from 100 pairs of female *An. albimanus* salivary glands using the Trizol reagent following supplier's instructions (Gibco BRL Life Technologies, Carlsbad, CA, USA).

This RNA was used as a template to amplify, by 3'RACE (3'RACE System, Gibco BRL, Life Technologies) a cDNA fragment between the intern specific primer and the 3'-end (Frohman *et al.*, 1988). The amplification conditions were: 94 °C for 2 min; 94 °C for 45 s, 52 °C for 50 s, 72 °C for 1 min 30 s; 72 °C for 7 min. Thirty-five cycles were conducted. The amplified product (GP35ANOAL/3R) was cloned using the AdvanTAGE PCR cloning system (Clontech, Palo Alto, CA, USA) and sequenced by the Big Dye System (ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v2.0, Perkin Elmer Biosystems, Foster City, CA, USA) and an ABI Prism DNA sequencer (ABI PRISM 377 DNA Sequencer, Perkin Elmer Biosystems).

Screening of a salivary gland cDNA library

To obtain the complete cDNA sequence of GP35, a female *An. albimanus* salivary gland cDNA library constructed in the λ ZAP Express vector (Stratagene Ltd, La Jolla, CA, USA) (Montero-Solis *et al.*, 2004) was screened. To obtain a specific probe the fragment GP35 ANOAL/3R was radioactively labelled with α -[³²P]dATP using the Random Primed DNA labelling Kit (Boehringer Mannheim Biochemica, Germany) (Feinberg & Vogelstein, 1983). For the primary screening, about 6×10^5 plaque forming units (pfu) were transferred from the agar plate to duplicate nylon membranes (Hybond-N⁺, Amersham Pharmacia Biotech Ltd, UK). Membranes were hybridized with α -[³²P]dATP-GP35ANOAL/3R as previously described (Sambrook *et al.*, 1989). Ten positive clones from a third screening were selected. The *in vivo* excision of these clones was performed as indicated by the supplier (Stratagene Ltd) and sequenced.

Northern blot and reverse transcription-polymerase chain reaction analysis

A Northern blot assay was performed using about 3 μ g of total RNA of salivary glands as described (Fourney *et al.*, 1988) and the α -[³²P]dATP – GP35 probe (571–GP35 probe). The RT-PCR analysis was carried out with about 900 ng of DNase-treated total RNA (DNaseI–RNase free, Gibco BRL Life Technologies), isolated from *An. albimanus* eggs, first to fourth instar larvae, pupae, and female and male adult salivary glands, as well as from blood-fed females at different times after blood ingestion. These total RNAs were isolated with Trizol, as before. First-strand cDNA synthesis was performed using an adapter primer and the SuperScript II, RNase H⁻ (Gibco BRL Life Technologies). After the reverse transcription step (42 °C for 50 min), heat inactivation of RT (70 °C for 20 min) and subsequent elimination of DNA contaminant with DNaseI–RNase free, 30 amplification cycles were performed as follows: 95 °C, 5 min once; 95 °C, 30 s; 67 °C, 45 s; 72 °C, 1.5 min; and an end extension at 72 °C for 5 min, using the specific primers GP35 sense (5'-GCGAGTGATGCTCCGAGGGG-3', positions 168–187) and GP35 anti-sense (5'-CGATTTGGAGCAGGAAC-TATCGG-3', positions 716–739), to obtain a fragment of 571 bp (571–GP35). Actin amplification was performed as expression control using specific primers: sense 5'-ACATGGAGAAAATCT-GGCACCACA-3' and anti-sense 5'-ACAGCTTTTCTTTGAT-GTCGCGAA-3', to obtain a fragment of $\approx$ 400 bp. Amplification conditions were: 95 °C, 5 min, once; cycles 95 °C, 30 s; 50 °C, 45 s; 72 °C, 1.5 min, 30 times, and an end extension at 72 °C for 5 min. To determine if the amplification products obtained corresponded to the GP35 and actin genes, both fragments were cloned at the pCR2.1® vector (TA cloning® Kit, Invitrogen Life Technologies, Carlsbad, CA, USA) and sequenced.

In silico analysis

GP35 sequence analysis and comparisons were performed using BLASTN and BLASTX programs (Altschul *et al.*, 1997) from the National Center Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>). Protein sequence alignments and relations were determined using the CLUSTAL W program (Multiple Sequence Alignment) (Higgins *et al.*, 1992; Thompson *et al.*, 1994; Felsenstein, 1988) available at Workbench SDSC site (San Diego, CA) (<http://seqtool.sdsc.edu>) (<http://workbench.sdsc.edu>). Predictions of signal peptide (Bendtsen *et al.*, 2004), subcellular localization

(Emmanuelsson *et al.*, 2000), cleavage site (Nielsen *et al.*, 1997), and post-translational modifications (PTMs), as well as N-glycosylation (Blom *et al.*, 2004), O-GalNAc (mucin type) glycosylation (Hansen *et al.*, 1998; Julenius *et al.*, 2005), and determination of serine, threonine and tyrosine phosphorylation sites (Blom *et al.*, 1999) of GP35, from its amino acid sequence, were carried out using programs of CBS Prediction Servers (<http://www.cbs.dtu.dk/services>) of the Technical University of Denmark DTU. Other PTMs, such as CK2 PHOSPHO SITE *Casein kinase II phosphorylation*, PKC PHOSPHO SITE *Protein kinase C phosphorylation*, TYR PHOSPHO SITE *Tyrosine kinase phosphorylation*, ASN GLYCOSYLATION *N-glycosylation sites* were carried out using ScanProsite program (<http://www.expasy.org/cgi-bin/prosite/ScanView.cgi>). Databases of protein families, domains, and functional sites were consulted using InterProScan server (<http://www.ebi.ac.uk/InterProScan>) (Mulder *et al.*, 2003) and ProDom (<http://prodrom.prabi.fr/prodom/current/html/home.php>).

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