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Biochemical and Biophysical Research Communications 327 (2005) 29–34
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Molecular identification of a myosuppressin receptor from the malaria mosquito *Anopheles gambiae*☆

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Received 23 November 2004
Available online 8 December 2004

Abstract

The insect myosuppressins ($X_1DVX_2HX_3FLRFamide$) are neuropeptides that generally block insect muscle activities. We have used the genomic sequence information from the malaria mosquito *Anopheles gambiae* Genome Project to clone a G protein-coupled receptor that was closely related to the two previously cloned and characterized myosuppressin receptors from *Drosophila* [Proc. Natl. Acad. Sci. USA 100 (2003) 9808]. The mosquito receptor cDNA was expressed in Chinese hamster ovary cells and was found to be activated by low concentrations of *Anopheles* myosuppressin (TDVDHVFLRFamide; EC_{50}, 1.6×10^{-8} M). The receptor was not activated by a library of 35 other insect neuropeptides and monoamines, including neuropeptides that resembled myosuppressin in their C-terminal moiety, such as PDRNFLRFamide (*Anopheles* FMRFamide-3), other *Anopheles* FMRFamide peptides, or neuropeptide F-like peptides, showing that the receptor was quite selective for myosuppressin. These results also showed that the myosuppressin receptor needs a much larger portion than the C-terminal FLRFamide sequence for its activation. The insect myosuppressins are often grouped together with the insect FMRFamides under the name FaRPs (FMRFamide-related peptides). However, this is not justified anymore, because the insect myosuppressin receptor/ligand couple is both functionally and evolutionarily fully unrelated to the insect FMRFamide receptor/ligand couple. To our knowledge, this is the first report on the molecular identification of a mosquito neuropeptide receptor.

Keywords: G protein-coupled receptor; Neuropeptide; FMRFamide; Myosuppressin; Leucomyosuppressin; *Anopheles*; Mosquito; Malaria

The insect myosuppressins are neuropeptides with the C-terminal sequence X_1DVX_2 HX_3 FLRFamide (where X_1 is pQ, P, T; X_2 is D, G, V; and X_3 is V, S) [1]. The insect myosuppressins obtained their name because they inhibit most insect visceral muscles, including those that are involved in the passage of food along the insect alimentary canal [1–5]. The myosuppressins have been isolated from a variety of insects and it can be assumed that they occur in all insect species [1–4,6–10].

The C-terminal four amino acid moiety of the insect myosuppressins (FLRFamide) resembles that of the FMRFamide neuropeptides. However, the actions of myosuppressins are often different from those of the insect FMRFamides [1,11] and also their preprohormones have a quite different organization (one immature myosuppressin sequence is contained in the myosuppressin preprohormones, while typically a large number of FMRFamide sequences—up to 24—are contained in the insect FMRFamide preprohormones) [12,13], suggesting that the two neuropeptide genes are not evolutionarily related.

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank Data Bank with Accession No. AY 345586.

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.11.139

The FMRFamide preprohormone from the malaria mosquito *Anopheles gambiae* contains a neuropeptide sequence, PDRNFLRFamide (*Anopheles* FMRFamide-3), that resembles a myosuppressin (Cazzamali et al., in preparation). *Anopheles* FMRFamide-3 acts as a genuine FMRFamide on the *Anopheles* FMRFamide receptor (Cazzamali et al., in preparation). It would be important to see whether *Anopheles* FMRFamide-3 also has myosuppressin properties, which would intermingle the insect FMRFamide and myosuppressin systems. This, however, would require the cloning of the *Anopheles* myosuppressin receptor. We have previously cloned and identified (''deorphanized'') the first two insect myosuppressin receptors from the fruitfly *Drosophila melanogaster* [14]. Here, we describe the cloning and functional characterization of a myosuppressin receptor from *Anopheles*. This is, to our knowledge, the first report on the molecular identification of a mosquito neuropeptide receptor.

Materials and methods

Total RNA was isolated from adult *A. gambiae* (strain KWA, kindly supplied by Drs. N. Hill and P. Aiyenuro, London School of Hygiene and Tropical Medicine, UK) using TRIzol Reagent (Life Technologies) and treated with DNAse using the DNA-free kit (Ambion). cDNA was synthesized using the SMART RACE cDNA Amplification Kit (Clontech). For 3′ RACE, the sense primer 5′-CGTGATGGACGTGCTGGCGTTGGTCAA-3′ and the nested sense primer 5′-GGACCGGTGGATGGCGGTGCCG-3′ (corresponding to positions 1080–1108 and 1190–1212 of Fig. 1) were used. For 5′ RACE, the antisense primer 5′-GCTGCCAAAGATGCAC ACCAGCAGGCAG-3′ followed by the nested antisense primer 5′-GGCCCCATCCTCATCGCCTGCCAG (corresponding to positions 174–201 and 57–81 of Fig. 1) were used. The PCR program was 94 °C for 3 min, then 20 cycles touchdown, 94 °C for 30 s, 68 °C for 45 s, decreasing 0.5 °C per cycle, 72 °C for 3 min, followed by 25 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 3 min.

The coding region was amplified using the sense primer 5′-CACCATGAGCTCGACGGAATTGG-3′ and the antisense primer 5′-GGACGTCCCGGCGTGCGCTA-3′ (the underlined nucleotides correspond to positions 1–19 and 1281–1301 of Fig. 1), cloned into pCR4-TOPO (Invitrogen), sequenced, and cloned into the pIRES-hrGFP-1a/Neo-module (Stratagene), using *Eco*RI. Cell culture, cell transfection, and the bioluminescence assay were performed as in [14–17].

DNA sequence comparisons were carried out using the Lasergene software package (DNASTAR). ClustalW was used for protein sequence alignments (www.npsa-pbil.ibcp.fr).

Results

We have previously identified two myosuppressin receptors from *Drosophila* [14]. Blasting of their sequences against the genomic database sequences from the malaria mosquito (*A. gambiae*) Genome Project (http://www.ncbi.nlm.nih.gov/BLAST/Genome/Fly-Blast.html) revealed the sequence of a putative mosquito myosuppressin receptor. To obtain the cDNA, encoding this receptor, we carried out PCR, and 5′ and 3′RACE PCR, using primers directed against the exons of the putative myosuppressin receptor gene, and cDNA from adult *A. gambiae* as a template.

The receptor cDNA contains a polyadenylation signal and a poly(A)$^+$ tail. The start codon is preceded by several in-frame stop codons (Fig. 1). The cDNA codes for a protein of 427 amino acid residues, containing seven transmembrane helices, which is characteristic for G protein-coupled receptors (Fig. 1).

A comparison of the cDNA of Fig. 1 with the genomic sequence of *A. gambiae* revealed a small number of nucleotide differences, which, however, did not result in amino acid residue differences (Table 1). This comparison also showed the presence of two introns in the receptor gene (Table 2).

An alignment of the mosquito receptor with the two *Drosophila* myosuppressin receptors showed 47–48% amino acid residue identity (52–53% in the transmembrane region) and 57–58% similar residues (63–64% in the transmembrane region) (Fig. 2). Furthermore, all three receptors had two introns with the same intron phasings in common, showing that the three receptor genes are evolutionarily closely related (Fig. 2; Table 2).

We stably expressed the coding region of the mosquito receptor gene (Fig. 1) in Chinese hamster ovary (CHO) cells and established clonal cell lines that expressed the receptor efficiently. These cells also stably expressed the promiscuous G protein, G-16 [15]. Two days before the bioassay, we transiently transfected the cells with DNA, coding for apoaequorin and 3 h before the assay, we added coelenterazine to the cell culture medium. An activation of G-16-coupled receptors in these pretreated cells, would lead to an IP_3/Ca^{2+}-mediated bioluminescence response, which could easily be measured and quantified [14–17].

We tested a library of 35 insect neuropeptides and biogenic amines on the transfected cells. Low concentrations of *Drosophila* myosuppressin (TDVDHVFLR Famide) activated the receptor (EC_{50}, 1.6×10^{-8} M) (Fig. 3), whereas the other neuropeptides and neurohormones, including those that resembled *Drosophila* myosuppressin in their C-terminal moieties, such as the *Anopheles* FMRFamides (we tested SALDKNFM RFamide, PDRNFLRFamide, STGSGYMRFamide, and AGNLMRFamide), short neuropeptide F-1 (AQ RSPSLRLRFamide), and perisulfakinin [EQFDDY (SO_3H)GHMRFamide] [1,11,18], did not give a response (all tested up to 10^{-5} M). This showed that the receptor is specific for myosuppressin.

Searching of the genomic database from *A. gambiae* (http://www.ncbi.nlm.nih.gov/BLAST/Genome/Fly-Blast.html) revealed only one gene coding for a myosuppressin preprohormone, which is in agreement with

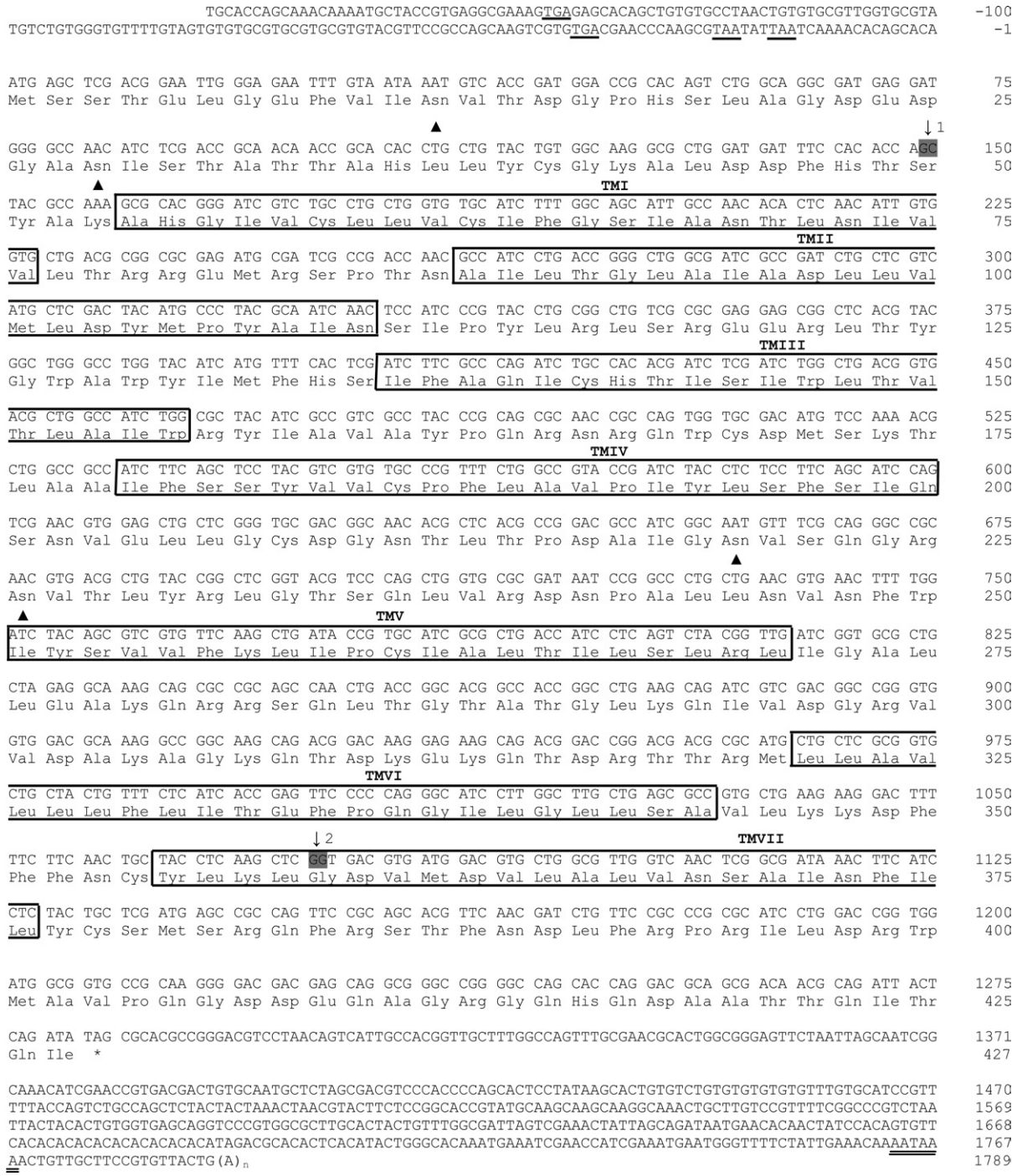

Fig. 1. cDNA and deduced amino acid sequence of the receptor gene. The nucleotides are numbered from the 5′ to 3′ end and the amino acid residues are numbered, starting with the first ATG (start) codon in the open reading frame. The introns are marked by arrows and numbered 1 and 2. The two nucleotides, bordering each intron, are highlighted in grey. The stop codons in the 5′ untranslated region are underlined. The putative polyadenylation signal in the 3′ untranslated region is underlined twice. The translation termination codon is marked by an asterisk. The seven transmembrane helices of the receptor protein are boxed and marked TM I-VII. Four potential glycosylation sites, obeying the NXT/S consensus sequence, are marked by filled triangles.

previous findings [18]. The myosuppressin contained in this preprohormone is identical to *Drosophila* myosuppressin. TDVDHVFLRFamide, therefore, is the probable intrinsic ligand for the mosquito myosuppressin receptor.

Blasting of the genomic database from *A. gambiae* with the three receptor sequences from Fig. 2 revealed only one receptor gene with significant amino acid sequence and intron position identities. *A. gambiae*, therefore, appears to have only one myosuppressin receptor.

Table 1
Nucleotide differences between the receptor cDNA and the corresponding genomic sequences from the *A. gambiae* Genome Project

Position of the nucleotide in the cDNA	Type of nucleotide in the gene	Type of nucleotide in the cDNA	Change in amino acid
120	C	T	—
165	T	C	—
213	G	A	—
349	T	C	—
546	G	C	—
564	C	T	—
823	T	C	—

Table 2
Intron/exon boundaries of the receptor gene

Intron	5′donor	Size (bp)	3′acceptor	Intron phase
1	AG gtaagca....	3290	.... atttcag C	2
	Ser		Ser	
2	G gtaagtg....	4435	.... gtttcag GT	1
	Gly		Gly	

Discussion

The insect myosuppressins ($X_1DVX_2HX_3FLRF$ amide) have a C-terminal moiety that resembles those of other insect neuropeptides, such as the insect FMRFamides. Especially the *Anopheles* FMRFamide family has a peptide member, PDRNFLRFamide (*Anopheles* FMRFamide-3), that has five amino acid residues, including the whole C-terminal FLRFamide moiety, in common with the intrinsic *Anopheles* myosuppressin (TDVDHVFLRFamide) [18]. Nevertheless, this FMRFamide was not able to activate the mosquito myosuppressin receptor, clearly illustrating that the mosquito myosuppressin receptor needs a longer peptide sequence for peptide recognition and activation, and perhaps even the whole TDVDHVFLRFamide sequence of *Anopheles* myosuppressin. A similar observation has previously been made for the two *Drosophila* myosuppressin receptors [14]. The *Drosophila* FMRFamide receptor, in contrast, appeared to be less selective and high concentrations of myosuppressin and short neuropeptide F-1 (10^{-6} M or ca. 100× higher concentrations than those of the FMRFamides) were able to activate the receptor [17]. Again, a similar observation has

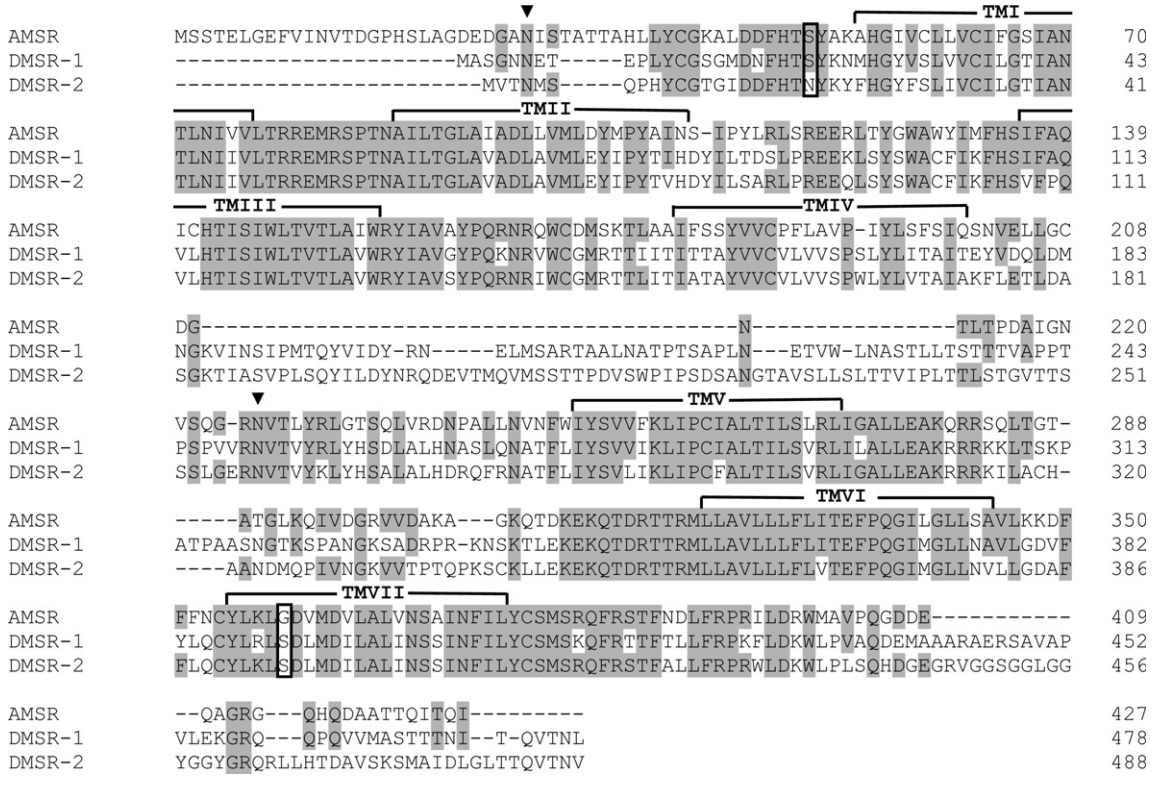

Fig. 2. Amino acid comparisons between the *Anopheles gambiae* myosuppressin-like receptor (AMSR) and the two *Drosophila* myosuppressin receptors (DMSR-1 and -2) published previously [14]. Amino acid residues that are identical between the mosquito and at least one of the *Drosophila* receptors are highlighted in grey. The seven transmembrane helices are indicated by TM I–VII. The positions of the two common introns are indicated by vertical boxes. The filled triangle indicates the glycosylation sites common for all three receptors. Gaps are introduced to optimize the alignments.

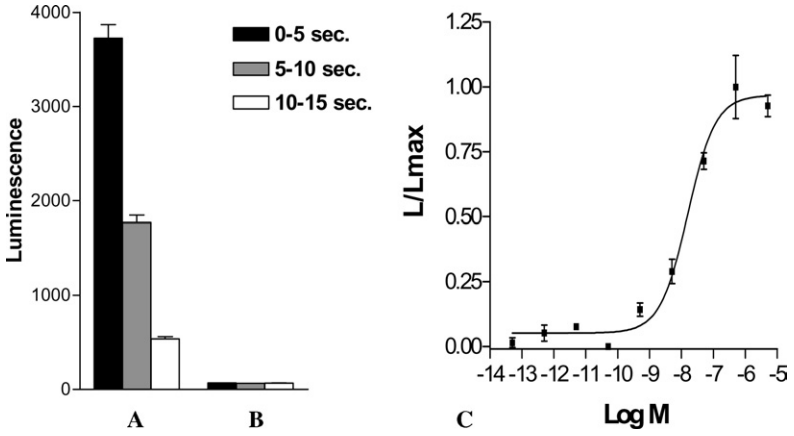

Fig. 3. Functional expression of the mosquito receptor gene. CHO cells, expressing G-16 and apoaequorin (CHO/G-16); or CHO cells, expressing G-16, apoaequorin and the receptor cDNA (CHO/G-16/AMSR), were compared. The vertical bars indicate SEMs, which sometimes are smaller than the symbols used. In these cases only the symbols are given. (A) Strong bioluminescence responses 0–5, 5–10, and 10–15 s. after addition of 5×10^{-7} M *Anopheles* myosuppressin to CHO/G-16/AMSR cells. (B) Lack of bioluminescence responses 0–5, 5–10, and 10–15 s. after the addition of 5×10^{-7} M *Anopheles* myosuppressin to CHO/G-16 cells. (C) Dose–response curve of the effects measured in (A). About 35 insect neuropeptides and biogenic amines did not activate the receptor. For a list, see [14] and the peptides mentioned in the text. The biogenic amines were octopamine, tyramine, L-dopa, dopamine, adrenaline, noradrenaline, histamine, and serotonin (all tested at 10^{-5} or 10^{-4} M).

been made for the *A. gambiae* FMRFamide receptor (Cazzamali et al., in preparation). These observations in two insect species, then, suggest that the insect FMRFamide receptor is less specific and that it only needs smaller portions of the peptide sequence for activation, whereas the insect myosuppressin receptor clearly is quite selective for myosuppressins.

The insect myosuppressin receptors are not evolutionarily related to the insect FMRFamide receptors (there are no significant sequence identities). Furthermore, as explained in the Introduction, the insect myosuppressin preprohormones are not evolutionarily related to the insect FMRFamide preprohormones [1,11]. The myosuppressin and FMRFamide receptor/ligand couples, therefore, are two separate systems that have originated independently during evolution. This is important to mention, because the two peptide systems have often been grouped together (as the FaRPs or FMRFamide-related peptides [1,11]). To avoid any further confusion, however, one should abandon the term "FaRPs". Furthermore, the insect myosuppressins should not be named FLRFamides (as has often been done [1,11]), since *Anopheles* FMRFamide-3 (PDRN FLRFamide) cannot activate the *Anopheles* myosuppressin receptor and, in fact, is a genuine FMRFamide peptide, activating the *Anopheles* FMRFamide receptor (Cazzamali et al., in preparation).

Anopheles has apparently only one myosuppressin receptor and a BLAST search of the recently published genomic database from the honeybee *Apis mellifera* (http://www.ncbi.nlm.nih.gov/genome/guide/bee/) gave a similar result for the honey bee. The two myosuppressin receptors found in *Drosophila* [14], therefore, have probably originated by a recent gene duplication after the split of the mosquito and fruitfly ancestors (approximately 250 million years ago) [19].

Acknowledgments

We thank Drs. Nigel Hill and Patricia Aiyenuro, London School of Hygiene and Tropical Medicine, UK, for a kind gift of *A. gambiae* mosquitos, and Drs. S. Rees and J. Stables (GSK, Stevenage, UK) for supplying cell line CHO/G-16, Sarah P. Dubédat for typing the manuscript, and Lundbeck Foundation, Novo Nordisk Foundation, and Carlsberg Foundation for financial support.

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