Anopheles gambiae Croquemort SCRBQ2, expression profile in the mosquito and its potential interaction with the malaria parasite *Plasmodium berghei*

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Abstract

The scavenger receptor family comprises transmembrane proteins involved in the recognition of polyanionic ligands. Several studies have established that members of this family are involved both in immunity and in developmental processes. In *Drosophila melanogaster*, one of the best characterized scavenger receptors is Croquemort, which participates in the recognition of apoptotic cells in the embryo. Although comparative genomic studies have revealed the presence of four orthologs of this receptor in the malaria vector *Anopheles gambiae*, little is known about their function. We have investigated the expression pattern of the four Croquemort orthologs during the mosquito life cycle. Croquemort transcripts SCRBQ2 and SCRBQ4 are expressed at all the developmental stages, while expression of Croquemort transcripts SCRBQ1 and SCRBQ3 is more restricted. We have also investigated the expression of the four Croquemort orthologs in the

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different organs of the adult female. Croquemort transcript SCRBQ2 is highly expressed in the *A. gambiae* female midgut. SCRBQ2 midgut gene expression was up-regulated after a non-infected or a *Plasmodium berghei*-infected blood meal, compared to its expression in midguts of sugar-fed females. Interestingly, knockdown of SCRBQ2 expression by dsRNA injection resulted in a 62.5% inhibition of oocyst formation, suggesting that SCRBQ2 plays a role in *Plasmodium–* mosquito interactions.

Keywords

Croquemort; Scavenger receptor; *Anopheles*; CD36; RNAi

1. Introduction

The scavenger receptor (SR) family comprises transmembrane proteins with multiple domains that participate in the recognition of a broad range of polyanionic ligands, including modified or oxidized low-density lipoproteins (oxLDL), bacteria and apoptotic cells (Pluddemann et al., 2006). The SR family includes eight independent classes (A–H) based on their multidomain structure (Murphy et al., 2005; Pluddemann et al., 2007), however, there is not a common domain to all SRs. Several studies have established that members of the SR family are involved both in immunity (Gough and Gordon, 2000; Mukhopadhyay and Gordon, 2004; Peiser et al., 2002) and in developmental processes (Murphy et al., 2005).

In insects, one of the best characterized SRs is Croquemort, a member of the class B of the SR family. This class includes three members: CD36, the scavenger receptor class B type I (SR-BI) and the lysosomal integral membrane protein II (LIMP-II) (Febbraio et al., 2001; Murphy et al., 2005). Croquemort is a CD36-family homolog that was first described in *Drosophila* (Franc et al., 1996), where it is expressed in the plasma membrane of hemocytes. Croquemort expression is coincident with the first wave of apoptosis in the embryo, functioning as an essential receptor for phagocytosis of apoptotic corpses (Franc et al., 1999). RNAi-mediated silencing of this gene in the *Drosophila* S2 cell line decreased their ability to internalize *Staphylococcus aureus*, but not *Escherichia coli*, indicating that this molecule can also act as a phagocytic receptor for Gram-positive bacteria (Stuart et al., 2005). Recently, a proteomic analysis of a *Drosophila* cell line infected with Flock House virus indicated that Croquemort expression was up-regulated in response to the viral infection (Go et al., 2006).

There are four orthologs (SCRBQ1–4) of *Drosophila*'s Croquemort in *Anopheles gambiae*, which appear to correspond to a specific gene expansion in the mosquito (Christophides et al., 2002). Of these, Croquemort transcript SCRBQ2 (AGAP010133) was first identified among the transcripts (ESTs) expressed by the *A. gambiae* hemocyte-like 4A3A (Dimopoulos et al., 2000) and 4A3B (Dimopoulos et al., 2002) cell lines. Recently, a microarray assay identified the expression of three Croquemort transcripts in the midguts of fourth-instar *A. gambiae* larvae. In this study, the Croquemort SCRBQ2 transcript was found to be enriched in the gastric caeca and in the hindgut/Malpighian tubules versus its

expression in whole larvae (Neira Oviedo et al., 2008). Changes in Croquemort SCRBQ2 mRNA abundance have also been described in the midguts of female *A. gambiae* mosquitoes upon *Plasmodium berghei* ookinete invasion (Dong et al., 2006), but the role, if any, of this molecule during parasite infection remains unknown.

Here we report on the developmental expression profile and organ expression in adult females of the four *A. gambiae* Croquemort orthologs and on the effect of Croquemort SCRBQ2 gene silencing on the development of *P. berghei* in the mosquito, as an initial approach to explore Croquemort gene function during parasite infection.

2. Materials and methods

2.1. Mosquito rearing

A. gambiae Keele strain mosquitoes were maintained on a 10% sugar solution at 27 °C and 80% humidity with a 12-h light/dark cycle according to standard rearing procedures (Benedict, 1997). dsRNA-injected, blood-fed and *P. berghei*-infected mosquitoes were kept at 19 °C under the same rearing conditions.

2.2. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR assays were used to determine the expression profile of the four Croquemort orthologs along the mosquito developmental cycle and in the different organs of the adult female. Additional RT-PCR assays, provided as Supplementary material, were also used to determine the expression profile of the four Croquemort orthologs in response to different feeding conditions as well as to assess multiple gene-knockdown in RNAi assays. 1–2 μg of DNase-treated total RNA (Turbo DNAse, Ambion, Austin, Texas, USA), isolated from *A. gambiae* embryos, first to fourth-instar larvae, pupae, and whole sugar-fed females and from midguts, ovaries, and carcass (whole body minus organs) of sugar-fed adult females were reverse-transcribed using SuperScript III (Invitrogen, Carlsbad, California, USA). Croquemort transcript abundance was determined using specific oligonucleotide primers corresponding to an internal region of each Croquemort sequence. All Croquemort sequences were retrieved from the *A. gambiae* database (http://agambiae.vectorbase.org/index.php), (Lawson et al., 2007). Gene-specific primers were: SCRBQ1-specific primers (accession no. AGAP0 10132): SCRBQ1-F, 5′-ACGAGCGTGGTATGGTATCGT-3′; SCRBQ1-R, 5′-CACTGGATTCAGCGTCGTCA-3′. SCRBQ2-specific primers (accession no. AGAP010133): SCRBQ2-F, 5′-AGTTCCGTACGAAGAGCTACAAAA-3′; SCRBQ2-R, 5′-ACACGCATTACTAACGAAACAAAA-3′. SCRB Q3-specific primers (accession no. AGAP008179): SCRBQ3-F, 5′-TGGACGTTTCGAGGTGCAA-3′; SCRBQ3-R, 5′-CATCTCGAACCGATG CTCCT-3′. SCRBQ4-specific primers (accession no. AGAP003373): SCRBQ4-F, 5′-GTGAAACGCGTCTTCCTGC-3′; SCRBQ4-R, 5′-ACTACCATTGCGATGCCACA-3′. The *A. gambiae* ribosomal protein (AgS7) (Salazar et al., 1993) amplification product was used as expression control. AgS7-specific primers (accession no. AGAP010592): AgS7F, 5′-TTCGTTGTGAACCCAAATAAAAATC-3′ and AgS7R, 5′-TGCGGCTTCAGATCCGAGTTC-3′. Amplification for all transcripts was performed as follows: 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s (30 cycles) with a final extension at 72 °C for 7 min. All PCR products were cloned either in the

pGEM-T Easy vector (Promega) or in the TOPO-4 vector (Invitrogen) and sequenced to confirm their identity. The results are representative of two independent biological replicates.

2.3. Real-time quantitative PCR

Real-time PCR was used to determine Croquemort SCRBQ2 transcript abundance in different mosquito organs and in response to different feeding conditions as well as to assess gene-knockdown in RNAi assays.

RNA samples were treated with Turbo DNase (Ambion, Austin, Texas, United States) and reverse-transcribed as above. Real-time quantification was performed using SYBR Green PCR Master Mix (Applied Biosystems) and the 7300 Real-Time PCR System (Applied Biosystems). All real-time PCR reactions were performed in triplicate. Specificity of the reactions was assessed by analysis of melting curves for each data point. Serial dilutions of a standard cDNA sample from whole *A. gambiae* females (six concentrations, two technical replicates each) were run along with the experimental cDNA to calculate a standard curve using the ABI Prism 7300 Sequence Detection Software (Applied Biosystems). Subsequently, cDNA templates were normalized using the ribosomal protein S7 gene. The AgS7 and SCRBQ2 Croquemort-specific primers used for all assays are the same as described above. The results are representative of at least two independent biological replicates.

2.4. RNAi gene-silencing assays

Gene-silencing assays were used to explore potential Croquemort SCRBQ2 gene function during *P. berghei* infection. A fragment of the SCRBQ2 transcript was amplified with specific primers flanked at their 5′ end by the T7 promoter sequence. dsRNA was produced from the PCR-amplified gene fragment using the *in vitro* transcription MEGAscript RNAi kit (Ambion) according to the manufacturer's instructions. dsGFP (Green Fluorescent Protein) was used as a negative control. SCRBQ2 Croquemort-specific primers were: SCRBQ2-F, 5′-TAATACGACTCACTATAGGGAGGTTTGGCTGGTTTGTTGG-3′, SCRBQ2-R, 5′-TAATACGACTCACTATAGGGGTCCCGTTACAGCAGTTTGG-3′. GFP-specific primers were: GFP-F: 5′-TAATACGACTCACTATAGGGTGTTCCATGGCCAACACTTGTCAC-3′, GFP-R: 5′-TAATACGACTCACTATAGGGTTGGAAAGGGCAGATTGTGTGGAC-3′.

To perform the RNAi-mediated gene-silencing assays, *A. gambiae* females (4–6 days-old) were cold-anesthetized and inoculated with dsSCRBQ2 or dsGFP (control). About 140 nl of each dsRNA (4 μg/μl) were injected into the thorax of the mosquitoes using a nano-injector (Nanoject II; Drummond Scientific, Broomall, Pennsylvania, United States).

Gene-silencing was verified in the midguts and carcasses 48, 72 and 96 h after dsRNA injection by real-time PCR using the SCRBQ2- and AgS7-specific primers as described above. Given the sequence similarity of the four Croquemort genes, a semi-quantitative RT-PCR assay of the four genes was performed using cDNA from the midguts 72 h after dsGFP

or dsSCRBQ2 injection to explore the possibility of multiple knockdowns involving one of the other Croquemort orthologs.

For *Plasmodium* infection assays, 3–4 days after dsRNA injection, GFP-injected and SCRBQ2-injected mosquitoes were fed on a single mouse infected with the *P. berghei* ANKA 2.34 strain per biological replicate. The midguts of blood-fed mosquitoes were dissected on ice 12–15 days after ingestion of infected blood. Only midguts from mosquitoes that ingested blood, as indicated by egg development, were analyzed. Mosquito midguts were stained with 0.2% mercurochrome and oocyst numbers per mosquito midgut were determined using a light microscope (Nikon). A total of 10 biological replicates (injecting 100 mosquitoes for each group in each replicate) were performed.

2.5. Statistical analysis

Averages from biological replicates are presented for the expression quantification experiments. The sum of rank test (Mann–Whitney U test) was used to test difference of continuous variables between groups. All analyses were two-tailed and p 0.05 was considered significant. In particular, to evaluate the effect of SCRBQ2 silencing, the sum of rank test was used to test the difference in the number of oocysts between dsSCRBQ2-(experimental) and dsGFP-injected (control) mosquitoes. Infection prevalence was defined as the proportion of mosquitoes with one or more oocysts. The percentage of inhibition in oocyst formation was calculated as follows: % inhibition = (control mean oocyst number – experimental mean oocyst number)/control mean oocyst number × 100. All statistical analyses were performed using the R software (R Development Core Team, 2007).

3. Results

3.1. Croquemort genes in A. gambiae

In contrast to the single *Drosophila* gene, there are four Croquemort genes (SCRBQ1–4) in the *A. gambiae* genome. Of these, the protein encoded by Croquemort transcript SCRBQ2 (AGAP010133) has the highest identity to its *Drosophila* counterpart (42%), while the gene products of Croquemort transcripts SCRBQ1 (AGAP010132), SCRBQ3 (AGAP008179), and SCRBQ4 (AGAP003373) have a 37%, 35% and 36% identity, respectively. Alignment of the predicted amino acid sequences using the ClustalW algorithm (Thompson et al., 1994) revealed the presence of conserved residues. In particular, several cysteines (256, 285, and 345), prolines (93, 350, 356, 374, 387, and 423) and glycines (92, 130, 185, 210, 230, 261, and 371) that were used to align the *Drosophila* Croquemort protein with other members of the CD36 superfamily (Franc et al., 1996) are also present in the *Anopheles* amino acid sequences. Likewise, a motif formed by 13 amino acids (amino acids 350–362) that has been suggested by (Franc et al., 1996) as relevant in these proteins (P I/V Y I/L S F/L P H F Y/L L A D/S) is also partially conserved in the *Anopheles* Croquemort proteins. In particular, the last nine amino acids are strongly conserved in the sequences (Fig. 1). The functional significance of this motif is currently under investigation (N. Franc, personal communication).

3.2. Developmental profile of Croquemort SCRBQ1–4 genes expression

We designed oligonucleotides to amplify a specific fragment of each mRNA to determine their expression profile at different developmental stages by RT-PCR (Fig. 2). SCRBQ1 was expressed most strongly in larval stages but its expression could not be detected in the embryo. SCRBQ2 was expressed evenly at all developmental stages. Croquemort transcript SCRBQ3 was expressed at four developmental stages: mainly in third instar larvae and weakly in first and fourth-instar larvae as well as in adult females. SCRBQ4 expression was detected in all the developmental stages, but it was strongest in third and fourth-instar larvae. These expression profiles were replicated in two independent RNA samples.

3.3. Transcriptional profile of Croquemort SCRBQ1–4 genes in the different organs of A. gambiae

The expression profile of these genes was analyzed in the midguts, ovaries and carcasses (whole bodies minus organs) of sugar-fed mosquitoes. As shown in Fig. 3, all the transcripts were detected in the midguts, although the expression of SCRBQ2 was noticeably higher than that of the other SCRBQ genes. SCRBQ2 expression was also detected in the ovaries and carcasses, while SCRBQ4 was also expressed in the ovaries.

We decided to focus on Croquemort transcript SCRBQ2 (AGAP010133) since it is strongly expressed in the mosquito midgut, an organ where close interaction between the parasite and the mosquito occurs. Besides, SCRBQ2 has the highest identity to the *Drosophila* protein and previous studies had reported transcriptional changes for this gene upon *P. berghei* invasion of the mosquito midgut (Dong et al., 2006).

3.4. Croquemort SCRBQ2 mRNA is mainly expressed in the midgut of A. gambiae mosquitoes

The expression profile of the Croquemort SCRBQ2 mRNA in female mosquitoes, as determined by real-time PCR assays, is shown in Fig. 4. The gene is expressed in all the organs of sugarfed *A. gambiae* females, but the mRNA is most abundant in the midgut. The transcript was also detected in the ovaries, salivary glands and carcass, its abundance being 36%, 44% and 55% of that observed in midguts, respectively. The differences in transcript abundance in the different organs with respect to the midguts were statistically significant ($p < 0.001$).

3.5. Croquemort SCRBQ2 mRNA expression is up-regulated in the midgut of blood-fed A. gambiae

As determined by real-time PCR assays, Croquemort SCRBQ2 expression was up-regulated (1.9-fold increase, $p = 0.004$) in the midguts of blood-fed mosquitoes 24 h after blood feeding, compared to its expression in midguts of sugar-fed mosquitoes. Gene expression was also up-regulated (2.3-fold increase, $p = 0.002$) in midguts from mosquitoes 24 h after feeding on a *P. berghei*-infected blood meal (Fig. 5A). The expression of the gene was moderately up-regulated in the carcass of blood-fed mosquitoes (1.7-fold increase with respect to its expression in the carcass of sugar-fed females, $p = 0.002$) and in the carcasses of mosquitoes infected with *P. berghei* (1.4-fold increase, $p = 0.002$) (Fig. 5B). We

confirmed that the differences we observed were not due to the different rearing temperatures of the mosquitoes, as no increase in the expression of SCRBQ2 was apparent by RT-PCR in sugar-fed mosquitoes that were kept for 24 h at 19 °C (data not shown). RT-PCR assays exploring the changes of the other three SCRBQ genes under different feeding conditions showed that SCRBQ2 is the only gene consistently up-regulated after blood- and infectious-blood feeding (Supplementary figure S1).

3.6. Double-stranded Croquemort SCRBQ2 RNA effectively reduces transcript abundance in female A. gambiae mosquitoes

To determine the level of RNAi-mediated gene silencing, midguts and carcasses were dissected 48, 72 and 96 h after dsRNA injection, and SCRBQ2 gene transcript abundance was determined by real-time PCR. At 48 h after the injection, Croquemort expression in the midguts of *A. gambiae* females injected with dsSCRBQ2 was reduced by 62% ($p < 0.001$) with respect to that observed at the same time point in control mosquitoes injected with dsGFP. A similar decrease in Croquemort transcript expression was observed 72 and 96 h after the injection of dsSCRBQ2 (55% reduction in both, $p < 0.001$). Overall, this represents a ~60% decrease in Croquemort SCRBQ2 expression in dsSCRBQ2-injected mosquitoes at all the time points examined, when compared to its expression in the midguts of dsGFP-injected (control) mosquitoes (Fig. 6A). Similarly, Croquemort expression in the carcasses of dsSCRBQ2-injected mosquitoes decreased by about 40% at the three time points ($p < 0.001$) (Fig. 6B).

The effect of dsRNA injection in Croquemort transcript levels was also determined in mosquitoes that were fed on *P. berghei*-infected mice 72 h after dsRNA injection. In the midguts, transcript abundance was reduced by 71% ($p = 0.002$) 96 h after dsRNA injection (24 h after infectious-blood feeding) (Fig. 6C). Similarly, in the carcass, SCRBQ2 expression was reduced by 33% ($p = 0.002$).

Possible knockdown of the other three Croquemort orthologs in the midgut by dsSCRBQ2 was explored by semi-quantitative RT-PCR 72 h after injection of dsGFP and dsSCRBQ2 (Supplementary figure S2). The expression of all three Croquemort genes in the midgut was markedly inferior to that of SCRBQ2. No differences were observed in the expression level of SCRBQ1 and SCRBQ3. However, SCRBQ4 could be weakly observed in dsGFP- but not in dsSCRBQ2-injected mosquitoes, suggesting that co-suppression by dsSCRBQ2 might occur.

3.7. Reduction of Croquemort SCRBQ2 transcript abundance affects P. berghei oocyst formation

To determine whether the loss of SCRBQ2 would affect oocyst development in the mosquito, we performed RNAi-mediated gene silencing and quantified the number of *P. berghei* oocysts in the mosquito midgut. There was a statistically significant difference ($p = 0.035$, Mann–Whitney *U* test) in the number of oocysts observed between mosquitoes injected with dsSCRBQ2 and those injected with dsGFP. Among the ten assays performed, 4386 oocysts were observed in 151 dsGFP-injected mosquitoes (mean oocyst number per mosquito = 29.0) and 2723 oocysts were present in 162 dsSCRBQ2-injected mosquitoes

(mean oocyst number per mosquito = 16.8). This corresponds to a 42.1% inhibition in oocyst formation. Given that infection levels were variable across the experiments, we performed a subgroup analysis, testing experiments separately depending on the level of infection occuring in either of both groups (cut-off: median infection 30 oocysts per midgut). Among the four experiments with higher infection levels, 2501 oocysts were observed in 38 dsGFP-injected mosquitoes (mean oocyst number per mosquito = 65.8) and 1652 oocysts were present in 67 dsSCRBQ2-injected mosquitoes (mean oocyst number per mosquito = 24.6). There was a statistically significant reduction ($p < 0.001$, Mann–Whitney U test) in the number of oocysts observed in dsSCRBQ2-injected mosquitoes with respect to dsGFP-injected mosquitoes (Fig. 7). This corresponds to an *in vivo* inhibition of 62.5% in oocyst formation. Among the six experiments with lower infection levels, there was also a marginally significant difference among the control and experimental groups (16.7 oocysts in dsGFP-injected mosquitoes versus 11.3 oocysts in dsSCRBQ2-injected mosquitoes, $p = 0.05$).

The prevalence of infection was 78.1% for dsGFP-injected mosquitoes and 69.8% for dsSCRBQ2-injected mosquitoes ($p = 0.12$). In the high infection group the prevalence of infection was 89.4% for dsGFP-injected mosquitoes and 79.1% for dsSCRBQ2-injected mosquitoes ($p = 0.27$), while in the low infection group the prevalence of infection was 74.3% for dsGFP-injected mosquitoes and 63.1% for dsSCRBQ2-injected mosquitoes ($p = 0.11$).

4. Discussion

Gene expansion can lead to diversification and the development of new species-specific functions (Christophides et al., 2002). Comparative genomics of *D. melanogaster* and *A. gambiae* have revealed numerous gene expansions in both genomes. In *A. gambiae*, the fibrinogen-domain (FBN) family (Zdobnov et al., 2002) and the carboxylesterases and cytochrome P450s families – both of them involved in insecticide resistance – (Ranson et al., 2002) are two examples. *Drosophila*'s Croquemort, a class B scavenger receptor, has four orthologs in the *A. gambiae* genome, which is another example of gene expansion (Christophides et al., 2002). Conceivably, the gene diversification of this family represents a particular adaptation to mosquito biology, since there are also four Croquemort orthologs in *Aedes aegypti* (Waterhouse et al., 2007).

Expression analyses evidenced differences in the developmental profiles of these four transcripts. Croquemort transcript SCRBQ2 had an almost constant expression level irrespective of the life stage, which suggests that changes in the transcriptional profile of this gene might not be developmentally regulated. However, for Croquemort transcripts SCRBQ1, SCRBQ3 and SCRBQ4, a difference in transcript abundance during the mosquito development cycle was observed. SCRBQ1 and SCRBQ4 expression was slightly up-regulated in late larval stages, while SCRBQ3 transcript was present mainly in third instar larvae and its expression in other developmental stages was more restricted, in contrast to the other Croquemort transcripts. Recently, data obtained from a microarray assay also identified the expression of Croquemort transcripts SCRBQ1, SCRBQ2, and SCRBQ3 in the midguts of fourth-instar *A. gambiae* larvae, however, Croquemort transcript SCRBQ4 was

not detected in these assays (Neira Oviedo et al., 2008). We detected the presence of all four Croquemort transcripts in fourth-instar larvae, although we did not perform dissections of individual organs. The use of whole larvae in our assays may explain why we were able to detect the presence of transcript SCRBQ4 in fourth-instar larvae.

The precise function of the four Croquemort orthologs during the different life cycle stages of *A. gambiae* remains to be established. If expressed in the hemocytes, they might be involved in apoptotic cell clearance during the remodeling process of the larvae, as was previously documented in *Drosophila* (Franc et al., 1996; Franc et al., 1999). Additionally, they could be acting as scavenger receptors for lipoprotein complexes (Turunen, 1985) which could be used for lipid metabolism in the larval midgut. This is an interesting possibility since transcript SCRBQ1 is expressed in the anterior midgut, the only region in which transcripts involved in lipoprotein transport have been identified. Transcripts SCRBQ1 and SCRBQ3 are also expressed in the posterior midgut, a region in which lipid metabolism and absorption apparently take place (Neira Oviedo et al., 2008). Whether the different Croquemort genes have redundant or overlapping functions or whether they perform diverse roles in the mosquito awaits investigation.

Scavenger receptors bind a broad range of polyanionic ligands, in particular modified low-density lipoproteins (LDL) (McGuinness et al., 2003). In vertebrates, CD36 binds oxidized LDL, modified LDL, long chain fatty acids, and anionic phospholipids (Febbraio et al., 2001). Both in *Drosophila* and in *Anopheles*, Croquemort proteins have a CD36 domain. Therefore, the possibility exists that Croquemort could be acting as a scavenger receptor for any or several of these molecules, particularly in the ovaries, where it could be scavenging for lipids that would be useful for egg production. Croquemort might be performing the same function in the fat body, as it is also expressed in the carcass (whole body minus midgut, ovaries and salivary glands) of the mosquito.

We focused on the SCRBQ2 transcript (AGAP010133), since it has the highest identity to *Drosophila*'s Croquemort and importantly, it had been shown to change its expression profile in relation to *P. berghei* invasion (Dong et al., 2006). Besides, SCRBQ2 was the ortholog predominantly expressed in adult females (Fig. 2) and it is also the gene that has the highest expression level in the midgut (Fig. 3).

Compared to its expression in midguts of sugar-fed mosquitoes, Croquemort SCRBQ2 expression was up-regulated in the midguts of blood-fed mosquitoes. Gene expression was also up-regulated in *A. gambiae* midguts 24 h after receiving a blood meal infected with *P. berghei*. Previously, microarray data indicated that Croquemort transcript SCRBQ2 was up-regulated in the midguts of *P. berghei*-infected mosquitoes upon ookinete invasion (Dong et al., 2006). In contrast, our data show a similar up-regulation of the SCRBQ2 transcript in response both to normal blood feeding and blood infected with *P. berghei*. However, it is not uncommon for real-time PCR and microarray data to vary due to differences in technical procedures, such as data normalization and the amount of change in gene expression (Morey et al., 2006). Our results indicate that increase in SCRBQ2 transcript abundance in the mosquito midguts appears to be more a physiological response to blood feeding than a

specific response to the malaria parasite crossing the midgut epithelium, which usually occurs 18–24 h after the infectious-blood meal (Beier, 1998; Dimopoulos et al., 2002).

Also, we cannot rule out the possibility that some of the Croquemort SCRBQ2 transcripts detected in the midguts could correspond to those expressed by hemocytes that remain attached to the midgut (Abraham et al., 2005; Osta et al., 2004). If expressed in *A. gambiae* hemocytes, this molecule could be acting as a phagocytic receptor for apoptotic cells (Franc et al., 1999) or as a phagocytic receptor for Gram-positive bacteria, as observed in a *Drosophila* cell line (Stuart et al., 2005). Supporting the latter hypothesis, microarray data has shown that SCRBQ2 expression is up-regulated in response to bacterial challenges (Dimopoulos et al., 2002).

During *Plasmodium* development in the mosquito, there is close interaction between the parasite and the midgut. RNAi-mediated gene silencing was used to assess potential functions of the Croquemort SCRBQ2 gene *in vivo*. Knockdown of gene expression resulted in a significant reduction (~60%, $p < 0.001$) in the number of *P. berghei* oocysts in the midgut of *A. gambiae*, compared to the number of oocysts in the control (GFP-injected) group, suggesting that SCRBQ2 participates in parasite development. Similarly, there were differences in the infection prevalence between the GFP- and SCRBQ2-injected mosquitoes, although they did not reach statistical significance.

Even though it is very weakly expressed in the midgut, it is possible that SCRBQ4 might have also been silenced in dsSCRBQ2-injected mosquitoes. Nevertheless, SCRBQ2 is substantially more abundant than SCRBQ4 in the midgut, which supports the fact that the results we observed are mainly due to the knockdown of SCRBQ2. The precise role that Croquemort SCRBQ2 plays in parasite development remains to be determined.

In conclusion, we present the expression profile of four Croquemort transcripts along the life cycle of *A. gambiae*. In particular, Croquemort transcript SCRBQ2 is highly expressed in the midguts of adult sugar-fed females, and is up-regulated after a blood meal. RNAi knockdown experiments suggest that Croquemort SCRBQ2 participates in the establishment of *Plasmodium* parasites in the mosquito midgut.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SCRBQ3     1  MGGCCSNWSPTGKRLCALGSTTALCVFALALGLVWPALIWQIAKREFVLEPGTEVYKNWI
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CG4280    59  EAPIPIYLSFYMFNWTNPEDIRNPD-IKPNFVEMGPYTFLEKHKKENYTFYDN-ATVAYY
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SCRBQ1    61  DGEVPIFFDIYLFHWTNPEQIRDPN-VRPNFVQMGPYVFSERHERGMVSFNDN-DTITFN
SCRBQ3    61  EPPIDTYLELYLWNWTNADAYLT---EKPHLEQLGPYTFREVHERVNLKWNDN-DTLTFQ
SCRBQ2    58  RTPIPMYLEVYFFNWTNPDEVKTKNGTKPHFVEMGPYTFSEVHERVNLVWNAN-NTVTYE
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CG4280   117  ERRTWFFDPERSNG-TLDDMVTAAHAITATVADEMRDQRKIVKKIIN--FMLNHEGGKLY
SCRBQ4    75  KKSLYYFDEEGSNG-SLDDVISSINIVAVSAAKRSKYWGYLKQKGVS--LGLNVYEQKIN
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SCRBQ3   117  QRRIWYHVPELSAGDYETDRVVTINPVLLTVGYALR-NEPEFLFYVDGIIMLNGLATTPF
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CG4280   174  VTKPVGEWIFEGYQDNITDF-LNLFNTTK-------IDIPYKRFGWLADRNESLTYDGLF
SCRBQ4   132  VVKTAGELLFDGYEDNMVLMGKHMFDAD---------EVPFDRVGWFYTRNNSADLIGHY
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SCRBQ3   176  YDVLVREMIFEGYDDTLLLTNLLALLALLPEESRPPIDLPPYDRFGWFFGRNGSETYDGTF
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CG4280   226  TIHTGTDDISNLGRLTHWNGKSETGFYEMPCGIVNGTTGDMFPPK----MNVNDEITIFA
SCRBQ4   183  NVHTGVEDIGMLGKMGEWNYKPRTDFFEGTCGMLNGSAGEFYPPG----LSKERPIELFT
SCRBQ1   237  QMGTGTDNHINTGVMRQWNNAPQVPNYRGVCGQVRGSAGEVWPPMGRNLGDNIRPLNLFL
SCRBQ3   236  TIGTGKDSVYNTGVMRLWNGANATDYYRGECGRIRGTTGEVWPPWGRTLTGTPPSVSVFA
SCRBQ2   226  TMRTGADGLESMGFLTQWNGSPNTGMYRGKCGEVYGTSGELWPAS----SNVPANITLFP
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CG4280   282  TDACRFMNLRPRGTYENHGLTATKWVGTEETLDSGENYPNQACFCDEA--RFDECPKTGV
SCRBQ4   239  PDMCRSLPLDFEEEVTIHGLKAYKYSGDRRAIDNGTLYPETACFSAG------EIVPSGV
SCRBQ1   297  PDLCSAITLRHEREFTVHGLDGELWVGDARNFDNGHTIPETECQCTSP-IDQCPFYRPGV
SCRBQ3   296  PDVCSSVTLEYAEEMERYGIDGLRWIGTDRVFDNGLHYPETECQCTAEDVADCPLLDNGA
SCRBQ2   282  SDICRSITLQGREQVSLYNIQGTKYVGDDRVFDNGVKYPEASCWCNSN-PTQCPDLKPGV
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CG4280   340  VECKACRDKAPIYSSFPHFYLADQSYVDAVSGMKPEKEKHEFFLAVEPITGVPVQVHGRI
SCRBQ4   293  LNISSCRFGTPVFVSFPHYYGADPFYLDQVEGLSPSKDKHQFYMSMEPTTSVPLDVAARL
SCRBQ1   356  LDVSECKFGAPLVVSYPHFYLAHPSYRTAVTGMEPDRAKHEFRFALHPFSGIPMAANGRI
SCRBQ3   356  MDVSRCKFGAPATVSYPHFYLANESYLKGISGMQPNEKEHRFEMELEPYTGVPLGVRAQL
SCRBQ2   341  FNASACKYGSPTFVSFPHFYLADESYQTAVTGLRPNQTEHEFYMAIEPSTGIPLDVRAQL
                *   *  *     *     *                *       *          *

CG4280   400  QINMMIEPDDDFDIYRGVQKVLMPMFWFDQYAELSSELASKAKLAINLSSYGIIFGYSMI
SCRBQ4   353  QLNIMIEPYENINLFSDVKRVFLPVLWFEQHVLMPSELAGEIGQALTIPSIVRLCGIAMV
SCRBQ1   416  QYNMHLR-DNGMALFRDVPDVIIPAFWIEQRMVLTKDIADDLKVQLL-------------
SCRBQ3   416  QVNLDVK-QYGMTLLKGIPEVMLPVLWFRQTASLTEELADDIKV----------------
SCRBQ2   401  QINEHLQPIKGFSFYEHVPDVMVPMIWFRQRATLTQELAEQAKLALALPSLGIYVAVFFG
                                              *

CG4280   460  AFASVFLITGITLTVTKKWVRRTPEDEDILTN
SCRBQ4   413  VFGALMLLW-IPLERTFFRRRRVA--------
SCRBQ1        --------------------------------
SCRBQ3        --------------------------------
SCRBQ2   461  SIGIIMTSVFLFCSIKKWSQSAEVVPYEELQN
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Fig. 1.

Sequence alignment of the four *A. gambiae* Croquemort predicted proteins (SCRBQ1–4) and *Drosophila* Croquemort (accession number CG4280). Identical residues are shaded black, while conserved groups are shown with a gray background. Conserved prolines, cysteines and glycines are indicated with an asterisk. A 13-amino acid motif that was originally described in *Drosophila*'s Croquemort and that is present in members of the CD36 superfamily is shown in a box. The last nine amino acids are strongly conserved both in *Drosophila* and in *A. gambiae*.
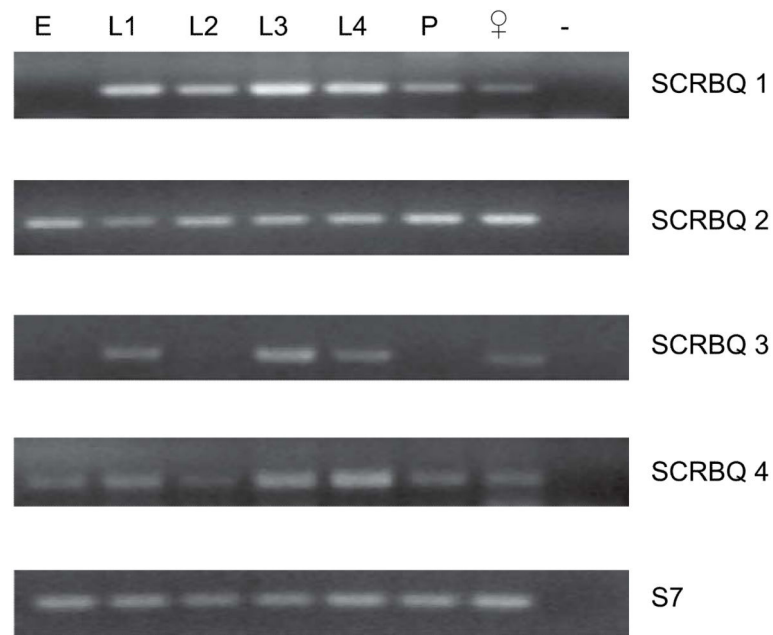
Fig. 2.
Developmental profile of the four Croquemort transcripts (SCRBQ1–4) in the *A. gambiae*
mosquito. E, embryos; first (L1), second (L2), third (L3) and fourth (L4) instar larvae; pupae
(P); ♀, females; –, negative control (no cDNA). The *A. gambiae* S7 ribosomal protein
served as a loading control. The expression profile obtained from a second independent
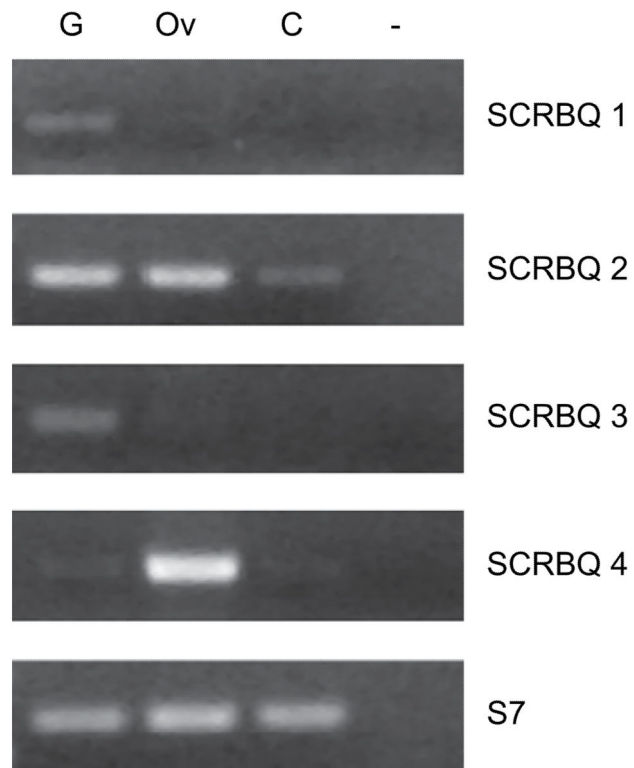RNA sample reproduced these results.

Fig. 3.
Transcriptional profile of the four Croquemort genes in the different *A. gambiae* organs. G, midguts; Ov, ovaries; C, carcass (whole body minus organs); –, negative control (no cDNA). The *A. gambiae* S7 ribosomal protein served as a loading control. These results were reproduced using a second independent RNA sample.
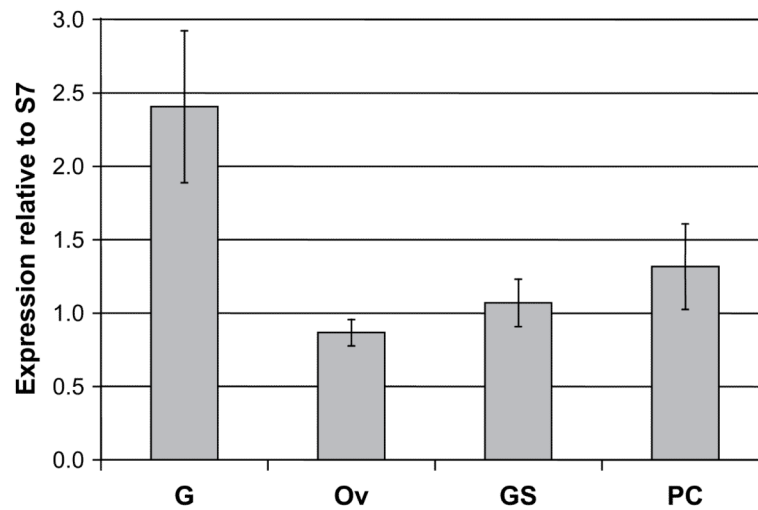
Fig. 4.
Expression profile of the Croquemort SCRBQ2 (AGAP010133) transcript in the different *A. gambiae* organs. Real-time quantitative PCR analysis of the SCRBQ2 Croquemort transcript. The *A. gambiae* S7 ribosomal protein mRNA served as a quantification standard. Bars indicate 95% confidence intervals. G, midguts; Ov, ovaries; SG, salivary glands; PC, carcass (whole body minus midgut, ovaries and salivary glands).
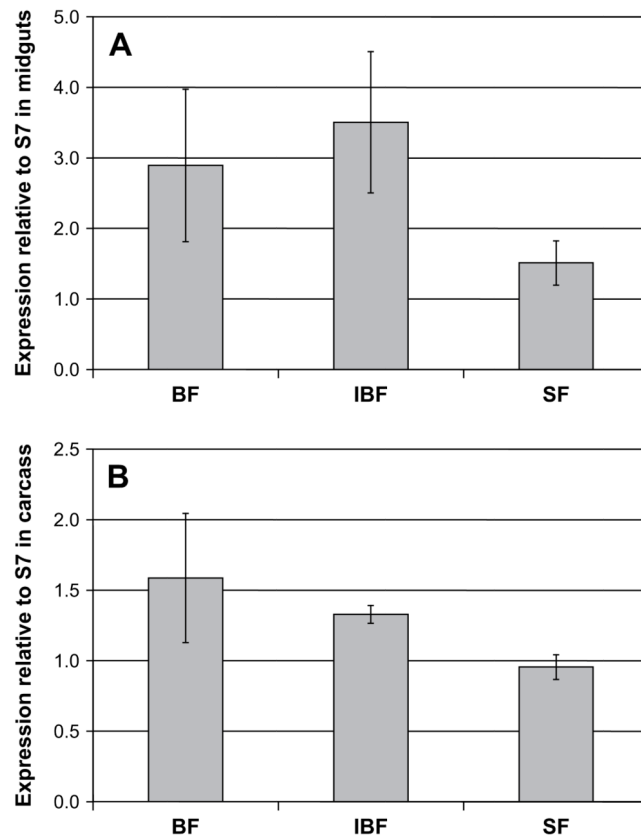
Fig. 5.
Expression profile of the Croquemort SCRBQ2 (AGAP010133) transcript in the *A. gambiae* mosquito under different feeding conditions. Real-time quantitative PCR analysis of the SCRBQ2 Croquemort transcript expression in response to a non-infected or infected blood meal. Transcript abundance was determined in the (A) midguts and (B) carcasses (whole body minus midgut) 24 h after a non-infectious-blood meal (BF) or a *P. berghei*-infected blood meal (IBF). Sugar-fed (SF) mosquitoes were used as a reference. The *A. gambiae* S7 ribosomal protein mRNA served as a quantification standard. Bars indicate 95% confidence intervals.
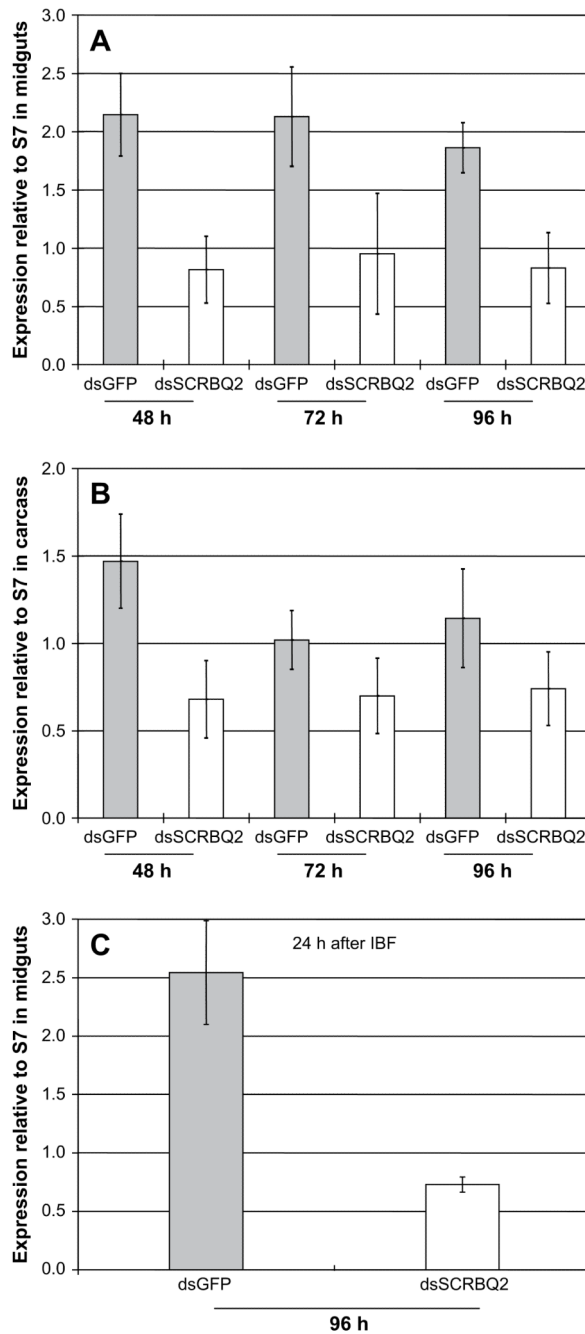
Fig. 6.
RNAi-mediated knockdown of Croquemort transcript SCRBQ2 in the *A. gambiae* mosquito.
Real-time quantitative PCR analysis of the SCRBQ2 Croquemort transcript expression in
the (A) midguts and (B) carcasses of *A. gambiae* females injected with dsSCRBQ2 or
dsGFP RNA. Samples were obtained 48, 72 and 96 h after dsRNA injection and analyzed by
real-time RT-PCR. (C) Croquemort expression in the midguts of *A. gambiae* female
mosquitoes injected with dsSCRBQ2 or dsGFP and fed 72 h later with *P. berghei*-infected
blood. Samples were obtained 96 h after dsRNA injection (24 h after infectious-blood

feeding). The *A. gambiae* S7 ribosomal protein mRNA served as a quantification standard. Bars indicate 95% confidence intervals.
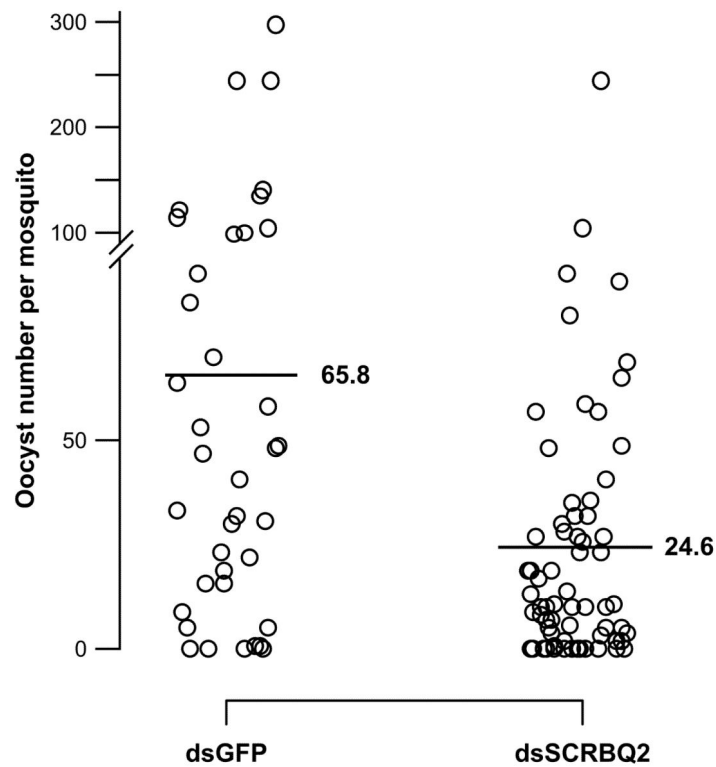
Fig. 7.
RNAi knockdown of Croquemort transcript SCRBQ2 decreases the number of *P. berghei* oocysts in midguts of *A. gambiae* mosquitoes. The midguts of dsSCRBQ2- and GFP-injected (control) mosquitoes were dissected 12–15 days after a *P. berghei* infectious-blood meal to determine the number of oocysts in each midgut. The mean number of oocysts is shown with a horizontal line. There was a statistically significant reduction of 62.5% ($p <$ 0.001, Mann–Whitney *U* test, two-tailed) in the number of oocysts observed in dsSCRBQ2-injected mosquitoes with respect to dsGFP-injected mosquitoes. Data was pooled from four independent biological replicates with a median infection   30 oocysts. A break and change in the scale of the vertical axis were made for clarity purposes.