Published in final edited form as:

Insect Mol Biol. 2011 August ; 20(4): 541–552. doi:10.1111/j.1365-2583.2011.01087.x.

Characterization of the oxysterol-binding protein gene family in the yellow fever mosquito, *Aedes aegypti*

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Abstract

The oxysterol-binding protein (OSBP) and related proteins (ORPs) are sterol-binding proteins that may be involved in cellular sterol transportation, sterol metabolism and signal transduction pathways. Four ORP genes were cloned from *Aedes aegypti*. Based on amino acid sequence homology to human proteins, they are *AeOSBP*, *AeORP1*, *AeORP8* and *AeORP9*. Splicing variants of *AeOSBP* and *AeORP8* were identified. The temporal and spatial transcription patterns of members of the *AeOSBP* gene family through developmental stages and the gonotrophic cycle were profiled. *AeORP1* transcription seemed to be head tissue-specific, whereas *AeOSBP* and *AeORP9* expressions were induced by a blood meal. Furthermore, over-expression of *AeORPs* facilitated [³H]-cholesterol uptake in *Aedes aegypti* cultured *Aag-2* cells.

Keywords

Oxysterol-binding protein; cholesterol; gene expression; sterol transport

Introduction

The oxysterol-binding protein (OSBP) was first discovered as a cytosolic receptor that binds various oxysterols with high affinity in mouse fibroblast cell cultures (Taylor *et al.*, 1984). Cloning of the rabbit OSBP led to the identification of a large family of similar OSBP-related proteins (ORPs). To date, twelve members in human (Lehto *et al.*, 2001) and seven members in the yeast, *Saccharomyces cerevisiae*, were reported (Beh *et al.*, 2001). In insects, only the *Drosophila* OSBP has been reported to date (Alphey *et al.*, 1997) and there are four oxysterol-binding protein orthologous members (Ma *et al.*, 2010).

All of the OSBP and ORPs contain a minimal oxysterol-binding domain at the C-terminus. Some members of the ORPs also contain the pleckstrin homology (PH) domain, which can sequester ORPs to phosphatidylinositol phosphates (PIP) enriched in the plasma membrane (PM) and Golgi apparatus (Chang *et al.*, 2006; Ngo *et al.*, 2010). In addition, most human ORPs have a conserved FFAT motif (two phenylalanines in an acidic tract; the consensus sequence is EFFDAXE) between the oxysterol-binding domain and the PH domain. The FFAT motif may target ORPs to the endoplasmic reticulum (ER) through interaction with the vesicle-associated membrane protein (VAMP)-associated protein (VAP) (Wyles & Ridgeway, 2004; Furuita *et al.*, 2010).

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OSBP binds cholesterol as well as 25-hydroxycholesterol (Wang *et al.*, 2005; Wang *et al.*, 2008) in the evolutionarily conserved ligand pocket (Suchanek *et al.*, 2007). It is known that most of the human ORPs can bind to either 25-hydroxycholesterol or 22(R)-hydroxycholesterol (Olkkonen & Hynnen, 2009). The protein crystal structure of full-length yeast ORP Osh4 (also known as Kes1) is the only ORP protein structure solved to date. Protein structures show that the recombinant Osh4 is bound to 25-hydroxycholesterol, 20-hydroxycholesterol, ergosterol, and 7-hydroxycholesterol (Im *et al.*, 2005). A model was proposed that sterol and membrane binding promote Osh4 conformational changes that facilitate a sterol transfer (Im *et al.*, 2005). It is speculated that ORPs are involved in, at least in lower eukaryotes, sterol translocation between membranes (Olkkonen & Hynnen, 2009). Several mammalian ORPs have been linked to cellular sterol metabolism. Over-expression of human ORP2 lacking the PH domain resulted in increasing efflux of cholesterol to all acceptors and the down-regulation of cholesterol esterification in Chinese hamster ovary cells (Laitinen *et al.*, 2002). In addition, OSBP is also found to play a direct role in signal transduction pathways and cell cycle regulation (Olkkonen *et al.*, 2006). Wang *et al.* (2005) presented evidences that OSBP serves as a sterol sensor through coordinating the activity of two phosphatases (tyrosine phosphatase and serine/threonine phosphatase) to control the extracellular signal-regulated kinase (ERK) signaling pathway. Most of the previous work was focused on yeast and human, and relatively little is known about ORPs in insects. Insect ORPs should not play a role in regulating cholesterol synthesis like their yeast or mammalian homologs since insects do not synthesize cholesterol *de novo* (Behmer & Nes, 2003). Recently, Ma *et al.* (2010) reported that the OSBP-mediated sterol transport pathway is critical for *Drosophila* spermatogenesis.

Cholesterol is not only a lipid molecule that plays important structural and functional roles in the cellular membrane, but also the precursor for synthesizing several oxidized sterols (Chang *et al.*, 2006), such as 7-dehydrocholesterol (Gilbert, 2004), a critical intermediate of ecdysteroid biosynthesis. Ecdysteroids are made from cholesterol in the prothoracic glands and other steroidogenic organs, such as ovaries, testes, and epidermis (Canavoso *et al.*, 2001). Ecdysone is one of the most important hormones in the life cycle of an insect.

As most hematophagous insects, mosquitoes have to obtain sufficient exogenous cholesterol from their diets (Svoboda & Feldlaufer, 1991). In the larval stage, cholesterol was absorbed in the midgut and then transferred to the fat body for storage. During the pupal stage, the stored cholesterol may be mobilized and transported from fat body to other tissues. AeSCP-2, one of the intracellular cholesterol carrier proteins, is highly expressed in the midgut and may play important roles in lipid uptake and metabolism (Krebs & Lan, 2003; Blitzer *et al.*, 2004; Dyer *et al.*, 2008), but it is still not clear if there are other players in this process.

In this study, we cloned four *OSBP/ORP* genes from *Aedes aegypti* and the different splicing variants were found. To understand the function of OSB/ORPs in *Aedes aegypti*, it is critical to be aware of the temporal and spatial transcription pattern of those genes in different developmental stages as well as in females after a blood meal. To demonstrate the protein function *in vivo*, each of the *AeORPs* were over-expressed in *Aedes aegypti* cultured cells, *Aag-2* and the effect on [³H]-cholesterol incorporation *in vivo* was analyzed. This study presents the first report on mosquito OSBP/ORPs and their potential function *in vivo*.

Results

AeOSBP and AeORP genes in *Aedes aegypti*

Four genes in the OSBP family, encoding six proteins (Fig.1), were identified via homology analysis of the *Aedes aegypti* genomic sequence in the VectorBase

(<http://www.vectorbase.org/>) using the yeast Osh4 amino acid sequence. Primers were designed to clone the coding region of the genes. Based on the amino acid sequence homology to human ORPs, they are *AeOSBP* (AAEL005852), *AeORP1* (AAEL012056), *AeORP8* (AAEL010483), and *AeORP9* (AAEL011953) (Fig. 1A). All of the *AeOSBP* and *AeORPs* contain the OSBP-related domain, through which they bind to sterols. Transcripts of *AeOSBP* and *ORPs* were found in the EST database in the VectorBase, except for *AeOPR1* that did not have a corresponding EST in the VectorBase.

The annotated *AeOSBP* in the published genomic sequence contains only the oxysterol-binding domain (AAEL005952). In contrast, the *AeOSBP* homologs in *Anopheles* and *Culex* genomes all contain an additional PH domain. Homology search of the *AeOSBP* N-terminal PH domain was performed using the *Culex OSBP* N-terminal PH domain sequence. AAEL005862, a gene annotated as “unknown” upstream of the AAEL005852, was found to be highly homologous to the N-terminal PH domain in both *Culex* and *Anopheles OSBPs*. Primers (Table 1) matched to the predicted 5' coding region of AAEL005862 and to the 3' of AAEL005852 were used to amplify the full-length *AeOSBP*. Seven sequenced cDNA clones of the *AeOSBP* N-terminal PH domain sequence all matched the predicted exons of AAEL005862, indicating that AAEL005862 should be the N-terminal region of the *AeOSBP*. Two cDNA sequences of the seven *AeOSBP* N-terminal PH domain clones from a 4th instar larvae cDNA library contained an extra 90 bps, giving rise to a longer version of the *AeOSBP* variant without corresponding ESTs in the VectorBase. Based on our PH domain cDNA sequences and several ESTs in the VectorBase, the entire span of *AeOSBP* is estimated to be 89,831 bps long (AAEL005862 and AAEL005852) containing 10 exons (Fig. 1B). *AeOSBP* has two isoforms derived from the same gene (Fig. 1A), the longer form, *AeOSBP-B*, (accession number JF309338) contains an extra 30 amino acids of glycine-rich sequence at the N-terminal compared to the short form, *AeOSBP-A* (accession number JF309337).

AeORP8 has two variants based on cDNA clones obtained in this study. *AeORP8-B* variant (accession number JF297970) (Fig. 1) may possibly be generated through alternative splicing or through the use of different promoters, but only the shorter transcript, *AeORP8-A*, was annotated in the genome. ESTs matched to the cDNA sequences of both *AeORP8-A* and *-B* were found in the VectorBase. Therefore, *AeORP8* should contain 10 exons (Suppl. Fig. 1) and exon 5 is where the alternative splicing occurs.

Alignment of the amino acid sequences showed that *AeOSBP* and *AeORPs* contain the oxysterol-binding domain with the conserved motif, EQVSHHPP. *AeOSBP* contains the PH domain at the N-terminus and the FFAT motif (EFYDAQE), which has a single amino acid substitution compared to the consensus sequence EFFDAxE (Loewen *et al.*, 2003). *AeORP8* is the only member of the mosquito OSBP family that has a transmembrane domain (Fig. 1A). When compared to *AeORP8-A*, *AeORP8-B* contains an extra 154 amino acid sequence between the PH domain and the oxysterol-binding domain (Fig. 1A). *AeORP1* and *AeORP9* contain only the oxysterol-binding domain (Fig. 1A).

***AeOSBP* and *AeORPs* showed distinct temporal transcription patterns**

The levels of gene transcription of the internal control (*rpL8*) did not change significantly in eggs through day 1 adult stages based on the Ct values (Table S1), thus the relative mRNA levels of *AeORPs* vs. *rpL8* reflected the changes in the transcription levels of all *AeORPs*. *AeORPs* showed varied temporal transcription patterns based on the data collected from all developmental stages. *AeOSBP* transcription level was very low in eggs. There were significantly higher levels of *AeOSBP* transcription (≥ 2.8 -fold, $p < 0.05$) in 1st-3rd instar larvae than that of in the eggs (Fig. 2A, 1st-3rd), and a further 10-fold increase ($p < 0.05$) in newly molted 4th instar through the middle 4th instar larval stage was detected (Fig. 2A, 4th

0–54h). In the late 4th instar *AeOSBP*, transcription increased 6 fold compared to early 4th instar larvae (Fig. 2A, 4th 60–66h vs. 0–54h) and reached the peak level in newly molted pupae (Fig. 2A, pupae 0h). *AeOSBP* mRNA levels decreased 6 hours after pupation but were up-regulated again in newly emerged adult mosquitoes (Fig. 2A, adults).

AeORP1 mRNA levels increased approximately 10 fold in adults, although *AeORP1* transcription was low throughout larval and pupal stages (Fig. 2B). In the case of *AeORP8*, one forward primer (Table 1) was designed within the conserved region to detect both of the transcripts (i.e. the total *AeORP8* mRNA). Another forward primer (Table 1) was designed specifically for *AeORP8B* transcripts. The results from RT-qPCR analyses showed that levels of *AeORP8-B* transcripts in larvae were almost equal to the total *AeORP8* mRNA (Fig. 2C), indicating that *AeORP8-B* was the major transcript of *AeORP8* in larvae. In pupae and adults, there were no significant differences ($p>0.05$) in *AeORP8-B* and total *AeORP8* transcription levels (Fig. 2C, pupae and adults). *AeORP9* was transcribed at stable levels throughout the larval stages ($p=0.078$), with significantly higher levels in the pupae and adults ($p=0.003$ and 0.005 , respectively) (Fig. 2D).

In larvae, *AeOSBP* and *AeORP9* were the two major transcripts of *AeORPs* when the overall levels of transcriptions were compared (Fig. 2A–D, larval stages). *AeOSBP* transcription levels were significantly higher (2 to 20 fold) than other *ORPs* in newly molted pupae (Fig. 2A–D, pupal stage). *AeORP1* is the major transcript of all *ORPs* in newly emerged adults (Fig. 2A–D, adults). The transcription level of *AeORP8* was lowest among all of the *AeORPs* throughout larval and pupal stages, but its mRNA level was 8 fold higher in adults than that of in larvae or in pupae (Fig. 2C).

***AeOSBP* and *AeORP1* showed tissue-specific transcription patterns**

AeOSBP was highly transcribed in the midgut of newly formed pupae, although there were only slightly higher ($p=0.016$) levels of transcription in the head than in the thorax/abdomen of 24 hour-old 4th instar larvae (Fig. 3A). In sugar-fed female adults, *AeOSBP* mRNA levels were significantly lower ($p=0.02$) in the midgut and the thorax/abdomen compared to the ovary (Fig. 4A, before BM). *AeORP1* showed highly consistent head-specific transcription patterns (Fig. 3B and 4B). *AeORP1* transcript was undetectable in the midgut of 24 hour-old 4th instar larvae and sugar-fed female adults (Fig. 3B and 4B, before BM). The transcription of *AeORP8* and *AeORP9* was ubiquitous and there was no significant difference between different larval and pupal tissues (Fig. 3C and E). In sugar-fed female adults (Fig. 4C, before BM), the mRNA level of *AeORP8* was higher in the head and the ovary than that of in the midgut and the thorax/abdomen ($p=0.011$). *AeORP8-B* showed similar transcription levels in the head and the midgut compared to total *AeORP8* mRNA levels in larvae (Fig. 3C and D, Fig. 4C and D). However, *AeORP8-B* mRNA levels were significantly lower in the pupal thorax/abdomen ($p=0.038$) (Fig. 3D) and the ovary of sugar-fed female adults ($p=0.036$) (Fig. 4D, before BM) compared to corresponding total *AeORP8* levels (Fig. 3C and 4C). The results indicated that *AeORP8-A* was expressed as the main transcript of *AeORP8* gene in the pupal thorax/abdomen and the adult ovary. *AeORP9* was mainly transcribed in the head and the ovary of sugar-fed female adults. (Fig. 4E, before BM)

The transcription of *AeOSBP*, *AeORP8-A* and *AeORP9* were up-regulated after a blood meal

To study the potential roles of *AeORPs* in the female mosquito reproductive cycle, female adults were fed a blood meal 4 days after they emerged. The mRNA levels were analyzed before a blood meal and at 24 h, 48 h, and 72 h after the blood meal. Since the expression of ribosomal protein genes changes during vitellogenesis (Niu & Fallon, 2000), *AeActin-1* gene (Ibrahim *et al.*, 1996) was chosen to verify the consistency in the levels of transcription of

the internal control (*rpL8*) for RT-qPCR analysis in blood-fed females. The levels of *rpL8* gene expression did not change significantly in sugar-fed and blood-fed adult females based on the Ct values of *rpL8* and *Actin-1* at 24 h, 48 h, and 72 h after a blood meal (Suppl. Table 2). Therefore, the relative mRNA levels of ORPs vs. *rpL8* reflected the changes in transcription levels of all ORPs presented in figures 4 and 5. *AeOSBP* transcription was up-regulated significantly at 24 h ($p=0.045$) and reached peak levels ($p=0.002$) with 8 fold increase at 48 h after the blood meal (Fig. 5). *AeORP1* mRNA levels were constant during the gonotrophic cycle (Fig. 5). *AeORP8* and *AeORP9* mRNA levels were significantly higher at 48 h after the blood meal compared to that of before the blood meal (Fig. 5). However, the transcription of *AeORP8-B* was stable, which suggests *AeORP8-A* transcription was induced by the blood meal. The transcription of *AeOSBP* decreased significantly at 72 h after the blood meal from the peak levels at 48 h (Fig. 5), but the transcription of *AeORP9* was still significantly higher ($p<0.05$) than that of before the blood meal.

To determine the spatial transcription patterns of ORPs during vitellogenesis, tissue samples were collected at 48 h after a blood meal and the relative quantities of ORP mRNAs were analyzed. Blood meal-induced transcription of *AeOSBP* was significant in the thorax/abdomen ($p=0.040$) and the ovary ($p=0.048$), while the ovary had the highest level of up-regulated transcription (Fig.4A, BM 48 h). *AeORP1* mRNA levels in the head, which were at least 50 fold higher than that of in other tissues, did not change significantly during vitellogenesis (Fig. 4B, BM 48 h), but *AeORP1* mRNA levels did increase significantly (9-fold, $p=0.002$) in the thorax/abdomen (Fig. 4B, BM 48 h). Transcription of total *AeORP8* and *AeORP8-B* were increased slightly in the midgut after the blood meal ($p=0.032$ and 0.008 , respectively), but dramatically in the thorax/abdomen (6–8 fold, $p=0.001$ and 0.005 , respectively) during vitellogenesis (Fig.4C&D, BM 48 h). *AeORP9* was up-regulated (5 fold, $p=0.019$) in the thorax/abdomen, but not significantly in the ovary ($p=0.165$) (Fig.5E). Overall, spatial transcription patterns showed that *AeOSBP*, *AeORP1*, and *AeORP8-B* significantly increased in thorax/abdomen at 48 h after a blood meal, whereas *AeOSBP* was the only OSBP/ORP genes up-regulated in the ovary by the blood meal. The results were not conflicting with the observation in overall transcription levels of *AeORP1* and *AeORP8-B* in the whole body samples (Fig. 5), since most of their mRNAs were present in the head (Fig. 4B and D).

Over-expression of AeORPs in Aag-2 cells facilitated cellular cholesterol incorporation

To study the role of AeORPs in sterol homeostasis, the oxysterol-binding domain of AeOSBP (Table 1, forward primer F2) and the full length open reading frame (ORF) of four AeORPs (including the two alternative spliced forms, AeORP8A and AeORP8B) were cloned into a constitutive expression vector pIE1^{hr} (Lan *et al.*, 1999) and transiently transfected separately into Aag-2 cells. Transcription of each AeORPs were verified via RT-PCR that indicated dramatically increased levels of each AeORP mRNA in the transient transfected Aag-2 cells compared to empty pIE1^{hr} vector transfected cells (Fig. S2). *AeORP1* and *AeORP8-B* transcripts were undetectable in empty pIE1^{hr} vector transfected cells (Fig. S2), indicating *AeORP1* and *AeORP8-B* expression might be cell type specific. Cells over-expressing an AeORP showed significantly higher ($p<0.001$) cellular levels of [³H]-cholesterol, ranging from 130 % –180 % relative to the empty pIE1^{hr} vector transfected cells (Fig. 6). There were no significant differences between over-expression of *AeORP8-A* and *AeORP8-B* in enhancing cellular cholesterol incorporation (Fig. 6), indicating that the variants may function similarly *in vivo*.

Discussion

AeOSBP and AeORP genes in *Aedes aegypti*

In this study, four genes from the OSBP family were identified in *A. aegypti*. The homologous genes, *DmOSBP*, CG1513, CG5077, and CG3860 were also reported in *Drosophila* (Alphey *et al.*, 1997; Ma *et al.*, 2010). In other species of insects, such as Coleoptera (*Tribolium castaneum*), Hymenoptera (*Apis mellifera*), and Lepidoptera (*Bombyx mori*), the same number of ORP genes are annotated in their perspective genomes. Two isoforms of AeOSBP and AeORP8 were identified via cDNAs isolated in this study and ESTs in the VectorBase. Complementary DNA sequences prove that *AeOSBP* is mis-annotated in the genomic sequence, which showed that AeOSBP has homologous domain architectures (Fig. 1) to that of *DmOSBP* (CG6708), *AgOSBP* (AGAP010893), and *CqOSBP* (CPIJ005418).

AeORP8-A is the only annotated transcript from AeORP8 gene in the *Aedes aegypti* genome and EST sequence of *AeORP8-B* was not found in the VectorBase. *ORP8* in *Anopheles* (AGAP003484) has two predicted splicing variants, whereas *Drosophila* CG5077, the homolog of *AeORP8*, has five predicted splicing variants, including long isoforms containing a PH domain and a short isoform without a PH domain. Two forms of human ORP8 were detected by western blot (Yan *et al.*, 2008). The results indicate that splicing variants of ORP8 genes seem to be a common feature in the ORP family. On the other hand, AeORP8 is the only member containing the transmembrane domain in the AeOSBP family (Fig. 1A), which implies its likely localization to the ER membrane as in the case for the human ORP8 (Yan *et al.*, 2008).

It is still unknown whether there are other splicing variants of insect *ORPs*. AeORP9 is homologous to one of the CG1513 splicing variants from *Drosophila*. CG1513 has another isoform with a PH domain, but the corresponding isoform is not found in the *A. aegypti* genomic sequence or ESTs in the VectorBase. The ORP9 orthologs in *Anopheles* contain both the PH domain and the oxysterol-binding domain, indicating the existence of a longer form of ORP9 in other mosquito species. In addition, it is possible that the PH domain of AeORP9 is missing in the annotated genomic sequence due to gaps in the coverage.

In yeast, each ORP gene performs distinct nonessential functions (Beh *et al.*, 2001; Levine *et al.*, 2001); however, simultaneous knockout of all seven ORPs is lethal in yeast (Beh *et al.*, 2001). Knockout of ORPs individually in *Drosophila* did not show detectable phenotypes for all 3 *DmORPs* except for *DmOSBP* (Ma *et al.*, 2010), suggesting *DmOSBP* family proteins have essential and redundant functions. The AeOSBP protein share 64% sequence identity with *DmOSBP*, but *DmOSBP* does not have any reported splicing variants. Compared to other AeORPs, AeOSBP contains a FFAT motif, which may target the protein to the ER membrane interacting with VAP. *DmOSBP* also has a FFAT motif interacting with FAN, a testis-specific VAP protein, to mediate sterol requirement in spermatogenesis (Ma *et al.*, 2010). Whether AeOSBP interacts with VAP needs further investigation.

The PH domain has high affinity binding to PIPs (Ngo *et al.*, 2010) and might have regulatory function in sterol metabolism in humans (Lehto *et al.* 2001). PH domains of various yeast ORPs contribute to their differing subcellular localizations (Levine *et al.*, 2001), suggesting that the PH domain in ORPs is directly linked to divergent functions of ORPs. Lagace *et al.* (1997) showed that over-expression of the rabbit OSBP decreases cholesteryl ester synthesis through the PH domain in Chinese Hamster ovary (CHO) cells. However, over-expression of a short variant of human ORP4 (ORP4S) inhibits low-density

lipoprotein (LDL)-derived cholesterol esterification in CHO cells (Wang *et al.*, 2002). The exact roles of the PH domain in insect OSBP/ORP are still elusive.

Overall, the numbers of ORP genes identified in insects are the same as that in *Caenorhabditis elegans*, but lower than that found in yeast or human. Reduction of ORP genes in the arthropod lineage compared to the low eukaryote such yeast might be due to the loss of the sterol biosynthesis pathway in arthropods (Behmer & Nes, 2003) since many of the ORP family members are involved in the regulation of sterol biosynthesis (Lagace *et al.*, 1997; Wang *et al.* 2005; Olkkonen *et al.*, 2006).

AeOSBP and AeORPs showed specific transcription patterns

AeOSBP mRNA levels increase dramatically during larval-pupal ecdysis and adult eclosion (Fig. 2A). Surprisingly, it presents mainly in the midgut of newly molted pupae (Fig. 5A). Late 4th instar larvae and pupae are in non-feeding stages. Therefore, high levels of *AeOSBP* in the midgut of newly molted pupae may not function in mediating sterol uptake from the diet, but possibly is involved in other physiological processes during metamorphosis, such as midgut remodeling. *AeORP1* expression showed highly head-tissue specificity (Fig. 5B), which is consistent with the transcription profiles of *AgORP1* (Pinto *et al.*, 2009). Similarly, transcription data in FlyAtlas (<http://flyatlas.org/>) showed that the CG3860 (*DmORP1*) mRNA is enriched in *Drosophila* brain and thoracicoabdominal ganglion. Studies in human ORP genes also found that *ORP1* transcription is sterol-responsive in neuronal cells (Laitinen *et al.*, 1999). It is possible that *AeORP1* functions in mosquito neuronal cells. The cellular localization of *AeORP1* needs to be investigated to determine the cellular specificity of *AeORP1* expression. The transcription of *AeORP8* and *AeORP9* during larval and pupal stages lacks tissue specificities (Fig. 3C-E), which suggests their housekeeping functions.

Sequential events take place in different tissues during vitellogenesis. The blood meal digestion and nutrient absorption occurs within 30 hours after the blood meal (Rudin & Hecker, 1979), lipid transfers from the fat body peaks at 40 hours after the blood meal (Van Heusden *et al.*, 1997), whereas lipid uptake in the ovary reaches the maximal level at 48 hours after the blood meal (Troy *et al.*, 1975). Whether tissue-specific up-regulation of mosquito ORPs changes during different phases of vitellogenesis needs further investigation. During the gonotrophic cycle, the transcription of *AeOSBP* and *AeORP9* were highly induced and peaked at 48 hours post the blood meal, during which the dry weight and total lipid content of ovaries increased sharply (Troy *et al.*, 1975). Our data showed that *AeOSBP* and *AeORP9* transcription are mainly induced in the thorax/abdomen and the ovary at 48 h after a blood meal. However, microarray data from Sanders *et al.* (2003) show *AeOSBP* was also induced in the midgut at 24 h after a blood meal, the same as microarray data for *AgOSBP* (AGAP010893) (Marinotti *et al.*, 2005). Transcription of *AgORP9* (AGAP005510) also showed to be significantly increased from 24 h to 72 h after a blood meal in the ovary (Marinotti *et al.*, 2005). Sixty percent of blood meal-derived fatty acid and sixty-two percent of blood meal-derived cholesterol are deposited into the ovary, respectively (Dyer *et al.*, 2008). Lipophorin, which delivers a majority of lipids (about 90%) to developing oocytes, is involved in intercellular transportation of cholesterol (Sun *et al.*, 2000; Jouni *et al.*, 2003). As cytosolic proteins, *AeOSBP* and *AeORP9* may fulfill the role of intracellular cholesterol transporters and/or sterol storage in developing oocytes. The sterol transport function of *AeOSBP* and *AeORP9* is also suggested in *Aag-2* cells (Fig. 6).

Intracellular sterol transport may involve two main pathways: the vesicular and non-vesicular transport (Hao *et al.*, 2002; Prinz, 2007; Mesmin & Maxfield, 2009; Lev, 2010). Several cytosolic protein families such as the intracellular sterol carrier protein-2 (SCP-2), the steriodogenic acute regulatory protein (StAR), and the ORPs may be involved in the non-vesicular sterol transport pathway (Fairn & McMaster, 2008). Previous studies have

shown that AeSCP-2 plays a role in cellular cholesterol uptake (Lan & Massey, 2004, Blitzer *et al.*, 2004, Dyer *et al.*, 2008, Radek *et al.*, 2010). However, knockdown of AeSCP-2 expression *in vivo* does not completely abolish cholesterol uptake (Blitzer *et al.*, 2004, Dyer *et al.*, 2008), suggesting other cytosolic proteins might also be involved in cellular cholesterol uptake. Over-expression of AeOSBP or any of the AeORPs in cultured cells leads to significantly increased cellular cholesterol uptake (Fig. 6). The results imply that all members of the AeORP family may participate in the cellular cholesterol transport pathway, which is different from the scenario of the AeSCP-2 family in which AeSCP-2 and AeSCP-2 like-2 selectively aid cholesterol and fatty acid uptake, respectively (Dyer *et al.*, 2008). However, the mechanism of OSBP/ORPs-mediated cellular cholesterol uptake is unknown. The over-expressed ORPs may increase the diffusion of membrane-bound free cholesterol to ER facilitating the incorporation of cellular cholesterol, which has been reported for the yeast Osh4 (Raychaudhuri *et al.*, 2006). ORPs may interact with other proteins to enhance the transfer of membrane-bound free cholesterol that in turn mediates cellular cholesterol up take (Im *et al.*, 2005; Du *et al.*, 2011). ORPs may increase the expression of other lipid transport proteins or lipid metabolic enzymes via signal transduction pathways that in turn increased cholesterol uptake (Romeo & Kazlauskas 2008; Hynynen *et al.*, 2009). ORPs may bind to oxysterols that affect partitioning of sterols in the Golgi and other organelles, which indirectly influence the cellular cholesterol distribution (Ngo & Ridgway, 2009). Whether each member of the AeORP family has the same mode of action or each protein acts via different mechanisms is unknown. All *AeORPs* except *ORP1* are transcribed ubiquitously (Fig. 2 and 3), whereas *AeOSBP*, *ORP8A*, and *ORP9* are expressed in *Aag-2* cells constitutively (Fig. S1). It is unknown whether members of the AeORP family form complex to aid intracellular cholesterol trafficking (Zhou *et al.*, 2010). The molecular mechanisms of insect OSBP/ORP-facilitated cholesterol transfer need to be addressed in the future.

Experimental Procedures

Chemicals

Chemicals and reagents were purchased from Sigma (St Louis, MO, USA), Fisher Scientific (Pittsburgh, PA, USA) and ICN (Costa Mesa, CA, USA) unless otherwise mentioned. [1,2-³H (N)]-cholesterol (40 Ci/mMol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA).

Experimental insects

The yellow fever mosquitoes, *Aedes aegypti* (Rockefeller strain), were reared at 26°C in 70–80% humidity at a light:dark cycle = 14:10. Larvae hatching during a 15-min period were collected and fed with fish food (TetraMin, Tetra Holding, Inc., Blacksburg, VA, USA). Adult mosquitoes were fed with 10% glucose. In addition, female adults were fed defibrinated rabbit blood (HemoStat Laboratory, Dixon, CA) 4 days after emergence.

Pharate fourth instar larvae were staged by physical appearance using the visible dark black hairs of fourth stadium larva that are wrapped around the body under the thorax and abdominal cuticle of the third instar (Christophers, 1960). Larvae selected by these criteria ecdysed during a 1-hour period. Pharate fourth instars were selected and transferred into a new container.

Cell cultures

The *Ae. aegypti* Aag-2 cell line was maintained in Eagle's medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (E5 complete medium) at

28°C under a 5% CO₂ atmosphere (Lan *et al.*, 1993). Cells were maintained in T75 flask and passed every 7 days with a 1:19 dilution.

RNA extraction and cDNA synthesis

Ten staged animals were washed with ddH₂O, rinsed once with DEPC-H₂O, and excess water was blotted off using clean Kimwipes. Cleaned animals or tissues were homogenized with a micropestle in 1 ml of Trizol reagent (Invitrogen), and total RNA was extracted according to the manufacturer's recommendations. The quantity of the total RNAs was determined by Nano Drop 1000 (Thermo Scientific) at 260 nm. Five microgram of total RNA was decontaminated from the genomic DNA (gDNA) twice using TURBO DNaseI (Ambion, Austin, TX, USA) at 37°C for 30 min. The gDNA-free RNA samples were quantified once again using the Nano Drop 1000 at 260 nm. Complementary DNA (cDNA) was synthesized from 500 ng gDNA-free total RNA using the high capacity cDNA synthesis kit (Applied Biosystems, Foster city, CA, USA).

Gene cloning

The mosquito ORPs genes were identified via sequence analysis of the genomic sequence. *OSBP* and *ORP* genes were cloned from *Aedes aegypti* via PCR using gene-specific primers (Table 1) based on the predicted genes in the genome. The cDNA library from 4th instar larvae was used as the PCR template. PCR products of AeORPs coding regions were cloned into the pBluntZero vector (Invitrogen) and sequenced. The genes were named based on the homology to human ORPs.

Reverse transcription quantitative real-time PCR (RT-qPCR)

The primers for RT-qPCR were listed in Table 1. All reactions were performed in triplicate in 15 µl volume using iQ SYBRGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). A 50 µl master mix was prepared containing 25 µl SYBRGreen, 1 µl of each 10 µM primer stock and 23 µl of diluted cDNA equivalent to 10 ng of total RNA. Reactions were performed using MyiQ real-Time PCR Detection system (Bio-Rad Laboratories) at 95°C for 3min followed by 40 cycles of 95°C for 1 min and 54°C for 45s. Threshold cycle (C_t) was generated automatically by MyiQ software. The relative transcription level of AeORPs was normalized to ribosomal protein L8 (rpL8). The experiments were performed in 3 replicates for each sample.

[³H]-cholesterol incorporation in transiently transfected *Aedes Aag-2* cells

The coding region of AeOSBP and AeORPs were cloned to pIE1^{hr} expression vectors (Lan *et al.*, 1999) respectively, using the BglII and SalI sites. The plasmids were sequenced to confirm the correctly inserted ORPs and purified using Plasmid Maxi Kit (Qiagen, Germantown, MD, USA). *Aag-2* cells were seeded at 5×10⁵/ml in 35mm diameter dish (Corning Inc. Corning, NY, USA) overnight. Cells were transfected with 8 µg of an over-expression plasmid with 16 µl Lipofectin (Invitrogen) in 1 ml transfection medium (E5 complete minus FBS and antibiotics) for 5 h as described (Vyazunova & Lan, 2010). Then, transfection medium was changed to E5 complete medium. At 24 hours the post transfection, the cells were incubated for another 24 h in the steroid-free E5 medium (Lan *et al.*, 1993). At 48 h the post transfection, media was replaced with 1ml of sterol-free E5 medium containing 0.33µCi [³H]-cholesterol/ml (ARC, St.Louis, MO, USA) and incubated for 16 h. The labeled cells were washed twice with 2 ml cold PBS and then lysed in 100 µl PBS with 1% Triton X-100. The [³H]-cholesterol was extracted with 1 ml hexane:isopropanol (v:v = 3:2) and the extracts were centrifuged at 12,000 rpm for 10 min to pellet the denatured proteins. The [³H]-cholesterol containing organic phase were decanted and air dried. The radioactivities were countered in 5 ml Ultimate Gold

scintillation fluid (Perkinelmer Inc., Waltham, MA, USA) in a liquid scintillation counter. The protein pellets were air dried overnight to remove the residue solvent. The dried proteins were redissolved in 200 μ l 0.2 M KOH at 65 °C and the protein concentrations were determined using the BCA kit (Thermo scientific).

Statistical analysis

Student's t-Test was performed between two samples. One-way ANOVA followed by Turkey HSD Test was performed among different samples. GraphPad Prism (Version 4.0, GraphPad software, La Jolla, CA) was used to perform the statistical analysis and generate the figures. The difference is significant only when $p < 0.05$. All the experiments were replicated at least three times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institute of Health grant (#5R01AI067422) to Q.L. The authors thank Chinese Scholarship Council for fellowship (File No. 2008622013) to Q.F.

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Figure 1.

Aedes aegypti ORP protein family and genomic structure of *AeOSBP*. (A) Domain organizations of AeOSBP and AeORPs proteins. **EQVSHHPP**: conserved motif in oxysterol binding domain; **FFAT**: conserved motif for ER membrane interaction. (B) Genomic architecture of *AeOSBP*. Exons (number 1–10) are connected by introns (the solid line). AeOSBP-A: exon 1 and 3–10, AeOSBP-B: exon 1–10.

Figure 2.

The temporal transcription profiles of *AeOSBP* and *AeORPs* during different developmental stages. **(A)** *AeOSBP*. **(B)** *AeORP1*. **(C)** *AeORP8*. **(D)** *AeORP9*. **1st, 2nd, 3rd, 4th**: First to fourth instar larvae; **h**: hours post molting to the stage; **M**: Male; **F**: Female. Bars= mean $\pm$ S.D. (n=3).

Figure 3. Spatial transcription profiles of *AeOSBP* and *AeORPs* during different developmental stages. (A) *AeOSBP*. (B) *AeORP1*. (C) *AeORP8*. (D) *AeORP8-B*. (E) *AeORP9*. **4th-24h**: Fourth instar 24 hours post molting to the stage; **h**: hours post molting to the stage; **PM**: male pupae; **PF**: female pupae; **AT**: thorax/abdomen without the alimentary tract. Bars= mean $\pm$ S.D. (n=3).

Figure 4. Tissue-specific transcription of *AeOSBP* and *AeORPs* genes before a blood meal (**BM**) and 48 h post the blood meal (**BM 48 h**). (**A**) *AeOSBP*. (**B**) *AeORP1*. (**C**) *AeORP8*. (**D**) *AeORP8-B*. (**E**) *AeORP9*. **AT**: thorax/abdomen without the alimentary tract. Bars= mean $\pm$ S.D. (n=3).

Figure 5.

The transcription of *AeOSBP* and *AeORPs* genes in female adults before and after a blood meal **BM**: blood meal; **h**: hours post the blood meal. Bars= mean $\pm$ S.D. (n=3).

Figure 6. Cellular [³H]-cholesterol levels in *Aag-2* cells over-expressing different pIE1^{hr}/ORPs. Bars= mean ± S.D. (n=3).

Table 1

List of primer sequences

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon
Cloning			
AeOSBP	F1: ATGTCGGAAGTGGTGCCCTC F2: ACGCTACGGGTAGCTCCTCA	TTAGAAAATGTCGGGACACTTGG	
AeORP1	ATGTTTGGTCTTTCTATCGTTTCATCC	TCAAAATATGTCCAGTTCGAACG	
AeORP8	ATGAAATCTTTCTAGGAATCTCGG	TTACGAACTACCGCTGTCACCT	
AeORP9	ATGCAAATTGTGTTTGCGAC	CTATCTTTTACTATCACGTCTCTCCAG	
RT-qPCR			
rpL8	TACCTGAAGGGAACCGTCAAGCAA	ACAATGGTACCTTGGGGCATCAGA	173
actin-1	CCCTGAAGTACCCCAATGAGC	CCATGTCATCCCAGTTGGTG	51
qOSBP	AGCCGCACAGTGCAAAGACACT	TGTCCGGTCGCATTTCGTACGTT	139
qORP1	AGCTTTCTGCAGATGACCGAA	ACCGCGAATGCCGACACATACTT	110
qORP8	AACGGCAGCCGAACGTTCTACATT	TGCAACTGATGCAGAAGCCATCCT	103
qORP8B	GCTACCTAATGAAGTACCTTCCGCCA	TACCGCCAGGTACAGCTGTTGAAA	134
qORP9	TCGACAAAGCAACGAACGCGAA	TCCACGATTCGCCCACTGATTGA	123