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COPI-mediated blood meal digestion in vector mosquitoes is independent of midgut ARF-GEF and ARF-GAP regulatory activities

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Abstract

We have previously shown that defects in COPI coatamer proteins cause 80% mortality in blood fed *Aedes aegypti* mosquitoes by 96 hr post-feeding. In this study we show that similar deficiencies in COPII and clathrin mediated vesicle transport do not disrupt blood meal digestion and are not lethal, even though both COPII and clathrin functions are required for ovarian development. Since COPI vesicle transport is controlled in mammalian cells by upstream G proteins and associated regulatory factors, we investigated the function of the orthologous ADP-ribosylation factor 1 (ARF1) and ARF4 proteins in mosquito tissues. We found that both ARF1 and ARF4 function upstream of COPI vesicle transport in blood fed mosquitoes given that an ARF1/ARF4 double deficiency is required to phenocopy the feeding-induced mortality observed in COPI coatamer deficient mosquitoes. Small molecule inhibitors of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) are often transitory, and therefore, we investigated the role of five *Ae. aegypti* ARF-GEF and ARF-GAP proteins in blood meal digestion using RNA interference. Surprisingly, we found that ARF-GEF and ARF-GAP functions are not required for blood meal digestion, even though both vitellogenesis and ovarian development in *Ae. aegypti* are dependent on GBF1 (ARF-GEF) and GAP1/GAP2 (ARF-GAPs) proteins. Moreover, deficiencies in orthologous COPI regulating genes in *Anopheles stephensi* mosquitoes had similar phenotypes, indicating conserved functions in these two mosquito species. We propose that based on the need for rapid initiation of protein digestion and peritrophic membrane formation, COPI vesicle transport in midgut epithelial cells is not dependent on ARF-GEF and ARF-GAP regulatory proteins to mediate vesicle recycling within the first 48 hr post-feeding.

Keywords

vesicle transport; secretion; vitellogenesis; mortality

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1. INTRODUCTION

Studies in mammalian cells have shown that COPI vesicle transport functions in retrograde transport between the Golgi and ER membrane compartments, whereas the COPII vesicle transport system is primarily required for anterograde transport and clathrin vesicles function in endocytosis (Kirchhausen, 2000). This canonical view of vesicle transport in eukaryotes is likely too limited as COPI vesicle transport has also been shown to control targeting of GPCRs to the plasma membrane, secretion of soluble proteins, and protein targeting to early endosomes and lipid droplet (Guo et al., 2008; Hsu et al., 2009; Rennolds et al., 2008; Styers et al., 2008). Indeed, we have recently found that blood fed *Aedes aegypti* mosquitoes are extremely sensitive to defects in COPI coatomer proteins, which leads to a ~80% mortality by 96 hr post blood meal (PBM) if any one of six coatomer proteins is deficient (Isoe et al., 2011). COPI coatomer protein knockdown disrupts blood meal digestion in the mosquito midgut by disabling protease expression, inducing premature defecation of the blood meal, and allowing midgut contents to leak into the hemocoel. Moreover, our ultrastructural cytological studies revealed that maintenance of rough endoplasmic reticulum (ER) whorls in midgut epithelial cells of female mosquitoes is α COPI-dependent (Zhou et al., 2011). These data suggest that highly specialized COPI secretory functions may be required in mosquito midgut epithelial cells to facilitate the dynamic metabolic challenge of blood meal digestion, which may have been an evolutionary adaptation to a hematophagous lifestyle.

The structure and function of protein components in the COPI vesicle transport system have been well characterized in mammalian cells and shown to consist of seven coatomer proteins (α , β , β' , γ , δ , ϵ , ζ) mediating ER budding and vesicle self-assembly. Small G proteins, known as ADP-ribosylation factors (ARFs), activate COPI transport by recruiting coatomer proteins to the ER membrane (Beck et al., 2009). Based on orthologous genes in other organisms, the *Ae. aegypti* genome encodes the two COPI activator proteins called ARF1 and ARF4 (Donaldson and Jackson, 2011), three ARF guanine nucleotide exchange factors (ARF-GEFs) known as GBF1, BIG1, and ARNO, and two ARF GTPase activating proteins (ARF-GAPs) named GAP1 and GAP2. We recently synthesized and characterized numerous small molecule inhibitors of the ARF-GEF protein GBF1 to study their role in controlling COPI functions in *Ae. aegypti* and *An. stephensi* mosquitoes. We found that two of our GBF1 inhibitors, GCA-8 and GCA-17, disrupted follicular development in blood fed *Ae. aegypti* mosquitoes, whereas five others were highly toxic to 2nd instar larvae of *Ae. aegypti* and *An. stephensi* (GCA-5, GCA-9, GCA-12, GCA-16, GCA-18) (Mack et al., 2012). One limitation of this chemical biology approach was the transitory effect of these GBF1 inhibitors on blood digestion in female mosquitoes, which made it difficult to study long term effects on fecundity and egg viability.

To understand how mosquito COPI vesicle transport is regulated in response to blood feeding, we performed a systematic molecular genetic analysis to investigate the relative contributions of ARF, ARF-GEF, and ARF-GAP proteins on blood digestion and ovarian development. Our results show that both ARF1 and ARF4 stimulate COPI-mediated blood digestion in *Ae. aegypti* and *An. stephensi* mosquitoes. However, unlike COPI vesicle transport in mosquito fat body and ovary tissue, upstream ARF-GEF and ARF-GAP activity is not required to initiate or complete blood meal digestion. The significance of this surprising finding is discussed within the context of acute dynamic processes required to rapidly secrete digestive proteases and peritrophic matrix components into the mosquito midgut shortly after feeding.

2. MATERIALS AND METHODS

2.1. Mosquito rearing

Aedes aegypti and *Anopheles stephensi* mosquitoes were reared in environmental chambers (28°C, 70% RH, and a 16:8 light:dark photoperiod). *Ae. aegypti* larvae were fed a mixture of tropical flakes (TetraMin, Blacksburg, VA), rat chow, and liver powder. *An. stephensi* larvae originated from Dr. Michael Riehle (University of Arizona) were fed cat food pellets. Adult mosquitoes of both species were maintained on 10% sucrose. Four-day-old mosquitoes were allowed to feed human blood donated by the American Red Cross (Tucson, AZ) supplemented with fresh ATP (5.0 mM final concentration) through an artificial parafilm membrane feeder.

2.2 Double-strand RNA (dsRNA) synthesis and microinjection

The gene-specific dsRNA was designed as described previously (Isoe et al., 2009a). Briefly, the T7 RNA polymerase promoter sequence was added to the 5' end of each dsRNA primer (Supplemental table 1). PCR was carried out using GoTaq Green Master Mix (Promega, Madison, WI) with mosquito cDNA derived from whole mosquitoes or midgut tissues. The PCR products were cloned into the pGEM-T easy vector (Promega), and the sequences were verified using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA) at the Genomics Analysis Technology Core facility (The University of Arizona, Tucson, AZ). *In vitro* dsRNA synthesis was performed with the purified PCR product using MEGAscript Kit (Ambion, Austin, TX) following the manufacturer's instructions. The purified dsRNA was resuspended in nuclease-free water at 5.0 µg/µl and stored at -80°C until use. Cold-anesthetized one to two-day old female mosquitoes were injected with 400 ng dsRNA using a Nanoject II microinjector (Drummond Scientific Company, Broomall, PA) unless noted otherwise. Injected mosquitoes were allowed to feed on human blood three days post injection. Fully blood-fed mosquitoes were used and maintained on 10% sucrose, and mortality was scored daily. In experiments shown in figure 4, dsRNA injected mosquitoes were fed with bovine serum albumin (Sigma-Aldrich, St. Louis, MO) or amino acid solution (Sigma-Aldrich) dissolved in mosquito feeding buffer (100 mM NaHCO₃ and 150 mM NaCl, pH 7.2) with a red food color, instead of human blood. Only fully fed mosquitoes inspected under a microscope were used for all mortality studies.

2.3. Time-lapse videography

Mosquitoes were injected with dsRNA targeting γ COPI, ARF1, ARF4, or FLUC control three days prior to blood feeding. Ten fully engorged mosquitoes were then immediately transferred to a glass liquid scintillation vial (20 mL capacity) that was capped with a nylon mesh cover. To precisely determine the time of premature defecation and death of individual mosquitoes as a function of blood meal feeding, we video recorded from 0–60 hours PBM using a SONY Handycam digital camera with a 80 gb internal memory chip. The camera was positioned 30 cm from the glass scintillation vials using a tripod on a table in an environmentally controlled mosquito rearing room (28°C, 70% RH, and a 24:0 light:dark photoperiod). The data captured on the internal memory chip was transferred to a desktop hard drive every 20 hours, which resulted in two ~15 min. gaps in the 60 hour data set. The video was analyzed by Adobe Premiere Pro software (Adobe Systems, San Jose, CA) to score for mortality and the data plotted as hourly time points. During the experiments mosquitoes were allowed to feed on 10% sucrose through the nylon mesh cap. The experiments shown in figure 3 were replicated three times.

2.4. Measuring knock down efficiency of dsRNA

Knockdown efficiency of each injected dsRNA was verified by real-time quantitative PCR (QRT-PCR) as described previously (Isoe et al., 2009a). Briefly, total RNA was isolated from dissected fat body and midgut tissues of individual mosquitoes at 24 h PBM for all genes examined. All RNA samples were treated with DNase I to remove any residual genomic DNA, and cDNA was synthesized using reverse transcriptase and oligo-dT. QRT-PCR was performed using Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA) by Real-Time PCR (7300 Real-Time PCR System, Applied Biosystems). Primers used for the QRT-PCR are listed in Supplemental Table 1. Relative expression from each gene was calculated at 24 hr PBM (96 hr post-injection) using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) and reported as mean values of 10 independent biological replicates in Supplemental Table 2.

2.5. Western blotting

Vitellogenin proteins present in mosquito ovaries were detected as described previously (Isoe et al., 2011; Isoe et al., 2009a). Briefly, western blot analysis was performed by SDS–PAGE using a 12% acrylamide separation gel and a 3% stacking gel. The resolved proteins were transferred to a nitrocellulose membrane (Odyssey Nitrocellulose, LI-COR Biosciences, Lincoln, Nebraska) and treated with Western Blot Signal Enhancer (Thermo Scientific). A mouse monoclonal antibody against *Ae. aegypti* vitellogenin was gift from Dr. Alexander Raikhel (University of California Riverside). A rabbit polyclonal antibody against *An. gambiae* vitellogenin was kindly provided by Dr. Eric Marois (Institut National de la Santé et de la Recherche Médicale, France). Anti α -tubulin mouse monoclonal antibody was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). The primary antibodies were each diluted 1:1000. The secondary antibodies were diluted 1:10,000 and were either IRDye 800CW goat anti-rabbit secondary antibody (LI-COR) or IRDye 800CW goat anti-mouse secondary antibody (LI-COR). The immunoreactive protein bands were detected with an Odyssey Infrared Imaging System. NewBlot™ Nitro Stripping Buffer (LI-COR) was used to remove antibodies as needed.

2.6. Follicle Length Determination

Ovaries were dissected from blood fed mosquitoes at 48 hr PBM and the follicles were carefully separated with a forceps. Photographs were taken of the follicles using a dissecting microscope. A total of five representative follicles were measured from five ovary pairs in each experimental sample.

2.7. Statistical Analysis

GraphPad Software (San Diego, CA) was used for Kaplan–Meier analysis with log rank significance test to analyze mortality data, for unpaired student's *t* test to analyze the efficiency of RNAi-mediated knockdown, and for one-way ANOVA followed by Tukey's multiple comparison test to detect significant differences in follicle length. Asterisks in figures indicate level of significances based on these statistical analyses (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *NS*, not significant).

3. RESULTS

3.1. Blood feeding-induced mortality is selective for deficiencies in COPI vesicle transport

We have previously shown that *Ae. aegypti* mosquitoes undergo blood feeding induced mortality if they are deficient in any of six COPI coatomer proteins (α , β , β' , γ , δ , ζ) (Isoe et al., 2011). Since vesicle transport in mammalian tissue culture cells has been shown to require COPI, COPII, and clathrin mediated vesicle transport (Kirchhausen, 2000), we tested

if deficiencies in COPII and clathrin coatamer proteins also caused blood feeding-induced mortality in mosquitoes. Based on bioinformatic analysis, we isolated *Ae. aegypti* genes encoding the Sec23 COPII coatamer protein (XP_001652783) and the clathrin heavy chain (CLTC) protein (XP_001656876). We then prepared dsRNA targeting each of the corresponding transcripts and injected three day old *Ae. aegypti* mosquitoes with 400 ng of dsRNA as described in Materials and Methods.

As shown in Figure 1A and Table 1, dsRNA targeting transcripts encoding the γ COPI, Sec23, and CLTC coatamer proteins induced 78% mortality in the γ COPI dsRNA injected mosquitoes by 96 hr PBM, consistent with previous findings (Isoe et al., 2011). We also observed that sugar-fed mosquitoes injected with the γ COPI dsRNA died at a faster rate than Fluc control (Figure 1B). However, deficiencies in Sec23 and CLTC proteins was not lethal under these same conditions (<3% mortality). This COPI selective effect was not due to insufficient Sec23 and CLTC knockdown, since ovarian development was inhibited to the same extent at 48 hr PBM in all three experimental samples (figure 1C). Representative midgut dissections revealed that unlike γ COPI dsRNA injected mosquitoes, Sec23 and CLTC dsRNA injected mosquitoes do not interrupt blood meal digestion by premature expulsion of the midgut contents at 24 hr PBM (figure 1D). This may explain why COPI deficiencies, but not COPII or clathrin deficiencies, selectively induce blood feeding mortality since midgut blood meal expulsion is linked to lethality (Isoe et al., 2011). Note that injecting *Ae. aegypti* mosquitoes with very high levels of Sec23 or CLTC dsRNA (>1000 ng) resulted in increased blood feeding-induced mortality, however the mechanism of lethality is unclear given that blood digestion and excretion was normal (data not shown).

3.2. ARF1 and ARF4 regulate COPI vesicle transport in blood fed mosquitoes

ARF1 and ARF4 are the two major G proteins known to regulate COPI coatamer assembly in mammalian cells (Beck et al., 2009), and the orthologous genes are conserved in *Ae. aegypti* (ARF1; XP_001652515 and ARF4; AY431368). Based on the functions of ARF1 and ARF4 in other organisms (Donaldson and Jackson, 2011), we hypothesized that RNAi-mediated knock down of ARF1 and/or ARF4 expression would phenocopy the blood feeding-induced phenotype seen with COPI coatamer deficiencies (Isoe et al., 2011). As shown in figure 2, injecting high levels of ARF1 or ARF4 dsRNA (1000 ng dsRNA) had no significant effect on survival over a 10 day period in either sugar fed (figure 2A) or blood fed (figure 2B) mosquitoes. However, sugar-fed mosquitoes injected with a dsRNA mixture of ARF1 (500 ng) and ARF4 (500 ng) died at a significantly faster rate than control mosquitoes (figure 2A). Blood feeding-induced mortality identical to a γ COPI coatamer knockdown was also observed (compare figures 1B and 2B). As shown in Table 1, statistical analysis using the Kaplan-Meier method showed that an ARF1/4 double knockdown caused significant mortality in blood-fed mosquitoes compared to mosquitoes injected with ARF1, ARF4, or Fluc dsRNA at both 4 days and 7 days PBM ($P < 0.001$, log-rank analysis). These results indicate that ARF1 and ARF4 each contribute to the regulation of COPI vesicle transport in midgut tissue of blood fed mosquitoes, which is consistent with the coordinate induction of γ COPI, ARF1, and ARF4 transcript levels in midgut tissue at 6 PBM (data not shown).

To determine if ARF1 and ARF4 are required for blood meal metabolism in other tissues, we investigated ovarian development and vitellogenin protein accumulation in ARF1 and ARF4 deficient mosquitoes. Even though individual ARF1 and ARF4 deficiencies had no effect on blood meal digestion in midgut tissue (figure 2B), these same ARF1 and ARF4 deficiencies severely inhibited ovarian development (figure 2C). Western blotting of ovary tissue extracts from dsRNA-injected mosquitoes with a vitellogenin-specific antibody revealed that vitellogenin protein accumulation was significantly reduced in the ovaries of ARF1 deficient mosquitoes as compared to Fluc dsRNA injected mosquitoes (figure 2D).

We also observed a decrease in the level of ovarian vitellogenin protein in ARF4 deficient mosquitoes. Not surprisingly, vitellogenin protein accumulation is completely inhibited at 48 hr PBM in surviving ARF1/4 double knockdown mosquitoes (40% survival at 48 hr PBM).

Since each data point used for the survival curves shown in figures 1A and 2B were obtained at 24 hr intervals as a function of blood feeding, it was possible that we missed important temporal differences between the lethal phenotypes in γ COPI and ARF1/4 deficient mosquitoes. Therefore, we used time-lapsed videography to record mortality continuously over a 60 hr timespan (Supplemental Video S1 and S2). By plotting percent survival at hourly intervals, it can be seen in figure 3A that control γ COPI dsRNA injected mosquitoes reached 50% survival (LT50) at 32.0 hrs PBM under these conditions (24 hr light cycle). This decrease in survival was associated with blood meal expulsion in the γ COPI deficient mosquitoes as compared to the Fluc dsRNA injected mosquitoes (supplemental video S1). As shown in figure 3B and supplemental video S2, the LT50 in blood fed ARF1/4 double deficient mosquitoes was 31.5 hrs PBM, and both groups of mosquitoes displayed blood meal expulsion in the first 24 hr PBM (figure 3C). Note that under these 24 hr light cycle growth conditions, ARF1 deficiencies resulted in higher mortality than in ARF4 dsRNA injected mosquitoes ($P < 0.05$, log-rank analysis), suggesting that COPI vesicle transport in midgut tissues is more dependent on ARF1 than ARF4 function as seen in other cell systems (Donaldson and Jackson, 2011).

3.3. Feeding-induced mortality in γ COPI and ARF1/4 deficient mosquitoes is protein-dependent

To determine if midgut engorgement alone is sufficient to stimulate feeding-induced mortality in mosquitoes lacking an intact COPI vesicle transport system, we fed γ COPI and ARF1/4 dsRNA injected mosquitoes feeding buffer (100 mM NaHCO₃ and 150 mM NaCl, pH 7.2) containing 5.0 mM ATP and inert red dye. As seen in figure 4 and Table 2, the mortality rate of these buffer-fed mosquitoes was comparable to sugar fed mosquitoes, and significantly lower than blood fed mosquitoes. Using this buffer feeding assay, we then prepared meals containing an amino acid mixture (30 mg/ml), or bovine serum albumin (BSA) protein at 30 mg/ml or 200 mg/ml in feeding buffer, to determine if one or both of these nutrients stimulated feeding-induced mortality. We found that BSA, but not an amino acid mixture, stimulated feeding-induced mortality to nearly the same extent as blood feeding, with 200 mg/ml BSA being more toxic than 30 mg/ml in these γ COPI deficient mosquitoes (figure 4A and Table 2). As shown in figure 4B, when we tested ARF1/4 deficient mosquitoes using these same artificial meals, we found again that BSA in feeding buffer resulted in a significant increase in feeding-induced mortality compared to feeding buffer alone or feeding buffer containing amino acids (Table 2). Taken together these data support the conclusion that initiation of the protein digestion process in the midgut of fed mosquitoes is required to cause feeding-induced mortality, which is likely dependent on activation of the COPI vesicle transport system. Moreover, since we found that the feeding induced mortality rates in γ COPI and ARF1/4 deficient mosquitoes are nearly identical (Table 2), these data indicate that ARF1 and ARF4 are necessary and sufficient to activate COPI coatamer assembly in midgut epithelial cells.

3.4. Recycling of COPI vesicles is not required in midgut epithelial cells of blood fed mosquitoes

The canonical view of COPI coatamer complex assembly in mammalian cells is that guanine nucleotide exchange factors (GEFs) are required to initiate COPI coatamer assembly by ARF proteins through stimulation of GDP/GTP exchange (Beck et al., 2009). Moreover, COPI coatamer disassembly is thought to be dependent on the activity of GTPase

activating proteins (GAPs), which stimulate GTP hydrolysis in ARF proteins to facilitate vesicle recycling. To determine which GEFs and GAPs regulate ARF1 and ARF4 activity in midgut epithelial cells of blood fed mosquitoes, we isolated cDNA sequences corresponding to the three known ARF-GEFs (GBF1, ARNO, BIG1) and two known ARF-GAPs (GAP1, GAP2) encoded in the *Ae. aegypti* genome. As shown in Table 1, 100% of the mosquitoes injected with dsRNA targeting coding sequences of the ARF-GEF protein GBF1 (XP_001653922) survived at 4 days and 7 days PBM, whereas only 25.7% and 4.4% of ARF1/4 deficient mosquitoes survived at these same two time points. Lack of blood feeding-induced mortality in GBF dsRNA injected mosquitoes was not due to insufficient knockdown in midgut tissue since 10 of 10 mosquitoes showed an average of 88.9% decrease in GBF1 transcript levels (Table S2). Moreover, we found that oocytes in GBF1 dsRNA injected mosquitoes did not reach maturity by 48 hr PBM (Figure 5A), confirming we had achieved a functional GBF1 knockdown. As seen in Figure 5B, quantitative analysis of follicle length showed significantly smaller follicles in GBF1 dsRNA injected mosquitoes as compared to Fluc dsRNA injected mosquitoes at this same time (Figure 5B).

We next tested if ARF1 and ARF4 functions in midgut epithelial cells are regulated by the ARF-GEF proteins BIG1 (XP_001663199) and ARNO (XP_001661955). As shown in Table 1, injection of BIG1 and ARNO dsRNA failed to elicit blood feeding induced mortality in these mosquitoes, despite robust knockdown efficiency in midgut tissue (Table S2). The same result was observed using both double (BIG1/ARNO) and triple (GBF1/BIG1/ARNO) knockdowns of these three ARF-GEF proteins (Table 1). However, unlike the defect in ovarian development we observed in GBF1 deficient mosquitoes, deficiencies in BIG1 and ARNO did not affect ovarian development (Figure 5). These results demonstrate that while none of the ARF-GEF proteins are required for blood digestion by midgut epithelial cells, GBF1 is in fact required for ovarian development.

Lastly, we investigated the role of ARF-GAP proteins in disassembly of COPI coatomer complexes in blood fed *Ae. aegypti* mosquitoes by knocking down expression of the GAP1 (XP_001661085) and GAP2 (XP_001648239) genes. As shown in Tables 1 and S2, efficient single and double knockdown of GAP1 and GAP2 transcripts did not stimulate blood feeding induced mortality, suggesting that these two ARF-GAP proteins are not required for blood meal digestion. In contrast, ovarian development was significantly reduced in GAP1/2 double knockdowns based on follicle length ($P < 0.001$), suggesting that the GAP1 and GAP2 proteins have redundant functions in this process (figure 5).

3.5. COPI vesicle transport processes in *An. stephensi* and *Ae. aegypti* are evolutionarily conserved

To gain a better understanding of COPI vesicle transport during blood meal digestion and vitellogenesis by vector mosquitoes, we examined the functional role of COPI coatomer and COPI regulatory proteins in *An. stephensi* mosquitoes. The orthologous DNA coding sequences for the *An. stephensi* γ COPI, β' COPI, ϵ COPI, ARF1, ARF4, and GBF1 genes were kindly provided by Dr. Zhijian Tu (Virginia Polytechnic Institute and State University). Primers used for the dsRNA synthesis are listed in Supplemental Table 1. One-day old *An. stephensi* adult females were microinjected with dsRNA and fed on human blood through a membrane feeder 3 days after microinjection. We recorded daily mortality, photographed midguts and ovaries at 24 hr and 48 hr PBM, and determined the level of vitellogenin proteins in ovaries at 48 h PBM using a rabbit polyclonal vitellogenin antibody raised against *An. gambiae* (a generous gift from Dr. Eric Marois, Institut National de la Santé et de la Recherche Médicale, France). As shown in Figure 6, injection of dsRNA targeting the *An. stephensi* β' COPI and γ COPI coatomer transcripts stimulated blood feeding-induced mortality that was similar to that of *Ae. aegypti* mosquitoes injected with orthologous β' COPI and γ COPI dsRNA (Isoe et al., 2011). In contrast, deficiencies in the

An. stephensi ϵ COPI coatomer subunit, ARF1, ARF4, and the ARF-GEF protein GBF1, did not decrease survival after blood feeding, whereas a double deficiency in ARF1/4 did cause feeding-induced mortality (figure 6A, 6B).

To determine if the blood digestion phenotypes of ARF1/4 deficient *An. stephensi* and *Ae. aegypti* are functionally equivalent, we examined midgut morphologies at 24 hr and 48 hr PBM and monitored blood meal expulsion using a filter paper assay as previously described (Isoe et al., 2011). As seen in figure 6C, ARF1/4 deficient *Ae. aegypti* mosquitoes display the same blood meal expulsion during the first 24 hrs PBM as reported for γ COPI deficient mosquitoes (Isoe et al., 2011), however this was not the case for ARF1/4 deficient *An. stephensi* mosquitoes at the same time point. In fact, we observed that blood meal digestion appeared to be delayed in ARF1/4 deficient *An. stephensi* mosquitoes as compared to control Fluc dsRNA injected mosquitoes, based on the amount of blood retained in the midguts at 24 hr and 48 hr PBM.

Lastly, we examined the effect of ARF1, ARF4, and GBF1 deficiencies on ovarian development and vitellogenin accumulation in blood fed *An. stephensi* mosquitoes as shown in figure 7. Similar to what we observed in ARF1 and ARF4 deficient *Ae. aegypti* mosquitoes (see figure 2), ovarian development in ARF1 and ARF4 deficient *An. stephensi* mosquitoes was severely restricted, even though these single deficiencies did not result in blood feeding induced mortality. Moreover, GBF1 function was found to be required for ovarian development, but not blood meal digestion, in *An. stephensi* mosquitoes, indicating that regulation of COPI vesicle transport is a conserved process in vector mosquitoes.

4. DISCUSSION

Blood meal digestion in *Ae. aegypti* mosquitoes requires secretion of numerous proteolytic digestive enzymes from midgut epithelial cells, including trypsins and trypsin-like proteases, as well as carboxypeptidases and aminopeptidases (Brackney et al., 2010; Isoe et al., 2009a; Isoe et al., 2009b; Noriega et al., 2002; Noriega et al., 1996). Based on our earlier results showing that RNAi-mediated suppression of midgut protease gene expression decreased rates of blood meal digestion and reduced fecundity (Isoe et al., 2009a), we knocked down expression of COPI coatomer subunits to inhibit protease secretion as a means to more effectively block blood meal digestion. As reported in Isoe et al. (Isoe et al., 2011), we discovered that a COPI coatomer deficiency resulted in blood feeding induced mortality. To better understand how mosquito COPI vesicle transport is regulated in response to blood feeding, we performed extensive molecular genetic analyses in this study to investigate the role of the COPI regulatory proteins in blood meal metabolism.

The clathrin, COPII, and COPI vesicle transport systems in eukaryotic cells function together to transport ER synthesized proteins to cellular destinations, including secretion at the plasma membrane. The basic protein machinery and intracellular functions of these three vesicle transport systems were first described over 25 years ago (Rothman and Wieland, 1996), however cell-specific functions of these vesicle transport systems under homeostatic and regulated conditions is less well understood (Glick and Luini, 2011). To determine if the observed blood feeding induced mortality in COPI deficient *Ae. aegypti* was due to general defects in midgut protein secretion, or a specific vesicle transport process, we compared the effect of coatomer protein knock downs targeting the COPI, COPII, and clathrin systems. Using a dsRNA injection regimen that produced the same level of midgut transcript knockdown of all three coatomer proteins (>85% repression in 90% of injected mosquitoes, Table S2), we found that only COPI defects led to blood feeding induced mortality. Phenotypic analysis of midgut and ovary tissues in these dsRNA injected mosquitoes at 48 hr PBM, revealed that while all three deficiencies blocked ovarian development, only a

COPI deficiency disrupted midgut digestion (Figure 1). This COPI-selective phenotype has been shown to be critical for formation of large rough ER whorls in midgut epithelial cells of unfed mosquitoes in that whorls do not form in α COPI deficient mosquitoes (Zhou et al., 2011). In this earlier study, we found that under normal conditions, rough ER whorls disassemble in response to blood (or amino acid) feeding at a time that is coincident with early trypsin protein synthesis and secretion (Zhou et al., 2011). Based on our finding that COPI coatomer deficiencies causes disruption of the midgut epithelial cell membrane in response to blood feeding (Isoe et al., 2011), unwinding of rough ER whorls at the onset of feeding may be essential for epithelial cell membrane integrity and protein secretion. If COPII and clathrin vesicle formation are not essential for COPI associated events in blood digestion, then it would explain why COPII and clathrin deficiencies are not lethal to blood fed mosquitoes.

A long-term goal of our research is to discover metabolic targets that could be selectively used to control vector mosquitoes in regions of high disease transmission. Since disrupting protein subunit interactions required for COPI coatomer formation by small molecule inhibitors could be challenging, we used molecular genetic approaches to characterize the role of seven COPI regulatory proteins in blood meal digestion. As shown in Table 1, the only single, double, or triple, RNAi knockdowns able to phenocopy a γ COPI knockdown was a double deficiency in the regulatory G proteins ARF1 and ARF4. Deficiencies in the three known ARF-GEF proteins GBF1, ARNO, and BIG1 alone or in combination with ARF1 or ARF4, had no effect on survival in blood fed mosquitoes. The same result was found with deficiencies in the two ARF-GAP proteins GAP1 and GAP2. The requirement that a double deficiency in ARF1 and ARF4 was needed to phenocopy a γ COPI deficiency was consistent with several other reports showing that ARF1 and ARF4 cooperate in COPI vesicle transport (Ben-Tekaya et al., 2010; Volpicelli-Daley et al., 2005), most likely through formation of a heterodimer complex involved in vesicle scission (Beck et al., 2011). With regard to affects in ovarian development, single knockdowns of ARF1, ARF4, and GBF1 were shown to significantly decrease follicle size and vitellogenin accumulation in the ovaries of both *Ae. aegypti* (figures 2 and 5) and *An. stephensi* (figure 7). Based on the proposed function of COPI vesicle transport in lipid droplet homeostasis in other organisms (Guo et al., 2008; Hsu et al., 2009; Rennolds et al., 2008; Styers et al., 2008), one explanation for these ovarian phenotypes in ARF1, ARF4, and GBF1 deficient *Ae. aegypti* and *An. stephensi* mosquitoes is a defect in fat body metabolism, which could directly or indirectly interfere with vitellogenesis.

The most surprising result of these studies was the finding that ARF-GEF and ARF-GAP functions are not required for blood meal digestion, even though deficiencies in GBF1 and GAP1/GAP2 were shown to disrupt ovarian development. COPI coatomer assembly on membranes is dependent on Golgi membrane anchored ARF1 and ARF4 in the active GTP bound form. Following cargo loading and vesicle scission, COPI vesicles attach to target membrane receptors, which stimulates ARF-GAP mediated activation of the intrinsic ARF GTPase activity to generate the inactive ARF1 and ARF4 in the GDP bound form (Beck et al., 2009). The entire process repeats when ARF-GEF mediated exchange of GDP for GTP in ARF1 and ARF4 reinitiates COPI coatomer assembly on the Golgi membrane. This vesicle recycling process by ARF-GAP and ARF-GEF regulatory proteins provides an efficient means to promote ongoing vesicle transport and protein secretion.

What explains the observation that blood meal digestion - but not ovarian development - is independent of ARF-GAP and ARF-GEF regulatory functions? Studies have shown that within 30 minutes of feeding, rough ER whorls in *Ae. aegypti* midgut epithelial cells have been disassembled (Zhou et al., 2011), and by 90 minutes post-feeding, pre-existing early trypsin (AaET) transcripts have been translated to produce secreted AaET protein that is

detectable in the midgut lumen (Brandon et al., 2008). Moreover, by 3 hrs PBM, expression of numerous genes in midgut epithelial cells are highly induced, including the AaSPI, AaSPVI, and AaSPVII midgut protease genes, which are increased >100-fold compared to unfed mosquitoes (Brackney et al., 2010). This rapid increase in protein synthesis, secretion, and gene expression in midgut epithelial cells of blood fed mosquitoes, results in a 90% decrease in serum albumin protein in the lumen by 24 hr PBM (Isoe et al., 2009a). Based on the time scale of these dynamic protein secretory processes in midgut epithelial cells of blood fed mosquitoes, we propose that COPI vesicle recycling by ARF-GEF and ARF-GAP proteins may be occurring but is not rate-limiting. Compare this to homeostatic conditions in mosquito fat body cells, which rely on COPI functions to continually secrete proteins into the hemolymph and to synthesize lipid droplets in the absence of blood feeding. This idea is supported by the observation that sugar fed COPI coatomer and ARF1/ARF4 deficient *Ae. aegypti* (figure 1 and 2) and *An. stephensi* (figure 6) mosquitoes do in fact die at a faster rate than control Fluc dsRNA injected mosquitoes.

Since dsRNA microinjection indiscriminately drives gene silencing in multiple tissues simultaneously, it is difficult to know with certainty if undeveloped oocytes in COPI deficient mosquitoes are due to defects in blood meal digestion in the midgut, vitellogenin synthesis in the fat body, vitellogenin deposition into oocytes, or uncharacterized defects in other tissues. The COPI coatomer and ARF1/4 knockdown phenotype appears to be mainly due to digestion defects in the midgut as premature expulsion of the blood meal is observed by 24 hr PBM. In contrast, GBF1, Sec23 and CLTC dsRNA injected mosquitoes show normal progression of blood meal digestion with regard to defecation, suggesting that the primary defect in vitellogenesis is the result of altered vitellogenin synthesis and/or uptake by the fat body and ovary, respectively. Given the diverse phenotypes associated with COPI and ARF1/ARF4 deficiencies, which include inhibition of digestive enzyme expression, leakage of midgut contents into the hemocoel, and premature defecation (Isoe et al., 2011), it is possible that midgut cells undergo apoptosis at greater rates than occurs normally. Further studies are required to determine rates of midgut epithelial cell apoptosis in mosquitoes with defect in COPI proteins as compared to control mosquitoes.

In summary, we found that blood feeding induced mortality in γ COPI coatomer deficient *Ae. aegypti* and *An. stephensi* mosquitoes can be reproduced by deficiencies in the ARF1 and ARF4 regulatory G proteins. This finding may provide an opportunity for selective metabolic inhibition by targeting the intrinsic GTPase activity in these mosquito proteins. Moreover, we showed that the ARF-GEF protein GBF1, but not ARNO or BIG1, is the upstream regulator of ARF1 and ARF4 activity in blood fed mosquitoes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- COPI vesicle transport, but not COPII or clathrin, is required for blood digestion.
- ARF1 and ARF2 are the primary COPI regulatory G proteins in mosquito midgut cells.
- The ARF-GEF regulatory protein GBF1 is required for ovarian development.
- Blood meal digestion in vector mosquitoes is not dependent on vesicle recycling.

Figure 1. Blood feeding induced mortality in mosquitoes injected with dsRNA targeting COPI, COPII, and Clathrin coatamer subunits

(A) Survival curves of mosquitoes injected with 400 ng γ COPI (COPI), Sec23 (COPII), clathrin heavy chain (CLTC) dsRNA or Fluc control dsRNA at 2 day post eclosion and blood fed at 3 days post-injection and followed for 7 more days (10 days post injection). Kaplan–Meier survival analysis showed that γ COPI knockdown caused significant mortality in blood-fed mosquitoes, compared with mosquitoes injected with Sec23, CLTC, or Fluc dsRNA ($P < 0.001$, log-rank analysis). The number of surviving mosquitoes each day for 10 days post-injection is shown as mean $\pm$ SEM using data from three independent biological experiments. (B) Survival curves of mosquitoes injected as in A except maintained on sugar pads without blood feeding. Kaplan–Meier survival analysis showed that γ COPI knockdown caused significant mortality in sugar-fed mosquitoes, compared with mosquitoes injected with Sec23, CLTC, or Fluc dsRNA ($P < 0.001$, log-rank analysis). (C) Representative photos of mosquito ovaries removed from Fluc, γ COPI, Sec23, and CLTC dsRNA-injected mosquitoes at 48 h PBM. (D) Representative photos of mosquito midguts removed from Fluc, γ COPI, Sec23, and CLTC dsRNA-injected mosquitoes at 24 h PBM.

Figure 2. Blood feeding induced mortality in mosquitoes injected with dsRNA targeting the ARF1 and ARF4 COPI regulatory proteins

(A) Survival curves of sugar-fed mosquitoes injected with dsRNA against ARF1, ARF4, ARF1/4 or Fluc control. Injected mosquitoes were maintained on sugar for 10 days post-injection. The results are reported as mean $\pm$ SEM using data from three independent biological experiments. Kaplan–Meier survival analysis showed that ARF1/4 double knockdown caused significant mortality in sugar-fed mosquitoes, compared with mosquitoes injected with ARF singly, or Fluc dsRNA ($P < 0.001$, log-rank analysis). (B) Survival curves of blood-fed mosquitoes injected as in A. Kaplan–Meier survival analysis showed that ARF1/4 double knockdown caused significant mortality in blood-fed mosquitoes, compared with mosquitoes injected with ARF singly, or Fluc dsRNA ($P < 0.001$, log-rank analysis). (C) Representative photos of mosquito ovaries from Fluc, ARF1, ARF4, and ARF1/4 dsRNA injected mosquitoes at 48 h PBM. (D) Western blot analysis of vitellogenin protein

present in ovaries of blood-fed mosquitoes at 48 h PBM. Protein loading was monitored using an anti- α tubulin antibody.

Figure 3. Analysis of time-lapsed videography data collected from mosquitoes injected with dsRNA targeting γ COPI, ARF1, and ARF4

Mosquito behavior was recorded for 60 hr PBM under standard conditions of temperature and humidity sugar feeding ad libitum. Survival curve data were compiled from three independent biological experiments. (A) Hourly survival curves of mosquitoes injected with Fluc or γ COPI dsRNA. Kaplan–Meier survival analysis showed that a loss of γ COPI by RNAi caused significant mortality, compared with mosquitoes injected with Fluc dsRNA ($P < 0.001$, log-rank analysis). See supplemental data Video S1. (B) Hourly survival curves of ARF1, ARF4, and ARF1/4 dsRNA injected mosquitoes. The ARF1/4 double RNAi knockdown showed significant mortality, compared with mosquitoes injected with ARF1 or ARF4 dsRNA alone ($P < 0.001$, log-rank analysis). See supplemental data Video S2. (C) A screen capture of time-lapse movie of *Ae. aegypti* mosquitoes injected with dsRNA

targeting ARF1, ARF4, and ARF1/4 at 24 hr PBM. More fecal drops were observed on a bottom of the glass liquid scintillation vial in dsRNA targeting both ARF1/4 than ARF1 or ARF4 alone. Arrow is pointing to fecal drops.

Figure 4. Feeding affects on mortality in mosquitoes injected with dsRNA targeting γ COPI, ARF1, and ARF4

(A) Mortality studies with mosquitoes injected with dsRNA against γ COPI three days prior to feeding the indicated meals; feeding buffer containing BSA at two different concentrations (30 mg/ml or 200 mg/ml), an amino acid mixture, feeding buffer alone, human blood, or 10% sugar. The number of surviving mosquitoes each day is shown as mean $\pm$ SEM using data from three independent biological experiments. (B) Mosquitoes were injected with ARF1/4 dsRNA and subjected to the same feeding regimens as in (A).

Figure 5. Representative photos of ovaries at 48 h PBM isolated from *Ae. aegypti* mosquitoes injected with the indicated dsRNA species

Mosquitoes were injected dsRNA targeting the ARF guanine nucleotide exchange factors (GBF1, BIG1, and ARNO), the ARF GTPase activating proteins (GAP1 and GAP2), with or without dsRNA targeting ARF1 or ARF4. (A) Photographs of representative ovaries dissected from GBF1, BIG1, ARNO, ARNO/BIG1, GAP1, GAP2, GAP1/GAP2, and Fluc dsRNA-injected mosquitoes at 48 h PBM. (B) For follicle length measurement, ovaries were dissected from the injected mosquitoes in 1X PBS under a microscope at 48 h post blood meal. Follicles were separated from the ovarian tissue and digitally captured. A total of 25 follicles were measured for each experimental condition (five follicles each from five injected mosquitoes) and the resulting data were statistically analyzed by one-way ANOVA. Asterisks indicate significant differences (** $P < 0.01$; *** $P < 0.001$; NS, not significant compared to the Fluc dsRNA control mosquitoes). The error bars indicate SEM.

Figure 6. Mortality and midgut phenotypes of *An. stephensi* mosquitoes injected with dsRNA targeting γCOPI, ARF1, and ARF4

(A) Survival curves of sugar-fed *An. stephensi* mosquitoes injected with dsRNA targeting β'COPI, γCOPI, εCOPI, ARF1, ARF4, ARF1/4, GBF1, or Fluc. Injected mosquitoes were maintained on sugar for 10 days post-injection. The results are shown as mean ± SEM using data from three independent biological experiments. (B) Survival curves of blood-fed *An. stephensi* mosquitoes injected as above in (A). Kaplan–Meier survival analysis showed that β'COPI, γCOPI, and ARF1/ARF4 knockdown caused significant mortality in blood-fed mosquitoes as compared with mosquitoes injected with Fluc control dsRNA ($P < 0.001$, log-rank analysis). (C) Representative photos of *Ae. aegypti* and *An. stephensi* midguts

dissected from ARF1/4 and Fluc dsRNA-injected mosquitoes at 24 and 48 h PBM. Also shown are representative photos of fecal drops on filter paper removed at 24 and 48 h PBM from vials containing five fully engorged blood-fed mosquitoes, which had been injected 3 days earlier with ARF1/4 or Fluc dsRNA.

Figure 7. Ovarian development and vitellogenin accumulation in *An. stephensi* mosquitoes injected with the indicated dsRNA species

(A) Representative photos of mosquito ovaries (48 h PBM) from Fluc, ARF1, ARF4, ARF1/4 and GBF1 dsRNA injected mosquitoes. (B) Western blot analysis of vitellogenin protein present in ovaries of blood-fed mosquitoes at 48 h PBM. Protein loading was monitored using an anti- α tubulin antibody.

Table 1Survival analysis of dsRNA injected *Ae. aegypti* mosquitoes

Injected dsRNA	4 days PBM	7 days PBM	[†] Statistics
Fluc	100.0 ± 0.0	100.0 ± 0.0	-
γCOPI	22.1 ± 7.2	6.7 ± 4.2	$P < 0.001$
ARF1	91.9 ± 2.2	87.7 ± 0.0	<i>NS</i>
ARF4	93.0 ± 0.3	86.0 ± 0.7	<i>NS</i>
ARF1/ARF4	25.7 ± 5.3	4.4 ± 2.6	$P < 0.001$
GBF1	100.0 ± 0.0	100.0 ± 0.0	<i>NS</i>
ARNO	100.0 ± 0.0	97.4 ± 2.6	<i>NS</i>
BIG1	100.0 ± 0.0	97.8 ± 2.2	<i>NS</i>
ARNO/BIG1	100.0 ± 0.0	100.0 ± 0.0	<i>NS</i>
GBF1 / ARNO/BIG1	100.0 ± 0.0	97.2 ± 2.8	<i>NS</i>
GBF1/ARF1	94.9 ± 7.3	89.2 ± 5.1	<i>NS</i>
GBF1/ARF4	97.6 ± 2.4	90.0 ± 2.2	<i>NS</i>
GAP1	100.0 ± 0.0	100.0 ± 0.0	<i>NS</i>
GAP2	100.0 ± 0.0	100.0 ± 0.0	<i>NS</i>
GAP1/GAP2	100.0 ± 0.0	97.8 ± 2.2	<i>NS</i>
GAP 1/GAP2/ARF1	90.3 ± 2.3	87.9 ± 1.7	<i>NS</i>
GAP 1 /GAP2/ARF4	94.7 ± 2.7	89.3 ± 3.0	<i>NS</i>

The number of surviving mosquitoes for 4 d and 7 d PBM is shown as mean ± SEM using data from three independent biological experiments

[†]Kaplan-Meier analysis with a log-rank significance test was used to analyze mortality curves compared with Fluc control.

Table 2Effect of different food types on the survival of dsRNA injected *Ae. aegypti* mosquitoes

Injected dsRNA Food types	γ COPI		ARF1/ARF4	
	LT ₅₀	[†] Statistics	LT ₅₀	[†] Statistics
Sugar	-	-	-	-
Feeding buffer	10	NS	-	NS
Amino acid	10	NS	-	NS
Blood	5	$P < 0.001$	6	$P < 0.001$
BSA (30 mg/ml)	7	$P < 0.001$	8	$P < 0.001$
BSA (200 mg/ml)	6	$P < 0.001$	7	$P < 0.001$

LT₅₀ indicates the median lethal time (day).[†] Kaplan–Meier analysis with a log-rank significance test was used to analyze mortality curves compared with sugar-fed control.