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Induction of actin gene expression in the mosquito midgut by blood ingestion correlates with striking changes of cell shape

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Abstract

Ingestion of a blood meal by the female mosquito *Anopheles gambiae* (L., Diptera: Culicidae), results in a dramatic distention of the midgut epithelium. Here we report that these events correlate with a transient increase of actin mRNA and protein abundance. The newly synthesized actin may provide a pool of actin protein needed to remodel epithelial cell cytoarchitecture. We also document changes in midgut epithelial cell morphology. Upon blood ingestion, the columnar cells flatten accompanied by the loss of microvilli on the luminal side and the unfolding of the labyrinth on the basal side. These changes correlate with the large increase of epithelial surface area needed to accommodate the blood meal. Actin gene expression, actin synthesis and cell morphology all return to the pre-feeding state by 24 h after blood intake.

Keywords

actin; *Anopheles gambiae*; midgut; microvilli; blood feeding

1. Introduction

Anopheles gambiae (L., Diptera: Culicidae) is one of the major vectors for human malaria, a disease that kills between 1 and 2 million people every year (Snow et al., 2005). Transmission is initiated when the mosquito ingests a blood meal from a parasitemic individual. In the mosquito, the parasite follows a complex developmental program that includes transformation into multiple morphological forms and the crossing of the midgut and salivary gland epithelia (reviewed by Gosh et al., 2000). Ingestion of a blood meal also triggers a number of dramatic morphological and biochemical changes in the midgut epithelium. The volume of blood

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ingested by the mosquito is very large (2 to 3 ul; Calheiros et al., 1998), in excess of its own body weight. Ingestion of a blood meal induces the secretion of the chitin-containing peritrophic matrix (PM), which is a thick acellular layer that completely surrounds the blood meal (Jacobs-Lorena and Oo, 1996; Tellam et al., 1999; Shao et al., 2001). In anophelines, there is evidence to suggest that PM components are stored in secretory vesicles and released into the lumen upon blood ingestion (Devenport et al., 2004). Blood uptake also triggers a complex program of gene expression that includes the activation and/or up-regulation of genes involved in blood digestion and nutrient uptake (Muller et al., 1993; Lemos et al., 1996; Noriega and Wells, 1999; Holt et al., 2002; Dana et al., 2005; Marinotti et al., 2006).

We used the differential display approach to identify genes that are activated in the midgut epithelium by blood ingestion. Two genes were found to be significantly up-regulated at 3 h after blood meal. One is *An. gambiae* carboxypeptidase (*AgCP*) gene. *AgCP* mRNA is rapidly (~ 3 h) and strongly (~ 10 fold) induced by blood ingestion (Edwards et al., 1997). The second gene, *An. gambiae* cytoskeletal actin, is the focus of the present communication. Relative changes of actin mRNA and protein expression in the mosquito midgut before and after ingestion of a blood meal correlate with prominent changes of midgut cell architecture.

2. Materials and methods

2.1. Mosquitoes

An. gambiae G3 strain were raised as described by Lemos et al., (1996).

2.2 Differential display

Differential display was used to identify mRNAs whose abundance increased after blood ingestion as described in detail by Edwards et al., (1997). Briefly, duplicate RNA samples were prepared from female guts prior to (0 h) and at 3 and 24 h after a blood meal. Two independent RNA samples were analyzed for each time point for a total of 6 samples. Each sample was amplified by RT-PCR using random primers and the resulting fragments were separated by electrophoresis on a 6% polyacrylamide sequencing gel. DNA bands preferentially amplified in the 3-hour samples were isolated and re-amplified by PCR using the same primers. To verify increased expression at 3 h, these fragments were used as probes for Northern blots of RNAs from pre- and post-blood fed mosquitoes. PCR fragments that by visual inspection gave stronger signals with RNAs from the 3 h sample compared to 0 and 24 h samples were used as probes to screen an *An. gambiae* cDNA library. The selected clones were sequenced and further characterized (Lemos et al., 1996).

2.3. Northern blot analysis

Midguts were dissected at different times after a blood meal and immediately transferred to a tube floating in a dry ice/ethanol bath. Samples were stored at -85 °C until use. RNA was extracted and analyzed on Northern blots as described (Lemos et al., 1996). The midguts were isolated from females from a single blood-feeding. Approximately 0.6–0.8 ug RNA/midgut (Edwards et al., 1997) were obtained and 5ug (equivalent to 6 midguts) were loaded on the gel for electrophoretic separation and subsequent transfer to GeneScreen membrane for hybridization with the appropriate probe. The actin probe was made to a full length actin cDNA, isolated from an *An. gambiae* cDNA library. Five ug of *An. gambiae* cDNA large mitochondrial rRNA subunit was used as a loading control (Lemos et al., 1996). A total of 3 independent experiments were performed yielding reproducible results.

2.4. Western blot analysis

Guts were dissected as for Northern blot analysis. The guts were thoroughly rinsed with saline to remove the blood-derived luminal proteins, thus avoiding high protein background and potential interference with Western blots. For immunoblotting, 20 guts were suspended in 80 μ l of 1X Laemmli buffer (Laemmli, 1970), boiled for 10 min and 24 μ l (equivalent to 6 guts) were separated by electrophoresis using 3 millimeter-thick acrylamide gels (Ausubel et al., 2001). Ten μ l of pre-stained protein molecular weight standard solution (Sigma) were also loaded. Initially gels were run in duplicate, one to assess protein mass by Coomassie Blue staining, and the other for immunoblotting. In later experiments, the same gel was first stained with Coomassie Blue and then processed for Western blotting according to Dionisi et al., (1995).

Immunoblotting was as described by Ausubel et al., (2001), with minor modifications. Blocking was in 20 ml of 1% powdered milk in filtered 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.1% Tween (TTBS) for 1 h with gentle agitation. The membrane was rinsed 3 times for 5 min each with about 30 ml TTBS with gentle agitation. The membrane was then incubated for 1 h with gentle agitation in 20 ml of rabbit anti-actin (Sigma, A-2066; Lot # 045H4802) diluted 1:200 in filtered TTBS, followed by 3–5 washes as above. The membrane was then incubated for 1 h in 20 ml of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, A-9919; Lot # 076H4858), diluted 1:10,000 in filtered TTBS and washed as above. Detection of alkaline phosphatase was as described (Dionisi et al., 1995). Three replicate Western blot experiments were performed on midgut proteins isolated from females from a single blood-feeding.

The rabbit anti-actin (Sigma, A-2066) was raised against a synthetic oligopeptide made to 11 amino acid residues at the actin C-terminus (Tam, 1988). This particular amino acid sequence is present in nearly all actins and conserved in most species (Vandekerckhove and Weber, 1984; Altschul et al., 1997). Immunoblotting, performed by Sigma, showed that the purified product recognized the 42 kDa actin band and localized actin in several species ranging from humans to amoeba (Sigma Immuno-File, A-2066).

2.5. Electron microscopy

Midguts from female *An. gambiae* were dissected at different times after a blood meal. The guts were fixed according to Sieber et al., (1991), with the following modifications. Guts were fixed for 2 hr with 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4 and 4% (w/v) sucrose, and postfixed for 2 h in 1% osmium tetroxide in 0.1 M phosphate buffer. The tissues were block-stained in 0.5% aqueous uranyl acetate for 6 h at 4 °C, dehydrated in ascending concentrations of ethanol and embedded in Epon 812 (Sieber et al., 1991). Thick sections of the middle region of the midguts were examined by light microscopy to confirm that microvilli were sectioned longitudinally. Ultrathin sections were stained with 2% uranyl acetate and lead citrate, and then examined with a Zeiss CEM902 electron microscope (Oberkochen, Germany).

3. Results

Using mRNA differential display analysis, we compared transcripts in midguts dissected before a blood meal with those in midguts at 3 and 24 h after blood feeding. DNA fragments that appeared to be preferentially amplified in the 3 h sample were excised from the gel, re-amplified and used as probes on Northern blots to confirm the initial observations. A total of 94 candidate PCR fragments were screened in this way. Of these, the DNA from three bands hybridized on Northern blots preferentially with RNA from the 3 h sample. These three probes were used to screen a midgut cDNA library. Sequencing of the corresponding cDNAs indicated that one encoded a carboxypeptidase (Edwards et al., 1997) and that the other two encoded a

cytoplasmic actin, named act1D (Salazar et al., 1994; GenBank accession number XM_001230771). The identity of this cDNA was established by sequencing, its expression pattern and the organization of the gene, all of which were most similar to the characterized cytoskeletal actin, act5C (GenBank accession number P84185) in *Drosophila* (Fyrberg et al., 1980; Sodja et al., 1982; Fyrberg et al., 1983; Salazar et al., 1994).

Changes in actin mRNA abundance as a function of time after a blood meal were assessed by Northern blot analysis of RNA extracted from midguts at different times after a blood meal (Fig. 1A). Mitochondrial rRNA provided a convenient and accurate loading control because the number of mitochondria does not significantly change within the first 24 h after a blood meal (Lemos et al., 1996). As expected, actin mRNA was abundant in midguts of non-blood fed mosquitoes. Actin mRNA abundance consistently increased soon after blood ingestion, peaked at around 4 to 6 h, and slowly decreased to levels present prior to the blood meal. At peak expression (4 ~ 6 h), gut actin mRNA abundance was about 2.5-fold higher than prior to a blood meal (Fig. 1B). Taking into account the substantial basal amounts of actin mRNA, this up-regulation represents a significant increase of actin mRNA content in response to blood ingestion.

Next, we sought to determine whether the observed changes in actin mRNA accumulation correlated with increased protein abundance. Proteins extracted from midguts dissected before and at different times after a blood meal were fractionated by gel electrophoresis. Luminal contents were washed away prior to protein extraction to ensure that any observed changes of actin content reflected changes occurring within the gut tissue. An identical aliquot of each sample was analyzed on polyacrylamide gel and stained with Coomassie Blue to verify that equal amounts of protein were loaded on each lane (Fig. 2, lower panel). The same gel was destained (Dionisi et al., 1995) and used for Western blot analysis with anti-actin antibody (Fig. 2, upper panel). As shown in Fig. 2, there was a modest increase in actin protein content that displayed nearly identical time profile of synthesis as that of the mRNA (Fig. 1). The two slower migrating, cross-reacting protein bands (Fig. 2, upper panel) may be a product of post-translational modifications of actin or may represent expression of actin-related proteins (ARPs), some of which are involved in actin filament organization and assembly (Schafer and Schroer, 1999; Muller et al., 2005).

Mosquitoes routinely ingest blood meals that exceed their own body weight (Calheiros et al., 1998). To accommodate this large volume of blood, the midgut inflates dramatically (Fig. 3), due to a remarkable distention of the epithelial cells and the muscle wall of the midgut. Measurements of the ultrastructural changes, including microvillar reorganization, in the midgut of hematophagous insects after a blood meal have been studied (Billingsley, 1990). Although the sequence and pattern of changes in these insects after blood meal ingestion are similar, differences in timing and extent of such changes, however, appear to be somewhat species specific (Houk and Hardy, 1982). Thus, it was important to analyze changes in *An. gambiae* female midguts as a function of time in order to demonstrate that any morphological alterations observed correlated temporally with molecular changes in actin gene transcription and translation as well as with the induction of AgCP after a blood meal ingestion.

Electron microscopy was used to investigate more closely the changes of cell morphology (Fig. 4, A–D). Morphometric analysis of cells such as those shown in Fig. 4A and B (note that both panels are shown at the same magnification) indicated that blood ingestion caused a 5- to 6-fold reduction in cell height, from 38 ± 6.2 μm to 6.8 ± 1.9 μm . These data are based on measurements made on 25 cells with similar size nuclei coming from 3 different midguts dissected prior to and 15 min after a blood meal. The increase in surface area on the basal side is provided by partial unfolding of the highly convoluted basal labyrinth (Fig. 4A and B). Although difficult to quantify, the thickness of the basal lamina appears to decrease by about

2-fold to accommodate the expansion. On the luminal side, the increase of surface area appears to be provided by reduction of microvillar size (Fig. 4A and B) and density (Fig. 5). The loss of microvilli is rapid and extensive (Fig. 5). As the volume of the bolus decreases by digestion and diuresis, the midgut starts reverting to its original shape with concomitant gain of microvillar density (Fig. 5). As noted previously (Billingsley and Rudin, 1992), the number of apical vesicles drops dramatically within the first 15 min of feeding (Fig. 4C and D), indicating the release of stored molecules. Recent experiments indicate that these vesicles contain components of the peritrophic matrix and digestive enzymes (Devenport et al., 2004).

4. Discussion

Actins are ubiquitous proteins found in all eukaryotic cells. They are highly conserved in evolution at both the nucleic and amino acid sequence levels (Fyrberg et al., 1980; Zafar and Sodja, 1983; Mounier et al., 1987; Hadden and Sodja, 1994). In contrast to the high conservation of their amino acid sequence, actins have a broad spectrum of functions. These include determination of cell shape, axonal guidance, muscle contraction, cell and chromosome movement, cytokinesis, cytoplasmic streaming, phagocytosis and intracellular signaling (Machesky and Insall, 1999; deRuvrois and Goffinet, 1999; Pollard et al., 2000; Fox, 2001). Most of these functions depend on the organization of actins into filaments and their interactions with a large repertoire of actin-binding proteins (Pollard and Cooper, 1986; Higgs and Pollard, 2001).

In intestinal microvilli, organization of actin filaments into bundles by proteins such as the microvillar-specific villin, provide structural rigidity (Bretscher, 1983; Cooper, 2000). The apical surface of the human small intestinal cell typically has several thousand such microvilli, increasing the absorptive surface area by about 10- to 20-fold (Cooper, 2000). This increase, however, does not appear to involve any major morphological changes in intestinal microvillar organization of mammals after food ingestion.

Blood ingestion by hematophagous insects triggers drastic reprogramming of gene expression. For instance, secretion of the mosquito protective peritrophic matrix and synthesis of most mosquito digestive enzymes is activated by feeding. Thus it was not unexpected that the differential display screen identified a carboxypeptidase as one of the genes induced by the blood meal (Edwards et al., 1997). The identification of actin as a gene induced early after the blood meal was initially surprising. However, the observed dramatic morphological changes of cell shape, and therefore of the cytoskeleton, that result from the blood meal provide a rational explanation for the observed induction of actin gene expression. We hypothesize that the remodeling of the cytoskeleton may involve actin degradation followed by its re-synthesis using the newly transcribed actin mRNA as a template. cDNA microarray analysis of transcripts isolated female *An. gambiae* prior to and at several time intervals after blood-meal ingestion, indicated that nearly half of the genes induced or up-regulated in expression within the first 5 h interval after blood ingestion, were involved in extracellular and intracellular structure, including the cytoskeletal actin (Dana et al., 2005). In diapausing or in diapausing cold-stressed adult mosquito, *Culex pipiens*, upregulation of 2 actin genes was observed as well as the cytoskeletal actin filament redistribution, especially prominent in the midgut (Kim et al., 2006).

The Western blot (Fig. 2, upper panel) indicates one to two higher molecular weight proteins cross-reacting with the actin antibody. These may be due to post-translational changes of actin itself or to the presence of actin-related proteins ARP2 and 3, both of which are required as an ARP2/3 complex in spatial and temporal control of actin assembly by binding to the first actin monomer of the nascent actin filament (Beltzner and Pollard, 2004; Muller et al., 2005). ARP 2 and 3 are cytoplasmic sharing >60% sequence identity and >90% sequence similarity with

the conventional actin (Muller et al, 2005). The weaker antigen-antibody interaction may reflect insufficient sequence identity in amino acid residues critical for such interactions or lower amounts of antigen. Although not detected in our studies, it is possible that the expression of other genes encoding cytoskeletal proteins involved in microvilli re-organization, such as villin, fimbrin and myosin is similarly regulated.

A tantalizing alternative hypothesis for induction of actin gene expression is based on several pieces of experimental evidence, which indicates that cytoskeletal remodeling is causally connected with changes in gene expression. Several reports of data suggest that alteration of cytoskeleton/cell shape alter the expression of lactoferrin (Close et al., 1997), of plasminogen activator inhibitor type-1 (PAI-1) (Providence et al., 1999) and of serum response factor (SRF) (Sotiropoulos et al., 1999). In case of the PAI-1 and SRF genes, the data demonstrated a direct link between remodeling/reorganization of cytoarchitecture and gene expression. Upregulated actin gene expression and the redistribution of actin filaments in *Cx. pipiens* in diapause and/or cold stress is thought to fortify the cytoarchitecture, increasing chances of survival during frigid winter temperatures (Kim et al., 2006). It is possible that the observed actin filament redistribution may be involved in additional functions, required for survival under diapause and/or cold conditions. Thus, in our case, the induction of actin, carboxypeptidase, other digestion-related genes, and the secretion of peritrophic matrix components by blood ingestion may also be causally linked to the observed cytoskeletal changes.

All measurements of gene expression were made with whole midguts, which include both epithelial and smooth muscle cells. Hence, it was not possible to determine the contribution of each cell type to the total actin mRNA and protein pool. Nonetheless, microscopy of gut sections suggests that the total cytoplasmic mass contributed by the muscle cells is small relative to that of the epithelial cells, suggesting that our measurements are representative of the changes of actin gene expression mostly in the midgut epithelial cells. Moreover, the gene identified by our studies is nearly identical to the ubiquitous cytoplasmic *An. gambiae* actin, characterized in detail by Salazar et al., (1994). Sequencing of this gene, named act1D (GenBank accession number XM_001230771) indicated that it has 99.2% sequence identity with the cytoplasmic/cytoskeletal *Drosophila* actin, act5C (GenBank accession number P84185) in addition to its gene structure and expression profile (Fyrberg et al., 1980; Sodja et al., 1982; Fyrberg et al., 1983; Salazar et al., 1994).

The large increase of midgut surface area induced by the blood feeding could be provided by new membrane synthesis or by unfolding of the microvilli and basal labyrinth. We favor the latter alternative because new membrane synthesis is likely to be too slow to accommodate the immediate need for gut expansion. The present results support the model that increased surface area is provided on the luminal side by reduction in microvillar size and number, and on the basal side by the partial disassembly of the basal labyrinth. Moreover, fusion of the apical vesicles with the luminal membrane during exocytosis (Fig. 4C and D), may also contribute to the increase of membrane surface. We note that the increased levels of actin mRNA and protein, which peak around 4 h after a blood meal, precede the reappearance of microvilli (Fig. 5). The increase in actin synthesis after ingestion of a blood meal may provide a pool of G actin for reassembly of microvilli.

Whereas morphological changes in midguts of several hematophagous insects after blood ingestion have been observed (Rudin and Hecker, 1979; Houk and Hardy, 1982; Billingsley, 1990; Okuda et al., 2002), we provide for the first time a quantitative and temporal assessment of the morphological changes incurred after ingestion of a blood-meal (Fig. 5), while correlating them with the expression of actin, a key component of the cytoskeleton. As in the cases of genes mentioned above, it is possible that the changes observed in the actin dynamics

may trigger reprogramming of gene activity after blood ingestion. The next challenge will be to elucidate at the molecular level, the mechanisms that regulate these changes.

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Fig. 1.

Actin mRNA abundance increases in response to blood ingestion. (A) Total midgut RNA (5 ug) was fractionated by electrophoresis on a denaturing agarose gel and transferred to a membrane. Bound RNAs were hybridized with actin and with mitochondrial rRNA probes (loading control). Time before (0 h) and after a blood meal is indicated above each lane. (B) The actin hybridization signal was quantified by densitometry and normalized to the loading control.

Fig. 2. Actin protein abundance increases modestly after a blood meal. Protein equivalent to six midguts was fractionated by electrophoresis on SDS-polyacrylamide gel and stained with Coomassie Blue (lower panel). The same gel was then electroblotted onto a membrane and the 42 kDa actin protein detected with anti-actin antibody (upper panel).

Fig. 3.

Size comparison of midguts dissected before and after blood ingestion. The two images provide visual evidence for the dramatic distension caused by the ingestion of a blood meal. The top is a gut from non-blood fed and the bottom from a blood-fed mosquito. Both guts were photographed together.

Fig. 4.

Electron microscopy reveals dramatic changes of epithelial cell morphology in response to blood ingestion. (A) Cross section of a midgut cell from a female *An. gambiae* mosquito dissected before blood feeding. The luminal surface (bottom) is densely lined by long microvilli (MV), the nucleus (N) is centrally located, and the cytoplasm is rich in mitochondria (Mt). An extensive labyrinth network (L), covered by a basal lamina (BL), is visible at the basal side (top). (B) Cross section of a midgut cell 15 min after blood feeding. The basal labyrinth is stretched and only scattered microvilli remain at the luminal side. Note that panels A and B are shown at the same magnification. (C) Cross section of a midgut before blood feeding shown at higher magnification (luminal side). A large number of secretory vesicles

(SV) are found near the midgut lumen. (D) Cross section of a midgut cell 15 min after feeding shown at the same magnification as in C. Secretory vesicles are no longer visible and the microvilli are sparser. Bars = 2 μ m.

Fig. 5.

Blood ingestion correlates with a striking decrease of microvillar density. Electron micrographs such as those shown in Fig. 4 were prepared from sections of midguts dissected at different times after a blood meal and the number of microvilli per unit length was measured. Note very rapid loss of microvilli per unit length after blood ingestion. The insert shows an expanded view of the early time points, to clarify the timing of early events. The data were generated for each time point from eight micrographs representing three different midguts. Each micrograph was measured by two persons.