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Two insulin-like peptide family members from the mosquito *Aedes aegypti* exhibit differential biological and receptor binding activities

Zhimou Wen[a,1], Monika Gulia[a], Kevin D. Clark[a], Animesh Dhara[b], Joe W. Crim[c], Michael R. Strand[a,*], Mark R. Brown[a,*]

[a] Department of Entomology, University of Georgia, USA
[b] Neuroscience Division of the Biomedical Health Sciences Institute, University of Georgia, USA
[c] Department of Cellular Biology, University of Georgia, USA

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ABSTRACT

Insects encode multiple ILPs but only one homolog of the vertebrate IR that activates the insulin-signaling pathway. However, it remains unclear whether all insect ILPs are high affinity ligands for the IR or have similar biological functions. The yellow fever mosquito, *Aedes aegypti*, encodes eight ILPs with prior studies strongly implicating ILPs from the brain in regulating metabolism and the maturation of eggs following blood feeding. Here we addressed whether two ILP family members expressed in the brain, ILP4 and ILP3, have overlapping functional and receptor binding activities. Our results indicated that ILP3 exhibits strong insulin-like activity by elevating carbohydrate and lipid storage in sugar-fed adult females, whereas ILP4 does not. In contrast, both ILPs exhibited dose-dependent gonadotropic activity in blood-fed females as measured by the stimulation of ovaries to produce ecdysteroids and the uptake of yolk by primary oocytes. Binding studies using ovary membranes indicated that ILP4 and ILP3 do not cross compete; a finding further corroborated by cross-linking and immunoblotting experiments showing that ILP3 binds the MIR while ILP4 binds an unknown 55 kDa membrane protein. In contrast, each ILP activated the insulin-signaling pathway in ovaries as measured by enhanced phosphorylation of Akt. RNAi and inhibitor studies further indicated that the gonadotropic activity of ILP4 and ILP3 requires the MIR and a functional insulin-signaling pathway. Taken together, our results indicate that two members of the *Ae. aegypti* ILP family exhibit partially overlapping biological activity and different binding interactions with the MIR.

1. Introduction

In vertebrates, insulin, IGFs, and relaxins are members of the ILP superfamily that regulate complex physiological processes including metabolism, growth, longevity, and fertility (Halls et al., 2007; Taguchi and White, 2008; Belfiore et al., 2009). Insulin and IGFs are differentially processed from prepropeptides, and preferentially bind structurally related but distinct receptor tyrosine kinase receptors (Taniguchi et al., 2006). Binding of insulin to the IR activates the phosphoinositol 3 kinase/Akt pathway (the canonical insulin-signaling pathway) while binding of IGFs to the IGFR activates the MAPK pathway although extensive cross talk also occurs between these ligands, receptors, and their downstream signaling pathways. Relaxins in contrast bind GPCRs, which activate multiple signaling pathways.

Phylogenetically advanced insects like the fruit fly, *Drosophila melanogaster*, silkmoth, *Bombyx mori*, honeybee, *Apis melifera*, and mosquitoes (*Aedes aegypti* and *Anopheles gambiae*) also encode multiple, structurally distinct ILPs, but only one homolog of the vertebrate IR that activates the insulin-signaling pathway (Wu and Brown, 2006; Ament et al., 2008; Toivonen and Partridge, 2009; Teleman, 2010). Overexpression of any of the seven ILPs encoded by *Drosophila* during larval development results in increased body size (Ikeya et al., 2002), whereas ablation of the brain neurosecretory cells that produce four of the ILPs (1, 2, 3 and 5) or genetic deletion of ILP1–5 reduces metabolic activity and size (Rulifson et al., 2002; Broughton et al., 2005; Zhang et al., 2009). A recent com-

Abbreviations: BSA, bovine serum albumin; ILP, insulin-like peptides; DIR, *Drosophila* insulin receptor; GPCR, G-protein-coupled receptor; IGFs, insulin-like growth factors; IGFR, insulin growth factor-like receptor; IR, insulin receptor; MAPK, mitogen-activated protein kinase; MIR, mosquito (*Aedes aegypti*) insulin receptor; PBM, post-blood meal; RNAi, RNA interference; RT-PCR, reverse transcriptase polymerase chain reaction; rqRT-PCR, relative quantitative RT-PCR.

* Corresponding authors at: University of Georgia, Department of Entomology, 413 Biological Sciences Building, Athens, GA 30602, USA. Tel.: +1 706 583 8237/1 706 542 2217; fax: +1 706 542 2279.
E-mail addresses: mrstrand@uga.edu (M.R. Strand), mrbrown@uga.edu (M.R. Brown).
[1] Current address: Syngenta Biotechnology Inc., Research Triangle Park, NC 27709, USA.

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prehensive study of gene-knockouts for all seven ILPs in *Drosophila* further indicates the concurrent loss of *dilp1-6* adversely affects development, stress resistance, and lifespan more than deletion of individual *dilps* (Grönke et al., 2010): a finding which lends additional evidence for the redundant and synergistic activation of the insulin-signaling pathway. Because mammalian insulins also activate this pathway in insects (Fernandez et al., 1995; Yamaguchi et al., 1995; Riehle and Brown, 1999; Wu and Brown, 2006; Gu et al., 2009), they are often viewed as structurally and functionally equivalent homologs of insect ILPs (Teleman, 2010). On the other hand, insect ILPs exhibit differences in expression patterns and secretion (summarized by Wu and Brown, 2006; Yang et al., 2008; Géminard et al., 2009; Okamoto et al., 2009; Veenstra, 2009; Grönke et al., 2010; Teleman, 2010), suggesting the function of individual ILP members is not fully overlapping. In addition, little is known about the actual binding affinities of insect IRs for different ILPs. Thus, it remains unclear whether all insect ILPs are functionally redundant insulin homologs or high affinity ligands for insect IRs.

Most mosquitoes must feed on blood from a human or other vertebrate host to mature eggs. This aspect of mosquito biology is of great interest, because many species transmit disease-causing pathogens when acquiring a blood meal. Recent studies also indicate that insulin-signaling impacts several processes following blood feeding including the cascade of events required for the production of yolk and its uptake into primary oocytes (Roy et al., 2007; Luckhart and Riehle, 2007; Brown et al., 2008; Arik et al., 2009; Sim and Denlinger, 2008). The yellow fever mosquito, *Aedes aegypti*, encodes eight ILP genes of which five (ILP1, 3, 4, 7 and 8) are expressed in the brain (Riehle et al., 2006). Ingestion of a blood meal triggers the release of these ILPs and other neuropeptides, which stimulate the ovaries to produce ecdysteroid hormones (Brown et al., 1998, 2008). These ecdysteroids in turn stimulate the fat body, which is the primary metabolic organ of insects, to synthesize and release yolk proteins produced from the nutrients acquired in the blood meal. Yolk proteins are then packaged into primary oocytes (Roy et al., 2007).

We previously determined that synthetic ILP3 elevates carbohydrate and lipid storage by the fat body in sugar-fed females, and exhibits potent gonadotropic activity as measured by stimulation of ecdysteroid production by the ovaries and yolk uptake by oocytes of blood-fed females (Brown et al., 2008). We also showed that ILP3 binds the MIR with high affinity (IC_{50} = 5.9 nM), while knockdown of the MIR by RNAi greatly reduces ILP3 gonadotropic activity. Earlier studies showed that bovine insulin also stimulates ecdysteroid production by ovaries through insulin signaling (Riehle and Brown, 1999). ILP3, however, exhibits gonadotropic activity at much lower concentrations, and bovine insulin poorly competes ILP3 binding to the MIR (Brown et al., 2008). Taken together, these data indicate that ILP3 exhibits insulin-like activity and suggest bovine insulin is comparatively a weak ligand for the MIR. In the current study, we addressed whether other ILP family members are functionally similar or distinct from ILP3 by producing and characterizing the activity of ILP4. Our results indicate that ILP4 and ILP3 exhibit partially overlapping biological activities and distinctly different binding affinities for the MIR.

2. Experimental procedures

2.1. Mosquitoes

The UGAL strain of *A. aegypti* was used in all experiments. Mosquitoes were reared as previously described (Riehle et al., 2006). Adult females were blood fed on anesthetized rats.

2.2. ILP4 synthesis

The A and B chains of ILP4 were synthesized using standard Fmoc chemistry with the exception that norleucine was substituted for the single methionine in the B chain to negate the possible effects of oxidation on bioactivity (Supplementary Fig. 1). Cross-linking of the chains to form mature ILP4 followed by purification to homogeneity was accomplished as previously described for the biosynthesis of ILP3 (Brown et al., 2008). The structure of ILP4 was then confirmed by MALDI-TOF mass spectroscopy followed by lyophilization and storage at −20 °C. ILP3 used in the study was produced as previously described (Brown et al., 2008). Both ILPs were rehydrated in water followed by dilution in *Aedes* saline (7.5 g NaCl, 0.35 g KCl, and 0.21 g CaCl2 to 1 L, pH 6.5) for use in bioassays.

2.3. Metabolic assays

Newly emerged adult females were provided water only for five days followed on day 6 by access to a 10% sucrose solution for 30 min. Immediately thereafter, females with swollen abdomens indicative of feeding were decapitated and injected with different concentrations of ILP4 or ILP3. Normal, intact mosquitoes (non-decapitated) served as a positive control while mosquitoes decapitated and injected with *Aedes* saline alone served as the negative control. After 24 h at 27 °C, females were frozen (−80 °C), and later processed to measure stored glycogen and lipid as described (Brown et al., 2008).

2.4. Yolk uptake and ovary ecdysteroid production assays

Yolk uptake assays were conducted using blood-fed females decapitated within 1 h PBM. Decapitated mosquitoes were then injected with different concentrations of ILP4 or ILP3 in 0.5 μl of saline, followed 24 h later by dissection and measurement of yolk deposition per egg using the long axis of each oocyte (Brown et al., 1998). Decapitated females injected with saline alone served as the negative control, and intact (non-decapitated) blood-fed females injected with saline served as the positive control.

Ecdysteroid production by ovaries was determined using a well-established *in vitro* bioassay (Sieglaff et al., 2005). Briefly, 2 or 4 pairs of ovaries from non-blood-fed females were incubated with serially diluted ILP3 or ILP4 in modified *Aedes* saline (140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 12.5 mM HEPES, 2.5 mM trehalose, 0.5 mM $MgCl_2$, and 0.9 mM $NaHCO_3$, pH 7.0) for 6 h at 27 °C followed by quantification of ecdysteroids in the medium by radioimmunoassay (RIA) using an ecdysteroid antiserum (4919) kindly donated by P. Poncheron (Université P. et M. Curie, Paris, France) (Sieglaff et al., 2005). To determine the commitment period for ILP activation, ovaries from non-blood-fed females were incubated as above with optimal ILP doses (ILP4, 20 pmol or ILP3, 10 pmol/ovary set) for different time periods. At the end of this period, medium was collected (50 μl) for the ecdysteroid RIA, and replaced with medium containing no ILP. After 6 h of total incubation time, medium from the second period was collected and ecdysteroid amounts determined as described above. For the inhibitor experiments, ovaries were set up as above and incubated for 6 h with ILP4 or ILP3 plus different concentrations of the IR inhibitor hydroxyl-2-napthalenylmethyl phosphonic acid – trisacetoxymethyl ester (HNMPA-(AM_3), EMD). The concentration of HNMPA that reduced ecdysteroid production by 50% (IC_{50} value) was calculated by non-linear regression using Sigma Plot (Brown et al., 2008).

2.5. Receptor binding and cross-linking assays

ILP3 and ILP4 were iodinated (^{125}Iodine, PerkinElmer) using the lactoperoxidase-hydrogen peroxide method and purified by HPLC (Crim et al., 2002). Membranes were extracted from 500 to 600 ovary pairs (4–7-day-old non-blood-fed females) as described previously (Brown et al., 2008). Unlabeled ILP (30 μl at 10X final concentration of 0.1 nM to 20 μM) and ^{125}I-ILP (~200–300 pM, ~400 K cpm/150 μl) were added to binding buffer (50 mM HEPES (pH 7.6), 1X Hanks balanced salt solution, 3% BSA and 0.1× Roche protease inhibitors) in 1.5 ml polypropylene microtubes (triplicate tubes/concentration) followed by addition of ovary membranes (20 μl) to a final volume of 300 μl. Samples were rotated overnight at 4 °C, centrifuged, and the supernatants removed by aspiration. The pelleted membranes were then rinsed with ice-cold binding buffer, re-centrifuged, and the supernatant again removed. Cross-competitive binding experiments were set up similarly with unlabeled ILP3 and ^{125}I-ILP4 and vice versa. Raw counts per minute in the pellet for each sample were converted to percent total binding, and the data from a minimum of three independent assays were analyzed by non-linear regression as described above to generate curves and IC_{50} values.

Binding assays, set up as described above, were also conducted for cross-linking experiments. After overnight incubation, membranes were pelleted and rinsed 2× with binding buffer minus BSA and protease inhibitors. Membranes were then dispersed in the same buffer (240 μl) followed by addition of 30 μl of *Bis*[sulfosuccin-imidyl] suberate and *Bis*[sulfosuccin-imidyl] glutarate (Aculytix, Rockford, IL) freshly prepared in water to achieve a 10 μM (ILP4) or 100 μM (ILP3) final concentration of each cross-linking agent. Sample tubes were then rotated for 1 h at 4 °C, and the reaction stopped by the addition of 100 mM Tris buffer (pH 7.5). Isolated pellets were then mixed with sample buffer, frozen, and later subjected to SDS-PAGE, immunoblotting and autoradiography as previously described (Brown et al., 2008). The MIR was detected using an anti-MIR primary antibody (377E, 1:6000), a peroxidase-conjugated goat-anti-rabbit secondary antibody (Sigma, 1:15,000) and chemiluminescence substrate (GE Amersham ECL Advance