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Lipophorins can adhere to dsRNA, bacteria and fungi present in the hemolymph of the desert locust: A role as general scavenger for pathogens in the open body cavity

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ABSTRACT

Desert locusts are characterized by a highly sensitive and effective RNA interference (RNAi) response. Moreover, delivery of dsRNA into the open body cavity will elicit potent silencing effects throughout the body. On the other hand, many other insect species, such as *Bombyx mori* and *Drosophila melanogaster*, lack the ability to efficiently spread the RNAi-signal. In this study, we demonstrated that, in the serum of the desert locust, lipophorins adhere to dsRNA-fragments. Lipophorins can be subdivided into high density and low density lipophorins (HDLp and LDLp), according to their buoyant density, and we showed that both types of lipophorins can interact with dsRNA fragments. Furthermore, in the presence of (gram-positive) bacteria or fungi, LDLp induce the formation of pathogen aggregates, while no clear aggregation effects were detected in the presence of HDLp.

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1. Introduction

In contrast to vertebrates, insects lack an adaptive immune system. Therefore, in insects, recognition of pathogen associated molecular patterns (PAMPs), molecules associated with groups of pathogens that are not regularly present in the host, plays a major role in the control and clearance of pathogens after an infection. For instance, double stranded (ds)RNA fragments, which are typically produced during the viral replication cycle, can activate the antiviral immune response, while lipopolysaccharides (LPS), peptidoglycans and β-1,3-glucan represent PAMPs for gram-negative bacteria, gram-positive bacteria and fungi, respectively (Tsakas and Marmaras, 2010).

Several studies have identified RNA interference (RNAi) to play an important role in the control of viral infections in flies and mosquitoes (Galiana-Arnoux et al., 2006; Wang et al., 2006; Keene et al., 2004; Campbell et al., 2008). RNAi is triggered by the cellular presence of dsRNA fragments and results in post-transcriptional gene silencing due to siRNA-guided cleavage of complementary RNA molecules (Hammond, 2005). However, RNAi can also be employed to silence endogenous genes of the host cell, making it a highly effective loss-of function tool in reverse genetic studies and giving it potential to contribute to the development of novel insect control strategies (Huvenne and Smagghe, 2010).

Viruses are obligatory intracellular pathogens. Yet, after cell lysis, viral particles and dsRNA fragments are released into the insect's circulatory system. In many insect species, dsRNA-fragments in the open body cavity (hemocoel) can elicit RNAi effects that spread throughout the body, a process that is termed systemic (sys)RNAi (Whangbo and Hunter, 2008). For this, dsRNA must be delivered to the target cells. The ability to systemically spread the RNAi-signal varies strongly among insect species (Belles, 2010; Terenius et al., 2011). For instance, several lepidopteran insect species, such as the tobacco hornworm *Manduca sexta* and the silkmoth *Bombyx mori*, as well as the fruit fly *Drosophila melanogaster*, lack the ability to efficiently spread the RNAi-signal throughout the body (Belles, 2010; Terenius et al., 2011; Miller et al., 2008). On the other hand, the desert locust *Schistocerca gregaria* and the red flour beetle *Tribolium castaneum* are characterized by a highly sensitive and robust sysRNAi-response (Miller et al., 2008; Wynant et al., 2012).

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dsRNA-stability in the hemocoel and delivery to the target cells might be crucial factors determining the success of sysRNAi. In *M. sexta*, it was shown that dsRNA is enzymatically degraded in the hemolymph (Garbutt et al., 2013), while lipophorins (the insect lipoproteins) adhere to dsRNA in the hemolymph of *B. mori* (Sakashita et al., 2009). Nevertheless, lipophorins are best characterized for their ability to transport lipophilic compounds in a hydrophilic environment. They can be subdivided into high density lipophorins (HDLp) and low density lipophorins (LDLp) according to their buoyant density range. Two proteins are present in lipophorin complexes, i.e. Apolipophorin 1 (Apo1) and Apolipophorin 2 (Apo2), with a molecular mass of 220–250 kDa and 70–85 kDa, respectively. In LDLp, a third exchangeable Apolipophorin 3 (Apo3) can also be found. The function of Apo3 is to transport lipids (especially diacylglycerol (DAG)) from sites of lipid storage in the fat body to sites of utilization of energy, such as flight muscles. The transition from HDLp to LDLp is regulated by the adipokinetic hormone (AKH) (reviewed in Rodenburg and Van der Horst (2005)). Whilst, mammalian lipoproteins are typically characterized as a shuttle for dietary energy, more recent studies illustrated that they also play an important role in innate immunity by scavenging for bacterial, fungal and viral PAMPs (Han, 2010). Likewise, in insects, Apo3 has been found to be a key player in defense against fungal (Whitten et al., 2004) and bacterial pathogens (Cheon et al., 2006; Ma et al., 2006).

Whereas the importance of locust lipophorins in energy transport has been widely investigated, we now demonstrate for the first time that lipophorins belonging to a single insect species can adhere to dsRNA, bacteria and fungi present in the hemolymph.

2. Materials and methods

2.1. Rearing of S. gregaria, Sarcophaga crassipalpis, Acheta domesticus and Periplaneta americana

Gregarious *S. gregaria* were reared under crowded conditions with controlled temperature (32 ± 1 °C), light (13 h photoperiod) and relative humidity (40–60%). The locusts were kept at high density (>200 locusts/cage) in wooden cages and fed daily with fresh cabbage and dried oat flakes. Mature female locusts deposited their eggs in pots with moistened sterile sand mixture (7 parts sand, 3 parts peat and 1 part water). These pots were collected once a week and set apart in empty cages, resulting in pools of 1st instar locusts, which differed at most 1 week in age. To synchronize the locusts, they were separated directly after their final moult.

Flesh flies (*S. crassipalpis*) were reared under laboratory conditions at 24 °C and 14 h photoperiods. The flies were daily fed with sugar water and cow liver. Three times a week, pieces of liver containing eggs were separated, resulting in pools of flies, which differed maximum 3 days in age.

The house cricket (*A. domesticus*) and the American cockroach (*P. americana*) were also reared at 24 °C and 14 h photoperiods. The crickets were fed daily with cabbage, oats and dog food (Frolic) while the cockroaches were fed with oat and dog food (Frolic). Whereas all cockroaches were reared in the same cage, mature female crickets deposited their eggs in pots with moistened sterile sand mixture (3 parts sand, 1 parts peat and 1/2 part water). These pots were collected once a week and set apart in empty cages, resulting in pools of crickets, which differed maximum 1 week in age.

2.2. Collection of serum

After amputating one leg of an adult desert locust, a hemolymph sample was collected with a capillary. Serum was prepared by leaving the hemolymph for 1 min at room temperature. Next, the clot was removed by centrifuging at 3000 rpm for 5 min. The resulting supernatant (serum) was collected in a new tube and stored at −20 °C.

2.3. Incubation of dsRNA in serum and visualization of the dsRNA

Double stranded RNAs were synthesized by using the MEGAscript RNAi kit, according to the manufacturer's specifications and as described by Wynant et al. (2012). To 10 μl serum sample, we added 150 ng of dsRNA. *S. gregaria* (*Sg*)-Ringer solution (1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) was used to dilute the samples prior to adding dsRNA. As a control, 150 ng of dsRNA was added to 10 μl *Sg*-Ringer solution. After the indicated incubation time, 2 μl of 6× loading dye (Fermentas) was applied. Thereafter, the samples were analyzed by 1% agarose [containing GelRed™ (VWR)] electrophoresis. The dsRNA was visualized with a ProXima 2500 imager (Isogen Life Science) under UV-light.

2.4. Production of Cy3-dsRNA

We produced Cy3-labelled dsRNA by using the Silencer®siRNA labelling kit (Ambion), according to the manufacturer's specifications. The labelled dsRNA molecules were separated from the unbound Cy3-dyes via ethanol precipitation. The Cy3-labelling was confirmed by assessing the fluorescence with an Ethan DIGE imager (GE healthcare), following 1% agarose gel electrophoresis [without adding GelRed (Fermentas)]. The dsRNA concentration and labelling efficiency were determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.).

2.5. Purification of the gel mobility shift band

After incubation of (in total) 1.5 μg dsRNA in 150 μl diluted serum sample (1/10 and 1/20), the gel mobility shift band was cut out and stored in MagnaLyser Green beads containing tubes (Roche). Next, 1 ml MilliQ water was added to the samples and the agarose gel was fractioned with the MagnaLyser apparatus (Roche). The agarose matrix was subsequently separated by centrifugation. After collecting the supernatants, the remaining agarose in the supernatants was removed via a second centrifugation step. Thereafter, the sample was concentrated by RotaVap lyophilisation (FTS Systems, Inc.).

2.6. SDS–PAGE

The samples were subsequently analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), prepared with NuPAGE Bis-Tris Mini Gels (Life technologies Co.). The staining was performed with SimplyBlue Safe stain (Life technologies Co.) according to the manufacturer's specifications. The molecular mass ladder was SeeBlue Plus2 Pre-Stained Standard (Life technologies Co.).

2.7. Determining the amino acid sequences by Edman degradation

Following the separation of the protein bands by SDS–PAGE (NuPAGE Bis-Tris Mini Gels, Life technologies Co.), the proteins were electrotransferred to a polyvinylidene fluoride (PVDF) membrane with the Cell II™ Blot module (Life Technologies Co.). After electrotransfer, the PVDF membranes were stained with amino black staining solution (amido Black (0.5% w/v), isopropanol (25% v/v) and acetic acid (10% v/v)) for 2 min. Destaining was done by several soakings in deionized water. The corresponding amino acid

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