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REVIEW

Apolipophorins and insects immune response

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Abstract

Insect lipoproteins, called lipophorins, are non-covalent assemblies of lipids and proteins serving as lipid transport vehicles. The protein moiety of lipophorin comprises two glycosylated apolipoproteins, apolipophorin I (apoLp-I) and apolipophorin II (apoLp-II), constantly present in a lipophorin particle, and an exchangeable protein, apolipophorin III (apoLp-III). ApoLp-III is an abundant protein occurring in hemolymph in lipid-free and lipid-bound state and playing an important role in lipid transport and insect innate immunity. In immune response apoLp-III serves as a pattern recognition molecule. It binds and detoxifies microbial cell wall components, *i.e.*, lipopolysaccharide, lipoteichoic acid, and β-1,3-glucan. ApoLp-III activates expression of antimicrobial peptides and proteins, stimulates their antimicrobial activity, participates in regulation of the phenoloxidase system and in hemolymph clotting. In addition, the protein is involved in cellular immune response, influencing hemocyte adhesion, phagocytosis and nodule formation, and in gut immunity. Although apoLp-III is the best studied apolipophorin in insect immunity so far, a literature review suggests that all the three apolipoproteins, apoLp-I, apoLp-II and apoLp-III, function together in a coordinated defense against pathogens.

Key Words: lipophorin; apolipophorin III; insect immunity; *Galleria mellonella*

Introduction

Insect lipoproteins, called lipophorins, are well-studied complexes of multifunctional molecules. These non-covalent assemblies of lipids and proteins serve as lipid transport vehicles. Lipophorins have a structure similar to mammalian lipoproteins. They possess a hydrophobic core composed of nonpolar lipid constituents, surrounded by a monolayer of amphiphilic phospholipids (PL) and apolipoproteins. The protein moiety of lipophorin comprises two glycosylated apolipoproteins, arising from a common precursor preproapolipophorin, i.e. apolipophorin I (apoLp-I, ca 240 kDa) and apolipophorin II (apoLp-II, ca 80kDa) which are present in a 1:1 molar ratio in lipophorin particles (Ryan *et al*., 1984; Weers *et al*., 1993; Blacklock and Ryan, 1994; Ryan and Van der Horst, 2000; Marinotti *et al*., 2006).

The fat body, a functional analog of mammalian liver, is the site of apoLp-I, apoLp-II and lipid synthesis as well as lipid storage, and assembling of lipoprotein particles. Lipophorins are released as high or very high density lipoproteins into the hemolymph, depending on the insect species (Prasad *et al*., 1986; Venkatesh *et al*., 1987; De Capuro and De Bianchi, 1990; Weers *et al*., 1992; Van Heusden *et al*., 1998). One of the major roles of lipoprotein is lipid transport during larval development and long-distance flight of insects. Adipokinetic hormones (AKHs), which are stored in the secretory granules of neuroendocrine cells (*corpus cardiaca*), are released during flight activity and are involved in lipid mobilization (Beenakkers *et al*., 1985; Van der Horst, 2003). AKHs trigger conversion of the fat body triacylglycerol (TAG) stores into diacylglycerol (DAG) by a specific lipase. The insect lipids are then released as DAG and, after being assembled with apoLp-I and apoLp-II, form high-density lipophorins (HDLp). Two proteins, apoLp-I and apoLp-II, are constantly present in a lipophorin particle, whereas the third protein, apolipophorin III (apoLp-III), is an exchangeable molecule in these complexes. Association of DAG and apoLp-III with HDLp converts them to low-density lipophorins (LDLp), which serve as transport vehicles for lipids in hemolymph to a target tissue.

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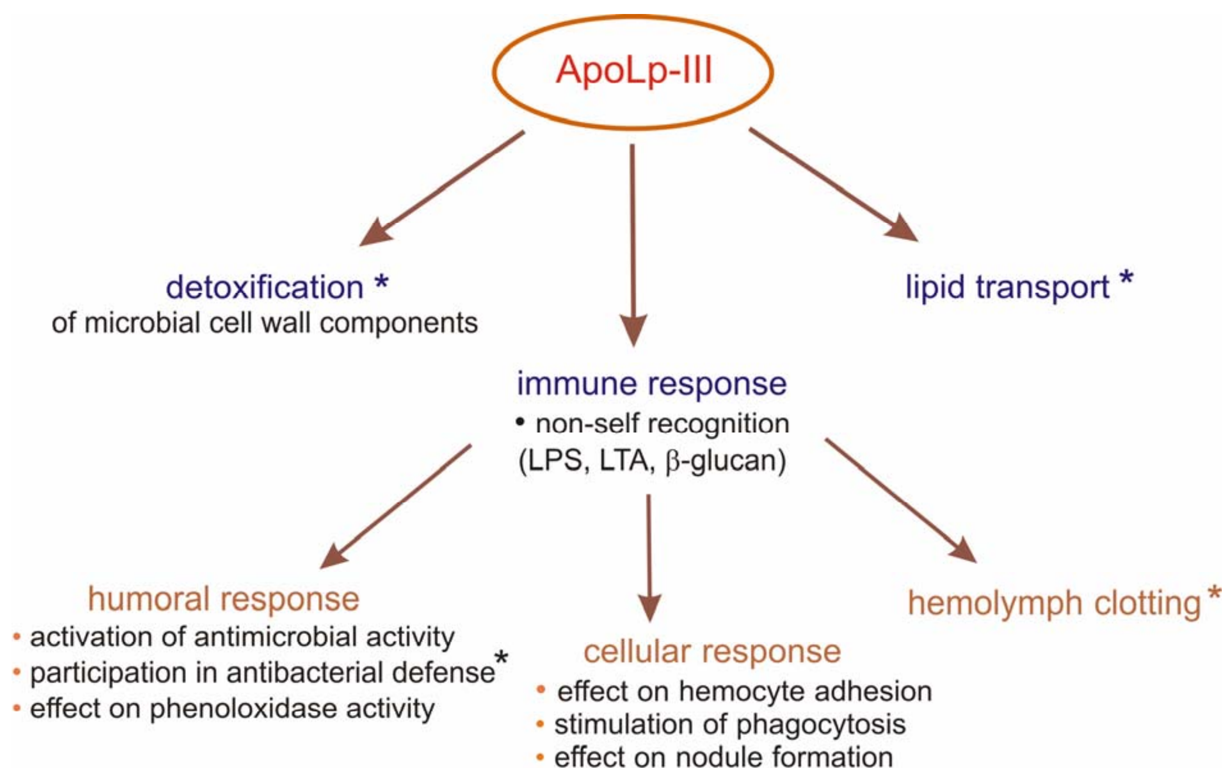

Fig. 1 Main functions of apolipophorin III in insects. (*) denotes processes in which lipophorin particles or apoLp-I/II are involved

From 14 to 16 molecules of apoLp-III can be associated with the LDLp surface (Kawooya *et al*., 1984, 1986; Wells *et al*., 1987; Van der Horst *et al*., 1991). After release of a lipid load, apoLp-III dissociates from the complex and together with HDLp can be reused for another cycle of DAG transport (Weers *et al*., 1992; Blacklock and Ryan, 1994; Soulages *et al*., 1995; Ryan and Van der Horst, 2000; Niere *et al*., 2001).

Among apolipophorins, especially apoLp-III is considered to be an important factor of insect immunity. The review summarizes available data on apoLp-III involvement in insect immune response (Fig. 1).

Apolipophorin III and lipid interactions

ApoLp-III was detected in different insect tissues, *e.g.,* eggs, fat body, hemocytes, and hemolymph. This protein was found in hemolymph plasma of larvae, pupae, adults as well as in the molting fluid. It is a water-soluble and heat-stable protein of molecular mass 17-20 kDa depending on the insect species (Table 1). ApoLp-IIIs contain no cysteine, some of them are glycosylated in Orthoptera species, but other, e.g. in Lepidoptera species, lack this modification (Kawooya *et al*., 1984; Chino and Yazawa, 1986; Chung and Ourth, 2002). The studies on *L. migratoria* and *G. mellonella* have shown that the protein can occur as isoforms, differing in pI values (Chino and Yazawa, 1986; Van der Horst *et al*., 1991; Wiesner *et al*., 1997; Zdybicka-Barabas and Cytryńska, 2011). The apoLp-III molecule is formed by a bundle of five antiparallel amphipathic α-helices organized in an up-and-down topology, which are connected by short hinged loop regions (Breiter *et al*., 1991). This bundle motif is a stable arrangement of the protein, which allows it to exist in hemolymph. A majority of residues in the molecule interior are hydrophobic, while hydrophilic residues are exposed to the aqueous environment of hemolymph. Although the degree of amino acid sequence identity of apoLp-IIIs from evolutionally divergent species is low, the three-dimensional structure of these proteins in their lipid-free state shows a striking similarity in molecular architecture, which is connected with the physiological functions of apoLp-III. Physiologically, the protein occurs in a lipid-free or lipid-bound state that readily converts from one to the other depending on the metabolic setting. The low intrinsic stability of the helix bundle in the lipid-free state probably facilitates interaction with lipid surfaces. During the formation of the complex between apoLp-III and lipid, considerable conformational changes in the protein were observed, *i.e.*, helix-helix interactions were replaced by helix-lipid interactions in the lipid-bound open conformation (Wientzek *et al*., 1994; Raussens *et al*., 1995). The lipid-bound state is the active form of

Table 1 Properties of apolipophorin III of selected insect species

	Molecular mass [(a,b)] (Da)	Number of residues	pI [(a,b)]/ isoforms	Reference
Lepidoptera				
Acherontia atropos	17247[b] 20 kDa[a]	161		Surholt *et al*., 1988
Bombyx mori	18420 18378[b]	164	8.04[b]	Yamauchi *et al*., 2000
Diatraea grandiosella	17964[b]	165	6.8[a]	Burks *et al*., 1992
Galleria mellonella	18075.5[a] 18075[b]	163	6.38[b] 6.5[a] 5.9[a] 6.1[a]	Weise *et al*., 1998 Zdybicka-Barabas and Cytryńska, 2011
Heliothis virescens	18 kDa[a] 17965.9[a]			Chung and Ourth, 2002
Hyalophora cecropia	18 kDa[a]			Telfer *et al*., 1991
Hyphantria cunea	18.3 kDa[a] 18344[b]	165	6.23[b]	Kim *et al*., 2004
Manduca sexta	18364 18380[b]	166	5.88[b]	Kawooya *et al*., 1984
Spodoptera exigua	16523[b]	149	6.25[b]	Rizwan-Ul-Haq *et al*., 2011
Spodoptera litura	18.3 kDa[a]	166		Kim *et al*., 1998
Thitarodes pui	18606[a]	171	5.61[b]	Sun *et al*., 2012
Orthoptera				
Acheta domesticus	17248[b] 17.2 kDa[a]	161		Smith *et al*., 1994 Strobel *et al*., 1990
Locusta migratoria	20488[a] (Glc) 17583[a] (non-Glc) 17327[b]	162-164	5.35[a] /5.43[a] 5.10[ba]/5.11[a] 4.98[a]	Van der Horst *et al*., 1984; 1991 Weers *et al*., 2000 Chino and Yazawa, 1986
Coleoptera				
Derobrachus geminatus	18039[b] 18 kDa[a]	165		Smith *et al*., 1994
Diptera				
Anopheles gambiae	19247[b]	170	4.82[b]	Gupta *et al*., 2010

[a] molecular mass and isoelectric point (pI) determined empirically; [b] theoretical molecular mass and pI; Glc – glycosylated; non-Glc – non-glycosylated

the protein and occurs when apoLp-III associates with lipid-enriched lipophorins. It has been demonstrated that interaction of apoLp-III with model phospholipid vesicles, composed of dimyristoylphosphatidylcholine (DMPC), transforms them into discoidal particles surrounded by apoLp-III (Weers *et al*., 1999, 2000; Weers and Ryan, 2003; Vasques *et al*., 2009; Narayanaswami *et al*., 2010; Wan *et al*., 2011). The interaction of the protein with membrane lipid bilayers does not affect their permeability and occurs via polar and/or electrostatic forces at the bilayer surface without penetration of the hydrophobic core of the bilayer (Zhang *et al*., 1993; Sahoo *et al*., 1998). The packing defects in the phospholipid bilayer generate sites of apoLp-III binding, which was reported for various DMPC and sphingomyelin (SM) vesicles (Chiu *et al*., 2009). It is known that lipid binding involves the hydrophobic surface of the helix bundle. Upon lipid binding, apoLp-III molecule undergoes conformational changes and its hydrophobic interior is exposed to the lipid environment, which allows hydrophobic amino acid side-chains to gain direct access to the lipid surface (Wientzek *et al*., 1994; Garda *et al*., 2002; Sahoo *et al*., 2002). A plausible model of the interactions of apoLp-III with model phospholipid particles is based on reorientation in the bundle of α-helices, *i.e.*, three

of them move away from the two others (Breiter *et al*., 1991; Ryan and Van der Horst, 2000; Van der Horst *et al*., 2001, 2002). It has been suggested that the lipid binding is initiated at one end of the helix bundle. Different models have been proposed for description of the initial binding steps. One model has been suggested for *L. migratoria* apoLp-III by Breiter *et al*. (1991), where directed helix-lipid contact is made possible by conformational opening of the bundle involving 'hinged' loops connecting helices 2 and 3 and helices 4 and 5. The resulting exposure of its interior permits formation of a stable interaction with hydrophobic patches on lipophorin particles which appear as a function of DAG enrichment. In turn, Kawooya *et al*. (1986) have proposed that *M. sexta* apoLp-III recognizes potential lipid surface binding sites via one of its end and then Breiter *et al*. (1991) have shown that loop A connecting helices 1 and 2 and loop C between helices 3 and 4 play this role. Furthermore, in the structure of apoLp-III of *L. migratoria, M. sexta*, and *Thitarodes pui* short additional helices (called 4' and 3') located at one end of the α-helix bundle are present. They adapt an orientation nearly perpendicular to the long axis of the bundle (Wang *et al*., 1997; Narayanaswami *et al*., 1999; Fan *et al*., 2003; Sun *et al*., 2012). Because they are exposed to the solvent, it has been suggested that these short helices play a critical role in initiating the lipid binding with apoLp-III. It is believed that repositioning of the first and last helices in the molecule is an essential step in the binding process of apoLp-III, facilitating further opening of the hydrophobic helix bundle interior and separation of all helices from each other, which allows the protein to spread out on the lipid surface (Narayanaswami *et al*., 1996; Garda *et al*., 2002; Sahoo *et al*., 2002). It has been demonstrated that apoLp-III of *M. sexta, L. migratoria* and *G. mellonella* were unable to bind lipid surfaces when helix 1 and helix 5 were tethered by a disulfide bond (Garda *et al*., 2002; Sahoo *et al*., 2002; Leon *et al*., 2006a). Based on spectroscopic analyses, Raussens *et al*. (1995) inferred that the helices of *M. sexta* apoLp-III in the lipid-bound state are oriented perpendicularly to fatty acyl chains.

ApoLp-III shares similarities in the structure and in the mechanism of lipid binding with the N-terminal domain of human apolipoprotein E (apoE) and apolipoprotein A-I (apoA-I). The structure of the 22 kDa N-terminal domain in apoE3 in the lipid-free state comprises four amphipathic α-helices with buried hydrophobic residues and exposed hydrophilic residues (Aggerbeck *et al*., 1988; Wilson *et al*., 1991). This domain can alter its conformation in a manner similar to apoLp-III on the lipoprotein particle. Like the human apolipoproteins, apoLp-III forms discoidal complexes with phospholipid liposomes in which extended helices of the protein are wrapped around nanodiscs (Saito *et al*., 2004; Hatters *et al*., 2006; Davidson *et al*., 2007; Narayanaswami *et al*., 2010).

Involvement of apolipophorin III in insects immunity

The level of apoLp-III in hemolymph of immune-challenged insects*, e.g., G. mellonella*, *Heliothis virescens, Plutella xylostella*, undergoes changes, depending on the insect species and the pathogen/parasite, which indicates participation of apoLp-III in immune response against microbial infections (Chung and Ourth, 2002; Song *et al*., 2008; Zdybicka-Barabas and Cytryńska, 2011). It has been postulated that during an early step of insect immune response an interaction of apoLp-III with lipids occurs and that apoLp-III in the lipid-bound state is involved in insect immunity (Wiesner *et al*., 1997; Dettloff *et al*., 2001a,b). A relationship between the lipid transport and immune function of apoLp-III has been shown in crickets. It appeared that the two processes compete for the protein, as after flight, the crickets became less able to fight bacterial infection (Adamo *et al*., 2008).

Recognition of ***non-self***

Proper recognition of invading pathogens by the immune system is a necessary prerequisite for activation and mounting of effective humoral as well as cellular immune response. It has been documented that apoLp-III binds microbial cell wall components, such as Gram-negative bacteria lipopolysaccharide (LPS), Gram-positive bacteria lipoteichoic acids (LTA), and fungal β-1,3-glucan (Dunphy and Halwani, 1997; Halwani *et al*., 2000; Pratt and Weers, 2004; Whitten *et al*., 2004; Leon *et al*., 2006a, b; Ma *et al*., 2006). Due to this property, apoLp-III is considered as a pathogen recognition receptor (PRR).

In their work, Halwani *et al*. (2000) reported on interaction of *G. mellonella* apoLp-III with LTAs of *Bacillus subtilis*, *Enterococcus hirae*, and *Streptococcus pyogenes*. They also demonstrated that *E. hirae* LTA promoted binding of apoLp-III to *E. hirae* cells. In addition, binding of LTAs by apoLp-III prevented loss of plasmatocytes caused by *B. subtilis* LTAs in *G. mellonella* larvae. Our recent study has demonstrated binding of *G. mellonella* apoLp-III to different Gram-positive and Gram-negative bacteria. The results suggested that, in addition to LTAs, apoLp-III interacted with other cell surface components of Gram-positive bacteria, since it bound to some of the tested bacteria lacking LTAs in their cell walls, *e.g*., *B. circulans* (Zdybicka-Barabas and Cytryńska, 2011).

Lipid A and the carbohydrate part of the LPS molecule are involved in interaction with apoLp-III. The binding causes conformational changes in the apoLp-III molecule, leading to rearranging and opening of the bundle of α-helices, similar to binding to lipid surfaces upon interaction with lipoprotein complexes (Leon *et al*., 2006a, b). Recently, a model of interaction between *G. mellonella* apoLp-III and *E. coli* LPS aggregates has been proposed. ApoLp-III disaggregated LPS by forming protein-LPS complexes. According to this model, the initial step is binding of apoLp-III to the LPS carbohydrate moiety through ionic interactions. The second step, leading to strong lipid A binding, is likely to be driven by hydrophobic interaction. It has been calculated that four apoLp-III molecules can interact with 24 molecules of *E. coli* LPS. Analysis of similar complexes formed between apoLp-III and *K. pneumoniae* LPS revealed that the complexes

contained three apoLp-III and nine LPS molecules (Oztug *et al*., 2012).

Whitten *et al*. (2004) demonstrated binding of apoLp-III to β-1,3-glucan, a fungal cell wall component. Moreover, they showed that the survival rate of *G. mellonella* larvae injected with conidia of entomopathogenic fungus *Metarhizium anisopliae* coated by apoLp-III was higher in comparison with that one in larvae challenged by non-coated conidia, indicating a protective role of apoLp-III against fungal infection. Binding of *G. mellonella* apoLp-III to the cell surface of different yeasts and conidia of filamentous fungi has also been demonstrated (Zdybicka-Barabas *et al*., 2012). An analysis of *in vitro* treatment of *Candida albicans*, *Zygosaccharomyces marxianus*, and *Fusarium oxysporum* with apoLp-III revealed morphological and metabolic activity changes, suggesting a role of this protein not only in fungi recognition but also in antifungal activity of hemolymph.

ApoLp-III as a signaling molecule and its role in antimicrobial activity induction

As reported by Dettloff *et al*. (2001a), shortly after injection into *G. mellonella* hemocoel, biotinylated apoLp-III was detected in the immune-competent hemocytes, suggesting functioning of apoLp-III as a signaling molecule in insect hemolymph. In accordance with this finding, *G. mellonella* bacterial challenge led to formation of apoLp-III-lipid complexes, assembled into LDLp which were taken up by granulocytes (Dettloff *et al*., 2001b; Niere *et al*., 2001).

It was reported that apoLp-III potentiated antimicrobial activity in insect hemolymph. An injection of apoLp-III into the hemocoel of *G. mellonella* larvae led to an increase in hemolymph lysozyme and anti-*E. coli* activity, similar to bacterial challenge (Wiesner *et al*., 1997; Halwani and Dunphy, 1999; Niere *et al*., 1999). The constitutive presence of apoLp-III in hemolymph and the fact that intrahemocoelic injection of apoLp-III (recombinant or purified from insects) resulted in a negligible increase in the soluble apoLp-III fraction in hemolymph, ruled out hormone- or cytokine-related activity of the protein. Evidence provided by Dettloff *et al*. (2001b) for LDLp formation after bacterial challenge and uptake of lipid-bound apoLp-III by the hemocytes, indicated that activation of the immune system by apoLp-III might be rather connected with conformational changes and an increase in the lipid-bound fraction of the protein in hemolymph. Intensification of hemolymph antimicrobial activity after apoLp-III injection could result from activation of antimicrobial gene expression, which was demonstrated in apoLp-III-challenged *Hyphantria cunea*. In this insect, apoLp-III injection induced the expression of lysozyme and cecropin-like peptides (hyphancins). In addition, apoLp-III was detected in *H. cunea* granulocytes which underwent degranulation and degradation upon *E. coli* immune challenge. The authors postulated a relationship between a local discharge of apoLp-III from the granulocytes in response to bacterial challenge and activation of immune response (Kim *et al*., 2004).

The existence of immune signals upstream of cell-bound receptors has been postulated by Rahman *et al*. (2006). They found that lipophorin particles mediated recognition and inactivation of LPS and bacteria in immune-challenged flour moth *Ephestia kuehniella* larvae. Moreover, an association of pattern recognition receptors, lectins as well as regulatory proteins activating prophenoloxidase with sub-population of lipophorin particles has been demonstrated (Rahman *et al*., 2006).

Detoxification of non-self components

The ability of apoLp-III to bind microbial cell wall components, *e.g*., LPS, implies participation of the protein in detoxification processes. Dunphy and Halwani (1997) demonstrated that *G. mellonella* apoLp-III bound to LPS isolated from the outer membrane of insect pathogenic bacteria *Xenorhabdus nematophilus*, which reduced the LPS toxicity and prevented *G. mellonella* hemocyte damage. The role of lipophorins, in which apoLp-III is an exchangeable component, in LPS detoxification was suggested by Kato *et al*. (1994a, b) in their study on *Bombyx mori*. The results indicated that formation of the lipophorin-LPS complex in *B. mori* hemolymph, similar to the lipoprotein-LPS complex in mammalian serum, caused a striking decrease in LPS biological activity, reflected by significant reduction in cecropin gene inducibility. As demonstrated by Ma *et al*. (2006), antibodies against LPS-binding proteins, such as immulectin-2, cross-reacted with proteins associated with purified lipophorin particles formed in *G. mellonella* hemolymph *in vitro* upon LPS addition. The results also indicated that lipophorin particles responded to LPS by forming insoluble aggregates sequestering LPS into non-toxic complexes.

ApoLp-III can also be considered as an LTA-neutralizing protein, since binding of *G. mellonella* apoLp-III to *B. subtilis* LTAs prevented loss of plasmatocytes caused by LTA, indicating protection of the insect against the toxin (Halwani *et al*., 2000).

Antimicrobial activity of apoLp-III and synergistic action with defense peptides

Our study has revealed that *G. mellonella* apoLp-III exhibits antibacterial activity against certain Gram-positive and Gram-negative bacteria. Among the most susceptible bacteria were *Salmonella typhimurium*, *K. pneumoniae*, *B. circulans*, and *Listeria monocytogenes* (Zdybicka-Barabas and Cytryńska, 2011). Interestingly, apoLp-III also inhibited significantly growth of *Legionella dumoffii* cultured in a medium supplemented with choline (Palusinska-Szysz *et al*., 2012). AFM imaging and analysis of apoLp-III-treated bacteria revealed considerable alterations of the structure and nanomechanical properties of the cell surface, e.g. roughness, elasticity, and adhesion (Fig. 2) (Zdybicka-Barabas *et al*., 2011). The results underline the important role of apoLp-III in insect antibacterial defense, similarly to the role demonstrated for mammalian apoE in immune

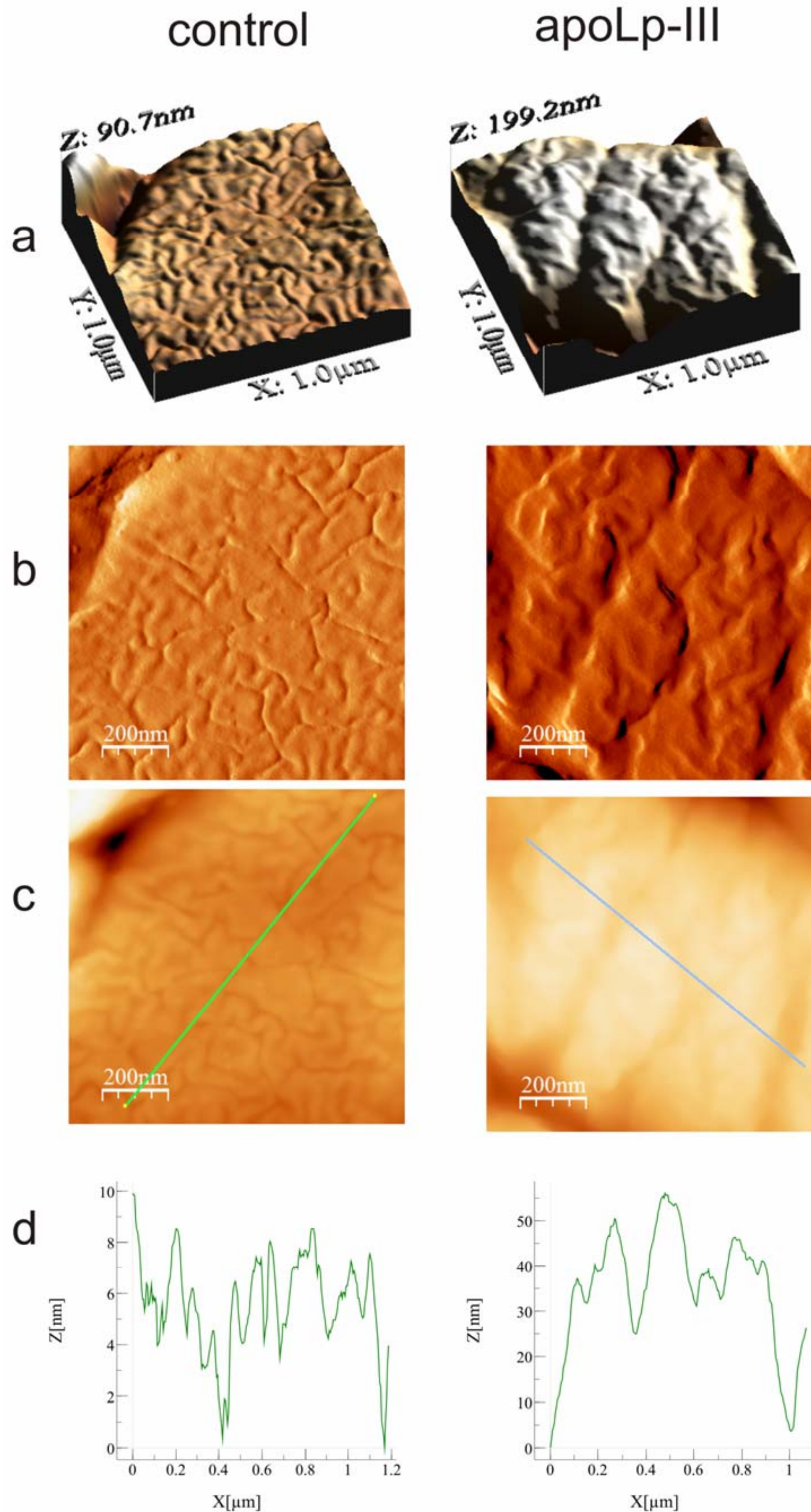

Fig. 2 AFM analysis of *K. pneumoniae* cell surface alterations after treatment with *G. mellonella* apoLp-III. Three dimensional (a), amplitude (b), and topography (c) images are presented. Section profiles corresponding to lines in (c) are demonstrated in (d).

response against *K. pneumoniae* and *L. monocytogenes* (Roselaar and Daugherty, 1998; Bont *et al*., 1999).

In *G. mellonella,* it was demonstrated that apoLp-III acted synergistically with other defense proteins and peptides against bacteria. Synergistic action of apoLp-III and lysozyme against *M. lysodeikticus* was suggested by Halwani and Dunphy (1999) on the basis of experiments on apoLp-III and EWL. In addition, they demonstrated an increase in hydrophobicity and the negative charge of the bacterial cells treated with apoLp-III, which could, to some extent, explain why apoLp-III enhances the antibacterial activity of cationic defense proteins and peptides. Recently, we have presented evidence for increasing *G. mellonella* lysozyme muramidase activity in the presence of apoLp-III, leading to an increase in lysozyme perforating activity of the *E. coli* cell membrane. Our research indicated that three defense factors present constitutively in *G. mellonella* hemolymph, namely apoLp-III, lysozyme, and anionic peptide 2, act in synergy against bacteria. ApoLp-III increases the enzymatic (muramidase) activity of lysozyme, whereas anionic peptide 2 seems to stimulate the non-enzymatic lysozyme activity (Zdybicka-Barabas *et al*., 2012, 2013). Moreover, an increase in cecropin A anti-*E. coli* activity in the presence of apoLp-III has been demonstrated (Park *et al*., 2005).

In addition to enhancing antibacterial activity of other defense proteins and peptides, involvement of apoLp-III in regulation of phenoloxidase activity in *G. mellonella* has been reported; however a possible mechanism of this phenomenon has not been explained yet (Halwani *et al*., 2000; Park *et al*., 2005).

Recently, Gupta *et al*. (2010) have reported on apoLp-III participation in midgut immune defense of *Anopheles gambiae* against *Plasmodium berghei*. In *A. gambiae* G3 females, invasion of *P. berghei* ookinetes triggered a strong transcriptional activation of apoLp-III in the midgut epithelial cells. Expression of apoLp-III in these cells stimulated antiplasmodial response, while silencing of apoLp-III by systemic injection of dsRNA greatly increased *Plasmodium* infection.

Contribution of apoLp-I and apoLp-II, in addition to apoLp-III, in insect defense mechanisms against pathogens has been recently suggested (Hanada *et al*., 2011). It has been found that ApoLp, consisting of apoLp-I and apoLp-II, of the *B. mori* silkworms' hemolymph is involved in resistance against *Staphylococcus aureus* infection by suppressing the expression of virulence genes encoding α- and β-hemolysin. Moreover, ApoLp also decreased expression of *saeRS* and RNAIII, important for activation of these hemolysin genes. It is possible that apoLp-I and apoLp-II, together with apoLp-III, function in coordinated antimicrobial defense in insects.

Role of apoLp-III in cellular immune response

Analysis of the properties of *G. mellonella* hemocytes after treatment with apoLp-III *in vitro* and after injection of apoLp-III into larval hemocoel revealed impaired adhesion of plasmatocytes and subpopulation of granulocytes to glass slides (Zakarian *et al*., 2002; Whitten *et al*., 2004). Since the ability to adhere to and spread on non-self surfaces is essential for hemocytes involved in cellular immune response, the finding suggested a role of apoLp-III in this arm of insect immunity. In the same study, delayed removal of apoLp-III-coated *B. subtilis* cells from the hemolymph was reported. Given the reduced hemocyte adhesion upon apoLp-III treatment, the authors postulated that apoLp-III may down-regulate nodule formation and/or phagocytosis (Zakarian *et al*., 2002). On the other hand, binding of apoLp-III to yeast cells (*S. cerevisiae*) enhanced the phagocytic activity of *G. mellonella* hemocytes *in vitro*, suggesting importance of apoLp-III opsonizing activity for effective clearance of the invaders (Wiesner *et al*., 1997). Similarly, the findings described by Whitten *et al*. (2004) presenting more effective *in vivo* nodule formation in larvae injected with apoLp-III could point towards a stimulating role of apoLp-III in cellular response. In support of this idea are the results presented by Son and Kim (2011) on the role of apoLp-III in activation of cellular response in diamondback moth, *P. xylostella*. Knockdown of apoLp-III expression by RNA interference caused a significant decrease in the apoLp-III level and resulted in considerable suppression of hemocyte nodule formation in response to bacterial challenge. Injection of recombinant apoLp-III to *P. xylostella* larvae parasitized by an endoparasitic wasp *Cotesia plutellae* restored the hemocyte activity. In addition, apoLp-III reduced pathogenicity of entomopathogenic bacteria *X. nematophila* toward *P. xylostella* larvae (Son and Kim, 2011). Although the exact role of apoLp-III in cellular immune response is difficult to define clearly on the basis of available data, it seems that the effect of the protein activity depends on the pathogen.

Involvement in clot formation

Clot formation can be considered as an integral part of insect immune response, because in addition to sealing wounds and limiting loss of body fluids, a clot entraps microbes at the wound site, thereby preventing invading the hemocoel. Moreover, upon activation of the PO system, the entrapped pathogens can be more easily killed and eliminated. Lipophorins have been identified as a common clotting factor in several insect species, *e.g. G. mellonella*, *Tenebrio molitor*, *L. migratoria*, *Periplaneta americana*, and *Leucophaea maderae* (Duvic and Brehélin, 1998; Altincicek *et al*., 2008; Dushay, 2009). The presence of apoLp-I and apoLp-I/II in the *Anopheles gambiae* and *Drosophila melanogaster* clots, respectively, has also been reported (Scherfer *et al*., 2004; Agianian *et al*., 2007).

Proteomic analysis revealed that apoLp-III, together with other apolipoproteins, was a component of *G. mellonella* net-like coagulation structures containing endogenous extracellular nucleic acids. Moreover, apoLp-III was detected among specific RNA-binding proteins, suggesting its role in extracellular RNA-mediated immune response (Altincicek *et al*., 2008).

Apolipophorin III and entomopathogens

One of the strategies developed by entomopathogenic organisms to cope with the host immune system is decreasing of the apoLp-III level. Reduction of the protein level is achieved by suppression of apoLp-III expression, which was described in *P. xylostella* parasitized by the entomopathogenic wasp *C. plutellae* (Son and Kim, 2011). Another way involves proteolytic degradation of apoLp-III by extracellular proteinases produced by entomopathogenic bacteria during infection, e.g. *Pseudomonas aeruginosa* elastase B and protease IV in infected *G. mellonella* larvae (Andrejko *et al*., 2005, 2008, 2013; Andrejko and Mizerska-Dudka, 2012). A very interesting strategy developed by entomopathogenic nematodes *Steinernema feltiae* infecting *G. mellonella* larvae was reported by Brivio *et al*. (2005, 2010). Hemolymph of the infected larvae was depleted of humoral immune factors which were attracted by and adsorbed to specific nematode surface molecules. Among proteins removed in this way from the insect hemolymph, a lipopolysaccharide binding protein (LBP), a peptidoglycan recognition protein LB (PGRP-LB), gloverin-like peptide and apoLp-III were identified. Removal of the immune factors, including apoLp-III, from the insect hemolymph by *S. feltiae* seems to protect nematode symbiotic bacteria *X. nematophila*, which kill the insect host and establish suitable conditions for reproduction of the nematodes (Brivio *et al*., 2005, 2010).

Summary

Apolipophorin III is a multifunctional insect protein involved in lipid transport and immune response. In addition, its role during programmed cell death has been described in *M. sexta* skeletal muscles and neurons (Sun *et al*., 1995). In insect immune response apoLp-III serves as a pattern recognition molecule. It binds and detoxifies microbial cell wall components, i.e. LPS, LTA, and β-1,3-glucan. ApoLp-III activates expression of antimicrobial peptides and proteins, stimulates their antimicrobial activity, and participates in regulation of phenoloxidase activity in insect hemolymph. In addition, the protein is involved in cellular immune response, influencing hemocyte adhesion, phagocytosis and nodule formation, and in gut immunity. Reduction of the apoLp-III level by entomopathogens through *e.g.,* suppression of apoLp-III expression and/or degradation of the protein by entomopathogen proteases seems to be a common strategy to avoid host immune response and indicates that apoLp-III is an important component of insect immunity. Although apoLp-III is the best studied apolipophorin in insect immunity so far, a literature review suggests that all the three apolipoproteins, apoLp-I, apoLp-II and apoLp-III, function together in a coordinated defense against pathogens.

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