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Immunolocalization of cuticular proteins in Johnston's organ and the corneal lens of *Anopheles gambiae*

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ABSTRACT

Previous work with EM immunolocalization examined the intracuticular placement of several antibodies directed against cuticular proteins (CPs) in various structures of *Anopheles gambiae*. Those structures had long stretches of fairly uniform cuticle. We have now used 19 antibodies directed against members of five CP families on two adult structures with considerable complexity, Johnston's organ and the corneal lens of the compound eye. We also localized chitin with colloidal-gold labeled wheat germ agglutinin. Twelve of these antibodies recognized structures in Johnston's organ. Only 6 were detected in the outer pedicel wall, but the internal structures were more complex with distinct distributions of members of the five CP families in six different structures. The corneal lens had four distinct regions of laminar cuticle. Thirteen of the 15 members of the CPR family were detected, none from the other CP families. Specific antibodies were localized to different regions and in different laminae within a region. The specificity of deployment of cuticular proteins revealed in this study is helping to explain why *An. gambiae* allocates about 2% of its protein coding genes to structural CPs.

1. Introduction

Anopheles gambiae allocates almost 2% (295) of its protein coding genes to structural cuticular proteins (CPs). In attempts to learn why there are so many CPs, we have examined the precise location of a small number of these proteins within the cuticle with EM immunolocalization (Vannini et al., 2014b, 2015; Vannini and Willis, 2016). The structures we examined had long stretches of seemingly identical cuticle and the epitopes detected for individual CPs covered large areas of these structures. We thought that we might gain additional information by using our available antibodies on two adult structures with special features. Johnston's Organ (JO), in the second antennal segment, also known as the pedicel of the antenna (Harbach and Knight, 1980), transmits information about wing beat frequency from nearby mosquitoes and contains many distinct cuticular structures (Clements, 1999; Hart et al., 2011). The corneal lens of the compound eye has laminae that are far better defined than what we saw in the surface cuticle.

The CPs of *An. gambiae* have been classified into 13 families based on sequence characteristics (For review see Willis, 2010; Willis et al., 2012). Chitin binding has been demonstrated for members of six of these families, CPR, CPAP1, CPAP3, CPCFC, CPFL and TWDL (Rebers and Willis, 2001; Jasrapuria et al., 2010; Tang et al., 2010; Dong et al., 2016).

The CPR family with 164 members in *An. gambiae* is the largest CP family in every other arthropod studied and has been divided into two major groups, RR-1 and RR-2. A convenient tool for recognizing these groups is the Web Site CutProtFam-Pred (http://aias.biol.uoa.gr/CutProtFam-Pred/home.php; Ioannidou et al., 2014). We recently used 13 antibodies against members of the CPR family and learned that the two hypotheses about the utilization of these groups, separation by type of cuticle or by exclusive location in exo- or endocuticle, were each somewhat incorrect. Rather some RR-2s, while restricted to hard cuticle, were found in both exo- and endocuticle, and one RR-1 protein was detected in hard cuticle (Vannini and Willis, 2016).

We had also published results of EM immunolocalization using antibodies against individual members of the CPF, CPLCG, and CPCFC families (Vannini et al., 2014b, 2015). Thus we had a collection of 19 antibodies that we thought might give us fresh insights, if used against the cuticle in the two specialized structures that are the focus of this report, JO and corneal lens.

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Results using antibodies against CPR130, CPR152 and CPAP3-C are reported here for the first time. CPR130 failed to give significant results in our earlier analysis. An analysis of transcript levels in whole animals across development revealed that transcripts from *CPR152* were present at much higher levels in male than in female pharate and newly eclosed adults (Togawa et al., 2008). *In situ* hybridization revealed that its mRNA was restricted to JO (unpublished observations), so its precise location in JO was of special interest. CPAP3-C is an ortholog of gasp (also known as obstr-C) in *Drosophila melanogaster*, where it participates in the formation of the tracheal lumen (Behr and Hoch, 2005; Tiklová et al., 2013). We wondered if it might play a role in the lumen of JO.

Our analysis of CPs in different adult structures by tandem mass spectrometry identified 79 CPs in JO and 90 in the corneal lens, both preparations including surrounding cuticle (Zhou et al., 2016). These data too revealed the need to learn more about how individual CPs contribute to cuticle.

The CPs of mosquitoes present a challenge to EM immunolocalization and to MS analyses because many proteins come from sequence clusters, groups of almost identical genes (Cornman et al., 2008; Cornman and Willis, 2008, 2009). We have used three antibodies that recognize from 6 to 10 proteins within a sequence cluster, because no suitable unique peptide could be obtained. Two other antibodies recognize proteins from two distinct genes that code for identical proteins. Another peptide is found in two otherwise unrelated proteins. Problems with such similar genes have been discussed in some detail in prior publications (Vannini and Willis, 2016; Zhou et al., 2016).

The results demonstrate that we were correct in anticipating that the two new structures we have examined would provide new information about the utilization of CPs.

2. Materials and methods

2.1. Mosquito rearing

Eggs were obtained from the colony of *An. gambiae* (G3 strain) maintained in the insectary of the University of Georgia Entomology Department. Larvae were reared in a 12/12 L/D photoperiod at 27 °C, fed ground Koi food (Foster and Smith Aquatics, Rhinelander, WI USA), and adults had access to water and an 8% fructose solution. To obtain developmentally synchronized pharate adults, pupae were collected at hourly intervals, separated by sex and maintained in small groups until they reached the desired age. Adults were collected on the morning after emergence (d 0) and prepared for fixation.

2.2. Antibody production

Sixteen of the 19 primary antibodies tested in this study were used in previous studies by Vannini et al. (2014b, 2015) and Vannini and Willis (2016). All the antibodies, except for the anti-3RC Cluster and anti-CPCFC1, were raised against synthetic peptides at Genscript Inc. (Piscataway, NJ) and affinity purified. Peptides were selected to avoid similarity to other *An. gambiae* CP sequences using blastp at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). See Supplementary File 1 for further information.

Anti-CPR130, anti-CPR152 and anti-CPAP3-C were used here for the first time. Two isoforms are known for CPAP3-C and the antibody was designed to recognize both of them (see sequences in Supplementary File 2).

The colloidal-gold conjugated secondary antibodies (Sigma–Aldrich Co., St. Louis, MO) were 10 nm goat-anti-mouse and 5

Table 1
Details about structure tested, antibody dilution ([ ]) and size of gold particles used for immunolocalization. Antibodies against names of members of the CPR family are in color, green for RR-1 group, brown for RR-2 and blue for unassigned. AR = antigen retrieval process used. Gray boxes indicate where positive label was found. Numbers refer to figure numbers and letters to figure panel where results are shown.

Primary antibodies	[1°Ab]	2°Ab [1"50]	Antenna								Corneal lens
			Antennal flagellum	Second antennal segment							
				Pedicel wall	Johnston's Organ						
					Basal plate	Prongs	Septa	Terminal filaments	Cap	Scolopidia lumen	
Anti-CPR12=13	[1:100]	anti-rabbit 10nm gold									11C
Anti-CPR22	[1:100]	anti-rabbit 10nm gold									13C
Anti-CPR59/70	[1:100]	anti-rabbit 5nm gold	2B	3A	4C	5A	6A				12A
Anti-CPR60	[1:100] AR	anti-rabbit 10nm gold	2A	3C	4B	5B					11B
Anti-CPR61	[1:100]	anti-rabbit 10nm gold									11D
Anti-CPR75	[1:100]	anti-rabbit 10nm gold									13A
Anti-CPR125	[1:100]	anti-rabbit 5nm gold	2G,H	3D							13D
Anti-CPR130	[1:100] AR	anti-rabbit 10nm gold				5C					12D
Anti-CPR133=153	[1:100]	anti-rabbit 10nm gold					6B		8B		
Anti-CPR140	[1:100]	anti-rabbit 5nm gold			4F						13E
Anti-CPR151	[1:100]	anti-rabbit 10nm gold							8C		13B
Anti-CPR152	[1:100]	anti-rabbit 5nm gold			4E		6C	7C	8D	9C,D	
Anti-2RA Cluster	[1:100]	anti-rabbit 10nm gold	2C				6D				12C
Anti-2RB Cluster	[1:100]	anti-rabbit 5nm gold									13F
Anti-3RC Cluster	[1:10K]	anti-rabbit 10nm gold	2D	3B	4D	5D	6E				12B
Anti-CPAP3-C	[1:100]	anti-rabbit 5nm gold						7B	8E	9E,F	
Anti-CPCFC1	[1:5K]	anti-rabbit 5nm gold	2E	3E			6F				
Anti-CPF3	[1:100]	anti-mouse 10nm gold							8F		
Anti-CPLCG3/4/5	[1:20K]	anti-rabbit 5nm gold	2F	3F							
WGA	[1:10] 15 nm gold				4A			7A	8A	9A,B	11A