Localization of the Sodium Dependent Cation-Chloride Cotransporter in Aedes aegypti

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Abstract

Aedes aegypti (Yellow Fever Mosquito) is a vector for many diseases, including Zika and yellow fever. With the increase in mosquito resistance to insecticides, scientist have sought out new molecular targets for insecticides. One potential protein target is the sodium dependent cation-chloride cotransporter (CCCs) which may play a role in salt and water balance in both larval and adult life stages of *A. aegypti*. As larvae, mosquitoes live in aquatic conditions that have a lower salt concentration compared to the concentration inside the organism. Under these conditions, water enters the larvae lowering the salt concentration in their body fluid (hemolymph). As adults, mosquitoes live in terrestrial environments. Therefore, water leaves and salt enters the mosquito’s body leading to a higher hemolymph-salt concentration. The sodium dependent CCCs are thought to carry salt into and out of the mosquitoes’ body, suggesting that they may regulate the hemolymph salt concentration in the organism. However, little is known about the function of the sodium dependent CCCs. To evaluate their properties, we conducted experiments to localize sodium dependent CCCs in mosquito tissues. We developed new polyclonal antibodies designed to bind specifically to two variations of the sodium dependent CCCs: aeCCC2 and aeCCC3. These new antibodies reacted with proteins in larvae, adult male, and adult female whole body tissue. However, the apparent size of the proteins was larger than the predicted size of 130 kDa. When the samples were exposed to urea, which disaggregates protein complexes, and PNGase F, which cleaves sugar residues off proteins, the apparent size of the protein detected by the antibodies did not decrease. We are now using these antibodies to localize aeCCC2 and aeCCC3 in larval and adult renal tissue, which are known to be involved in salt transport.

Background

Mosquitoes have three versions of the Na^+ dependent cation-chloride cotransporter (aeCCC), which move ions across epithelial tissue in renal organs. However, limited research has been done on the function of each transporter and how it relates to the molecular physiology in this insect. Previous research conducted in the lab showed that the aeCCC1 is more phylogenetically related to the functionally characterized Na^+, K^+, $2Cl^-$ cotransporters (NKCC). One study, has shown that the NKCC protein in *Drosophila melanogaster* is required for normal renal tubule function (Rodan et al. 2012). The renal tubule, like the Malpighian tubule, is used to secrete ions and water from the body and into the environment. Because these two proteins are closely related, aeCCC1 is probably found on the basolateral side of secretory tissue as seen in the Drosophila’s NKCC. The aeCCC2 and the aeCCC3 are closely related to transporters in *Anopheles gambiae*, another mosquito species; however, very little research has been done on these transporters in mosquitoes or in other insects(Figure 1). Previous lab work has shown that aeCCC2 and aeCCC3 are more localized in the adult hindgut and in the larval anal papillae, respectively, which absorb ions and water from the environment to the body. This suggests that aeCCC2 and aeCCC3 may be absorptive cotransporters, found on the apical side of the hindgut and anal papillae. However, sequence similarity does not necessarily dictate physiology. In order to determine the functions of the CCC proteins, it is necessary to determine where each protein is more heavily expressed and what kind of environmental factors increase expression.

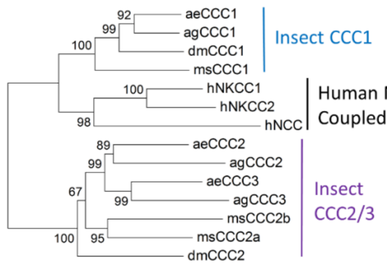

Figure 1. Phylogenetic analysis of CCC amino acid sequences in predicted transmembrane domains using Clustal W alignment and neighbor-joining method (Mega 4 program)

Methods

Mosquito Rearing *A. aegypti* larvae and adults were reared and maintained as described (Pannabecker et al., 1993). Eggs (Liverpool) were hatched under a vacuum filter to induce low O_2. Larvae were raised in a container filled with fresh water (1.5 in) in a controlled environment at 28˚C and a light:dark cycle of 14:10 hr. Larval food consists of 3 parts finely ground TetraFin fish flakes (Tetra, Melle, Germany) and 1 part Yeast Extract (Spectrum Chemical). The adults mosquitoes were fed 10% sucrose/tap water using an inverted test-tube plugged with cotton.

Tissue Collection Sugar-fed mosquitoes and 4th instar larval renal organs (Malpighian Tubules, hindgut, and anal papillae) were dissected as described (Pannabecker et al., 1993). Whole body samples were immediately placed in a collection tube containing 50µL of 1% Triton-X 100 and 1% SDS in Ringer Solution (in mM: NaC1 150, HEPES 25, KC13.4, CaC12 1.7, NaHCO3 1.8, MgC12 0.6, and glucose 5). Protein concentrations were determined with the bicinchoninic acid method (BCA Reagent, Pierce Chemical).

Western Blot Proteins were separated on 7.5% Tris–HCl gels and transferred to PVDF membranes. Blots were blocked for 1 h in phosphate buffered saline with 0.1% Tween 20 (PBT) 5% bovine serum albumin (BSA), and exposed to primary antibody for 3 hr with gentle agitation. Unpurified antiserum was used at dilutions ranging from 1:1000 to 1:5000. Purified aeCCC2 and aeCCC3 antibody was used at a 1:1000 and 1:5000 dilution, respectively, of a 1 mg/ml stock solution. Blots were washed 3 times for 5 min in PBT 5% BSA, 3 times for 5 min in PBT, exposed to HRP-conjugated anti-rabbit IgG secondary antibody (Sigma Chemical, 1:5000 dilution) and the washes were repeated. Detection was found by using chemiluminescence (ChemiDoc MP, Bio Rad).

Antibody Constuct

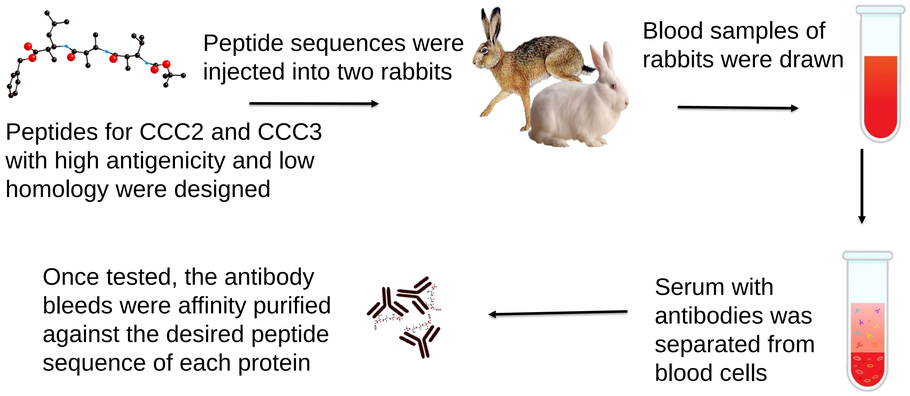

Figure 2. Flowchart of the process of creating polyclonal antibodies in rabbits

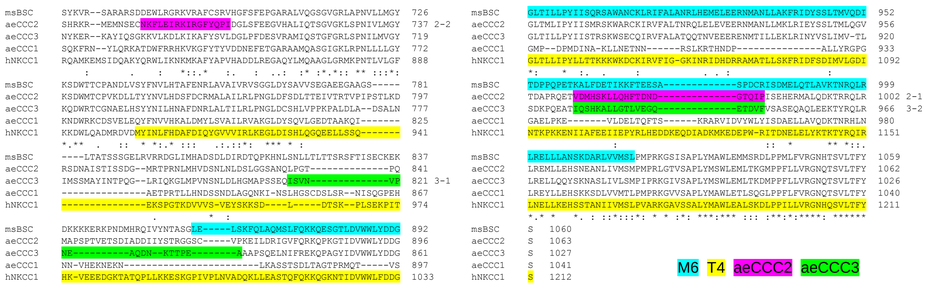

Figure 3. Amino acid sequence of antibodies M6 and T4 in comparison to the developed antibodies

Results

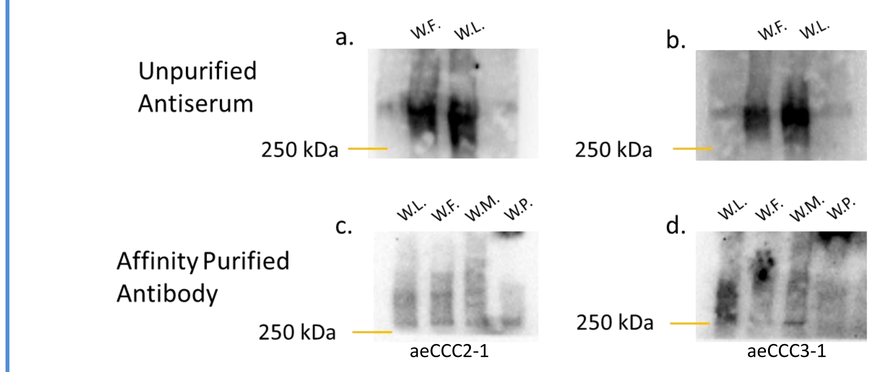

Figure 4. Western blots of whole body samples from adult female mosquitoes (W.F.), 4th instar larvae (W.L), adult males (W.M.), and whole pupae (W.P.) Image a. and c. come from the same rabbit and b. and d. come from the other. The images were exposed for A) 6 seconds, B) 15 seconds, C) 50 seconds, D) 50 seconds.

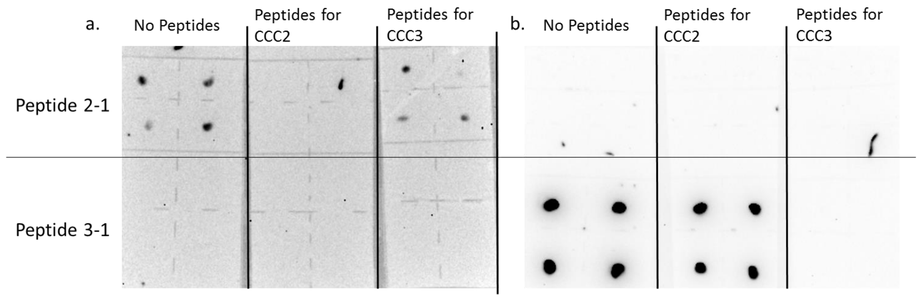

Figure 5. Peptide competition Dot Blots. Image a. is the aeCCC2 antibody while image b. is the aeCCC3 antibody. Peptides used in producing the aeCCC2 and aeCCC3 antibodies were spotted onto PVDF film. When blocking, the peptides were added to the purified antibody such that the antibodies would bind to the free peptide before binding to the PVDF film. The images were exposed at 70 sec.

Conclusion

- Images suggest that the protein is greater than 250 kDa, which reflects similar results when using the M6 antibody
- The purified antibody binds the to correct peptide
- The purified antibody is competed off by the free peptides
- When comparing CCC3 expression, we see that the protein expression is lighter in adult female and males than in larvae.
- CCC2 is equally expressed in all stages of the mosquito life cycle

Future Research

- Because these are initial results, more blots need to be produced in order to confirm protein expression between larvae and female adults
- The apparent size of the protein is larger than the predicted size of 130 kDa.
- Where are these proteins expressed in different tissues? We predict that CCC2 will be present in hindguts of larvae and adult female and that CCC3 will be present in anal papillae of larvae
- A peptide competition experiment was conducted with the bleeds. The figure suggest that the lower band was bound by the CCC2 antibodies. However, this experiment needs to reexamined with the affinity purified antibodies.

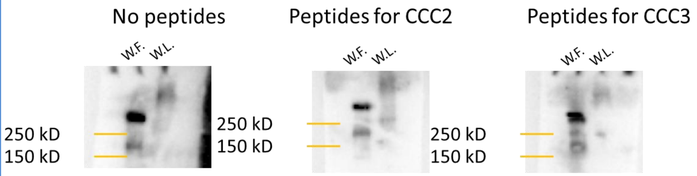

Figure 6. Peptide competition Western Blots of adult female mosquitoes (W.F.) and 4th instar larvae (W.L). Image Blots were washed with an unpurified antiserum When blocking, the peptides were added to the antibody such that the antibodies would bind to the free peptide before binding to the PVDF film. The images were exposed at 45 sec.

Reference

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