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Expression and Evolution of the cation-coupled cotransporters in Aedes aegypti Larvae
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Abstract

Mosquitoes are the most dangerous animals in the world, causing over 1 million human deaths annually by transmitting diseases such as Zika, malaria and dengue fever. A potential mosquito control strategy is to interfere with their unique osmoregulatory processes that include taking in salt and water with each bloodmeal and transitioning from aquatic larvae to terrestrial adults. We studied one group of osmoregulatory proteins, known as cation-coupled cotransporters (CCCs), using quantitative PCR. We measured the expression of Aedes aegypti CCCs in larvae exposed to varying levels of NH4Cl, and transport variants (aeCC3) were upregulated in 1 mM NH4Cl, which was associated with waste products. In initial trials we found that in a separate project, we studied the family of membrane proteins that the CCCs belong to, the solute carrier family 12 (SLC12). By making phylogenetic trees, we assessed the evolution of the SLC12 family from a basal eukaryote. We also evaluated Aedes CCCs in the context of other animal CCCs. We found that aeCC2 and aeCC3 seem to result from an insect specific gene duplication event, and thus are not closely related to vertebrate transport proteins.

Introduction

Background

We have observed that knockdown of the Na+/K+/2Cl− transporter (NKCC, or aeCC1) and aeCC2 caused higher levels of hemolymph NH4Cl in Ae. aegypti larvae (1). As larvae live in aquatic environments that are often polluted with high concentrations of ammonia, it is probable that some protein is responsible for NH4 movement in larvae. NH4 can also substitute for K+ in some cation-coupled cotransport, so these cotransporters serve as potential candidates for NH4 movement. At its most ancestral root, the NKCC seems to originate in animals. Other members of the SLC12 superfamily of proteins, such as CCC-interacting proteins (CIP1) and K+ cotransporters (CCTs), are seen in animals as well as other eukaryotes, including fungi and protists. Insects’ Na-dependent CCC duplicated after the divergence of vertebrates from invertebrates, and a second duplication event is responsible for the third copy of CCC (2, Fig. 1). In mosquitoes, aeCC3 is seen significantly more in larval than in adults (3,4), and this suggests that aeCC2 and aeCC3 may have a functional difference.

Methods

RNA isolation, cDNA synthesis, and qPCR: RNA was isolated from fourth instar larvae using Trizol reagent and protocol recommended by the manufacturer (ThermoFisher). RNA samples were purified with TURBO DNA-free (Ambion) and Clean & Concentrator (Zymo) according to manufacturer instructions. The concentrations of the RNA samples were measured by spectrophotometry. Total RNA (2.5 μg) was reverse transcribed to cDNA using the Takara reverse transcriptase kit (Taqman reverse transcriptase kit) under the following conditions: 50 μl reaction, 25 °C for 10 min, 42 °C for 30 min, 95 °C for 5 min, 4 °C infinite hold. Template RNA without reverse transcriptase added to the reactions served as the negative control. Relative levels of transcript were quantified using SYBR Green (ABI), with primers for ribosomal protein S5 (aeRsp5) as the endogenous control and primers to detect aeNKCC, aeCC2, and aeCC3. The template-free cDNA was used as the negative control. The qPCR ran as follows: 50 °C for 2 min, 95 °C for 30 min, 40x (95°C for 15 seconds, 60 °C for 1 min), with dissociation stage (95 °C for 15 seconds, 60 °C for 1 min, 95 °C for 15 seconds, 60 °C for 15 seconds) on the 7500 RT PCR System (ABI). DNA levels were quantified using dCT values and compared by ANOVA.

Phylogenetic Analysis: Sequences with similarity to Aedes aegypti NKCC or CCC were obtained by BLAST search (NCBI) by queries with phyly of interest. Transmembrane domains were isolated using TOPCONS and sequence alignment in MAFFT. Preliminary trees were built in MAFFT by neighbor-joining on all gap-free sites and WAG substitution model (100 bootstrap trials).

Results

Did the duplication event responsible for insect CCC3/3 occur in crustaceans?

- Homo sapiens NP_001139435 1
- Hyatella azteca XP 018006465 1
- Mus musculus NP_000580355 1
- Portunus trituberculatus ANU07831 1
- Callicrates sapidus AAF07502 1
- Homo sapiens NP_0003932 1
- Homo sapiens NP_0003931 1
- Manduca sexta Mse008440
- Anopheles gambiae AAG001557
- Aedes aegypti AAL006180
- Daphnia pulex EPK74118 1
- Manduca sexta Q24579 1
- Manduca sexta Mse014748
- Aedes aegypti AAEL008988
- Anopheles gambiae AGAP003274
- Anopheles gambiae AGAP003275

are suggestively more closely related to insect species than NKCC. Malacostraca group more closely to insect CCC and NKCC. Malacostraca group separate from the insect CCC3/3 and branchiopods.

Phylogenetic data: Insects experienced a basal duplication event to the ancestral NKCC, and mosquito and lepidoptera experienced a second duplication event later in their evolution.

- Vertebrates experienced a gene duplication to their NKCC lineages independent of the arthropod duplication event.
- Crustacean NKCC did not experience a gene duplication event, and it may be that this ancestral NKCC gene has changed more to meet the needs of crustaceans as a result.
- Neither fungi nor protists have Na-dependent chloride cotransporters, and instead have CIP1 proteins, another member of the SLC12 family. These results agree with those published in (5).

qPCR data: There is no significant difference between these conditions, but aeCC3 trends towards being expressed less in the 1mM and 5mM conditions than in the control condition.

Conclusion/Future Questions

- Future Questions:
  - Would a higher concentration of NH4Cl induce changes in expression of aeCC3?

Will NH4Cl treatment upregulate any cation-coupled cotransporters in Aedes aegypti larvae?

- aeNKCC1
- aeCC2
- aeCC3

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