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Cloning and functional characterization of the *Anopheles albimanus* DMT1/NRAMP homolog: Implications in iron metabolism in mosquitoes

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Abstract

In addition to its wide role in metabolism, iron in insects has been implicated in vitellogenesis and the immune response. The NRAMP family comprises a well-conserved family of divalent cation transporters in metazoans. To gain insight on the role of NRAMP in *Anopheles albimanus*, we cloned a cDNA encoding a 571-residue protein (AnaNRAMP) with the structural features defining the NRAMP family. AnaNRAMP mRNA induced $^{59}Fe^{2+}$ incorporation when injected into *Xenopus* oocytes. Western blot analysis revealed that AnaNRAMP is expressed in the head, midgut and at high levels in Malpighian tubules of unfed female mosquito. Upon blood feeding, AnaNRAMP levels were reduced in the midgut whereas they increased in the Malpighian tubules. Using immunolocalization by transmission electron microscopy, AnaNRAMP was localized in the membrane of the intra-cellular concretions or spherites of the Malpighian tubule principal cells. Taken together, our results suggest an important role of AnaNRAMP in iron transport and indicate a role of the mosquito Malpighian tubule as an important organ for iron homeostasis.

Keywords: *Anopheles*; Malaria; Iron transport; Gonotrophic cycle; Malpighian tubules; Hematophagous insects

1. Introduction

Iron participates in many vital processes in insects, including the vitellogenic process (Kurama et al., 1995; Nichol et al., 2002). In *Anopheles gambiae* laboratory strains, refractoriness to *Plasmodium* infection is associated with an increased capacity to generate reactive oxygen species (ROS) (Lanz-Mendoza et al., 2002; Kumar et al., 2003), a biological process tightly linked to Fe and Mn metabolism (Culotta et al., 2005). Understanding divalent cation transport in these insects may provide physiological clues to design alternative strategies for vector control aimed at interrupting pathogen development by limiting iron availability.

The *D*ivalent *M*etal *T*ransporter 1/*N*atural *R*esistance *A*ssociated *M*acrophage *P*rotein (NRAMP) family comprises of a group of proton-coupled divalent cation transporters widely distributed in prokaryotes and in eukaryotes (Cellier et al., 1995, 2001). Proteins of the NRAMP family are mainly involved in Fe and Mn transport, but they have also been implicated in transporting other divalent cations such as Zn, Co, and Ni (Gunshin et al., 1997). In mammals, NRAMP 2 (also known as Dmt1, Dct1, Slc11a2) is the main nontransferrin-bound

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Fe^{2+} transporter from the gut lumen (Hentze et al., 2004), and the G185R mutation is associated with microcytic anemia in Belgrade rats and the *mk* mouse (Su et al., 1998). NRAMP1 (Slc11a1), the NRAMP2 paralog is expressed in macrophages and has been implicated in depriving intracellular pathogens of iron and manganese inside the phagocytic vacuole. The G169D NRAMP1 mutation is associated with increased susceptibility to intracellular infections in mice (Forbes and Gros, 2001). *Malvolio*, the NRAMP homolog in *Drosophila* is expressed in the nervous system, macrophages, midgut and Malpighian tubules (Rodrigues et al., 1995; Folwell et al., 2006) and has been implicated in the fly taste behavior (Rodrigues et al., 1995; D'Souza et al., 1999).

Due to its role in divalent cation metabolism, the participation of NRAMP in mosquito physiology requires documentation. We have cloned the cDNA of the NRAMP homolog in the mosquito *Anopheles albimanus*, a major malaria vector in Mexico and Central America (Rodríguez, 2005), characterized its iron transport capacity and analyzed its protein expression pattern in adult mosquitoes. Our results indicate that AnaNRAMP is a functional iron transporter that may be involved in mobilization of this cation primarily in the midgut and Malpighian tubules.

2. Materials and methods

2.1. Mosquitoes

The *White striped* strain of *An. albimanus* (Chan et al., 1994) were obtained from the Centro de Investigación de Paludismo in Tapachula, Chiapas, Mexico. Mosquitoes were reared in a 12 h:12 h light:dark cycle at 70% relative humidity and 25 °C, and fed with 10% sucrose solution *ad libitum*.

2.2. cDNA cloning, sequencing and sequence analysis

A pair of oligonucleotide guessmers AaF 5′-GGC AAC ATC GAG TCG GAT ATG CAG-3′ and AaR 5′-ATC ATG CCG CAC AAC CTG TAC CTG-3′ were designed based on the amino acid positions 95–102 and 271–278 of the *malvolio* protein (Accession ♯P49283), the *Drosophila melanogaster* NRAMP homolog. The gene region was chosen based on the sequence conservation determined by Clustal W 1.6 multiple sequence alignments of highly homologous regions between *Homo sapiens* (P49279 and P49281), *Mus musculus* (P49282), *Caenorabditis elegans* (U23525.1), and *Saccharomyces cerevisiae* (P38925) NRAMP family members; cDNA was obtained from oligo d-T18 primed reverse transcription of total RNA obtained from *White stripe* females using Superscript II RNAseH- (Gibco, UK). Conditions for PCR amplification were 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM AaF and AaR primers and 1 U of Taq Polymerase (Gibco,UK); for 35 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min). Once sequenced, the oligonucleotides, BAMP 5′-CGG GAT CCA ATG ATG CCC ACT CCT T-3′ and ERAMP 5′-GGA ATT CGC AGG CTG GAG CTA TTT T-3′ were used to amplify the 5′ and 3′ cDNA region, respectively, with the SMART RACE amplification kit (Clonetech, US) according to manufacturer's instructions. The 5′ and 3′ RACE products where cloned into the pCR_II TOPO vector (Invitrogen, UK).

The full length AnaNRAMP cDNA was generated by independent amplification of the 5′ and 3′ RACE products using the plasmid clones pCRII-5RS11, pCRII-3RS7 respectively. The primers Funi 5′-GGAATTC TGG TAT CAA CGC A-3′ and FuFor 5′-GAT GTA CTG ATA ACC AAA GG-3′ amplified the 1.1 kp 5′ RACE product, whereas Funi and FuRev 5′-CCT TTG GTT ATC AGT ACA TC-3′ amplified 1.4 Kb 3′RACE product. Funi is based on the long universal primer sequence of the SMART RACE amplification system and has an *Eco RI* site (underlined). FuFor and FuRev are complementary primers based in the overlapping region of 5′ and 3′ products. After amplification, PCR products were diluted 1000 fold and mixed 1:1 and used as PCR template using the Funi primer to yield a single 2.6 Kb product that was cloned in the pCRII-TOPO system to generate pCR-AnaNRAMP-FL (see supplementary Fig. 1. Genbank Accession EF153100). To search for iron responsive elements (IRE) in AnaNRAMP mRNA, a weight matrix was generated using Consensus (V.6a) (Hertz and Stormo, 1999) using as input known IRE sequences described by Nichol et al. (2002). The weight matrix was used to scan the AnaNRAMP mRNA sequence with Patser (Hertz and Stormo, 1999).

2.3. Evaluation of $^{59}Fe^{2+}$ transport in Xenopus oocytes

The full length AnaNRAMP cDNA was obtained by *Eco* RI digestion of pCR-AnaNRAMP-FL and was subcloned in the pcDNA3 vector (Invitrogen) to generate pcSlc11S7. The product encoded by pcSlcS7 has a Proline in position 440. To generate pcSlc11S8-Leu, the *Xho* I fragment from pcR-3RS8 replaced the corresponding segment in pcSlc11S7. A similar plasmid, pSPORT-DMT1 (kindly provided by Mathias A. Hediger, Institute of Biochemistry and Molecular Medicine, BERN, Switzerland.) containing the DMT1/NRAMP2 rat homolog was used as positive control (Gunshin et al., 1997). Plasmids (1 μg) were linearized with *Not* I and used for *in vitro* transcription with mMessage mMachine (Ambion Inc.) according to manufacturer instructions. The RNA was extracted with phenol/chloroform; ethanol precipitated and resuspended in DEPC-treated distilled H_2O. In all, 50 nL mRNA (0.5 μg μL^{-1}) was injected in stage V–VI *Xenopus laevis* oocytes. Negative control oocytes were injected with an equal volume of dH_2O. Injected oocytes were maintained in 3 mM pyruvate supplemented ND96 media at 18 °C for 4–5 days. Fe^{2+} incorporation was evaluated by incubating samples of 12 oocytes for 1 h in 10 μM ferrous ion ($^{59}Fe^{2+}$, as ferrous citrate salt) at 20 °C,