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Isolation and characterization of the cDNA encoding DH_{31} in the kissing bug, *Rhodnius prolixus*

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ABSTRACT

Rhodnius prolixus undergoes a period of rapid diuresis after ingesting large blood meals. Neurohormones with either diuretic or anti-diuretic activity control diuresis by acting on several tissues including the Malpighian tubules. One of the neurohormones that potentially plays a role in diuresis is diuretic hormone 31 (DH_{31}) which belongs to the insect calcitonin-like family of diuretic hormones. Here we determine the complete cDNA sequences of three *Rhopr-DH_{31}* splice variants (*Rhopr-DH_{31}-A, Rhopr-DH_{31}-B and Rhopr-DH_{31}-C*) and characterize their expression in unfed fifth-instar *R. prolixus*. Reverse transcriptase-PCR demonstrates that *Rhopr-DH_{31}* is predominantly expressed in the central nervous system (CNS) of unfed fifth-instars. However, the expression of the three splice variants differs with *Rhopr-DH_{31}-B* expression being the highest followed by *Rhopr-DH_{31}-A* and *Rhopr-DH_{31}-C*, as determined using semi-quantitative Southern blot analysis. Fluorescent *in situ* hybridization reveals that *Rhopr-DH_{31}* is expressed in a variety of cells in the CNS, including some neurosecretory cells.

1. Introduction

Rhodnius prolixus is a blood-feeding hemipteran which is confined to drier savannah areas of Central and South America (Dujardin et al., 1998; Feliciangeli et al., 2004; Monroy et al., 2003; Ramsey et al., 2000). This insect is a principal vector of Chagas' disease, an incurable illness damaging the human heart and nervous system, caused by the parasite *Trypanosoma cruzi* (Koberle, 1968). *Rhodnius prolixus* ingests large blood meals and then undergoes a period of rapid diuresis. Infection occurs when *R. prolixus* releases protozoans in urine that it deposits near the site of feeding (see Prata, 2001). Diuresis, the process of urine production, involves a variety of processes and tissues. These include ion and water movement across the epithelium of the anterior midgut and the Malpighian tubules, and muscle contractions of the anterior midgut, hindgut and dorsal vessel which facilitate mixing of the blood meal, mixing of the haemolymph, and the expulsion of waste (Coast et al., 2001, 2005; Donini et al., 2008; Te Brugge et al., 2005, 2008, 2009). Neurohormones with either diuretic or anti-diuretic activity control the function of Malpighian tubules (see Coast et al., 2002; see Schooley et al., 2005). Diuretic hormones (DHs) cause acceleration in primary urine production, whereas anti-diuretic hormones (ADHs) either stimulate fluid reabsorption in the hindgut or reduce Malpighian tubules secretion (see Coast et al., 2002). Insect DHs include serotonin (5-hydroxytryptamine) and various families of neuropeptides such as the calcitonin-like DHs (CLDH), kinin-like DHs, corticotropin-releasing factor (CRF)-like DHs and CAPA-like DHs (see Coast et al., 2002; Furuya et al., 2000; Kean et al., 2002; Maddrell et al., 1991; Te Brugge et al., 1999, 2002, 2005, 2009).

One of the neurohormones that may play a role in diuresis in *R. prolixus* is referred to as diuretic hormone 31 (DH_{31}), and this belongs to the CLDH family of insect peptides (Te Brugge et al., 2005). The first CLDH was identified in the Pacific beetle cockroach, *Diploptera punctata* (Furuya et al., 2000). Since then, CLDHs have been identified in several insects, as well as crustaceans and chelicerates (Table 1) (Christie, 2008; Christie et al., 2010; Coast et al., 2001, 2005; Gard et al., 2009; Li et al., 2008; Schooley et al., 2005). Here, we annotated CLDHs in the southern house mosquito, *Culex quinquefasciatus* and the honey bee varroa mite, *Varroa destructor* following a BLAST search of their genome databases. CLDHs are 31 amino acids in length in arthropods, with the exception of *Ixodes scapularis* and *V. destructor* (predicted) where they contain 34 amino acids. These peptides show a high degree of amino acid identity and are amidated at their carboxyl termini. Invertebrate CLDHs are less similar to vertebrate calcitonin but share the C-terminal Gly-X-Pro-NH_2 (Furuya et al., 2000). CLDHs have been shown to stimulate fluid secretion by Malpighian tubules (Coast et al., 2001; Furuya et al., 2000; Maddrell et al., 1991; Te Brugge et al., 2002, 2005; Te Brugge and Orchard, 2008), to have potent

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Table 1
Structures of mature DH_{31} peptides (deduced or sequenced) from 16 species.

Species	Peptide structure	Reference
Insects		
Rhodnius prolixus[1]	GLDLGLSRGFSGSQAAKHLMGLAAANYAGGP-NH_2	This study, Te Brugge *et al.*, 2008
Diploptera punctata[1]	GLDLGLSRGFSGSQAAKHLMGLAAANYAGGP-NH_2	Furuya et al., 2000
Apis mellifera[1]	GLDLGLSRGFSGSQAAKHLMGLAAANYAGGP-NH_2	Schooley et al., 2005
Tribolium castaneum[2]	GLDLGLGRGFSGSQAAKHLMGLAAANFAGGP-NH_2	Li et al., 2008
Bombyx mori	AFDLGLGRGYSGALQAKHLMGLAAANFAGGP-NH_2	Schooley et al., 2005
Drosophila melanogaster	TVDFGLARGYSGTQEAKHRMGLAAANFAGGP-NH_2	Coast et al., 2001
Anopheles gambiae[3]	TVDFGLSRGYSGAQEAKHRMAMAVANFAGGP-NH_2	Coast et al., 2005
Aedes aegypti[3]	TVDFGLSRGYSGAQEAKHRMAMAVANFAGGP-NH_2	Schooley et al., 2005
Nasonia vitripennis	GLDLGLNRGFSGSQAAKHLMGLAAANYAGGP-NH_2	Predicted
Acyrthosiphon pisum	GLDLGLSRGYSGTQAAKHLMGMAAANFAGGP-NH_2	Predicted
Nilaparvata lugens	GLDLGLSRGFSGSQAAKHLMGLAAANYAAGP-NH_2	Predicted
Culex quinquefasciatus[3]	TVDFGLSRGYSGAQEAKHRMAMAVANFAGGP-NH_2	This study, Predicted
Crustaceans		
Daphnia pulex	GVDFGLGRGYSGSQAAKHLMGLAAANYAIGP-NH_2	Gard et al., 2009
Homarus americanus[2]	GLDLGLGRGFSGSQAAKHLMGLAAANFAGGP-NH_2	Christie et al., 2010
Chelicerates		
Ixodes scapularis	AGGLLDFGLSRGASGAEAAKARLGLKLANDPYGP-NH_2	Christie et al., 2008
Varroa destructor	SNGMLDFGLARGMSGVDAAKARLGLKYANDPYGP-NH_2	This study, Predicted

Amino acids that are shared by all sequences are highlighted in gray.
[a]Identical sequences.
[b]Identical sequences.
[c]Identical sequences.

natriuretic activity (Coast et al., 2005) and to increase dorsal vessel and hindgut contractility (Te Brugge et al., 2008). Moreover, Rhopr-DH_{31} causes an increase in cAMP concentration and contraction frequency in the anterior midgut (Te Brugge et al., 2009). Since these processes are associated with diuresis, CLDHs might play an important role in post-feeding diuresis in *R. prolixus*.

In the present study, complete cDNA sequences of three *Rhopr-DH_{31}* splice variants (*Rhopr-DH_{31}-A*, *Rhopr-DH_{31}-B* and *Rhopr-DH_{31}-C*) were obtained, which encode a mature peptide that is 100% identical to that determined previously by MALDI-TOF mass spectrometry for Rhopr-DH_{31} (Te Brugge et al., 2008). Northern blot hybridization was performed to confirm the approximate size of the DH_{31} gene (*DH_{31}*) transcripts. Reverse transcriptase-PCR (RT-PCR) analysis also confirmed *Rhopr-DH_{31}* expression in the central nervous system (CNS). Semi-quantitative analysis using Southern blot hybridization to determine the relative expression of the three splice variants within the CNS revealed that *Rhopr-DH_{31}-B* expression was the highest followed by *Rhopr-DH_{31}-A* and *Rhopr-DH_{31}-C* in fifth-instar *R. prolixus*. Using *Rhopr-DH_{31}-A* partial cDNA sequence to design a probe, fluorescent *in situ* hybridization (FISH) was performed to localize the cell-specific expression of *Rhopr-DH_{31}*, revealing a number of cells distributed throughout various regions of the CNS.

2. Methods

2.1. Animals

Fifth-instar *R. prolixus* of both sexes were taken from a long standing colony at the University of Toronto Mississauga. Insects were maintained at high relative humidity in incubators at 25 °C and were fed on rabbits' blood. All the tissues used were dissected from 6 weeks post-fed (as fourth-instars) insects in nuclease-free phosphate-buffered saline (PBS) (Sigma–Aldrich, Oakville, ON, Canada) and were either used immediately or stored at −20 °C in RNAlater™ RNA stabilization reagent (Qiagen Inc., Mississauga, ON, Canada).

2.2. Screening of fifth-instar CNS cDNA library

Degenerate forward primers (dh31forward1, dh31forward2, dh31forward3 and dh31forward4) (Supplementary Table S1) were designed using the Rhopr-DH_{31} peptide sequence (Te Brugge et al., 2008). These primers were used along with plasmid reverse primers (DNR-LIB REV110 and DNR-LIB REV77) (Supplementary Table S1) to obtain a partial *Rhopr-DH_{31}* cDNA sequence. Next, modified 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR reactions were performed. The partial *Rhopr-DH_{31}* cDNA sequence was used to design gene-specific forward and reverse primers for 3′ and 5′ RACE, respectively. For 5′ RACE, semi-nested PCRs were performed using two plasmid forward primers (DNR-LIB FOR1 and DNR-LIB FOR2) and four gene-specific reverse primers (DH31 5race1, DH31 5race2, DH31 5race3 and DH31 5race4) (Supplementary Table S2). One plasmid reverse primer (pDNR-LIB 3–25 REV) and four gene-specific forward primers (FOR1DH31, FOR2DH31, FOR3DH31 and FOR4DH31) were used to perform the semi-nested PCRs for 3′ RACE (Supplementary Table S3). The forward and reverse primers were used successively in order to selectively amplify the specific products. PCR product of each reaction was either column purified using EZ-10 Spin Column PCR Products Purification Kit (Bio Basic Inc., Markham, ON, Canada) or gel extracted using EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham, ON, Canada) and used as a template for the subsequent reaction. Plasmid DNA isolated from a *R. prolixus* CNS cDNA library was used as the template for the above reactions (Paluzzi et al., 2008). All PCR reactions were performed using GeneAmp® PCR System 9700 (Applied Biosystems) Thermocylcer. Temperature-cycling profiles remained constant and were based on the following profile: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50–60 °C (depending on the primers used) for 30 s and 72 °C for 1 min. A final 10 min extension at 72 °C was also included. Products generated after the final PCR were gel extracted, cloned using the pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA), and the plasmid DNAs isolated from the overnight cell cultures and sequenced at the SickKids DNA Sequencing Facility (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada).

Northern blot analysis revealed that the size of transcripts was comparatively larger (see below) than that obtained following the 5′ and 3′ RACE PCR reactions. *In silico* analyses confirmed that a considerable region at the 3′ end could not be cloned using the 3′ RACE reactions and thus the following approach was adopted to obtain the complete *Rhopr-DH_{31}* cDNA sequence for all three splice variants. *Rhopr-DH_{31}* 3′ UTR sequence was predicted using the *R. prolixus* preliminary genome assembly and used to design forward and reverse gene-specific primers for PCR (Supplementary Table S4). CNS single-stranded cDNA was used as a template for these PCR reactions (for cDNA synthesis, see Section 2.5).

2.3. cDNA sequence analysis

The deduced Rhopr-DH_{31} prepropeptide sequences were examined for potential signal peptides using SignalP 3.0 (Bendtsen et al., 2004) and potential ubiquitination sites using UbPred (Radivojac et al., 2010). The intron–exon boundaries of *Rhopr-DH_{31}* were determined using BLAST (Altschul et al., 1990) search results obtained from the *R. prolixus* preliminary genome assembly (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=insects – last accessed on April 12, 2010) and confirmed using Genie, online software for splice site prediction (Reese et al., 1997). The three Rhopr-DH_{31} variant prepropeptide sequences (products of transcript variant A – HM030716, transcript variant B – HM030715 and transcript variant C – HM030714) and their homologous sequences from other insect species, including *Drosophila melanogaster transcript variant A* (NM_078790.3), *D. melanogaster transcript variant C* (NM_164825.2), *Bombyx mori* (NP_001124379.1), *Anopheles gambiae* (EAA01397.3/XP_321755.3),