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OKB, a novel family of brain-gut neuropeptides from insects

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ABSTRACT

In insects, neuropeptides play a central role in the control of most physiological processes. The knowledge and characterization of new neuropeptide families, is of interest on the fields of Genetics, Genomics, Neurobiology, Endocrinology and Evolution. This knowledge also provides the tools for the design of peptidomimetics, pseudopeptides or small molecules, capable of disrupting the physiological processes regulated by the signaling molecules and their receptors. This is a promising target for a novel generation of insecticides. Using database searches, mass spectrometry and RACE-PCR, we identified a neuropeptide precursor transcript encoding a new family of insect neuropeptides in the hemipteran *Rhodnius prolixus*. We named this precursor Orcokinin B, because is originated by the alternative splicing of the Orcokinin gen. EST and genomic data suggests that Orcokinin B is expressed in the nervous system and gut from several insect species, with the exception of *Drosophila* sp. (Diptera) and *Acyirthosiphon pisum* (Hemiptera). Mass spectrometry and RT-PCR confirmed the expression of Orcokinin B in brain and anterior midgut of *R. prolixus*. Furthermore, we identified orthologues of this new family of peptides in genomic and EST databases from Arachnids and Crustaceans.

1. Introduction

In insects, neuropeptides play a central role in the control of most physiological processes. The biologically active neuropeptides are produced as large precursors, which are cleaved and further modified to yield mature peptides. Mature neuropeptides are secreted to the extracellular environment to exert their physiological role. The insect neuropeptides and its receptors are a promising target for a novel generation of insecticides, which offer improved selectivity and environmental compatibility (Scherkenbeck and Zdobinsky, 2009). The knowledge of neuropeptide system provides the tools for the design of peptidomimetics, pseudopeptides or small molecules capable of disrupting the physiological processes regulated by the signaling molecules and their receptors. Thus, the structural and functional characterization of the neuropeptidomes from pest insects is the first requirement to develop strategies to replace or complement conventional neurotoxic insecticides.

The triatomine *Rhodnius prolixus* is one of the main vectors of Chagas' disease, which is caused by the protozoan *Trypanosoma cruzi*. Chagas' disease is an important human disease, with 10 million people affected in Central and South America (http://www.who.int/mediacentre/factsheets/fs340/en/). The medical importance of triatomine insects in the transmission of Chagas' disease motivated the sequencing of *R. prolixus* genome, and the generation of EST data (http://rprolixus.vectorbase.org/).

The identification of neuropeptide-encoding genes in genomes has mainly relied in homology searches, using orthologous sequences as queries. This strategy has allowed the identification of structurally related molecules in different species (see for example Hauser et al., 2010; Hewes and Taghert, 2001; Hummon et al., 2006;

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Li et al., 2008; Ons et al., 2011; Roller et al., 2008), but the drawback is the detection of functional molecules of different sequence and, therefore, the discovery of new molecular families.

Among the arthropods neuropeptides, Orcokinins (OK) occur in several species (see Christie, 2008; Christie et al., 2010; Ons et al., 2011). Although the physiological function of OK has not been extensively studied in insects, recent results demonstrate its implication in the regulation of ecdysteroidogenesis in the lepidopteran *Bombyx mori* (Yamanaka et al., 2011), mean while a previous report suggests a role in the control of circadian locomotor activity in the cockroach *Leucophaea maderae* (Hofer et al., 2005). In this paper, we report the identification, expression and characterization of the neuropeptide precursor transcript *RhoprOKB* in *R. prolixus*. *RhoprOKB* is originated by alternative splicing of the OK gen, resulting in a new family of mature neuropeptides expressed in the anterior midgut and nervous system. EST data suggests that this new family of peptides is widely occurring in insect, arachnids and crustaceans.

2. Material and methods

2.1. Insect rearing

A colony of *R. prolixus* was maintained in our laboratory in a 12 h light/dark schedule at 28 °C. Insects were weekly fed on chickens.

2.2. RhoprOKB transcript identification and characterization

An EST database from *R. prolixus* anterior midgut (http://rhodnius.iq.ufrj.br) was used for the homology based search, performed by local TBLASTN by using BLOSUM62 and *RhoprOKA* sequence as query. The EST detected was used to design specific primers to sequence the complete open reading frame of the *RhoprOKB* transcript by using RACE-PCR. For the 3′ region we used the primer OKB-T7 fwd (5′TAATACGACTCACTATAGGGTAGACGGTGTTATCGTAGAG-3′) and the 3′Genracer primer from the GeneRacer® Kit (Invitrogen, Argentina). For sequencing of the 5′ region we used the primers OKA/B Fwd: 5′-GATGATCAACATGCTGTCGT-3′ and OKB rev 5′-AATGACCTCCACCCAAACCAT-3′.Template cDNA was prepared from the anterior midgut of adult *R. prolixus* at different times post-feeding using the guanidine thiocyanate method. RACE was performed using the GeneRacer® Kit with SuperScript® III RT (Invitrogen, Argentina). PCR fragments were cloned using the pGEM-T Easy Vector System (Promega, Argentina) and sequenced.

For prediction of convertase cleavage sites the rules described for insect neuropeptide precursors (Veenstra, 2000) were followed.

2.3. RT-PCR tissue profiling

Tissues were micro-dissected from adult *R. prolixus* at different times post-feeding. Total RNA was isolated from tissues using Trizol (Ambion, Sao Paulo, Brazil). cDNA was synthesized using High Capacitv cDNA Reverse Transcription Kit (Applied Biosystems, Sao Paulo, Brazil), following manufacturer instructions. An aliquot of the cDNA from each tissue was used as a template to perform the subsequent PCR reactions. The following temperature cycling parameters were used to amplify the *RhoprOKA* and *RhoprOKB* fragments in parallel reactions: 94 °C 10 min (1 cycle); 94 °C 45 s, 55 °C 45 s, 72 °C 1.5 min (40 cycles); 72 °C 10 min (1 cycle). For the amplification of *Rhopr*OKA fragment we used the OKA/B forward primer (GATGATCAACATGCTGTCGT) and the OKA reverse primer (AATGATGTGGCTCACTTT). For the amplification of *Rhopr*OKB fragment, a seminested PCR reaction was performed. For the primary reaction we used the OKA/B forward primer and the OKB reverse primer (AATGACCTCCACCCAAACCAT). The second PCR reaction was performed using the primers OK B forward (TAGACGGTGTTATCGTAGAG) and OKB reverse. For a positive control to test the quality of the template cDNA, a 320 bp fragment from Ribosomal protein L41 (*RhoprRL41*) was amplified using a forward primer with the sequence 5′-ATGAGGTACGGTGAATAGTTTC-3′, and a reverse primer with the sequence 5′-TTCTCTTCTCACGAATGAGCA-3′. The procedure was repeated three times using independent biological samples.

2.4. Mass spectrometry (MS)

MS analysis has been performed as described previously (Ons et al., 2009). Briefly, 20 *R. prolixus* brains were placed in 100 μl methanol/water/acetic acid (90, 9, 1, v/v/v), sonicated and centrifuged. The collected supernatant was placed over vacuum to remove organic solvents, re-diluted in 20 μl 0.1% trifluoroacetic acid (TFA) and de-salted using a C18 extraction disc (Varian, Darmstadt, Germany) activated with 80% acetonitrile (ACN)/0.1% TFA. Organic solvent was then removed and sample re-diluted in 20 μl 0.1% TFA.

Nano-RP LC separation was performed on a Dionex nano-LC system (LC Packings). Pre-column were self-packed using Dr. Maisch Reprosil-Pur 120 ODS-3 (Dr. Maisch, Ammerbuch-Entringen, Germany). Analytical column were self-packed with C18 RP material, (Vydac MS218, 5 μm particle size and 300 Å pore size, Vydac, Hesperia, CA). Brain samples were loaded onto the pre-column at a flow rate of 10 μl/min in loading buffer (0.1 v/v TFA in water) for 10 min. After valve switching peptides were eluted by a linear gradient of 10–60% v/v solvent B (80% v/v ACN, 0.1% v/v TFA in water) in 180 min. The flow rate was 0.3 μl/min. The gradient was then raised from 60 to 100% of solvent B in 10 min. The eluate was mixed with α-cyano-4-hydroxicinamic acid (CHCA, 10 mg/ml in 70% ACN 01% TFA) containing Glu-fibrinogen peptide (10 fmol/μl; Sigma) as internal standard, and directly spotted on a steel MALDI target plate using a Probot microfraction collector (LC Packing). Matrix was delivered with a flow rate of 0.9 μl/min and fractions were spotted at intervals of 15 s onto stainless steel LC-MALDI plates (Applied Biosystems/MDS Sciex).

MALDI-TOF- MS/MS was performed on an ABI 4800 analyzer (Applied Biosystems/MDS Sciex). Each spot was first analyzed by MS. Spectra in MS were recorded between 600 and 4000 m/z. Job-wide interpretation of the MS data allowed the 15 highest peptides in intensity (with signal to noise ratios $\geq$30) for sequencing in MSMS mode. 1000 (3800 J) and 5000 (4500 J) laser shots were applied for MS and MS/MS, respectively. Collision energy was set to 1×10^{-6} Torr, with potential difference between the source acceleration voltage and the collision cell set at 1 kV. An eight point plate model calibration was performed with the Calibration Mixture 5 kit for Proteomics Analyzer (Applied Biosystems/MDX Sciex).

MS/MS spectra were processed and peak lists were generated. De novo sequencing was first performed automatically by Peaks Studio 3.1 software (Bioinformatic Solutions, Ontario, Canada) with the following settings: enzyme: none; parent ion mass error tolerance: 0.1 Da; fragment ion mass error tolerance: 0.1 Da. Manual analysis has been performed in the spectra that mass-match the peptides predicted from *RhoprOKB* gene, identifying accurate series of b-and y- ions, and a-, z-ions and immonium ions confirming sequences.

2.5. Software

Signal peptide sequences were predicted with the SignalP server (Bendtsen et al., 2004) (http://www.cbs.dtu.dk/services/SignalP/). Protein sequence alignment was carried out using BioEdit 7.0.9. We searched the nucleotide collection (nr/nt), the non-human and non-mouse ESTs databases and Whole Genome Sequence (WGS)

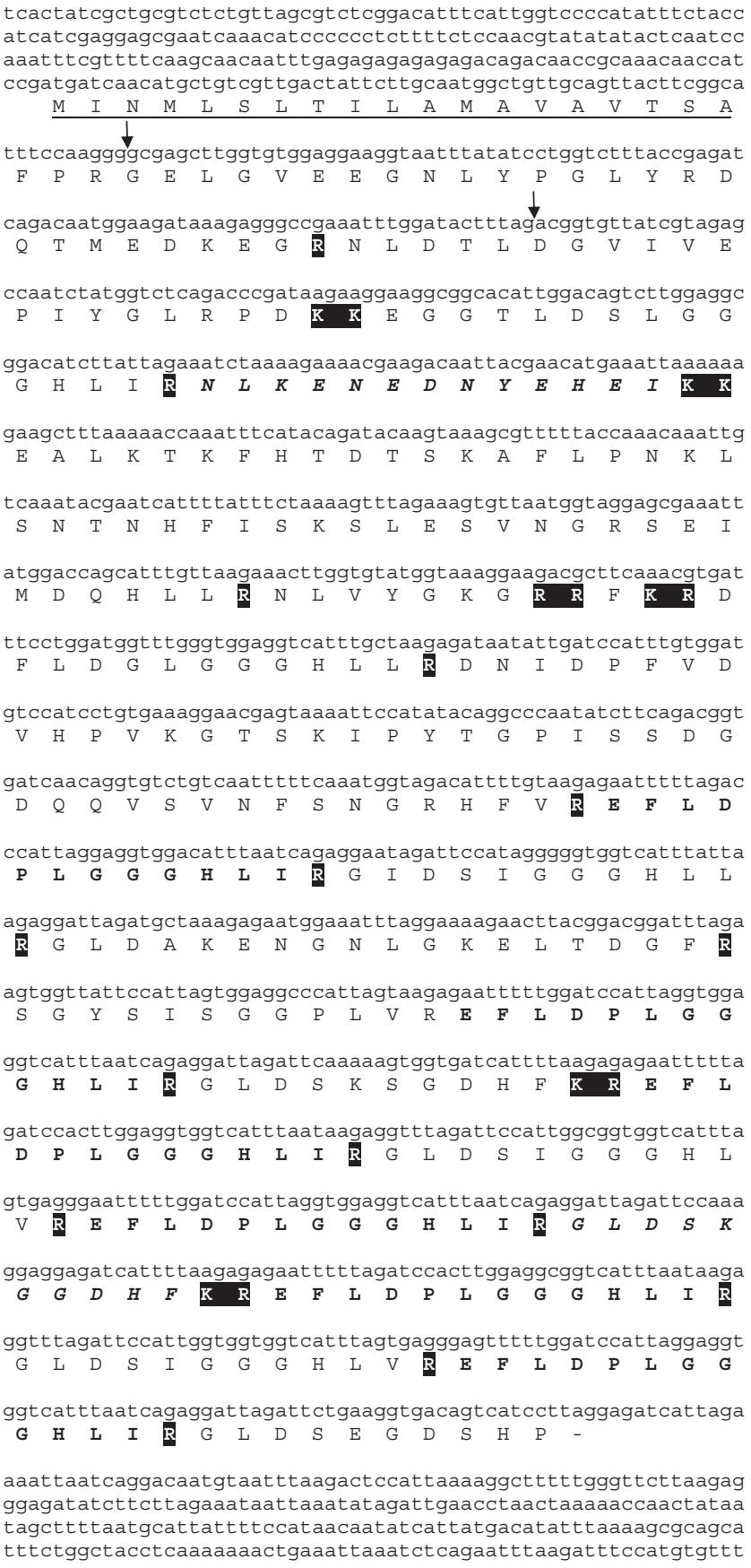

Fig. 1. Complete nucleotide and amino acid sequence of *RhoprOKB* transcript, and mature peptides predicted from the sequence. Predicted signal peptide sequence is underlined. The mono and dibasic post-translational cleavage sites are highlighted in black. Peptides identified by MS/MS are in bold face, mass-match identified peptides are in italic face. Arrows indicate introns. M + H^+ for each peptide are indicated.

ttttatttacttaaaaattttatgcattcatacaaattatatatatttttttccatatat
tgtagctcattagtaataatataataataaaaccgcttt

Predicted mature peptides encoded:

RhoprOKB1: FPRGELGVEEGNLYPGLYRDQTMEDKE-amide (3141.49)

RhoprOKB2: NLDTLDGVIVEPIYGLRPD (2099.10)

RhoprOKB3: EGGTLDSLGGGHLI (1325.67)

RhoprOKB4: ***NLKENEDNYEHEI*** (1646.73)

RhoprOKB5: EALKTKFHTDTSKAFLPNKLSNTNHFISKSLESVNGRSEIMDQHLL (5226.70)

RhoprOKB6: NLVYGK-amide (692.41)

RhoprOKB7: DFLDGLGGGHLL (1213.62)

RhoprOKB8: DNIDPFVDVHPVKGTSKIPYTGPISSDGDQQVSVNFSNGRHFV (4670.29)

RhoprOKB9: **EFLDPLGGGHLI** (1267.67)

RhoprOKB10: GIDSIGGGHLL (1038.56)

RhoprOKB11: GLDAKENGNLGKELTDGF (1877.92)

RhoprOKB12: SGYSISGGPLVR**EFLDPLGGGHLI** (2441.28)

RhoprOKB13: GLDSKSGDHF (1062.48)

RhoprOKB14: GLDSIGGGHLV (1024.54)

RhoprOKB15: GLDSKGGDHF (1032.47)

RhoprOKB16: GLDSEGDSHP (1013.42)

Fig. 1. (*continued*).

databases from invertebrates using the TBLASTN program (http://www.blast.ncbi.nlm.nih.gov). For the construction of the alignments showed in Fig. 4 we used sequences with the following Genebank™ accession numbers: Rhimi OKA + B: CK189083; Dappu OKA + B FE416856, Rhopr OKB: JF761320; RhoprOKA + B: FJ167860; Anoga OKB: BX067041; Anoga OKA: XM320317; Apime OKB: BI504477; Apime OKA: DB747167; Bommo OKB: FY059912; Bommo OKA + B: AB298932; Acypi OKA: XM001947427; Trica OKB: XM001810367.

3. Results

In our previous comprehensive characterization of the *R. prolixus* neuropeptidome, we identified a neuropeptide precursor gene encoding Orcokinin-like peptides (*RhoprOKA*) (Ons et al., 2011). Using *RhoprOKA* sequence as query, we have performed a TBLASTN search in an EST database from *R. prolixus* anterior midgut (http://rhodnius.iq.ufrj.br). This search revealed the presence of a partial open reading frame (ORF) containing the first two exons of *RhoprOKA* (encoding the signal peptide) followed by a third exon, absent in the *RhoprOKA* isoform. To obtain the complete sequence of the transcript, we performed RACE-PCR on cDNA from *R. prolixus* anterior midgut. The cloned cDNA (*RhoprOKB*, Genbank™ accession number: JF761320) spans 1719 nucleotides, with an ORF encoding a prepropeptide of 394 amino acid residues (Fig. 1). From these, the first 53 amino acids are identical to the *RhoprOKA* precursor, including the predicted signal peptide and a cleavage site at amino acids 19–20. Dibasic or monobasic cleavage sites can be predicted from the sequence to generate 21 putative mature peptides (Fig. 1). Among them, 11 show the characteristic GGGHLX (X = L, I, V) C-terminus domain and 7 display the characteristic N-terminus domain GX_1DX_2 (X_1 = L, I; X_2 = S, A) (Fig. 2). Six of the predicted peptides are

Fig. 2. Ten novel *RhoprOKB* peptides with the characteristic domains are showed. Amino acid residues that are similar (grey) ore identical (black) in at least five peptides are highlighted.

"spacer" peptides, namely peptides occurring in the precursor sequence flanked by endopeptidase recognition sites, but that lack the characteristic repetitive domain of the core peptides (Wegener and Gorbashov, 2008) (Fig. 1). The presence of a C-terminus G residue implies that two of the putative peptides might be amidated. A peptide with sequence EFLDPLGGGHLI is repeated 6 times in the precursor. The presence of this peptide was detected by MS/MS in extracts from *R. prolixus* brains (Fig. 3), confirming the expression of the precursor.

Interestingly, previously predicted OKs from arachnids (Christie, 2008) and crustaceans (Christie et al., 2010, 2011; Dircksen et al., 2011) encode the OKA and OKB peptides in the same transcript, resulting in hybrid OKA + B precursors (see Fig. 4). *Daphnia pulex*, the only crustacean with sequenced genome, shows two putative genes encoding OK peptides (Dircksen et al., 2011). Both precursors give rise to OKA and OKB mature peptides (OKA + B transcripts). An analysis of genomic and EST databases from insects, however, reveals that OKA and OKB prepropeptides are produced by alternative splicing of a single gene (Fig. 5).

TBLASTN searches in insects genomic sequences reveal the presence of genes expressing OKB related peptides in *Anopheles* sp., *Aedes aegypti*, *Culex quinquefasciatus* (Diptera), *Nasonia vitripenis*, *Apis mellifera* (Hymenoptera), *B. mori* (Lepidoptera), *Pediculus humanus* (Phthiraptera) and *Tribolium castaneum* (Coleoptera) (See Fig. 4 for the sequence alignment of OKA and OKB precursors in Arthropod species). An analysis of the mature peptides encoded in OKB transcripts in different insect species reveals the consensus sequence $X_1DXX_1GGGX_2LX_3$ (X_1 = I or L; X_2 = N or V; X_3 = I, L or V) for OKB peptides (Fig. 6). OKB sequences could not be found in any of the *Drosophila* species with sequenced genome (Diptera), or in the pea aphid *Acyrthosiphon pisum* (Hemiptera). OKA has been previously identified in all the previously mentioned insect species, with the exception of species of the Genus *Drosophila* and *T. castaneum*. Interestingly, OKA transcripts described for *B. mori* (Roller et al., 2008) conserve a fragment encoding for a peptide with the OKB characteristic sequence (NLDPLGGGNLV), being essentially an OKA + B precursor (Fig. 4). The OKB peptide present in the OKA + B precursor is encoded in an exon that is absent from the OKB isoform. Sequence alignment of OKA, OKB and OKA + B prepropeptides from arthropods shows the structural conservation among isoforms (Fig. 4). Altogether, these observations suggest that OKA and OKB transcripts could be originated in arthropods as an internal duplication and posterior mutation of an ancestral gene. In insects, a splicing site occurs, rising OKA (or OKA + B in *B. mori*) and OKB transcripts (Fig. 4).

Screening of insect EST databases using TBLASTN with *RhoprOKB* as query, reveals the presence of OKB transcripts in the guts of Holometabola and Hemimetabola Orders: *Dendroctonus ponderosae* (GO493469; E = 0.048; Coleoptera), *Epiphyas postvittana* (EV807453; $E = 1\ e^{-07}$), *Ostrinia nubilalis* (GH998356; $E = 4\ e^{-18}$), and *Spodoptera frugiperda* (DY791627; $E = 8\ e^{-11}$) (Lepidoptera), *Locusta migratoria* (CO860835; $E = 8\ e^{-16}$; Orthoptera) and *Reticulitermes flavipes* (FL637349S; $E = 4\ e^{-24}$; Isoptera). Recent results from Yamanaka et al. (2011) also suggest the expression of OKB transcripts in the gut of *B. mori* (see discussion).

To determine the expression pattern of *RhoprOKA* and *RhoprOKB* genes, RT-PCR tissue profiling was performed (n = 3 independent trials). The quality of the template cDNA was evaluated by the amplification of a 320 bp fragment from the *RhoprRL*41 gene, as a positive control. Results demonstrate that, whereas *RhoprOKA* is expressed only within the central nervous system (CNS), *RhoprOKB* expression was confirmed in CNS and anterior midgut. No PCR product was detected in the other tissues tested (posterior midgut, hindgut, salivary glands, Malpighian tubules, fat body, ovaries and testes) (Fig. 7).

4. Discussion

In this paper we describe OKB, which constitute a novel neuropeptide family in arthropods. Insects have two different transcripts encoding OKA and OKB mature neuropeptides, occurring by alternative splicing of the OK gene, whereas the other arthropod groups express OKA and OKB peptides in the same transcript, rising OKA + B precursors. The data suggests that OKA and OKB transcripts were originated by events of intragenic duplication and further mutation. The existence of OKA + B precursors in arachnids and crustaceans, the structural similarity among both precursors in insects (Fig. 4), and the fact that a lepidopteran as *B. mori* possesses OKB molecules within the OKA transcript, supports this hypothesis.

In insects, OKB shows a widespread occurrence in Hemimetabola (Isoptera, Hemiptera, Orthoptera, Phthiraptera) and Holometabola (Coleoptera, Diptera, Hymenoptera, Lepidoptera). However, some derived insects such as the sequenced species of *Drosophila* (Diptera), and also the Hemiptera *A. pisum*, seems to have lost the OKB molecules. Whereas *Drosophila* might have lost the whole OK gene, *A. pisum* conserves the OKA precursor gene, and *T. castaneum* has lost OKA molecules, but conserves OKB. This would indicate that OK signaling system might have been abandoned, as a whole or in part, by different species independently during evolution.

Alternative splicing is a way to generate neuropeptide diversity. Our previous work on the *R. prolixus* neuropeptidome has showed splicing variants of another neuropeptide precursor gene, which originates different molecules in a tissue-specific pattern (Ons et al., 2011). Isoforms A and B of Calcitonin-like diuretic hormone (CT-like-DH) where found in nervous system, encoding a signal peptide and a mature peptide with diuretic activity (Te Brugge

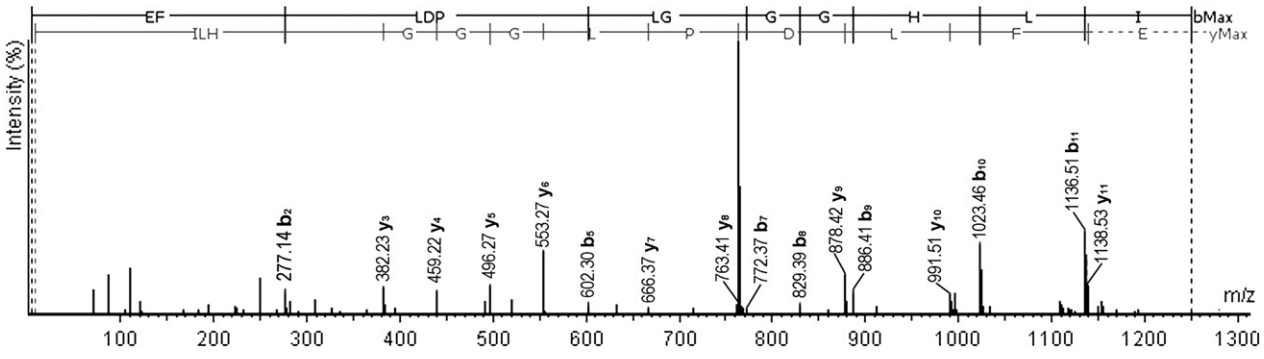

Fig. 3. MS/MS spectrum verifying the presence of the peptide EFLDPLGGGHLI in *R. prolixus* brain extracts. Prominent fragment ions are labeled.

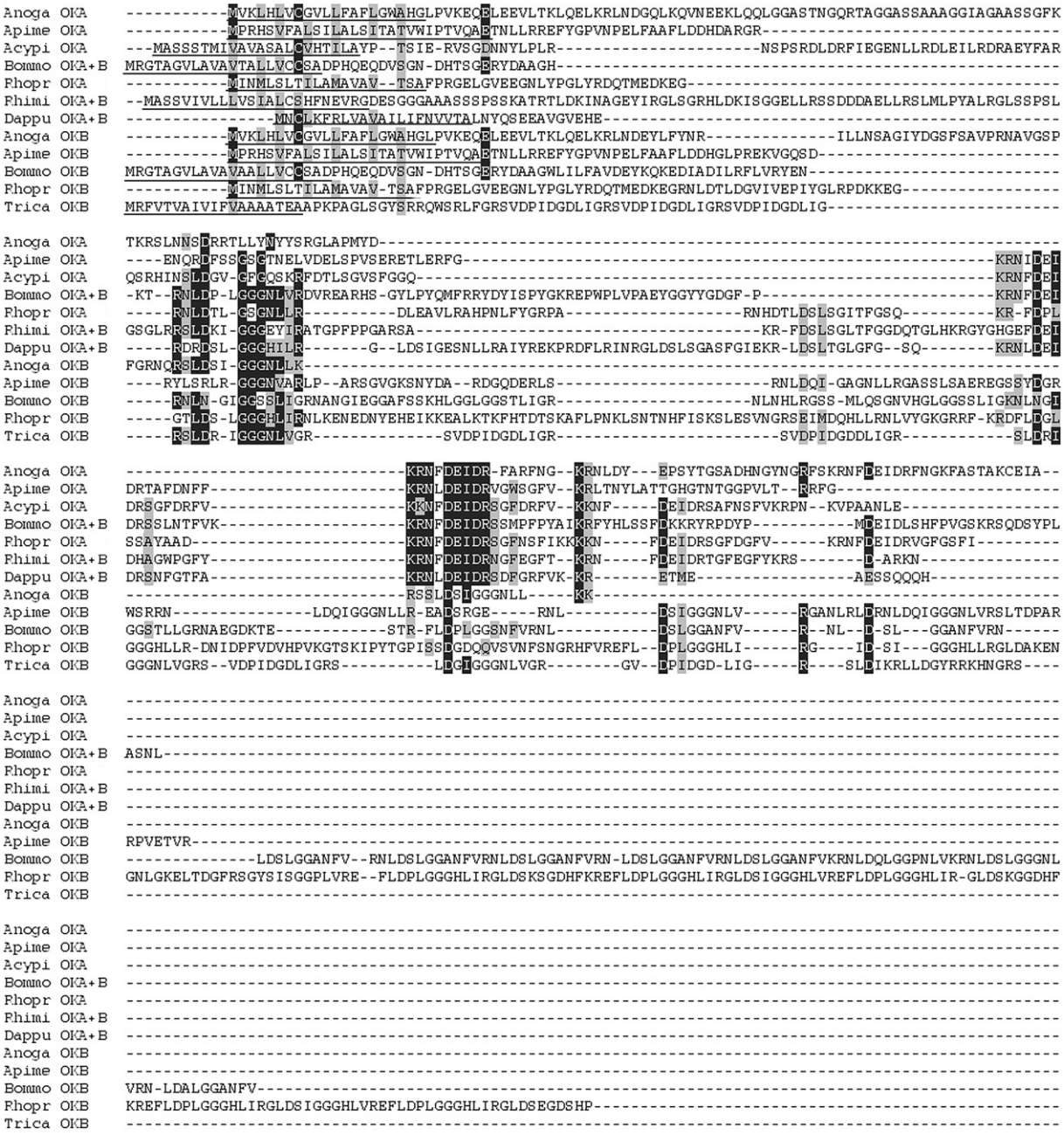

Fig. 4. Alignment of the OKA, OKA + B and OKB preprohormones from arthropods. Anoa: *Anopheles gambiae*, Apime: *Apis mellifera*, Bommo: *Bombyx mori*, Pedu: *Pediculus humanus*, Rhopr: *Rhodnius prolixus*, Acypi: *Acyrthosiphon pisum*, Rhimi: *Rhipicephalus microplus*, Dappu: *Daphnia pulex*, Trica: *Tribolium castaneum*. Amino acid residues that are similar (grey) ore identical (black) in at least seven preprohormones are highlighted. Predicted signal peptides are underlined.

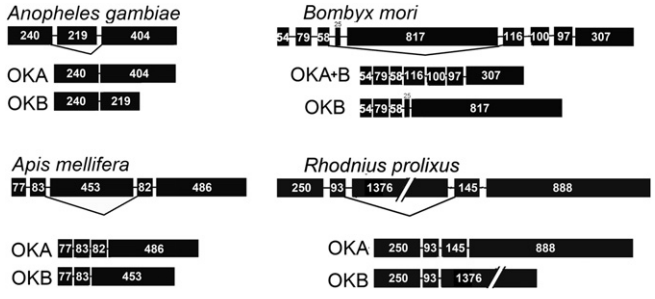

Fig. 5. Structure of the Orcokinin neuropeptide precursor gene in insect species. The numbers into each exon (black boxes) indicates its size in nucleotides. Lines represent introns. For every insect species studied OKA (OKA + B in *B. mori*) and OKB transcripts are formed by alternative splicing of the Orcokinin gene.

et al., 2005). Isoform C, however, is expressed in testes, without the characteristic hallmarks of a neuropeptide precursor (i.e signal peptide, putative mature peptides flanked by basic processing sites). This example and the present findings on OKB, indicate that internal duplication, alternative splicing and differential expression, may constitute mechanisms for the evolution of the hormonal control on different physiological processes by structurally related molecules.

Our experimental results in *R. prolixus* and the searches in EST databases, suggests that OKB is expressed in the gut from insects of several orders. Whereas in other insects endocrine cells are found throughout the midgut (Zitnan et al., 1993; Veenstra et al., 1995), previous reports suggested that in *R. prolixus* endocrine cells were only present in the posterior midgut (Billingsley and Downe, 1986; Te Brugge et al., 1999, 2001; Tsang and Orchard, 1991). Our findings

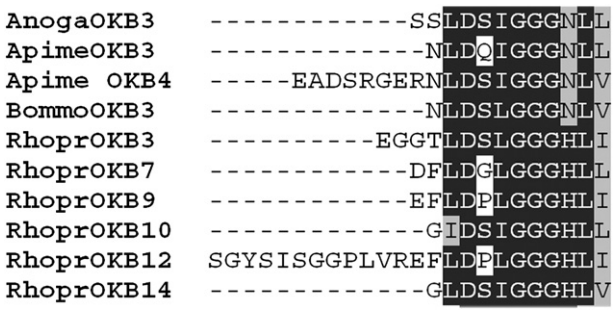

Fig. 6. Alignment of OKB mature peptides with the characteristic domain in insect species. Amino acid residues that are similar (grey) ore identical (black) in at least five peptides are highlighted.

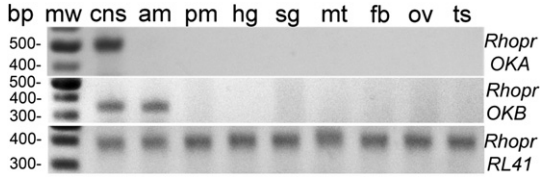

Fig. 7. RT-PCR of *RhoprOKA* and *RhoprOKB* in adult *R. prolixus*. A fragment of *RhoprOKA* is PCR amplified in cDNA from CNS. A fragment of *RhoprOKB* is amplified in CNS and anterior midgut (AM) cDNAs. MW: molecular weight marker; PM: posterior midgut; HG: hindgut; SG: salivary glands; MT: Malpighian tubules; FB: fat body; OV: ovaries; TS: testes. *RhoprRL41* was PCR amplified from tissues as a positive control to test the quality of the template cDNAs ($n = 3$ biological replicates).

are the first report that cells from anterior midgut express a neuropeptide precursor gene in *R. prolixus.*

Recently, Yamanaka et al. (2011) performed immunohistochemistry (IHC) and in situ hybridization (ISH) analysis in order to study the expression pattern of OKs in *B. mori.* For the IHC analysis, they used an antibody developed against a characteristic region of *BmoriOKA*, whereas for ISH authors report the use of an RNA probe spanning the 3 firsts exons. In their study, ISH revealed expression of OK mRNA in the nervous system and in endocrine cells of the anterior and central midgut, while immunoreactive cells were only present in the nervous system. This apparent contradiction can now be explained based on our data; ISH detects both *BmoriOKA* and *BmoriOKB* transcripts, whereas IHC only detect OKA peptides. This result suggests a differential expression pattern of OKA and OKB in the nervous system and midgut from *B. mori*. Similarly, our tissue profiling experiment demonstrate that the expression of *RhoprOKA* is restricted to CNS, whereas *RhoprOKB* is expressed in CNS and anterior midgut. Although further experiments should be performed to determine the physiological role of *RhoprOKB*, its expression in anterior midgut suggests an implication in the regulation of processes related to feeding and diuresis.

5. Conclusions

We describe OKB, which constitute a novel family of neuropeptides in arthropods. OKB is expressed as an alternative splicing variant of the previously described OKA neuropeptide precursor gene. In insects, alternative splicing give rise to OKA and OKB transcripts. The analysis of the data suggests that OKA and OKB transcripts originate from intragenic duplication and mutation. In *R. prolixus*, OKB is expressed in CNS and anterior midgut, whereas the expression of *RhoprOKA* is restricted to CNS.

The finding of a new family of neuropeptides in arthropods is an important contribution in the fields of neuroendocrinology. It also provides new putative target sites for a novel generation of insecticides based in the knowledge of the physiology of signaling molecules and their receptors.

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