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Characterization and baculovirus-directed expression of a myosuppressin encoding cDNA from the true armyworm, *Pseudaletia unipuncta*

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Abstract

Insect myosuppressins are a highly conserved sub-family of peptides which are primarily characterized by the ability to suppress contraction of visceral muscles in a variety of insect species. We have isolated a cDNA from the true armyworm, *Pseudaletia unipuncta*, that encodes a prohormone containing a peptide identical to *Manduca*FLRFamide. We have shown that this myosuppressin gene appears to be expressed in late larval and adult insects. In *Manduca sexta*, a number of extended-FLRFamide peptides have previously been purified including *Manduca*FLRFamide, F7D (DPSFLRFamide), F7G (GNSFLRFamide) and two larger peptides F24 and F39 that contain the shorter *Manduca*FLRFamide sequence at their C-terminus. Comparison with the true armyworm prepropeptide characterized here identifies F24 and F39 as partially processed products from the same precursor. Expression in the true armyworm was shown by in situ hybridization to occur in over 150 cells throughout the adult brain and nerve cord, and also to occur in both open and closed endocrine type cells of the gut. Overexpression of the *P. unipuncta* FLRFamide cDNA from a baculovirus vector in cabbage looper caterpillars was used to assess the potential for myosuppressin expression as a means of enhancing virus efficacy. Viral expression of the armyworm prohormone cDNA resulted in raised levels of RFamide-like products in the hemolymph of infected insects, but the products were found to be chemically distinguishable from authentic mature peptide and probably represent partially processed hormone.

Keywords: FMRFamide-related peptide; Baculovirus; Neuropeptide; Insect

1. Introduction

The FMRFamide-related peptides (or FaRPs) represent a diverse and widely distributed family of peptides based upon structural similarity to the tetrapeptide FMRFamide [25]. FMRFamide itself was first isolated based on its cardioexcitatory activity in the clam *Macrocallista nimbosa*, and many of the FaRPs have since been shown to have effects on muscle activity in a variety of invertebrate systems, including insects (for review see [33]). Most of these are stimulatory [1,34], however the sub-family of FaRPs found in insects known as the myosuppressins has complex effects, showing the ability to inhibit contractions of a variety of visceral muscles [5,7,15,22,26] as well as stimulating some skeletal muscles [12,28] and even affecting secretions from the gut [8,20].

The first characterized myosuppressin, leucomyosuppressin (LMS), was a decapeptide isolated from the cockroach *Leucophaea maderae*, based on its ability to inhibit spontaneous contractions of cockroach hindgut [9]. Similar molecules have now been isolated from many species and these have the common sequence XDVXHXFLRFamide. Some are known by terminology based on function and called myosuppressins, while others that were isolated based on structural similarity to FMRFamide are referred to as FLRFamides (eg. the molecule found in the hawkmoth *Manduca sexta* is called *Manduca*FLRFamide [12]).

The first myosuppressin cDNA to be characterized was isolated from the cockroach, *Diploptera punctata*, and found to encode a precursor peptide containing the mature LMS decamer within it, along with the appropriate cleavage and processing signals required for production of the mature peptide [3]. Unlike the large multipeptide precursors encod-

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ing FMRFamide-related peptides previously characterized in molluscs [30] nematodes [31] or insects [32], the myosuppressin precursor contained only a single FaRP. This appears to be true of the fly myosuppressin as found in the *Drosophila melanogaster* genome, and in a lepidopteran, where a myosuppressin has also been shown to be encoded in a separate precursor product [1]. Thus the myosuppressins appear to represent a distinct class of FaRPs, expressed independently from the multipeptide FaRP genes already known in many arthropods.

The role(s) of myosuppressins in insect physiology have also been investigated through studies of their expression using antibodies and nucleic acid probes. In the cockroach, *D. punctata*, LMS was found to be expressed in both the central and stomatogastric nervous systems as well as by endocrine-like cells in the midgut [3,6]. Dromyosuppressin-specific antibodies were used to show myosuppressin peptide in cells and fibers throughout the nervous system of the blow fly, *Phormia regina*, as well as fibers in the crop and crop duct [26]. Similar widespread staining in the nervous system is produced with this antibody in *D. melanogaster*, as well as staining of processes on the crop and crop duct and additional staining of two rectal cells in the hindgut [17]. The extent of myosuppressin expression in these examples indicates they may have many functions, but the strong association with the stomatogastric nervous system and gut specifically suggest involvement with feeding and digestion.

Due to the diverse and powerful effects seen on visceral and skeletal muscle activity in insects, in addition to likely effects on the gut and feeding, the myosuppressins represent promising targets and/or tools for disruption of insect biology in efforts to control pests. However, these peptides are characterized by modified elements requiring post-translational processing, including endo- and exo-nuclease cleavage from the precursor, amidation by peptidylglycine α-amidating monooxygenase (PAM) and cyclization to pyroglutamate at the N-terminus. Thus the prospect of manipulating pest physiology by expressing such products in vivo is dependent on the capabilities of the cellular processing machinery. We set out to test the potential for in vivo peptide production using baculovirus expression in a lepidopteran model (the cabbage looper *Trichoplusia ni*). To obtain a cDNA encoding a lepidopteran myosuppressin, we isolated and characterized a cDNA from another lepidopteran species, the true armyworm, *Pseudaletia unipuncta*. Overexpression of the *P. unipuncta* FLRFamide cDNA resulted in the presence of a FaRP product in the hemolymph which was shown to be different from authentic mature myosuppressin.

2. Materials and methods

2.1. cDNA library screening

A degenerate oligonucleotide was synthesized that codes for the peptide sequence of *Manduca*FLRFamide using a Biosearch model 8750 DNA synthesizer at the Core Facility for Protein/DNA Chemistry (Queen's University, Kingston, ON). The sequence was selected using a *D. melanogaster* codon bias: 5′-GCCGAAICGIAGGAAIGAGTGCACCA-CATCCTG-3′. The oligonucleotide was end-labeled with γ^{32}P-ATP and purified over a NENSORB column (NEN). Labeled oligonucleotide was used to screen 30,000 plaques from a cDNA library (Stratagene) that was synthesized using RNA extracted from adult *Pseudaletia unipuncta* brains [10]. All standard molecular biological techniques were performed according to [29]. Potential positives were purified by secondary and tertiary screening, before excision of plasmid (Stratagene). Plasmid inserts were sequenced using a Taq Dye-deoxy terminator cycle sequencing kit and analysis of the products was done on an ABI 373A DNA sequencer (Applied Biosystems) at the Core Facility for Protein/DNA Chemistry (Queen's University).

2.2. RNA extraction and Northern blotting

PolyA$^+$ RNA was extracted from batches of 100 brains representing different developmental stages of *P. unipuncta* using a Quickprep mRNA Purification Kit (Amersham Pharmacia Biotech). For each developmental point, 5 μg of RNA was denatured with glyoxal-dimethyl sulfoxide, and then separated on an agarose gel and transferred to Hybond N$^+$ nylon (Amersham). A cDNA fragment encompassing the myosuppressin precursor peptide coding region was ^{32}P-labeled by random priming and hybridized to the RNA in Quikhyb hybridization solution (Stratagene). After washing, the membrane was exposed to X-ray film and the resulting autoradiogram scanned with an ImageQuant computing densitometer (Molecular Dynamics). Membranes were also hybridized to a ^{32}P-labeled cDNA representing the *D. melanogaster* α-tubulin coding region, and the data used to normalize myosuppressin mRNA levels.

2.3. Whole-mount in situ hybridization

A DIG-labeled RNA probe representing the *P. unipuncta* FLRFamide cDNA was made by linearizing a plasmid containing residues 1 to 554 in Fig. 1. Antisense RNA was transcribed from the template in the presence of digoxigenin-UTP using T7 RNA polymerase (Roche).

P. unipuncta tissues were fixed in freshly prepared 4% paraformaldehyde in PBS (0.13 M sodium chloride in 0.1 M sodium phosphate buffer, pH 7.0) for 2 h at room temperature, then rinsed in PBS for 5 min, and stored in PBST (PBS with 0.3% Triton X-100) at 4°C. Fixed tissues were pretreated with 0.2N HCl for 20 min, 2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) for 30 min at 70°C, proteinase K at 37°C for 30 min, then refixed in 4% paraformaldehyde for 20 min. Tissues were acetylated in acetic anhydride/TEA (0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0), rinsed with PBS and then prehybridized in 50% formamide, 0.3 M NaCl, 20 mM Tris, pH

7.5, 1 mM EDTA, 1× Denhardt's solution, 0.1 M DTT, with 0.5 μg/ml yeast tRNA. Hybridization was carried out overnight at 50°C in fresh solution with 10% dextran sulfate and 100 ng/ml DIG-labeled probe. Two washes in 0.3 M NaCl, 0.01 M $NaPO_4$, 0.01 M Tris pH 6.8, 5 mM EDTA, and 50% formamide were followed by treatment with 20 μg/ml RNase A to remove unhybridized probe. Bound probe was detected by incubation with a 1:200 dilution of anti-DIG peroxidase conjugated antibody (Roche), followed by a wash in PBST and incubation in PBS containing 0.2% β-d(+)glucose, 0.04% NH_4Cl, and 0.02 mg/ml 3,3′ diaminobenzidine with glucose oxidase (2.5 U/ml). After development, tissues were dehydrated through an ethanol series and cleared in xylene, followed by mounting in Canada Balsam. Photomicroscopy was performed on a Leica model DMRF microscope.

2.4. Baculovirus expression

The entire *P. unipuncta* FLRFamide cDNA (Fig. 1) was excised from the cloning vector (pBluescript) and ligated into the transfer vector for the Bac-to-Bac Baculovirus Expression System, pFastBac1 (Life Technologies). The cDNA was transposed to bacmid as directed by the supplier and then transfected and amplified in Sf9 cells. Viral stocks in EX-CELL 405 medium (JRH Biosciences), were injected into final instar *Trichoplusia ni* caterpillars using 5000 PFU in 5 μl. Control infections were done in the same way using wild type *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) or a Bac-to-Bac recombinant (vGUS) supplied with the Bac-to-Bac Expression System carrying the gene for β-glucuronidase (*gusA*). Injected insects were maintained at 24°C/16 h (light) and 20°C/8 h (dark) until being bled on ice by removal of two prolegs. Collected hemolymph was cleared of hemocytes by centrifugation at 14,000 × g for 2 min at 4°C, and then the supernatant frozen at −70°C. Peptides were extracted from hemolymph by extraction with 5 volumes acid/methanol (90% methanol/9% acetic acid/1% water). Samples were centrifuged for 30 min at 14,000 × g, the supernatant removed from the gelled precipitate, and the pellet re-extracted with the same volume acid/methanol. Combined supernatants were dried in a Savant Speed-Vac concentrator with no heat and stored at −20°C.

2.5. Sep-Pak separation

C_{18} Sep-Pak cartridges were used to crudely separate the peptides in the dried hemolymph samples, as well as *Manduca*FLRFamide standard, for radioimmunoassay (RIA) or for high performance liquid chromatography (HPLC). This was achieved by resuspending the samples in 1 ml dH_2O containing 0.1% trifluoroacetic acid (TFA). The samples were then passed 3 times through a C_{18}-containing Sep-Pak cartridge which had been prepared by sequential application of 10 ml of 100% methanol, 10 ml dH_2O followed by 3 ml dH_2O containing 0.1% TFA and 10 μg bovine serum albumin. The column was then washed with 8 ml of dH_2O containing 0.1% TFA and the retained materials on the Sep-Pak cartridge were eluted sequentially with 5 ml of varying percentages of acetonitrile containing 0.1% TFA. An aliquot from each of the eluted fractions was dried down using a Savant Speed-Vac concentrator (with no heat) and resuspended for RIA analysis.

2.6. Reversed-phase high performance liquid chromatography (HPLC)

Dried samples of hemolymph from third and fourth day vPsuFLRFamide-infected insects were resuspended in dH_2O containing 0.1% TFA, then passed through a C_{18} Sep-Pak cartridge as described above. The 60% eluent was evaporated to dryness using a Savant Speed-Vac concentrator and resuspended in HPLC buffer (9% ACN plus 0.1% TFA) for analysis on a Brownlee RP-18 Spheri 5 (C_{18}) HPLC column (4.6 mm × 22 cm). Peptides were eluted using a linear gradient of acetonitrile containing 0.1% TFA from 9–60% over 35 minutes starting 5 minutes after injection of the sample. The column was then washed with a further linear gradient from 60–80% acetonitrile containing 0.1% TFA over the next 5 minutes and held at 80% for 30 minutes. One minute fractions were collected. Aliquots were taken from each of the one minute fractions and dried using a Savant Speed-Vac concentrator with no heat. Synthetic *Manduca*FLRFamide standards were also run on the column for comparisons of elution times.

2.7. Radioimmunoassay (RIA)

Radioimmunoassay was performed on dried, processed hemolymph samples and on C_{18} Sep-Pak or HPLC samples. Dried samples were resuspended in RIA buffer and assayed for FMRFamide-related peptides (FaRPs) using an RIA, as described previously by [23]. To determine fmole equivalents for *Manduca*FLRFamide the RIA was run using synthetic *Manduca*FLRFamide as standard. The FMRFamide antiserum was purchased from DiaSorin (Stillwater, MN), with FMRFamide (Peninsula Laboratories, Belmont, CA), and *Manduca*FLRFamide (Queen's University Core Facility) used to provide standard curves. A dilution curve of the immunopositive material produced a parallel curve to that of dilution of standard indicating that there was no interference from the samples.

3. Results

3.1. cDNA characterization

Approximately 30,000 plaques of a true armyworm brain cDNA library were screened with an oligonucleotide representing the *Manduca*FLRFamide peptide sequence and a

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                    5'-CAAACTATTCAGCAACA ATG GCT TTG GGC GGT AAT GGT AAC CAC GTG 47
                                         Met Ala Leu Gly Gly Asn Gly Asn His Val 10

GCG GTA GTG TGC CTG GTG CTG GCG TGC GCG AGC GTG GCG CTG TGT GCG CCC GCG CAG CTC 107
Ala Val Val Cys Leu Val Leu Ala Cys Ala Ser Val Ala Leu Cys Ala Pro Ala Gln Leu 30

TGC GCC GGC GCC GCC GAC GAC GAC CCC AGG GCG GCG CGC TTC TGC CAG GCG CTC AAC ACC 167
Cys Ala Gly Ala Ala Asp Asp Asp Pro Arg Ala Ala Arg Phe Cys Gln Ala Leu Asn Thr 50

TTC CTC GAA CTG TAT GCT GAG GCT GCT GGC GAG CAG GTG CCC GAG TAT CAA GCC CTG GTC 227
Phe Leu Glu Leu Tyr Ala Glu Ala Ala Gly Glu Gln Val Pro Glu Tyr Gln Ala Leu Val 70

CGT GAC TAC CCT CAG CTG CTC GAC ACC GGC ATG AAG AGA CAA GAC GTC GTA CAC TCG TTC 287
Arg Asp Tyr Pro Gln Leu Leu Asp Thr Gly Met Lys Arg Gln Asp Val Val His Ser Phe 90

CTG CGC TTC GGC CGC CGG CGC TGA CCTGTCGCGTCCCCGCAGCGGCCCCGCCGCGTCCACGCCCAACACCC 358
Leu Arg Phe Gly Arg Arg Arg ---                                                  97

GCAACCCACACGCAGCGACCAGGCTTAGATTGTTTGCTAATATAAACTACATTTAGTTCTTTACGTTATTTCGTCCAAA 437
ATATTTAAAAAGTTCCTTTATAATGTAAGGCCGTAACCAAATGCTAGGTATCCGAACTGCGAAATGAAAAAAAAAAACA 516
TCCATTCATCGATGCGATGTCAACAACAACTGACGTCTAAACCTCTCTCTCCAAATTTAATAGACTAGAATTAGAACTT 595
ATTATAATGAAATGTACTTATTGCTAAAGTATTAATAGTTTTTAAGATGTAATTAAACGATCTTGTTGTCAACATTTTT 674
GTGGCATTTCATTTGATAAGTGTAAGAATATTTAAATAATTATTATGATGTTTTAAATAAATAAAGTACGTATTTATGA 753
CAAAAAAAAAAAAAAAAAAAA-3'                                                         775
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Fig. 1. Nucleotide sequence of a *P. unipuncta* FLRFamide cDNA and the deduced amino acid sequence of the peptide precursor. The numbering for the sequences is shown on the right. The amino acid sequence of mature *Manduca*FLRFamide is shown in bold type. Dibasic amino acids for potential cleavage sites are shown in italics and a glycine residue available for amidation is double underlined. A potential polyadenylation signal near the 3′-end is underlined. This sequence has been deposited in the GenBank database (accession no. AF390443).

hybridizing clone isolated that was completely sequenced on both strands. The sequence revealed contains an open reading frame (ORF) encoding the *Manduca*FLRFamide peptide within a larger putative precursor (Fig. 1). The deduced precursor is 97 amino acids in length (10.5 kDa) with the mature *Manduca*FLRFamide sequence located at the C-terminal end between potential cleavage signals KR and RR (Fig. 1). A glycine residue necessary for amidation of the FLRF terminus by peptidylglycine α-amidating monoxygenase (PAM) [4] follows the mature peptide sequence. This sequence also begins with a glutamine residue, which would be expected to be modified to pyroglutamate as is found in mature *Manduca*FLRFamide isolated from *M. sexta* [12].

The start site for the precursor shown in the figure was assigned based on its favorable context as compared to *D. melanogaster* start sites, as analyzed by [2]. There are no in-frame stop codons located upstream in the cDNA characterized, but the length of the isolated cDNA (775 bp) was found to be shorter than the size of the mRNA calculated from Northern blots (1,100 bp), so there is likely additional sequence 5′ to that shown in Fig. 1 in the complete mRNA. However, the selection of the start site shown is supported not only by its context, but also by the similarity in size of the resulting precursor compared to that of the cockroach and fly myosuppressin precursors (97 amino acids compared to 96 and 100 amino acids respectively), and by the presence of a good potential secretory signal sequence [35] in the N-terminal region of the precursor (with cleavage likely between amino acids 23 and 28 (Fig. 1). There are two overlapping polyadenylation signals located 15 base pairs from the poly A tail (Fig. 1).

3.2. *Expression of P. unipuncta* FLRFamide mRNA

RNA (Northern) blot hybridization analysis was used to assess expression in *P. unipuncta* brain of the *P. unipuncta* FLRFamide mRNA over a period of time from late larva to early adult in the life cycle. A single mRNA species of 1.1 kb was observed for all the developmental stages examined and the relative level of expression of this species at each time point was determined by comparison against the level of a constitutive mRNA species, α-tubulin (Fig. 2). The *P. unipuncta* FLRFamide mRNA was found to be expressed at a low level in the late stage larva, followed by a drop during

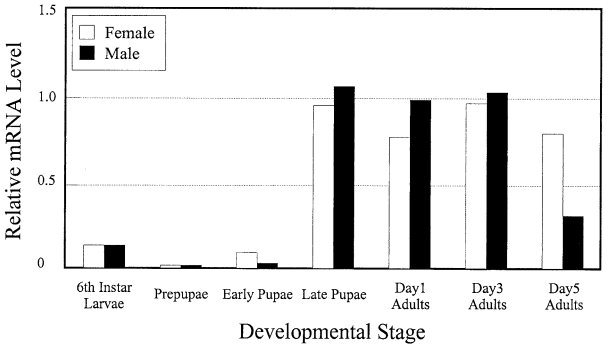

Fig. 2. Expression of the *P. unipuncta* FLRFamide mRNA. PolyA$^+$ RNA from 100 brain batches of *P. unipuncta* at different stages of development was separated on an agarose gel, blotted to membrane and hybridized to labeled *P. unipuncta* FLRFamide cDNA fragment. The mRNA levels for each stage were quantitated by densitometry and normalized against levels of α-tubulin determined by reprobing with a fragment of the *D. melanogaster* α-tubulin gene. The mRNA samples for 6th instar larvae and prepupae were prepared from mixed-sex insects.

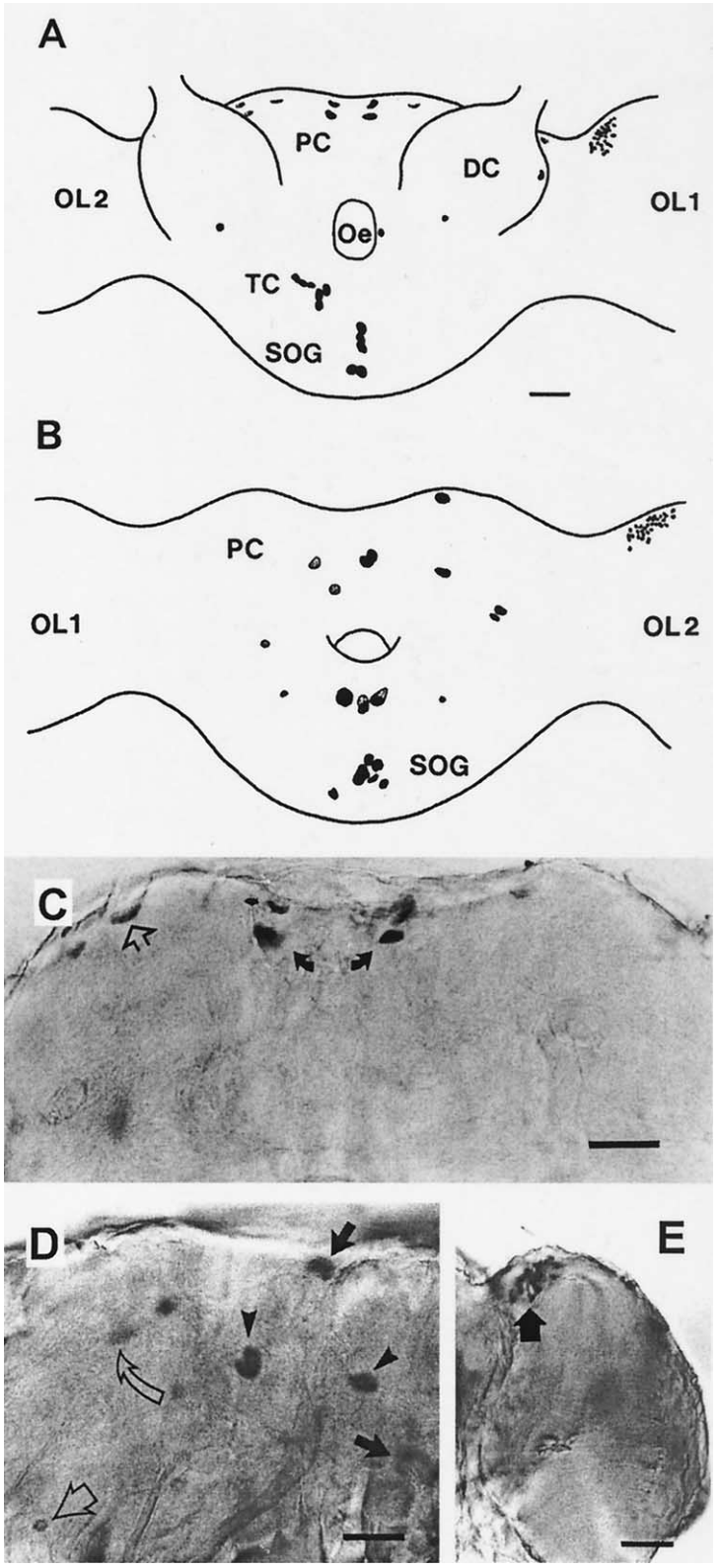

Fig. 3. *P. unipuncta* FLRFamide mRNA producing cells in the brain and subesophageal ganglion (SOG) revealed by in situ hybridization with a DIG-labeled cDNA fragment. A. Composite camera lucida drawing of stained cells in a frontal view of the anterior brain and SOG. Filled cells exhibited intense staining after hybridization with a fragment of labeled *P. unipuncta* FLRFamide cDNA, while stippled cells were more weakly stained. B. Frontal view of the posterior brain and SOG. DC, deutocerebrum; PC, protocerebrum; TC, tritocerebrum; OL, optic lobe; Oe, oesophagus. C. Actual view of a whole mount preparation of the anterior brain. Filled arrows indicate cells that are intensely stained and open arrows those that were more weakly stained. D. Whole mount preparation of the posterior brain. The most intensely stained cells are shown by filled arrowheads. E. Whole mount preparation of the optic lobe, showing a cluster of intensely staining cells. Scale bar = 50 μm.

early pupation, and subsequent increase to a peak at late pupation and early adulthood.

In situ hybridization was then used to localize expression of the *P. unipuncta* FLRFamide mRNA in specific tissues of newly emerged adults, the time when expression was seen from the Northern blots to be strong in the brain. The *P. unipuncta* adult nervous system is composed of the fused brain and subesophageal ganglion (SOG), the prothoracic ganglion, the pterothoracic ganglion (made up of the fused mesometa plus the first and second abdominal ganglia), four abdominal ganglia and a terminal ganglion. Expression of *P. unipuncta* FLRFamide mRNA was detected in more than 150 cells in the adult nervous system by hybridization with a DIG-labeled fragment of the cloned cDNA. No differences were observed based on the sex of the individuals examined. The greatest number of positive cells was visualized in the brain/SOG complex as summarized in Fig. 3(A and B). Groups of strongly expressing cells were visible in the pars intercerebralis and posterior protocerebrum (Figs. 3C and D), as well as the tritocerebrum and SOG. A tight cluster of 30 to 40 small cells were also stained in each optic lobe of the brain (Fig. 3E).

In the nerve cord, small numbers of discrete cells were visible in each of the ganglia examined, as summarized in Fig. 4A. The prothoracic ganglion contained two groups of weakly staining cells in all specimens examined (Fig. 4B), while the remaining ganglia generally displayed cells with more intense staining (Fig. 4C–E).

Expression of the *P. unipuncta* FLRFamide mRNA was also detected throughout the adult midgut (Fig. 5). The greatest numbers of positively staining cells were located in the anterior and central regions of the midgut (Fig. 5B), with the cells being much smaller than those in the CNS. Two morphological types of cells were observed, with the closed type endocrine cells having a rounded shape and open type cells displaying apical cytoplasmic processes (Fig. 5).

3.3. FaRP expression in caterpillar hemolymph

To assess the potential for baculovirus-based myosuppressin overexpression in insects, the *P. unipuncta* cDNA encoding *Manduca*FLRFamide was transferred into the Bac-to-Bac baculovirus expression system and recombinant virus generated for infection of a suitable host insect. Caterpillars of the cabbage looper, *T. ni*, were utilized since they are highly susceptible to infection by this virus [18]. FMRFamide-related peptide levels were quantified in the hemolymph of final instar caterpillars and those infected with several different baculoviruses. A four day infection period was used to compare the levels of FaRPs in uninfected, wild type AcMNPV-infected, vGUS-infected, and vPsuFLRFamide-infected animals, using a FMRFamide RIA with a *Manduca*FLRFamide standard curve. There was no significant difference between the control animals ($p > 0.5$), while vPsuFLRFamide-infected animals had significantly elevated levels ($p < 0.001$) of FMRFamide-like immunoreactivity (Fig. 6A).

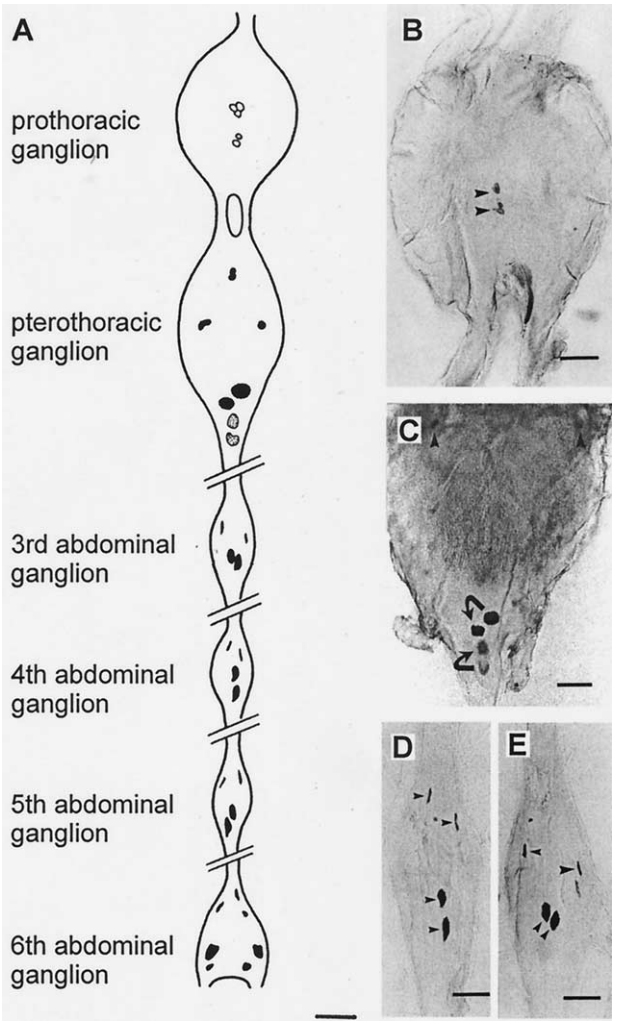

Fig. 4. *P. unipuncta* FLRFamide mRNA producing cells in the thoracic and abdominal ganglia revealed by in situ hybridization with a DIG-labeled cDNA fragment. A. Composite camera lucida drawing of stained cells in the ganglia examined. Filled cells exhibited intense staining after hybridization with a fragment of labeled *P. unipuncta* FLRFamide cDNA, stippled cells were more moderately stained, and open cells were weakly stained. Scale bar = 100 μm. B. Actual view of a whole mount preparation of the prothoracic ganglion. Arrowheads indicate two clusters of very weakly staining cells. C. Whole mount preparation of the pterothoracic ganglion. Arrowheads mark positively stained lateral cells in the midganglion and curved arrows indicate larger cells in the posterior region. D. and E. Whole mount preparations of third and fourth ganglia of the ventral nerve cord. Arrowheads indicate positively stained cells. Scale bar = 50 μm.

A time course study of vPsuFLRFamide infection was then used to follow FaRP production over 5 days of infection (Fig. 6B). In the control, there were no significant changes in levels of FMRFamide-like immunoreactivity over 5 days post-infection in vGUS-infected animals ($p > 0.5$), while vPsuFLRFamide-infected animals showed significantly elevated levels within 48 hours post-infection ($p < 0.001$). FaRP levels resulting from vPsuFLRFamide

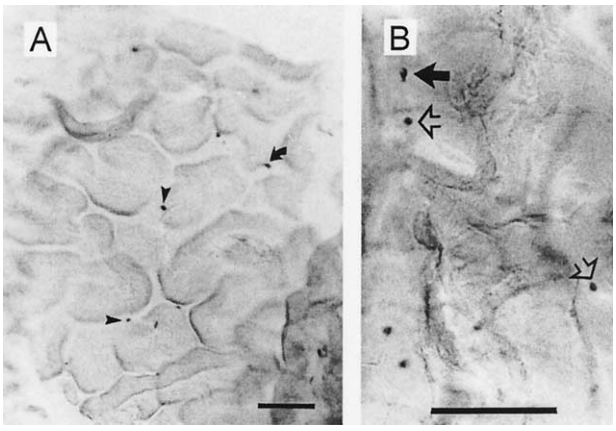

Fig. 5. *P. unipuncta* FLRFamide mRNA producing cells in the gut revealed by in situ hybridization with a DIG-labeled cDNA fragment. A. Whole mount preparation from the posterior midgut. The arrow indicates an open type endocrine cell and arrowheads indicate closed type cells. B. Whole mount preparation from the anterior midgut. The filled arrow indicates an open type endocrine cell and the open arrows indicate closed type endocrine cells. Scale bar = 50 μm.

infection continued to rise over the 5 days post-infection (Fig. 6B).

To investigate the identity of the FaRP products resulting from vPsuFLRFamide infection, Sep-Pak cartridge separation was used to compare the properties of the RIA-positive material with synthetic control *Manduca*FLRFamide. Elution from cartridges with variable concentrations of acetonitrile revealed that the synthetic standard and the RIA positive fractions from hemolymph eluted with different percentages of acetontrile (Fig. 7). Most of the FaRPs measured by RIA in the blood of PsuFLRFamide-infected animals eluted in the 50% fraction, with a small amount in the 60% acetonitrile cut. This is different from the *Manduca*FLRFamide standard which elutes in the 30 and 40% acetonitrile cuts from the Sep-Pak cartridge.

The difference in Sep-Pak elution profile was examined more closely and confirmed by using HPLC followed by RIA for FaRPs (Fig. 8). RIA detection of fractions separated on a C_{18} HPLC column with an acetonitrile gradient from 9–80% revealed that the *Manduca*FLRFamide standard eluted earlier (fraction 26) under these conditions than the RIA-positive material from the vPsuFLRFamide-infected hemolymph (Fractions 28 and 29). These two results taken together indicate that the material in the hemolymph of the vPsuFLRFamide-infected insects measured using the RIA is not the same as *Manduca*FLRFamide.

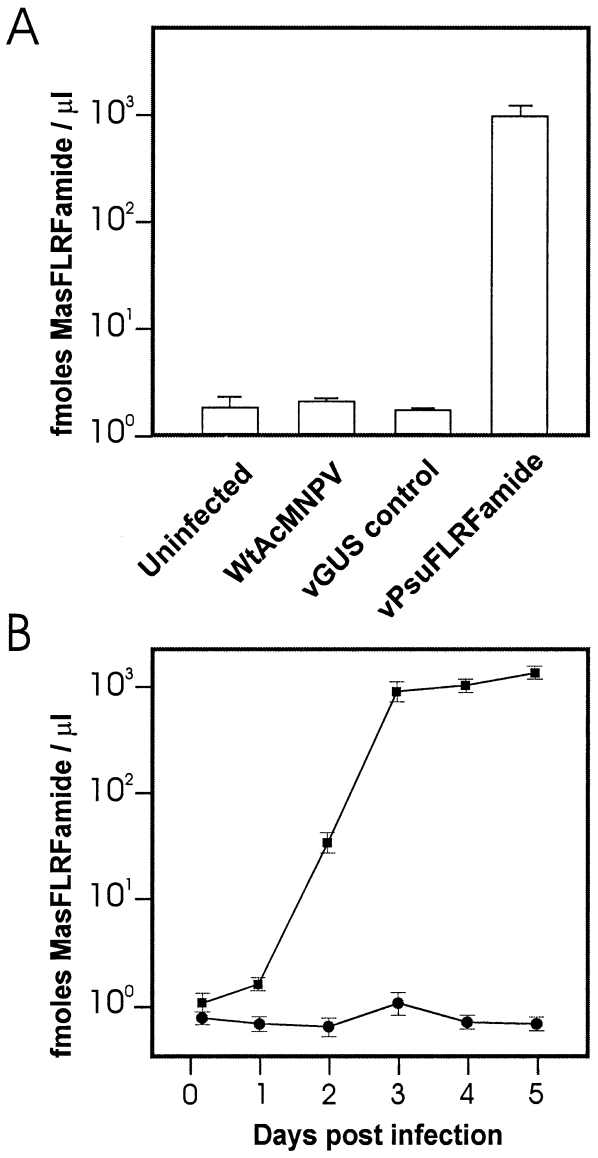

Fig. 6. Quantification of FaRP levels in the hemolymph of *T. ni* caterpillars infected with recombinant baculoviruses. A. Final instar caterpillars were infected with recombinant virus by injection of 5000 PFU and then bled after 4 days. Hemolymph extracts were assayed by RIA using an FMRFamide antibody and FaRP levels deduced by comparison to a standard curve using *Manduca*FLRFamide. FaRP levels resulting from infection with a virus construct carrying the *P. unipuncta* FLRFamide cDNA (vPsuFLRFamide) were approximately three orders of magnitude greater than those resulting from infection with the same expression vector carrying the β-glucuronidase gene (vGUS control), wild type *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV), or no infection. B. Final instar caterpillars were infected with recombinant virus and hemolymph extracts assayed after varying time periods. ■ = vPsuFLRFamide; ● = vGUS control. Note the log scale on the y-axis, especially in B which shows FaRP titres continuing to rise up to 5 days post-infection.

4. Discussion

We have isolated the cDNA for a lepidopteran myosuppressin from the true armyworm *P. unipuncta*. The deduced precursor shows *Manduca*FLRFamide to be the only predicted RFamide produced from this gene (Fig. 1). Homologous myosuppressin peptides having the common sequence XDVXHXFLRFamide have been isolated from many species in various insect orders, including the Blattodea, Orthoptera, Lepidoptera, and Diptera. At present, only three cDNAs are known for genes from this family

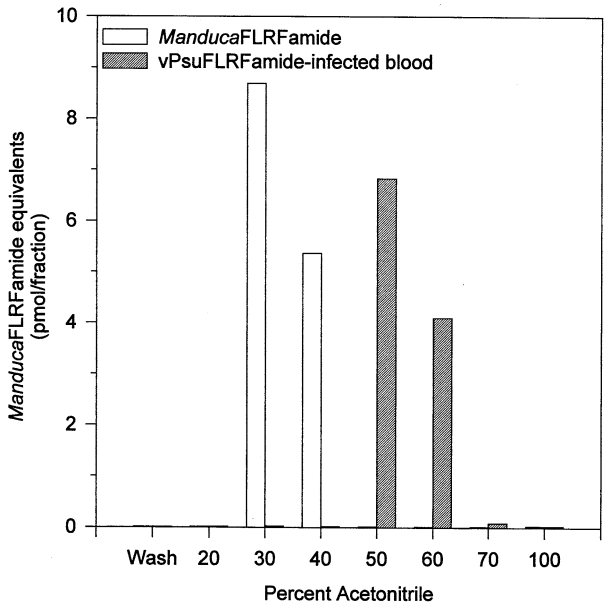

Fig. 7. Sep-Pak separation of FaRPs produced in the hemolymph of *T. ni* caterpillars infected with vPsuFLRFamide baculovirus. Dried hemolymph samples were resuspended in dH_2O with 0.1% TFA and applied to C_{18} Sep-Pak for 3 passages through the column. After washing, retained materials were eluted sequentially with increasing percentages of acetonitrile containing 0.1% TFA. Aliquots of each fraction were tested for FaRPs by RIA and the amount in picomoles graphed (gray bars). Synthetic *Manduca*FLRFamide was also bound and eluted for comparison (white bars).

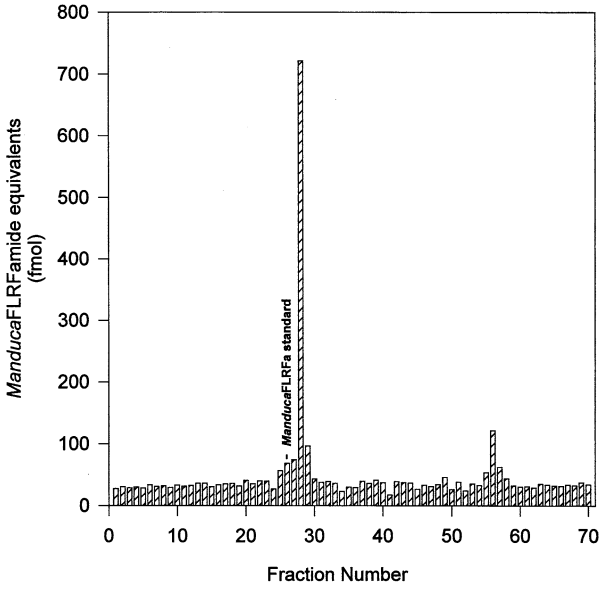

Fig. 8. HPLC separation of FaRPs produced in the hemolymph of *T. ni* caterpillars infected with vPsuFLRFamide baculovirus. Dried samples from infected caterpillars after 3 and 4 days were passed through a C_{18} Sep-Pak cartridge and the 60% eluent evaporated to dryness and resuspended in HPLC buffer. Samples were loaded on a Brownlee RP-18 Spheri 5 (C_{18}) column and eluted with a 9–60% gradient of acetonitrile over 35 minutes. Fractions were dried down and assayed for FaRPs by RIA, with quantities (in femtomoles) shown on the graph. A sample of synthetic *Manduca*FLRFamide was similarly run for comparison of elution time (as marked).

(from the Diptera [*D. melanogaster* sequence CG6440], Lepidoptera [this paper], and Blattodea [3]). Comparison of the deduced preprohormones from all three shows each to have an approximately 100 amino acid precursor containing the decamer sequence at its C-terminus (Fig. 9). The mature peptide for each follows a KR cleavage signal and precedes a glycine (for amidation) and dibasic cleavage signal before the stop codon. The sequences of the mature peptides themselves are highly conserved, containing seven of ten residues in common.

In the lepidopteran *M. sexta*, a number of other FLRFamide peptides have been purified from the nervous system and the gut in addition to the myosuppressin decamer designated *Manduca*FLRFamide [11]. These include two unique heptapeptides F7D (DPSFLRFamide) and F7G (GNSFLRFamide), as well as two larger peptides F24 and F39, which were identified using insects parasitized by a braconid wasp [13,19]. The two longer peptides F24 and F39 contain the shorter *Manduca*FLRFamide sequence at their C-terminus, and it is clear by comparison with the true armyworm prepropeptide characterized here, that they are partially processed products from the same precursor. The heptapeptides must be encoded by another as yet unidentified FLRFamide encoding gene.

The expression of the *P. unipuncta* FLRFamide mRNA was found to occur in the brain/SOG complex and ventral nerve cord in adult *P. unipuncta* (Figs. 3–4) as well as in both open and closed endocrine type cells of the gut (Fig. 5). This is similar to the case of the homologous LMS peptide in the cockroach *D. punctata*, in which mRNA expression indicated LMS to be a brain-gut peptide [7], and is also consistent with the isolation by Miao et al. [19] of *Manduca*FLRFamide peptide from both nervous and gut tissues of *M. sexta*.

Myosuppressin peptides show a unique spectrum of effects on various types of insect tissues, both visceral and skeletal. In general, the effects on visceral tissues tend to be inhibitory, while peptide effects on skeletal muscle are primarily stimulatory or biphasic, depending on concentration. This is illustrated by considering the activities of two of the best studied myosuppressin peptides. In the Lepidoptera, *Manduca*FLRFamide has been shown to have stimulatory effects on skeletal muscles such as the dorsal longitudinal muscle of *M. sexta* [12]. It also produces increased force and amplitude of neurally evoked contractions of the external ventral protractor muscle of the locust [14]. In contrast, the same peptide produces inhibitory effects on visceral muscles such as the moth midgut [5] or locust midgut [15]. The orthopteran myosuppressin *Schisto*FLRFamide, also shows stimulatory effects in the ventral protractor muscle assay [14] and biphasic effects in the extensor tibiae assay [27], while producing inhibitory effects on various visceral tissues, including locust heart [27], midgut [15], and oviduct [22]. In the locust, it has been proposed that different receptor types in the visceral and skeletal tissues are likely

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D. punctata      MKHLVIVLIGVLT-VLLACAPRRAAAVPPPQCSSNMLEDISPRFRKICAALSSIYDLSMA 59
D. melanogaster  MSFAQFFVACCLAIVLLAVSNTRAAVQGPPLCQSGIVEEMPPHIRKVCQALENSDQLTSA 60
P. unipuncta     MALGGNGNHVAVVCLVLACAS-VALCAPAQLCAG--AADDDPRAARFCQALNTFLELYAE 57
                 *          :. ::** :   *    .  * .    :  *:  :.* **..  :*

D. punctata      MEAYLEDKC---VRENTPLMDNGVKRQDVDHVFLRFGRRR 96
D. melanogaster  LKSYINNEASALVANSDDLLKNYNKRTDVDHVFLRFGKRR 100
P. unipuncta     AAGEQVPEYQALVRDYPQLLDTGMKRQDVVHSFLRFGRRR 97
                   .    :    * :   *:..  ** ** * *****:**
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Fig. 9. Alignment of myosuppressin precursors deduced from cDNA sequences of *Diploptera punctata*, *Drosophila melanogaster* and *Pseudaletia unipuncta*. Symbols beneath the aligned sequences indicate when amino residues are identical (*), or a conserved substitution (:) or a semi-conserved substitution (.) has resulted. Dibasic sites for potential proteolytic processing of the precursor are indicated in bold and the mature peptide that would be released upon processing is double-underlined (the terminal glycine is necessary for amidation).

responsible for these divergent responses to the locust myosuppressin *Schisto*FLRFamide [14].

In light of the above, the myosuppressins appear to be multifunctional peptides, with potential roles in activities such as flight and egg laying through the stimulation of skeletal muscle, and conversely involved in the activities of visceral tissues such as gut, oviduct and crop through inhibitory actions. Their potential role in digestion has also recently been found to be more complex than a simple myotropic contribution with the discovery that the cockroach myosuppressin, LMS, is capable of stimulating the release of digestive enzymes in the gut [8,20]. A coordinated overall role for the diverse activities of the FLRFamides has also been proposed to support the physiological changes accompanying ecdysis by Miao et al. [19].

Based on their wide-ranging effects on insect physiology, the myosuppressins represent a promising tool for pest control strategies in insects. One potential application is through expression by baculovirus biopesticides, as a means for producing more rapid effects on insect physiology upon virus infection and thus improving virus efficacy. Our experiments with *T. ni* caterpillars, demonstrate that infection with a recombinant baculovirus carrying the *P. unipuncta* FLRFamide cDNA under the control of the polyhedrin promoter produces significant increases in the levels of RFamide-like material in the blood of infected insects (Fig. 6). However, HPLC analysis of the dominant FaRPs present showed that they failed to elute at the same time points as synthetic peptide standards. Inaccurate or incomplete processing of the peptide precursor represents the most likely reason for the discrepancy seen, although interestingly, amidation must have occurred, because the amide is essential for detection in the RIA. Similar results have been found with baculovirus expression of other insect neuropeptides in both caterpillars and cultured cells [16,21,24]. Expression in *T. ni* caterpillars of a cDNA encoding the pheromone biosynthesis activating neuropeptide (PBAN) from *Helicoverpa zea* resulted in active product in the blood, but Western blotting showed many immunopositive products [16]. As with *P. unipuncta* FLRFamide cDNA expression, the products in the blood were amidated. In cell culture, expression of *Bombyx mori* prothoracicotropic hormone (PTTH) was found to be inefficient using the complete PTTH precursor cDNA, but was successful when a chimeric construct encoding a signal peptide fused to the mature PTTH sequence was used [21]. The same was seen with PBAN expression as well. In the case of ion transport peptide from *Schistocerca gregaria*, baculovirus expression in cultured cells resulted in improper cleavage of the precursor (11 amino acids from the site of cleavage in *S. gregaria*). However, processing was correctly carried out when the cDNA was expressed in stably transformed insect cell lines. Thus, for high level expression of exogenous myosuppressins in lepidopteran larvae, optimized constructs expressing mature sequences may be required.

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