Insulin-like peptides in *Spodoptera littoralis* (Lepidoptera): Detection, localization and identification

Sandrien Van de Velde ^{a,1,*}, Liesbeth Badisco ^{b,1}, Ilse Claeys ^b, Peter Verleyen ^b, Xi Chen ^b,
Luc Vanden Bosch ^b, Jozef Vanden Broeck ^b, Guy Smagghe ^{a,*}

^a Laboratory of Agrozoology, Department of Crop Protection, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

^b Laboratory of Developmental Physiology, Genomics and Proteomics, Department of Animal Physiology and Neurobiology, Zoological Institute, K.U.Leuven, Naamsestraat 59, B-3000 Leuven, Belgium

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Abstract

Insulin is an extensively studied peptide hormone in mammals. However, insulin is not restricted to vertebrates, but has also been identified in invertebrates, among whom several insect species. These insulin-like peptides (ILPs) show structural and—at least some—functional homology with mammalian insulin and act through a conserved pathway. Yet many aspects of insulin function in insects remain to be unveiled. We analyzed the presence of ILPs in the cotton leafworm, *Spodoptera littoralis*, at two levels: (1) cellular localization of ILPs in whole tissues of the central nervous system from *S. littoralis*, and (2) detection and identification of ILPs at nucleotide level. To our knowledge, nothing about the presence of ILPs in *S. littoralis* has been described so far. By whole mount *in situ* immunolocalization, we localized bombyxin-like material in *S. littoralis* in four pairs of *pars intercerebralis* cells and in the *corpus cardiacum-corpora allata* complexes. In addition, we have cloned two different *S. littoralis* ILP precursor cDNAs by a combination of PCR and RACE. The corresponding precursor polypeptides ('*Sl*-ILPP1' and '*Sl*-ILPP2') show significant sequence homology with precursors for bombyxin and other bombyxin-related peptides. Our results strongly suggest that the *S. littoralis* ILPs belong to the category of bombyxin-analogs.

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1. Introduction

Bombyxin, isolated from the silkworm *Bombyx mori*, was the first insulin-like molecule identified in insects (Nagasawa et al., 1984). Bombyxin was originally identified as the *Bombyx* small prothoracicotrophic hormone (4K-PTTH), as it exerted prothoracicotrophic activity in a related silkworm species, *Samia cynthia ricini* (saturniid silkworm), but surprisingly not in *Bombyx* itself. Extensive research on the presence of bombyxins and other

insulin-like peptides (ILPs), also designated as insulin-related peptides (IRPs), in other insect species followed (for review, see Claeys et al., 2002; Wu and Brown, 2006). The organization of insect ILP precursors is very similar to that of vertebrate insulin, consisting of a secretory signal peptide, a B-chain, an interconnecting C-peptide and an A-chain (Adachi et al., 1989; Lagueux et al., 1990; Iwami et al., 1996a; Brogiolo et al., 2001; Krieger et al., 2004). ILP precursors are post-translationally modified to form the mature ILPs consisting of the A- and B-chain, joined by disulfide bonds. In mammals, insulin itself originates from specialized pancreatic cells, but other mammalian ILPs and insulin-like growth factors (IGFs) are secreted from several other tissues as well. In insects, the nervous system appears to be the

* Corresponding authors. Fax: +32 9 2646239.

E-mail addresses: sandrien.vandavelde@ugent.be (S. Van de Velde), guy.smagghe@ugent.be (G. Smagghe).

¹ Equally contributing authors.

primary source of ILPs (Krieger et al., 2004). Additionally, ILP transcripts have been localized in the insect epidermis, the fat body, the gut, the prothoracic glands, the pterotheca, the thoracic muscle, the ovaries, the testes, the mature oocyte, the silk gland, and the Malpighian tubules (Kromer-Metzgen and Lagueux, 1994; Iwami et al., 1996b; Broughton et al., 2005). Insect ILPs bind in the target organs to receptors displaying similarity with the vertebrate insulin receptor. Insulin receptor homologs were characterized for a few insect species, like the fruitfly *Drosophila melanogaster* (Fernandez-Almonacid and Rosen, 1987) and the mosquito *Aedes aegypti* (Graf et al., 1997). Binding of ILPs to their receptor activates an evolutionarily conserved signal transduction cascade (Claeys et al., 2002). Presently, the endocrinology of insect ILPs is in a discovery phase compared to the state of knowledge for insulins and insulin-related peptides in vertebrates. Mammalian insulin is very well-known for its key role in glucose homeostasis. For insects on the contrary, reports on the involvement of ILPs in carbohydrate metabolism are rare (Satake et al., 1997; Rulifson et al., 2002; Broughton et al., 2005). Most research has focused on the role of ILPs in controlling cell, organ and total body size and growth (Chen et al., 1996; Brogiolo et al., 2001; Ikeya et al., 2002; Nijhout and Grunert, 2002; Malaterre et al., 2003; Nijhout, 2003; Saito and Iwabuchi, 2003; Stern, 2003; Goto et al., 2005; LaFever and Drummond-Barbosa, 2005). Additionally, ILPs appear to be involved in controlling life-span (Clancy et al., 2001; Tatar et al., 2001). Several studies suggest a role for ILPs in insect reproduction physiology (Iwami et al., 1996b; Fullbright et al., 1997; Böhni et al., 1999; Drummond-Barbosa and Spradling, 2001; Tatar et al., 2001; Broughton et al., 2005). This offers new perspectives for pest control, since reproduction is a promising target for the development of new pest management strategies. Moreover, a stimulatory effect of ILPs on ecdysteroidogenesis at prothoracic gland and ovarian level has been described for a few insect species (Nagasaki et al., 1984; Graf et al., 1997; Nagata et al., 1999; Tu et al., 2002; Manière et al., 2004).

In this paper we present the first evidence for the presence of ILPs in the cotton leafworm, *Spodoptera littoralis*. This polyphagous noctuid species is an economically important caterpillar in agriculture and horticulture that damages at least 87 economically important plant species belonging to 40 families distributed all over the world (Alford, 2000). For the related *Spodoptera* species, the fall armyworm *Spodoptera frugiperda*, the presence of specific receptors for bombyxin (Fullbright et al., 1997) and the presence of ILP-binding proteins (Doverskog et al., 1999; Andersen et al., 2000) have already been described, but no reports on ILPs as such have been made for any *Spodoptera* species so far. This paper describes our search for ILPs in *S. littoralis* at two levels: cellular localization of ILPs in tissues of the central nervous system, and detection and identification of ILPs at the nucleotide level.

2. Materials and methods

2.1. Insect rearing

A continuous colony of the cotton leafworm *S. littoralis* was maintained under standard laboratory conditions of $23 \pm 2^\circ\text{C}$, $60 \pm 5\%$ relative humidity and a 16 h-light photoperiod, and with larvae being fed *ad libitum* on a Poitout-based artificial diet (Smaghe et al., 2000).

2.2. Whole mount in situ immunolocalization

Section 2 is based on previous work of Verleyen et al. (2004). After water-anaesthetization of *S. littoralis* last instars, brains, subesophageal ganglia (SOG) and corpora cardiaca–corpora allata (CC–CA) complexes were dissected in insect Ringer solution and immediately transferred in glass tubes containing the fixative, 2% paraformaldehyde in 10 mM phosphate-buffered saline (PBS). Samples were stored overnight at 4°C . Fixative was removed and tissues were carefully washed for 5 h with PBS at 4°C , and eventually PBS was replaced by a perforating and blocking solution containing 4% Triton[®] X-100, 2% normal porcine serum (NPS) and 2% bovine serum albumin (BSA) in PBS. Tubes were kept overnight on a shaker at 4°C . Next, the solution in the tubes was replaced by a 1:500 dilution of primary antibody in 0.4% Triton[®] X-100, 2% NPS and 2% BSA in PBS. Three different antisera were tested: rabbit polyclonal antiserum against bombyxin A1 A-chain, against *Locusta migratoria* insulin-related peptide (LIRP) A-chain and against *Drosophila* ILP2 (DILP2) A-chain. The antisera were a kind gift of Prof. M.R. Brown (University of Georgia, Athens, GA). Each primary antiserum was tested on 2×10 tissue complexes. Samples were placed overnight on a shaker at 4°C . Tissues were again washed for 5 h with PBS and then incubated overnight with a 1:20 dilution of FITC-conjugated secondary antibody in PBS. Tissues were washed 2 times with PBS and transferred on microscopy slides. Anti-fading reagent (0.1% para-phenylenediamine in 90% glycerol) was added and a cover glass was placed on the tissues. Slides were wrapped with aluminum foil and stored at -20°C till further use. A first selection was made under a Leica MZ 16F fluorescence microscope. Only intact tissues were kept for further analysis under a Zeiss LSM 510 META confocal microscope.

2.3. Detection of *Spodoptera* ILPs at RNA level: PCR

Spodoptera littoralis last instar larvae were decapitated and collected heads were directly frozen in liquid nitrogen. Until further processing, samples were stored at -80°C . Heads were homogenized and total RNA extraction was performed using the 'RNeasy[®] lipid tissue mini kit' (Qiagen) in combination with a DNase treatment ('RNase-free DNase set', Qiagen). The resulting RNA served as a template for cDNA synthesis using the 'SuperScript[™] III Reverse Transcriptase' protocol (Invitrogen). An initial PCR was performed on cDNA with a degenerate primer-set (Sigma) derived from the amino acid sequences of ILPs from the lepidopterans *Bombyx mori* (silkworm), *Agrilus convolvuli* (sweet potato hornworm), *Samia cynthia ricini* (saturniid silkworm) and *Manduca sexta* (tobacco hornworm). Fifty microlitres of PCRs were performed containing 5 μl of cDNA template, 5 μl of PCR buffer (Fermentas), 5 μl (10 mM) of dNTPs, 5 μl of each primer, and 0.25 μl of Taq-polymerase (Fermentas). Hot-start PCR was run for 35 cycles. The thermal cycling profile consisted of a denaturation step of 1 min at 94°C , a primer annealing step of 1 min at 50°C , and an extension step of 1 min at 68°C . After the last cycle an additional extension step of 7 min at 72°C was applied. PCR amplification products were analyzed by horizontal agarose gel electrophoresis and purified using the 'GenElute Gel Extraction Kit' (Sigma). PCR products were subcloned into a pCR 2.1 TOPO vector (TOPO TA cloning kit, Invitrogen), and nucleotide sequencing was performed using the 3130 Genetic Analyzer (Applied Biosystems). Sequence alignments were done with the ClustalW program (Thompson et al., 1994).

Nucleotide sequence translations were performed with the ExpASY translate tool. BLAST (Altschul et al., 1990) programs were used to search databases for sequence homologies.

2.4. Further identification of *Spodoptera* ILPs: RAcE

In order to obtain complete cDNA sequences, RAcE (rapid amplification of cDNA ends) was employed (Frohman et al., 1988). The RAcE protocol was carried out according to the instructions of the 'BD Smart RAcE cDNA Amplification Kit' (BD Biosciences, Clontech). The adapter primers were included in the kit. Specific 5' and 3' RAcE primers (Sigma) were derived from the sequences of the previously obtained (2.3) PCR fragments. The resulting RAcE fragments were then separated by horizontal agarose gel electrophoresis, purified, cloned and sequenced according to the same protocols as outlined above (2.2). Nucleotide sequence translations were performed with the ExpASY translate tool. Putative cleavage sites were predicted using a combination of SignalP (Nielsen et al., 1997; Bendtsen et al., 2004) and SIGFIND (Reczko et al., 2002) for cleavage of the signal peptide, and NeuroPred (Southy et al., 2006) for cleavage of the C-peptide. The mass of the resulting—putative—active peptides was computed with MassLynx Molecular Mass Calculator (Windows NT). BLASTx (Altschul et al., 1997) was used to search databases for sequence homologies. Sequence alignments were done with ClustalW (Thompson et al., 1994).

3. Results

3.1. Cellular localization of ILPs in *Spodoptera littoralis* brain–SOG–CC–CA complexes

Cellular localization of ILPs in brain–SOG–CC–CA complexes of *S. littoralis* last instar larvae was examined by whole mount immunostaining using three different primary antisera: rabbit polyclonal antiserum against bombyxin A1 A-chain, against LIRP A-chain and against DILP2 A-chain. Only the antiserum directed against bombyxin A1 A-chain led to a positive result. ILP-like immunoreactivity was observed in four pairs of *pars intercerebralis* cells, in the CC–CA complex, and in the connectives between the brain and the CC–CA complex (Fig. 1).

3.2. Molecular cloning of two *Spodoptera littoralis* ILP precursor cDNAs and precursor analysis

PCR was performed on *Spodoptera* cDNA with a degenerate primer-set derived from the amino acid sequences of ILPs from the lepidopterans *B. mori*, *A. convolvuli*, *S. cynthia ricini* and *M. sexta*. Fig. 2 shows the primer design. Taking the common structure of pro-insulin into consideration, namely the B–C–A order from NH₂- to COOH-terminus, the primer sequences were chosen as follows:

- (1) → 5'-TAY TGY GGI CGI CAY CTI GC-3' (B-chain forward)
- (2) ← 5'-RCA RCA YTC RTC IAC DAT ICC-3' (A-chain reverse)
with Y = (T,C), I = (T,C,A,G), R = (A,G) and D = (A,G,T).

This yielded two fragments: a fragment of 151 bp and a fragment of 207 bp. These fragments were cloned and

Fig. 1. Whole-mount immunolocalization of ILPs in the central nervous system of *Spodoptera littoralis*. Cellular localization of ILPs was examined using primary antibodies against bombyxin A1 A-chain. Immunofluorescent staining was observed in four pairs of median neurosecretory cells, in the CC–CA complex, and in the connectives between the brain and the CC–CA complex. No positive staining was observed when using antibodies against LIRP A-chain and against DILP2 A-chain. Abbreviations used: CC, corpus cardiacum; CA, corpus allatum; MNSC, median neurosecretory cells.

sequenced. After translation, conserved regions of the ILP B-chain and A-chain were found in both fragments. BLAST searches for homology indeed confirmed significant sequence similarity with known ILP precursors.

To obtain the entire nucleotide sequences of the two putative *S. littoralis* ILP precursors, 5' and 3' RAcE were performed. The specific 5' and 3' primers were chosen as follows:

For the 151 bp fragment:

5' primer: 5'-ACGATGCCCTCGTTTGCTCGAAC-3'
3' primer: 5'-CCAAGGCATCAGGGCTCTGAACGGC-3'

For the 207 bp fragment:

5' primer: 5'-ATCCTCGACGCCGCTCCACGGCCAA-3'
3' primer: 5'-GCCCAGTTTCAGACTTTATGCCTGGCG-3'

This yielded a 5' RAcE fragment of 327 bp and a 3' RAcE fragment of 189 bp for the initial 151 bp PCR product, and a 5' RAcE fragment of 323 bp and a 3' RAcE fragment of 409 bp for the 207 bp PCR product. By combining the overlapping RAcE fragments, the cDNA sequences encoding the entire precursor polypeptides were obtained. Their nucleotide sequences and corresponding deduced amino acid sequences are shown in Fig. 3. For convenience, we designate from now on these two different *S. littoralis* ILP precursors 'Sl-ILPP1' and 'Sl-ILPP2'.

Chain B

```

bomE1      1 --EANVAHHYCGRHLANTLADLCWD-TSV---
mas        1 -----HCGRHLARTLADLCY-----
bomB11     1 --QEVARITYCGRHLANTLAYVCFG-VE---
bomG1      1 --QEEVARRYCGRHLAVTMADLCFG-VQFD--
bomC1      1 --QTASQFYCGDFLARTMSSLCW--SDMQ--
bomF1      1 QELGGSRRYCGRHLAQTMAVLCWIGIDEMSAE
bomD1      1 --ASEEGHIYCGRVLAYKMADLCWR-AGFE--
bomA3      1 ---DTPHVCYGRRLAIMLSYLCNDQYLM---
sacA3      1 ---DTPHVCYGRRLAIMLSYLCNDQY----
sacA2      1 ---DTAHVCYGRRLATMLHYVCDNQYQV---
sacB2      1 ---YCGRVLADTLAYLCP-----
agc        1 -----NNYCGRHLSETLAYMCPLEGAS--
consensus 1   e  h  YCGrHLa  tlaylCw

```

Chain A

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bomE1      1 --GVVDECCIQPCTLDVLA---YC--
bomB11     1 GPGVVDECCFRPCKLEVLKSFFFFC--CD-
sacB2      1 --GVVDECCYNSCTLDVLLS---YC--
bomF1      1 --GIIDECCIQACTRDVLLS---YC--
bomD1      1 --GIIDECCIQPCTNDVLLS---YC--
bomG1      1 --QGIIDECCLVPCITNVLS---YC--
bomC1      1 --GVVDECCYRPTCTLDVLS---YCDN
bomA3      1 --QGIIDECCNKPCTEDELLG---YC--
sacA3      1 --GIIDECCNKPCTEDELLG---YC--
sacA2      1 --GIVECCNKPCTEDELLG---YCYK
agc        1 --GVVDECCYQSCITDELLT---YC--
mas        1 --GVIDECCRACTLDLEFT---YC--
consensus 1   GivDECC  rpCtldvLls  yC

```

Fig. 2. Primer design for initial PCR on *Spodoptera littoralis* cDNA. This figure shows ClustalW multiple sequence alignments of the B-chains and of the A-chains of a number of lepidopteran insulin-like peptides. Conserved regions are highlighted and consensus sequences of the B- and A-chain are shown. The primer-set was designed against the underlined regions in the consensus sequences. *Abbreviations used:* bomE1, bomB11, bomG1, bomC1, bomF1, bomD1, bomA3 = bombyxin E1-A3; mas = *Manduca sexta* insulin-like peptide; sacA3, sacA2, sacB2 = *Samia cynthia* insulin-like peptide A3-B2; agc = *Agrius convolvuli* insulin-like peptide.

The first completed cDNA sequence in Fig. 3 is 467 bp long and contains an open reading frame (ORF) of 88 amino acids (AA) (*SI-ILPP1*). In this ORF, we recognized an ILP B-chain of presumably 27 AA, and an ILP A-chain of presumably 20 AA. The predicted cleavage sites where (1) the putative secretory signal peptide (18 AA) and (2) the interconnecting C-peptide (19 AA) are cleaved, are shown in Fig. 3.

The second completed cDNA sequence in Fig. 3 is 660 bp long and contains an open reading frame (ORF) of 105 AA (*SI-ILPP2*). Again, an ILP B-chain (27 AA) and an ILP A-chain (21 AA) were found, displaying moderate (B-chain) to high (A-chain) sequence similarity with those of *SI-ILPP1*. The ILP B-chains share 9 (33%) identical AA residues, plus 10 (37%) conserved or semi-conserved substitution residues. The ILP A-chains share 12 (60%) identical residues, plus 4 (20%) conserved or semi-conserved substitution residues. The computed secretory signal peptide of *SI-ILPP2* is 17 AA long, and the C-peptide 36 AA long. The signal peptides of *SI-ILPP1* and *SI-ILPP2* display 47% AA sequence identity, though a 1 AA-gap was needed to optimize the alignment. When aligning the C-peptides, the best score was obtained when

the shorter C-peptide of *SI-ILPP1* was compared to the back part (3' end) of the *SI-ILPP2* C-peptide. In this case, 5 identities and 4 conserved/semi-conserved substitutions were found, after allowing a 1 AA-gap. The alignment files for the *SI-ILPP1* and *SI-ILPP2* signal peptides, B-chains, C-peptides and A-chains individually are shown in Fig. 4.

Fig. 5 depicts the mature active peptides (i.e. '*SI-ILP1*' and '*SI-ILP2*') formed out of these two precursors, thus containing only their respective B- and A-chain, joined by disulfide bonds. The interconnections between the cysteine residues were drawn by analogy with a common pattern of disulfide bond formation in bombyxins and other lepidopteran ILPs. The computed mass is 5192 Da for *SI-ILP1* and 5348 Da for *SI-ILP2*.

BLASTx searches for homology were performed for the entire precursor polypeptides *SI-ILPP1* and *SI-ILPP2*. *SI-ILPP1* shows the highest homology with the precursor for bombyxin B2 (46% identities; 61% positives). *SI-ILPP2* shows the highest homology with the precursor for *Agrius convolvuli* bombyxin-related peptide A (35% identities; 48% positives). *SI-ILPP1* and *SI-ILPP2* both show second highest homology with the precursor for *Samia cynthia ricini* bombyxin A3 homolog (44% identities and 55% positives for *SI-ILPP1*; 33% identities and 48% positives for *SI-ILPP2*). Fig. 6 presents the multiple sequence alignment of the entire precursor sequences for *SI-ILP1*, *SI-ILP2*, and their respective highest and second highest scoring BLAST hits.

4. Discussion

In this paper, we report on the first attempts to identify insulin-like peptides of the cotton leafworm *S. littoralis*. To our knowledge, nothing about the presence of ILPs in *S. littoralis* has been described so far.

We have localized ILP-like material in the central nervous system of *S. littoralis*. Positive immunofluorescent staining was observed in four pairs of *pars intercerebralis* cells, when using primary antibodies against bombyxin A1 A-chain. No positive immunofluorescence was observed when using antibodies against LIRP A-chain and against DILP2 A-chain, demonstrating the significant difference in structure of lepidopteran ILPs versus orthopteran and dipteran ILPs. Our results are in line with those reported by Ishizaki and Suzuki (1994), who found that four pairs of dorsomedial neurosecretory cells are the major site of bombyxin synthesis in the *B. mori* brain. Analogous findings were reported for other lepidopterans such as *A. convolvuli* (Iwami et al., 1996a) and *M. sexta* (El-Salhy et al., 1983). Moreover, medial neurosecretory cells appear to be important sites for ILP gene expression in other insect orders as well. Small clusters of dorsomedial neurosecretory cells are immunostained by ILP antisera in different life stages and species of Diptera (Duve and Thorpe, 1979; Zitnan et al., 1993; Krieger et al., 2004), Orthoptera (Raabe, 1986; Hansen et al., 1990; Claeys, 2005), Coleoptera (Sevala et al., 1993) and Hemiptera

SI-ILPP1

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ctaatacgactcactatagggcaagcagtggtatcaacgcagagtacgcggggacattca 60
agcttcagcggttgaaacagtcacatctaccagttcttcaacccctcatcatcatgaag 120
                                     M K
ttctacatcgctctttgccttgatcctggcctgtgctgcatgctacgcttctcggaagga 180
F Y I V F A L I L A C A A C Y A S R E G
                                     ↑
actgatttctactgcggacggaacctggctatcactatagcaaaattatgctataattct 240
T D F Y C G R N L A I T I A K L C Y N S
                                     *
gagatcgctaagcgtgatgcgggctggtggttgctccccaaggcatcagggtctgaac 300
E I A K R D A G W W L P P Q G I R A L N
ggcgttcgaggcaaacgaggcctgttgacgagtgctgcgataaacctgctcccttaat 360
G V R G K R G P V D E C C D K P C S L N
                                     * * *
gaactcctgtcttactgctaacttggtccgatgttgctgtaaatgttgattgtaataa 420
E L L S Y C -
                                     *
atgagatttatctcttgtaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 467

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SI-ILPP2

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ctaatacgactcactatagggcaagcagtggtatcaacgcagagtacgcggggatacatct 61
ataaaactttccctaaaaatcacaccgcagctgaaacgtaaaacatgaagcttatcctggta 121
                                     M K L I L V
tctgtcttctgacctggcgtatgctgcagaaaccagaccccgatcctgtgtgga 181
S C L L T L A Y A A A N Q T P V I L C G
                                     ↑
cggcagctggctaaccgtagagtattgctgtgttatgggaaagaatatgtcaataagagg 241
R Q L A N A R V L L C Y G K E Y V N K R
                                     *
acaaggatcagcccagtttcagactttatgcctggcgaaaaaatgaatgaaatcgattgg 301
T R I S P V S D F M P G E K M N E I D W
ccgtggagcgggctcgaggatccttaagtccaactggtcacgatacaagagagacgga 361
P W S G R R G S L S A N W S R Y K R D G
ctggtcgacgagtgctgcctcaagccctgcactaccgacgtcatccttaactactgctaa 421
L V D E C C L K P C T T D V I L N Y C -
                                     * * *
acgaaaatcaaccttaaccaaagaaaatcagaatgaagacaaaaattttctaacgcaag 481
aaagtgtcgaaaaacttgccgcaacctgagaagacttaagcaagatggcgctcatttca 541
ttctgaacgctaatagtacgtatattttagatgtaagctagttacgtaactagtccac 601
ataaataattgtatatattttactataaacctaataaacgaccgaaaaaaaaaaaaa 660

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Fig. 3. Nucleotide sequences of two *Spodoptera littoralis* ILP precursor (SI-ILPP1 and SI-ILPP2) cDNAs and deduced amino acid sequences (ExPASy translate tool). Complete cDNA sequences resulted from a combined approach of PCR and RAcE. ILP B-chains and A-chains are shaded. Conserved cysteine residues are marked by an asterisk (*). Putative signal peptide cleavage sites are indicated by an arrow (↑). Lysine–arginine cleavage sites flanking both termini of the C-peptides are boxed (KR). 5' RAcE primer sequences are in bold and underlined. 3' RAcE primer sequences are underlined.

(Sevala et al., 1992). In addition, we observed ILP-like material in the *Spodoptera* CC–CA complex, which is known as a major storage and release site for ILPs, and in the connectives between the brain and the CC–CA complex. These findings were also reported by El-Salhy et al. (1983), Hansen et al. (1990), Sevala et al. (1993), Krieger et al. (2004) and Claeys (2005).

Furthermore, we have cloned two distinct *S. littoralis* ILP precursor cDNAs. To our knowledge, the corresponding precursor polypeptides, SI-ILPP1 and SI-ILPP2, are the first two ILP precursors ever reported for *S. littoralis*. Initial PCR on *S. littoralis* cDNA using a degenerate primer-set based on the amino acid

sequences of ILPs from the lepidopterans *B. mori*, *A. convolvuli*, *S. cynthia ricini* and *M. sexta*, yielded two amplified fragments, respectively, 151 bp and 207 bp. Previously, we tried to amplify *S. littoralis* brain cDNA using an available primer-set based on the nucleotide sequence of *L. migratoria* insulin-related peptide, LIRP. However, this attempt failed, again demonstrating the divergence between locust ILPs and lepidopteran ILPs. These PCR results are consistent with our above mentioned results of the whole mount experiments, where anti-LIRP antibodies did not react with any ILP-like material in the central nervous system, whereas a positive result was obtained with anti-bombyxin antibodies.

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Sl-ILPP1 signal peptide      MKFYIVFALI-LACAACYA 18
Sl-ILPP2 signal peptide      MKLILVSCLLTLAYAAA-- 17
                               **: :* .*: ** *.

Sl-ILPP1 B-chain            SREGTDFYCGRNLAITIAKLCYNSEIA- 27
Sl-ILPP2 B-chain            -NQTPVILCGROLANARVLLCYGKEYVN 27
                               .: .: ***:** :. ***..* .

Sl-ILPP1 C-peptide          -----DAGW-WLPPQGIRALNGVRG 19
Sl-ILPP2 C-peptide          TRISPVSDFMPEGKMNEDWPWSGRGSLSANWSRY 36
                               :.* * :* :* *

Sl-ILPP1 A-chain            -GPVDECCDKPCSLNELLSYC 20
Sl-ILPP2 A-chain            DGLVDECCCLKPCTTDVILNYC 21
                               * ***** **: :.*.

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Fig. 4. ClustalW amino acid sequence alignment of *Sl*-ILPP1 and *Sl*-ILPP2 signal peptides, B-chains, C-peptides and A-chains. Consensus symbols: (*) = identical residues; (:) = conserved substitutions; (.) = semi-conserved substitutions. The conserved six cysteine residues are shaded.

Sl-ILP1 :

Average Mass = 5191.8774, Monoisotopic Mass = 5188.3055
N-Terminus = H, C-Terminus = OH

1 SREGT DFYCG RNLAI TIAKL CYNSE IA

■1 GPVDE CCKDP CSLNE LLSYC

Sl-ILP2 :

Average Mass = 5348.2384, Monoisotopic Mass = 5344.5521
N-Terminus = H, C-Terminus = OH

1 NQTPV ILGGR QLANA RVLLC YGKEY VN

■1 DGLVD ECCLK PCTTD VILNY C

Fig. 5. Prediction of the structure of the mature active insulin-like peptides *Sl*-ILP1 and *Sl*-ILP2. Disulfide bonds between cysteine residues were drawn by analogy with a common pattern of disulfide bond formation in bombyxins and other lepidopteran ILPs. The molecular mass of *Sl*-ILP1 and *Sl*-ILP2 was computed with MassLynx Molecular Mass Calculator (Windows NT).

The complete ILP precursor cDNAs were obtained by RAcE using specific primers derived from the sequences of the previously obtained 151 bp and 207 bp PCR fragments. *Sl*-ILPP1 and *Sl*-ILPP2 differ from each other in size and sequence. The A-chain of *Sl*-ILPP1 appears to be 1 amino acid residue shorter than the A-chain of *Sl*-ILPP2. We can state with almost a 100% certainty that we predicted these A-chains correctly, since we are almost 100% certain that we predicted the cleavage of the C-peptide correctly. Paired basic amino acids (Lys-Arg) appear to be highly conserved proteolytic cleavage sites for C-peptides from lepidopteran ILP precursors (Kimura-Kawakami et al., 1992; Iwami et al., 1996a) and even mammalian (Bell et al., 1980) insulin precursors. In fact, this is also visible from our alignment file (Fig. 6), where the precursors

for *Samia cynthia ricini* bombyxin A3 homolog, *Bombyx mori* bombyxin B2, and *Agrius convolvuli* bombyxin-related peptide A also display this particular Lys-Arg cleavage motif. The *Sl*-ILPP1 and *Sl*-ILPP2 C-peptides differ strongly in length and show a rather low degree of similarity, but this was not entirely unexpected since C-peptide sequences do not appear to be well conserved during evolution (Claeys et al., 2002). The B-chains of *Sl*-ILPP1 and *Sl*-ILPP2 have the same length. That is, of course, if we assume that our prediction of the signal peptide cleavage site is correct. The applications we used (SignalP and SIGFIND) were not entirely unanimous, but this appeared the most plausible prediction. A common trend for ILPs is that they show high similarity in the A-chains, but lower, moderate similarity in the B-chains. This is indeed also apparent for *Sl*-ILP1 and *Sl*-ILP2. The conserved six cysteine residue pattern, found throughout the insulin superfamily (Claeys et al., 2002), is likewise present in *Sl*-ILP1 and *Sl*-ILP2. The relative positions of these essential residues in *Sl*-ILP1 and *Sl*-ILP2 are identical to that of all members of the family. Another characteristic feature is the conservation of residues that contribute to the hydrophobic core of insulins, and conservation of their relative positions. Conserved hydrophobic residues were found at the following positions (the numbering relates to *Sl*-ILP1, according to the alignments presented in Fig. 4): A3 (Val), A16 (Leu or Ile), A19 (Tyr) for the A-chains, and B13 (Leu) and B20 (Leu) for the B-chains. Furthermore, conservation of glycine at A1, glutamate at A5 and glycine at B10 was observed (Lagueux et al., 1990; Iwami et al., 1996a; Claeys et al., 2002). BLAST searches for homology confirmed structure and sequence similarity between the *S. littoralis* ILP precursors and known ILP precursors, mainly from other lepidopterans.

In conclusion, this paper presents the first evidence for the presence of insulin-like peptides (ILPs) in the cotton leafworm, *S. littoralis*. We have (1) localized ILPs in the central nervous system of *S. littoralis*, and (2) cloned two *S. littoralis* ILP precursor cDNAs. Our results demonstrate the sequence and structure similarity between *S. littoralis*

Fig. 6. ClustalW multiple sequence alignment of the precursors for SI-ILP1, SI-ILP2, and their respective highest and second highest scoring BLASTx hits. Abbreviations used: BXA3_SAMCY = *Samia cynthia ricini* bombyxin A3 homolog precursor; BXB2_BOMMO = *Bombyx mori* bombyxin B2 precursor; BXRA_AGRCO = *Agrius convolvuli* bombyxin-related peptide A precursor. Consensus symbols: (*) = identical residues; (:) = conserved substitutions; (.) = semi-conserved substitutions. Conserved cysteine residues are shaded.

ILPs and bombyxins. It is worth mentioning, however, that the primer-sets for detection of ILPs at cDNA level, and the antibodies used for ILP immunolocalization, are derived from a small select group of known ILPs. Thus, the choice of primer sets and antibodies probably had an important influence on our results. In order to detect a possible larger spectrum of *S. littoralis* ILPs, other primer and antibody choices should be considered. In the near future, we plan to perform quantitative real-time PCR studies of ILP transcripts for various tissues and life stages of *S. littoralis*. The ultimate challenge however will be to determine the *in vitro* and *in vivo* functional roles of *Spodoptera* ILPs (or insect ILPs in general).

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