Molecular cloning and characterization of cDNA for insect biogenic peptide, growth-blocking peptide

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Abstract Growth-blocking peptide (GBP) is an insect biogenic peptide that prevents the onset of metamorphosis from larva to pupa. A cDNA coding for GBP is described. Mixed oligonucleotides derived from a GBP peptide sequence were used to generate amplified DNA by the polymerase chain reaction (PCR). Based on the sequence of the amplified DNA, a 41 bases oligonucleotide was designed for screening a cDNA library which was constructed from the armyworm *Pseudaletia separata* larvae parasitized with the parasitic wasp *Cotesia kariyai*. The cloned cDNA for GBP was 809 base pairs in length. An open reading frame of 429 base pairs encodes a pre-pro-peptide of 143 amino acid residues in which GBP is localized at the C-terminal region, and other three peptides including a putative signal peptide and appropriate processing sites for endoproteolytic cleavage precede the GBP sequence. Northern blot analyses demonstrate the presence of a 800-base mRNA transcript in fat body and 2.5-kilobase transcript in brain and nerve cord, suggesting the possibility that the transcription of GBP gene is regulated in a tissue-dependent manner. This interpretation was supported by isolating a GBP cDNA fragment from cDNA pool of brain-nerve cords. GBP mRNA is constantly expressed in both parasitized and non-parasitized last instar larvae and there is no difference in the levels of the mRNA between both larvae, thus indicating that parasitism may effect on translational or posttranslational level to elevate plasma GBP concentration.

Key words: Growth-blocking peptide; Parasitism; Insect; cDNA cloning; Brain–nerve cord; Fat body

1. Introduction

Endoparasitic insects disrupt the metamorphosis of host insects with the development often arrested in the larval stages. For example, last instar larvae of the armyworm *Pseudaletia separata* parasitized with the parasitoid wasp *Cotesia kariyai* do not initiate metamorphosis to pupae, and the wasp larvae emerge from the host larvae about 11 days after parasitization. Studies on the mechanisms whereby parasitism causes suppressed metamorphosis of host insects revealed that a novel peptide, growth-blocking peptide (GBP), occurred in the plasma (haemolymph devoid of cells) of parasitized last instar larvae of the host armyworm [1]. Injection of about 20 pmol of GBP into non-parasitized armyworm during early last larval instar retards larval growth and causes a delay in pupation for more than a few days through repression of plasma JH esterase activity [2]. Further studies demonstrate that GBP not only exists in the plasma of parasitized last (6th) instar larvae, but also in the plasma of non-parasitized penultimate (5th) instar larvae. From these results, it was proposed that GBP might be a hormone-like peptide which coordinates, along with JH, the regulation of larval characteristics in lepidopteran insects [3,4]. This hypothesis has recently been strengthened by quantifying GBP concentrations in the plasma during the late stages of larval development of the parasitized and non-parasitized host armyworm [5]. The highest level of plasma GBP titer detected on Day O of the penultimate instar gradually decreased throughout larval growth except for the temporary increase on Day O of the last larval instar. After parasitization on Day O last larval instar, plasma GBP titer increased within one day. The elevated level of plasma GBP persisted for only one day, declined gradually, rose again and then declined. These occasional elevations of plasma GBP levels are likely to disturb the parasitized larvae in normal development in the parasitized larvae because a single injection of GBP into non-parasitized larvae during early last larval instar has caused a delay in pupation.

Although physiological significance of GBP has been emphasized by these studies, we were largely ignorant about the mechanism by which parasitism increases plasma GBP concentrations in the armyworm larvae. A previous study demonstrated that the elevation of plasma GBP levels by parasitism can be reproduced by injection of the wasp symbiotic virus, polydnavirus [6,7], which is normally injected by female wasps into the host at oviposition [8]. Furthermore, it has been demonstrated that plasma GBP concentrations are also increased by transferring non-parasitized larvae of the armyworm from 25°C to 10°C. In order to examine the mechanism by which plasma GBP levels are elevated by stress such as polydnavirus infection and low temperature, detailed investigations concerning the nature of GBP gene are warranted.

The present study was conducted to isolate a cDNA clone encoding GBP towards understanding the above mechanism. We report herein the characterization of the GBP cDNA from the armyworm as well as the expression of GBP mRNA in this insect.

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Abbreviations: GBP, growth-blocking peptide; JH, juvenile hormone; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SSC, standard saline citrate; AA, amino acid; bp, base pair; kb, kilobase.

2. Experimental procedures

2.1. Animals

Armyworm *P. separata* were reared on an artificial diet at 25 ± 1°C with a photoperiod of 16 h light:8 h dark. Parasitization by *C. kariyai*

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was carried out by exposing prospective hosts (Day O last instar larvae of *P. separata*) to female wasps. After a single oviposition was observed, the parasitized larva was quickly removed in order to avoid superparasitization and then reared on an artificial diet. Adult wasps were maintained with honey. Penultimate instar larvae undergoing ecdysis between 2 and 2.5 h after lights on were designated as Day O last instar larvae.

2.2. Chemicals

Radioactively labelled reagents were obtained from NEN Research Products (Dupont). Oligonucleotides were synthesized on a model 392 DNA synthesizer (Applied Biosystems). Radiolabelling of oligonucleotides was performed by using T4 polynucleotide kinase with [γ-^{32}P]ATP [9], and cDNA fragments were labelled using the random prime labelling method with [α-^{32}P]dCTP [10].

2.3. RNA isolation

After collecting haemolymph from last instar larvae of the armyworm for isolation of haemocytes, other tissues such as brain, nerve cord, fat body and midgut were dissected and washed well with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2). The pellet resulting from centrifugation at 300 × *g* for 5 min was used as a haemocyte preparation [11]. Immediately after isolating host tissues, they were frozen in liquid nitrogen and total RNA was isolated by the method of Chomczynski and Sacchi [12].

2.4. PCR amplification

The amplification reaction was conducted according to the procedure slightly modified from the method of Strub and Water [13]. Standard PCR was done in a 100 μl volume containing 50 ng of cDNA, 1 × PCR buffer (Perkin-Elmer/Cetus), 200 μM dNTPs, 0.01% gelatin, 2.5 units Amplitaq DNA polymerase (Perkin-Elmer/Cetus), and 100 pmol of each oligonucleotide primer set. Temperature-cycling profiles were generally based on the following model: 40 cycles at 95°C for 1 min, 63°C for 2 min and 72°C for 3 min.

2.5. cDNA synthesis and cloning

Total RNA was prepared by homogenizing gut-eviscerated bodies of Day 2 last instar larvae of the armyworm 2 days after parasitization. Polyadenylated mRNA was purified from total RNA using the Quick Prep mRNA purification system (Pharmacia). Synthesis of cDNA from 2 μg of poly (A)$^+$ RNA was done by the method of Gubler and Hoffman [14] with a cDNA synthesis kit (Amersham). The cDNA was size-fractionated to remove fragments <500 bp, ligated to λgt 10 arms and packaged into capsids using a commercial kit, λgt 10 cDNA cloning system (Amersham). For initial screening, the recombinant phages were plated on *E. coli* NM514 (Amersham) at about 3,000 plaque forming units/90-mm Petri dish, and plaques were transferred to nylon membranes (Hybond N$^+$, Amersham). A ^{32}P-labelled synthetic oligonucleotide (41 bases) corresponding to the portion of GBP sequence was used to screen the cDNA library. The cDNA inserts were subcloned into M 13 (mp 18) phage vector (Pharmacia) using a commercial ligation kit (Takara).

2.6. DNA sequencing

The nucleotide sequences of the cDNA inserts or PCR amplified DNA fragments from appropriate sublones were determined by a Taq Dye Primer Cycle Sequencing kit (Takara) using DSQ 1000 DNA sequencer (Shimadzu). Computer assisted sequence analysis was done with GENETYX-MAC Ver. 6.2.0 (Software Development Co., Tokyo).

2.7. Northern blot hybridization

Ten microgram of total RNA was denatured with glyoxal and dimethyl sulfoxide and separated by 1.0% (w/v) agarose gel electrophoresis in 10 mM sodium phosphate (pH 7.0). The RNAs were transferred to nylon membranes and hybridized at 65°C in a hybridization solution containing ^{32}P-labelled GBP cDNA for 15 h. The membranes were washed with 2 × SSC containing 0.1% SDS for 30 min, then twice with 0.1 × SSC containing 0.1% SDS each for 30 min, both at 60°C, according to the protocols of Sambrook at al [15]. The membranes were exposed to Kodak X-OMAT AR film (Eastman Kodak Co.) for 6–12 h at −80°C with intensifying screen (Dupont). Sizes of the RNAs were estimated with RNA markers (0.24–9.5 kb RNA ladder, GIBCO BRL).

3. Results

3.1. PCR using degenerate oligonucleotides

The GBP cDNAs were cloned by a combination of PCR and cDNA library screening. To isolate the coding sequence of an internal portion of the GBP, oligonucleotide primers were designed to amplify the GBP peptide sequence (pentacosa peptide). The degenerate sequences for the primers corresponding to both ends of GBP diagrammed in Fig. 1 (primers A1 and A2) were deduced from the amino acid (AA) sequence of the peptide. Each primer carries a 5′ extension that provides *Eco*RI restriction recognition sequences for cloning the DNA fragments.

PCR amplification was done on a cDNA pool synthesized from mRNA extracted from the eviscerated bodies of the parasitized armyworm larvae. A 91 bp product was isolated and

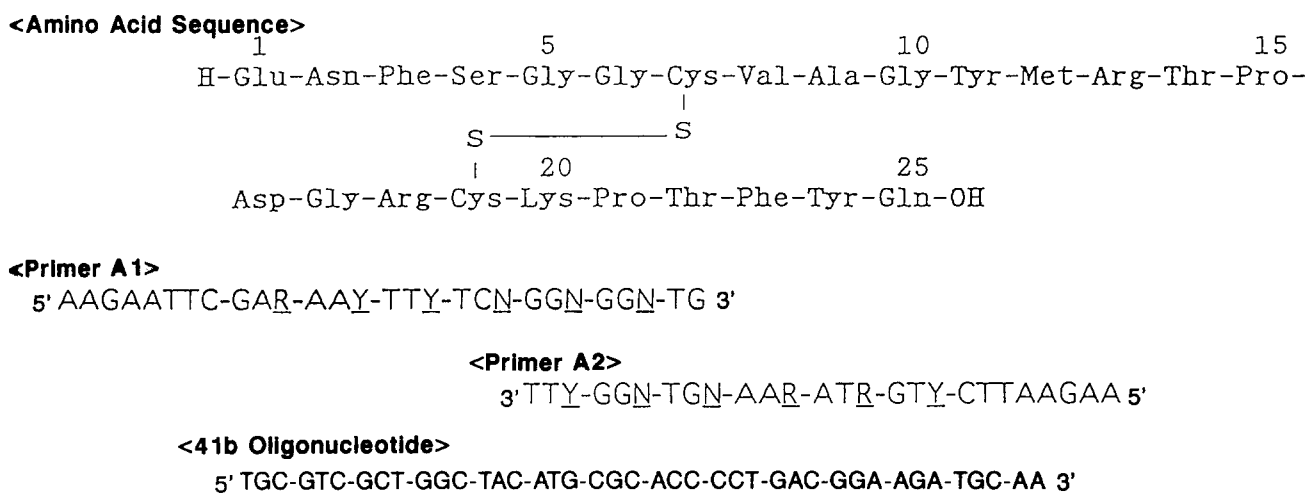

Fig. 1. Design of oligonucleotide primers for PCR based on an internal segment of the GBP. Primers A1 and A2 are degenerate oligonucleotides used to amplify an internal segment of the sequence. A 41 base oligonucleotide represents the resulting concensus sequence obtained and was used to screen a λgt 10 cDNA library. R represents A or G; Y represents C or T; N represents A, C, G or T.

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       10         20         30         40         50         60
CTTCAGTCAAGAGAAAATTATCTGAAGAGTCGTGACACGTACATAATAATTTCACAAAAT
                                                    Met

       70         80         90        100        110        120
GAAATTAACTATTTCCATTTTATTTTGTGTTATTTTAACACTGCAATATAACGGTGCAGA
 LysLeuThrIleSerIleLeuPheCysValIleLeuThrLeuGlnTyrAsnGlyAlaAsp

      130        140        150        160        170        180
TGGAAAACTGAAGGATTTATTCGGGAAAATCCATGATTCAGTACACGGAACAGCAGACAA
 GlyLysLeuLysAspLeuPheGlyLysIleHisAspSerValHisGlyThrAlaAspLys
   ↑
      190        200        210        220        230        240
GGTAAAAGAAGATTTGAATAGCTTGTTTCATCCAAATGACAAGAATCAACAGGGAAACAA
 ValLysGluAspLeuAsnSerLeuPheHisProAsnAspLysAsnGlnGlnGlyAsnAsn

      250        260        270        280        290        300
CGATGCATCCTCGAATATTCACTTTGCTGATTCGGAAGAAAACACTGACGCTGCTAAGAA
 AspAlaSerSerAsnIleHisPheAlaAspSerGluGluAsnThrAspAlaAlaLysLys

      310        320        330        340        350        360
GCCAGATGAAGTAACTCCTGCAACAACGACCACTACTACTGCAGCGCCAGCAGTACCCAA
 ProAspGluValThrProAlaThrThrThrThrThrThrAlaAlaProAlaValProAsn
    <Primer B1>  5' CCTGCAACAACGACCACT 3'
      370        380        390        400        410        420
TGCACCTTCGGACAATCCCACGACGCTTGCACCGTCTACCACAACTAAAGATGGCCGCGA
 AlaProSerAspAsnProThrThrLeuAlaProSerThrThrThrLysAspGlyArgGlu

      430        440        450        460        470        480
GAACTTTTCAGGCGGATGCGTCGCTGGCTACATGCGCACCCCTGACGGAAGATGCAAACC
 AsnPheSerGlyGlyCysValAlaGlyTyrMetArgThrProAspGlyArgCysLysPro

      490        500        510        520        530        540
CACCTTCTAGCAATTAATTACTTAATATCGACATGTCTTGGTAATGTATCTTTGTGTAAC
 ThrPhe***GlnLeuIleThr***
                              3' CTGTACAGAACCATTACA 5'  <Primer B2>
      550        560        570        580        590        600
TGTATTTTTATTTCTCGTTAATTTTTTTCTTACCATTAGCAATTTACCGGCTGCATATCA

      610        620        630        640        650        660
TTTGATTTTATGTTAACAATGTAAGTAATATATTGTTATTTTATGGCATTTATCTGTGCA

      670        680        690        700        710        720
CGTGTTAAACATCCATAGTTGAAAATATTTGTTTATTCCTTTTTGTTGTTATTAGGGTTG

      730        740        750        760        770        780
TTGTAGTTTGTATGCGTGAAATAAAATACTTCTACAGGTCTTATAAATTTATTAATAAAA

      790        800        810
TTATCGCTTGAACAGTAAATCAATTAAAT
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Fig. 2. Nucleotide and deduced amino acid sequences of cloned cDNA for GBP. A full length and *Pst*I-digested fragments (341 bp and 468 bp) of cDNA insert were subcloned into M13 mp 18 vector. The sequence determinations were carried out starting from universal forward and reverse primers present in the multiple cloning site of M13 mp 18. The sequence of GBP is underlined. Polyadenylation signals are double underlined. Dibasic amino acid pair is marked by thick bar. The most likely site for cleavage of a signal sequence is indicated by an arrow. Primers B1 and B2 (bold letters) primed PCR product is dotted-underlined. Two base differences among the PCR products are shown with asterisks above the nucleotide sequence. Both sites are changed to 'T'.

cloned into M13 vector. A series of the resulting clones were then sequenced. The DNA sequencing confirmed the presence of GBP mRNA sequence in the amplified DNA fragments. Based on the nucleotide sequence of the amplified region, a third 41 bases oligonucleotide excluding the primer portions was prepared. This third oligonucleotide was then used to screen a λgt 10 cDNA library generated from the cDNA pool described above. Four positive cDNA clones were obtained, bearing the cDNA inserts ranging from 0.75 to 0.85 kb in lengths.

3.2. The GBP mRNA sequence

The cDNA clone with the longest 0.85 kb cDNA insert was sequenced 100% in both directions. Fig. 2 shows the GBP cDNA sequence and predicted primary structure of the protein. After the first ATG, there is a 429 bp open reading frame followed by an untranslated 322 bp region including two consensus polyadenylation signals (AATAAA). The open reading frame beginning at residue 59 and ending at 487 encodes an apparent precursor polypeptide consisting of 143 AA. The amino acid sequence and composition of the 22 residues from Met-1 to Gly-22 match well with the consensus for signal peptides for secretion [16,17]. The amino acid sequence of GBP was found at a carboxyl proximal domain of the deduced precursor polyprotein. A potential site for dibasic endoproteolytic cleavage (Lys-Lys) is found at position 296 to 301 where is located in between the putative signal peptide and GBP (Fig. 2); thus, two peptides other than GBP are likely to be released from the precursor through posttranslational processing. One of the peptides which lies close to an N-terminal region of GBP, contains extremely high content (49%) of Pro and Thr. Computer-aided search of the National Biomedical Research Foundation protein data bank failed to detect any significant matches between these peptide sequences and the published data.

GBPs purified from the plasma of parasitized and non-parasitized armyworm larvae consist of 25 AA and 23 AA residues, respectively [2,5]. The characterized cDNA encodes GBP consisting of 23 AA residues. To examine whether cDNAs encoding 25 AA GBP occur in the cDNA pool which was used for constructing the library, primers (B1 and B2) were designed based on the DNA sequences flanking the GBP sequence (Fig. 2) and PCR amplification of the cDNAs were performed. A product of about 220 bp was isolated and cloned. Sequence

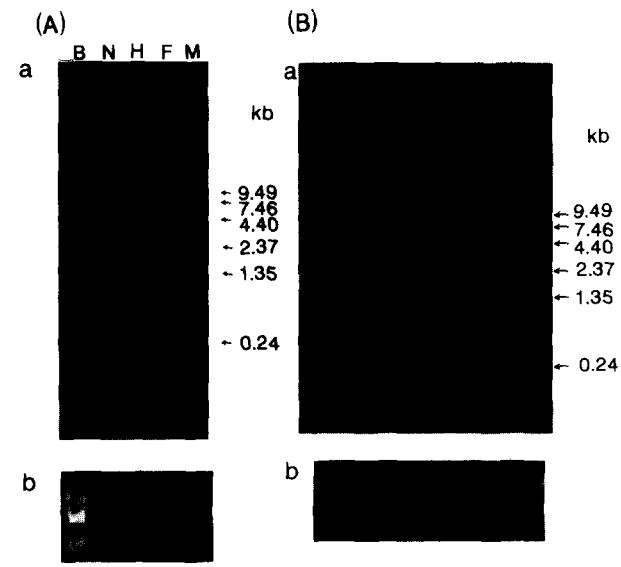

Fig. 3. Expression of the gene encoding the GBP precursor. (A,a) brain (B), nerve cord (N), haemocytes (H), fat body (F) and midgut (M) were collected from Day 1 penultimate instar larvae of nonparasitized armyworm. 10 μg of total RNA was electrophoresed on each lane and analyzed by Northern hybridization using GBP cDNA as a probe. (A,b) Portion of the duplicate gels was stained for ribosomal RNA stained with ethidium bromide. (B,a) N0–24: total RNA prepared from eviscerated bodies of non-parasitized last instar larvae on Day 0 at indicated time. P6–24: total RNA prepared from last instar larvae at indicated time after parasitization on Day 0 (2 h after light on). Total RNA was loaded at 10 μg per lane for a Northern blot analysis. (B,b) Portion of the duplicate gels was stained for ribosomal RNA with ethidium bromide.

analyses of the resulting fifteen clones indicated that two clones bear base mismatch in the region of GBP portion but none of clones have a conventional tyrosine codon at the 24th amino acid residue as shown in Fig. 2.

3.3. Northern blot analyses

To investigate the expression of GBP in various tissues, a Northern blot analysis was carried out using total cellular RNAs from non-parasitized larval tissues. The analysis using ^{32}P-labelled GBP cDNA probe showed the presence of GBP RNAs in the fat body, brain and nerve cord as shown in Fig. 3A. No hybridization bands were seen with RNAs from hemocytes and midgut. Although the hybridization band (approximately 800 b) observed with the fat body RNA corresponds closely with the predicted mRNA size based on sequence analyses of the GBP cDNA, approximately 2.5 kb of the positive band both in brain and nerve cord is much longer than that of GBP mRNA in fat body. In order to examine whether the hybridzed 2.5 kb mRNA encodes the sequence of GBP, mRNAs extracted from brain-nerve cords were reverse transcribed and PCR amplification of the cDNAs was done by using the B1 and B2 primers. A DNA band with the predicted size was amplified. This band was cloned and then sequenced. This sequence was demonstrated to be exactly identical with that of the characterized cDNA shown in Fig. 2 (dotted-underlined).

As we previously reported, after parasitization on Day 0 last larval instar, plasma GBP titer increased within one day [5]. During this period, it was interested to determine whether GBP mRNA levels correlate with plasma GBP peptide concentration. As shown in Fig. 3B, the constant levels of GBP mRNA were detected in both parasitized and non-parasitized last instar larvae, indicating that parasitism had no effect on GBP mRNA levels.

4. Discussion

We have determined the sequence of a cDNA encoding GBP of the armyworm *P. separata* and demonstrated that GBP is first synthesized as a polyprotein precursor in a manner analogous to those observed with most of the neuroactive peptides such as enkephalin [18], opiomelanocortin [19], glucagon [20] and tachykinin precursor [21] in mammals. In insects, it has been found that neuropeptides such as diapause hormone and allatostatin are first synthesized as precursors [22,23]. These precursors often produce multiple peptides with distinct biological activities [24].

Some of neuropeptides have different precursor structures, and the generation of different types of precursor mRNA is regulated in a tissue-specific manner by alternative RNA splicing through the inclusion or exclusion of an each exon sequence [25–27]. The GBP mRNA is transcribed both in brain-nerve cord and fat body, and the mRNA present in brain-nerve cord (2.5 kb) is much longer than that in fat body (0.8 kb) as shown is Fig. 3A. Although we have not sequenced the full length of cDNA for brain-nerve cord GBP, examination of nucleotide sequence of the DNA fragment amplified by PCR revealed the presence of GBP mRNA sequence in the cDNAs reverse transcribed from brain-nerve cord RNA. Hence, it is possible to speculate that the brain-nerve cord GBP cDNA correspond to a variant species of GBP mRNA different from the fat body mRNA which has been characterized in the present study. Obviously, analysis of the brain-nerve cord cDNA for GBP is certainly required to elucidate this question.

The amino acid sequence for the first 23 residues of GBP deduced from the cDNA sequence is identical with that of GBP purified from the plasma of the armyworm larvae, except that the nucleotide sequence for the 24 th amino acid is the TAG stop codon. However, GBP purified from the parasitized armyworm larvae consists of 25 AA resides, containing 24th Tyr and 25th Gln, in addition to the 23 AA GBP from the nonparasitized larvae. If a G at nucleotide position 490 of the cloned GBP cDNA replaces a T or C, or the TAG is unconventionally decoded as Tyr, then Tyr is inserted at the position of 24th AA and followed by 25th Gln (CAA in the cDNA). Thus, this peptide sequence have a structural property identical with GBP purified from the parasitized larvae. In fact, there is evidence that the UAG codon of tobacco mosaic virus can be read during infection by tobacco Tyr tRNA [28,29]. Furthermore, GBP peptide sequence predicted from the cDNA analyses is followed by three more amino acide, Leu-Ile-Thr. In the preliminary AA sequencing performed on a larger amount of purified GBP C-terminal fragment, we have detected 26th Leu and 27th Ile (unpublished data). From these results and observations, it would be reasonable to propose the following two hypotheses: First, in the parasitized armyworm larvae, TAG codon for the 24th amino acid might be changed to TAT or TAC codon, otherwise TAG codon might unconventionally be decoded as Tyr. Second, an intact and mature GBP in the plasma of the parasitized armyworm larvae might consist of 28 amino acid residues.

Northern blot analysis demonstrated that there was no difference in the levels of GBPmRNA in parasitized and nonparasitized larvae. Thus, the kinetics of the mRNA in the both larvae suggest that parasitism does not effect on transcriptional levels but on translational or posttranslational levels to elevate plasma GBP concentration (Fig. 3B).

As we stated above, GBP was initially found in the plasma of the parasitized host armyworm larvae whose development was halted at the last larval stage. The developmental arrest and the elevation of plasma GBP levels can be reproduced by injection of polydnavirus particles [4,8]. Therefore, it is reasonable to speculate that the modification of 23 AA GBP to give rise to longer than 25 AA peptide, might be caused during polydnavirus infection following parasitization with parasitoid wasps. The preliminary experiments show that the 23 AA GBP has about 70% of physiological activities of the 25 AA GBP. It is apparent that, if polydnavirus infection causes the above modification of GBP structure as well as the elevation of plasma GBP levels, the development of parasitized larvae is quite effectively retarded.

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