Insect cytokine growth-blocking peptide (GBP) regulates insect development

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Abstract

Growth-blocking peptide (GBP) was first identified in the hemolymph of the host armyworm, *Pseudaletia separata*, whose growth is halted in the last instar larval stage by parasitization with the parasitoid wasp, *Cotesia kariyai*. Studies on the mechanism of growth retardation by GBP revealed that GBP titers in hemolymph fluctuate synchronously with dopamine levels: GBP and dopamine peaks coincide with each larval molt period, during which larvae temporarily cease moving and feeding. The fact that GBP induced the elevation of dopamine concentration in the hemolymph was demonstrated in armyworm larvae by GBP injection. Dopamine elevation was also observed in insects exposed to various stress conditions such as parasitization and chilling. These results, together with the fact that dopamine itself inhibits larval growth, indicate that GBP induces growth retardation via the elevation of dopamine levels. Further, we demonstrated that the diapause-inducing influence of short day length also elevates dopamine concentrations in hemolymphs and the brain-central nervous system (Br-CNS) of the cabbage armyworm, *Mamestra brassicae*. The elevation of dopamine levels contributes to the onset of pupal diapause. We therefore proposed that a GBP-dopamine system contributes to the control of growth rates in insects. Recent studies by ourselves and other laboratories have found more than 10 homologous peptides in various insect species. These peptides have diverse functions: larval growth retardation, paralysis induction, cell proliferation, immune cell stimulation and cardioacceleration. As demonstrated, GBP itself exerts most of these functions, so it is reasonable to conclude that GBP and GBP-like peptides widely present in insects should be regarded as insect cytokines with a variety of functions.

Key words: Cytokine; growth-blocking peptide (GBP); dopamine; growth; development

INTRODUCTION

Insects, like all animals, respond to a variety of stimuli and adapt to external environmental changes by controlling their morphology and physiology. In particular, in insects, the distinguishing features are programmed into their life cycles: dramatic morphological changes in the developmental process of metamorphosis, and obligatory or facultative diapause (Nijhout, 1994). Such drastic changes in the developmental or physiological processes have attracted a large number of entomologists to study the endocrinology that controls these changes. Since Kopec demonstrated that the larval brain of *Lymantria dispar* releases a factor necessary for pupation (Kopec, 1922), a variety of insect hormones have been identified, isolated, and most of their structures demonstrated: ecdysone (E), 20-hydroxyecdysone (20E), juvenile hormone (JH), prothoracotrophic hormone (PTTH), and diapause hormone (DH). Information about some insect hormones is as extensive as for their mammalian counterparts (Gade al., 1997). In contrast to insect hormones, however, insect cytokines have received little attention.

Although cytokines initially drew our attention mainly as important mediators of immunity, cytokine research has become increasingly relevant in many different areas of the biological sciences in the past 25 years (Pedersen and Hoffman-Goetz, 2000). Today we recognize that cytokines play key roles in a variety of physiological processes such as inflammation, cell growth, tissue remodeling, and development (Hanada and Yoshimura, 2002; Eswarakumar et al., 2005; Klagsbrun and Eichmann, 2005; McGeechay and Anderton, 2005).

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Abbreviations: Br-CNS, brain-central nervous system; Br-SG, brain-subesophageal ganglion; DH, diapause hormone; DOPA, 3,4-dihydroxy-L-phenylalanine; GBP, growth-blocking peptide; PSP, plasmatocyte spreading peptide

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Most of these studies have been performed using mammals or mammalian cells, and new cytokines and receptors continue to be identified. Compared with mammalian cytokines, little is known about insect cytokines. Although some cytokine-like molecules and receptors have been assigned by homology searches and genetic analysis in *Drosophila*, until recently, no insect cytokines had been substantially identified in insects by biochemical research (Muskavitch and Hoffmann, 1990; Shilo and Raz, 1991; Neuman-Silberberg and Schupbach, 1993; Schweitzer et al., 1995; Raffery and Sutherland, 1999). The first family of insect cytokines was referred to as ENF peptides, based upon the unique N-terminal consensus sequence, Glu-Asn-Phe-. Growth-blocking peptide (GBP) is the founding member of this peptide family (Strand et al., 2000; Aizawa et al., 2002). The following is a short review of our studies on the cytokine GBP, which was initially identified in the last instar larvae of the armyworm, *Pseudaleitia separata*, parasitized by its parasitoid wasp, *Cotesia kariyai*.

**DISCOVERY OF GROWTH-BLOCKING PEPTIDE (GBP)**

Endoparasitoid wasps lay their eggs in the body cavity of other insects where the progeny will feed (Beckage, 1985). After oviposition, the eggs and larvae of the wasps could be attacked by the defense system of the oviposited insects; however, when these insects are permissive hosts for the wasps, the wasp eggs and larvae are rarely killed by the host defense system. Once the parasitoid larva circumvent the host defense system, they have to absorb enough nutrition to mature and emerge from the host larval body. As a consequence of parasitoid development, the growth of the host insect is often retarded (Vinson and Iwantsch, 1980). Therefore, when the relationship between parasitoid wasps and their permissive hosts was evolutionally established, the immune system and growth regulatory system of the host insect fell under the control of the wasps. Such a successful one-sided relationship has allowed us to analyze how the host insect is manipulated by its parasites. At the end of the 1980s when we started to study the parasitization strategy of endoparasitoid wasps at the molecular level, most researchers in this field focused their attention on the growth retardation and/or immune suppression that are usually observed in parasitized host insects (Vinson, 1990; Lawrence and Lanzrein, 1993; Strand and Pech, 1995).

We focused on growth retardation of the parasitized host insect. Last instar larvae of the armyworm *Pseudaleitia separata* parasitized by the wasp *Cotesia kariyai* do not initiate metamorphosis, and this developmental disturbance allows the wasps to complete their larval growth and emerge while the host is still in the larval stage; otherwise, the wasp larvae would be trapped in the sclerotized pupal cuticle. In most lepidopteran larvae, the burst of hemolymph JH esterase activity early in the last instar correlates with a decline in plasma JH, after which PTTH is released and initiates a cascade of events leading to pupation (Hammock, 1985). We revealed that hemolymph JH esterase activity in the last instar larvae was completely blocked by parasitization; however, the blockage of JH esterase activity was probably not due to inhibition of this enzyme but repression of its gene expression. We have identified the peptidergic factor(s) that repress the increase of JH esterase expression in the hemolymph of parasitized larvae (Hayakawa, 1990). This repressive factor was purified by measuring its repressive activity toward JH esterase expression when injected into nonparasitized early last instar larvae of the armyworm. Four consecutive analytical reversed phase HPLC runs were sufficient to purify the responsible peptide (Hayakawa, 1990). Injection of this peptide into early last instar larvae blocked the elevation of plasma JH esterase activity and, consequently, disturbed the normal development of the larvae; therefore, this peptide was named growth-blocking peptide (GBP) (Hayakawa, 1991, 1995; Hayakawa and Yasuhara, 1993).

Peptide sequencing and fast atom bombardment mass spectrometry showed that GBP is a 25 amino acid peptide with one disulfide bond between two cysteine residues: Glu-Asn-Phe-Ser-Gly-Gly-Cys-Val-Ala-Gly-Tyr-Met-Arg-Thr-Pro-Asp-Gly-Cys-Lys-Pro-Thr-Phe-Tyr-Gln (Hayakawa, 1991). Soon after the primary structure of GBP was reported, seven homologous peptides that have paralytic effects were isolated from the hemolymph of lepidopteran larvae (Skinner et al., 1991, 1993). All these paralytic peptides consist of 23 or 24 amino acid residues, and sequence homology between
GBP and the paralytic peptides is approximately 80% (Hayakawa, 1995; Kamimura et al., 2001). No other homologous peptide was reported for the next six years, until 1997 when plasmatocyte-spreading peptide (PSP) was found as a member of the same peptide family (Clark et al., 1997). This peptide induces plasmatocytes to spread on foreign surfaces in vitro. Three years later, another homologous peptide was isolated as a cardioactive peptide (Furuya et al., 1999). We conducted parallel experiments using GBP and PSP to determine whether these peptides have distinct or multiple biological activities. Both peptides exhibited significant growth retardation and paralytic activity (Strand et al., 2000). Further, they induced plasmatocytes to spread on a culture plate. Based on these results, we propose that all these peptide family members are insect cytokine homologs that likely have multiple biological activities. Based on the consensus sequence of their N termini, we further proposed that these molecules be referred to as members of the "ENF" peptide family (Strand et al., 2000).

GROWTH BLOCKING ACTIVITY OF GBP

We demonstrated that the injection of 20 pmol of chemically synthesized GBP into nonparasitized armyworms during the early last larval instar clearly retards larval growth and causes a delay in pupation through repression of plasma JH esterase activity. On the basis of this result, together with the fact that GBP was found in the parasitized larval hemolymph, we concluded that GBP is one of the main causes of parasitization-induced growth retardation. We initially hypothesized two possibilities for the origin of GBP; from the wasp or host. Subsequent studies demonstrated that GBP exists not only in the hemolymph of parasitized last instar larvae, but also in the hemolymph of nonparasitized larvae, which indicates that GBP is not a peptide of the parasitoid wasp but a gene product of the armyworm genome (Hayakawa, 1992; Hayakawa et al., 1995). Using anti-GBP monoclonal antibody, GBP concentrations in the hemolymph of parasitized and nonparasitized larvae were measured. In nonparasitized larvae, the hemolymph GBP concentration was highest in day 0 penultimate instar larvae and declined gradually with larval development, although a temporary increase was observed on day 0 of last instar larvae, suggesting that the GBP titer is relatively higher during the younger larval stage than during the last instar stage (Ohnishi et al., 1995; Hayakawa et al., 1998). The GBP titer continued to decrease during the last larval instar to less than 10 nm after day 1 in last instar larvae. Unlike the decrease seen in nonparasitized last instar larvae after final larval ecdysis, the GBP titer of parasitized larvae increased within one day after parasitization; however, the elevation of hemolymph GBP persisted for only one day, declined gradually, rose again, and then kept declining (Fig. 1). The increased level of GBP was not maintained for a long period and gradually declined; however, the lowest level of GBP is higher than that of nonparasitized larvae. Further, this characteristic pattern of fluctuating hemolymph GBP titer after parasitization allowed us to speculate that the occasional elevations of GBP level are likely to disturb normal larval development. This speculation was partly supported by the fact that a single injection of GBP into nonparasitized early last instar larvae retards larval growth and delays pupation. Further, we demonstrated that the characteristic pattern of fluctuating hemolymph GBP titer was also observed in last instar larvae whose development was retarded by transfer from 25°C to 10°C (Ohnishi et al., 1995).

![Graph showing GBP and dopamine concentrations](image-url)

Fig. 1. GBP (●) and dopamine (○) concentrations in hemolymphs of 4th, 5th, and last instar larvae of the armyworm Pseudaletia separata. Day 0 last instar larvae were parasitized by Cotesia kariyai 7 h after lights on. Parasitization caused a three-fold increase of GBP titers within one day (●). Each point represents the mean of 7–10 independent determinations.
Under this cold stress condition, the body weights of larvae remained lower than those of larvae growing at 25°C and pupation was delayed for 11 days. During the prolonged last instar larval period, the plasma GBP level was much higher than that in control larvae and decreased gradually with six peaks. Therefore, the GBP concentration in hemolymph is increased under various stress conditions.

MECHANISMS OF GBP-INDUCED GROWTH BLOCKING

We observed that GBP retarded growth through the repression of hemolymph JH esterase activity. Although this implies that growth retardation is due to a relatively high level of JH in the hemolymph, the mechanism by which GBP represses hemolymph JH esterase activity is totally unknown. We directed our attention to previous reports that demonstrated the contributions of biogenic amines to insect neuroendocrinology and developmental physiology: the monophenolamine octopamine, which has been reported to have allatostatic activity on the corpora allata of the cockroach, Diploptera punctata (Thompson et al., 1990), and octopamine, dopamine, and serotonin, which increase cyclic AMP levels in the corpora cardia of the American cockroach, Periplaneta americana (Downer et al., 1984). In Manduca sexta, dopamine stimulates JH and JH acid synthesis in the corpora allata in vitro in early last instar larvae, but inhibits synthesis in the later stage (Granger et al., 1994, 1996). Further, dopamine undergoes developmental changes with four peaks that coincide with the two molts, pupation, and adult emergence in Drosophila melanogaster (Martinez-Ramirez et al., 1992).

In order to elucidate the pathway of GBP action, we analyzed biogenic amines in armyworm larvae that were parasitized by parasitoid wasps or injected with GBP. HPLC analysis of hemolymph with electrochemical detection indicated a difference between dopamine levels in parasitized and nonparasitized larvae. Concentrations of other biogenic amines such as octopamine and serotonin were unaffected by parasitization (Noguchi et al., 1995). In nonparasitized larvae, the dopamine level was highest at the last larval molt, and it decreased rapidly to one-sixth of the maximal level. Parasitization of day 0 last larval instars caused an approximately two-fold increase in dopamine levels within one day, and the elevated levels were maintained throughout the last larval instar period. The elevation of hemolymph dopamine levels was reproduced by injection of GBP: hemolymph dopamine levels were three times higher in GBP-injected larvae than in control PBS-injected larvae one day after injection; therefore, hemolymph dopamine elevation in parasitized larvae is reasonably ascribed to the effect of GBP.

We tested the effect of dopamine on larval development by injection of 2.6 nmol dopamine (the natural dopamine concentration in hemolymph of day 0 last instar larvae soon after ecdisis). Injection of dopamine into nonparasitized larvae once each day from day 0 to day 3 of the last instar decreased weight gain and delayed pupation by a few days in 70% of tested larvae. Similar growth retardation was observed when a D2 receptor agonist, (-)-quinpirole, was injected into larvae, although injection of a D1 agonist, (+)-SKF38393, had no effect (Noguchi et al., 1995). These results suggest that dopamine plays an important role in the retardation of larval development through binding to D2-type dopamine receptors. However, we do not know the mechanism by which activation of D2 receptors disturbs the normal development of armyworm larve.

We examined which organ contributes to the elevation of hemolymph dopamine. In insects, two separate pools of dopamine are present in the nervous system and the integument (Evans, 1980). In the insect nervous system, dopamine is the most abundant monoamine and it may serve as a neurotransmitter and neuromodulator (Martin et al., 1984; Orchard, 1984; Davis and Pitman, 1991; Goldstein and Camhi, 1991). In addition, an extremely high concentration of dopamine is present in the integument, where it serves as an essential intermediate of cross-linking agents in cuticle formation throughout insect development (Hopkins et al., 1984; Andersen et al., 1995). Measurement of dopamine concentrations in these two tissues demonstrated that the total amount of dopamine in the integument was approximately 1,000 times greater than that in the larval brain of the armyworm. The dopamine pool in the integument is also approximately 50 times bigger than that in the hemolymph, thereby suggesting that the GBP-in-
duced elevation of hemolymph dopamine could be from dopamine in the integument (Noguchi and Hayakawa, 1996). This speculation was partly confirmed by time-course studies during in vitro culture experiments. Integument containing radiolabeled dopamine was incubated in Grace’s medium both with and without 5% sodium dodecyl sulfate (SDS) in order to follow the release of radiolabeled dopamine into the medium. In the absence of SDS, radiolabeled dopamine was released into the medium in a linear fashion for the first 30 min, and at 45 min, its release reached a plateau. In contrast, when 5% SDS was added to the incubation medium, radiolabeled dopamine was continuously leaked into the medium without reaching a plateau, suggesting that the observed release of dopamine into Grace’s medium was not due to artificial leakage but to actively regulated release from the integument (Noguchi and Hayakawa, 1996).

**ROLE OF A GBP-DOPAMINE SYSTEM IN PUPAL DIAPAUSE INDUCTION**

Changes in hemolymph GBP and dopamine concentrations during the late larval stage of the armyworm are shown in Fig. 1. Both titers fluctuate synchronously during larval development. We are especially interested in the fact that GBP and dopamine peaks coincide with each molt period, during which larvae temporarily cease moving and feeding (Fig. 1) (Ohnishi et al., 1995; Noguchi and Hayakawa, 1997). These results, together with the previous observation that GBP causes rigid paralysis in injected armyworm larvae, allow us to speculate that a GBP-dopamine system might participate in the control of the induction of diapause (Noguchi and Hayakawa, 1997, 2001; Noguchi et al., 2003).

To assess this speculation, we measured dopamine concentrations in the hemolymph, integument, and brain-central nervous system (Br-CNS) of diapause- and non-diapause-destined prepupae and pupae of the cabbage armyworm, *Mamestra brassicae* (Noguchi and Hayakawa, 1997). In all tissues, dopamine levels were significantly higher in diapause-destined insects than in non-diapause-destined insects. The difference between dopamine levels in both animals was biggest during pupal molt: the concentration was quadrupled in every tissue. These results suggest that a high concentration of dopamine in these tissues relates to the induction of diapause. To determine whether dopamine could participate in triggering pupal diapause in *Mamestra* pupae, we increased dopamine levels in pupae that had been reared under non-diapause-destined conditions, such as long day length. By feeding *Mamestra* last instar larvae DOPA, the dopamine level was elevated several times in all the tested tissues: hemolymph, integument, and Br-CNS. Treatment with DOPA resulted in developmental retardation in more than 60% of the tested pupae: about 30% of the pupae metamorphosed into adults at around two months after onset of the wandering stage, and another 30% of the pupae metamorphosed at around four months. In contrast to the DOPA-treated pupae, all control pupae had metamorphosed into adults by two weeks after onset of the wandering stage (Fig. 2) (Noguchi and Hayakawa, 1997). Therefore, these data strongly suggest that dopamine concentrations in hemolymph and Br-CNS play an important role in controlling the induction of diapause in *Mamestra* pupae. Although we have not revealed the mechanism by which a high concentration of dopamine induced a diapause-like state, it is reasonable to expect that dopamine must have an inhibitory effect on the secretion of PTTH from brain.

![Fig. 2. Number of cabbage armyworms metamorphosed to adults after onset of the wandering stage. Control insects fed an artificial diet (Insecta-LF, Nihon Nosan Kogyo Co. Ltd) (open bars) and test insects fed an artificial diet containing 1% (w/w) DOPA during last larval instar.](image-url)
ROLE OF THE GBP-DOPAMINE SYSTEM IN EGG DIAPAUSE INDUCTION

As another example, we analyzed the mechanism of egg diapause of the silkworm, *Bombyx mori*. The silkworm enters diapause at an early embryonic stage before dermal differentiation is completed. High temperature (25°C) and long day length act on the embryos to program the resulting mature adult females to lay diapause eggs, whereas low temperature (15°C) and short day length produce non-diapause eggs. Although it has been reported that diapause-destined conditions, high temperature and long day length, act on the subesophageal ganglion (SG) to induce it to secrete DH, which acts on developing oocytes to induce diapause in the resulting embryos, the mechanism by which such diapause-destined conditions induces the secretion of DH is unknown. We measured dopamine concentrations in hemolymph and Br-SGs of silkworm larvae and pupae that were incubated under either diapause-destined or non-diapause-destined conditions. In both tissues, dopamine concentrations were constantly higher in diapause-type than in non-diapause-type insects. The difference between Br-SG dopamine levels in diapause- and non-diapause-type insects was particularly great at two and three days after pupation (Fig. 3). Dop decarboxylase (DDC) activities were measured in Br-SGs of diapause- and non-diapause-type insects. DDC activities were 2-4 times higher in diapause-type than in non-diapause-type insects during the first few days after pupation, with the greatest point of difference coinciding with the greatest difference in Br-SG dopamine levels (Fig. 3). Further, DDC mRNA levels were significantly higher in the diapause type at the same time, indicating that the increase in DDC activity in diapause-type insects was due to the enhancement of DDC gene expression.

After we confirmed that dopamine concentrations are higher in diapause-type than in non-diapause-type silkworms, as in the case of the cabbage armyworm, we examined the effect of elevated dopamine levels on the induction of egg diapause. Feeding DOPA to larvae or the injection of DOPA (or dopamine) into day 2 pupae increased dopamine concentrations in Br-SGs of day 3 pupae. Female adults whose dopamine concentrations were elevated by either treatment laid diapause eggs at frequencies of over 60%, even when they had been placed under non-diapause conditions, short day length at 15°C, when they were in the embryonic stage. As the elevation of Br-SG DH mRNA levels in diapause-type females a few days after pupation has been reported (Sato et al., 1993; Xu et al., 1995), we measured Br-SG DH mRNA levels in non-diapause-type females after treatment with dopamine or DOPA. Injection with DOPA or dopamine produced an approximately three-fold increase in DH mRNA levels in day 3 pupae 20 h after injection, indicating that the dopamine-induced embryonic diapause of the silkworm occurs through the increased level of DH during the early pupal stage (Noguchi and Hayakawa, 2001).

It has been reported that dopamine functions as a neurotransmitter and inhibits the release of the crustacean hyperglycemic hormone from the eyestalk neuroendocrine complex of the red swamp crayfish, *Procambarus clarkii* (Sarojini et al., 1995a). Further, dopamine has been found to an-
agonize the ovary-stimulating action of serotonin in *P. clarkii* (Sarojini et al., 1995b). Moreover, it is known that dopamine has a regulatory effect on prolactin secretion from vertebrate pituitary cells; a high concentration of dopamine inhibits prolactin release, whereas a low concentration of dopamine stimulates prolactin secretion (Shin et al., 1997; Youngren et al., 1999). Although we do not know at present whether such dopamine functions are related to the induction of diapause in the silkworm and cabbage armyworm, it is reasonable to speculate that dopamine has regulatory effects on DH and PTTH secretions; a high concentration of dopamine stimulates DH secretion in the silkworm pupal Br-SG while inhibits PTTH secretion from cabbage armyworm brains.

**GBP AND GBP HOMOLOGS IN INSECTS**

Since GBP was found in *P. separata* larvae in 1990, more than 10 homologous peptides have been reported as the factors responsible for different functions, such as paralysis induction (Skinner et al., 1991, 1993), hemocyte stimulation (Clark et al., 1997; Yu et al., 1999), cell proliferation (Hayakawa and Ohnishi, 1998), and cardioacceleration (Furuya et al., 1999). All these peptides contain 23–25 amino acid residues, and sequence homology between GBP and other homologs is approximately 80% (Strand et al., 2000; Aizawa et al., 2001; Clark et al., 2001). The structural similarities between these peptides strongly suggest that they are all members of a common peptide family, yet different functions have been ascribed to most of them in the species in which they were originally identified. As mentioned above, we have demonstrated that GBP itself has multiple functions, such as growth regulation (Hayakawa, 1990, 1991), paralysis induction (Strand et al., 2000), hemocyte stimulation (Strand et al., 2000; Matsuno et al., 2003), cell proliferation (Hayakawa and Ohnishi, 1998) and morphogenesis (Tsuzuki et al., 2005a, b). Recent studies using various deletion mutants of GBP demonstrated that the minimum structure of GBP containing mitogenic activity is 2–23 GBP, whereas that with hemocyte-stimulation activity is 1–22 GBP (Aizawa et al., 2001). Based on these observations, it is reasonable to expect

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**Fig. 4.** Alignment of *Bombyx mori* paralytic peptide genes (GBP orthologous genes). The underlined C-terminal sequences are active paralytic peptides. The sequence designated as “proPP-full” with the ENF-amino terminal was determined by Kamimura et al. (2001), and two other homologs with DNF-amino terminals were identified by BLAST searches of the *Bombyx mori* genome database for this sequence.
that the multifunctional properties of GBP are mediated by different types of GBP receptors.

One question concerning GBP has long remained unanswered; whether this peptide family is specific to lepidopteran insects, because all family members have been found only in Lepidoptera. This question was answered just recently by identifying similar cytokines in Diptera (Hayakawa, 2003). Although the primary structures of the cytokines in dipteran insects are not highly homologous to those of lepidopteran GBP and its homologs, their biological functions seem to be conserved. Further, BLAST searches of the Bombyx mori genome database for GBP revealed two novel GBP homologs whose amino terminal sequences are Asp-Asn-Phe- (D-N-F-) (Fig. 4). Although GBP and its homologs were designated as the ENF peptide family, as mentioned in the Introduction, the name is unsuitable. However, we believe that insect cytokines with similar multifunctions should be categorized into the same family and that they occur broadly throughout insect species.

REFERENCES


Insect Cytokine Growth-Blocking Peptide


