Bombyxin is a growth factor for wing imaginal disks in Lepidoptera

H. Frederik Nijhout* and Laura W. Grunert

Department of Biology, Duke University, Durham, NC 27708

Edited by Donald F. Steiner, University of Chicago, Chicago, IL, and approved October 14, 2002 (received for review September 9, 2002)

The mechanisms that control the growth rate of internal tissues during postembryonic development are poorly understood. In insects, the growth rate of imaginal disks varies with nutrition and keeps pace with variation in somatic growth. We describe here a mechanism by which the growth of wing imaginal disks is controlled. When wing imaginal disks of the butterfly *Precis coenia* are removed from the larva and placed in a standard nutrient-rich tissue culture medium they stop growing, suggesting that nutrients alone are not sufficient to support normal growth. Such disks can be made to grow at a normal rate by supplementing the culture medium with an optimal concentration of the steroid hormone 20-hydroxyecdysone and with hemolymph taken from growing larvae. The growth-promoting activity of the hemolymph is caused by a heat-stable factor that can be extracted from the CNS and appears to be identical to the neurohormone bombyxin, a member of the insulin family of proteins. Synthetic bombyxin stimulates growth at concentrations as low as 30 ng/ml, and specific antibodies to bombyxin completely remove growth-promoting activity from the hemolymph. Bombyxin evidently acts together with 20-hydroxyecdysone to stimulate cell division and growth of wing imaginal disks. It appears that the level of bombyxin in the hemolymph is modulated by the brain in response to variation in nutrition and is part of the mechanism that coordinates the growth of internal organs with overall somatic growth.

Bombyxin was first identified as a neurosecretory hormone in the silk moth, *Bombyx mori* (1). Despite two decades of work, we are still in the dark about bombyxin's natural function in insect biology. Bombyxin was isolated because of its ability to stimulate ecdysone secretion by the prothoracic glands of the moth *Samia cynthia* and was therefore believed to be the long-sought prothoracicotropic hormone of insects (2, 3). Unfortunately, bombyxin was subsequently shown not to stimulate ecdysone secretion in *B. mori* itself and does not appear to be the natural prothoracicotropin. The actual prothoracicotropic hormone has since been identified and clearly has no structural relationship to bombyxin (4). Bombyxin is a member of the insulin family of peptides, and its structure and molecular biology have been extensively studied (2, 5–11). Because of bombyxin's similarity to insulin, its possible role in carbohydrate metabolism has been studied (12, 13). The similarity of bombyxin to insulin and insulin-like growth factors has also led some authors to suggest that bombyxin may play a role in the regulation of growth (14). The work reported below shows that bombyxin functions as a growth factor for wing imaginal disks.

Methods

All larval and tissue cultures were maintained at a constant temperature of 27°C. Wing imaginal disks were removed from 12- to 36-h-old fifth-instar larvae of *Precis coenia*. Larvae were surface-sterilized by immersion in a 0.2% solution of benzalkonium chlorides (Matheson) for 15 min and dissected in sterile lepidopteran saline. Disks were immediately placed in a small Petri dish containing Grace's insect tissue culture medium (Invitrogen/GIBCO 11605-094) supplemented with 10% FCS and 10% microbial inhibitors (Invitrogen/GIBCO antibiotic-antimycotic 15240-096), and any adhering fat body and epidermis were carefully removed. Disks were subsequently transferred to 300 μl of the same medium in a 24-well culture plate (Corning/Costar 3524). In all cases, disks were cultured for 24 h before transfer to a test medium to allow any factors carried over with the disk to decay. All cultures were maintained in a sealed chamber (Billups-Rothenberg/Labequip, Del Mar, CA) under an atmosphere of 95% oxygen and 5% carbon dioxide and placed on a shaker platform rotating at 60 rpm. Cultures were maintained for 24–48 h in 200–300 μl of test medium, consisting of the above modification of Grace's medium with the addition of 20-hydroxyecdysone, heat-treated hemolymph, and/or synthetic bombyxin II.

Hemolymph to be tested was collected from early fifth-instar larvae of *P. coenia* and *Manduca sexta*. Hemolymph was collected into an ice-cold test tube, which was immediately immersed in boiling water for 10 min, followed by centrifugation at 12,000 $\times$ *g* for 10 min. The supernatant was transferred to a sterile test tube and stored at 4°C. This hemolymph retained its growth-promoting activity for at least 4 months.

Dry weights were determined by rinsing disks in lepidopteran saline, placing them on a small tared piece of aluminum foil, carefully drawing off all adhering saline, and drying in a 60°C oven until a constant weight was reached ($\approx$48 h). Protein was quantified by means of the bicicroninic acid method (BCA Protein Assay, Pierce), using BSA as a standard. Protein content of disks was found to be $\approx$75% of dry weight. Mitotic figures were observed after staining with either 4′,6-diamidino-2-phenylindole (Sigma) or Schiff reagent. Antibody immobilization was done by using protein-A affinity columns (Seize-X immunoprecipitation kit, Pierce). To remove antigen, hemolymph samples were passed over a column three times, each pass followed by elution of the bound material and reactivation of the column. Pure synthetic bombyxin II was obtained as gifts from Shinji Nagata and Hiroshi Kataoka (University of Tokyo, Tokyo) and Akira Mizoguchi (Nagoya University, Nagoya, Japan). Antibodies to bombyxin were gifts from Akira Mizoguchi. Of the four antibodies we tested, two (AN-I and GP-Pab) recognized antigens in the hemolymph of *M. sexta* and *P. coenia*.

Results

During the last larval instar of *P. coenia*, the wing imaginal disks grow at an exponential rate (Fig. 1). Mitoses occur at a low but continuous rate during this time period, with a cell doubling time of 30 $\pm$ 6 h (Fig. 1). By contrast, the protein doubling time of the wing imaginal disks is 48 $\pm$ 7 h, indicating that the cell size and nuclear-cytoplasmic ratio decreases during growth. This finding is consistent with the observation that the density of nuclei in the epithelium of a wing imaginal disk increases as the disks grow (Fig. 2 *A* and *B*). The epithelial cells of the wing imaginal disk are thin and spindle-shaped, with a nuclear bulge that is wider than the rest of the cell. Cell number increases faster than the

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: DILP, *Drosophila* insulin-like protein; IDGF, imaginal disk growth factor.

*To whom correspondence should be addressed. E-mail: hfn@duke.edu.

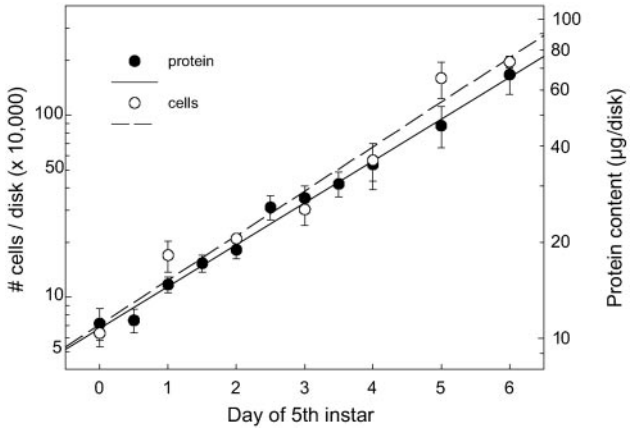

Fig. 1. Growth of the forewing imaginal disks of *P. coenia in vivo* during the last (fifth) larval instar. Size was measured as total protein content and cell number. Each point is the mean of four to six specimens; bars are SEM. Growth is exponential, but the doubling time for cell number is shorter than the doubling time for protein content.

surface area of the disk, so nuclei become arranged in a broad band between the apical and basal surfaces of the epithelium.

We have developed an *in vitro* tissue culture method that supports a normal rate of growth of the wing imaginal disks of

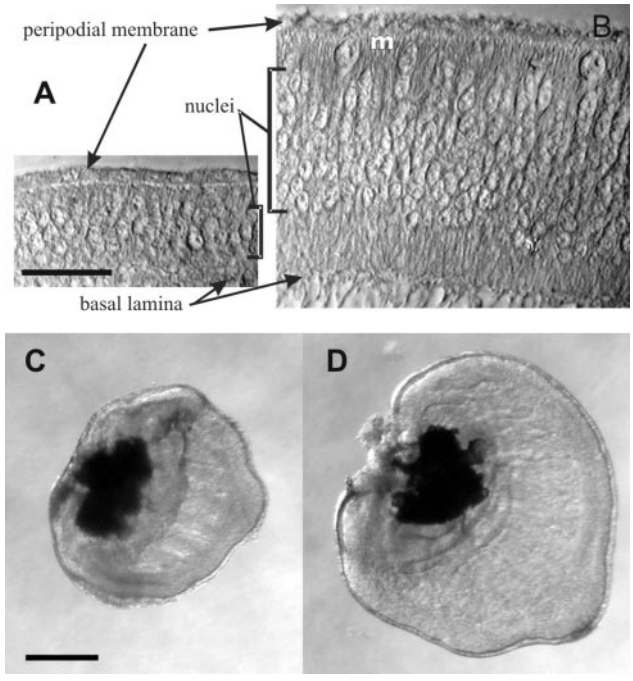

Fig. 2. (*A* and *B*) Sections through wing imaginal disks on day 1 (*A*) and day 5 (*B*) of the fifth larval instar. Each epithelium of the wing imaginal disk is a monolayer of narrow spindle-shaped cells. The number of nuclei in a given area increases with time because cells divide faster than the rate at which the surface area increases. Cells become taller instead of wider and nuclei become crowded and arranged at several levels through the thickness of the epithelium. Nuclei undergoing mitosis migrate to the apical surface (m). Both images are at the same magnification; scale bar is 100 μm. (*C* and *D*) Left and right wing imaginal disks taken from a 24-h fifth-instar larva. (*C*) Disk was cultured for 48 h in unsupplemented Grace's medium. (*D*) Disk was cultured for 24 h in unsupplemented medium, followed by 24 h in medium supplemented with 0.1 μg/ml 20-hydroxyecdysone and 10% active hemolymph. Difference between these two disks thus represents 24 h of stimulated growth *in vitro*. Both images are at the same magnification; scale bar is 0.35 mm.

Table 1. Requirement for hemolymph and 20-hydroxyecdysone for growth of wing imaginal disks in culture

Ecdysone	No	Yes	No	Yes
Hemolymph	No	No	Yes	Yes
Initial weight*	19.0 ± 2.3	21.2 ± 2.5	16.78 ± 1.6	18.8 ± 2.8
Final weight	18.3 ± 2.5	23.8 ± 3.3	17.0 ± 1.5	39.2 ± 5.7
Change	−4.7%	+11.9%	+3.1%	+115.6%

Disks were held in culture medium for 40 h without supplement or supplemented with 0.1 μg/ml 20-hydroxyecdysone and/or 10% heat-treated hemolymph.
*In μg dry weight ± SEM (n = 6 in all cases).

P. coenia. Supplementation of culture medium with both 20-hydroxyecdysone and heat-treated hemolymph from early fifth-instar larvae of either *P. coenia* or *M. sexta* was found to be an absolute requirement for normal growth (Table 1; Fig. 2 *C* and *D*). We found that there is an optimal concentration of 20-hydroxyecdysone (0.1 μg/ml) that supports normal growth (Fig. 3). This concentration is well below that required for molting and corresponds to the measured levels of ecdysone during the growth phase of the last larval instar of *M. sexta* (15). A similar low concentration of 20-hydroxyecdysone is required for the growth of wing imaginal disks of *B. mori* (16) and is involved in the regulation of development of the eye imaginal disk of *M. sexta* (17), although it does not appear to be required for imaginal disk growth in *Trichoplusia ni* (18). At this optimal concentration of 20-hydroxyecdysone, isolated wing imaginal disks grow at a rate similar to that observed *in vivo* (Fig. 3).

In life, wing imaginal disk growth is strictly proportional to the growth rate of the larva and varies with variation in nutrition. When larvae of *Precis* are starved, growth of the wing imaginal disks ceases within 4–6 h (19). When wing imaginal disks from starved larvae were put into a fully supplemented culture medium with active hemolymph and 20-hydroxyecdysone, normal growth resumed. This finding suggests that the growth-promoting activity of the hemolymph may fluctuate with the nutritional state and growth rate of the larva. We therefore tested whether hemolymph from starved larvae could support growth of wing imaginal disks. Wing imaginal disks of 11 individuals were cultured in medium supplemented with hemolymph from larvae starved for 24 h. The growth of these disks

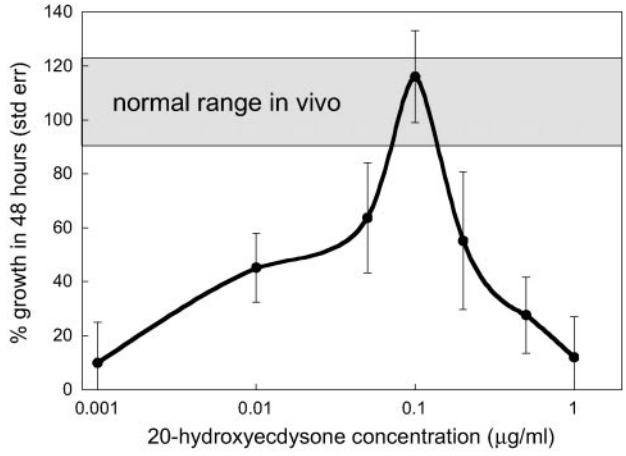

Fig. 3. Dose–response curve for 20-hydroxyecdysone in culture medium supplemented with 10% active hemolymph. Each point is the mean of 6–12 wing imaginal disks; bars are SEM. There is a narrow optimal 20-hydroxyecdysone concentration of 0.1 mg/ml that promotes normal growth. This concentration is well below the ecdysone concentration that stimulates the molting cycle (0.6–1.0 mg/ml).

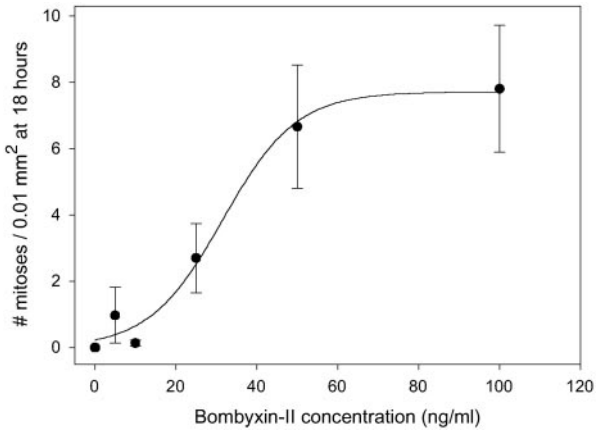

Fig. 4. Dose–response curve for bombyxin II in culture medium supplemented with 0.1 mg/ml 20-hydroxyecdysone. Each point is the mean of three to five wing imaginal disks; bars are SEM.

was compared with that of 10 disks cultured in medium supplemented with hemolymph from normally feeding larvae. After 24 h in culture growth was measured as a percentage change in total protein content of the disks compared with corresponding contralateral disks taken at the beginning of the experiment. With hemolymph taken from normally growing larvae disks grew 68% (±12%); with hemolymph from starved larvae growth was 13% (±9%). Evidently hemolymph from starved larvae supported little growth of wing imaginal disks.

Many of the protein components of hemolymph in insects are synthesized by the fat body, and conditioning of the culture medium by culturing fat body for 24 h often enhances the growth of cultured insect cells. In *Drosophila melanogaster*, the fat body is known to produce a growth factor for imaginal disks cells (20). Hence we investigated whether the fat body of *Precis* was the source for our putative growth factor. Conditioning the culture medium by culturing fat body for 24 h, followed by culture of imaginal disks (with or without the continued presence of fat body), did not support the growth of imaginal disks, even in the presence of an optimal concentration of 20-hydroxyecdysone. However, adding homogenates of brains of *P. coenia* (two brain-equivalents per 300 μl of medium) strongly stimulated wing imaginal disk growth in the presence of ecdysone. The same result was obtained with brain homogenates and homogenates of subesophageal ganglia of *Manduca*, but not with homogenates of subesophageal ganglia of *Precis*. Evidently the active factor is not a general component of nervous tissue, because homogenates of subesophageal ganglia of *Precis* were inactive. These results show that the putative growth factor is not species-specific, because *Manduca* brains can stimulate normal growth in *Precis* wing imaginal disks, and that species may differ in the source(s) of this factor.

Of the insect neurosecretory hormones, the one most likely to act as a growth factor is the hormone bombyxin, because bombyxin is a member of the insulin family, and other members of this family play a critical role in the control of growth in *Drosophila* (20, 21). We found that synthetic bombyxin II, a member of the A family of bombyxins, could substitute for active heat-treated hemolymph and supported normal growth of the wing imaginal disks in culture. A dose–response curve for synthetic bombyxin II is shown in Fig. 4. It appears that concentrations of 30–50 ng/ml ($\approx 7 \times 10^{-9}$–10^{-8} M) are mitogenic. The BM-N4 cell line of *B. mori* responds to bombyxin by a change in morphology and an increase in size at concentrations of 10^{-10}–10^{-8} M (≈0.48–48 ng/ml) (22). The effective concentration of bombyxin II for *Precis* is not unreasonable, considering it is probably somewhat different from the native bombyxin of *Precis*. In the absence of 20-hydroxyecdysone, bombyxin II failed to stimulate growth of cultured wing imaginal disks. Recombinant human insulin and insulin-like growth factor II were inactive in our culture system.

Two antibodies to bombyxin (AN-I, a mouse monoclonal, and GP-PAb, a guinea pig polyclonal) recognized an antigen in the hemolymph of *P. coenia* (by indirect ELISA). We immobilized these antibodies on protein A affinity columns and passed active heat-treated hemolymph over them. We found that this procedure made the hemolymph incapable of supporting growth or mitosis in cultured wing imaginal disks (no growth by protein assay in 11 disks). Activity was restored by the addition of 50 ng/ml synthetic bombyxin II. Thus antibodies to bombyxin completely removed the activity from our hemolymph. This finding suggests that our active factor is sufficiently like bombyxin II to be recognized by a specific mAb. Moreover, the experiment shows that there are no additional active factors in the hemolymph that are not recognized by antibombyxin.

Discussion

The Molecular Biology of Bombyxins. The neurohormone bombyxin, together with the steroid hormone 20-hydroxyecdysone, is required for normal growth of wing imaginal disks of the butterfly *P. coenia*. Bombyxins are insulin-like proteins with molecular masses between 4,500 and 5,000 Da. Like insulin, bombyxin is a heterodimer, with A and B chains connected by disulfide bonds. In *B. mori*, there are at least 38 bombyxin genes (a few of which are pseudogenes), and these are classified into five families (named A–E), based on sequence similarities (2, 9, 10, 23–25). Bombyxin genes have also been identified in *Samia cynthia* (Saturniidae), which has six genes, and *Agrius singulatus* (Sphingidae), which appears to have three genes. In both the latter species the bombyxin genes belong to the same two families, related to the A and B families of *B. mori* (9). Several of the bombyxins are neurosecretory products (6, 26), although the locations of expression of most of the bombyxins have not been determined.

In *D. melanogaster* there are seven insulin-like proteins, DILP-1 to DILP-7 (21). DILP-1 to DILP-5 have substantial structural similarity to each other whereas DILP-6 and DILP-7 are different from each other and from the rest. None of the *Drosophila* insulins appear to be particularly closely related to the lepidopteran bombyxins (other than in possessing the characteristic insulin motifs). The sources of the DILPs are quite diverse: mRNAs of *dilp-2*, *dilp-3*, and *dilp-5* are expressed in cerebral neurosecretory cells, and *dilp-7* is expressed in neurosecretory cells of the ventral nerve cord. *dilp-4* and *dilp-6*, by contrast, appear to be expressed primarily in the gut (21). Thus in *Drosophila*, not all insulin-like molecules are neurosecretory, which suggests that the lepidopteran bombyxins may also have a diversity of sources, possibly associated in some way with their function.

The Control of Growth in Imaginal Disks. Most of our knowledge of the control of growth in imaginal disks comes not from studies on intact disk, but from work with cell lines derived from imaginal disks. Insulin is required for growth of an imaginal disk-derived cell line in *Drosophila*, but here there is no requirement for ecdysone. The *Drosophila* insulins work in conjunction with a group of proteins named imaginal disk growth factors (IDGFs) (20). These growth factors are secreted by cultured imaginal disk cells themselves and are also expressed abundantly in the fat body, which is believed to be their main source in intact animals. IDGFs have a molecular mass of ≈52,000 Da and are related to the enzyme chitinase. Both insulin and IDGFs are required for growth in an imaginal disk-derived cell line of *Drosophila*, and unlike the case in *Precis*, human insulin is fully active and evidently can substitute for the native *Drosophila* insulin (20, 27).

There are clearly several important similarities and differences

between *Precis* and *Drosophila* in the regulation of imaginal disk growth. Both require an insulin-like protein, but *Drosophila* requires IDGFs produced by the fat body, whereas *Precis* requires the steroid hormone ecdysone, produced by the prothoracic glands. It is not clear at present whether the conditions required for the growth of an imaginal disk-derived cell line are the same as those required for intact imaginal disks. Cultures of intact imaginal disks of *Drosophila* appear to require juvenile hormone (JH) for normal growth (28), but this is not the case for the imaginal disk-derived cell line in which the effects of insulin and IDGFs have been studied (20). JH is not required for wing imaginal disk growth in *Precis*. Indeed, during the fifth-larval instar, high levels of JH inhibit growth of the wing imaginal disks *in vivo* (29).

The Role of Insulin and Ecdysone in Growth. Not only are insulin-like proteins required for the growth of wing imaginal disks, but the insulin receptor signaling pathway is also a key regulator of overall growth and body size in *Drosophila* (21, 30–36). Overexpression of one of the *Drosophila* insulin genes causes an increase in organ and body size caused by an increase in cell size and cell number (20). By contrast, partial loss-of-function mutations in the insulin receptor, as well as in other molecules of the insulin signaling pathway, cause an increase in development time and a severe, but proportional, reduction in body size (21, 34).

Localized overexpression of the insulin receptor leads to localized cell proliferation and overgrowth of cells (21). It has been noted that mutational deficiencies in the insulin signaling pathway cause a reduction in body size that is similar to that obtained by starvation or by growth on a nutrient-deficient diet, suggesting that the insulin pathway is involved in mediating the growth response to variation in nutrition (30, 34, 37). It appears that the growth of imaginal tissues does not respond directly to the level of nutrients in the hemolymph. Instead, the level of nutrients is sensed by the brain or the fat body, depending on the species, and this organ regulates the growth of internal tissues through the secretion of growth factors.

It is interesting that both *Precis* and *Drosophila* require two kinds of growth factors for normal growth: a neurosecretory insulin-like protein and an additional factor (either ecdysone or IDGF). Whereas the insulins (bombyxins and DILPs) probably act via similar mechanisms, it is unlikely that the ecdysone and the IDGFs have their effect via related mechanisms, because steroids and proteins signal in fundamentally different ways.

The differences between these regulatory mechanisms may simply be caused by evolved differences between these species, but they may also be caused in part by differences in the way tissues grow within an individual. In *Drosophila* there are two types of larval tissues. Tissues that will not be carried through to the adult stage (e.g., the epidermis) grow by cell enlargement and endomitosis, whereas prospective imaginal tissues (e.g., imaginal disks, histoblasts, neuroblasts) grow by cell division. Growth in these two types of tissues appears to be controlled through different mechanisms (37). All tissues appear to require insulin-like molecules for normal growth (21), but only the imaginal disks require specific growth factors from the fat body (20, 37). In the Lepidoptera, by contrast, most larval cells are carried through to the adult and most larval tissues grow by cell division. Epidermal cells undergo a round of cell division at each molt, and imaginal disk cells grow and divide continuously throughout the intermolt period (Fig. 1). In Lepidoptera both epidermal cells and imaginal disk cells require ecdysone for growth, but the two cell types differ in their requirement for bombyxin, as this hormone does not appear to be required for epidermal cell growth *in vitro* (38).

Thus whereas in *Drosophila* the insulins play a fundamental role in growth of all tissues, this does not appear to be the case in the Lepidoptera. It is possible that in *Precis* ecdysone and bombyxin control different aspects of growth of the wing imaginal disks. Bombyxins could be involved primarily with cytoplasmic growth and cell enlargement, whereas ecdysone is mitogenic, in accordance with its role as a mitogen in epidermal cells.

The Role of Nutrition. The growth rate of insects is regulated, in part, by nutrition. It is becoming increasingly clear that growing cells and tissues do not respond directly to varying or reduced levels of circulating nutrients. Instead, the cellular response to nutrition is indirect and is regulated by variation in the level of growth factors (35, 36). In *Drosophila* both the fat body and the CNS appear to sense nutrient levels in the hemolymph and, in response, regulate the growth of tissues through the secretion of specific growth and mitogenic signals (35, 37). The identity of the factors from the fat body has not yet been established, but they are most likely to be the IDGFs. The CNS regulates growth via the secretion of neurosecretory insulin-like protein in both *Precis* and *Drosophila* (30, 35, 36, 39). In *Drosophila*, the expression of two of the three neurosecretory DILPs varies with nutrient availability (35), and these may therefore act as the mediators between nutrition and somatic growth.

In Lepidoptera, the growth of wing imaginal disks is tightly coordinated with somatic growth. When larvae of *P. coenia* are starved, their wing imaginal disks cease to grow within about 4–6 h (19). Wing imaginal disks from such starved larvae can be induced to grow normally *in vitro* by the addition of ecdysone and bombyxin to the culture medium, suggesting that insulin signaling may be involved in the regulation of growth in response to nutritional variation. The principal changes in hemolymph nutrients that occur when larvae of *M. sexta* are starved are sharp declines in the concentrations of glucose and trehalose (40). Within 6–12 h after the onset of starvation, glucose and trehalose decline to about half and one-third, respectively, of their value in feeding larvae. Hemolymph carbohydrates may thus be accurate indicators of the nutritional state of the larva. This hypothesis finds circumstantial support from recent experiments done with *B. mori*. Starvation of *Bombyx* larvae causes a decrease in the titer of bombyxin, and injection of glucose stimulates bombyxin secretion (13). In addition, injection of bombyxin causes a lowering of the carbohydrate level in the hemolymph of *Bombyx* (12). These results have been taken to indicate that bombyxin plays a role in carbohydrate metabolism, but they are also consistent with the hypothesis that bombyxin level is a reflection of the nutritional state of the insect. It is possible that bombyxin may have an important role in the regulation of metabolism, in addition to its effect as a mitogen in wing imaginal disks.

Variation in bombyxin level may be the mechanism that adjusts wing imaginal disk growth to nutritive input in *Precis*. It will be interesting to discover whether ecdysone concentration also varies with nutrition and how this mechanism of regulation has evolved to account for the differences between the Lepidoptera and higher Diptera.

We thank Knut Schmidt-Nielsen for critical reading of the manuscript. This work was supported by Grant IBN-9728727 from the National Science Foundation.

1. Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Ishizaki, H., Mizoguchi, A., Fujiwara, Y. & Suzuki, A. (1984) *Science* **226,** 1344–1345.
2. Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Mizoguchi, A., Fujiwara, Y., Suzuki, A., Takahashi, S. Y. & Ishizaki, H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5840–5843.
3. Nijhout, H. F. (1994) *Insect Hormones* (Princeton Univ. Press, Princeton).
4. Nagasawa, H., Kataoka, H. & Suzuki, A. (1990) in *Molting and Metamorphosis,* eds. Ohnishi, E. & Ishizaki, H. (Springer, Berlin), pp. 33–48.
5. Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H. & Suzuki, A. (1991) *Agric. Biol. Chem.* **55,** 73–86.

6. Mizoguchi, A., Ishizaki, H., Nagasawa, H., Kataoka, H., Isogai, A., Tamura, A., Suzuki, A., Fujino, M. & Kitada C. (1987) *Mol. Cell. Endocrinol.* **51,** 227–235.
7. Mizoguchi, A., Hatta, M., Sato, S., Nagasawa, H., Suzuki, A. & Ishizaki, H. (1990) *J. Insect Physiol.* **36,** 655–664.
8. Ishizaki, H. (1989) in *Ecdysone: From Chemistry to Mode of Action,* ed. Koolman, J. (Thieme, New York), pp. 204–210.
9. Iwami, M. (1995) in *Molecular Mechanisms of Insect Metamorphosis and Diapause*, eds. Suzuki, A., Kataoka, H. & Matsumoto, S. (Industrial Publishing and Consulting, Tokyo), pp. 65–74.
10. Nagata, K., Hatanaka, H., Kohda, D., Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H., Suzuki, A. & Inagaki, F. (1995) in *Molecular Mechanisms of Insect Metamorphosis and Diapause*, eds. Suzuki, A., Kataoka, H. & Matsumoto, S. (Industrial Publishing and Consulting, Tokyo), pp. 87–96.
11. Nagata, K., Maruyama, K., Nagasawa, H., Urushibata, I., Isogai, A., Ishizaki, H. & Suzuki, A. (1992) *Peptides* **13,** 653–662.
12. Satake, S., Masumura, M., Ishizaki, H., Nagata, K., Kataoka, H., Suzuki, A. & Mizoguchi, A. (1997) *Comp. Biochem. Physiol.* **118,** 349–357.
13. Masumura, M., Satake, S., Saegusa, H. & Mizoguchi, A. (2000) *Gen. Comp. Endocrinol.* **118,** 393–399.
14. Iwami, M., Furuya, I. & Kataoka, H. (1996) *Insect Biochem. Mol. Biol.* **26,** 25–32.
15. Wolfgang, W. J. & Riddiford, L. M. (1986) *Dev. Biol.* **113,** 305–316.
16. Kawasaki, H. (1988) *Special Bull. Coll. Agric. Utsunomiya Univ.* **63,** 1–4.
17. Champlin, D. T. & Truman, J. W. (2000) *Development (Cambridge, U.K.)* **127,** 3543–3551.
18. Lynn, D. E., Miller, S. G. & Oberlander, H. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2589–2593.
19. Miner, A. L., Rosenberg, A. J. & Nijhout, H. F. (2000) *J. Insect Physiol.* **46,** 251–258.
20. Kawamura, K., Shibata, T., Saget, O., Peel, D. & Bryant, P. J. (1999) *Development (Cambridge, U.K.)* **126,** 211–219.
21. Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R. & Hafen, E. (2001) *Curr. Biol.* **11,** 213–221.
22. Tanaka, M., Kataoka, H., Nagata, K., Nagasawa, H. & Suzuki, A. (1995) *Reg. Pept.* **57,** 311–318.
23. Kawakami, A., Iwami, M. N., Nagasawa, H., Suzuki, A. & Ishizaki, H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6843–6847.
24. Kondo, H., Ino, M., Suzuki, A., Ishizaki, H. & Iwami, M. (1996) *J. Mol. Biol.* **259,** 926–937.
25. Maruyama, K., Hietter, H., Nagasawa, H., Isogai, A., Tamura, S., Suzuki, A. & Ishizaki, H. (1988) *Agric. Biol. Chem.* **52,** 3035–3041.
26. Nogueira, B. V., Muehleisen, D. P., Whisenton, L. R., Gray, R. S. & Bollenbacher, W. S. (1997) *J. Insect Physiol.* **43,** 47–53.
27. Bryant, P. J. (2001) in *Novartis Foundation Symposium*, eds. Bock, G., Cardew, G. & Goode, J. (Wiley, New York) Vol. 237, pp. 182–199.
28. Davis, K. T. & Shearn, A. (1977) *Science* **196,** 438–440.
29. Kremen, C. & Nijhout, H. F. (1998) *J. Insect Physiol.* **44,** 287–296.
30. Edgar, B. A. (1999) *Nat. Cell Biol.* **1,** E191–E193.
31. Weinkove, D. & Leevers, S. J. (2000) *Genet. Dev.* **10,** 75–80.
32. Oldham, S., Böhni, R., Stocker, H., Brogiolo, W. & Hafen, E. (2000) *Philos. Trans. R. Soc. London B* **355,** 945–952.
33. Leevers, S. J. (2001) *Curr. Biol.* **11,** R209–R212.
34. Johnston, L. A. & Gallant, P. (2002) *BioEssays* **24,** 54–64.
35. Ikeya, T., Galic, M., Belawat, P., Nairz, K. & Hafen, E. (2002) *Curr. Biol.* **12,** 1293–1300.
36. Britton, J. S., Lockwood, W. K., Li, L., Cohen, S. M. & Edgar, B. A. (2002) *Dev. Cell* **2,** 239–249.
37. Britton, J. S. & Edgar, B. A. (1998) *Development (Cambridge, U.K.)* **125,** 2149–2158.
38. Hiruma, K., Hardie, J. & Riddiford, L. M. (1991) *Dev. Biol.* **144,** 369–378.
39. Rulifson, E. J., Kim, S. K. & Nusse, R. (2002) *Science* **296,** 1118–1120.
40. Dahlman, D. L. (1973) *Ann. Entomol. Soc. Am.* **66,** 1023–1029.