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Abstract

BACKGROUND: The insulin/IGF-1 signaling pathway controls cellular and organismal growth in many multicellular organisms. In Drosophila, genetic defects in components of the insulin signaling pathway produce small flies that are delayed in development and possess fewer and smaller cells as well as female sterility, reminiscent of the phenotypes of starved flies. RESULTS: Here we establish a causal link between nutrient availability and insulin-dependent growth. We show that in addition to the Drosophila insulin-like peptide 2 (dilp2) gene, overexpression of dilp1 and dilp3-7 is sufficient to promote growth. Three of the dilp genes are expressed in seven median neurosecretory cells (m-NSCs) in the brain. These m-NSCs possess axon terminals in the larval endocrine gland and on the aorta, from which DILPs may be released into the circulatory system. Although expressed in the same cells, the expression of the three genes is controlled by unrelated cis-regulatory elements. The expression of two of the three genes is regulated by nutrient availability. Genetic ablation of these neurosecretory cells mimics the phenotype of starved or insulin signaling mutant flies. CONCLUSIONS: These results point to a conserved role of the neuroendocrine axis in growth control in multicellular organisms.
Nutrient-Dependent Expression of Insulin-like Peptides from Neuroendocrine Cells in the CNS Contributes to Growth Regulation in *Drosophila*

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Summary

Background: The insulin/IGF-1 signaling pathway controls cellular and organismal growth in many multicellular organisms. In *Drosophila*, genetic defects in components of the insulin signaling pathway produce small flies that are delayed in development and possess fewer and smaller cells as well as female sterility, reminiscent of the phenotypes of starved flies.

Results: Here we establish a causal link between nutrient availability and insulin-dependent growth. We show that in addition to the *Drosophila* insulin-like peptide 2 (*dilp2*) gene, overexpression of *dilp1* and *dilp3-7* is sufficient to promote growth. Three of the *dilp* genes are expressed in seven median neurosecretory cells (m-NSCs) in the brain. These m-NSCs possess axon terminals in the larval endocrine gland and on the aorta, from which DILPs may be released into the circulatory system. Although expressed in the same cells, the expression of the three genes is controlled by unrelated cis-regulatory elements. The expression of two of the three genes is regulated by nutrient availability. Genetic ablation of these neurosecretory cells mimics the phenotype of starved or insulin signaling mutant flies.

Conclusions: These results point to a conserved role of the neuroendocrine axis in growth control in multicellular organisms.

Introduction

Regulation of cell, organ, and body growth is controlled by intrinsic and extrinsic factors [1, 2]. The insulin/IGF signaling pathway plays a key role in the control of growth in vertebrates and invertebrates [3]. In mammals, the primary role of insulin and the insulin receptor is energy homeostasis by the regulation of blood glucose levels [4]. But mutations in the human insulin receptor gene also cause embryonic growth retardation [5, 6]. The primary growth regulatory function in mammals, however, is mediated by the IGF-1 and IGF-2 growth factors and the IGF-1 receptor [7]. In *Drosophila*, there is a single insulin-like receptor, and it regulates postembryonic growth, reproduction, and aging [8]. In vertebrates and invertebrates, intracellular signal transduction from the insulin/IGF receptors depends on insulin receptor substrate (IRS) proteins [9]. In mammals, loss of IRS1 function causes severe reduction in embryonic and postembryonic growth [10, 11]. Loss of IRS2 leads only to a moderate reduction in growth, but mice become hyperglycemic and contain increased body fat, and females are sterile [12]. In *Drosophila*, flies mutant for *chico*, which encodes the single *Drosophila* homolog of IRS1-4, are developmentally delayed, have a severely reduced body size and increased fat, and females are sterile [13]. This demonstrates a striking conservation of the role of the insulin/IGF signaling pathway during evolution.

Several lines of evidence suggest a link between the activity of the insulin/IGF signaling pathway and nutrient availability. The female sterility and the growth retardation phenotypes of IRS2 knock-out and IRS1 knock-out mice, respectively, are similar to those observed in starved mice [7, 14]. In *Drosophila*, growth retardation, reduced body weight due to fewer and smaller cells, and female sterility are phenotypes not only characteristic of *chico* mutant flies but also of flies that have been starved during development [13, 15]. In fact, oogenesis is blocked during stem cell divisions and before the onset of vitellogenesis in starved flies and in *chico* mutant flies [16]. Furthermore, starvation reduces the activity of the insulin signaling pathway in vivo [17]. In nematodes, starvation or mutations in the insulin signaling pathway arrest development at the so-called Dauer stage [18, 19]. Moreover, caloric restriction and mutations in the insulin/IGF1 signaling system extend lifespan in vertebrates and invertebrates [8]. It is not known, however, whether the link between nutrient availability and the activity of the insulin signaling pathway is direct, and how the nutritional status is translated into insulin/IGF receptor activity in the target tissues.

One obvious hypothesis for a connection between nutrient availability and insulin/IGF activity is that nutrients control the expression of the insulin/IGF growth factors. In mammals, blood glucose levels not only regulate the release of insulin from the pancreatic β cells, but also regulate the *insulin* gene expression via an autocrine loop [20]. The regulation of IGF-1 levels that control postnatal growth is more complex. Growth hormone synthesized by the pituitary controls IGF-1 expression in the liver, accounting for approximately one-third of the postnatal growth promoting activity. Growth also depends on GH-independent expression of IGF-1 and GH activity that is independent of IGF-1 [3, 7].

The role of insulin-like growth factors in invertebrates in the regulation of growth and their regulation in response to starvation is less well defined. In *Drosophila*, the search for insulin-like genes (*dilps*) in the genome revealed seven genes that show highly regulated temporal and spatial expression [21, 22, 23]. Ubiquitous overexpression of one of the *dilp* genes, *dilp2*, is sufficient to increase body size. This growth-promoting activity of DILP2 is dependent on the insulin receptor signaling pathway. Three *dilp* genes are expressed in small cell clusters in the central region of the brain [23]. Here we show that these three genes are expressed in the same median neurosecretory cells (m-NSCs) possessing axon...
The presence of seven dilp signaling pathway in the Drosophila genome raises the question of whether the different DILPs possess different functions. It is possible, for example, that some of the DILPs act as receptor antagonists, as has been postulated for some of the insulin-like peptides in C. elegans [24]. We tested whether DILP1 and DILP3-7, like DILP2, also promote growth when expressed ubiquitously throughout development. As shown in Figure 1, expression of each of the dilp genes under the control of the weak, ubiquitous armadillo-Gal4 (arm-Gal4) driver caused a statistically significant increase in body size. The largest increase in body size was observed by dilp2 overexpression resulting in a 51% weight gain in males (Figure 1A). Constitutive, high levels of expression of the dilp genes using an actin-Gal4 driver caused lethality in the case of dilp2 and slightly higher weight increases with the other dilp constructs (data not shown). Interestingly, expression of the human insulin gene either under heat shock or actin control did not cause a significant increase in body size or lethality (data not shown), despite its known growth-promoting effect in tissue culture [25]. It is possible that preproinsulin is not processed correctly in Drosophila and that insulin exerts its effect only when added as processed peptide. Our results indicate that all the DILPs possess the ability to promote growth and thus function as insulin receptor agonists. The identification of the reason behind the differences in growth promoting potency of various DILPs awaits further characterization of their biochemical properties and the analysis of specific loss-of-function mutations.

DILP2, 3, and 5 Are Expressed in the m-NSCs
The dilp2, 3, and 5 genes are expressed in bilaterally paired clusters of cells in the median region of the larval brain [23]. To test whether these three genes are expressed in the same group of cells, we performed double in situ hybridization. As shown in Figures 2A–2C, expression of dilp2, 3, and 5 genes was indeed detected in the same two clusters of cells. The position of these cell clusters in the pars intercerebralis of the larval brain suggests that they correspond to the m-NSC clusters that stain with an anti-Bombyxin antibody [26]. Indeed, the expression of GFP under the control of the dilp2 cis-regulatory elements (see below) permits the visualization of the axonal projections of the dilp expressing cells (Figures 2D–2F). Axon projections are seen in the corpora cardiaca of the ring gland and on the aorta, the site from which neuropeptides are released into the hemolymph. Therefore, dilp2, 3, and 5 are coexpressed in the cluster of m-NSCs identified in larger insects and in Drosophila. Although expressed in the same cells, the temporal expression pattern of dilp 2, 3, and 5 in the m-NSCs differs (Figures 2G–2L). While dilp2 is expressed already in the first instar stage (data not shown), dilp2 and dilp5 expression is detectable in the second instar stage, while dilp3 expression starts at the mid to late third instar stage. The successive activation of dilp genes in the m-NSCs correlates with the increasing growth that occurs during the last larval instar.

The Expression of dilp2, 3, and 5 in the m-NSCs Is Independently Regulated
The dilp2, 3, and 5 genes are located together with dilp1 and dilp4 in a gene cluster spanning 26 kb on the third chromosome. To search for enhancer elements controlling the expression of the three genes in the m-NSCs, we constructed a series of lacZ reporter genes containing various amounts of upstream genomic sequences (Figure 3). Upstream fragments of 1.0 kb, 1.7 kb, and 450 bp derived from the dilp2, 3, and 5 genes, respectively, were sufficient to drive reporter gene expression specifically in the m-NSC. Thus, each of the three genes possesses its own m-NSC-specific enhancer. A further dissection of these upstream sequences revealed that a 394 bp fragment located at position –540 to –146 of dilp2 is sufficient to recapitulate the expression of dilp2 in the m-NSCs. This construct, however, is no longer expressed in imaginal discs, suggesting that different enhancer elements control expression in this tissue. The cis-regulatory elements that control dilp3 expression are more complex. The m-NSC-specific expression depends on two separate elements located between –763 to –1167 and between +1 to –165, including the putative transcription start site. The fragment located between

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**Figure 1. Constitutive Ubiquitous Overexpression of dilp1–7 Is Sufficient to Increase Body Size**

The weights of male (A) and female (B) control (arm-Gal4) and arm-Gal4; UAS-dilpx flies are shown. Overexpression of each of the dilp genes leads to a statistically significant increase (P < 0.002) in body weight.
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**Figure 3. Promoter Analysis of dilp2, 3, and 5 Genes**

(A) Schematic representation of the genomic region containing dilp1-5 in the 67C1-2 chromosomal region [23]. The black horizontal bars represent the largest fragments used in the promoter dissection shown in (B).

(B) Enlargement of the upstream region of dilp2, dilp3, and dilp5. The horizontal bars represent the upstream regions included in the different reporter gene constructs. The line on the right indicates the relative strength of lacZ expression in the m-NSCs. In the case of dilp3, the expression in the gut muscle is also indicated. For a more detailed description of the expression pattern, see the Results section. For the precise extent of the fragments, see Experimental Procedures.

sequences required for m-NSC expression of dilp2, 3, and 5 did not reveal obvious stretches of similarity that may identify a common m-NSC-specific enhancer element in the different genes. It therefore appears that even within this small group of cells, the three dilp genes are regulated by different combinations of transcription factors.

**The Expression of dilp3 and 5, but Not dilp2, Is Regulated by Nutrient Availability**

A causal link between starvation-induced reduction in growth and reduced insulin receptor activity may involve the regulation of circulating DILP levels by nutrient availability. To test this hypothesis, we examined the expression of dilp2, 3, and 5 in third instar larvae that have been starved for 24 hr. In nonstarved larvae, we detected dilp2, 3, and 5 mRNA transcripts of dilp2, 3, and dilp5 in the m-NSCs. In late third instar larvae, dilp2 (J), dilp3 (K), and dilp5 (L) are expressed.

Surprisingly, sequence comparison of the genomic
part of the nutrient-dependent regulation of growth is mediated by the regulated expression of the dilp3 and dilp5 genes.

Targeted Ablation of m-NSCs during Late Third Instar Stage Mimics the Phenotype of Reduced Insulin Signaling Pathway Function

During larval development, the seven dilp genes are expressed in a variety of different tissues in addition to the m-NSCs [23]. To begin to address the role of DILP production in the m-NSCs, we wanted to specifically ablate these cells. We used our dilp2-Gal4 line, which is exclusively expressed in the m-NSCs starting at the late third instar stage (Figure 2D and data not shown), to drive expression of the proapoptotic gene reaper (rpr) in these cells [27]. dilp2>rpr flies are viable but eclose one day later than control flies. Freshly eclosed flies show a slight but significant reduction in body weight (Figures 5A and 5B). The size difference is also observed in the reduced wing area (Figure 5C). Interestingly, the largest difference between control and dilp2>rpr flies was observed in the size of the abdomen of females. This difference becomes further enhanced during the first three days of adult life. During this phase, egg production is stimulated by feeding and mating in wild-type females. Comparison of the ovaries of three-day-old wild-type and dilp2>rpr females revealed a striking difference in ovary size. Each ovary is composed of approximately 15 ovarioles [28]. Ovarioles are oocyte tubes containing stem cells at the tip and oocytes of increasing maturity toward the oviduct. While wild-type females possess multiple vitellogenic oocytes in each ovariole, dilp2>rpr females possess at most a single vitellogenic oocyte. This reduced size of the ovary of dilp2>rpr flies is reflected in the reduced fecundity of these females. While control flies lay on average 60 eggs per day, dilp2>rpr females produce only 10 eggs per day (Figure 5F). The dilp2>rpr flies exhibit a developmental delay, reduced body size, and reduced fecundity of females owing to a partial block in production of vitellogenic oocytes. These phenotypes resemble those of weak mutations in the genes coding for components of the insulin signaling pathway. While chico females are almost completely sterile and severely reduced in size, certain combinations of Inr alleles produce females very similar to the dilp2>rpr flies [23, 29]. It is possible that the partially penetrant phenotype of m-NSC ablation is due to the late onset of expression of the dilp2-Gal4 driver line, resulting in only a partial ablation of the m-NSCs. Since we also detect dilp5 expression in the follicle cells of the female ovary (Figures 5G and 5H), this m-NSC independent source of DILP may provide an alternative explanation for the partially penetrant phenotype of m-NSC ablation.
Figure 5. Ablation of m-NSCs Reduces Growth and Female Fertility

(A) A control (215-3 dilp2Gal4/UAS-GFP, left) and an m-NSC ablated female (215-3 dilp2Gal4/UAS-rpr, right). Note the reduction in overall body size and wing size, and the substantially reduced abdomen.

(B) The weight of m-NSC ablated male and female flies is reduced compared to the weight of dilp2Gal4/UAS-GFP control flies.

(C) The wing area of m-NSC ablated males is reduced compared to control flies. The reduction is due to a reduction in cell number and cell size (data not shown).

(D) Ovary of a three-day-old 215-55 dilp2Gal4/UAS-GFP virgin female. Each ovariole possesses mature eggs and vitellogenic oocytes (arrow).

(E) Ovary of a three-day-old 215-55 dilp2Gal4/UAS-rpr virgin female possesses mature eggs and immature and vitellogenically arrested oocytes (arrow). Arrested oocytes are at stage 9-10.

(F) The fecundity of dilp2Gal4/UAS-rpr flies is dramatically reduced. Dilp2Gal4/UAS-GFP females lay approximately 60 eggs/day, while dilp2Gal4/UAS-rpr females lay on average 10 eggs/day.

(G and H) Localization of dilp5 mRNA transcripts in the follicle cells on the wild-type adult ovary. (G) Control sense probe revealing unspecific staining in the nurse cells. (H) dilp5 anti-sense probe reveals dilp5 transcripts in the follicle cells of stage 10 oocytes.

Discussion

Nutrient-dependent regulation of growth and reproduction is observed in all multicellular organisms. The results presented here provide further support for an evolutionary conserved signaling pathway involved in this regulation. We show that expression of each of the seven dilp genes in Drosophila is sufficient to promote growth, and that their different function is primarily associated with their different spatial and temporal regulation of expression.

In mammals, insulin secretion from the pancreatic β cells is regulated by the concentration of glucose in the blood, and thereby regulates energy homeostasis in response to food intake [4]. Embryonic and postembryonic growth is regulated by IGF-1 production that in part depends on GH synthesized from the pituitary [3, 7]. In insects, the m-NSCs appear to play a role in both functions. The release of insulin-like peptides from the corpora cardiaca in Bombyx is regulated by carbohydrate levels in the hemolymph [30] in a way analogous to the release of glucose from pancreatic β cells in mammals. We have shown that the expression of dilp3 and dilp5 is repressed by food withdrawal. Furthermore, ablation of the m-NSCs results in growth retardation. These results are consistent with the recent study of Rulifson et al. (2002) showing that early Reaper-induced ablation of the m-NSC cells using a multimerized dilp2-Gal4 construct severely reduced growth and led to a concomitant increase in glucose and trehalose levels in the hemolymph of these animals [31]. Given the data from Bombyx and Drosophila together, we suggest that the m-NSCs possess functions similar to those of the pancreas and the pituitary in mammals.

In insects, the growth regulatory function of DILPs is 2-fold. First, circulating levels of DILPs in the hemolymph activate growth in the target tissues by the activation of the insulin receptor PI3K pathway in each cell [2, 32].
This action is complemented by the local production of DILPs in the target tissues in a manner similar to the expression of IGF-1 in target tissues. Second, DILPs exert their effect on growth indirectly. The m-NSCs project their axon terminals into the ring gland where ecdysone and JH are synthesized. The stimulation of ecdysone synthesis by insulin-like peptides is well documented in many insects [33, 34, 35]. Furthermore, JH levels are reduced in long-lived insulin receptor mutant flies, suggesting that DILPs also regulate JH synthesis [36]. Through the regulation of the levels of one or both of these two hormones, DILPs may regulate growth indirectly. Under starvation conditions, reduced JH levels may result in the premature increase in ecdysone titer in third instar larvae, leading to the precocious initiation of metamorphosis and thus producing flies with fewer cells. Alternatively, a precocious rise in ecdysone titer may be caused by the increase in the local concentration of DILPs in the ring gland due to the increased retention of insulin-like peptides in the corpora cardiaca during starvation, as observed in Bombyx [30].

In nematodes, nutrient availability regulates the developmental program and fertility without having a direct effect on cell size or cell number. In the absence of food, the larvae enter the immature long-lived Dauer stage [37]. This response is controlled by two pathways, the insulin signaling pathway and the daf-4/TGF-β pathway [19]. Each of these pathways acts nonautonomously in the nervous system, and they converge on the nuclear hormone receptor daf-12 [38]. This implies an intermediate steroid or lipid hormone signal. Indeed, daf-9 that acts genetically between daf-2 and daf-4 signaling encodes a cytochrome P450 enzyme involved in steroid and fatty acid metabolism [39]. It is interesting to note that the two activities of the Prothoracicotropic hormone (PTTH) that regulates ecdysone synthesis in Bombyx involve a member of the TGF-β superfamily and an insulin-related peptide synthesized in distinct sets of neurosecretory cells in the brain [40]. Therefore, it is likely that the nutrient-dependent growth regulation in nematodes and Drosophila is conserved in spite of the absence of an autonomous requirement of insulin signaling in cell growth in C. elegans.

Egg maturation is blocked by starvation in many species. In insects, ecdysone produced by the ovary is required for yolk protein production in the fat body and oocyte maturation [41, 42]. Ecdysone production is stimulated by insulin-like peptides in vitro and in vivo [40]. Ablation of m-NSCs significantly slows down oogenesis. In humans, brain-specific knockouts of the insulin receptor or IRS2 also block oocyte maturation by affecting the synthesis of gonadotropins [43]. Furthermore, steroidogenesis in the female gonad is required for oocyte maturation and is regulated by the expression of IGF-1 in different gonadal cells [44]. Interestingly, we also detect expression of dilp5 in the ovarian follicle cells in Drosophila. Local production of DILP5 may stimulate ecdysone production in the female ovary directly. The similar roles of insulin-related peptides in growth regulation, energy homeostasis, and oogenesis in nematodes, insects, and mammals are striking. How far the underlying mechanisms are also conserved remains to be investigated.

Experimental Procedures

In Situ Hybridization

RNA in situ hybridization using DIG-labeled probes was performed essentially as described [23]. Probes were derived from genomic DNA encompassing the coding regions of dilp2, 3, 5, and 7, respectively. Two-probe in situ hybridizations using DIG-labeled and Fluorescein-labeled probes were done as described [45].

Recombinant DNA Construction

UAS constructs of dilp1 and dilp3-7 were prepared by inserting PCR-amplified genomic DNA encompassing the coding regions (primer sequences are available by request) of each of the dilp genes into the pUAST vector. For human insulin, a cDNA containing the entire coding region (NM_000207) was used. Several independent transgenic lines were obtained for each construct.

For the dilp promoter analysis, we amplified the 5’ genomic regions of dilp2, 3, and 5 by PCR and cloned the resulting fragments into BlueScript II or pCRII-Topo (Invitrogen). These promoter fragments are inserted into the pX72 transformation vector, which contains a nuclear LacZ reporter [46]. Position and length of the fragments used with respect to the putative transcription start site: pXD215-1 (–540 to +1); pXD215-2 (–146 to +1); pXD215-3 (–983 to –146); pXD215-4 (–983 to –540); pXD215-5 (–540 to –146); pXD311-1 (–1167 to +1); pXD311-2 (–763 to –165); pXD311-3 (–1688 to –165); pXD311-5 (–1688 to –763); pXD311-6 (–1688 to –1167); pXD311-7 (–1167 to –165); pXD311-9 (–763 to –165); pXD311-10 (–1101 to +1); pXD311-11 (–1167 to –763; –165 to +1); pXD51XB (450 bp fragment including dilp5 TATA box).

Germline transformation and X-Gal staining was performed as previously described [47]. 215-5dilp2-Gal4 has a tandem duplication of the enhancer fragment used in the 215-5 construct (–540 to –146) fused to the –63 to +62 fragment. 215-3dilp2-Gal4 contains the enhancer fragment used in the 215-3 construct. Both dilp2-Gal4 lines express Gal4 exclusively in the m-NSC and in six cells of lateral ventral ganglion. The UAS-rpr.C line [48] was obtained from P. Galant.

Weight Determination and Starvation Protocols

Embryos from crosses of UAS-dilp1-7, UAS-hin5, and hs-Gal4 flies were collected for 24 hr and then subjected to 1 hr heat shock at 37°C every 12 hr until eclosion in a temperature- and humidity-controlled incubator. Crosses with flies carrying the actin-Gal4 or the armadillo-Gal4 driver were carried out at 25°C without temperature shifts. Only tubes containing 30–50 progeny were used for the analysis to avoid overcrowding. 14–20 flies were collected after eclosion and weighed 24 hr later on a precision balance (Mettler Toledo MX5). Statistical analysis was performed after eliminating the 10% highest and lowest values. The mean weights and the standard deviations were determined, and statistical significance was determined with a two-sided t test.

For the starvation experiments, mid third instar larvae (72 hr AEL) were collected and incubated for 24 hr at 25°C in an empty glass vial covered with a water-soaked foam stopper. Under this condition, all larvae crawled on the foam stopper of the inverted tube. Larval development proceeded; however, the size of imaginal discs was smaller than that of the normally fed controls. After starvation, larvae were immediately dissected and fixed for further analysis.

Ablation Experiments

For the m-NSC ablation experiment, four UAS-rpr females were crossed with one dilp2-Gal4 male. Offspring females from non-crowded tubes were reared for 3 days. Body weight was measured as described above. Fecundity was measured by counting pupae from single pair matings of dilp2-rpr females with y w males.

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