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Short Article

Drosophila ALS Regulates Growth and Metabolism through Functional Interaction with Insulin-Like Peptides

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SUMMARY

In metazoans, factors of the insulin family control growth, metabolism, longevity, and fertility in response to environmental cues. In *Drosophila*, a family of seven insulin-like peptides, called Dilps, activate a common insulin receptor. Some Dilp peptides carry both metabolic and growth functions, raising the possibility that various binding partners specify their functions. Here we identify dALS, the fly ortholog of the vertebrate insulin-like growth factor (IGF)-binding protein acid-labile subunit (ALS), as a Dilp partner that forms a circulating trimeric complex with one molecule of Dilp and one molecule of Imp-L2, an IgG-family molecule distantly related to mammalian IGF-binding proteins (IGFBPs). We further show that *dALS* antagonizes Dilp function to control animal growth as well as carbohydrate and fat metabolism. These results lead us to propose an evolutionary perspective in which ALS function appeared prior to the separation between metabolic and growth effects that are associated with vertebrate insulin and IGFs.

INTRODUCTION

Members of the insulin-like peptide (ILP) family are found in a wide range of metazoans, where they control carbohydrate metabolism, tissue growth, reproduction, and longevity. The functional separation between insulin-like growth factor (IGF) and insulin signaling, as seen in mammals, dates back 600 million years, as the two types of molecules are already present in the lower metazoan tunicate phylum (Sherwood et al., 2006). Insulin and IGF-1 carry different biological functions, in part through their binding to closely related receptors, the insulin receptor (IR) and the IGF-1 receptor (IGF-1R), respectively. In contrast to insulin, which is produced and stored in specific endocrine tissues and is released by a highly regulated process, vertebrate IGF-1 accumulates in body fluids, where it associates with a large array of binding molecules globally referred to as IGF-binding proteins (IGFBPs). These play important though cryptic functions in controlling the biological activity of IGF-1 (Rosenfeld et al., 2000; Duan and Xu, 2005). Vertebrates have six bona fide IGFBPs, which directly bind IGF-1 and form stable binary complexes in the circulating blood. These binary complexes comprise 10% of the total plasma IGF-1. Most of the remaining plasma IGF-1 is bound up into ternary complexes comprising one molecule of IGFBP-3 or IGFBP-5 and one molecule of acid-labile subunit (ALS), leaving only 1% of plasma IGF-1 free (Boisclair et al., 2001). The binding of IGF partners greatly enhances the half-life of IGF-1 but also restrains its ability to interact with its receptor, IGF-1R, leading to the formation of a large reservoir of circulating IGF-1. The ALS partner is essential for the stabilization of circulating IGF-1, as deficiencies in the *ALS* gene in both mouse and human lead to a drastic reduction in plasma levels of IGF-1 and IGFBP-3 (Domene et al., 2005, 2007). Ternary IGF-1 complexes have been proposed to contribute to the functional separation between IGF-1 and insulin by preventing illegitimate interaction between high blood concentrations of IGF-1 and the insulin receptor. While in vivo studies of IGFBP and ALS in mammals have revealed some key functions of these molecules in controlling the physiology of IGF-1, a full understanding of how these regulations take place in complex organisms is still lacking (Duan and Xu, 2005; Domene et al., 2005).

Insects provide a simpler evolutionary alternative, possessing a single insulin-like system that represents a possible ancestor of the dual insulin/IGF system (Wu and Brown, 2006). In *Drosophila*, seven insulin-like peptide (Dilp)-encoding genes have been identified. These interact genetically with a unique insulin receptor called dInR. The *dilp* genes are expressed in different larval tissues, suggesting that they carry specific functions. At least three of them (*dilp2*, *3*, and *5*) are expressed in two symmetric clusters of seven neurosecretory cells called the insulin-producing cells (IPCs), located in each brain hemisphere. Ablation of these neurons leads to dramatically smaller body size with increased levels of circulating carbohydrate (trehalose in insects) in the hemolymph, indicating that the Dilp peptides produced in brain IPCs control both carbohydrate homeostasis and tissue growth (Brogiolo et al., 2001; Rulifson et al., 2002). Interestingly, growth and trehalose level defects are rescued by ectopic expression of one Dilp, Dilp2, suggesting that at least this molecule carries both metabolic and growth functions (Rulifson et al., 2002). However, individual genetic analysis of *dilp* genes, which should yield

important information toward the resolution of Dilp functions, has not yet been performed.

In the current study, we tested the possibility that specific complexes between the Dilps and binding partners may restrict their interactions with dInR, thereby specifying their functions. Both a putative ALS ortholog (dALS; Colombani et al., 2003) and a possible candidate for an ILP-binding protein (encoded by the *Imaginal morphogenesis protein-Late 2* [*Imp-L2*] gene; Garbe et al., 1993) have been identified in *Drosophila*. The Imp-L2 protein is a member of the immunoglobulin superfamily, which shares slight homology with the mammalian IGF-binding protein-related protein 1 (IGFBP-rP1/IGFBP-7; Yamanaka et al., 1997). It can bind human IGF-1 in vitro (Sloth Andersen et al., 2000) and functions as a growth inhibitor in *Drosophila* (B.H., H. Stocker, and E. Hafen, unpublished data). We now present evidence that Dilp2 forms a trimeric complex with the *Drosophila* ALS and Imp-L2 proteins. We further demonstrate that dALS is required for the growth and metabolic functions of the Dilps. This indicates that the formation of a trimeric complex containing dALS and some Dilp peptides is a prerequisite for both metabolic and growth control in flies.

RESULTS

We previously identified *CG8561* as a candidate gene encoding a putative *Drosophila* ortholog of the vertebrate ALS protein, which we called *dALS* (Colombani et al., 2003). The dALS protein contains a series of 21 leucine-rich repeats (LRRs) that also form the core of the vertebrate ALS. Based on sequence similarity and the presence of LRRs, two additional related sequences were found in the *Drosophila* genome (see Table S1 available online). We examined the expression levels of all three genes in larval tissues and in normally fed or starved animals. We had previously established that *CG8561* is exclusively expressed in two larval tissues that play important roles in growth and metabolic regulation: the 14 IPCs in the brain, and the fat body (FB), a larval tissue that shares some functions with the vertebrate liver and fat (Colombani et al., 2003). Remarkably, *dALS* expression in the FB is suppressed under amino acid restriction (as determined by qPCR on *ppl>Slif*^{A} larvae; see below), a finding reminiscent of the strong downregulation of the vertebrate *ALS* gene observed in the liver under starvation (Colombani et al., 2003). The two other related genes did not show clear expression in any of the larval tissues, nor did they show nutrition-regulated expression. We therefore focused our analysis on *CG8561* (Table S1).

dALS, Dilp2, and Imp-L2 Are Found in a Common Protein Complex

We first addressed whether dALS is able to form stable complexes with *Drosophila* insulin-like peptides. In mammals, IGF-1 binds to a variety of IGFBPs, which serve as adaptor molecules for the binding of ALS and the formation of a stable trimeric complex. Therefore, we hypothesized that Imp-L2, a possible *Drosophila* ILP-binding protein (Garbe et al., 1993; Sloth Andersen et al., 2000), might be required for the interaction between Dilps and dALS. To test this possibility, S2 cells were transfected with various combinations of tagged Dilp2-, dALS-, and Imp-L2-expressing constructs. dALS-myc (dALS^{M}) was immunoprecipitated from cell lysates, and the immunoprecipitates were tested for the presence of Dilp2 and/or Imp-L2 by western blotting. We observed that dALS and Imp-L2 physically interact in the absence of Dilp2 but that dALS and Dilp2 do not interact directly. However, coexpressing Imp-L2 together with dALS and Dilp2 allowed coprecipitation of Dilp2 with dALS (Figure 1A). These results indicate that the three proteins form a trimeric protein complex and that Imp-L2 serves as a molecular bridge between Dilp2 and dALS. Dilp2 and Imp-L2 did not coprecipitate in control experiments in which dALS was replaced with the unrelated protein eIF4E, indicating that the interaction is specific for dALS (Figure 1B). Interactions between Dilp3 and Dilp5, two other Dilps expressed in the IPCs, and dALS were also tested using the same experimental protocol. A specific coprecipitation of dALS and Dilp5 was observed in the absence of Imp-L2. Interestingly, the coexpression of Imp-L2 suppressed the interaction, suggesting that dALS and Dilp5 interact through a distinct Dilp-binding protein (Dilp-BP) present in S2 cells that is outcompeted by Imp-L2 for binding to dALS (Figure 1C). Under the same conditions, no interaction was observed between dALS, Dilp3, and Imp-L2 (data not shown).

The physical interaction between dALS and Dilp2 was further confirmed in vivo. Expression of the tagged protein dALS^{M} in the FB leads to its accumulation in vesicular structures suggestive of the secretory pathway (Figure 2A), as well as in the larval hemolymph, indicating that it behaves like a lymph-circulating secreted protein (Figure 2B). We tested the presence of circulating complexes containing dALS and Dilp2 in coprecipitation experiments using larval hemolymph. The presence of Dilp2 in the dALS immunoprecipitate and, conversely, the presence of dALS in the Dilp2 immunoprecipitate were indeed revealed by western blotting (Figure 2C). Therefore, dALS can form circulating complexes with several Dilps and Dilp-BPs, including the product of the *Imp-L2* gene.

dALS Is Required for Proper Growth Regulation

To analyze the role of dALS in the control of Dilp function in greater detail, we investigated the effects of tissue-specific loss or gain of function of *dALS* on larval growth and metabolism. For this purpose, we utilized *UAS*-driven lines allowing Gal4-induced tissue-specific expression of normal (dALS) or myc-tagged (dALS^{M}) dALS protein, as well as *dALS* RNAi (*dALSi*) to silence *dALS* expression. Gal4 lines that either partially or totally recapitulate the expression pattern of the *dALS* gene were used: *pumpless-Gal4* (*ppl-Gal4*), which expresses Gal4 in the larval FB, *dilp2-Gal4*, which expresses Gal4 specifically in the brain IPCs, and a recombinant line that expresses *Gal4* in both tissues. Expressing a *dALSi* construct with *ppl-Gal4* lowered the total larval *dALS* mRNA level by 45% (Figure 3A). Interestingly, altered *dALS* expression levels in the FB of growing larvae led to changes in adult size: *dALS* overexpression reduced adult male mass by 14%, whereas *dALS* silencing increased the mass by 12% (Figure 3B). Under these conditions, no delay in larval development was observed, but the larval growth rate was accelerated in RNAi-expressing lines and reduced in *dALS*-expressing lines (Figure 3C).

Fat body cells can be efficiently used to produce and secrete large amounts of proteins in the hemolymph. To functionally test the interaction between dALS and Dilps in vivo, we used ectopic

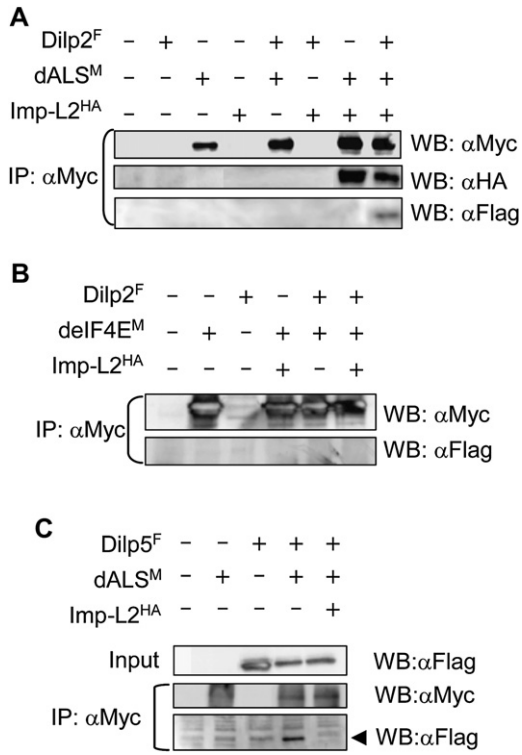

Figure 1. Physical Interaction between dALS and Dilp2 In Vitro and In Vivo

(A) Coimmunoprecipitation of dALS, Imp-L2, and Dilp2 proteins from total *Drosophila* S2 cell extracts. Myc-tagged dALS was precipitated following single, double, or triple transfections, and the immunoprecipitates were analyzed by western blotting (WB) with anti-FLAG ($Dilp2^F$), anti-Myc ($dALS^M$), or anti-HA ($Imp\text{-}L2^{HA}$) antibodies.

(B) Absence of coimmunoprecipitation of Dilp2 with a control protein (delF4E). Myc-tagged delF4E was precipitated following single, double, or triple transfections, and the immunoprecipitates were analyzed by WB with anti-FLAG ($Dilp2^F$) or anti-Myc ($delF4E^M$) antibodies.

(C) Coimmunoprecipitation of dALS and Dilp5 from total *Drosophila* S2 cells extracts. Myc-tagged dALS was precipitated following single, double, or triple transfections, and the immunoprecipitates were analyzed by WB with anti-FLAG ($Dilp5^F$) or anti-Myc ($dALS^M$) antibodies. Black arrowhead indicates the FLAG-Dilp5 band.

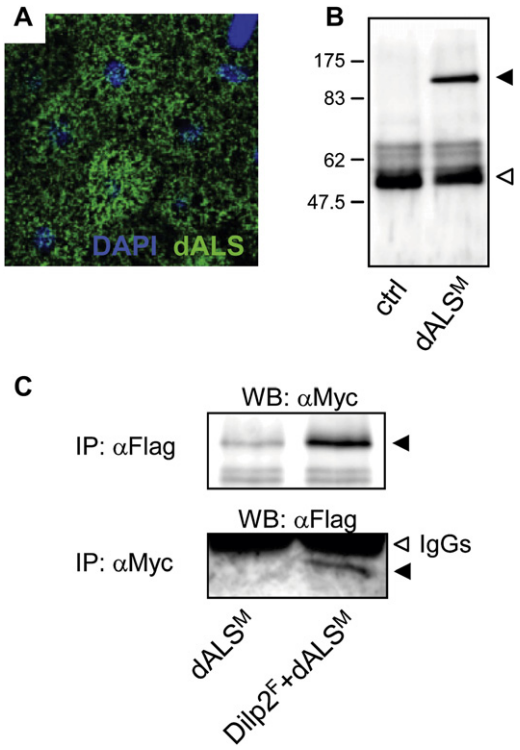

Figure 2. dALS Is a Secreted Protein that Interacts with Circulating Dilp2

(A) When overexpressed in the fat body with the *ppl-Gal4* driver (*ppl>*), myc-tagged dALS ($dALS^M$) appears in small punctate structures in the cytoplasm of fat cells that are characteristic of vesicular structures.

(B) The $dALS^M$ protein produced in the fat body is found in the larval hemolymph. Immunoprecipitates and western blotting of $dALS^M$ protein from the hemolymph of *ppl>dALS*^{M} larvae are shown. Black arrowhead, $dALS^M$; white arrowhead, background bands.

(C) Coimmunoprecipitation of dALS and Dilp2 from third-instar larval hemolymph extracts. $dALS^M$ without or with FLAG-tagged Dilp2 ($Dilp2^F$) was expressed in the larval fat body using the *ppl-Gal4* driver. Tagged proteins were immunoprecipitated from larval hemolymph, and the precipitates were analyzed by western blotting (WB) to detect either $dALS^M$ or $Dilp2^F$ (black arrowhead). Mouse anti-Myc IgG light chains revealed by anti-mouse secondary antibodies are indicated by white arrowhead.

expression of a FLAG-tagged version of Dilp2 ($Dilp2^F$) together with dALS in the FB. Although Dilp2 is normally not expressed in FB cells, ectopic expression of $Dilp2^F$ in this tissue leads to a systemic increase in adult mass, suggesting that these cells are capable of producing and secreting functional $Dilp2^F$ (Figure 3B; see also Supplemental Experimental Procedures). Interestingly, coexpression of dALS and $Dilp2^F$ in FB cells significantly reduced the adult mass increase observed upon $Dilp2^F$ expression alone, indicating that dALS counteracts the effect of Dilp2 on the control of animal growth (Figure 3B). Neither overexpressing nor silencing *dALS* in the brain IPCs had any effect on growth (Figure S1A). In accordance with these results, *dALS* or *dALSi* expression in both the FB and the IPCs resulted in growth phenotypes indistinguishable from FB expression alone (data not shown). Together, these data suggest that dALS acts as a negative regulator of the larval growth rate by counteracting Dilp function and that dALS function is required in the FB. No clear function associated with *dALS* expression in the IPCs could be identified by our analysis.

dALS Is Required for Proper Regulation of Energy Homeostasis

Our experiments also revealed that dALS antagonizes the function of fly Dilps in the control of carbohydrate and fat metabolism. The disaccharide trehalose is the main circulating carbohydrate in the insect hemolymph. The control of trehalose levels by the Dilps and the glucagon-like adipokinetic hormone (AKH) shows striking parallels to the control of glycemia in vertebrates (Broughton et al., 2005; Kim and Rulifson, 2004; Rulifson et al., 2002). In measuring total trehalose in larval hemolymph, we observed a 25% increase in animals expressing *dALS* in the FB and a 21% reduction in animals with FB-specific *dALS* silencing (Figure 4A). Additionally, FB-specific expression of *dALS* reduced larval di- and triacylglycerol (DAG+TAG) levels,

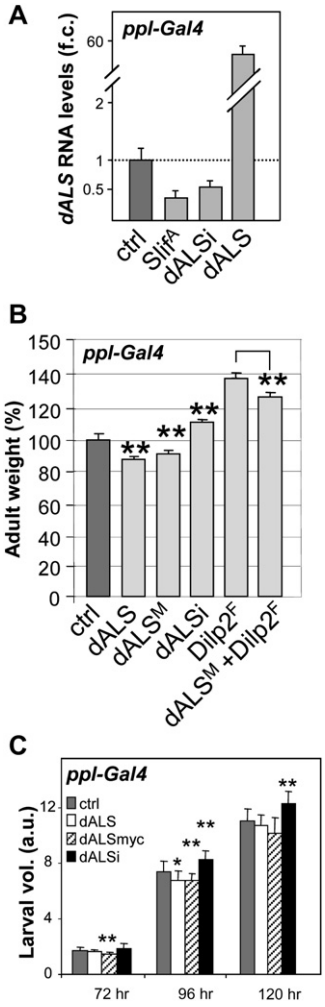

Figure 3. dALS Is a Secreted Protein that Controls Growth Nonautonomously

For all experiments, the *ppl-Gal4* driver was used to direct expression in the larval fat body.

(A) Measurements of larval *dALS* transcripts by quantitative RT-PCR. *dALS* overexpression (*dALS*) and silencing (*dALSi*) conditions are shown relative to control (all crosses at 29°C, except *ppl>Slif*^{A} at 25°C). Fold changes (f.c.) are indicated, and *Act-5C* is used as an internal reference. *dALSi* does not modify transcription of two closely related genes (*CG5195* and *CG7896*), and no clear off-targets were detected using computer analysis, validating this construct for *dALS* loss of function. *ppl>Slif*^{A} larvae present limited amino acid import in their fat body, mimicking a state of food deprivation (Colombani et al., 2003).

(B) Adult weight measurements in conditions of modified *dALS* expression. Varying *dALS* expression in the larval fat body modifies growth: *dALS* overexpression (*dALS* and *dALS*^{M}) decreases adult mass, whereas *dALS* silencing (*dALSi*) increases it. The mass increase induced by *dilp2*^{F} expression is partially rescued by *dALS*^{M} expression. (29°C; n = 30; **p < 0.01; bracket indicates genotypes compared for p value calculation.)

(C) Measurement of larval growth rate in arbitrary units (a.u.). Changes in larval weight are detectable as early as 72 hr after egg laying and increase throughout larval development. (29°C; n = 5 × 30; *p < 0.05, **p < 0.01.)

Error bars represent SEM.

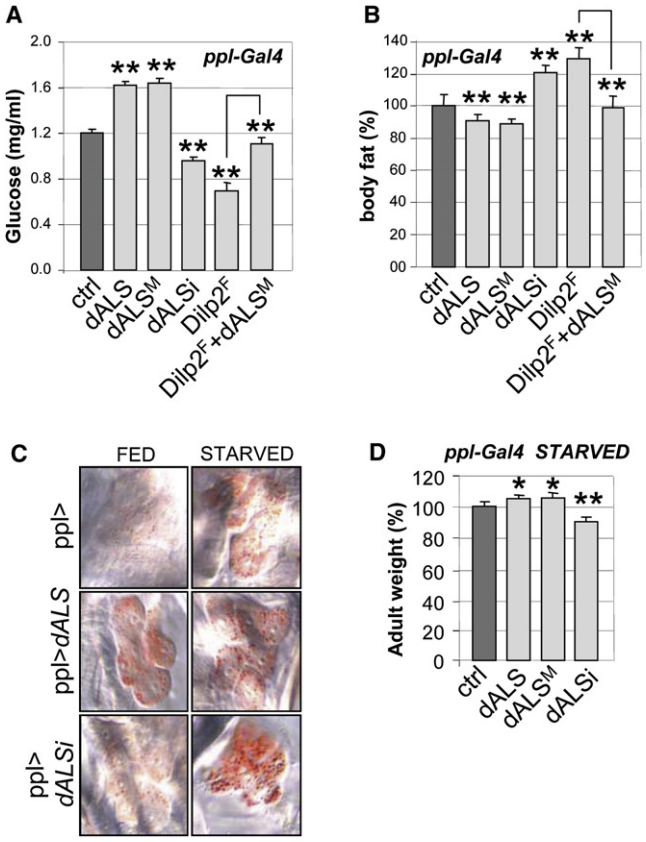

Figure 4. dALS Is Required for Metabolic Regulation and Becomes Limiting for Growth upon Nutritional Stress

For all experiments, the *ppl-Gal4* driver was used to direct expression in the larval fat body.

(A) Measurements of trehalose levels in the larval hemolymph. Trehalose levels were measured after conversion of hemolymph trehalose into glucose. *dALS* overexpression (*dALS* and *dALS*^{M}) increases trehalosemia, whereas *dALS* silencing (*dALSi*) decreases it. Moreover, coexpression of *dALS*^{M} with *dilp2*^{F} rescues the hypotrehalosemia induced by *dilp2*^{F} expression alone. (25°C; **p < 0.01.)

(B) Measurements of total triacylglycerol (TAG) in larvae. Increased *dALS* expression (*dALS* and *dALS*^{M}) leads to a reduction in larval TAG, whereas *dALS* silencing (*dALSi*) increases it. *dALS* expression fully rescues the TAG increase induced by *dilp2*^{F} expression alone. (25°C; **p < 0.01.) TAG levels are normalized to total protein levels.

(C) Lipid accumulation in oenocytes of fed and starved larvae from different genotypes shown using oil red O staining.

(D) Weight measurements of animals with modified *dALS* expression levels raised on yeast-deprived (0.1×) medium (starved). Under conditions of nutritional stress, *dALS* overexpression (*dALS* and *dALS*^{M}) increases adult mass, whereas *dALS* silencing (*dALSi*) decreases it. (29°C; n = 2 × 30; *p < 0.05, **p < 0.01.)

Error bars represent SEM.

whereas *dALS* silencing in the FB provoked a 25% increase in DAG+TAG (Figure 4B). Dilp2^{F} expression in the FB strongly reduced circulating trehalose levels and increased total fat content (Figures 4A and 4B). As was seen in the case of growth regulation, coexpression of dALS with Dilp2^{F} almost completely suppressed the metabolic changes observed upon Dilp2^{F} expression alone, suggesting an antagonistic genetic interaction between *dilp2* and *dALS* in metabolic regulation (Figures 4A and 4B).

The effects of dALS on metabolic control were confirmed using a readout for lipid mobilization. Upon starvation, a portion of

the lipids released from the FB accumulate in specialized cells called oenocytes (Gutierrez et al., 2007). This accumulation is easily detected using oil red O staining and serves as a sensitive indicator of lipid mobilization in fat cells. In normally fed animals, fat staining in oenocytes was barely detectable (Figure 4C). In animals subjected to 8 hr fasting on PBS/1% sucrose medium, a dramatic accumulation of fat droplets was observed in oenocytes, while nearby muscles and ectodermal cells remained unstained (Figure 4C; Gutierrez et al., 2007). Interestingly, *dALS* expression in fed larvae (*ppl>dALS*) triggered oenocyte lipid accumulation, thereby mimicking the effects of starvation, whereas *dALS* silencing (*ppl>dALSi*) had no visible effect (Figure 4C). This extends our previous observations and suggests that increased *dALS* expression generally inhibits insulin/IGF signaling (IIS), leading to the mobilization and release of lipid stores from fat cells, followed by their uptake into oenocytes.

Overall, this series of experiments demonstrates that dALS not only regulates growth rate but also antagonizes circulating Dilps in the control of energy metabolism.

dALS Function Becomes Limiting for Growth upon Nutritional Stress

Our previous experiments (Colombani et al., 2003) indicated that *dALS* expression in the FB varies with the nutritional status of the larva, suggesting that dALS might participate in the regulation of IIS in response to variations in nutritional conditions. We therefore tested the requirement for dALS function under limited nutrient conditions. Strikingly, manipulating *dALS* expression in the FB yielded opposite results in fed and starved animals. In particular, FB-specific *dALS* expression (*ppl>dALS*) did not increase lipid staining in oenocytes of starved larvae, while *dALS* silencing (*ppl>dALSi*) did (Figure 4C), indicating that dALS function is required to limit the extent of lipid remobilization during starvation.

We next measured the effect of varying *dALS* expression on the size of animals raised under limited nutrient conditions. For this purpose, larvae were raised on agar medium containing only 10% of the normal yeast content (1.7 g/l), a condition that significantly reduces adult size. Under these conditions, increased *dALS* expression partially compensated for the size defect, whereas *dALS* silencing further inhibited growth and decreased viability (Figure 4D). These results are again in contrast with the *dALS*-induced growth inhibition observed under normal food conditions and suggest that, although dALS counteracts Dilp function in normally fed animals, it becomes limiting for proper IIS activation under conditions of nutritional stress.

DISCUSSION

This work presents the functional characterization of an insulin/IGF binding partner in invertebrates and provides strong evidence for the formation of a trimeric complex involving Dilp2, dALS, and Imp-L2, a molecule with Dilp-binding protein function in *Drosophila*. No binding was observed between dALS and Dilp2 in the absence of Imp-L2, suggesting that, as with the trimeric IGF-1 complexes circulating in mammalian blood, the binding of dALS requires prior formation of a dimeric Dilp/Imp-L2 complex. Dilp5, another member of the ILP family in *Drosophila*, is also capable of forming a complex with dALS in cultured cells. Interestingly, the binding of Dilp5 and dALS is suppressed by excess Imp-L2, suggesting that one or more other Dilp-BPs produced in S2 cells compete with dALS binding for the formation of Dilp5 complexes. We propose that dALS may function as a common scaffold protein for different Dilp/Dilp-BP complexes in the hemolymph, with specific Dilp-BPs participating in the specialization of Dilp functions. At present, the technical difficulty of measuring the levels of endogenous Dilps in the hemolymph of *Drosophila* larvae precludes a detailed analysis of the types and amounts of circulating Dilp/Dilp-BP/dALS complexes.

No abnormal phenotypes were observed upon *dALS* overexpression or silencing in the brain IPCs. This could be due to a lack of sensitivity in our method, as we found that expressing $dALS^M$ in the 14 IPCs leads to very low accumulation of $dALS^M$ in the hemolymph as compared to its expression in the FB (data not shown). Conversely, silencing *dALS* in the IPCs does not reduce global *dALS* transcript levels, possibly because an important *dALS* transcription from FB cells is masking this effect. We also noticed that, when expressed in the IPCs, $dALS^M$ is not present in the same vesicular structures as Dilp2 (Figure S1C), suggesting that the two molecules are not found in a preassembled complex before being released into the hemolymph. Determination of the function of IPC-produced dALS will require further examination.

Our results point to a dual effect of dALS in the control of IIS that depends on nutritional status. We interpret this dual effect in light of the complex functions of IGFBPs and ALS in mammals. Under optimal nutritional conditions, Dilps are not limiting, and overexpression of *dALS* can induce the recruitment of more Dilps into stable but inactive trimeric complexes. If the release of active Dilp molecules is limited by the amounts of the various proteases that break apart the trimeric complexes, the net effect of *dALS* overexpression will be growth inhibition, as observed in vivo. In contrast, fasting leads to a general inhibition of IIS that may reveal a positive function for dALS: Dilp molecules becoming limiting, and *dALS* overexpression may increase the half-life of circulating Dilps and thereby enhance Dilp signaling (as long as the proteases are not limiting). Along these lines, the severe downregulation of *dALS* transcription observed under limited nutrient conditions (Colombani et al., 2003; Figure 3A) suggests that dALS participates in the adaptation of IIS to limited nutrition and the necessity of slowing down growth rate as well as carbohydrate and fat metabolism. Alternatively, the opposing results observed in starved versus fed conditions could be explained by the differential regulation of Dilp/dALS complexes involved in distinct regulations of IIS in response to nutritional conditions.

It has been proposed that in vertebrates, the formation of trimeric IGF/IGFBP/ALS complexes contributes to the functional separation between insulin and IGFs. Here we have provided evidence that such complexes are required for both the growth and metabolic functions carried out by the Dilps in *Drosophila*. Our work suggests an alternative scenario in which dALS, Imp-L2, and possibly additional Dilp-BPs participate in an ancestral function used for both metabolism and growth control.

EXPERIMENTAL PROCEDURES

Nomenclature

Gal4 driver lines were named after the gene used for *Gal4* expression specificity (for example *pumpless*, abbreviated *ppl*), followed by either -*Gal4* or > (*ppl-Gal4* or *ppl>*). The name *ppl>dALS* is therefore an abbreviation for the genotype *ppl-Gal4;UAS-dALS*, produced by the cross between the *ppl-Gal4* line and the *UAS-dALS* line.

Fly Stocks

The stocks *ppl-Gal4* (*ppl>*) (Colombani et al., 2003), *dilp2-Gal4* (*dilp2>*) (gift from E. Rulifson, University of California, San Francisco), *UAS-dALS*, *UAS-dALS^{M}*, *UAS-dALSi*, *UAS-dilp2^{F}*, and *UAS-Slif^{A}* (Colombani et al., 2003) were used. *UAS-Slif^{A}* allows reduced expression levels of the Slimfast amino acid transporter, thereby mimicking a state of amino acid deprivation (Colombani et al., 2003). For each gain- or loss-of-function experiment, a minimum of two separate lines were used (see Table S2 for details). Adult weight variations obtained upon *dALS* gain or loss of function using the *ppl-Gal4* driver were confirmed with an independent fat body driver (*cg-Gal4*, data not shown). Animals were reared at 25°C (unless otherwise indicated) on fly food containing, per liter: 17 g inactivated yeast powder, 83 g corn flour, 10 g agar, 60 g white sugar, 4.6 g nipagin M (in ethanol) (referred to as rich medium, or 1×). 0.1× medium contained 1/10 the normal amount of yeast powder.

Plasmid Constructs

pUAS-dALS and *pUAS-dALS-myc* (*pUAS-dALS^{M}*) constructs were generated by cloning an EcoRI/NotI fragment into the *pUASt* or *pUASt-myc* vectors (gift from L. Ruel, CNRS, Nice, France). A *dALS* cDNA covering the entire predicted coding region was obtained by fusing a partial *dALS* cDNA with the 5′ missing sequence amplified by PCR from genomic DNA. The *FLAG-dilp2* (*UAS-dilp2^{F}*) construct was created by PCR amplifying the *dilp2* coding sequence without the signal peptide sequence from the full-length cDNA clone EST GH11579 (obtained from Research Genetics). The resulting PCR product was then equipped with the hemagglutinin signal peptide sequence and a FLAG tag and inserted into *pUASt*.

The *UAS-dALSi* construct was generated by cloning a 697 bp fragment of the *dALS* cDNA (positions 2018–2715) into the pJM1084 hairpin vector (gift from J. Montagne, CNRS, Gifs/Yvette, France). The same *dALS* fragment was cloned (NheI/BamH1 and XbaI/BglII) in two opposite orientations, allowing the formation of a double-stranded hairpin *dALS* RNA under *UAS* control. Analysis of off-targets related to the *dALS* hairpin sequence revealed a single possible off-target on *CG11856* with very low gene specificity (0.16%; off-target size 19 bp). No changes in *CG11856* expression were detected upon *UAS-dALSi* induction using qPCR.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, two tables, and one figure and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/7/4/333/DC1/.

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