Growth control: **Invertebrate insulin surprises!**

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Recent work on *Drosophila* has provided new insights into how insulin signalling – conserved in mammals, flies and worms – regulates growth and cell division during development. Invertebrates have been found to possess more insulin-like ligands than predicted, some of which behave as receptor antagonists.

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Intercellular communication is essential for multicellular organisms to coordinate their development and complex physiological functions. When this communication occurs at a distance, extracellular ligands are employed to relay messages from one cell type to another that displays ligand-specific transmembrane receptors. Once the ligand is bound, a conformational change is transmitted to the intracellular domains of the receptor molecules, activating signalling cascades and, ultimately, altering cell behaviour. One such means of intercellular communication that has been studied in depth is the mammalian insulin signalling pathway.

Impaired insulin signalling in humans results in type II diabetes — a complex physiological disorder characterised by defective nutrient uptake and metabolism. Consistent with the complex nature of diabetes, insulin signalling influences numerous processes, including glucose uptake, metabolism, protein synthesis, growth and cell division. The insulin receptor is a tetramer of two extracellular alpha subunits, linked by disulphide bonds to two beta subunits, which traverse the cell membrane and have intracellular tyrosine kinase domains. When insulin binds, the tyrosine kinase domains of the receptor undergo transphosphorylation, resulting in autoactivation and trigger a signalling cascade initiated by the lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase) and including several serine/threonine kinases.

This signalling pathway, from the receptor to the target serine/threonine kinases, is conserved through evolution, and has been characterised extensively in the fruitfly *Drosophila* and the nematode worm *Caenorhabditis elegans*. Recent studies [1–4] of insulin signalling in invertebrates have provided new insights into its role in growth regulation [1], and also some surprises. Firstly, both flies and worms have been found to possess many more insulin-like ligands than expected [1–4]; and secondly, data from worms suggest that some insulin-like ligands antagonise, rather than activate, the insulin signalling pathway [2].

Growth regulation by insulin signalling in *Drosophila*

During development, the regulation of growth — mass increase — and cell division generates animals of consistent size, containing cells of constant size and number [5]. In *Drosophila*, the insulin signalling pathway regulates the growth of larval imaginal discs [6–8]. These epithelial bilayers are reorganised during metamorphosis to construct the adult epidermis; when insulin signalling is reduced, disc growth, disc size and also adult fly size are decreased. Detailed analyses in the imaginal discs have shown that reducing insulin signalling reduces both the size and number of cells. In contrast, when the activity of the pathway is artificially enhanced (by transgene expression), growth and cell size are increased, but cell number does not increase significantly. This observation suggests that activation of the pathway may not be sufficient to trigger cell division [8].

So far, studies of growth regulation in *Drosophila* have focussed on what happens when the activity of PI 3-kinase and downstream serine/threonine kinases is altered. Although certain mutant combinations of the *Drosophila* insulin receptor (DInr) have been reported to produce small flies [9,10], this phenotype has, until recently, not been analysed at the cellular level. This has changed with the experiments reported in a recent issue of *Current Biology* by Brogiolo *et al.* [1], who have examined the function of the DInr more closely. They have found that overexpression of DInr in *Drosophila* leads to an increase in growth, cell size and also cell number.

Brogiolo *et al.* [1] generated transgenic *Drosophila* in which DInr was overexpressed in the proliferating cells of the developing eye. They found that this resulted in the production of dramatic outgrowths, and eyes containing more, as well as larger, cells. This observation is important, as it implies that the pathway may branch at the level of the receptor, with a growth-promoting signal being transduced via the PI 3-kinase pathway, and a cell-division-promoting signal being transduced via a second signalling pathway. The observation that mitogen activated protein (MAP) kinase is activated in the heads of flies overexpressing activated DInr suggests that the Ras/MAP kinase pathway might transduce this second signal [1]. It should be noted, though, that overexpression of the DInr

increases growth more than it induces cell division, as it still increases cell size.

Drosophila insulin-like pepides

The alpha chain of DInr shares sequence similarity with mammalian insulin receptors, suggesting that DInr is activated by insulin-like ligands [9,10]. Brogiolo *et al.* [1] used homology searches of the *Drosophila* genome to identify putative DInr ligands, referred to as DILPs for '*Drosophila* insulin-like peptides' [1]. Given that the *Drosophila* genome has just one insulin receptor homologue, and fewer isoforms of the downstream signalling components than mammals, it is surprising that seven genes encoding DILPs were identified. These ligands have a domain structure similar or identical to mammalian insulin, including four consistently spaced cysteines within the A-chain, and consensus cleavage sites allowing proteolysis to generate active A-chain:B-chain dimers. Their amino acid sequences and domain structure are both more like mammalian insulin than the insulin-like growth factors, IGF1 and IGF2.

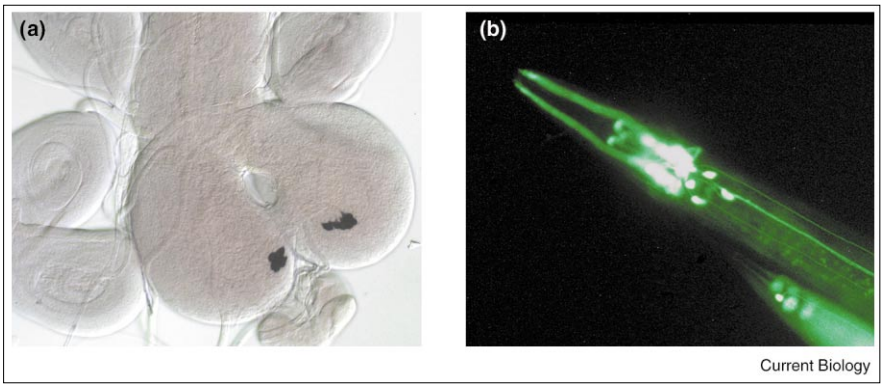
Ubiquitous overexpression of DILP2 — the family member that most closely resembles insulin — during disc development produced large flies [1]. Several observations suggest that DILP2 acts via DInr to increase growth. Firstly, the DILP2-induced large flies have more cells as well as larger cells. Secondly, mutation of one copy of *dinr* suppressed the DILP2-induced formation of large flies. And thirdly, the formation of large eyes by DInr overexpression was suppressed in flies heterozygous for a deletion that removes the genes for DILP1–5, suggesting that overexpressed DInr is dependent on the DILPs for its activity. And finally, embryonic overexpression of DILP2 was lethal and could also be suppressed by reducing DInr or downstream serine/threonine kinase activity. Given the postulated bifurcation of signalling from DInr (see above), it would be interesting to determine whether mutation of genes on the Ras/MAP kinase pathway also suppresses the DILP2-induced phenotypes.

As starvation during larval development also reduces growth, resulting in small flies, and as nutrients induce insulin secretion in mammals, it has been postulated that signalling via the *Drosophila* insulin pathway is nutrient-sensitive [6–8,11]. Furthermore, the larval fat body has been proposed as a nutrient-sensitive tissue that might secrete growth-promoting factors. But when the expression patterns of *dilp* mRNAs were examined by *in situ* hybridisation, no expression in the fat body was observed [1]. Highly localised expression patterns were observed, though. For instance, DILP2, DILP4 and DILP7 are expressed in the embryonic mesoderm and mid-gut; DILP2 is expressed at low levels in the imaginal discs; and DILP2, DILP3 and DILP5 are expressed at high levels in brain cells that may be neurosecretory (Figure 1a). Brogiolo *et al.* [1] speculate that DILPs produced by these cells may be transported to the ring gland, which contains the fly corpora cardiaca, then released from the copora cardiaca in a nutrient-dependent manner. It remains to be seen whether the transcription, expression and secretion of any or all of the *dilp* genes is nutrient-sensitive, but we now have the tools to make this possible. It will also be interesting to examine whether the forced overexpression of DILPs in one tissue is sufficient to drive the growth of another tissue, and if so, at what distances the DILPs can act.

A conserved role for insulin signalling in growth regulation

These results emphasise the striking conservation of insulin signalling function through evolution. Impaired insulin signalling in humans is classically associated with insulin resistance and type II diabetes, and can result from mutation of the insulin receptor (as well as other

Figure 1



Drosophila and *C. elegans* insulin homologues have neuronal expression patterns. (a) Several *Drosophila* insulin-like peptides are expressed in the brain. For example, *in situ* hybridisation reveals that *dilp3* is exclusively transcribed in seven cells in each larval brain hemisphere (shown in black), which may correspond to neurosecretory cells; *dilp2* and *dilp5* are transcribed in the same cells [1]. (b) Most of the *C. elegans* insulin homolgues examined are expressed in at least some amphid sensory neurons. For example, fusion of green fluorescent protein to the *ins-1* promoter reveals that *ins-1* is expressed in various neurons throughout the worm [2], including the amphid sensory neurons ASI and ASJ, which regulate dauer arrest [15].

molecules). In some clinical syndromes, however, increased insulin secretion can increase growth, and insulin deficiency can hinder growth. Furthermore, one human patient has been identified with a homozygous mutation in the insulin receptor that retards growth and reduces body size, causing a syndrome known as leprechaunism [12]. This observation provides compelling evidence that an evolutionarily conserved function of insulin signalling is the regulation of growth and body size.

C. elegans has 37 insulin-like ligands

Several *C. elegans* laboratories, like their colleagues working on *Drosophila*, have taken a bioinformatics approach to identifying putative ligands for the worm insulin receptor homologue, DAF-2 [2–4]. Extensive searches, based on sequence homology and structural predictions, have identified an insulin supergene family with a remarkable 37 genes: *ins-1–ins-37*. The ligands encoded by these genes have been sub-classified into four types, according to their predicted disulfide bond connectivity [2]. Of the resulting four groups, the gamma type includes mammalian insulin and IGFs, DILPs1–7 and eleven of the *C. elegans* ligands [2].

Why *C. elegans* should have so many insulin-like ligands is at present unclear — as in *Drosophila*, only one putative receptor has been identified. While functional redundancy is a possibility, some experiments suggest distinct functions for different ligands (see below). In addition, fusion of various *ins* gene promoter and enhancer regions to a reporter gene encoding green fluorescent protein (GFP) revealed specific and distinct expression patterns, consistent with the different genes being regulated by distinct cues, in order to provide distinct functions (Figure 1b) [2].

Insulin-like ligands as receptor antagonists in *C. elegans*

In *C. elegans*, insulin signalling regulates the formation of dauer larvae — stress-resistant, metabolically inactive and long-lived larvae, which normally form in response to overcrowding and starvation. When DAF-2 or downstream pathway components are mutated, dauer formation increases, even in the presence of food. Mutants which do reach adulthood have significantly extended adult lifespans, possibly because these adult worms have some dauer-like characteristics [13]. Conversely, when negative regulators of the pathway are mutated, the resulting worms have a reduced ability to form dauers, even when starved or crowded.

One approach to investigating *ins* gene function was to delete part of the *ins-1* gene. No effect on dauer formation or adult lifespan was observed in wild type or *daf-2* mutant worms, suggesting that *ins-1* function may be redundant with another *ins* gene [2]. As an alternative approach, worms were generated that overproduce INS molecules or human insulin. It was predicted that, like mutation of negative regulators of the DAF-2 pathway, the overproduction of INS molecules would reduce dauer formation. Instead, overproduction of INS-1 or INS-18 — the worm ligands most like human insulin — or of human insulin itself was found to enhance dauer formation, both in wild-type worms and in *daf-2* mutants, which already form dauers at an increased frequency. Furthermore, INS-1 overproduction slightly extended adult lifespan [2].

These observations are surprising, as they imply that INS-1, INS-18 and human insulin antagonise rather than activate DAF-2 signalling. When INS-9, INS-19, INS-22 and INS-31 were overproduced, they did not affect dauer formation. These ligands may be non-functional, or may influence biological processes not examined in these experiments. Other, as yet untested, INS peptides may behave as expected — as activators of DAF-2 signalling. But the ability of INS-1, INS-18 and human insulin to antagonise DAF-2 signalling still has to be explained. Some extracellular ligands activate their receptors at low concentrations, yet at high concentration, or after long-term treatment, downregulate signalling from the same receptor. To ensure that the apparent antagonism of DAF-2 signalling was not a result of chronic ligand overexpression, the transgene dosage was lowered [2]. The ability of INS-1 to enhance *daf-2* dauer formation persisted, even when only twice the wild-type *ins-1* gene dosage was used.

So it seems that at least some worm INS peptides can antagonise DAF-2 signalling. How might this occur? Human insulin, which certainly activates the human insulin receptor, also antagonises DAF-2 signalling. This finding suggests that the ability of INS-1 and INS-18 to antagonise DAF-2 signalling may not be a peculiarity of the ligands themselves, rather a consequence of what does (or does not) happen when these ligands bind to DAF-2. The structures of human insulin and the insulin receptor have been analysed in remarkable detail, and recently the three-dimensional structure of the entire receptor complexed with insulin has been determined by electron microscopy [14]. This information has provided molecular insights into how insulin binding brings the intracellular domains into sufficiently close proximity for transphosphorylation to occur. While it is possible that the structure of DAF-2 has evolved to allow exactly the opposite, this is hard to imagine! A more likely explanation is that the antagonistic ligands bind but do not activate DAF-2, and thereby compete with the true receptor agonists. Molecular modelling of the ligand and receptor domains and residues involved in ligand–receptor interaction and receptor activation process might prove informative here.

Another possibility is that, instead of the antagonism occurring at the level of the ligand–receptor interaction, it occurs further upstream. INS-1, INS-18 and human insulin might interact non-productively with molecules

involved in ligand processing and secretion, or with molecules homologous to the mammalian IGF-binding proteins that modulate ligand–receptor interactions. Whether such interactions occur only when the ligands are overproduced, or whether they reflect the true functions of INS-1 and INS-18 remains to be seen. It should also be noted that it has not been proven that any of the worm or fly insulins do actually bind to their assumed receptors. Simple biochemical analyses to examine whether INS-1 and INS-18 do bind and activate or inactivate DAF-2 will be useful. In addition, more extensive mutation of the worm *ins* genes should provide a more complete view of their function.

These analyses of insulin-like protein function in invertebrates have important implications for human insulin signalling. Firstly, might more extensive bioinformatic analysis of the human genome, using the techniques applied in *C. elegans* and *Drosophila*, identify more insulin-like molecules? And, secondly, if these molecules exist, might some of them act by antagonising, as opposed to activating, insulin signalling?

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