PERSPECTIVE

Insulin worms its way into the spotlight

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Obesity and the occurrence of diabetes are on the rise. Much of this is attributable to a more sedentary lifestyle and high caloric intake in industrialized countries and is a major cause of a variety of health problems. Obesity and diabetes are intimately linked to insulin, which increases glucose uptake in cells and serves as a primary regulator of blood glucose levels. Insulin has been an important target of investigation for decades, as indicated by its sequence determination by Sanger and colleagues in 1955 (Brown et al. 1955; Ryle et al. 1955; Murray-Rust et al. 1992), and is now the subject of renewed interest.

In addition to its role in glucose homeostasis (Saltiel and Kahn 2001), studies in the last several years have revealed a central role of insulin signaling in life span and aging in *Caenorhabditis elegans*. This surprising finding has allowed researchers to gain a foothold on a difficult, but important, biological problem. The control of life span by insulin signaling is not restricted to *C. elegans*, but now has been extended to equivalent pathways in *Drosophila* and mammals.

Because of these important roles in growth, development, and aging, insulin signaling is receiving scrutiny from scientists in diverse fields. Many downstream signaling components have now been identified and examined for their participation in metabolism and aging. Since the complete sequence of many organisms is now available, the identification of ligands has become easier and has revealed unexpected complexity. Sorting out the functions of these ligands will be an important aspect toward gaining a more complete understanding this signaling pathway. In this issue of *Genes & Development*, two papers (Hua et al. 2003; Li et al. 2003) address the structural and biological activities of some of these newly identified ligands. Ruvkun and colleagues (Li et al. 2003) examine *daf-28*, which is shown to be a divergent ligand that functions in the insulin pathway. Weiss and colleagues (Hua et al. 2003) examine the tertiary structure of another related *C. elegans* ligand, *INS-6*. They show that it forms the human canonical insulin structure, in spite of several changes of conserved residues,

and functionally binds the human insulin receptor. Since some insulin signals emanate from neurons and act elsewhere, the study of the insulin pathway in the context of the whole organism is going to reveal new insights not possible in cell culture.

Basic signaling features of the pathway

The elucidation of the insulin pathway in *C. elegans* arose from studies of the dauer pathway. Dauer is an alternative stage of development that arises from environmental stress and was one of the first pathways intensively studied by genetic dissection (Riddle and Albert 1997). The genes that control dauer formation group into two parallel pathways that converge on a common transcription factor. One branch of the pathway is defined by an insulin pathway, which signals through *DAF-2*, a transmembrane tyrosine kinase. The second branch of the dauer pathway is a TGF β -like pathway that includes *daf-7*, a ligand (Patterson and Padgett 2000) that acts synergistically with the *daf-2* pathway (Kimura et al. 1997). Both pathways converge on *daf-16*, which encodes a forkhead transcription factor that regulates transcription of downstream developmental and metabolic genes. Closely related human homologs of *daf-16* (FKHR and AFX) may be misregulated in some types of diabetes (Hua et al. 2003). A separate, nonoverlapping TGF β pathway controls body size in *C. elegans* (Gumienny and Padgett 2003).

In *C. elegans*, there is a single transmembrane insulin receptor, *daf-2*, which encodes a transmembrane tyrosine kinase (Kimura et al. 1997). In addition, a series of putative insulin-like receptor proteins have been identified recently in *C. elegans*. These proteins lack a kinase domain, but may antagonize *daf-2* signaling—their functions are unknown (Dlagic 2002). Upon stimulation of *DAF-2*, the kinase domain autophosphorylates and activates PI3K/*AGE-1*. In *Drosophila* and mammals, adaptor proteins link the receptors to downstream signaling components—these are known as insulin receptor substrates (IRS). Until recently, no homologs had been identified in *C. elegans*. However, *ist-1* and *aap-1* have been shown to participate in the *daf-2* pathway, but may not be required for all *daf-2* outputs (Wolkow et al. 2002). PI3K produces the second messenger, PIP3, which is required for activation of *PDK-1*, *AKT-1*, and *AKT-2*. This

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pathway functions to block the nuclear localization of *DAF-16*, and double mutants of *daf-2* and *daf-16* bypass the need for insulin (Fig. 1).

The insulin pathway affects life span

As the genetic work on the dauer pathway progressed, it became clear that the dauer pathway was involved with longevity. Kenyon and colleagues (1993) showed that *daf-2* mutants live about twice as long as wild type. When *daf-2* was molecularly cloned by Ruvkun and colleagues in 1997 (Kimura et al. 1997), the tie between longevity and insulin signaling was established. Subsequently, examination of other dauer signaling components were shown to also affect longevity, and when the corresponding genes were cloned, they were shown to encode insulin signaling components such as PI3K/*AGE-1* (Morris et al. 1996) and *DAF-16* (Lin et al. 1997; Ogg et al. 1997).

Life span extension by the insulin pathway requires the action of *daf-16*, which is the main downstream target (Lin et al. 1997; Ogg et al. 1997). *daf-16* encodes a

forkhead transcription factor, which translocates to the nucleus, depending on its phosphorylation level (Lin et al. 2001). The *daf-2* pathway prevents nuclear migration of *DAF-16* by promoting its phosphorylation. Disruption of the AKT phosphorylation sites on *DAF-16* promotes nuclear localization in wild-type animals, but does not affect life span (Lee et al. 2001; Lin et al. 2001). This result suggests that *daf-2* may have other outputs affecting longevity. Life span can be extended by perturbing germ cells and this extension requires *daf-16*. Germline activity regulates accumulation of *DAF-16*, but the nuclear localization patterns are different from the nuclear localization patterns modulated by *daf-2*. This suggests that germline signals act by modulating the activity of the insulin pathway (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002).

Gene structure of the *C. elegans* insulin ligands

While mutations affecting the insulin pathway downstream of *daf-2* have been identified and characterized, until recently little was known about the nature of the

Figure 1. Insulin signaling pathways in *C. elegans*. Environmental cues come through some neurons, such as ASI and ASJ, resulting in the production of some insulin proteins. These peptides act as agonists or antagonists on DAF-2. A kinase signaling cascade results in changes in DAF-16 phosphorylation and output of the pathway. The signaling cascade is highly conserved among nematodes, *Drosophila*, and mammals.

daf-2 ligand(s). Using a combination of sequence- and structure-based algorithms to screen the *C. elegans* genome, Pierce et al. (2001) identified 37 candidate genes encoding insulin-like peptides. Many of these new insulin genes are not detected by standard search algorithms. In fact, only 12 of these 37 candidate genes had been previously identified as insulin-like genes as small insertions and deletions alter the spacing of the conserved cysteine residues. Further, only 15 of the 37 insulin genes were identified by GeneFinder. However, all 37 of these predicted peptides contain the hallmarks of an insulin-like peptide, including a signal sequence, A chain peptide, and B chain peptide with the potential to form at least three disulfide bonds between conserved cysteine residues (Fig. 2).

The organization of many of these 37 insulin-like genes into genomic clusters of 3–7 genes suggests that at least some are the result of recent duplication events. Members of this gene family share 25%–40% amino acid

sequence identity. Extensive sequence divergence is also due to the presence or absence of a C-peptide fragment separating the B and A chains, as well as small insertions or deletions in the loops connecting the canonical α -helix motifs (Fig. 2). Thirty-four of the 37 predicted genes exhibit the conserved intron–exon structure found in vertebrate insulins, suggesting that the precursor to this gene family must have been present in a common ancestor 600 million years ago.

Do these new insulin genes fold properly?

The insulin-like peptides fall into four basic classes—the γ -class with the canonical 2 interchain and 1 intra A chain disulfide links (canonical insulin class), the β -class with an additional interchain disulfide link, the α -class which lacks the intra A chain disulfide link, and a rare class with multiple A and B chains (Fig. 2). Despite extensive sequence variation, computer modeling of pep-

Figure 2. Structure of the *C. elegans* ligand classes. (A) The three nematode classes of ligands based on probable peptide composition and cysteine bonds. (B) The classes of nematode ligand genes based on similarity of gene structure (adapted from Duret et al. 1998; Pierce et al. 2001). *ins-31* constitutes its own class with three repeats of the B and A peptide chains.

tide tertiary structure suggests that each class can adopt the canonical insulin-fold motif characterized by two parallel α -helices in the A chain crossed by a single α -helix in the B chain (Duret et al. 1998). The intra A chain disulfide link missing in the α -class peptides is substituted by the interaction of aromatic amino acid side chains in appropriate positions.

Given the sequence and structural diversity among the insulin genes, an important question is whether they actually fold properly and function as insulin-like molecules. Based on a variety of sequence motifs, *ins-1*, *ins-4*, and *ins-21* have been predicted to have the insulin fold (Pierce et al. 2001). Duret et al. (1998) modeled 10 *C. elegans* genes representing members from each class. These 10 were predicted to have the correct tertiary structure. Members of the α -class missing the intra A chain disulfide bond were postulated to form the correct structure by utilizing hydrophobic interactions between tyrosine and/or phenylalanines (Duret et al. 1998).

Direct evidence that the *C. elegans* insulin peptides assume the correct canonical confirmation has been lacking until Weiss and colleagues (Hua et al. 2003), in this issue of *Genes & Development*, provided experimental evidence. Weiss and colleagues (Hua et al. 2003) demonstrated that the *C. elegans* INS-6 peptide (a member of the β -class) can adopt the insulin-fold conformation and physically interact with the human insulin receptor. The 50-amino-acid INS-6 was chemically synthesized and allowed to fold. NMR analysis of this synthetic INS-6 indicates that it forms a single-folded conformation containing the three α -helices typical of insulin. Despite the fact that insulin and INS-6 share only 12 amino acid identities (including the six conserved cysteines), the insulin-fold conformation is stabilized by hydrophobic interactions that form the core of the structure. The exposed surface of INS-6, however, is topographically and electrostatically different from insulin. These differences are responsible for the monomeric nature of INS-6 and might be expected to affect the ability of INS-6 to interact with the insulin receptor.

INS-6 binding to the human insulin receptor was evaluated by its ability to competitively displace ^{125}I -insulin (Hua et al. 2003). While the single-chain INS-6 was a relatively weak competitor of dimeric insulin (~100-fold lower), it was a much stronger competitor than the human single-chain miniproinsulin (10-fold higher). In addition, INS-6 was shown to stimulate human insulin receptor autophosphorylation. Thus, despite limited primary structure similarities between human insulin and INS-6, both adopt similar tertiary structures and have similar receptor binding characteristics.

Genetic analysis of insulin ligands

While mutations affecting many downstream components of the insulin signaling pathway in *C. elegans* have been identified and characterized, none affecting the insulin ligands were known. Since 38 members of the insulin-like gene family have been identified in the *C. elegans* genome, the potential for functional redundancy

suggests that such mutations may be difficult to identify. The paper by Ruvkun and colleagues in this issue of *Genes & Development* (Li et al. 2003) represents the first molecular characterization of an insulin-like ligand in *C. elegans*—*daf-28*.

Partial characterization of some of the ligands has been performed using RNAi and overexpression studies (Pierce et al. 2001). Overexpression of *ins-1*, which has a putative C peptide fragment and proper cleavage sites (Fig. 2), enhances dauer arrest in weak *daf-2* mutants. A deletion of *ins-1* sequences does not enhance or suppress dauer formation, suggesting redundancy among some of the ligands. Likewise, overexpression of *ins-18*, which also contains a putative C peptide fragment, causes dauer arrest at 26°C in wild-type animals and enhances dauer arrest in *daf-2* animals. In contrast, many of the other insulin genes lacking predicted C peptide cleavage sites, such as *ins-9*, *ins-19*, *ins-22*, and *ins-31*, do not affect dauer arrest in wild-type or in *daf-2* animals. At high doses, *ins-9* causes some embryonic and L1 larval arrest while *ins-31* and *ins-19* cause some L1 arrest. As with *ins-1*, human insulin can stimulate the nematode insulin pathway, resulting in low levels of dauer arrest in wild type at 26°C. It also enhances dauer arrest in *daf-7* and *daf-2* at 20°C and is partially suppressed by *daf-16*.

C. elegans genomic sequences corresponding to *daf-28* were localized using an SNP mapping approach and found to encode a previously unidentified member of the insulin-like gene family. It was missed in the earlier searches because sequence of this region only recently became available. Comparison of wild-type and mutant *daf-28* genomic sequences showed that the semidominant sa191 allele contains a single base change that results in an arginine-to-cysteine substitution predicted to affect the proteolytic processing of the insulin-like peptide. Previous work has shown that mutations affecting peptide ligand maturation often have a poisoning effect on wild-type gene function resulting in the observed semidominant phenotype. Confirmation that the single base change associated with the sa191 allele is responsible for the *daf-28* semidominant phenotype was obtained by transformation rescue of the *daf-28* dauer arrest phenotype. This assay also demonstrated that *ins-4* and *ins-6* can substitute for *daf-28*, supporting the concept of functional redundancy among some insulin-like ligands (note that other insulin-like family members were unable to rescue the *daf-28* dauer arrest phenotype, including *ins-7*, *ins-9*, *ins-17*, *ins-21*, *ins-22*, and *ins-23*).

Insulin-like ligands are proposed to function as agonists or antagonists of *DAF-2*, the *C. elegans* insulin receptor. The sa191 allele of *daf-28* appears to antagonize *daf-2* activity, as evidenced by the nuclear localization of the forkhead transcription factor (*DAF-16*) that serves as the major output of the insulin signaling pathway. Interestingly, *daf-16* loss-of-function mutations only partially suppress the *daf-28* dauer arrest phenotype, while *daf-16* totally suppresses the *daf-2* phenotype, suggesting that *daf-28* may also function via an alternative, uncharacterized receptor pathway.

The analysis of temporal and spatial patterns of GFP

expression directed by the *daf-28* promoter demonstrates that *daf-28* is expressed in ciliated sensory neurons ASI and ASJ at an appropriate stage of larval development to function in the nematode dauer formation pathway. *Daf-28* expression is down-regulated in response to environmental conditions that promote dauer formation, suggesting that *daf-28* functions as an intermediate signal between environmental sensory input and the dauer formation pathway directed by *daf-2*. *Daf-28* expression in ASI and ASJ requires input from the TGF- β signaling pathway—*daf-28* expression is dramatically reduced in *daf-7* loss-of-function dauers (and less dramatically in *daf-1* loss-of-function dauers). This observation provides a mechanism for the interaction of these two signaling pathways in determining whether the worm will develop reproductively or enter the highly resistant dauer state.

Future issues

Why are there so many insulin ligands and how many more are there? Most of the ligand genes in *C. elegans* were not predicted to encode bona fide insulin molecules and were only identified using a multitude of search tools. This result highlights the difficulty in identifying all the ligands in one organism, particularly in divergent family members. Based on these observations, it is reasonable to speculate that additional ligand genes may be found in *C. elegans* through the use of more sophisticated searches or through molecular cloning of unknown genes.

In *Drosophila*, seven ligand genes have been reported (Brogiolo et al. 2001). Some of these genes were found using search algorithms that looked for conserved spacing of the four cysteines in the A chain. Again, the structure of the insulin family is divergent enough that new members are likely to be identified as more sophisticated bioinformatic tools develop and are applied to this problem. As in *C. elegans*, the functions of most of the *Drosophila* ligands are unknown, but their distinct expression patterns suggest additional complexity in their functions. In addition to affecting body size and growth control in *Drosophila* (Garofalo 2002; Oldham and Hafen 2003), mutations in insulin signaling components have been shown to affect longevity (Tatar et al. 2001).

In mammals, the insulin family is reported to have eight ligands, but additional ligand genes may be discovered as the genome is further analyzed. The roles of these eight ligands are just beginning to be examined (Nef and Parada 2000). Since many genes that regulate life span in invertebrates are components of the insulin pathway, it was of interest to examine longevity extension in insulin pathway mutants in mammals (Gems and Partridge 2001; Wolkow 2002). Since insulin receptor nulls in mice are lethal, heterozygous mice were examined. Heterozygous IGF-1 mutant female mice live 33% longer, while males live 16% longer (male life span increase is not statistically significant). These heterozygous mice are not dwarf, their energy metabolism is normal, and fertility is normal (Holzenberger et al. 2003). However, the heterozygotes show a greater resistance to oxidative

stress, a likely/possible determinant of aging. Further evidence for a connection between insulin signaling and life span comes from Laron mice. These mice live about 40%–55% longer than wild type and have less than 10% of normal IGF-1 levels, as well as other hormonal deficiencies (Coschigano et al. 2000). This implies that the insulin pathways connection to life span is an ancient invention and conserved among many phyla. A better understanding of this pathway will generate new therapies for diabetes, and open avenues for manipulating aging.

In addition to the obvious interest in further characterizing the core insulin pathway, two areas of study will be important to understand how this pathway intertwines metabolism and life span. First, where do insulins function and what role does each of the many family members contribute to these processes? Given that there are known interactions between neurons and insulin signaling, studies in the context of a whole organism, such as *C. elegans*, will be instrumental. However, the large number of ligands and paucity of mutations in them (or mutant phenotypes with RNAi) will be a hindrance to these studies. The second important area of focus will be the downstream effectors of the pathway. Almost no downstream genes regulated by *daf-16* are known, with the possible exception of Mn SOD (Honda and Honda 1999; Furuyama et al. 2000). What are the actual effectors that control cell size, metabolic homeostasis, and dictate longevity? With completely sequenced genomes, microarray technology, and whole genome RNAi analyses (Ashrafi et al. 2003; Kamath et al. 2003; Lee et al. 2003) readily available, answers to these questions should be forthcoming.

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