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, 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 includes DAF-2, a transmembrane tyrosine kinase. The second branch of the dauer pathway is a TGFβ-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 TGFβ 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 [Dlakic 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...
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
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-

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**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.
nentsoftheinsulinsignalingpathwayin
C.elegans
While mutations affecting many downstream compo-
turessandhavesimilarreceptorbindingcharacteristics.

Given the sequence and structural diversity among
the insulin genes, an important question is whether they
actually fold properly and function as insulin-like mol-
eules. Based on a variety of sequence motifs, ins-1, ins-
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 link missing in the α-class peptides is substi-
tuted by the interaction of aromatic amino acid side
chains in appropriate positions.

As the C. elegans insulin ligands and receptors have been
studied extensively, the ligands are known to include
insulin-like genefamilyhavebeenidentifiedinthe
C.elegans—daf-28
deletion of
ins-1, ins-
and
ins-21, 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 semidomi-
nant sa191 allele contains a single base change that res-
ults in an arginine-to-cysteine substitution predicted to
affect the proteolytic processing of the insulin-like pep-
tide. 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 respon-
sible for the daf-28 semidominant phenotype was ob-
tained by transformation rescue of the daf-28 dauer ar-
est phenotype. This assay also demonstrated that ins-4
and ins-6 can substitute for daf-28, supporting the con-
cept 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, includ-
ing ins-7, ins-9, ins-17, ins-21, ins-22, and ins-23].
Insulin-like ligands are proposed to function as ago-

ins or antagonists of DAF-2, the C. elegans insulin re-
ceptor. 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. Inter-
estingly, 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, uncharac-
terized receptor pathway.

The analysis of temporal and spatial patterns of GFP

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-genic analysis of insulin ligands
While mutations affecting many downstream compo-
nents 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 iden-
tify. 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
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The analysis of temporal and spatial patterns of GFP

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expression directed by the daft promoter demonstrates that daft-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. Daft-28 expression is down-regulated in response to environmental conditions that promote dauer formation, suggesting that daft-28 functions as an intermediate signal between environmental sensory input and the dauer formation pathway directed by daft-2. Daft-28 expression in ASI and ASJ requires input from the TGF-β signaling pathway—daft-28 expression is dramatically reduced in daft-7 loss-of-function dauer [and less dramatically in daft-1 loss-of-function dauer]. 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-1mutant 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 daft-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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