Published in final edited form as:

Int J Parasitol. 2006 March ; 36(3): 347–352. doi:10.1016/j.ijpara.2005.11.007.

The fork head transcription factor FKTF-1b from *Strongyloides stercoralis* restores DAF-16 developmental function to mutant *Caenorhabditis elegans*

Holman C. Massey Jr., Mahendra K. Bhopale, Xinshe Li, Michelle Castelletto, and James B. Lok*

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104, USA

Abstract

The purpose of this study was to determine whether *Strongyloides stercoralis* FKTF-1, a transcription factor of the FOXO/FKH family and the likely output of insulin/IGF signal transduction in that parasite, has the same or similar developmental regulatory capabilities as DAF-16, its structural ortholog in *Caenorhabditis elegans*. To this end, both splice variants of the *fkf-1* message were expressed under the control of the *daf-16a* promoter in *C. elegans* carrying loss of function mutations in both *daf-2* (the insulin/IGF receptor kinase) and *daf-16*. Under well-fed culture conditions the majority (91%) of untransformed *daf-2; daf-16* double mutants developed via the continuous reproductive cycle, whereas under the same conditions 100% of *daf-2* single mutants formed dauers. Transgenic *daf-2; daf-16* individuals expressing *fkf-1b* showed a reversal of the double mutant phenotype with 75% of the population forming dauers under well-fed conditions. This phenotype was even more pronounced than that of *daf-2; daf-16* mutants transformed with a homologous rescuing construct, *daf-16a::daf-16a* (56% dauers under well fed conditions), indicating that *S. stercoralis* *fkf-1b* can almost fully rescue loss-of-function mutants in *C. elegans* *daf-16*. By contrast, *daf-2; daf-16* mutants expressing *S. stercoralis* *fkf-1a*, encoding the second splice variant of FKTF-1, showed a predominantly continuous pattern of development identical to that of the parental double mutant stock. This indicates that, unlike FKTF-1b, the *S. stercoralis* transcription factor FKTF-1a cannot trigger the shift to dauer-specific gene expression in *C. elegans*.

Keywords

Strongyloides stercoralis; *Caenorhabditis elegans*; Dauer larva; Infective larva; Fork head transcription factor; Transgenesis

1. Introduction

Morphological, developmental and behavioral similarities between the infective third-stage larvae of parasitic nematodes (L3i) and dauer larvae of *Caenorhabditis elegans* have led to the hypothesis that the development of L3i and of *C. elegans* dauers may be regulated by common molecular and cellular mechanisms (Hotez et al., 1993; Blaxter and Bird, 1997; Blaxter, 1998; Burglin et al., 1998). This hypothesis has been bolstered by the discovery that dauer switching in *C. elegans* and the switch between homogonic and heterogonic developmental alternatives in the parasitic nematode *Strongyloides stercoralis* are controlled

by the homologous amphidial neurons (Bargmann and Horvitz, 1991; Ashton et al., 1998; Nolan et al., 2004). Our laboratory is investigating possible parallels between molecular mechanisms regulating dauer development in *C. elegans* and the development of L3i in *S. stercoralis*. In *C. elegans*, dauer arrest is controlled by three interacting cellular signal transduction pathways: a G protein coupled odorant receptor pathway that processes signals from a constitutively secreted dauer pheromone (Zwaal et al., 1997) and TGF- β - (Georgi et al., 1990; Bargmann and Horvitz, 1991; Estevez et al., 1993; Ren et al., 1996; Nolan et al., 2002) and insulin-like (Morris et al., 1996; Kimura et al., 1997; Ogg et al., 1997; Ogg and Ruvkun, 1998; Paradis and Ruvkun, 1998; Paradis et al., 1999; Li et al., 2003) pathways that transduce signals from sensory neurons in the amphids to multiple tissues that are remodeled during dauer development. We have discovered that orthologs of genes in all three of these signal transduction pathways are conserved in *S. stercoralis* (Massey et al., 2001, 2003, 2005) and we are investigating the possible role of insulin-like signaling in regulating L3i development in this parasite.

Under environmental conditions favoring continuous, reproductive development, output from the insulin-like pathway in *C. elegans* negatively regulates DAF-16, a forkhead, winged helix-class transcription factor, causing it to remain localized in the cytoplasm. Under dauer inducing conditions, insulin-like signaling ceases and DAF-16 assumes a nuclear localization where it shifts the pattern of gene expression to one favoring dauer arrest and lifespan extension. Loss-of-function mutations in *daf-16* result in a dauer deficient (*daf-d*) phenotype and suppress the dauer constitutive and lifespan extension phenotypes associated with mutations in all known upstream kinases in the insulin pathway, suggesting that negative regulation of *daf-16* is the major output of this pathway (Lin et al., 1997; Ogg et al., 1997).

Recently, we discovered *fkf-1*, an *S. stercoralis* ortholog of *daf-16* in *C. elegans* (Massey et al., 2003). The structures of *fkf-1* and *daf-16*, of their alternately spliced transcripts and of their predicted peptide products are similar. The predicted peptide sequences of FKTF-1A and DAF-16A1, for example, show the highest levels of homology (79.5% identity) in their DNA-binding or 'forkhead' domains. Lower levels of homology are predicted for the C-terminal (31.4% identity) and N-terminal (51.4% identity) domains of these proteins. The latter value excludes the serine rich region predicted at the N terminus of FKTF-1A (Massey et al., 2003). Like its *C. elegans* ortholog, *fkf-1* is expressed at similar levels throughout development (Ogg et al., 1997; Massey et al., 2003). The splice variants of the *daf-16* transcript are expressed in various tissues and anatomical sites that undergo remodeling during dauer development, each under the control of its own promoter, (Lee et al., 2001). The anatomical expression patterns of *fkf-1* are unknown at this time.

At present, the lack of functional genomic methods prevents direct assessment of *fkf-1* function in *S. stercoralis*. An alternative approach to obtaining information about the functional capabilities of *fkf-1* might be to characterize its ability to complement *daf-16* loss of function mutations in *C. elegans* by transgenesis. This approach has been used to assess functional conservation of promoter elements (Britton et al., 1999; Gomez-Escobar et al., 2002) and coding sequences (Britton and Murray, 2002) from other parasite genes. In the present paper, we have taken a similar approach with *fkf-1* from *S. stercoralis*. We showed that when expressed as a transgene, one of its alternately spliced transcripts could restore dauer-forming capability to *C. elegans* carrying a loss-of-function mutation in *daf-16*, thus demonstrating possible functional homology of the two genes.

2. Materials and methods

2.1. Genetic stocks of *C. elegans*

Starting cultures of the N2 (wild-type) strain of *C. elegans*, the single mutant *daf-2*(e1370) and the double mutant *daf-2*(e1370); *daf-16*(mg54) strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota). Worms from all genetic stocks as well as transgenic lines were maintained on NGM agar plates with *Escherichia coli* OP50 lawns using standard methods (Lewis and Fleming, 1995).

2.2. Transformation constructs

The vector pPV198 (Fig. 1) was made from the *C. elegans* vector pPD95.81 (gift from Andrew Fire). pPD95.81 was cut at the *Eco*R1 site between the *gfp* coding sequence and the *unc-54* 3' end cassette and the oligonucleotide sequence (5'-AAT TAC GCG TCC TAG GCC TG-3') adding restriction sites for *Stu*I, *Mlu*I, *Avr*2 upstream of *Eco*R1. The *C. elegans daf-16a* promoter (Ogg et al., 1997) was amplified from genomic DNA with the primers CeD16aprF and CeD16aprR tagged with restriction sites for *Bam*H1 and *Age*I, respectively. This was cloned into pPCRScript Amp SK+ (Stratagene), and the *Bam*H1, *Age*I fragment from this construct was inserted into pPV198 to create pPV199 (Fig. 1) according to the manufacturer's protocol. cDNAs encoding *daf-16a*, *fktf-1a* and *fktf-1b* were amplified by reverse transcriptase (RT)-PCR using the SuperScript First Strand Synthesis System[®] (Invitrogen) and the following primers in the PCR step: D16aMetF and D16TerR (*daf-16a*), FK1aMetF and FK1TerR (*fktf-1a*), and FK1bMetF and FK1TerR (*fktf-1b*). Sequences of these primers are given in Table 1. All forward and reverse primers used to amplify *daf-16* or *fktf-1* coding sequences contained an *Age*I or *Avr*2 restriction site, respectively, for cloning. Amplified coding sequences were cloned into pPCRScript Amp SK+(Stratagene). The cDNAs were cut from the pPCR-Script clones with *Age*I and *Avr*2 and ligated to pPV199 from which the *gfp* sequence had been removed with the same restriction endonucleases to make pPV200, pPV203 and pPV207 (Fig. 1).

2.3. DNA transformation of *C. elegans* and selection of transgenic lines

Caenorhabditis elegans of the *daf-2*; *daf-16* double mutant background were transformed by microinjection of the test constructs at a final concentration of 20 ng/μl into the distal gonad arms of L4 or young hermaphrodites. Plasmid pRF4 (a gift from M. Sundaram, University of Pennsylvania) containing the *rol-6* marker was co-injected with each transgene construct at a final concentration of 80 ng/μl. This transgene encodes a cuticular collagen with a dominant mutation resulting in the abnormal 'right roller' motility phenotype, allowing easy identification and manual selection of transformants (Kramer et al., 1990; Mello et al., 1991). Microinjected worms were reared in isolation, and transformants were selected from among their F1 progeny and re-plated. Progeny of individual microinjected worms that transmitted transgenes to the F2 generation and beyond were maintained as independent transgenic lines.

Cell-specific expression was indicated by *gfp* expression and viewed under a fluorescent microscope.

2.4. Assay for dauer switching in *C. elegans* mutants and transgenic lines

In order to assess effects of various transgenes on dauer switching in *C. elegans*, we used an assay for dauer switching based on that outlined previously (Nolan et al., 2002). This assay involves evaluating development of synchronized cohorts of worms under uniform non-dauer inducing conditions. Mutant strains used as controls in these experiments were stably transformed with the *rol-6* plasmid, pRF4. Briefly, 10–20 egg-laying transformant (roller) hermaphrodites were placed on 60 mm NGM agar plates with standard *E. coli* OP50 lawns

formed by drying 40 μ l of an overnight LB broth culture with cell density adjusted to 0.25% w/v. The worms were allowed to oviposit for 3–8 h and then removed from their respective plates. The aim was to accumulate a semi-synchronous population of 50–100 eggs from each line on an individual assay plate. The plates were then incubated at 25 °C for 65–70 h. At the end of this incubation, individual transformants were picked either to a separate plate or a clear area of their own plate and scored as non-dauer individuals (L4s or adults) or dauer larvae. These assessments were made based on radial constriction of the body and on filariform pharyngeal morphology and lack of pharyngeal pumping (Riddle and Albert, 1997). Mean proportions of dauer and non-dauer development were calculated from at least two replicate assays, conducted on different days, of at least two independently derived lines for each transgene construct. Proportions of dauer and non-dauer development in transgenic lines were compared to those in the *daf-2*; *daf-16* parental stock by χ^2 analysis, and *P* less than or equal to 0.05 was set as the criterion for significance.

3. Results

3.1. Expression of test constructs in *C. elegans* mutants

The *daf-16a* promoter::*gfp* fusion construct (pPV199, Fig. 1) was expressed in all life stages of *C. elegans* transformants. Bright fluorescence was seen in cells of the body wall, muscles, intestine and neurons but not in the pharynx or gonad (Fig. 2A–D). Messenger RNA encoding FKTF-1 was amplified by RT-PCR using *fkf-1*-specific primers in all lines created by transforming *daf-2*; *daf-16* double mutants with our *daf-16a*::*fkf-1a* (pPV203) or *daf-16a*::*fkf-1b* (pPV207) rescuing constructs (Fig. 2E). An RTPCR signal, presumably from the mutant *daf-16*(mg54) message prevented this type of confirmatory analysis in our control lines transformed with the homologous rescuing construct *daf-16a*::*daf-16a*.

3.2. Results of cross-species complementation experiments

Percentages of dauer and non-dauer development in wild type, mutant and transgenic *C. elegans* are shown in Fig. 3. Patterns of development in untransformed N2 (wild-type) worms, *daf-2* mutants and *daf-2*; *daf-16* double mutants transformed only with the *rol-6* encoding plasmid pRF4 were consistent with those reported previously for worms of these strains developing under well-fed conditions (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). Note especially the uniform *daf-c* phenotype of *daf-2* and the suppression of this phenotype by a second mutation in *daf-16*. Interestingly, the heterologous transgene construct *daf-16a*::*fkf-1b* resulted in partial restoration of constitutive dauer development in *C. elegans* *daf-2*; *daf-16* double mutants, indicating that FKTF-1b can partially replace DAF-16 function. χ^2 comparison of this result with proportion of dauer and non-dauer development in the parental *daf-2*; *daf-16* stock was highly significant (*P*<0.005). On the other hand, when worms from this strain were transformed with the *daf-16a*::*fkf-1a* construct they showed no appreciable change in their patterns of dauer switching compared with the parental *daf-2*; *daf-16* strain (*P*>0.9). Finally, transformation of the *daf-2*; *daf-16* double mutant with the homologous *daf-16a*::*daf-16a* construct also resulted in a statistically significant departure from the developmental pattern in the *daf-2*; *daf-16* parental stock (*P*<0.005), indicating partial complementation of the *daf-16* mutation.

4. Discussion

The *daf-2*; *daf-16* double mutant used in this study provided a convenient subject in which to study *fkf-1* function since restoration of *daf-16*-like activity in this genetic background could be easily quantified by assaying the frequency of dauer development in well-fed cultures. Another important methodological point is that the *daf-16a* promoter we derived and used in all transgene constructs in this study drove GFP expression primarily in body wall and intestine, a subset of tissues known to be remodeled during dauer development in

C. elegans (Ogg et al., 1997). This, along with the conspicuous lack of reporter expression observed in the gonad and pharynx of L1 transformants, is consistent with anatomical patterns of *daf-16a* promoter activation reported by others for *C. elegans* L1 (Ogg et al., 1997) and indicates that the promoter element we derived functioned normally and drove expression of the *fktf-1* transcripts in the appropriate cell and tissue types. The finding that *fktf-1b* complements a loss-of-function mutation in *daf-16* indicates that the product of this gene is capable of effecting the switch to dauer-specific gene expression in *C. elegans*. This result is not surprising given the highly conserved domain structure, putative Akt/PKB phosphorylation sites and high degree of sequence identity in the DNA binding domains of FKTF-1, DAF-16 and their orthologs in other systems (Massey et al., 2003). Indeed, Lee et al. (2001) have shown that the insulin-regulated forkhead transcription factor FKHL-1 from humans will partially complement mutations in *daf-16*. The fact that the *fktf-1b* gene product can be appropriately regulated and can shift gene expression in *C. elegans* to a pattern favoring the developmental and biochemical modifications involved in dauer arrest in the L3 supports the hypothesis that, under the control of insulin-like signaling, it regulates the strikingly similar process of heterogonic-to-homogonic life cycle switching in *S. stercoralis*. This, in turn, supports the hypothesis that parasitic nematodes have adapted the neural and cell signaling mechanisms governing dauer development in *C. elegans* to regulating the development of their morphologically and physiologically similar L3i (Hotez et al., 1993; Ashton et al., 1998; Blaxter, 1998; Burglin et al., 1998; Tissenbaum et al., 2000).

The *daf-2; daf-16* double mutant stock used in this study contains the *mg54* allele in *daf-16*. This mutation produces an amber stop mutation in the DNA binding domain of *daf-16a*, but it is not predicted to affect *daf-16b* splice variant. Our finding that *daf-16a* mutations can be complemented by *fktf-1b* is consistent with the findings of Lee et al. (2001) that the respective functions of the 'a' and 'b' splice forms of *daf-16* are determined not by differences in their intrinsic structures but rather by the tissue specificities of their individual promoters. *daf-16a* and *daf-16b* are functionally equivalent in pharyngeal remodeling when expressed under the control of the pharynx-specific *daf-16 β* promoter. Likewise the two splice forms are functionally equivalent when expressed under the control of the *daf-16a* promoter in gut and body wall tissues (Lee et al., 2001). On the other hand, the failure of *fktf-1a* to complement the *daf-16*(*mg54*) mutation may stem from a marked structural peculiarity predicted for the *S. stercoralis* protein. Conceptual translations predict a long serine-rich region at the N terminus of FKTF-1a. This motif is predicted to be absent in FKTF-1b and DAF-16b and much reduced in DAF-16a (Massey et al., 2003) and may prevent FKTF-1a from carrying out its developmental regulatory function in *C. elegans*. It should be possible to test this hypothesis in the *C. elegans* surrogate system by observing developmental phenotypes resulting from expression of chimeric gene constructs in which the sequence encoding the serine-rich domain is deleted from *fktf-1a* or appended to the coding sequence of *daf-16a* or *fktf-1b*.

Insulin-like signal transduction regulates lifespan as well as larval development in *C. elegans* (Kenyon et al., 1993; Lin et al., 1997; Apfeld and Kenyon, 1999; Kenyon, 2001; Murphy et al., 2003). In addition to dauer constitutive development, loss-of-function mutations in DAF-2 and other intermediates in the cellular kinase signaling cascade also result in lifespan extension. Similar to their suppression of upstream dauer constitutive phenotypes, mutations in *daf-16* also suppress the lifespan extension resulting from mutations in upstream signaling intermediates. Grant et al. (in press) have noted that extreme longevity is common to many parasitic nematodes and that the life cycles of Rhabditoid parasites such as *Parastrongyloides trichosuri* and *Strongyloides* spp. feature great disparities in longevity, with parasitic adults living for many weeks and their more ephemeral free-living counterparts living for a few days only. Our laboratory is currently

using the *C. elegans* surrogate system to investigate the capability of *fttf-1* and other putative insulin signaling elements from *S. stercoralis* to regulate lifespan.

Acknowledgments

This work was supported by grants from the National Institutes of Health (AI-50688 to J.B. Lok, AI-22662 to G.A. Schad and RR02512 to M. Haskins) and The Ellison Medical Foundation (Grant No. ID-IA-0037-02 to J.B. Lok). We thank Qiukan Chen, Mario Brenes, Linda Capewell, Andrea Freeman and Laura Leighton for technical assistance and, for providing *C. elegans* strains, the *Caenorhabditis* Genetics Center, University of Minnesota, which is funded by the NIH National Center for Research Resources.

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Fig. 1.

Cloning strategy for reporter and rescuing constructs. Starting material is the *Caenorhabditis elegans* vector pPD 95.81, kindly provided by A. Fire. Preliminary steps involve inserting a supplemental cloning cassette with restriction sites for *StuI* (S), *MluI* (M) and *Avr2* (Av) at the *EcoR1* site (E) upstream of the *unc-54* 3' untranslated region (UTR) to yield plasmid pPV198 and the *daf-16α* promoter at the *Bam* H1 (B) and *Age* 1 (A) sites upstream of the *gfp* coding sequence to yield the reporter construct, plasmid pPV199. Rescuing constructs containing coding sequences for *daf-16a* (pPV200), *fktf-1a* (pPV203) or *fktf-1b* (pPV207) were made by first removing the *gfp* coding sequence from pPV199 by digestion with *Age* 1 and *Avr2* and ligating the appropriate cDNAs, generated with *Age* 1- and *Avr2*-compatible ends by reverse transcriptase-PCR, at these same restriction sites.

Fig. 2.

Expression of transgenes in *Caenorhabditis elegans* strains. (A,B), DIC and fluorescence images, respectively, of N2 (wild type) L1 expressing *daf-16a::gfp*. Note generalized expression in body wall and intestine (I) and lack of expression in pharynx (P) and gonad (G). (C,D), DIC and fluorescence images, respectively detailing characteristic lack of *daf-16a::gfp* expression in gonad. (E), results of reverse transcriptase-PCR to detect *fktf-1*-specific mRNA in *daf-2; daf-16* transgenic lines. The constitutively expressed mRNA encoding the ribosomal protein small subunit RPS-21 was used as a loading control. Templates in lanes as follows: (1), untransformed parental *daf-2; daf-16* double mutant stock; (2–5) *daf-16a::fktf-1b* transformant lines 031003-3, 020403-16, 021805-10 and 170304-10, respectively; 6–7, *daf-16a::fktf-1a* transformant lines 020105-17 and 2251103-6, respectively.

Fig. 3.

Results of dauer developmental assay on mutant and transgenic *Caenorhabditis elegans* stocks. Data are pooled from three replicate experiments on at least two independently derived lines for each transgene. Numbers in parentheses indicate the total number of worms scored in all experiments. *Denotes statistically significant ($P < 0.005$) difference from proportions of dauer and non-dauer development in parental *daf-2; daf-16* stock.

Table 1

PCR primers used to amplify promoters and coding sequences for synthesis of transformation constructs (see Fig. 1) used for *Caenorhabditis elegans* complementation studies

Element	Primer designation	Sequence
<i>daf-16a</i> promoter	CeD16aprF	5'-GATGGATCCTCTGATTGGCTTCTCGTGGTG-3' ^a
	CeD16aprR	5'-TCTACCGGTATTTTCAGCCAAAGACGACGATG-3' ^b
<i>daf-16a</i> coding sequence	d16aMetF	5'-AATACCGGTAGACGTTATGATGGAGATGCTGG-3' ^b
	d16aTerR	5'-CGTTTTTGGTACCTAGGTCAGTGCTAATGATTTTACTATCTGG-3' ^c
<i>ftt-1a</i> coding sequence	fk1aMetF	5'-AATACCGGTAAGAGTAAAAATGAGTGGACATC-3' ^b
	fk1aTerR	5'-AAACCTAGGTTTGTACAACTTTTAGGAATC-3' ^c
<i>ftt-1b</i> coding sequence	fk1bMetF	5'-ATAACCGGTAAAAATGAATACATCCATTGTATCGG-3' ^b
	fk1bTerR	5'-AAACCTAGGTTTGTACAACTTTTAGGAATC-3' ^c

^a *Bam*H1 restriction site underscored.

^b *Age*I restriction site underscored.

^c *Avr*2 restriction site underscored.