Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor

Suzanne Paradis and Gary Ruvkun[1]

Department of Molecular Biology, Massachusetts General Hospital (MGH) and Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114 USA

A neurosecretory pathway regulates a reversible developmental arrest and metabolic shift at the *Caenorhabditis elegans* dauer larval stage. Defects in an insulin-like signaling pathway cause arrest at the dauer stage. We show here that two *C. elegans* Akt/PKB homologs, *akt-1* and *akt-2*, transduce insulin receptor-like signals that inhibit dauer arrest and that AKT-1 and AKT-2 signaling are indispensable for insulin receptor-like signaling in *C. elegans*. A loss-of-function mutation in the Fork head transcription factor DAF-16 relieves the requirement for Akt/PKB signaling, which indicates that AKT-1 and AKT-2 function primarily to antagonize DAF-16. This is the first evidence that the major target of Akt/PKB signaling is a transcription factor. An activating mutation in *akt-1*, revealed by a genetic screen, as well as increased dosage of wild-type *akt-1* relieves the requirement for signaling from AGE-1 PI3K, which acts downstream of the DAF-2 insulin/IGF-1 receptor homolog. This demonstrates that Akt/PKB activity is not necessarily dependent on AGE-1 PI3K activity. *akt-1* and *akt-2* are expressed in overlapping patterns in the nervous system and in tissues that are remodeled during dauer formation.

[*Key Words:* Insulin signaling; dauer; Fork head transcription factor; life span]

Received May 14, 1998; revised version accepted June 25, 1998.

An insulin receptor-like signaling pathway regulates *Caenorhabditis elegans* metabolism, development, and longevity (Kimura et al. 1997). This insulin receptor pathway is required for reproductive growth and normal metabolism. Mutations in the insulin/IGF-I receptor homolog *daf-2* (Kimura et al. 1997) or in the phosphoinositide-3-OH kinase (PI3K) homolog *age-1* (Morris et al. 1996) cause animals to arrest as dauers, shift metabolism to fat storage, and live longer (Kenyon et al. 1993; Larsen et al. 1995; Kimura et al. 1997). This regulation of *C. elegans* metabolism is similar to the physiological role of mammalian insulin in metabolic regulation. Insulin controls glucose homeostasis by changing the activity of key metabolic regulators, such as glucose transporters and metabolic enzymes (Avruch 1998). Mutations in the Fork head transcription factor DAF-16 completely suppress the dauer arrest, metabolic shift, and longevity phenotypes of *daf-2* and *age-1* mutants (Kenyon et al. 1993; Gottlieb and Ruvkun 1994; Larsen et al. 1995; Lin et al. 1997; Ogg et al. 1997), indicating that DAF-16 is a negatively regulated downstream target of *C. elegans* insulin receptor-like signaling. Mammalian homologs of DAF-16 may similarly be regulated by insulin receptor signaling to mediate the transcriptional effects of insulin (Ogg et al. 1997). Molecules that couple the DAF-2 insulin receptor-like protein and AGE-1 PI3K to the DAF-16 transcription factor have not been identified by previous extensive genetic screens.

Activation of the mammalian insulin receptor tyrosine kinase in response to insulin binding has been shown biochemically to affect multiple downstream targets that regulate cellular responses to insulin. Some of the major downstream effectors of insulin receptor activation include Ras and PI3K; these molecules have outputs to diverse biological processes, such as cell growth, gene expression, glycogen synthesis, protein synthesis, and glucose transport in response to insulin receptor activation (Kahn 1994).

Activated PI3K generates 3-phosphoinositides, such as phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P2) and PtdIns-3,4,5-P3 (Kapeller and Cantley 1994). PtdIns-3,4-P2 and PtdIns-3,4,5-P3 are thought to act as second messengers because their levels rise rapidly in response to growth factor signaling (Toker and Cantley 1997). PI3K has been implicated in a variety of cellular responses to growth factor signaling such as mitogenesis, receptor trafficking, regulation of the actin cytoskeleton, and cell survival (Carpenter and Cantley 1996). A few

[1]**Corresponding author.**
E-MAIL Ruvkun@frodo.mgh.harvard.edu; FAX (617) 726-6893.

downstream effectors of PI3K signaling have been identified biochemically and shown to bind to the 3-phosphoinositides generated by PI3K. These include the serine/threonine kinase Akt/PKB (also known as RAC) and other pleckstrin homology (PH) domain containing proteins, certain PKC isoforms, and SH2 domain-containing proteins (Toker and Cantley 1997).

Activation of Akt/PKB is dependent on PI3K activation—PtdIns-3,4-P2 and/or PtdIns-3,4,5-P3 bind to the amino-terminal PH domain of Akt/PKB and activate the kinase (Franke et al. 1997; Frech et al. 1997; Klippel et al. 1997). Phospholipid binding presumably causes a conformational and/or localization change that makes Akt/PKB more accessible to phosphorylation events that are necessary for activation (Alessi et al. 1997; Stokoe et al. 1997). Akt/PKB has been implicated in cellular responses to insulin signaling, such as glucose transporter translocation and expression and glycogen synthesis (Cross et al. 1995; Kohn et al. 1996). Akt/PKB has also been shown to have anti-apoptotic activities in response to signaling by various growth factor receptors including insulin and IGF-1 (Dudek et al. 1997; Kauffmann-Zeh et al. 1997; Kulik et al. 1997).

Although biochemical studies suggest that mammalian Akt/PKB transduces signals from PI3Ks associated with receptor tyrosine kinases such as the insulin receptor to downstream effectors, this has not been demonstrated by genetic analysis of signaling pathways in whole organisms. Such an analysis could reveal the key signaling inputs to Akt/PKB as well as major targets of Akt/PKB. We report the identification of two *C. elegans* Akt/PKB homologs, *akt-1* and *akt-2*. We establish the action of AKT-1 and AKT-2 in the DAF-2 insulin receptor-like signaling pathway by the isolation of an activating *akt-1* mutation in a screen for suppressors of an AGE-1 PI3K null allele and by genetic analysis of *akt-1* and *akt-2* inactivation and overexpression. We find that an activating mutation in *akt-1* or increased dosage of *akt-1*(+) can bypass the normal requirement for AGE-1 PI3K signaling but still partially depends on DAF-2 signaling, showing that *akt-1* is the major output of PI3K signaling but not the only output of the DAF-2 insulin-like receptor. We show that inactivation of *C. elegans* Akt/PKB signaling causes a dauer constitutive phenotype; we therefore conclude that Akt/PKB signaling is necessary for reproductive growth and metabolism. Null mutations in the DAF-16 Fork head transcription factor relieve the requirement for Akt/PKB signaling to repress dauer formation, indicating that DAF-16 is a major downstream target of Akt/PKB signaling.

Results

akt-1(mg144) *is an activating mutation in a* C. elegans *Akt/PKB homolog that relieves the requirement for AGE-1 PI3K signaling*

The almost complete genome sequence of *C. elegans* (Wilson et al. 1994) (A. Coulson, pers. comm.) reveals the presence of two Akt/PKB homologs that we named *akt-1* and *akt-2* (Fig. 1). An activating mutation (*mg144*) in *akt-1* was identified in a genetic screen for mutations that suppress the dauer arrest phenotype of the *age-1(mg44)* null mutant (Morris et al. 1996). This screen was designed to isolate reduction of function mutations in molecules negatively regulated by PI3K signaling, or gain of function mutations in molecules positively regulated by PI3K signaling. Ten independent suppressor mutations emerged from a screen of ~3800 haploid genomes. In addition to the dominant-activating *akt-1* mutation, two alleles of *daf-16*, a previously known negatively regulated target (Gottlieb and Ruvkun 1994; Larsen et al. 1995), were isolated [*daf-16(mg87)* (Ogg et al. 1997) and one other mutation that maps to the *daf-16* interval between *lin-11* and *unc-75* and is presumed to be allelic]. This suggests that the screen reveals genes that act in this insulin-like signaling pathway. Another dominant mutation, *mg142*, which suppresses multiple *age-1* alleles, and six mutations that vary in their ability to suppress multiple *age-1* alleles were also isolated in the screen.

The *mg144* mutation suppresses the three *age-1* alleles tested (Table 1; data not shown), including two classes of nonsense mutations (Morris et al. 1996) and one missense mutation in a conserved residue of PI3K. *mg144* is completely dominant for suppression of the dauer constitutive phenotype of *age-1(mg44)* [75.1% of the progeny of *age-1(mg44); mg144*/+ animals develop as nondauers, 24.9% arrest at the dauer stage, $n = 774$]. On its own, *mg144* does not have any obvious phentoypes (Table 1); the strain arrests as dauers on starved plates and on plates treated with pheromone, it moves normally, and has a normal vulva and brood size. Therefore *mg144* does not activate the AGE-1 PI3K signaling pathway to the point that normal dauer arrest is affected but does activate the pathway sufficiently to alleviate the requirement for AGE-1 PI3K outputs.

Using suppression of the dauer constitutive phenotype of *age-1(mg44)*, *mg144* was mapped to a region on chromosome V within 1.3 map units (m.u.) of the polymorphic STS marker *bP1* (Fig. 1A). This 1.3-m.u. region contains the *C. elegans* Akt/PKB homolog *akt-1* (Fig. 1A). Because an activating mutation in Akt/PKB is a good candidate to be a suppressor of an *age-1* PI3K null mutant, we determined the *akt-1* DNA sequence in the *mg144* strain by PCR amplification and direct sequencing. The *akt-1* gene in the *mg144* mutant strain bears an Ala183Thr substitution (Fig. 1D). *akt-1* is differentially spliced within the conserved kinase domain to generate the *akt-1a* and *akt-1b* isoforms with distinct kinase domain subregions IV, V, and VI (Hanks and Hunter 1995) (Fig. 1A,D) (92% identical, 238/258 amino acids over the entire kinase domain; 69% identical, 44/64 amino acids in the differentially spliced region). *akt-1a* is 58% identical to human Akt/PKBα overall (Fig. 1C,D). *akt-1* has a pleckstrin homology domain, kinase domain, and the two phosphorylation sites necessary for mammalian Akt/PKB activation (Alessi et al. 1996a), which are the hallmarks of the Akt/PKB family (Fig. 1D). The next most closely related mammalian kinase is rat PKCβ1,

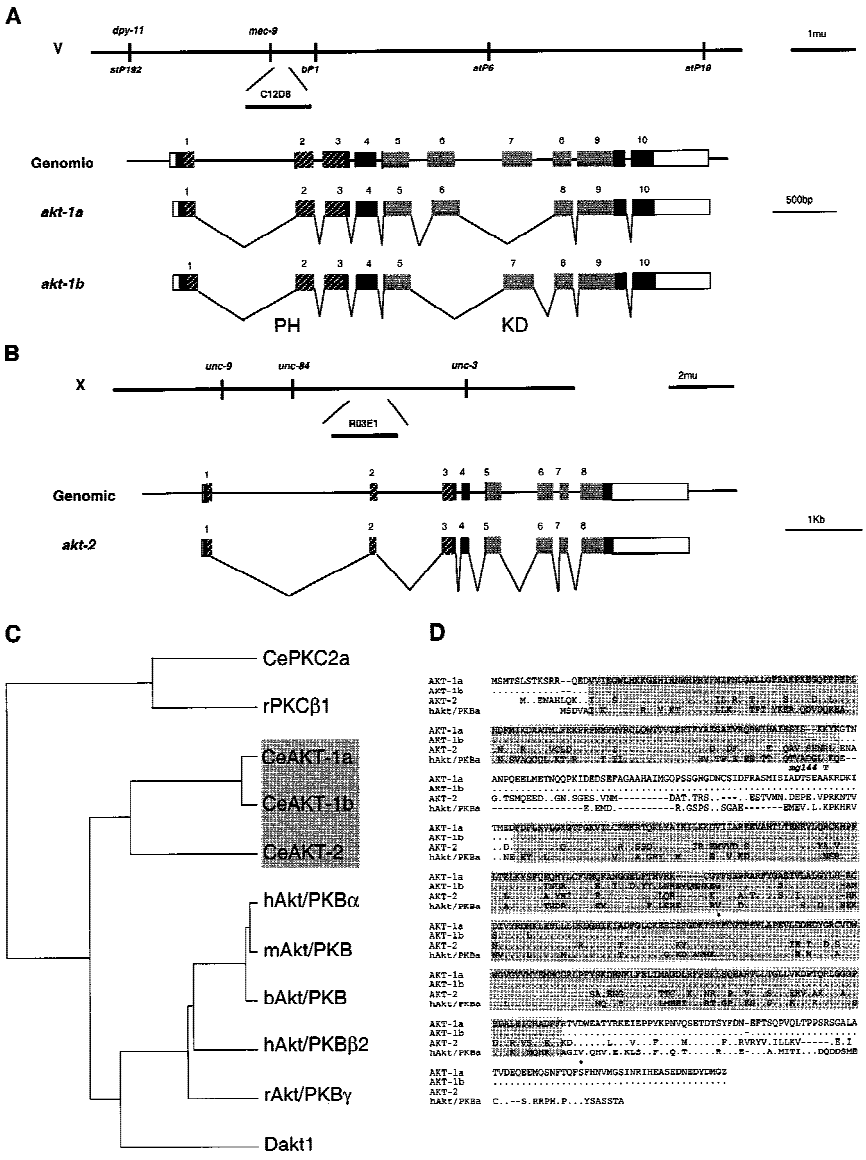

Figure 1. *akt-1* and *akt-2* encode Akt/PKB serine/threonine kinases. (*A*, *top*) Genetic and physical map of the *akt-1* region; *akt-1* is contained on cosmid C12D8. (*Bottom*) Exon/intron structure of *akt-1*. Coding regions are solid boxes, noncoding regions are open boxes, and introns are lines. The pleckstrin homology domain is indicated by hatched boxes (Musacchio et al. 1993), the kinase domain (Hanks and Hunter 1995) is indicated in gray. (*B*, *top*) Genetic and physical map of the *akt-2* region; *akt-2* is contained on cosmid R03E1. (*Bottom*) Exon/intron stucture of *akt-2*. All symbols are as in *A*. (*C*) Dendogram of Akt/PKB and PKC protein kinase families. PILEUP (GCG) was used to align the entire coding sequences of the indicated proteins. (Ce) *C. elegans* proteins; (r) rat; (h) human; (m) mouse; (b) bovine; (D) *D. melanogaster*. The GenBank accession numbers for the proteins used in the PILEUP are contained in parentheses: CePKC2a(U82935), rPKCβ1(M19007), hAkt/PKBα(M63167), mAkt/PKB(M94335), bAkt/PKB(X61036), hAkt/PKBβ2(M95936), rAkt/PKBγ(D49836), and Dakt1 (Z26242). The accession numbers for the proteins reported in the paper are contained in parentheses: AKT-1a (AF072379), AKT-1b (AF072380), and AKT-2 (AF072381). As an outgroup, rPKCβ1 (the closest non-Akt/PKB homolog to both *akt-1a* and hAkt/PKBα) and CePKC2a (the closest *C. elegans* homolog to rPKCβ1) were included in the PILEUP. The Akt/PKB homologs described in this report are indicated by the gray box. (*D*) AKT-1a, AKT-1b, AKT-2, and human Akt/PKBα (M63167) were aligned using PILEUP. Identical residues are indicated by dots; gaps introduced to align the sequence are indicated by dashes. The pleckstrin homology domain (Musacchio et al. 1993) is indicated by the amino-terminal shaded areas; the kinase domain (Hanks and Hunter 1995) is indicated by the carboxy-terminal shaded areas. The *mg144* Ala-183–Thr substitution is indicated as a T above the AKT-1a sequence (SEA**A**KRD). The AKT-1 and AKT-2 phosphorylation sites that correspond to the hAkt/PKBα Thr-308 and Ser-473 phosphorylation sites (Alessi et al. 1996a) are indicated as dots above the amino acid residue that is phosphorylated.

which is 38% identical to *akt-1a* overall. The *akt-1(mg144)* mutation is present in both splice forms of *akt-1* and is located in a region of the protein that links the amino-terminal pleckstrin homology domain to the carboxy-terminal kinase domain. This mutation is in a region that is not conserved between *C. elegans* and mammalian Akt/PKB and may reveal a negative regulatory region on *akt-1* because the *mg144* allele is an activating mutation (see below).

To confirm that the *mg144* suppression of *age-1* that is genetically linked to *akt-1* is due to a mutation in *akt-1*, we used a reverse genetic assay termed RNA interference (RNAi) (Fire et al. 1998; Rocheleau et al. 1997; Zhang et al. 1997) to decrease *akt-1* gene activity in an *age-1(mg44); akt-1(mg144)* strain. If a mutation in *akt-1* is responsible for the suppression of *age-1* observed in this strain, RNAi of *akt-1* in this strain should revert the suppression phenotype and result in a dauer constitutive phenotype. This experiment is conceptually similar to the classic genetic arguments that show that a *cis*-acting loss-of-function mutation can revert a gain-of-function mutation in the same gene. Inhibition of *akt-1* activity in an *age-1(mg44); akt-1(mg144)* strain reverts the *akt-1(mg144)* suppression phenotype (Table 2). Inhibition of *akt-1* activity in wild type does not induce dauer arrest (Table 2). Therefore we conclude that the *mg144*-activating mutation is a lesion in the *akt-1* locus.

The C. elegans *Akt/PKB homolog* akt-2 *functions redundantly with* akt-1 *to repress dauer formation and negatively regulate the DAF-16 transcription factor*

The *C. elegans* Akt/PKB homolog *akt-2* (Fig. 1B) is 55%

Table 1. *Effects of* akt-1(mg144) *on dauer formation*

Genotype of parent	Phenotype of progeny at 25°C (%)					
	L4 larvae and adult	dauer	partial dauer	dead eggs	other	*N*[a]
Wild type	100	0	0	0	N.D.	403
akt-1(mg144)	98.4	0	0	1.6	N.D.	314
sqt-1(sc13) age-1(mg44)[b]	0	82.2	0	3.2	14.6[c]	185
sqt-1(sc13) age-1(mg44); akt-1(mg144)[d,e]	88.2	0	0	0.2	11.6[f]	440
daf-16(m27); sqt-1(sc13) age-1(mg44)[d]	99.6	0	0	0.4	N.D.	269
unc-4(e120) age-1(m333)[b]	0	96.5	0	2.3	1.2	171
unc-4(e120) age-1(m333); akt-1(mg144)[d,g]	96.8	0	0	0	3.2	279
daf-2(e1370)	0	96.1	0	2.8	1.0	387
daf-2(e1370); akt-1(mg144)[h]	0	0	92.4	0	7.6	436
daf-16(m27); daf-2(e1370)	99.8	0	0	0	0.2	499

(N.D.) Not determined.
See Materials and methods for description of categories.
[a](*N*) Total number of animals scored. Numbers represent the summary of at least two trials of each genotype in at least two experiments performed on different days.
[b]These animals are the homozygous *age-1* progeny of *age-1*/balancer hermaphrodites.
[c]Nine of nine animals scored became dauers by 97 hr post-egg lay.
[d]These animals are the homozygous *age-1* progeny of homozygous *age-1* hermaphrodites.
[e]Rescoring of the entire assay at 96 hr post-egg lay: 90.5% of animals were gravid adults, 2.7% were sterile adults, 0.7% were dauers, and 6.1% were other (*N* = 147); *sqt-1(sc13) age-1(mg44)* animals remained arrested at the dauer stage.
[f]By 96 hr post-egg lay, 5/28 animals scored became gravid adults, 11/28 became dauers or partial dauers, 10/28 became sterile adults, and 2/28 remained as non-dauer-arrested larvae of various stages.
[g]Rescoring of the entire assay at 72 hr post-egg lay: 81.6% of animals were gravid adults, 17.8% were sterile adults, and 0.6% were other (*N* = 151); *unc-4(e120) age-1(m333)* animals remained arrested at the dauer stage.
[h]Rescoring of the entire assay at 96 hr post-egg lay: 11.5% of animals were gravid adults, 66.3% were sterile adults, 6.9% were dauers, 13.9% were partial dauers, and 1.4% were other (*N* = 418); *daf-2(e1370)* animals remained arrested at the dauer stage. In comparison, *daf-16(m27); daf-2(e1370)* animals formed 96.7% gravid adults, 2.9% sterile adults, and 0.4% other by 72 hr post-egg lay (*N* = 486).

identical to human Akt/PKBα overall and 35% identical to rat PKCβ1 overall. *akt-1* and *akt-2* are more closely related to each other (66% identity between *akt-1a* and *akt-2* overall) than to any other Akt/PKB homolog (Fig. 1C). Interestingly, *akt-2* only has the Thr308 phosphorylation site that is necessary for human Akt/PKBα activation by PDK1 (Alessi et al. 1997; Stokoe et al. 1997) but not the Ser473 phosphorylation site (Alessi et al. 1996a) (Fig. 1D) and yet clearly functions in the insulin-like signaling pathway (see below).

Reduction of both *akt-1* and *akt-2* activities revealed that they transduce insulin-like signals from the AGE-1 PI3K to the DAF-16 Fork head transcription factor. Inhibition of either *akt-1* or *akt-2* activity by RNAi does not cause dauer arrest (Table 2). Simultaneous inhibition of both *akt-1* and *akt-2* activities, however, causes nearly 100% arrest at the dauer stage (Table 2). We conclude that Akt/PKB signaling from either *akt-1* or *akt-2* is sufficient for reproductive development. This result indicates that *akt-1* and *akt-2* can function redundantly in the control of *C. elegans* reproductive development and metabolism and raises the possibility that various mammalian Akt/PKB isoforms could function redundantly as well. Significantly, the constitutive dauer arrest induced by inhibition of both *akt-1* and *akt-2* is fully suppressed by a null mutation in *daf-16* (Ogg et al. 1997) but is not suppressed by a null mutation in the Smad gene *daf-3* (Patterson et al. 1997) (Table 2), which places *akt-1* and *akt-2* upstream of *daf-16*. Because a null mutation in *daf-16* alleviates the need for *C. elegans* Akt/PKB signaling, the primary function of AKT-1 and AKT-2 is to antagonize DAF-16.

Genetic analysis of akt-1(mg144) *and overexpression of* akt-1*(+) and* akt-1(mg144)

akt-1(mg144) suppresses the dauer constitutive phenotype of three *age-1* alleles (Table 1; data not shown). The suppression of these *age-1* alleles by *akt-1(mg144)* is comparable with the suppression by *daf-16(m27)* (Table 1), a reduction of function allele (Lin et al. 1997). Because two of the *age-1* alleles that are suppressed by *akt-1(mg144)* are nonsense alleles, these data strongly argue that *akt-1* acts downstream of *age-1* and that activation of AKT-1 can bypass the normal requirement of upstream AGE-1 PI3K signaling.

The normal requirement of *age-1* activity for reproductive development is also bypassed by increased gene dosage of wild-type *akt-1*. Transgenic *age-1(mg44)* animals carrying a 7.3-kb *akt-1*(+) genomic region as a transgene can grow reproductively rather than arrest at the dauer stage (Table 3). This rescue is dependent on a conserved lysine residue implicated in mammalian Akt/PKB kinase activity (Franke et al. 1995) [*akt-1(KD);* Table 3]. In a similar experiment with *age-1(mg44)* animals carrying the same genomic region amplified from *akt-1(mg144),*

Table 2. *Effects of* akt-1(RNAi) *and* akt-2(RNAi) *on dauer formation*

Strain	dsRNA injected	Phenotype of progeny at 25°C (%)					
		L4 larvae and adult	dauer	partial dauer	dead eggs	other	N^a
Wild type	uninjected	99.8	0	0	0.2	N.D.	985
Wild type	*akt-1*	98.9	0	0	1.1	N.D.	542
Wild type	*akt-2*	97.3	0	0	2.7	N.D.	598
Wild type	*akt-1 + akt-2*	3.1	90.1[b]	4.2	1.0	1.6	2123
daf-16(mgDf50)	uninjected	99.0	0	0	1.0	0	968
daf-16(mgDf50)	*akt-1 + akt-2*	98.3	0	0	0.9	0.7	847
daf-3(mgDf90)	uninjected	98.9	0	0	1.1	N.D.	925
daf-3(mgDf90)	*akt-1 + akt-2*	11.6	85.8	2.0	0.6	N.D.	1051
age-1(mg44); akt-1(mg144)[c]	uninjected	87.5	0.2	0	1.9	10.5[d]	536
age-1(mg44); akt-1(mg144)[c]	*akt-1*	5.4	70.6	9.0	6.0	9.0	299
age-1(mg44); akt-1(mg144)[c]	*akt-2*	77.7	10.6	3.6	3.0	5.1	471
age-1(mg44)[e]	uninjected	0	82.2	0	3.2	14.6	185

(N.D.) Not determined.
See Materials and methods for description of categories.
[a](*N*) Total number of animals scored. Numbers represent the summary of progeny scored from at least two seperate injections of each genotype in at least two experiments performed on different days.
[b]Under the dissecting microscope animals were constricted and had a dark intestine like wild-type dauers. Using Nomarski optics, the dauers had dauer alae (10/10), but the pharynx was not a dauer-remodeled pharynx (10/10) and, unlike in dauers, pharyngeal pumping was observed in some larvae (4/8). On a given plate, some dauers appeared smaller than wild-type dauers.
[c]The full genotype of this strain is *sqt-1(sc13) age-1(mg44); him-8(e1489)*/+; *akt-1(mg144).*
[d]See Table 1 for discussion.
[e]These data are from Table 1 and were added here for ease of comparison. The full genotype of this strain is *sqt-1(sc13) age-1(mg44).*

less transgenic animals arrested as dauers than *age-1(mg44)* animals carrying the *akt-1*(+) transgene (Table 3), suggesting that the same 7.3-kb genomic region amplified from the *akt-1(mg144)* strain may be a more potent suppressor of *age-1(mg44)* than the *akt-1*(+) transgene. This data, like the RNAi of *akt-1(mg144)* using *akt-1* RNA, maps *mg144* to the 7.3-kb region of *akt-1* that includes the Ala-183-Thr substitution in AKT-1. Although multiple independent *akt-1(mg144)* transgenes are more potent suppressors of *age-1(mg44)* than *akt-1*(+) transgenes, which suggests that more *akt-1* gene activity is generated by *akt-1(mg144),* there is significant variation in the penetrance of suppression observed with different transgenes. In addition, even though *akt-1*(+) transgenes confer suppression of *age-1(mg44)* that is not observed with chromosomal *akt-1*(+), the penetrance of suppression of *age-1(mg44)* by either *akt-1*(+) or *akt-1(mg144)* transgenes is less than from *akt-1(mg144)*/+ heterozygotes or *akt-1(mg144)* homozygotes (see above; Tables 1 and 3). This may be attributable to mosaicism of *akt-1* gene expression from transgenic arrays or a saturation of *akt-1* gene function by high gene dosage.

Inactivation of *akt-1* and *akt-2* by RNAi causes dauer arrest, as do null mutations in *age-1*. Conversely, the dominant allele *akt-1(mg144)* or high gene dosage of *akt-1*(+) promotes reproductive growth even in an animal bearing an *age-1* null mutation. Therefore *akt-1(mg144)* has a similar genetic activity to increased *akt-1*(+) gene dosage and is an activating mutation, as opposed to a loss of function or dominant-negative mutation.

Because *akt-1* and *akt-2* function redundantly to repress dauer formation, we asked whether overexpression of *akt-2*(+) could also bypass the normal requirement of AGE-1 PI3K signaling. *age-1(mg44)* animals carrying the *akt-2*(+) transgene arrested as dauers whereas *age-1(mg44)* animals carrying the *akt-1*(+) transgene can bypass dauer (Table 3). Therefore either because of differences in the AKT-2 protein or differences in gene expression, high gene dosage of *akt-2* is not able to bypass the usual requirement for AGE-1 PI3K signaling.

A mutation in *daf-2* is suppressed more poorly by *akt-1(mg144)* than by a reduction of function mutation in *daf-16* (Table 1). The *age-1* alleles suppressed by *akt-1(mg144)* are null (Morris et al. 1996), whereas *daf-2(e1370)* is a temperature-sensitive mutation in the kinase domain (Kimura et al. 1997). This *daf-2* allele is suppressed completely by many *daf-16* alleles, including null alleles (Gottlieb and Ruvkun 1994; Larsen et al. 1995; Ogg et al. 1997). This result, in comparison to the robust suppression of *age-1* mutations by *akt-1(mg144)* (Table 1), suggests that AKT-1 is a major output of AGE-1 signaling and one of multiple outputs of DAF-2 signaling.

Overexpression of either *akt-1*(+) or *akt-1(mg144)* can bypass the need for DAF-2 signaling, whereas overexpression of *akt-2*(+) or *akt-1(KD)* does not alleviate the need for DAF-2 signaling (Table 3). *akt-1*(+) and *akt-1(mg144)* transgenes, however, are more efficient suppressors of the dauer constitutive phenotype of *age-1(mg44)* than of *daf-2(e1370)* (Table 3). This supports the model that AKT-1 is a primary output of AGE-1 signaling but is not the only output of DAF-2 signaling.

Life-span regulation

Reduction of zygotic *age-1* activity increases *C. elegans*

Table 3. *Effects of* akt-1 *and* akt-2 *transgenes on dauer formation*

Dauer constitutive mutation	Transgene	Phenotype at 25°C (%)			
		L4 larvae and adult	dauer	other[a]	*N*[b]
Wild type[c]	none	100	0	0	403
Wild type	*akt-1*(+)	97.6	0	2.4	454
Wild type	*akt-1(mg144)*	93.2	0	6.8[d]	414
Wild type	*akt-1(KD)*[e]	98.6	0	1.4	778
Wild type	*akt*-2(+)	99.3	0	0.7	570
age-1(mg44)[c,f]	none	0	82.2	17.8	185
age-1(mg44)[f]	*akt-1*(+)	66.7	32.0	1.3	228
age-1(mg44)[f]	*akt-1(mg144)*	88.3	7.6	4.1	341
age-1(mg44)[f]	*akt-1(KD)*[e]	0	100	0	58
age-1(mg44)[f]	*akt-2*(+)	0	100[g]	0	70
daf-2(e1370)[c]	none	0	96.1	3.9	387
daf-2(e1370)	*akt-1*(+)	35.3	63.1[h]	1.6	510
daf-2(e1370)	*akt-1(mg144)*	30.3	66.9	2.8	601
daf-2(e1370)	*akt-1(KD)*[e]	0	92.5	7.5	375
daf-2(e1370)	*akt-2*(+)	0	94.8	5.2	289

See Materials and methods for description of categories.
[a]This category includes dead eggs for the data imported from Table 1.
[b](*N*) Total number of transgenic animals scored. Numbers represent the summary of at least two separate trials from two independent transgenic lines of each genotype.
[c]These data are from Table 1 and were added here for ease of comparison.
[d]72 hrs post-egg lay: Of 17 of these animals, 9 were L4 larvae or adults and 8 remained as non-dauer-arrested larvae of various stages.
[e]*akt-1(KD)* is a kinase-defective mutation that changes a conserved lysine in the ATP binding site to a methionine and has been shown to cause mammalian Akt/PKB to be a nonfunctional kinase (Franke et al. 1995).
[f]The full genotype of this strain is *sqt-1(sc13) age-1(mg44).*
[g]2.9% of these worms were partial dauers (see Materials and Methods for description).
[h]7.4% of these worms were partial dauers (see Materials and Methods for description).

life span greater than twofold (Klass 1983; Larsen et al. 1995; Morris et al. 1996) (Table 4). Mutations in *daf-16* suppress this life-span increase (Dorman et al. 1995; Larsen et al. 1995). *akt-1(mg144)* does not suppress the *age-1(mg44)*-induced increase in life span (Table 4). Therefore *akt-1(mg144)* bypasses the need for AGE-1 signaling in reproductive development but does not activate normal aging pathways. It is possible that *akt-1(mg144)* does not subserve all the functions of the wild-type *akt-1*. *akt-2* or other as yet unidentified downstream effectors of *age-1* may be the pertinent signaling molecules for life-span regulation.

AKT-1/GFP and AKT-2/GFP are widely expressed

The expression patterns of both *akt-1* and *akt-2* were examined in transgenic animals containing a translational fusion of each genomic locus to green fluorescent protein (GFP) (Chalfie et al. 1994). The GFP fusion proteins contain the entire genomic coding region from either *akt-1* or *akt-2*, including 5′ upstream regulatory sequence, fused in frame at the carboxyl terminus to GFP. The AKT-1/GFP construct is sufficient to suppress the dauer constitutive phenotype of *age-1(mg44),* whereas the AKT-2/GFP construct is not (data not shown). This result is not unexpected because increased gene dosage of *akt-2*(+) does not suppress *age-1(mg44),* whereas increased gene dosage of *akt-1*(+) does (Table 3). AKT-1/GFP expression is first observed in late embryos and is maintained throughout the life of the animal. In postembryonic animals, AKT-1/GFP is expressed in the majority of head neurons including sensory neurons (Fig. 2A). Expression is also observed in motor neurons of the ventral and dorsal nerve cord and several other neuronal commissures throughout the body, and the tail neurons (Fig. 2B,C). The fusion protein is localized throughout the cell body and axonal and dendritic processes of neurons but is usually excluded from the nucleus (Fig. 2A–C). Additional tissues that consistently express AKT-1/GFP include neurons and muscle cells of the pharynx, the rectal gland cells, and the spermatheca (Fig. 2A,C,D). AKT-1/GFP expression was observed more variably in a variety of cell types including hypodermis, intestine, muscle, some of the P-cell descendants that form the vulva, and in the excretory canal.

Consistent with redundant roles of *akt-1* and *akt-2*, an AKT-2/GFP full-length protein fusion gene is expressed at the same times as AKT-1/GFP and in the same tissues that express AKT-1/GFP although AKT-2/GFP seems to be less abundant (Fig. 2E). These expression patterns are consistent with AKT-1 and AKT-2 functioning either in secretory neurons to regulate dauer arrest and metabolic shift or in the target tissues that are remodeled during dauer formation, such as the phayrnx, hypodermis, and intestine (Riddle and Albert 1997).

Discussion

The *C. elegans* Akt/PKB homologs AKT-1 and AKT-2 function to antagonize the DAF-16 transcription factor

Table 4. *Effects of* akt-1(mg144) *on life span extension of* age-1(mg44)

Strain	Life span (days)		
	mean	maximum	*N*[a]
Wild type	12	16	28
sqt-1(sc13) age-1(mg44)[b]	18	36	20
daf-16(m27); sqt-1(sc13) age1(mg44)[c]	14	16	32
sqt-1(sc13) age-1(mg44); akt-1(mg144)[c]	22	38	36

All mean life spans are significantly different ($P \leq 0.02$; see Materials and Methods) from each other except for *age-1(mg44)* vs. *age-1(mg44); akt-1(mg144).*
[a](*N*) Total number of animals scored. Numbers are from one representative experiment, which has been performed at least one other time and gave similar results.
[b]These animals are the homozygous *age-1* progeny of *age-1/* balancer hermaphrodites.
[c]These animals are the homozygous *age-1* progeny of *age-1* hermaphrodites.

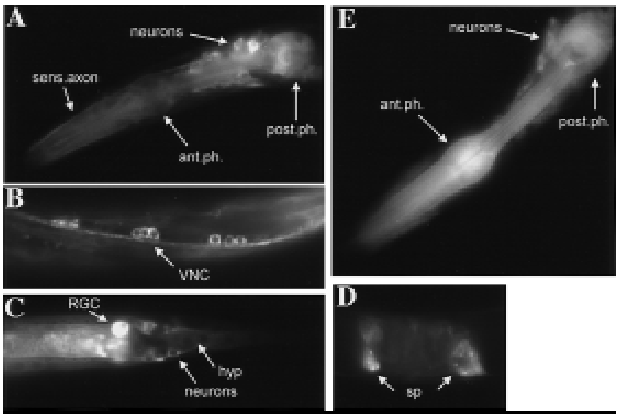

Figure 2. AKT-1/GFP and AKT-2/GFP expression. (*A–D*) AKT-1/GFP expression; (*E*) AKT-2/GFP expression. (*A*) AKT-1/GFP expression in the head of an L2 animal. Expression in the anterior and posterior bulb of the pharynx (ant.ph. and post.ph.) is shown; expression in the isthmus of the pharynx is also observed. Many neurons in the head express AKT-1/GFP; expression in the neuronal nuclei is not visible. Also, expression can be seen in sensory axons (sens.axon) that proceed to the nose of the animal. (*B*) AKT-1/GFP expression in the ventral nerve cord (VNC) of an L1 animal (anterior is to the *left*). Both cell bodies (nuclei do not appear to express AKT-1/GFP) and axons of the VNC are clearly visible. Animal appears twisted because of coinjection with the *rol-6* marker. (*C*) AKT-1/GFP expression in the tail of an L1 animal. The rectal gland cell (RGC), neurons of the tail with axons, and hypodermal cells (hyp) all clearly express AKT-1/GFP. AKT-1/GFP expression is not visible in the nuclei. (*D*) AKT-1/GFP expression in the spermatheca (sp) of an adult animal. (*E*) AKT-2/GFP expression in the head of an L4 animal. Expression in the anterior and posterior bulb of the pharynx (ant.ph. and post.ph.) is indicated; expression in the isthmus of the pharynx is also visible. Also shown is AKT-2/GFP expression in many neurons in the head; the nuclei appear to be excluded from AKT-2/GFP expression. AKT-2/GFP expression in the VNC, tail, and spermatheca was similar to that observed for AKT-1/GFP.

and repress the metabolic shift and growth arrest associated with the dauer stage. Both AKT-1 and AKT-2 transduce DAF-2 signals because both gene activities must be decreased to cause dauer arrest. Because a null mutation in *daf-16* bypasses the normal requirement for *C. elegans* Akt/PKB signaling, the primary function of AKT-1 and AKT-2 is to antagonize DAF-16. Interestingly, DAF-16 contains four consensus sites for phosphorylation by Akt/PKB (Alessi et al. 1996b) and three of these sites are conserved in the human DAF-16 homologs FKHRL1, FKHR, and AFX (Fig. 3C). AKT-1 and AKT-2 in *C. elegans* (and Akt/PKB in mammals) may exert their negative regulatory effect by directly phosphorylating DAF-16 (and FKHRL1, FKHR, and AFX in mammals) and altering its transcriptional regulatory function.

The activating mutation *akt-1(mg144)* as well as overexpression of *akt-1*(+) bypasses the normal requirement for AGE-1 PI3K signaling in the DAF-2 insulin receptor-like signal transduction pathway. These results demonstrate that *C. elegans* Akt/PKB gene activity is not strictly dependent on upstream *age-1* activity if Akt/PKB activity is increased. In the almost complete *C. elegans* genome sequence, AGE-1 is the only PI3K homolog of the type known to generate 3-phosphoinositides (C.A. Wolkow and G. Ruvkun, pers. comm.). If AGE-1 is the only protein able to generate 3-phosphoinositides in *C. elegans*, our results suggest that although normal AKT-1 signaling is dependent on 3-phosphoinositides, AKT-1 can become activated in their absence if gene dosage is increased or the *mg144* mutation is introduced.

Importantly, either activated *akt-1* or higher *akt-1*(+) gene dosage does not efficiently suppress mutations in the DAF-2 insulin receptor suggesting that *age-1* and *akt-1* constitute one major signaling pathway from DAF-2 and that other, as yet unidentified genes, constitute one or more parallel pathways. These pathways most likely converge on the DAF-16 Fork head transcription factor and negatively regulate its activity, as loss-of-function mutations in *daf-16* completely suppress both *daf-2* and *age-1* mutations (Gottlieb and Ruvkun 1994; Larsen et al. 1995), as well as inactivation of *akt-1* and *akt-2* signaling.

Although AKT-1 and AKT-2 appear to function redundantly in transduction of DAF-2/AGE-1 signals, increased *akt-1* gene dosage is a much more potent suppressor of *age-1* null mutations than increased *akt-2* gene dosage. A major distinction between AKT-1 and AKT-2 is that AKT-1 bears two phosphorylation sites (corresponding to Thr-308 and Ser-473 in human Akt/PKBα) (Fig. 1D) that are necessary for activation of Akt/PKB by upstream growth factor inputs (Alessi et al. 1996a), whereas AKT-2 only has the Thr-308 phosphorylation site (Fig. 1D). In mammals, Akt/PKB is phosphorylated at Thr-308 by PDK1 and at Ser-473 by the as yet unpurified PDK2 (Alessi et al. 1997; Stokoe et al. 1997). Therefore AKT-1 may couple to a PDK2-like kinase whereas AKT-2 cannot do so. AKT-1 and AKT-2 may also differ in other kinase inputs or in their substrates. Interestingly, at lower temperatures, the *akt-2*(+) transgene can supply sufficient Akt/PKB activity to weakly suppress the dauer arrest caused by *age-1(mg44)* (S. Paradis and G. Ruvkun, unpubl.). Temperature is a major modulator of dauer arrest (Riddle and Albert 1997). The penetrance of dauer arrest in most dauer constitutive mutants is increased at high temperatures (Riddle and Albert 1997), suggesting that some signals in the pathway are enhanced at low temperature. Therefore at low temperatures perhaps PDK1 signaling to AKT-1 and AKT-2 or signaling in pathways parallel to AGE-1/AKT-1/AKT-2 are enhanced, now allowing increased *akt-2*(+) gene dosage to weakly bypass the normal requirement for AGE-1 PI3K signaling.

Insulin-like and transforming growth factor-β (TGF-β) neuroendocrine signals regulate whether animals arrest at the dauer stage or grow to reproductive adults (Kimura et al. 1997; Riddle and Albert 1997). The TGF-β-like molecule DAF-7 is a probable neuroendocrine signal—it is expressed in the sensory neuron ASI that represses dauer arrest (Bargmann and Horvitz 1991) and its expression is regulated by dauer-inducing pheromone (Ren et

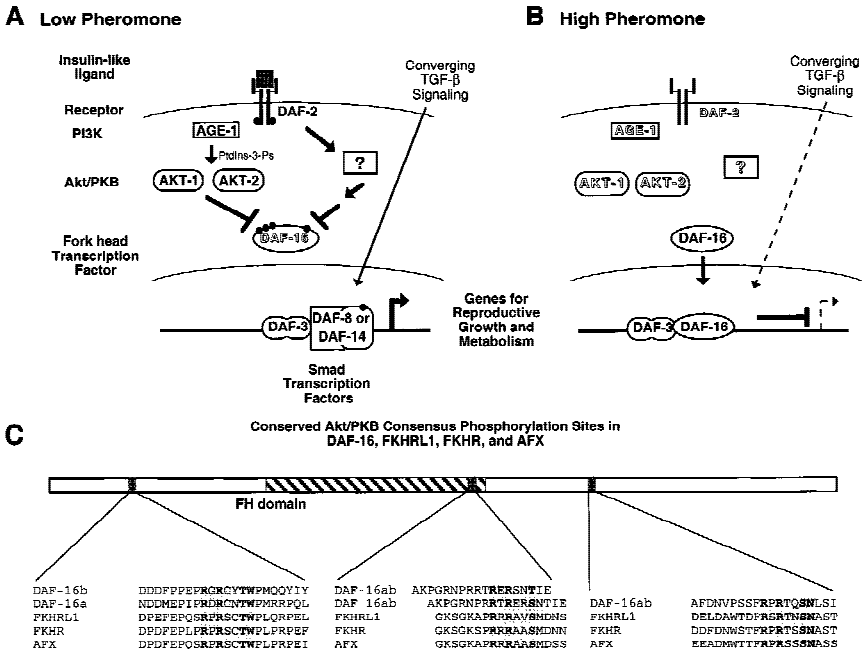

Figure 3. Model for regulation of dauer formation by AGE-1 PI3K activation of AKT-1/AKT-2. (*A*) Normal growth occurs in conditions of low pheromone as a result of DAF-2 insulin receptor-like signaling and converging TGF-β signaling; DAF-16 is possibly inactivated by phosphorylation in these growth conditions. (See text for details.) (*B*) Dauer arrest occurs in conditions of high pheromone that cause lack of DAF-2 insulin receptor-like signaling and converging TGF-β signaling; DAF-16 may repress transcription in these conditions. (See text for details.) (*C*) Location and sequence context of consensus Akt/PKB phosphorylation sites in DAF-16 and the human homologs FKHRL1, FKHR, and AFX. *daf-16* is differentially spliced to produce two gene products (DAF-16a and DAF-16b) that differ in the amino-terminal one-third of the protein but are identical (DAF-16ab) for the remainder of the protein. The consensus Akt/PKB phosphorylation site, RXRXXS/THyd (Alessi et al. 1996b), is boxed in gray and the amino acid residues identical in that site in all proteins are in boldface type. Other amino acid residues flanking these sites are also identical or show conservative substitutions in all proteins but are not in boldface type. The sites are located in the same relative regions of each protein, near the amino terminus, at the carboxy-terminal region of the Fork head DNA-binding domain (but downstream of the DNA recognition helix) and downstream of the Fork head domain. Note that two adjacent Akt/PKB consensus sites occur within the Fork head domain of DAF-16 and are shown aligned with a single Akt/PKB consensus site in FKHRL1, etc.

al. 1996; Schackwitz et al. 1996). Although the insulin-like ligand for the DAF-2 insulin-like receptor has not yet been identified, it may also be produced by secretory neurons and regulated by pheromone. Precedence from biochemical analysis predicts that DAF-2, AGE-1, AKT-1/AKT-2, and DAF-16 function in the same cells. It is not yet clear whether the DAF-2 signaling pathway acts in the target tissues that are remodeled in dauer larvae, such as the pharynx, hypodermis, and intestine, or in other signaling cells that in turn control target tissues. The broad expression pattern of *akt-1* and *akt-2* includes the nervous system, pharynx, and hypodermis. This expression pattern is consistent with a role for these genes either in sensory neurons that signal to repress dauer arrest or in the target tissues that receive the dauer repressing signal. The expression patterns of *daf-2* and *age-1* have not been reported; *daf-16* is widely expressed (Ogg et al. 1997) as are *daf-3* and *daf-4*, two genes that comprise the DAF-7 TGF-β signal reception pathway (Patterson et al. 1997). Mosaic or tissue-specific expression analysis will be required to demonstrate in which cell types the DAF-2 insulin-like and DAF-1/DAF-4 TGF-β signal transduction pathways act.

The role of AKT-1 and AKT-2 in regulating the metabolic shift and developmental arrest associated with dauer formation suggests the following model (Fig. 3). Under normal growth conditions (Fig. 3A), an insulin-like molecule binds to the DAF-2 insulin receptor kinase inducing autophosphorylation and recruitment of AGE-1 PI3K. A parallel pathway (or pathways) from the DAF-2 insulin receptor-like protein is also activated. The AKT-1 and AKT-2 kinases, as well as molecules from the parallel pathway, negatively regulate DAF-16 activity, possibly via phosphorylation. Phosphorylated DAF-16 could be inactive, function to activate genes required for reproductive growth and metabolism, or repress genes required for dauer arrest and energy storage. Other signaling molecules that are activated by DAF-2 must also converge downstream of AGE-1 (e.g., on DAF-16 or AKT-1/AKT-2) for proper regulation of metabolism and life span. The dauer arrest induced by loss of AGE-1 PI3K or AKT-1/AKT-2 activity implies that the loss of only one of these inputs to DAF-16 is sufficient to cause dauer arrest. Under dauer-inducing conditions, DAF-2, AGE-1, AKT-1/AKT-2, and other signaling pathways from DAF-2 are inactive and therefore DAF-16 is active, presumably because it is under-phosphorylated (Fig. 3B). Active DAF-16 either represses genes required for reproductive growth and metabolism or activates genes necessary for dauer arrest and energy storage.

The DAF-16 Fork head protein has been suggested to interact with the DAF-3, DAF-8, or DAF-14 Smad proteins to integrate converging TGF-β-like neuroendocrine signals with insulin-like signals (Ogg et al. 1997; Patterson et al. 1997). DAF-16 may form a complex with the DAF-3 Smad protein under dauer-inducing conditions to regulate these downstream genes (Ogg et al. 1997) and AKT-1/AKT-2 phosphorylation of DAF-16 may inhibit the formation of a Smad/Fork head complex during reproductive development.

Akt/PKB has been implicated in mammalian insulin receptor signaling that localizes glucose transporters to the plasma membrane (Kohn et al. 1996) and has been shown to regulate glycogen synthesis via direct phosphorylation of GSK3 (Cross et al. 1995); two events that are not transcriptional. Whereas there also may be such Akt/PKB outputs in *C. elegans*, the DAF-16 Fork head transcription factor represents the major output of DAF-2/AGE-1/AKT-1/AKT-2 insulin receptor-like signaling (Ogg et al. 1997). Similarly Akt/PKB action in the insulin/IGF-I anti-apoptotic pathway (Dudek et al. 1997; Kauffmann-Zeh et al. 1997; Kulik et al. 1997) may also converge on transcription factors related to DAF-16.

Our model, based on genetic evidence that Akt/PKB couples insulin receptor-like signaling to transcriptional output via the DAF-16 Fork head transcription factor in *C. elegans*, predicts that Akt/PKB will have transcriptional outputs in insulin-like signaling across phylogeny. Human homologs of DAF-16 may be the pertinent downsteam effectors of insulin signaling (Ogg et al. 1997). Two of the consensus Akt/PKB sites conserved in DAF-16 and its human homologs are located outside of the Fork head DNA-binding domain, and two sites are located in the highly basic W2 region of the Fork head domain that has been shown to mediate DNA phosphate backbone contacts (Clark et al. 1993) and possibly nuclear localization (Qian and Costa 1995) (Fig. 3C). Insulin-stimulated Akt/PKB phosphorylation of the W2 sites may affect DNA nuclear localization or binding, whereas the other conserved sites may affect transactivation. A recent report shows that Akt/PKB mediates insulin-dependent repression of the insulin-like growth factor binding protein-1 (IGFBP-1) gene in HepG2 cells via a conserved insulin response sequence (CAAAAC/TAA) (Cichy et al. 1998). Interestingly, DAF-16 binds to this same insulin response sequence in vitro (N. Nasrin, S. Ogg, G. Ruvkun, and M. Alexander-Bridges, pers. comm.). We propose that Akt/PKB mediates its transcriptional effects on insulin-responsive genes such as IGFBP-1 via the human homologs of DAF-16: FKHRL1, FKHR, and AFX.

Materials and methods

akt-1(mg144) *isolation and mapping*

A parent strain carrying the *sqt-1(sc13) age-1(mg44)* chromosome balanced with the crossover suppressor chromosome *mnC1* was mutagenized with ethylmethanesulfonate and more than 3800 haploid genomes screened in the F_2 or F_3 generation for zygotic or maternal effect suppressors of *age-1(mg44)* by scoring for animals that did not arrest at the dauer stage. Ten independent suppressor loci emerged from the screen, one of which is *akt-1(mg144).* Mapping was performed by crossing *sqt-1(sc13) age-1(mg44)*/+; *akt-1(mg144)*/+ males to the polymorphic strain RW7000 hermaphrodites. *sqt-1(sc13) age-1(mg44); akt-1(mg144)* animals were isolated and assayed by PCR for various STS polymorphisms on each chromosome as described (Williams 1995). Of nine animals, one had the chromosome V marker *bP1,* whereas markers from other chromosomes assorted independently; we therefore conclude that *mg144* is linked to chromosome V. An additional 30 *sqt-1(sc13) age-1(mg44); akt-1(mg144)* animals from this cross were assayed for the chromosome V markers *stP192, bP1, stP6,* and *stP18* (Fig. 1A). Of 30 animals, 2 carried both *stP6* and *stP18*. Because none carried *bP1,* the two-factor map distance between *bP1* and *mg144* is 1/39 recombinants, or 1.3 m.u. Additionally, this mapping experiment places *mg144* left of *stP6* and either left of *bP1* or close to *bP1* on the right.

Allele sequencing

Genomic DNA from *akt-1(mg144)* and *age-1(mg109)* strains was PCR-amplified and directly sequenced. Sequencing of the *age-1(mg109)* strain revealed that the previous report of this mutation is incorrect (Morris et al. 1996). The missense mutation in *age-1(mg109)* changes a conserved serine to an asparagine in the kinase domain (Ser-826-Asn, VLG**S**AKRP).

cDNA characterization

akt-1a gene structure was confirmed by sequencing of cDNAs (yk551d10, yk450a7 provided by Y. Kohara (National Institute of Genetics, Mishima, Japan) and CEESY35 provided by The Institute for Genomic Research (Rockville, MD) and by 5′ RACE (GIBCO) using a gene-specific primer to exon 6 for reverse transcription. *akt-1b* gene structure was confirmed by sequencing of a cDNA (yk131h3 provided by Y. Kohara) that confirms exon 5 through the 3′ UTR only and 5′ RACE (GIBCO) with a gene-specific primer to exon 7 for reverse transcription. The *akt-1a* and *akt-1b* messages analyzed by 5′ RACE were *trans*-spliced with an SL1 leader sequence 49 bp upstream of the first methionine. *akt-2* gene structure was confirmed by sequencing of a cDNA (yk232g7 provided by Y. Kohara) and 5′ RACE (GIBCO) using a gene-specific primer to exon 4 for reverse transcription. The *akt-2* message analyzed by 5′ RACE was *trans*-spliced with a SL1 leader sequence 7 bp upstream of the first methionine.

Scoring of akt-1(mg144) *effects on dauer constitutive mutations*

Gravid adults were allowed to lay eggs for 3 hr at 25°C. Progeny were scored at 48 hr post-egg lay for dead eggs, between 50–62 hr post-egg lay for all other categories, and some strains were rescored at 72 or 96 hr post-egg lay. For all assays performed for this study, the following scoring categories were used. Scoring with a dissecting microscope, ''partial dauer'' refers to animals that were arrested and had a dark intestine like *age-1* or *daf-2* dauers but were not as fully constricted as *age-1* or *daf-2* dauers; or animals that were arrested and constricted like *age-1* or *daf-2* dauers but did not have as dark an intestine as *age-1* or *daf-2* dauers. ''Other'' refers to animals that could not be classified as dauer, etc. because the animal was young, was male, had grossly aberrant morphology, or was dead. Sometimes this class was not determined because the F_2 generation was growing on the plate when the plate was scored.

Construction of akt-1 *and* akt-2 *transgenic lines and scoring of* akt-1 *and* akt-2 *transgene effects on dauer constitutive mutations*

A 7.3-kb PCR product of genomic DNA from the *akt-1*(+) or *akt-1(mg144)* genomic region containing 3.2 kb of 5′ upstream regulatory sequence (the next gene on the cosmid, transcribed in the same direction, ends ~5 kb 5′ to *akt-1*), 3.5 kb of coding sequencing containing introns and exons, and 0.6 kb of *akt-1* 3′ UTR was purified using QIAquick (Qiagen) and injected at 10 ng/ml with *ttx-3*::GFP at 50 ng/μl (pPD95.75-C40H5-GFP, O. Hobert, MGH and Harvard Medical School) as a coinjection

marker (Mello et al. 1991). *akt-1(KD)* was constructed and injected in the same manner except PCR primers were used to introduce the K → M mutation. A 5.9-kb PCR product of genomic DNA from the *akt-2*(+) genomic region comprising 2.1 kb of 5′ upstream regulatory sequence (because the cosmid was in shotgun sequencing status at the time so the location of a 5′ upstream gene could not be determined, a conservative amount of upstream regulatory sequence was used), 3.1 kb of coding sequence including introns and exons, and 0.7 kb of *akt-2* 3′ UTR was purified and injected as for *akt-1* constructs. Once transgenic lines were established in wild type, they were crossed into either *age-1(mg44)* or *daf-2(e1370)* mutant backgrounds. To score the arrays in a wild type background, a 3-hr egg lay was performed at 25°C. Transgenic progeny from the egg lay were scored ~48 hr post-egg lay as dauer or nondauer (L4 larvae, gravid adult, or sterile adult); some additional progeny were scored at ~72 hr post-egg lay. Initially, six independent *akt-1(mg144)* transgenes and four independent *akt-1*(+) transgenes were crossed into *age-1(mg44).* Five of six *akt-1(mg144)* transgenes were able to partially suppress *age-1(mg44),* whereas one of six did not. Four of four *akt-1*(+) trangenes partially suppressed *age-1(mg44).* To collect the data in Table 3, two independent *akt-1(mg144)* transgenes and two independent *akt-1*(+) transgenes in an *age-1(mg44)* background were used to perform a 5-hr egg lay at 25°C. Transgenic progeny from the egg lay were scored ~72 hr post egg lay as dauer or nondauer (gravid or sterile adult). To score *daf-2(e1370)* suppression by the various transgenes gravid adults were shifted to 25°C and allowed to lay a brood. Progeny were scored approximately 48 hr or 72 hr postshift for *akt-1(KD)* and *akt-2*(+) transgenic animals and ~96 hr postshift for *akt-1*(+) and *akt-1(mg144)* transgenic animals as dauer or non-dauer (gravid or sterile adult).

RNAi

The *akt-1* coding region was amplified from CEESY35 (The Institute for Genomic Research) by PCR (primers CMO24 5′-TTGTAAAACGACGGCCAG and CMO25 5′-CATGATTACGCCAAGCTC). The *akt-2* coding region was amplified by PCR from yk232g7 (Y. Kohara) using CMO24 and CMO25. RNA was transcribed using MEGAscript T3 and T7 kit (Ambion). Single-stranded RNAs were combined before injection and stored on ice during injection. Approximately 5 µg/µl double-stranded RNA for single-gene injections and ~2.5 µg/µl double-stranded RNA per gene for double-gene injections was injected into the gut of L4 hermaphrodites. Animals were allowed to recover for 24 hr at 20°C then moved to fresh plates and allowed to lay eggs for 24 hr at 25°C. Broods were scored for dead eggs day 1 post-egg lay, and for dauers or L4 larvae and adults day 2 post-egg lay.

Life span assays

Animals were synchronized by performing an egg lay at 25°C for 3 hr. Four days after egg lay, the animals were transferred to new plates (five animals/plate) and the animals were scored and moved away from their progeny every two days while the animals were laying their brood; animals were scored every 2–3 days subsequently and moved periodically to keep growth conditions mold free. Animals were scored as dead if they failed to respond to a gentle tap on the head and tail with a platinum wire. Life span is defined as day of egg lay ($t = 0$) to day the worm was scored as dead. A *t*-test was performed to compare mean life spans of each strain pairwise.

akt-1 *and* akt-2 *expression*

The AKT-1/GFP translational fusion was constructed as follows. A 6.7-kb PCR product of genomic DNA from *akt-1* genomic region comprising 3.2 kb of 5′ upstream regulatory region and 3.5 kb of coding region including exons and introns was fused by PCR in-frame to a GFP with *unc-54* 3′ UTR PCR product from pPD95.75 (A. Fire, Carnegie Institute of Washington, Baltimore, MD). This 5′ upstream regulatory region is the same as that used for the *age-1(mg44)* suppression experiment (see text). PCR products were purified using QIAquick (Qiagen) and injected with *rol-6* (pRF4, 100 ng/µl) as the coinjection marker (Mello et al. 1991). The AKT-2/GFP translational fusion was constructed similarly using a 5.2-kb PCR product from the *akt-2* genomic region comprising 2.1 kb of 5′ upstream regulatory region and 3.1 kb of coding region, including exons and introns. This 5′ upstream regulatory region is the same as that used for the *age-1(mg44)* suppression experiment (see text).

Acknowledgments

We thank Y. Liu for expert technical assistance; C.A. Wolkow, N. Nasrin, S. Ogg, and M. Alexander-Bridges for sharing results prior to publication; O. Hobert, B. Reinhart, G. Patterson, and J. Sze for assistance with cell identifications; Y. Kohara and The Institute for Genomic Research for providing cDNAs; C. Mello for advice on the RNAi technique; M. Robinson for statistical anaylsis; and members of the Ruvkun lab for helpful comments and suggestions and critical reading of the manuscript. Some of the strains were provided by the *C. elegans* Genetics Center, which is supported by the National Center for Research Resources. This work was supported by National Institutes of Health grant RO1AG14161.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked ''advertisement'' in accordance with 18 USC section 1734 solely to indicate this fact.

References

Alessi, D.R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B.A. Hemmings. 1996a. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15:** 6541–6551.

Alessi, D.R., F.B. Caudwell, M. Andjeclkovic, B.A. Hemmings, and P. Cohen. 1996b. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett.* **399:** 333–338.

Alessi, D.R., S.R. James, C.P. Downes, A.B. Holmes, P.R.J. Gaffney, C.B. Reese, and P. Cohen. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Curr. Biol.* **7:** 261–269.

Avruch, J. 1998. Insulin signal transduction through protein kinase cascades. *Mol. Cell. Biochem.* **182:** 31–48.

Bargmann, C.I. and H.R. Horvitz. 1991. Control of larval development by Chemosensory neurons in *Caenorhabditis elegans. Science* **251:** 1243–1246.

Carpenter, C.L. and L.C. Cantley. 1996. Phosphoinositide kinases. *Curr. Opin. Cell Biol.* **8:** 153–158.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* **263:** 802–805.

Cichy, S.B., S. Uddin, A. Danilkovich, S. Guo, A. Klippel, and T.G. Unterman. 1998. Protein kinase B/Akt mediates effects of insulin on hepatic insulin-like growth factor-binding protein-1 gene expression through a conserved insulin response sequence. *J. Biol. Chem.* **273:** 6482–6487.

Clark, K.L., E.D. Halay, E. Lai, and S.K. Burley. 1993. Co-crystal structure of the HNF-3/*fork head* DNA-recognition motif resembles histone H5. *Nature* **364:** 412–420.

Cross, D.A.E., D.R. Alessi, P. Cohen, M. Andjelkovich, and B.A. Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378:** 785–789.

Dorman, J.B., B. Albinder, T. Shroyer, and C. Kenyon. 1995. The *age-1* and *daf-2* genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*. *Genetics* **141:** 1399–1406.

Dudek, H., S.R. Datta, T.F. Franke, M.J. Birnbaum, R. Yao, G.M. Cooper, R.A. Segal, D.R. Kaplan, and M.E. Greenberg. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275:** 661–665.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391:** 806–811.

Franke, T.F., S.-I. Yang, T.O. Chan, K. Datta, A. Kaziauskas, D.K. Morrison, D.R. Kaplan, and P.N. Tsichlis. 1995. The protein kinase encoded by the *Akt* proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81:** 727–736.

Franke, T.F., D.R. Kaplan, L.C. Cantley, and A. Toker. 1997. Direct regulation of the *Akt* proto-oncogene product by phoshatidylinositol-3,4-bisphosphate. *Science* **275:** 665–668.

Frech, M., M. Andjelkovic, E. Ingley, K.K. Reddy, J.R. Falck, and B.A. Hemmings. 1997. High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J. Biol. Chem*. **272:** 8474–8481.

Gottlieb, S. and G. Ruvkun. 1994. *daf-2, daf-16,* and *daf-23:* Genetically interacting genes controlling dauer formation in *Caenorhabditis elegans*. *Genetics* **137:** 107–120.

Hanks, S.K. and T. Hunter. 1995. The eukaryotic protein kinase superfamily. In *The protein kinase facts book protein–serine kinases* (ed. G. Hardie and S. Hanks), pp. 7–47. Academic Press, San Diego, CA.

Kahn, C.R. 1994. Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* **43:** 1066–1084.

Kapeller, R. and L.C. Cantley. 1994. Phosphatidylinositol 3-kinase. *BioEssays* **16:** 565–576.

Kauffmann-Zeh, A., P. Rodriguez-Viciana, E. Ulrich, C. Gilbert, P. Coffer, J. Downward, and G. Evan. 1997. Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385:** 544–548.

Kenyon, C., J. Chang, E. Gensch, A. Rudner, and R. Tabtlang. 1993. A *C. elegans* mutant that lives twice as long as wild type. *Nature* **366:** 461–464.

Kimura, K., H.A. Tissenbaum, Y. Liu, and G. Ruvkun. 1997. *daf-2,* an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science* **277:** 942–946.

Klass, M. 1983. A method for the isolation of longevity mutants in the nematode *C. elegans* and initial results. *Mech. Aging Dev.* **22:** 279–286.

Klippel, A., W.M. Kavanaugh, D. Pot, and L.T. Williams. 1997. A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell. Biol.* **17:** 338–344.

Kohn, A.D., S.A. Summers, M.J. Birnbaum, and R.A. Roth. 1996. Expression of constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem*. **271:** 31372–31378.

Kulik, G., A. Klippel, and M.J. Weber. 1997. Antiapoptotic signalling by the insulin-like growth factor I receptor, phophatidylinositol 3-kinase, and Akt. *Mol. Cell. Biol.* **17:** 1595–1606.

Larsen, P. L., P. S. Albert, and D. L. Riddle. 1995. Genes that regulate both development and longevity in *Caenorhabditis elegans. Genetics* **139:** 1567–1583.

Lin, K., J.B. Dorman, A. Rodan, and C. Kenyon. 1997. *daf-16:* An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans. Science* **278:** 1319–1322.

Mello, C.C., J.M. Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in *C. elegans:* Extrachromosomal maintenance and integration transforming sequences. *EMBO J.* **10:** 3959–5970.

Morris, J.Z., H.A. Tissenbaum, and G. Ruvkun. 1996. A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhaditis elegans. Nature* **382:** 536–539.

Musacchio, A., T. Gibson, P. Rice, J. Thomson, and M. Saraste. 1993. The PH domain: A common piece in the structural patchwork of singalling proteins. *Trends Biochem. Sci.* **18:** 343–348.

Ogg, S., S. Paradis, S. Gottlieb, G.I. Patterson, L. Lee, H.A. Tissenbaum, and G. Ruvkun. 1997. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity singals in C. elegans. *Nature* **389:** 994–999.

Patterson, G., A. Koweek, A. Wong, Y. Liu, and G. Ruvkun. 1997. The DAF-3 Smad protein antagonizes TGF-β-related receptor signaling in the *Caenorhabditis elegans* dauer pathway. *Genes & Dev.* **11:** 2679–2690.

Qian, X. and R.H. Costa. 1995. Analysis of hepatocyte nuclear factor-3β protein domains required for transcriptional activation and nuclear targeting. *Nucleic Acids Res.* **23:** 1184–1191.

Ren, P., C.-S. Lim, R. Johnsen, P.S. Albert, D. Pilgrim, and D.L. Riddle. 1996. Control of C. elegans larval development by neuronal expression of a TGF-β homolog. *Science* **274:** 1389–1392.

Riddle, D.L. and P.S. Albert. 1997. Genetic and environmental regulation of dauer larva development. In C. elegans *II* (ed. D. L. Riddle, T. Blumenthal, B.J. Meyer and J.R. Priess), pp. 739–768. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rocheleau, C.E., W.D. Downs, R. Lin, C. Wittmann, Y. Bei, Y.-H. Cha, M. Ali, J.R. Priess, and C.C. Mello. 1997. Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. *Cell* **90:** 707–716.

Schackwitz, W.S., T. Inoue, and J.H. Thomas. 1996. Chemosensory neurons function in parallel to mediate a pheromone response in *C. elegans. Neuron* **17:** 719–728.

Stokoe, D., L.R. Stephens, T. Copeland, P.R.J. Gaffney, C.B. Reese, G.F. Painter, A.B. Holmes, F. McCormick, and P.T. Hawkins. 1997. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277:** 567–570.

Toker, A. and L.C. Cantley. 1997. Signalling through the lipid products of phophoinositide-3-OH kinase. *Nature* **387:** 673–676.

Williams, B.D. 1995. Genetic mapping with polymorphic sequence-tagged sites. In Caenorhabditis elegans *modern biological analysis of an organism* (ed. H.F. Epstein and D.C. Shakes), pp. 81–97. Academic Press, San Diego, CA.

Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Burton, M. Connell, J. Bonfield, T. Copsey, J. Cooper et al. 1994. 2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans . *Nature* **368:** 32–38.

Zhang, B., M. Gallegos, A. Puoti, E. Durkin, S. Fields, J. Kimble, and M. Wickens. 1997. A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390:** 477–484.