An insulin-like signaling pathway regulates development and lifespan in Caenorhabditis elegans. Genetic screens that identified many components of the C. elegans insulin pathway did not identify homologs of insulin receptor substrates or the phosphoinositide 3-kinase (PI3K) adaptor/regulatory subunit, which are both required for signaling by mammalian insulin/insulin-like growth factor 1 pathways. The C. elegans genome contains one homolog of each protein. The C. elegans versions of insulin receptor substrate (IST-1) and PI3K p50/p55 (AAP-1) share moderate sequence similarity with their vertebrate and Drosophila counterparts. Genetic experiments show that ist-1 and aap-1 potentiate C. elegans insulin-like signaling, although they are not required for signaling in the pathway under most conditions. Worms lacking AAP-1 activity because of the mutation aap-1(m889) constitutively arrest development at the dauer larval stage when raised at high temperatures. aap-1 mutants also live longer than wild-type animals, a phenotype observed in other C. elegans mutants with defects in DAF-2 signaling. Interestingly, IST-1 appears to be required for signaling through a pathway that may act in parallel to AGE-1/PI3K.

An insulin-like signaling pathway controls development and lifespan in Caenorhabditis elegans (1–3). Genetic studies have shown that the C. elegans insulin-like pathway utilizes many of the same components as the human insulin and insulin-like growth factor (IGF-I) pathways. C. elegans insulin-like signaling requires the genes daf-2, encoding an insulin/IGF-I receptor-like protein, age-1, a homolog of vertebrate p110 catalytic subunits of phosphoinositide 3-kinase (PI3K), akt-1 and akt-2, encoding AKT/protein kinase B-like proteins activated by the phospholipid products of PI3K, and pdk-1, encoding a PDK-1-like kinase also required for AKT/protein kinase B activation (1, 4–6). In both worms and vertebrates, insulin-like signaling antagonizes forkhead transcription factors DAF-16 in the worm and FKHR, FKHRL1, and AFX in vertebrates (7–11). Loss-of-function mutations in daf-2, age-1, akt-1, or pdk-1 cause constitutive developmental arrest at the dauer larval stage, a long-lived stress-resistant non-reproductive larval form specialized to survive in hostile environments. Weak mutations in these genes allow normal reproductive development but significantly extend adult lifespan (2, 12, 13). daf-16 activity is required for dauer arrest and long lifespan as daf-16 mutations suppress these phenotypes in daf-2 and age-1 mutants (2, 12, 14–17).

In vertebrates and Drosophila, insulin-like signaling utilizes adaptor proteins that link activated growth factor receptors to intracellular signaling pathways (18–20). Ligand binding activates the tyrosine kinase activity of the insulin and IGF-I receptors, resulting in the phosphorylation of the insulin receptor substrate (IRS). Phosphotyrosines on IRS act as binding sites for downstream targets of growth factor receptors such as PI3K and Ras/MAPK pathways. Biochemical and genetic studies in mammalian cells have shown that PI3K is a major component of signaling downstream from insulin and IGF-I receptors (21). The PI3K adaptor subunits p85, p55, p50α, and p50β bind to phosphotyrosines within a YXXM motif on IRS and recruit the p110 catalytic subunit to the membrane for access to the lipid substrates. In addition, the interaction between the adaptor and catalytic subunits activates the p110 lipid kinase activity (22, 23).

We have investigated the functions of two C. elegans genes, one homologous to mammalian PI3K adaptor subunits named aap-1 and the other homologous to IRS named ist-1. Genetic analysis demonstrates that aap-1 and ist-1 both function in the daf-2/insulin-like pathway. The physical interactions between the PI3K catalytic and adaptor subunits are conserved in the worm homologs despite low sequence conservation in the inter-subunit interaction domains. Previous studies have shown that dauer arrest in age-1 mutants is suppressed by a gain-of-function mutation in Akt/protein kinase B, akt-1(mg144g) (5). Both ist-1 and aap-1 are required for akt-1(mg144g) to suppress age-1 mutations. These findings indicate that the DAF-2/insulin receptor-like protein may activate both AGE-1 PI3K signaling and a parallel pathway that remains to be identified.
EXPERIMENTAL PROCEDURES

Growth and Maintenance of Strains—Worm stocks were grown on nematode growth medium following established protocols (24). The following strains were used: N2 Bristol (wild type); GR1346 (sqt-1 (sc13 age-1 (mg44)); akt-1 (mg144)); GM9 (fer-15 (b26); daf-2 (m41)); GM7 (aap-1 (m889); fer-15 (b26); daf-2 (m41)); and DR2227 (aap-1 (m889); fer-15 (b26)) (5). Life spans were determined as described previously (12) using the temperature-sensitive fer-15 (b26) mutation to prevent progeny production in the test populations. Early L4 animals grown at 15 °C were transferred to 25.5 °C for sterilization and were scored daily for adult survival.

Identification and cDNA Cloning of aap-1 and ist-1—The aap-1 (Y110a7a.10) and ist-1 (C54D1.3) gene structures were predicted using the GeneFinder and Blast gene prediction and analysis programs and were confirmed by sequencing partial cDNA clones (aap-1 (yk442b3; ist-1 (yk26d7; yk110e3)), which were obtained from the Genome Biology Laboratory at the National Institute of Genetics (Mishima Japan). The 5′ ends of these genes were cloned using 5′-RACE (Strat...
age-1 (m889) mutation causes dauer arrest and extends adult life span

| Genotype          | Dauer larvae (27 °C) | Adult life span (mean) (25.5 °C) |
|-------------------|----------------------|----------------------------------|
| Wild type         | <5                   | 18 days (n = 119)                |
| aap-1(m889)       | 100 (maternal effect)| 33 days (n = 138)                |
| def(2m41)         | 100                  | 34 days (n = 128)                |
| aap-1(m889); def(2m41) | 100              | 34 days (n = 137)                |

* All life span assays were done in the fer-15(b26) background to eliminate progeny from the survival tests.

AGE-1 amino terminus, this interaction was tested in vitro. When bound to beads, bacterially expressed AAP-1 co-precipitated an amino-terminal fragment of AGE-1(1–268) containing the predicted interaction domain (Fig. 1D). The AGE-1(1–268) fragment did not bind to beads lacking AAP-1. This evidence indicates that the intersubunit interaction domains of PI3K are conserved in AGE-1 and AAP-1 despite low sequence conservation.

An AAP-1:GFP translational fusion with 1.8 kb of upstream sequence (containing the presumptive promoter) was used to identify cells in which AAP-1 might function. Although AAP-1:GFP expression was weak, fluorescence was consistently observed in the intestine and in neurons and was occasionally observed in body wall muscles and the hypodermis (data not shown). Expression was observed at all developmental stages beginning with early embryos and continued through adulthood. AAP-1::GFP did not appear to perturb aap-1 or age-1 function in vivo, because AAP-1::GFP-containing animals appeared grossly normal and did not arrest at the dauer stage (data not shown). The expression pattern of aap-1 is similar to that of the age-1 effectors akt-1, pdk-1, and daf-16, which are also widely expressed throughout development (5–7). However, the GFP expression pattern may not represent the full expression pattern of the endogenous aap-1 gene.

AAP-1 Is Required for Full Signaling through the DAF-2 Pathway—PI3K signaling by AGE-1 in C. elegans is required for wild-type adult lifespan and to bypass dauer larval arrest (4, 12–14). Genetic analysis shows that a loss of aap-1 gene activity also lengthens adult lifespan and causes dauer arrest, consistent with a function in the same pathway as age-1. The aap-1(m889) mutation was identified in a screen for thermotolerant mutants (28) and was mapped to the left arm of chromosome I between unc-38 and dpy-5. An additional three-factor cross-mapped aap-1(m889) to a 0.34-map unit (242 kb) region between unc-38 and unc-63. The 41-kb YAC sequence between cosmids C30F8 and unc-63 contains 14 predicted genes including Y110A7A.10. The sequencing of Y110A7A.10 revealed an amber nonsense mutation in the m889 mutant at Trp-325 (TGG to TAG), which would result in a truncated protein that lacks the COOH-terminal 198 amino acids including the second SH2 domain (Fig. 1C).

At 25.5 °C, aap-1(m889) mutant adults have a mean lifespan of 33 days, nearly twice as long as the controls (Table I). When grown at 27 °C, aap-1(m889) animals arrest development as dauer larvae (Table I). Dauer arrest at 27 °C is rescued by wild-type maternal aap-1 activity (data not shown), similar to the maternal rescue of age-1 mutations (13, 14). The DAF-16 forkhead-like transcription factor is necessary for dauer arrest and long lifespan in age-1 pathway mutants as well as the phenotypes of aap-1(m889) mutants (28). In addition, the presence of aap-1(m889) in a double mutant does not affect daf-2(m41) adult longevity. This is consistent with placing aap-1 into the same pathway as age-1.

To further support the hypothesis that aap-1 functions in the...
same pathway as age-1, we tested whether the loss of aap-1 function by RNA interference (RNAi) could enhance weak mutations in age-1 or in daf-2, encoding an insulin receptor-like protein that activates AGE-1 PI3K (1). aap-1(RNAi) did not cause any detectable phenotype in wild-type animals grown at 25.5 or 27 °C, the restrictive temperatures for many mutations in the daf-2 pathway. However, we found that aap-1(RNAi) could enhance the phenotype of weak mutations in the daf-2 pathway. age-1(hx546) is a weak allele of age-1 that causes dauer arrest at 27 °C but not at 25.5 °C (17, 29). aap-1(RNAi) dramatically enhanced dauer arrest at 25.5 °C in the age-1(hx546) background (Fig. 3A). aap-1(RNAi) also enhanced dauer arrest in daf-2(e1370) mutants at a semi-permissive temperature (22 °C) (Fig. 3B). Together, these results show that aap-1 potentiates signaling by the daf-2/age-1 pathway.

A TGF-β pathway also controls C. elegans dauer arrest in parallel to the daf-2/age-1 pathway (30, 31). The gene daf-1 encodes the type I TGF-β receptor essential for signaling through this pathway (31). aap-1(RNAi) in the daf-1(m40) mutant at semi-permissive temperature did not enhance dauer arrest, indicating that aap-1 activity is not required for signaling via the TGF-β branch of the dauer pathway (Fig. 3B).

**IST-1 Is a Homolog of the Mammalian IRS Proteins That Acts in the DAF-2 Pathway**—A predicted protein distantly related to vertebrate IRS proteins was identified in the C. elegans genome (32), which we named ist-1 (insulin receptor substrate). IRS proteins contain amino-terminal PH and phosphotyrosine

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**Fig. 2. Structure of the C. elegans IRS homolog IST-1.** A, IST-1 gene structure showing PH and PTB domains (shaded boxes) and the position of a putative binding site for the AAP-1/AGE-1 heterodimer. B, alignments of the PH and PTB domains from C. elegans IST-1, Drosophila Chico, and human IRS-1. C, sequences surrounding tyrosine residues in IST-1, which may be potential docking sites for SH2 domain-containing proteins. Tyrosine residues are underlined and are in boldface.
binding (PTB) domains for binding to activated insulin and IGF-I receptors (19). The IRS carboxyl terminus contains tyrosine substrates for insulin/IGF-I receptor kinase that upon phosphorylation act as docking sites for downstream effectors including PI3K and Ras/MAPK. The C. elegans IST-1 protein contains recognizable PH and PTB domains, although the overall sequence similarity is low. The PH domain of IST-1 is only 13–21% identical to the PH domains of other IRS proteins. The IST-1 PTB domain is better conserved and is 29% identical to the PTB domain of human IRS-1. IST-1 contains 22 tyrosines, some of which may recruit downstream proteins containing SH2 domain (Fig. 2C). There is a single YXXM motif for AAP-1/AGE-1 PI3K binding (Tyr-530). IRS proteins from other species also contain at least one YXXM binding site for PI3K. In addition, Tyr-321 in the PTB domain matches consensus binding sites for Grb-2 (YY/I/VXF/L/A/V) and SH-PTP2 (YY/I/XV/I/L/P) (26, 33). To date, neither protein has been implicated as a component of the DAF-2/insulin-like signaling pathway, although C. elegans homologs have been characterized (34, 35).

To determine whether IST-1 functions in the DAF-2 pathway, RNA interference was used to inactivate ist-1 function. As with aap-1, reduced ist-1 function is predicted to decrease dauer arrest activity, resulting in constitutive arrest at the dauer larval stage. Arrest at the dauer larval stage was not observed in wild-type animals with ist-1(RNAi) when grown at either 27 or 25.5 °C (data not shown). However, ist-1(RNAi) enhanced dauer arrest in the age-1(hx546) background at 25.5 °C as observed with aap-1(RNAi) (Fig. 3A). This result is consistent with placing IST-1 in the DAF-2 pathway.

The DAF-2 Receptor May Activate Parallel Downstream Pathways—Phosphorylation of IRS by the insulin receptor provides docking sites for downstream pathways including PI3K and Ras/MAP kinase pathways. To date, DAF-2/insulin receptor signaling has only been shown to act through the AGE-1/PI3K pathway. However, evidence suggests that DAF-2 also acts through a parallel AGE-1-independent pathway. A gain-of-function mutation in akt-1 suppresses dauer arrest in age-1 null mutants but not in daf-2 mutants (5). The akt-1(mg144gf) mutation contains an A183T substitution in the linker region between the PH and kinase domains and may activate the AKT-1 kinase activity in the absence of phospholipids. The inability of akt-1(mg144gf) to suppress daf-2 mutants has been postulated to suggest that signaling bifurcates downstream of DAF-2 to activate both AGE-1/PI3K and a parallel pathway. In addition, Gems et al. (3) proposed that some daf-2 mutants (e.g. e1370) have reduced signaling in both parallel pathways, whereas others (e.g. m41) are affected in only one. Class 1 mutants like m41 are dauer-constitutive, thermotolerant, and long-lived. Class 2 mutants exhibit these traits but are more pleiotropic with high levels of embryonic and L1 arrest, de-

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**Fig. 3.** *aap-1* and *ist-1* are required for full activity of the *daf-2*/insulin-like pathway. Dauer arrest and arrest as sterile adults are shown at the percent of a population of animals with *aap-1* or *ist-1* loci inactivated by RNA interference. The progeny of animals injected with double-stranded RNA of the corresponding gene were grown at 25.5 °C (A) or at indicated temperature (B) for 72 h before dauer arrest was scored.
creased adult motility, abnormal gonad morphology, and decreased broods.

One scenario based on the modularity of insulin signaling in vertebrates is that IST-1 is necessary for the activation of parallel DAF-2 outputs. To test this possibility, ist-1 activity was inactivated in age-1(mg44);akt-1(mg144gf) double mutants. In this background, ist-1(RNAi) would cause dauer arrest only if IST-1 activates AGE-1-independent pathways. If the only function of IST-1 is to link AGE-1/PI3K to the DAF-2 receptor, akt-1(mg144gf) should suppress the phenotype of ist-1(RNAi) and then these animals will develop into fertile adults. In fact, ist-1(RNAi) caused significant dauer arrest in age-1(mg44);akt-1(mg144gf) mutants (Fig. 4). Interestingly, aap-1(RNAi) also mildly reversed the age-1(mg44);akt-1(mg144gf) phenotype. However, in this case, the majority of animals developed into dark sterile adults, an intermediate phenotype observed in animals with weakened DAF-2 pathway signaling (14). The observation that ist-1(RNAi) could revert the akt-1(mg144gf) phenotype in age-1(mg44) null mutants supports the hypothesis that the DAF-2 receptor can signal through multiple pathways.

**DISCUSSION**

**AAP-1 and IST-1 Potentiate Signaling Downstream from the DAF-2 Receptor**—Using a combination of forward and reverse genetic approaches, we have demonstrated that the *C. elegans* DAF-2/insulin receptor utilizes an IRS-like protein, IST-1, and a PI3K p55-like adaptor subunit, AAP-1, to activate downstream signaling pathways. The description and characterization of these genes is necessary for understanding how signals are transduced from the DAF-2 receptor to AGE-1, the PI3K p110 catalytic subunit. Our results show that both AAP-1 and IST-1 are required for full DAF-2 pathway signaling. However, the relatively subtle phenotypes revealed by RNAi and by the aap-1(m889) mutation suggest that these genes may not be essential for DAF-2 pathway signaling under normal growth conditions.

Mutations in aap-1 and ist-1 have not been identified in forward genetic screens for dauer arrest mutants. The aap-1(m889) allele causes both developmental and lifespan phenotypes similar to those caused by mutations in other daf-2 pathway genes. In addition, the RNAi phenotypes provide support for placing aap-1 and ist-1 into the daf-2 pathway. RNAi can phenocopy the loss-of-function phenotypes of most genes. However, genes expressed in the *C. elegans* nervous system are usually resistant to RNAi. Both daf-2 and age-1 act in the nervous system to regulate dauer arrest and lifespan, although dauer arrest can be regulated by daf-2 pathway activity in some non-neuronal cell types such as intestine and muscle (36, 37). The weak phenotypes resulting from aap-1 and ist-1 RNAi may reflect the inability to sufficiently remove the activities of the genes from the nervous system.

![Model for signaling downstream from DAF-2](image)

**FIG. 4.** *ist-1* is required for reproductive development when insulin-like signaling is mediated by gain-of-function AKT. Dauer arrest was scored in the progeny of age-1(mg44);akt-1(mg144gf) animals with ist-1(RNAi) or aap-1(RNAi). Animals were grown at 25.5 °C for 72 h before development was scored.

![Diagram](image)

**FIG. 5.** Model for signaling downstream from DAF-2. Insulin-like ligands may activate DAF-2, triggering tyrosine phosphorylation on YXXM PI3K target sites in the DAF-2 COOH terminus and in IST-1 (arrow). The products of AGE-1 PI3K activate downstream kinases, AKT-1/2, and PDK-1, antagonizing the DAF-16 forkhead transcription factor. The genetic analysis presented here suggests that IST-1 also activates a parallel signaling pathway that may impinge directly or indirectly on DAF-16 or AKT activity (dashed lines).
An alternative explanation for the weak RNAi phenotypes we observed is that AAP-1 and IST-1 are dispensable for DAF-2 pathway signaling in otherwise wild-type backgrounds. This would suggest that the AGE-1/p110 catalytic subunit can be activated directly by the DAF-2 receptor or redundantly by another mechanism. The finding that null mutations in the *Drosophila* PI3K adaptor subunit Dp60 are not as severe as null mutations in the p110 catalytic subunit (Dp110) supports this view (38). One explanation for these results may be that p110 has a low basal level of lipid kinase activity in the absence of receptor stimulation. In the fly and the worm, this low basal activity may be sufficient for normal or near-normal development under most conditions. Only when PI3K signaling is compromised in other ways, i.e. by mutations in the p110 catalytic subunit, is the requirement for the adaptor subunit revealed.

In the mouse, signals from the insulin receptor are transduced via IRS to several redundant PI3K adaptor subunits p85α, p55α, p50α (all transcribed from the same gene), p85β, or p55β (39). Recent genetic analysis of these adaptor proteins suggests that they act both positively and negatively with respect to insulin signaling. Knock-out mutants in either the gene encoding p85, p55, p50α, or p85β displayed increased insulin sensitivity as compared with wild type, suggesting that reductions in PI3K adaptor/regulatory protein activity is beneficial in this instance (27, 40, 41). We find that the lack of aap-1 activity weakens the daf-2 pathway, suggesting that negative inputs from PI3K adaptor subunits are not a general feature of insulin-like signaling pathways. The increased complexity and redundancy of the mammalian insulin pathways relative the *C. elegans* DAF-2 pathway may account for these phenotypic differences.

We found that aap-1(RNAi) slightly enhanced dauer arrest in the age-1(mg44); akt-1(mg144gf) background. We had expected that aap-RNAi would have no effect, because age-1(mg44) is a nonsense mutant that deletes the entire lipid kinase domain (4). In this background, aap-1 activity should be dispensable. One explanation for this result is that AAP-1 weakly potentiates all signaling by the DAF-2 receptor, perhaps by competing with negative regulators for binding to the DAF-2.

**IST-1 May Be Required to Activate Parallel Outputs of the DAF-2 Receptor**—Our genetic experiments in the *akt-1(mg144gf)* background suggest that the *C. elegans* IRS homolog, IST-1, is necessary to activate an AGE-1-independent output of the DAF-2 receptor (Fig. 5). In mammalian cells, IRS can couple to multiple outputs including the Ras/MAKP pathway and PI3K to mediate insulin receptor signaling (42). These proteins contain SH2 domains that recognize phosphotyrosines within specific amino acid motifs (33). For example, the p85 PI3K subunit binds to phosphotyrosine within a YXXM motif. The sequence of the *Drosophila* IRS protein, Chico, contains binding sites for PI3K, a known output of *Drosophila* insulin signaling as well as potential GR2B/DRK and SH-PTP2 binding sites (20). IST-1 contains a YXXM motif for PI3K binding and a potential SH-PTP2 consensus binding site (YV/LX/V/I/L/P) (26). SH-PTP2 binding to IRS appears to attenuate responses of mammalian cells to insulin, suggesting that this protein could antagonize DAF-2 signaling in the worm (43). In summary, we have characterized the genetic activities of the *C. elegans* homologs of mammalian PI3K adaptor/regulatory subunits, AAP-1, and IST-1. Reducing the functions of these genes in wild-type backgrounds did not disrupt development, suggesting that they may be dispensable for growth under normal conditions. However, an aap-1 nonsense mutation extends adult longevity. Both AAP-1 and IST-1 are required for development in the background of weak mutations in the insulin-like signaling pathway of the worm. By using a combination of reverse genetics and sensitized backgrounds, the roles of other proteins that potentiate signaling by the DAF-2 insulin pathway may be addressed.

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