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C. elegans SGK-1 Is the Critical Component in the Akt/PKB Kinase Complex to Control Stress Response and Life Span

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Summary

The DAF-2 insulin receptor-like signaling pathway controls metabolism, development, longevity, and stress response in *C. elegans*. Here we show that SGK-1, the *C. elegans* homolog of the serum- and glucocorticoid-inducible kinase SGK, acts in parallel to the AKT kinases to mediate DAF-2 signaling. Loss of *sgk-1* results in defective egg-laying, extended generation time, increased stress resistance, and an extension of life span. SGK-1 forms a protein complex with the AKT kinases, and is activated by and strictly depends on PDK-1. All three kinases of this complex are able to directly phosphorylate DAF-16/FKHRL1, yet have different functions in DAF-2 signaling. Whereas AKT-1 and AKT-2 are more important for regulating dauer formation, SGK-1 is the crucial factor for the control of development, stress response, and longevity. Our data also suggest the existence of a second pathway from DAF-2 to DAF-16 that does not depend on AKT-1, AKT-2, and SGK-1.

Introduction

The mammalian insulin signaling pathway affects multiple downstream targets mediating a variety of cellular responses to insulin (Lizcano and Alessi, 2002). Genetic studies have shown that a similar pathway exists in *C. elegans* that involves insulin-like ligands, the DAF-2 insulin/IGF-I receptor-like protein, the AAP-1 PI3K-like adaptor subunit, the AGE-1 PI3K-like catalytic subunit, the DAF-18 PTEN lipid phosphatase, and the PI3K-like dependent kinase PDK-1 which activates the Akt/PKB-like serine/threonine (S/T) protein kinases AKT-1 and AKT-2. AKT-1 and AKT-2 are candidates to antagonize the forkhead transcription factor DAF-16 to repress genes that regulate diapause, longevity, stress response, and energy storage, and to activate genes necessary for metabolism and reproductive growth (Kimura et al., 1997; Lee et al., 2003; Murphy et al., 2003). Reduction of *daf-2* gene activity or other genes that are positively regulated by *daf-2*, like *aap-1*, *age-1*, *pdk-1*, or *akt-1/-2*, cause a constitutive developmental arrest at the dauer larval stage (Daf-c) (Morris et al., 1996; Wolkow et al., 2002; Paradis and Ruvkun, 1998; Paradis et al., 1999). Reduction-of-function mutations in genes that antagonize *daf-2* signaling, such as *daf-18* and *daf-16*, suppress the dauer constitutive phenotype of *daf-2* and the other Daf-c mutants (Lin et al., 1997; Ogg and Ruvkun, 1998).

According to the current model of Akt/PKB activation in mammalian cells, phosphatidylinositol-3,4-diphosphate (PIP2) and/or phosphatidylinositol-3,4,5-triphosphate (PIP3) bind to the amino-terminal pleckstrin homology (PH) domain of Akt/PKB, resulting in the recruitment of Akt to the plasma membrane. PDK1 colocalizes with Akt/PKB to the cell membrane and activates it by phosphorylation (Alessi et al., 1996; Anderson et al., 1998). Activated Akt/PKB is capable of promoting cell survival by phosphorylating and inactivating the mammalian DAF-16 homolog FKHRL1 (Rena et al., 1999). However, in *C. elegans*, RNAi depletion of the Akt/PKB homologs *akt-1* and *akt-2* was not sufficient to shut down the DAF-2 insulin signaling pathway entirely (Paradis and Ruvkun, 1998). Moreover, inactivation of the Akt/PKB consensus phosphorylation sites in DAF-16 was not sufficient to induce dauer formation and life span extension (Lin et al., 2001). Therefore, other, yet-unidentified components have to exist downstream of *pdk-1* to essentially control metabolism, development, longevity, and stress response.

Serum- and glucocorticoid-inducible kinases (SGKs) are S/T protein kinases with approximately 55% sequence similarity to Akt/PKB. SGK expression is regulated by a variety of external stimuli, including growth factors like TGF-β and IGF-I, steroid or peptide hormones like insulin, cytokines, alterations in cell volume, osmotic changes, and cortical brain injury (Brunet et al., 2001). Recent studies revealed a role for SGK in cell survival signaling depending on PI3K (Brunet et al., 2001; Leong et al., 2003). SGK is thought to be activated by PDK1 at sites that are equivalent to the Akt/PKB phosphorylation sites. PDK1 uses its hydrophobic PIF binding pocket to interact with and activate SGK (Biondi et al., 2001). SGK may promote cell survival in parallel or complementary to Akt/PKB, since it participates in phosphorylating and inactivating FKHRL1 at sites different from those modified by Akt/PKB (Kobayashi and Cohen, 1999; Brunet et al., 2001).

Using reverse genetic approaches and biochemical analyses, we demonstrate that the *C. elegans* homolog of the serum- and glucocorticoid-inducible kinase, SGK-1, functions in parallel to AKT-1 and AKT-2 to mediate DAF-2 insulin receptor-like signaling. We show that SGK-1 is activated by and strictly depends on PDK-1, and binds to AKT-1 and AKT-2 to form a multimeric protein complex. We provide direct evidence that the AKT-1/AKT-2/SGK-1 complex transduces AGE-1/PI3K signals via PDK-1 to control the intracellular localization and activation of DAF-16/FKHRL1 by phosphorylation. In this respect, AKT-1/AKT-2/SGK-1 compete with a parallel branch within the DAF-2 pathway. Moreover, inactivation of *sgk-1*, but not of *akt-1* or *akt-2*, results in a

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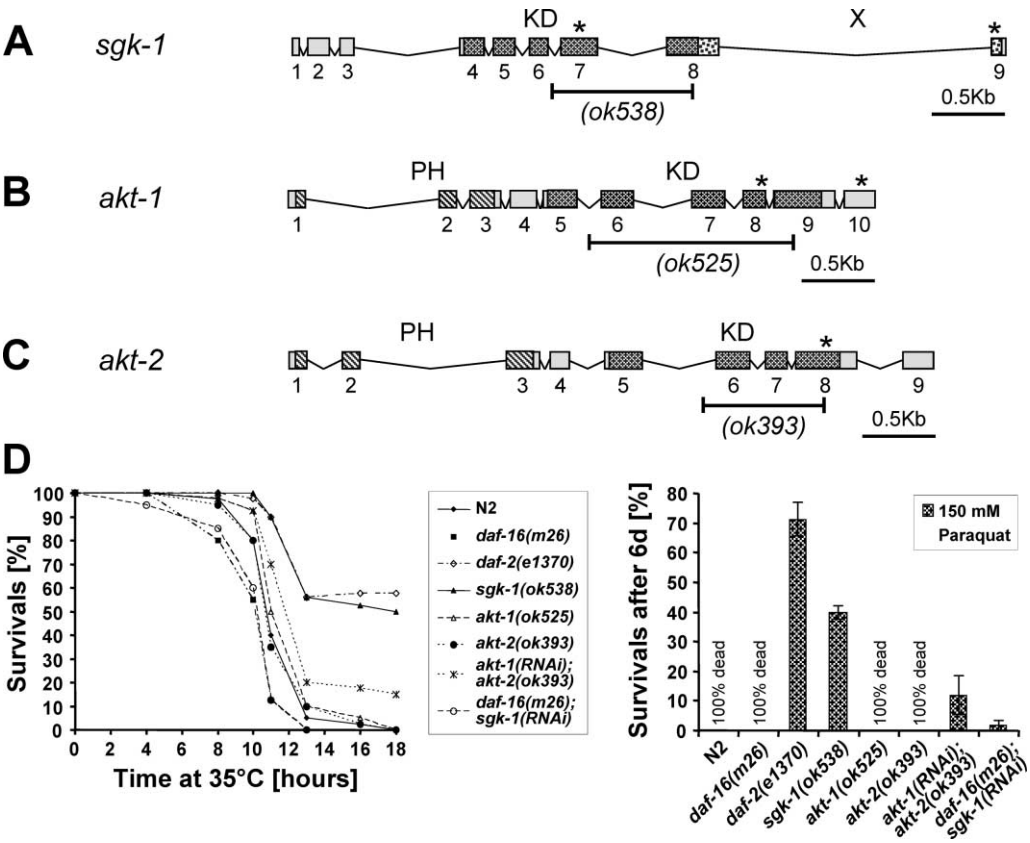


Figure 1. Genomic Organization and Stress Resistance of *sgk-1* Compared to *akt-1* and *akt-2*

(A–C) Locations of deletions in *sgk-1* (*W10G6.2*), *akt-1* (*C12D8.10*), and *akt-2* (*F28H6.1*). Coding regions are boxed, introns are lines. The kinase domain is indicated by checkered boxes, the X domain by dotted boxes. The deletions (*ok538, ok525, ok393*) are marked. The consensus sites on SGK-1, AKT-1, and AKT-2 corresponding to the hSGK and hAkt/PKB Thr^{308} and Ser^{473} phosphorylation sites are indicated by asterisks. (D) *sgk-1(ok538)*, but not *akt-1(ok525)* or *akt-2(393)*, is resistant to heat stress (35°C) and oxidative stress (paraquat). In stress assays, *sgk-1*(RNAi) behaves similarly to *sgk-1(ok538)* (Supplemental Figure S3).

retarded postembryonic development, defective egg-laying, extended life span, and increased stress tolerance. We conclude that SGK-1 is the critical kinase of the AKT-1/AKT-2/SGK-1 regulatory complex that controls aspects of development, longevity, and stress response.

Results

sgk-1 Mutants Have a Pleiotropic Developmental Phenotype

In mammals, activation of SGK is dependent on PI3K, suggesting that SGK might play a role in the insulin signaling pathway (Brunet et al., 2001). The *C. elegans* genome contains one open reading frame (*sgk-1*, *W10G6.2*) with strong sequence similarity to human SGK. Genefinder prediction indicates that SGK-1 contains a kinase domain which is 78% similar and 67% identical to the human SGK kinase domain, and an X domain with unknown function which bears 61% similarity and 25% identity to the human X domain (Figure 1A and Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/6/4/577/DC1]).

To compare *sgk-1* function with that of *akt-1* and *akt-2*, we characterized deletion alleles of all three genes. Previous reports had only described RNAi inactivation experiments of *akt-1* and *akt-2* and the *akt-1(mg144)* gain-of-function allele (Paradis and Ruvkun, 1998). *akt-1(ok525)* eliminates 1251 bp from exons 6, 7, 8, and part of exon 9 including the kinase domain (Figure 1B). *akt-2(ok393)* animals bear a 719 bp deletion removing the sixth, seventh, and part of the eighth exon (Figure 1C). Therefore, both *akt-1(ok525)* and *akt-2(ok393)* most likely represent strong loss-of-function or null alleles, since they lack most of the kinase domains that are critical for Akt/PKB activity. The *akt-2(ok393)* animals suffer from a weak egg-laying defect (11% of the animals die from a bag of worms phenotype [n = 111]). However, unlike mutants of *daf-2*, *age-1*, and *pdk-1*, *akt-2(ok393)* did not show an extended generation time (2.84 ± 0.06 [n = 30] compared to 2.8 ± 0.06 [n = 55] days in wild-type) or a reduced brood size (327 ± 25 [n = 19] compared to 336 ± 31 [n = 20] eggs in wild-type). In agreement with Paradis and Ruvkun (1998), we observed that *akt-2(ok393)* is not able to induce constitutive dauer formation at 26°C (0.5% dauers, Table 1C), whereas the *akt-1*(RNAi); *akt-2(ok393)* double mutant resulted in 98.3% dauer arrest already at 15°C (Table 1A).

Since in mammalian cells SGK and Akt/PKB are thought to act in parallel (Brunet et al., 2001), we wanted to determine whether *sgk-1* has a similar function in

Table 1. Effects of *sgk-1* on Dauer Formation

Strain	L4 Larvae and Adult	Dauer	Partial Dauer	L1 Arrest	Number[a]
A. Phenotype of Progeny at 15°C (%)					
wild-type	100	0	0	0	365
rrf-3(pk1426)	100	0	0	0	438
pdk-1(sa680)	95.8	4.2	0	0	341
pdk-1(sa680) sgk-1[b]	1.2[c]	98.8	0	0	234
akt-1[b]*; akt-2(ok393)*	1.7	98.3	0	0	920
akt-1[d]*; akt-2*[d]	0.2	99.8	0	0	800
akt-1[b]*; akt-2(ok393) sgk-1*[b]	1.9	98.1	0	0	306
akt-1[d]*; akt-2*[d] *sgk-1*[d]	0.2	99.8	0	0	2049
daf-7(e1372)	43.5	56.5	0	0	862
daf-7(e1372); sgk-1[b]	66.6	33.4	0	0	935
daf-11(m47)	93.1	6.9	0	0	495
daf-11(m47); sgk-1[b]	99.6	0.4	0	0	719
B. Phenotype of progeny at 20°C (%)					
wild-type	99.5	0	0	0.5	372
sgk-1[b]	98.8	0	0	1.2	260
daf-2(e1370)	99.6	0.4	0	0	748
daf-2(e1370); sgk-1[b]	90.9	9.1	0	0	343
age-1(mg44)[e]	58.9	2.1	36.9	2.1	488
age-1(mg44)[e]*; sgk-1*[b]	2.3	90.6	4.8	2.3	306
C. Phenotype of progeny at 26°C (%)					
wild-type	98.8	0	0	1.2	962
rrf-3(pk1426)	99.9	0.1	0	0	695
akt-1[d]	98.7	1.2	0	0.1	603
akt-2(ok393)	98.9	0.5	0	0.5	1112
akt-2[d]	99.0	0.4	0.2	0.4	504
sgk-1[d]	98.1	1.1	0.4	0.4	595
akt-1[d]*; sgk-1*[d]	66.1	29.6	1.3	3.0	308
akt-2(ok393) sgk-1[b]	97.9	2.1	0	0	678
akt-2[d] *sgk-1*[d]	96.1	3.5	0.2	0.3	646
daf-18(e1375)	100	0	0	0	651
daf-18(e1375); akt-1[b]*; sgk-1*[b]	91	8.9	0	0.1	982
daf-16(m26)	99.9	0	0.1	0	537
daf-16(m26); akt-1[b]*; sgk-1*[b]	99.3	0.2	0	0.5	844

[a] Total number of animals scored.
[b] All data with *akt-1*[b]*, akt-2*[b] and *sgk-1*[b] were done by RNAi by feeding.
[c] Animals were sterile.
[d] *rrf-3(pk1426)* was used for RNAi by feeding.
[e] The full genotype of this strain is *sqt-1(sc13) age-1(mg44)*.

the *C. elegans* DAF-2 signaling pathway. Therefore, we analyzed the phenotype of *sgk-1* RNAi-treated and *sgk-1(ok538)* mutant animals. *sgk-1(ok538)* harbors an 852 bp deletion removing the seventh and most of the eighth exon containing most of the kinase domain that is critical for SGK-1 activity (Figure 1A). Therefore, it can be supposed that *sgk-1(ok538)* represents a strong loss-of-function or null allele. We found that inactivation of *sgk-1* by either RNAi or deletion results in a very similar phenotype, characterized by an extended generation time (3.5 $\pm$ 0.17 and 3.3 $\pm$ 0.09 days, compared to 2.8 $\pm$ 0.06 [n $>$ 60] days in wild-type) which is mainly caused by an extension of the L2 larval stage, a strong egg-laying defect (73% and 66% [n $>$ 129] of the animals, respectively, die from a bag of worms phenotype), and, probably as a consequence, a reduced brood size (250 $\pm$ 30 and 224 $\pm$ 38 eggs, compared to 336 $\pm$ 38 in wild-type [n $>$ 20]). In addition, the animals are about 20% thinner at all larval stages up to the adult stage until they become Egl. Thus, RNAi treatment efficiently reduces *sgk-1* activity and phenocopies *sgk-1(ok538)* (see also Figure 2A). In summary, the pleiotropic phenotype of *sgk-1*(RNAi) or *sgk-1(ok538)* animals is reminiscent of that seen in mutants of other insulin signaling components like *daf-2*, *age-1*, and *pdk-1* (Gems et al., 1998; Morris et al., 1996; Paradis et al., 1999) and therefore might be consistent with *sgk-1* playing a role in DAF-2 signaling.

sgk-1 Expression Suggests a Role in the DAF-2 Insulin Receptor-like Signaling Pathway

To identify the expression pattern of *sgk-1*, we constructed a reporter which contains a *sgk-1::gfp* translational fusion with 3.1 kb of upstream regulatory sequence. In transgenic animals, *sgk-1::gfp* expression starts in late embryos just before hatching. At postembryonic stages, *sgk-1::gfp* is expressed in sensory (e.g., ASK, ADL, ASI) and many other neurons (Figures 2B and 2C). In the tail, *sgk-1::gfp* is expressed in the sensory neurons PHA and PHB and in the interneurons LUA (Figures 2B and 2D) (identified by costaining with DiI). *sgk-1::gfp* expression was also observed in the motor neurons of the ventral nerve cord (Figure 2E) and in the intestine (Figures 2B and 2F). In neurons, SGK-1::GFP is localized both in the cytoplasm and in the nucleus, whereas in the intestine it is exclusively cytoplasmic. Thus, the expression pattern and localization of SGK-1 closely resembles that of PDK-1 (Paradis et al., 1999),

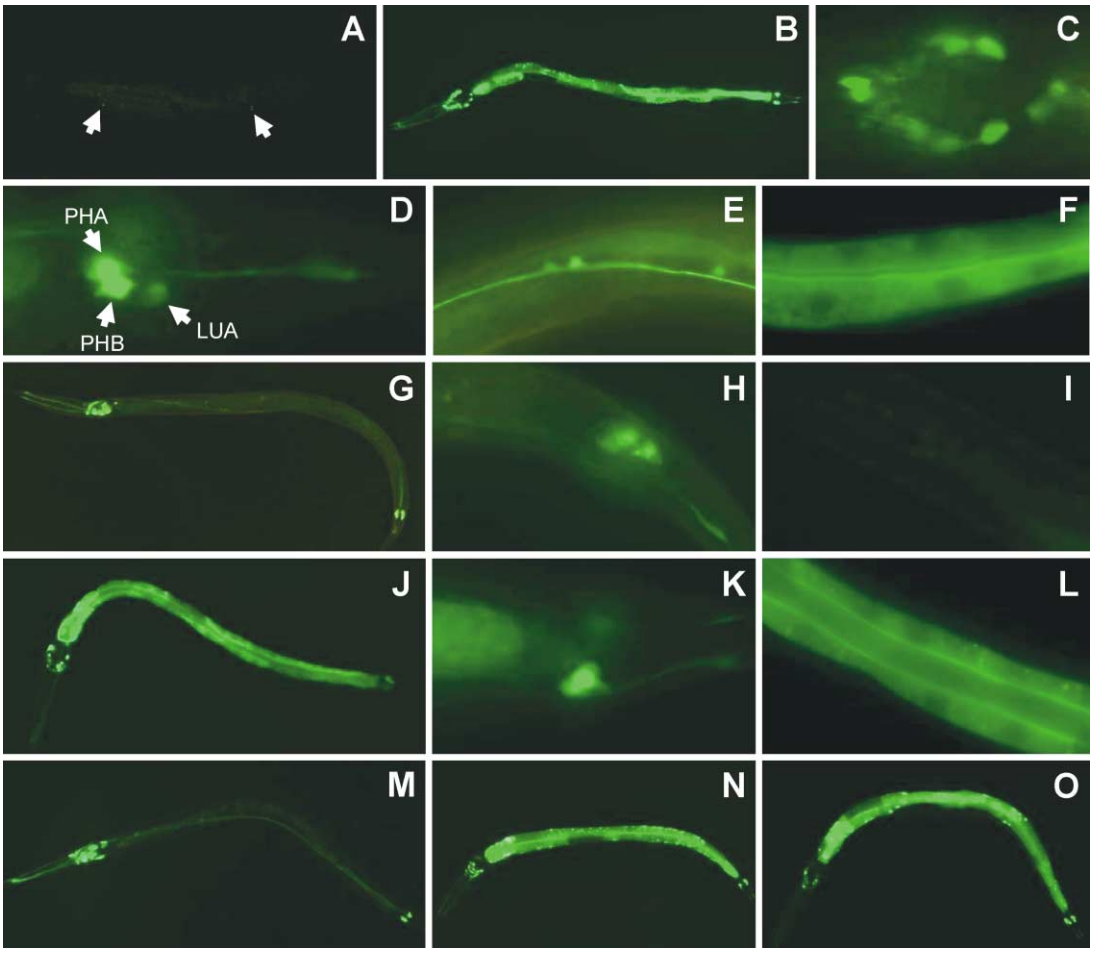


Figure 2. *sgk-1* Expression in Wild-Type and Various Mutants

(A) *sgk-1*(RNAi): *sgk-1::gfp* is completely downregulated in L4 larvae and adults, and strongly downregulated in younger larvae (L2 larva marked by arrows).

(B–F) wild-type: *sgk-1::gfp* is expressed in the cytoplasm and the nuclei of the majority of head neurons (B and C), the tail neurons PHA, PHB, and LUA (B and D), the ventral nerve cord including motor neurons (E), and the cytoplasm of the intestine (B and F).

(G–I and M) *sgk-1::gfp* expression in a *pdk-1(sa680)* (G–I) and *daf-2(e1370)* (M) background. SGK-1 is slightly diminished in neurons (H) and not detectable in the intestine (G, I, and M).

(J–L) *daf-16(m26): sgk-1::gfp* expression is indistinguishable from wild-type. Neither neuronal (J and K) nor intestinal (J and L) expression is affected by *daf-16(m26)*.

(N and O) *sgk-1::gfp* expression in a *daf-7(e1372)* (N) or *daf-11(m47)* (O) background is also indistinguishable from wild-type.

Same magnifications were used in (A) and (B), (G) and (J), (M)–(O); higher magnifications were used in (C)–(F), (H) and (I), and (K) and (L), always showing an L4 larva.

but is distinct from the cellular localization of AKT-1::GFP and AKT-2::GFP, which preferentially stain the cytoplasmic membrane (Paradis and Ruvkun, 1998; our unpublished data). This corresponds to SGK localization examined in cultured cells, since SGK, in contrast to Akt/PKB, does not contain a PH domain that serves as a membrane anchor. In summary, expression data are in line with a role for *sgk-1* in the DAF-2 signaling pathway (Riddle and Albert, 1997).

We also tested *sgk-1::gfp* in the genetic background of different insulin signaling-defective mutants. At 25°C, temperature-sensitive (ts) loss-of-function mutations in *daf-2(e1370)* or *pdk-1(sa680)* lead to strongly diminished SGK-1::GFP staining in the intestine. In the neurons, SGK-1 is also diminished, but still detectable (Figures 2G–2I and 2M). *sgk-1::gfp* expression in a *daf-16(m26)* mutant background is not affected (Figures 2J–2L). These observations indicate that SGK-1 localization and/or stability depend on DAF-2 and PDK-1 activity, but are independent of functional DAF-16. Therefore, our expression data suggest a role of SGK-1 in the DAF-2 pathway downstream of DAF-2 and PDK-1 and upstream of DAF-16.

Furthermore, we tested whether *sgk-1::gfp* expression is modulated by TGF-β and cGMP signaling that also contribute to dauer regulation in *C. elegans*. However, we found no alteration in a *daf-7(e1372)* (TGF-β-related ligand of the TGF-β-related dauer pathway) or a *daf-11(m47)* (guanylyl cyclase of the cGMP dauer pathway) mutant background (Figures 2N and 2O). This suggests that *sgk-1* expression is independent of these two alternative signaling pathways.

sgk-1 Acts Upstream of *daf-16* in the DAF-2 Pathway

Although we showed that *sgk-1* expression does not depend on TGF-β and cGMP signaling, nevertheless its function could be modulated by these pathways. Therefore, in dauer formation assays we tested if *sgk-1*(RNAi) genetically interacts with *daf-7* or *daf-11* mutants. *sgk-1* did not enhance the Daf-c phenotype of either *daf-7(e1372)* or *daf-11(m47)* at 15°C, but rather partially suppressed the dauer phenotype of both genes (Table 1A). This result is in line with some other mutants of the DAF-2 pathway that have also been shown to partly or even completely suppress mutants of the TGF-β or cGMP pathways (Inoue and Thomas, 2000; Ogg et al., 1997) due to cross-connections between all three signaling cascades. Taken together, our results indicate that *sgk-1* only affects the DAF-2 pathway.

In the absence of a dominant allele to decisively determine the genetic position of *sgk-1* within the DAF-2 pathway, we tested whether *sgk-1*(RNAi) mutants can synergize with weak mutants of the pathway. At 20°C and 26°C, only 0% and 1.1% of the *sgk-1*(RNAi) worms enter the dauer stage constitutively, even if we tested RNAi in the background of *rrf-3(pk1426)* (Tables 1B and 1C) that had been shown to increase sensitivity toward RNAi (Simmer et al., 2002). Therefore, deprivation of *sgk-1* alone is not sufficient to block PI3K signaling. *akt-1*(RNAi), *akt-2(ok393)*, or *akt-2*(RNAi) animals are also not able to induce dauer formation (Table 1C), whereas inactivation of *akt-1* and *akt-2* together results in a complete Daf-c phenotype already at 15°C as described above (Table 1A). Although the additional inactivation of *sgk-1* could not further enhance the already fully penetrant Daf-c phenotype, *sgk-1* does contribute to dauer control. In an *akt-1*(RNAi) background at 26°C, *sgk-1*(RNAi) increased dauer formation from 1.2% to 29.6%, and it also increased *akt-2(ok393)* or *akt-2*(RNAi) weakly from 0.5/0.4% to 2.1/3.5% (Table 1C). However, the combined inactivation of *akt-1* and *akt-2* is enough to completely block the signaling pathway, suggesting that these two genes are most important for the regulation of dauer entry. At 15°C, only 4.2% of *pdk-1(sa680)* animals enter the dauer stage. Additional inactivation of *sgk-1* results in a fully penetrant Daf-c phenotype (98.8% dauers and 1.2% sterile adults, Table 1A). Reduction of *sgk-1* activity also enhances the weak Daf-c phenotype of *daf-2* and *age-1* at 20°C. Whereas the Daf-c phenotype in *daf-2(e1370)* is enhanced moderately (from 0.4% to 9.1% Daf-c), *age-1(mg44)* background leads to a strong enhancement from 2.1% to 90.6% (Table 1B). Moreover, *sgk-1*(RNAi) together with *akt-1*(RNAi) can partially suppress the dauer defective (Daf-d) phenotype of *daf-18(e1375)* at 26°C (Table 1C). Therefore, we suggest that in DAF-2 signaling, *sgk-1* is genetically localized downstream of *daf-18*.

daf-16 is the only known downstream target of *akt-1* and *akt-2*. We also placed *daf-16* downstream of *sgk-1*, since at 26°C the Daf-d mutant *daf-16(m26)* fully suppressed the weak Daf-c phenotype of *sgk-1* (16.6% dauer formation and additional 12% partial dauer formation [n = 522]) which could be induced in the absence of food. Furthermore, *daf-16(m26)* was also able to suppress the Daf-c phenotype of *akt-1; sgk-1*(RNAi) (Table 1C). The placement of *sgk-1* upstream of *daf-16* was also corroborated by life span and stress tolerance experiments (see below).

Inactivation of *sgk-1*, but Not of *akt-1* or *akt-2*, Confers Longevity and Stress Resistance

Mutations in various genes of the DAF-2 signaling pathway, including *daf-2*, *age-1*, and *pdk-1*, confer a longevity phenotype. *sgk-1*(RNAi) mutants also have a substantially increased mean adult life span (63% increase at 25°C, see Table 2; due to the strong egg-laying defect of *sgk-1(ok538)* at 25°C, we used *sgk-1*(RNAi) for life span analyses). The life span extension induced by *sgk-1*(RNAi) is in the same range as that induced by *daf-2(e1370)* or *pdk-1(sa680)* animals and is even more pronounced than that observed for *age-1(mg44)* (Table 2). Longevity is further enhanced in double mutants of *sgk-1*(RNAi) with *daf-2(e1370)* or *age-1(mg44)*. In contrast, overexpression of *sgk-1* significantly shortens life span (Table 2). These data confirm that the level of *sgk-1* expression is a major determinant for longevity. *daf-16(mgDf50)*, a putative null allele of *daf-16*, fully suppresses the life span extension of *sgk-1* (Table 2), which is another confirmation that *daf-16* is the major output of *sgk-1*. Individual inactivation of neither *akt-1* nor *akt-2* had an effect on longevity (Table 2), and *akt-1*(RNAi); *akt-2(ok393)* animals only showed a weak life span extension by 19% (Table 2). This suggests that, in contrast to their strong input into dauer regulation, neither *akt-1* nor *akt-2* plays a major role in the control of life span. This was corroborated by the finding that life span of the triple mutant *akt-1*(RNAi); *akt-2(ok393) sgk-1*(RNAi) was not longer than that of *sgk-1*(RNAi) alone (Table 2), arguing for *sgk-1* being the main factor at this position of the pathway to control life span.

Mutants in a variety of components of the DAF-2 pathway confer increased stress tolerance for heat, oxidation, and various chemicals (Honda and Honda, 1999; Yanase et al., 2002). We found that *akt-1(ok525)* and *akt-2(ok393)* are not resistant to either increased temperature (35°C) or the oxidative stressors paraquat and H_2O_2. *akt-1*(RNAi); *akt-2(ok393)* animals only show a weak increase in stress resistance when compared to wild-type (Figure 1D and Supplemental Figure S4). In contrast, *sgk-1(ok538)* animals display a strong resistance to heat damage and oxidative stress and behave similarly to *daf-2* (Figure 1D and Supplemental Figure S4) and *age-1* (Yanase et al., 2002) mutants. The increased stress resistance of *sgk-1(ok538)* is completely suppressed by *daf-16(m26)* (Figure 1D). In conclusion, these results demonstrate that *sgk-1*, and not *akt-1* or *akt-2*, is crucial for the control of life span and stress response.

SGK-1 Strictly Depends on PDK-1 and Forms a Multimeric Complex with AKT-1 and AKT-2

High gene dosage of *akt-1*, but not of *akt-2*, is able to bypass the requirement for AGE-1 PI3K signaling via PDK-1 (Paradis and Ruvkun, 1998). To test the dependence of SGK-1 function on PDK-1 activity, we did similar experiments, but also tested a *sgk-1*(+) overexpressing strain in a *pdk-1(sa680)* background. The *sgk-1* transgene was not capable of suppressing the 96.9%

Table 2. Effects of *sgk-1* on Life Span

Strain	Mean Adult Life Span ± SEM 25°C (days)	Maximum Adult Life Span 25°C (days)	*P* Value against Wild-Type	Number[a]
wild-type	14.7 ± 0.3	23	-	110
sgk-1[b]	24.0 ± 0.4	33	<0.0001	147
Ex[sgk-1::gfp][c]	11.3 ± 0.2	18	<0.0001	103
daf-2(e1370)	26.0 ± 0.8	37	<0.0001	103
daf-2(e1370); sgk-1[b]	36.0 ± 0.9	65	<0.0001	108
age-1(mg44)[d]	20.7 ± 0.8	34	<0.0001	75
age-1(mg44)[d]*; sgk-1*[b]	30.0 ± 0.8	46	<0.0001	78
pdk-1(sa680)	25.5 ± 0.6	35	<0.0001	98
pdk-1(sa680) sgk-1[b]	*	*	*	*
akt-1(ok525)	15.5 ± 0.4	22	0.1642	100
akt-2(ok393)	14.2 ± 0.3	22	0.3717	101
akt-1[b]*; akt-2(ok393)*	17.5 ± 0.6	30	0.0005	132
akt-1[b]*; akt-2(ok393) sgk-1*[b]	22.7 ± 0.5	36	<0.0001	133
daf-16(mgDf50)	11.4 ± 0.2	17	<0.0001	101
daf-16(mgDf50); sgk-1[b]	11.4 ± 0.3	19	<0.0001	111

The corresponding life span curves are depicted in Supplemental Figure S2. SEM: standard error of the mean. **pdk-1(sa680) sgk-1*[b] animals are 100% dauer constitutive that prevented any analysis. Life span data with wild-type, *sgk-1*[b], *daf-16(m26)* and *daf-16(m26); sgk-1*[b] at 20°C are included in Supplemental Table S1, showing that *daf-16(m26)* partially suppresses the *sgk-1*(RNAi) life span phenotype at 20°C.

[a] Total number of animals scored.

[b] All data with *sgk-1*[b] and *akt-1*[b] were done by RNAi by feeding.

[c] Three transgenic lines were examined. Data of one representative line (BR2773) are provided.

[d] The full genotype of this strain is *sqt-1(sc13) age-1(mg44)*.

Daf-c phenotype observed in *pdk-1(sa680)* at 26°C (Figure 3A). On the other hand, overexpression of *akt-1* completely suppressed the *pdk-1* Daf-c phenotype, whereas overexpression of *akt-2* partially suppressed the *pdk-1* Daf-c phenotype (Figure 3A). These data suggest that SGK-1 cannot bypass the need for PI3K-dependent PDK-1 function. It also correlates with our data obtained from *sgk-1::gfp* localization experiments and the kinase assays (see below) showing that SGK-1 activity strictly depends on functional PDK-1 and DAF-2. In contrast, AKT-1, and to a lesser degree AKT-2, can also be activated through PDK-1-independent signals.

Since *akt-1*, *akt-2*, and *sgk-1* may act at the same genetic position, downstream of *pdk-1* and upstream of *daf-16*, it is possible that their encoded proteins might also interact physically. Therefore, we tested protein-protein interactions between SGK-1 and other components of *C. elegans* insulin signaling, including PDK-1, AKT-1, AKT-2, and DAF-16, in vitro and in vivo in *Saccharomyces cerevisiae*. In pull-down experiments, we found that both in vitro-translated PDK-1 and DAF-16 interact with bacterially expressed, GST-tagged SGK-1, AKT-1, and AKT-2. Our experiments also revealed that SGK-1, AKT-1, and AKT-2 interact with each other (Figure 3B). These results were confirmed by yeast two-hybrid studies. SGK-1, AKT-1, and AKT-2 also bind to each other in yeast, whereas the experiments provided no evidence that the kinases are able to homodimerize (Figure 3C). To map the interaction surface, we tested SGK-1, AKT-1, and AKT-2 proteins lacking their kinase domains. Elimination of the kinase domains did not abolish protein interactions (Figure 3C), suggesting that the N-terminal PH domain of AKT-1/AKT-2, and the N-terminal part and/or the C-terminal X-domain of SGK-1, are critical for protein binding. Interestingly, elimination of the kinase domain now allowed AKT-2 to homodimerize, suggesting that this domain somehow prevented dimerization of the full-length protein. Since any combination of AKT-1, AKT-2, and SGK-1 was capable of heterodimer formation, it is conceivable that all three kinases form a trimeric complex. We therefore incubated bacterially expressed AKT-1 and AKT-2 with immobilized SGK-1 and found that AKT-1 and AKT-2 bind SGK-1 simultaneously. Moreover, increasing AKT-1 concentration from 1- to 10-fold could not titrate AKT-2 from SGK-1 (Figure 3D), suggesting that AKT-1 and AKT-2 occupy different binding pockets on SGK-1. Taken together, these results strongly indicate that SGK-1, AKT-1, and AKT-2 form a multimeric protein complex that may be phosphorylated by PDK-1 for activation. Since all components of this complex interact with PDK-1 and DAF-16, the respective upstream- and downstream factors, we suggest that this kinase complex is phosphorylated by PDK-1 and contributes to DAF-16 phosphorylation and inactivation.

AKT-1/AKT-2/SGK-1 Is Activated by PDK-1 to Suppress DAF-16 by Direct Phosphorylation

Since SGK-1, AKT-1, and AKT-2 all contain a highly similar kinase domain, we tested if they are able to phosphorylate their candidate substrate DAF-16 in vitro. For this purpose, we constructed transgenic wild-type and *pdk-1(sa680)* mutant strains expressing *gfp*-tagged *akt-1*, *akt-2*, or *sgk-1*. The fusion proteins were immunopurified from worm extracts and used for kinase assays with purified DAF-16. Both AKT-1, AKT-2, and SGK-1 purified from a wild-type background phosphorylated DAF-16 (Figure 4A). In contrast, all three kinases purified from a *pdk-1* mutant background were not able to phosphorylate DAF-16 (Figure 4A). This demonstrates that functional PDK-1 is required to activate AKT-1, AKT-2, and SGK-1 in vivo. Each kinase in the AKT-1/AKT-2/SGK-1 complex has the potential of antagonizing DAF-16 by direct phosphorylation. Whether the loss of the

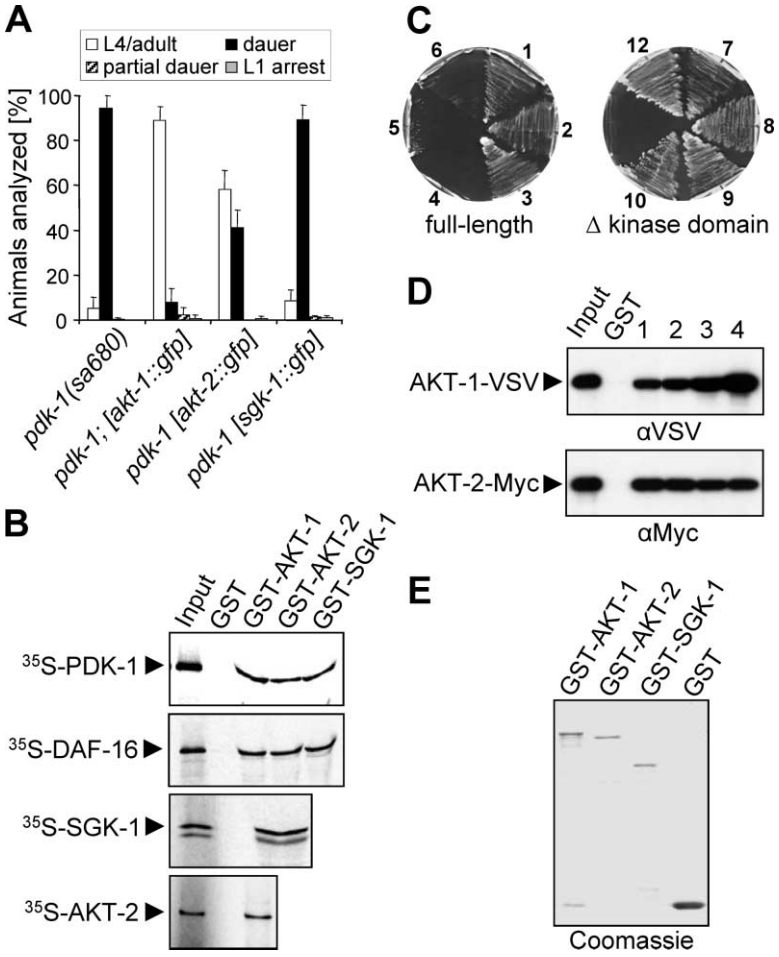


Figure 3. SGK-1 Fully Depends on Functional PDK-1, Interacts with PDK-1 and DAF-16, and Forms a Complex with AKT-1 and AKT-2

(A) Suppression of the *pdk-1* Daf-c phenotype (n = 570) was analyzed by overexpressing *akt-1::gfp*, *akt-2:gfp*, or *sgk-1:gfp*. The genotypes of the strains and the number of tested animals were: *pdk-1(sa680); byEx[akt-1::gfp]* (n = 379), *pdk-1(sa680) byEx[akt-2::gfp]* (n = 408), and *pdk-1(sa680) byEx[sgk-1::gfp]* (n = 442).

(B) In vitro pull-down assays. Reticulocyte lysates containing ^{35}S-labeled PDK-1, DAF-16, SGK-1, or AKT-2 generated by in vitro translation were probed with equal concentrations of glutathione-sepharose-coupled GST, GST-AKT-1, GST-AKT-2, and GST-SGK-1. Labeled proteins were visualized by SDS-PAGE followed by autoradiography.

(C) GAL4 yeast two-hybrid protein interaction studies. AKT-1-GAD (with GAL4 activation domain)/SGK-1-GBK (with GAL4 DNA binding domain) (1), AKT-2-GAD/SGK-1-GBK (2), AKT-2-GAD/AKT-1-GBK (3), AKT-1-GAD/AKT-1-GBK (4), AKT-2-GAD/AKT-2-GBK (5), SGK-1-GAD/SGK-1-GBK (6), AKT-1ΔKD-GAD (delta kinase domain)/SGK-1ΔKD-GBK (7), AKT-2ΔKD-GAD/SGK-1ΔKD-GBK (8), AKT-1ΔKD-GAD/AKT-2ΔKD-GBK (9), AKT-2ΔKD-GAD/AKT-2ΔKD-GBK (10), GAD/GBK as negative control (11), and T-antigen-GAD/human p53-GBK as positive control (12).

(D) Coimmunoprecipitation assays. Bacterial lysates containing equal concentrations of AKT-1-VSV and AKT-2-Myc were mixed and probed with glutathione-sepharose-coupled GST or GST-SGK-1. Precipitated proteins were visualized by SDS-PAGE followed by Western blotting with anti-VSV and anti-Myc antibodies. The assay was also repeated with increasing concentrations of AKT-1-VSV bacterial lysate to demonstrate that high amounts of AKT-1 are not able to displace AKT-2 from SGK-1; equal (1), double (2), 5-fold (3), and 10-fold (4) concentrations of AKT-1-VSV compared to AKT-2-Myc were used.

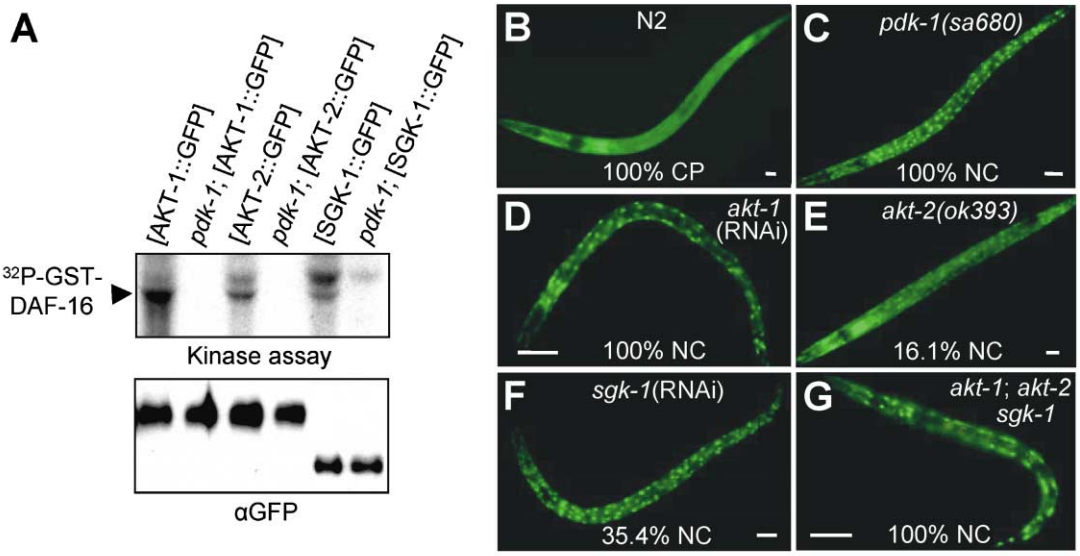
(E) A Coomassie blue-stained gel of the purified GST fusions GST-AKT-1, GST-AKT-2, GST-SGK-1, and GST used in (B) and (D) is shown.

SGK-1::GFP signal in the intestine in *pdk-1(sa680)* animals is the consequence of degradation of inactive SGK-1, or may be caused by an additional level of *sgk-1* expression control by *pdk-1* in certain tissues cannot be decisively concluded from this experiment.

To analyze in vivo how AKT-1, AKT-2, and SGK-1 individually affect DAF-16, we studied the intracellular localization of a DAF-16::GFP fusion protein in a variety of mutant backgrounds. Since overexpression of *daf-16* increases dauer formation at high temperatures (25°C; Henderson and Johnson, 2001), the assays were done at 15°C. At this temperature, DAF-16::GFP in wild-type animals is exclusively localized to the cytoplasm (Figure 4B). Upon interruption of DAF-2 signaling in *pdk-1(sa680)*, DAF-16 remains unphosphorylated and, as a consequence, migrates to the nucleus where it can induce dauer formation (Figure 4C). Upon deprivation of *akt-1*, DAF-16::GFP was also nuclearly localized in 100% of the animals. These animals either go into dauer or already arrest at the L1 larval stage (Figure 4D), underlining the crucial role of AKT-1 function in reproductive development. In contrast, 16.1% of *akt-2(ok393)* and 35.4% of *sgk-1*(RNAi) animals displayed nuclear localization of DAF-16. Accordingly, the vast majority of *akt-2(ok393)* animals become reproductive adults, and only very few animals went into dauer (Figure 4E). *sgk-1* (RNAi) induced nearly 50% dauer formation in the sensitized background of *daf-16::gfp* overexpression (Figure 4F). Taken together, depletion of *akt-1* has the most pronounced effect on DAF-16 localization and developmental function, and loss of *sgk-1* is more critical for DAF-16 cytoplasmic localization than *akt-2*. The ranking of the three kinases in DAF-16 localization corresponds to their role in dauer induction reported above. In double and triple mutant backgrounds, we discovered an additive role of the three kinases. In the absence of all three kinases, 100% of the animals showed nuclear localization of DAF-16, accompanied by 100% L1 arrest (Figure 4G). All double mutant combinations resulted in a weaker phenotype (data not shown). The additive effects of *akt-1*, *akt-2*, and *sgk-1* are another indication that all three proteins act together and have overlapping functions in the same signaling pathway.

AKT-1/AKT-2/SGK-1 Competes with a Parallel Branch of the DAF-2 Pathway

A previous study has reported that the disruption of the four Akt/PKB consensus phosphorylation sites in DAF-16 results in nuclear accumulation in wild-type animals, but is not sufficient to promote dauer formation or to



H Effects of *daf-16::gfp* transgenes on dauer formation at 25°C

daf-16(mu86) + background	*daf-16a::gfp (muEx128)*	*daf-16a::gfp/b^{KO} (muIs71)*	*daf-16a^{AM}::gfp (muEx116)*	*daf-16a^{AM}::gfp/b^{KO} (muEx151)*
-	0 (288)	0 (373)	0.8 (247)	4.8 (737)
akt-1^{a}	4.5 (36)	0.1 (1440)	14.2 (42)	13.3 (875)
akt-2^{a}	1.6 (123)	0 (1603)	13.1 (38)	13.7 (827)
sgk-1^{a}	2.1 (95)	0 (1232)	13.7 (51)	14.5 (633)
akt-1^{a}; akt-2^{a} sgk-1^{a}	16.7 (60)	1.4 (820)	22.6 (31)	15.1 (714)

Dauer formation is indicated in percent; all non-dauer animals are L4 larvae or young adults. Total number of animals scored are in brackets. Control: *daf-16(mu86)* shows 0% (527) dauer formation. [(a)] All data with *akt-1^{a}*, *akt-2^{a}* and *sgk-1^{a}* were done by RNAi by feeding. The *daf-16(mu86)* transgenic lines *muEx128*, *muIs71*, *muEx116* and *muEx151* were from Lin et al., 2001.

Figure 4. Inactivation of AKT-1, AKT-2, and SGK-1 Prevents DAF-16 Phosphorylation In Vitro and Affects Subcellular DAF-16 Localization and Dauer Formation In Vivo

(A) Kinase activity assays. AKT-1::GFP, AKT-2::GFP, or SGK-1::GFP from wild-type or *pdk-1(sa680)* worm lysates was immunoprecipitated with anti-GFP antibody and subjected to kinase analysis to determine the kinase activity on purified DAF-16a protein with [γ-^{32}P]ATP. Total amounts of AKT-1/AKT-2/SGK-1::GFP in immunoprecipitates were determined by Western blotting with anti-GFP antibody as a control.

(B–G) Localization of DAF-16::GFP in strains of various genetic background, all containing *zIs356[daf-16::gfp]*. Percentage of cytoplasmic (CP) or nuclear (NC) localization is indicated. Numbers of animals tested are: n = 300 (B), 320 (C), 302 (D), 253 (E), 319 (F), 389 [(G); genotype is *akt-1*(RNAi); *akt-2(ok393) sgk-1*(RNAi)]. Assays were done at 15°C. Animals with the ts-allele *pdk-1(sa680)* were shifted to 25°C for 12 hr before analysis. Animals were analyzed at equivalent time points during development. Scale bars: 25 μm.

(H) Effects of *daf-16::gfp* transgenes on dauer formation when *akt-1*, *akt-2*, *sgk-1*, or all three kinases are inactivated.

extend life span (Lin et al., 2001). It was concluded that the DAF-2 pathway must have additional outputs. We used the same transgenic strains to test them in *akt-1/akt-2/sgk-1* single and triple mutants. Only 0.8% of *daf-16(mu86)* animals expressing *daf-16a^{AM}::gfp*, a construct in which all four Akt/PKB phosphorylation sites are mutated, and 4.8% of *daf-16a^{AM}::gfp/b^{KO}*, a construct harboring an additional chain-terminating frameshift mutation in the *daf-16b*-specific exon (Lin et al., 2001), produced a Daf-c phenotype at 25°C (Figure 4H). These results are in agreement with Lin et al. (2001) and suggest that there are either additional phosphorylation site(s) in DAF-16 not targeted by the mutations or in a second unidentified protein that are needed for the efficient control of dauer entry. Most surprisingly, inactivation of *akt-1*, *akt-2*, and *sgk-1* together in *daf-16(mu86); daf-16a::gfp* or *daf-16(mu86); daf-16a::gfp/b^{KO}* animals also did not result in a fully penetrant Daf-c phenotype (16.7% and 1.4% dauers at 25°C; Figure 4H), although the same transgenes in a *daf-16(mu86); daf-2(e1370)* background caused 100% dauer formation (Lin et al., 2001). The inactivation of all three kinases in a wild-type background also resulted in almost 100% dauer formation already at 15°C (Table 1). This strongly indicates that DAF-2 signaling can bypass the need for AKT-1/AKT-2/SGK-1 to phosphorylate DAF-16a and/or DAF-16b under the conditions of this experiment. These data therefore suggest the existence of a second branch within the DAF-2 pathway that acts in parallel to AKT-1/AKT-2/SGK-1 and converges on DAF-16 or on a second unidentified protein necessary for the efficient regulation of dauer entry.

We repeated this experiment, but this time we tested the *daf-16* transgenes in which the four Akt/PKB phosphorylation sites had been inactivated (*daf-16a^{AM}::gfp* or *daf-16a^{AM}::gfp/b^{KO}* in a *daf-16(mu86); akt-1; akt-2 sgk-1* background). Dauer formation in *daf-16a^{AM}::gfp* was increased from 16.7 to 22.6%, and in *daf-16a^{AM}/b^{KO}::gfp*

was increased from 1.4 to 15.1% compared to the corresponding strains with intact Akt/PKB phosphorylation sites (Figure 4H). This suggests that the parallel branch of the DAF-2 pathway may use one or several of the Akt phosphorylation sites to prevent dauer entry, and the mutational inactivation of such sites increased the Daf-c phenotype. The mutations of the Akt phosphorylation sites also increased dauer formation of *akt-1*, *akt-2*, and *sgk-1* single mutants from 4.5/1.6/2.1% to 14.2/13.1/13.7%, independently confirming our previous results that all three kinases contribute to dauer formation (compare Table 1). Most significantly, *daf-16a^{AM}::gfp* or *daf-16a^{AM}::gfp/b^{KO}* transgenes in the *daf-16(mu86); akt-1; akt-2 sgk-1* mutant background did not induce a fully penetrant Daf-c phenotype (Figure 4H), whereas they led to 100% Daf-c in *daf-16(mu86)*; *daf-2(e1370)* (Lin et al., 2001). The most obvious explanation for this remarkably low Daf-c phenotype is that, although the AKT-1/AKT-2/SGK-1 branch of the DAF-2 signaling is shut off, in the presence of functional DAF-2 the second branch of the pathway is still active. One immediate conclusion from this result is that the DAF-2 pathway branches at or downstream of DAF-2, but upstream of AKT-1/AKT-2/SGK-1 to converge on DAF-16 or another unidentified protein. Therefore, only the inactivation of both AKT-1/AKT-2/SGK-1 and the second branch should result in complete dauer formation in a *daf-16(mu86); daf-16a::gfp* background.

Discussion

An intricate regulatory network is responsible for the coordination of DAF-2 signaling to control dauer formation and longevity. Tissue specificity (Libina et al., 2003) and yet-unidentified parallel signaling pathways (Paradis and Ruvkun, 1998) contribute to the complexity of DAF-2 signaling to regulate the localization and function of the forkhead transcription factor DAF-16. Here we show that *sgk-1*, the *C. elegans* SGK homolog, is activated by and strictly dependent on PDK-1 to regulate the phosphorylation, intracellular localization, and activity of DAF-16. AKT-1 and AKT-2 predominantly control dauer entry, whereas SGK-1, and not AKT-1 or AKT-2 as previously suggested, is the crucial kinase for the regulation of life span and stress response.

In mammals, it was concluded that Akt/PKB and SGK may act in parallel in a complementary rather than a redundant way to antagonize the function of the DAF-16 homolog FKHRL1 (Kobayashi and Cohen, 1999; Brunet et al., 2001). Here we provide evidence that the Akt/PKB homologs AKT-1 and AKT-2 even physically interact with SGK-1 to form a multimeric protein complex. This result was somewhat surprising, since Akt/PKB proteins are thought to be predominantly membrane bound, whereas human SGK has been shown to have a dynamic localization pattern and shuttles between cytosplasm and nucleus. Our observation that, depending on the genetic background, *sgk-1::gfp* is also predominantly localized to either nucleus or cytoplasm indicates that the same dynamics are also seen for the *C. elegans* SGK. Since both AKT-1, AKT-2, and SGK-1 are phosphorylated by PDK-1 and are themselves capable of phosphorylating DAF-16, their direct contact may reflect a temporary, regulatory interaction. This interaction obviously does not depend on the kinase domain that is highly similar in all three proteins. Interestingly, in a *daf-2* or *pdk-1* background, the *sgk-1::gfp* signal is most significantly reduced in the intestine, suggesting that DAF-16, the *sgk-1* target for the control of longevity, may no longer be inactivated by phosphorylation in this organ. DAF-16 activity in the intestine has recently been shown to be sufficient to extend the life span of *C. elegans* (Libina et al., 2003).

Our genetic data strongly support distinct yet overlapping roles for *akt-1*, *akt-2* and *sgk-1*. Whereas *sgk-1* is fully dependent on *pdk-1*, transgenic overexpression of *akt-2* can bypass *pdk-1* partially and *akt-1* overexpression can suppress loss of *pdk-1* entirely to prevent constitutive dauer formation. Therefore, AKT-1 and to some extent AKT-2, but not SGK-1, may also be activated by a mechanism that does not involve PDK-1.

Three parallel pathways contribute to the control of dauer formation. Yet our results indicate that *sgk-1* is not modulated by TGF-β or cGMP signaling and only participates in DAF-2 insulin signaling. In contrast to *akt-1* and *akt-2*, *sgk-1* seems to have a minor role in dauer control. Instead, *sgk-1* is involved in other developmental decisions, whereas we have no indication that the same may be true for *akt-1* or *akt-2*. Elimination of *sgk-1* function causes a pleiotropic phenotype that resembles that of other mutants in the DAF-2 pathway. *sgk-1* animals display an extended generation time and an egg-laying defect accompanied by reduced brood size. Moreover, *sgk-1* mutants showed a strongly increased stress resistance for both thermal stress and hyperoxidation. This is similar to the phenotype of *daf-2*, *age-1*, and *pdk-1* mutants (Paradis et al., 1999; Yanase et al., 2002). The stress tolerance is accompanied by a 63% extended life span that is further enhanced in *daf-2*; *sgk-1* and *age-1; sgk-1* double mutants. In contrast, neither *akt-1* nor *akt-2* mutants showed a similar phenotype, since even the double mutant *akt-1; akt-2* had only a minor influence on stress response and longevity.

We immunopurified GFP-tagged AKT-1, AKT-2, and SGK-1 from transgenic *C. elegans* to show that all three kinases are capable of phosphorylating DAF-16 when isolated from wild-type, but not when purified from *pdk-1* mutant animals. Although these experiments cannot fully exclude the possibility that in this assay phosphorylation of DAF-16 is mediated by an unknown, PDK-1-dependent kinase that coprecipitated with each of the GFP-tagged kinases, we consider this quite unlikely. There is also no genetic evidence for an additional factor downstream of AKT-1/AKT-2/SGK-1 and upstream of DAF-16. Phosphorylation results in cytoplasmic retention of DAF-16 and has also been shown to abolish its function as a transcription factor (Henderson and Johnson, 2001). Our in vitro results showing that AKT-1, AKT-2, and SGK-1 individually contribute to the phosphorylation of DAF-16 are supported by the analysis of DAF-16::GFP intracellular localization in several mutant backgrounds. *akt-1*, *akt-2*, and *sgk-1* single, double, and triple mutant background(s) all effected the in vivo localization of DAF-16. Interestingly, there is no simple correlation between the strength of the single mutants to cause DAF-16 nuclear localization and the

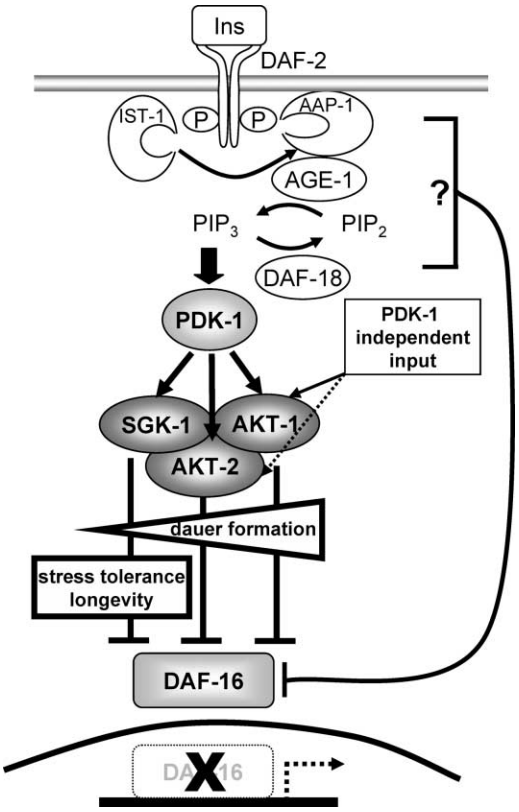


Figure 5. Model for the Function of SGK-1 and AKT-1/-2 Kinases in DAF-2 Insulin Signaling

Upon DAF-2 activation, AGE-1 and PIP3 activate PDK-1. PDK-1 phosphorylates AKT-1, AKT-2, and SGK-1 that form a kinase complex to antagonize DAF-16 by direct phosphorylation. The major importance of AKT-1 and AKT-2 to prevent DAF-16 from inducing the dauer program is indicated. SGK-1, but not the AKT kinases, inhibits DAF-16-induced longevity and stress tolerance. The figure is based on the model from Wolkow et al. (2002) and was expanded to include novel results obtained in this study. See text for details.

contribution of the same mutants to induce dauer formation in the double mutant strains. These differences may be due to AKT-1, AKT-2, and SGK-1 using different (complementary or overlapping) patterns of phosphorylation on DAF-16. It was recently shown that human Akt and SGK coordinately regulate the function of FKHRL1 by phosphorylating this transcription factor at distinct sites to specifically control nuclear import, DNA binding, or transactivation (Lee et al., 2001). The lack of a simple correlation between nuclear localization of DAF-16::GFP and dauer formation/longevity was also suggested recently (Lin et al., 2001).

Using the sensitized background of various transgenic *daf-16::gfp* strains, Lin et al. (2001) showed that the DAF-2 pathway inhibits dauer formation and extension of life span even when the four Akt/PKB phosphorylation sites in DAF-16 are mutated. They suggested that the AKT proteins may phosphorylate additional sites either in DAF-16 or in a second, unidentified protein. Our data using the same transgenic constructs further support such a model. In addition to AKT-1 and AKT-2, we show also that SGK-1 plays an important role in the phosphorylation and functional control of DAF-16. Furthermore, the data strongly support the existence of a second signaling pathway from DAF-2 to DAF-16 that is able to bypass the AKT-1/AKT-2/SGK-1 kinases. The existence of such a pathway has been proposed before (Paradis and Ruvkun, 1998). Our data show that in a *daf-16(mu86)* mutant supplemented with functional *daf-16::gfp* transgenes, the triple mutant *akt-1; akt-2 sgk-1* only results in weak dauer formation, suggesting that only part of DAF-2 signaling to DAF-16 is blocked. Only the elimination of DAF-2 shuts off signaling entirely, resulting in 100% dauer formation.

In summary, we showed by genetic and biochemical methods that *C. elegans* SGK-1 is the long-sought kinase in DAF-2 insulin signaling to control postembryonic development, life span, and stress response (Figure 5). We also show that SGK-1 is part of a protein kinase complex together with AKT-1 and AKT-2, whose components play distinct roles in transmitting phosphorylation signals from PDK-1 to DAF-16 for a variety of cellular and developmental decisions. AKT-1/AKT-2/SGK-1 mediate only one part of the DAF-2-dependent repression of DAF-16 function. Our data provide evidence that such an unidentified branch of the DAF-2 pathway indeed exists (Figure 5). Using the powerful genetics of *C. elegans*, a similar *daf-16::gfp* sensitized background can now be used in genetic screens to find the components of this second branch of the DAF-2 pathway.

Experimental Procedures

Strains and plasmids are described in the Supplemental Data at http://www.developmentalcell.com/cgi/content/full/6/4/577/DC1.

Rescue of *sgk-1(ok538)*, *akt-1(ok525)*, and *akt-2(ok393)*

sgk-1(ok538) (predicted gene *W10G6.2*), *akt-1(ok525)* (predicted gene *C12D8.10*), and *akt-2(ok393)* (predicted gene *F28H6.1*) were kindly provided by the *C. elegans* Gene Knockout Consortium. We outcrossed the strains two (*sgk-1*), four (*akt-1*), and four (*akt-2*) additional times. At 15°C, the *akt-1(ok525); akt-2*(RNAi) strain and the *akt-1*(RNAi); *akt-2(ok393)* strain already displayed a strong Daf-c phenotype which we rescued in dauer formation tests by introduction of a *akt-1::gfp* or a *akt-2::gfp* translational fusion transgene (BR3126, BR3030) (Supplemental Table S2). *akt-1*(RNAi); *sgk-1(ok538)* displayed a Daf-c phenotype at 26°C which we also rescued by introducing a corresponding *sgk-1::gfp* transgene (BR3063) (Supplemental Table S2).

sgk-1::gfp Expression

A *sgk-1::gfp* translational fusion was prepared by cloning the complete 4.2 kb genomic *sgk-1* locus (predicted gene *W10G6.2*) and 3.1 kb upstream regulatory sequence of the predicted start codon into pPD95.75 (A. Fire, Carnegie Institute of Washington, Baltimore, MD). 50 ng/μl pBY1763 (containing the *sgk-1::gfp* translational fusion) was injected into N2 and *daf-16(m26)* together with *rol-6* (pRF4, 50 ng/μl) as the coinjection marker (Mello et al., 1991). Three (two in *daf-16* background) independent transgenic lines (BR2773/76/77 and BR2979/91) were analyzed to determine the expression pattern of *sgk-1*. BR2773/76/77 were also used for life span experiments. To investigate *sgk-1* expression in *daf-2*, *pdk-1*, *daf-7*, and *daf-11* backgrounds, *byEx[sgk-1::gfp]* (BR2773) was crossed into *daf-2(e1370)*, *pdk-1(sa680)*, *daf-7(e1372)*, and *daf-11(m47)*, respectively.

Phenotypic Analysis

sgk-1, *akt-1*, and *akt-2* RNAi experiments by feeding using N2 or *rrf-3(pk1426)* (Simmer et al., 2002) were performed as described in Kamath et al. (2001). Double and triple RNAi experiments were done by mixing the bacterial cultures directly before seeding the NGM plates. To determine their generation time, adult animals were

placed on fresh plates to lay eggs for 2 hr at 20°C. The worms were removed and after 40 hr, 55 worms of the progeny were singled on fresh plates. Animals were scored after they laid the first egg. Brood size was determined as in Wong et al. (1995), and life span experiments were performed essentially as in Lakowski and Hekimi (1998). To determine mean adult life span at 25°C, animals were grown at 15°C until the L4 larval stage and then shifted to prevent dauer entry. RNAi treatment of the life span strains *akt-1*(RNAi); *akt-2(ok393)* and *akt-1*(RNAi); *akt-2(ok393) sgk-1*(RNAi) was started with L3 larvae of the F0 generation to bypass the problem of dauer entry in F1. Assays of stress resistance were performed essentially as described in Yanase et al. (2002). For heat stress assays (35°C), 2 × 20 young adults per each time point and each strain were used. For oxidative stress assays (paraquat), seeded NGM plates containing 150 mM paraquat were used. Per each strain, 3 × 20 young adults were tested and counted after 6 days. Dauer formation assays were performed as described in Paradis and Ruvkun (1998). Table 1 and Figure 4H combine the results of three independent experiments, each with similar results.

Protein Interaction Studies

For radioactive pull-down assays, GST-AKT-1, GST-AKT-2, and GST-SGK-1 were expressed in *E. coli* BL21(RIL) from pGEX plasmids and purified on glutathione-sepharose 4B. PDK-1, DAF-16a, SGK-1, and AKT-2 were in vitro translated from pCITE plasmids using the TNT coupled reticulocyte lysate transcription/translation system (Promega) and metabolically labeled with [^{35}S]methionine-[^{35}S]cysteine. The GST fusion proteins were immobilized on sepharose beads and were incubated with the reticulocyte lysates in buffer containing 10 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride (PMSF). Beads were washed several times with this buffer, denatured, and separated by SDS-PAGE. Dried gels were exposed to film. For coimmunoprecipitation experiments, GST-SGK-1 was immobilized on glutathione-sepharose beads and coimmunoprecipitated with AKT-1-VSV and AKT-2-Myc simultaneously, in buffer containing 1x PBS, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, and 2 mM PMSF. Coimmunoprecipitates were resolved on SDS-PAGE and analyzed by Western blot analysis using mouse anti-VSV or anti-Myc monoclonal antibody. Yeast two-hybrid protein interaction studies were performed using the MATCHMAKER GAL4 Two-Hybrid System 3 according to the manufacturer's instructions (Clontech). AKT-1, AKT-2, SGK-1, AKT-1ΔKD (delta kinase domain), AKT-2ΔKD, and SGK-1ΔKD were fused to GAL4 activation domain (pGADT7) and GAL4 DNA binding domain (pGBKT7) and transformed into yeast AH109.

Kinase Assays

Sonicated worm lysates from the following strains, *byEx[akt-1::gfp]*, *pdk-1(sa680)*; *byEx[akt-1::gfp]*, *byEx[akt-2::gfp]*, *pdk-1(sa680)*; *byEx[akt-2::gfp]*, *byEx[sgk-1::gfp]*, *pdk-1(sa680)*; *byEx[sgk-1::gfp]*, were incubated with rabbit anti-GFP polyclonal antibody in 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 10% glycerol, 1% Triton X-100, and 5 mM PMSF at 4°C for 3 hr. Samples were precipitated with protein G-agarose beads. Immunoprecipitates were subjected to protein kinase analysis and immunoblotting with rabbit anti-GFP polyclonal antibody. For kinase assays, immunoprecipitates were incubated with 2 μg bacterially expressed and purified GST-DAF-16a in 20 μl kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, and 5 μCi [γ-^{32}P]ATP at 25°C for 5 min. Samples were analyzed by SDS-PAGE and autoradiography.

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