Published in final edited form as:

Gene. 2001 November 28; 279(2): 137–147.

***kin-18*, a *C. elegans* protein kinase involved in feeding**

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Abstract

TAO1 and TAO2 are recently described protein kinases whose initial characterization has placed them at the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase kinase (MEKK) level of stress-responsive MAPK pathways. Because their physiological roles have not been identified, we sought to study their *C. elegans* homolog to learn more about their functions. *kin-18* encodes a previously uncharacterized protein in *C. elegans* whose catalytic domain shares over 60% identity with TAO1 and TAO2. We demonstrate that KIN-18 is a protein of 120 kDa whose promoter is active in the pharynx and intestine of *C. elegans*. To learn more about TAO/KIN-18 function, we studied how expression of constitutively active forms of TAO1 or KIN-18 would affect the physiology of intact worms. Strains of *C. elegans* expressing active forms of TAO1 or KIN-18 exhibit altered pharyngeal electrophysiology as measured by electropharyngeogram. These worms grow more slowly and lay fewer eggs, phenotypes that could result from reduced feeding. We have also identified a *C. elegans* gene that encodes a protein kinase similar to mammalian MAPK/ERK Kinase (MEK) 4 whose promoter is active in the pharynx. It is phosphorylated by TAO1 in vitro and physically interacts with TAO1.

Keywords

TAO1; TAO2; MAP kinase; MEK kinase; Stress response

1. Introduction

Mitogen activated protein kinase pathways contain evolutionarily conserved cascades consisting of a MAPK/ERK that is activated by a MAPK/ERK kinase (MEK) which in turn is activated by a MEK kinase. This generic MAPK module takes on different forms to produce parallel but interwoven MAPK signaling pathways that respond to different extracellular stimuli. These pathways have been found in yeast, plants, and animals.

TAO1 and TAO2 are mammalian protein kinases with similarity to the yeast Ste20p protein kinase (Hutchison et al., 1998; Chen et al., 1999). The initial characterization of TAO proteins places them at the MEKK level of the MAPK module activating stress-sensitive MEKs. The best evidence indicates that TAO1 and TAO2 act primarily on MEKs upstream of p38. However, attempts to place TAOs in a pathway by identifying physiological activators and downstream effectors in mammalian cells have proven difficult. As a result, we chose to study a TAO homolog in an intact organism to understand its effects on the animal's physiology.

The nematode *C. elegans* is commonly used for genetic manipulation and physiological characterization. Components of multiple MAPK pathways have been discovered in nematodes and assigned functions. Genetic analysis of the signaling that controls vulval induction in *C. elegans* revealed that the ERK/MAPK pathway played a crucial role (Hajnal et al., 1997). The MEK7/c-Jun-N-terminal kinase (JNK) pathway was recently implicated in neuronal control of coordinated movement (Kawasaki et al., 1999). Another potential MEK7 homolog, *mek-1*, plays a role in stress responses in *C. elegans* (Koga et al., 2000).

Searches of the GenBank database show that the sequences of TAO1 and TAO2 are homologous to that of *kin-18*, an uncharacterized gene in *C. elegans*. Analysis of *kin-18* may provide new insights into the function and roles of TAO proteins in mammals as well as establish a system to study a previously undefined MAPK pathway in *C. elegans*. Transgenic lines of worms expressing mutant forms of TAO1 and *kin-18* were used to explore function of TAO proteins and to determine if they might regulate MAPK cascades in nematodes. Phenotypic analysis of these worms shows that TAO1 and *kin-18* mutants exhibit altered feeding resulting in the perturbation of growth patterns.

Further analysis of the *C. elegans* genome reveals several uncharacterized ORFs that are similar to the stress sensitive MEKs of mammals. Cosmid F42G10 encodes a kinase 40% identical to MEK4. The promoter of this MEK is active in pharyngeal muscle and the protein is a substrate of TAO1 in vitro, supporting the hypothesis that this kinase may lie directly downstream of *kin-18* in a signaling pathway.

2. Materials and methods

2.1. Nematode strains and culture

Bristol N2 worms were used as the wild type strain for injection to create the transformants for the following constructs: *kin-18::GFP*, *myo-2::TAO1(1–416)*, *kin-18::kin-18(1–395)*. For these, the *rol-6(d)* containing plasmid, pRAK3, was co-injected as the marker (Davis et al., 1995). All plasmids were introduced at a concentration of 70 ng/μl by microinjection (Mello and Fire, 1995). Andy Fire kindly provided plasmids pPD95.75, pPD49.26, and pPD30.69 (Mello and Fire, 1995). For the *kin-18::GFP/rol-6* strain, 2.5 kb of the upstream 5' UTR as well as the first 30 bases of the first exon were amplified from a single N2 worm by PCR and inserted into the vector pPD95.75 such that the first ten amino acids of *kin-18* would be fused in frame to GFP. For the *myo-2::TAO1(1–416)* strain, PCR was used to generate the appropriate piece of TAO1 flanked by *NheI* sites, and this was inserted into pPD30.69. The Rol phenotype and PCR verified the presence of the extrachromosomal

array. For the *kin-18*: *kin-18*(1–395) strain, 2.5 kb of upstream 5' UTR along with the DNA encoding the first 395 amino acids (including introns) flanked by *NcoI* sites was amplified by PCR from a single N2 worm and was inserted into pPD49.26 in the *NcoI* site.

The *myo-2*: *TAO1*(1–416) and *kin-18*: *kin-18*(1–395) extrachromosomal arrays were integrated into the genome using gamma irradiation by exposure to a total of 2500 Rads over a total of 5 min. One integrated line of each was generated and each was backcrossed twice with a wild type N2 strain. The strains are DA1556 *adIs1556* [*myo-2*: *TAO1*(1–416) *rol-6(d)*] and DA1754 *adIs1754* [*kin-18*: *kin-18*(1–395) *rol-6(d)*]. The control integrated line is DP67, which has a *rol-6 unc-119*: *GFP* transgenic array integrated into the genome by radiation (*edIs1* [*rol-6 unc-119*: *GFP*]) (Maduro and Pilgrim, 1995). Other strains generated are DA1492 *adEx1492* [*myo-2*: *TAO*-WT *rol-6(d)*], DA1491 *adEx1491* [*myo-2*: *TAO*(D169A) *rol-6(d)*], and DA1746 *adEx1746* [*F42G10.2*: *GFP* *rol-6(d)*]. Worms were grown at 20°C using standard techniques on plates containing *E. coli* strains HB101 or DA837 (Davis et al., 1995) as indicated.

The Tc1 insertion *kin-18(pk71*: *Tc1*) was kindly provided by the Plasterk lab.

2.2. Growth and brood size assays

To measure the number of days it took for the bacteria on a plate to be depleted (plate starvation assay), a single larva in the L3 stage was placed on a 60 mm plate that had been seeded with DA837 bacteria that had been grown in liquid media comprised of LB with streptomycin (50 µg/ml) and M9 solution in a 1:3 ratio (LB:M9). Bacteria had been grown in the liquid media overnight at 37°C and stored at 4°C. Each plate was seeded with 120 µl of this bacterial suspension and the bacteria were grown on the plate at 37°C overnight. For analysis of worm size, eggs were collected by hypochlorite treatment (Emmons et al., 1979), synchronized to the L1 stage and placed on plates seeded with DA837. For brood size assays, single L2 stage worms were placed on plates seeded with DA837 and each worm was moved to a new plate each day until each worm laid zero eggs for 24 h. The resulting progeny on each plate were counted.

2.3. Electropharyngeogram

Single adult worms were placed in a liquid suspension of HB101 bacteria, which had been grown at 37°C overnight in a liquid suspension of LB with streptomycin (50 µg/ml) and M9 solution in a 1:3 ratio. Electrical recordings were accumulated in the absence of serotonin as previously described (Avery et al., 1995; Raizen and Avery, 1994). To calculate the excitation:repolarization (E:R) ratios, recordings of fifteen consecutive pumps of eight separate worms of each strain were analyzed.

2.4. Protein extraction and western blotting

Extraction of protein from N2 worms was as previously described (Gettner et al., 1995). 0.2 ml of pelleted N2 worms were lysed in 0.5 ml of ice cold immunoprecipitation buffer (50 mM Tris–Cl, pH 8.0; 150 mM NaCl; 1 mM MgCl₂; 1% Triton X-100) supplemented with a cocktail of protease inhibitors (50 µg/ml each leupeptin, aprotinin, and pepstatin; 1 mM phenylmethylsulfonyl fluoride). After the addition of 0.2 ml of packed quartz sand (Sigma),

the worms were alternately vortexed for 45 s and placed on ice for 30 s for a total of 5 min. The extract was transferred to a 1.5 ml microfuge tube and sedimented at 4°C at 1000 × g for 2 min, and the supernatant was then sedimented at 2500 × g at 4°C for 5 min. A final supernatant was obtained after a third centrifugation at 15000 × g for 30 min. Supernatant proteins were resolved by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies.

The polyclonal antibody S101 was raised against the peptide EKLEKELEELIAYQKK, which corresponds to amino acids 876–891 of KIN-18; polyclonal antibody S103 was raised against the peptide ERRPPLFSMNAMSALYHIAQ, which corresponds to amino acids 222–241 of KIN-18. The peptides were conjugated to *Limulus*-hemocyanin and injected into rabbits (Boulton and Cobb, 1991). Ten µl of S103 was used to immunoprecipitate KIN-18 from whole worm protein extracts. These immunoprecipitates were washed three times in a buffer containing 0.75 M NaCl and 20 mM Tris, pH 7.6 before being analyzed by SDS/PAGE, transferred to nitrocellulose membranes, and immunoblotted with S101.

2.5. cDNA isolation and transfections

Whole worm RNA was isolated using the TRI-Reagent (Molecular Resource Center). RNA was used for RT-PCR using the cDNA cycle kit (Invitrogen) to isolate a cDNA encoding the predicted ORF of F42G10.2. The cDNA was subcloned into the pCMV5 plasmid (Invitrogen) modified to attach an N-terminal Myc epitope into the encoded protein. HEK-293 cells were transfected with 2 µg DNA using calcium phosphate (Sambrook et al., 1989).

After 48 h, cells were lysed in detergent for kinase assays or by hypotonic lysis and douncing for coimmunoprecipitation as previously described (Hutchison et al., 1998). For coimmunoprecipitation experiments, immunoprecipitates were washed 3 × with 150 mM NaCl, 20 mM Tris, pH 8.0. They were then subjected to SDS-PAGE and immunoblotted as indicated.

2.6. Kinase assays

For kinase assays, proteins were immunoprecipitated with anti-Myc antibodies and harvested with protein A-Sepharose. Precipitates were washed 3 × with 0.75 M NaCl, 20 mM Tris, pH 8.0 and then incubated with 200 ng recombinant (His)₆-TAO1(1–416) for 45 min at 30°C in buffer containing 50 mM Tris, pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 100 µM ATP ([γ-³²P]ATP, 2–7 cpm/fmol). The protein A-Sepharose beads were washed with 1 ml of 50 mM Tris, 10 mM MgCl₂. Proteins were eluted from sedimented beads with electrophoresis loading buffer and analyzed by SDS-PAGE and autoradiography. Recombinant TAO1(1–416) was isolated from Sf9 cells as described (Hutchison et al., 1998).

3. Results

3.1. *kin-18* encodes a protein of about 120 kDa

kin-18 is located on cosmid T17E9 on chromosome III. While TAO1 and TAO2 contain 1001 and 1235 amino acids, respectively, ACeDB (A *C. elegans* Data Base) (Durbin and Thierry-Mieg, 1994) predicts *kin-18* to encode a protein containing 982 amino acids (accession number U11280). We were able to clone the cDNA by RT-PCR, and it agreed with the sequence and splicing predicted by ACeDB. The protein contains a putative kinase domain that shares 62% identity and 78% similarity with that of TAO1 (Fig. 1). Overall, the two proteins are 40% identical and 60% similar. The long C-terminal tails of approximately 700 amino acids do not contain any known protein binding domains, except a region to which a MEK3/6 binding site has been mapped (Chen et al., 1999). This region, residues 314–451 of TAO2, displays some conserved features with TAO1 and KIN-18. The balance presumably acts as a regulatory domain for the kinase, as a scaffold to which additional proteins bind, and to confer the appropriate localization of the protein.

Polyclonal antibodies S101 and S103 were generated against peptide sequences unique to the sequence of KIN-18. One peptide corresponded to amino acids 222–241 in the catalytic domain (S103) and the other to amino acids 876–891 in the C-terminal tail (S101). Each antibody recognized a protein of about 120 kDa, the appropriate size for a protein of 982 amino acids, on immunoblots of lysates of wild-type worms (Fig. 2A). Recognition of this 120 kDa band was blocked by preincubation of the antibodies with their corresponding peptides prior to immunoblotting (not shown). In addition, KIN-18 from whole worm lysates immunoprecipitated with S103 was detected when the precipitate was immunoblotted with S101 (Fig. 2B).

3.2. The *kin-18* promoter is active in the pharynx and intestine

To assess the expression pattern of the *kin-18* promoter in *C. elegans*, 2.5 kb of the 5' UTR of *kin-18* was fused to the GFP reporter sequence. The expression vector did not include a nuclear localization signal, and therefore the entire cell body of any cell expressing the *kin-18*:GFP fusion protein should be visible. Bright fluorescence indicative of *kin-18* promoter activity was observed in the pharynx, intestine, and a structure near the anus likely to be the anal sphincter (Fig. 3A). In addition, the H-shaped or excretory cell and several ring neurons also demonstrated *kin-18* promoter activity (Fig. 3A,B). In the pharynx, cells with *kin-18* promoter activity include the pharyngeal muscle of the metacarpus, the isthmus, and the terminal bulb (Fig. 3B). The pharyngeointestinal valve between the pharynx and intestine also exhibited *kin-18* promoter activity. Worms expressing lacZ with a nuclear localization signal from the *kin-18* promoter corroborated the GFP expression pattern observed (not shown).

3.3. Constitutively active TAO1 and KIN-18 affect worm growth and brood size

Because mammalian TAO1 activates kinases in cellular signaling cascades and the *kin-18* promoter is active in pharyngeal muscle, KIN-18 most likely regulates a signal transduction pathway in the pharynx of *C. elegans*. We generated strains of worms which express either wild type TAO1, kinase inactive TAO1, or a constitutively active truncation mutant,

TAO1(1–416) (Hutchison et al., 1998) under the control of the *myo-2* promoter. *myo-2* encodes a myosin heavy chain that is expressed solely in pharyngeal muscles (Okkema et al., 1993); thus the *myo-2* promoter directs TAO1 expression selectively to that site. We also constructed a strain in which the native *kin-18* promoter drives a truncated form of KIN-18, *kin-18: kin-18(1–395)*, thus expressing the catalytic domain of the kinase. This truncation mutant is analogous to constitutively active TAO1(1–416). The *myo-2: TAO1(1–416)* and *kin-18: kin-18(1–395)* extrachromosomal arrays were integrated into the genome by gamma irradiation.

When grown on the *E. coli* strain DA837, which is more difficult to ingest than the standard HB101 strain (Davis et al., 1995), both *myo-2: TAO1(1–416)* and *kin-18: kin-18(1–395)* worms grow more slowly, have a more starved appearance, and approach their full size at a more advanced age than the control. Strain DP67, with *rol-6(d) unc-119: GFP* integrated, shows no growth or egg laying defects and served as a control (Fig. 4). The worms expressing the active form of TAO1 have a lighter appearance as a result of fewer storage granules in their intestine, indicative of reduced food intake (Avery, 1993). The phenotype of the *kin-18: kin-18(1–395)* mutant is more severe than that of the *myo-2: TAO1(1–416)* worms; they grow significantly more slowly than the other strains. As observed by the appearance of the vulva, the *kin-18: kin-18(1–395)* worms also reach adulthood later (age 72 h) than the control (age 48 h). Also, the *kin-18: kin-18(1–395)* worms did not have eggs visible in their gonads until later in adulthood (age 96 h- Fig. 4A), although the vulvae were fully developed at 72 h (Fig. 4C). The retarded growth of the TAO1(1–416)- and KIN-18(1–395)-expressing strains is most evident during the larval stages, resulting in very small worms at the young adult stage. Strains with transgene arrays for either wild type TAO1 or TAO1(D169A) under the control of the *myo-2* promoter did not grow differently from control worms and did not exhibit the starved appearance (data not shown).

The *myo-2: TAO1(1–416)* strain takes 35% longer than the control to consume all the bacteria on a plate (plate starvation) and the *kin-18: kin-18(1–395)* mutants took twice as long (Fig. 5A). Because the time to plate starvation is directly related to the number of worms and the amount each eats, it appeared likely that the mutants were also producing fewer progeny. Brood size was assayed by counting the total number of viable eggs laid by each of ten worms of each strain (Fig. 5B). The brood sizes of the *myo-2: TAO1(1–416)* worms and *kin-18: kin-18(1–395)* worms were 25 and 60% smaller than that of the control strain. Brood sizes of TAO1(1–416) and *kin-18(1–395)* expression strains were significantly different from that of the control and from each other. (Student's *t*-test, $P < 0.001$). Again, *kin-18: kin-18(1–395)* mutants had a more severe phenotype than *myo-2: TAO1(1–416)* mutants, with a more starved appearance and slower growth and proliferation.

3.4. Expression of active TAO1 or active KIN-18 affects pharyngeal pumping

The amount eaten by worms directly correlates with their size, growth, and brood size. Because the promoter of *kin-18* is active in the pharynx, we thought that the *myo-2: TAO1(1–416)* and *kin-18: kin-18(1–395)* mutants might have altered feeding behavior. Worms eat by rhythmic pumping of the pharynx when food is present. Mutations affecting pharyngeal pumping impact feeding behavior and worm growth (Avery, 1993). Pumping is controlled

by pharyngeal muscle electrical activity, which can be assessed by the electropharyngeogram (EPG), which measures the current generated by pharyngeal muscle during pumping (Raizen and Avery, 1994). We observed that the *myo-2::TAO1(1–416)*- and *kin-18::kin-18(1–395)*-expressing worms exhibit EPGs distinct from the control worms. A blind test analysis of EPG recordings of these strains showed that EPGs of the *kin-18* and TAO1 truncation mutant strains could be distinguished from control recordings ($P < 0.01$). However, the mutant strains could not be distinguished from each other by this test. Representative EPG recordings are shown (Fig. 6A).

This alteration in the EPG was most apparent in the ratio of the height of the excitation peak to that of the repolarization peak (E:R). (Note that the ratio should be independent of worm size because both spikes are expected to be affected equally by differences in size (Raizen and Avery, 1994)). This ratio for the TAO1 and *kin-18* truncation mutant strains is significantly smaller than that of a control strain. To quantitate the E:R ratios, EPG recordings of 15 consecutive pumps by each of eight separate adult worms of each strain were analyzed (Fig. 6B). The E:R ratio of *myo-2::TAO1(1–416)* and *kin-18::kin-18(1–395)* worms was approximately 33% smaller than that of control worms. Both mutant strains were statistically significantly different from the control, and the *myo-2::TAO1(1–416)* and *kin-18::kin-18(1–395)* mutant strains had statistically significantly different E:R ratios from each other ($P < 0.01$), although the difference is modest. The pumping rate was not altered by expression of the mutant kinases as measured by visual inspection.

3.5. F42G10.2 encodes a possible downstream effector of KIN-18

Biochemical experiments indicate that TAO1 and TAO2 may be physiological stimulators of MEKs 3, 4, and 6 (Hutchison et al., 1998; Chen et al., 1999). Cosmid F42G10 is predicted to encode a protein that is related to mammalian stress-responsive MEKs, sharing 40% sequence identity with MEK4. Using GFP as a reporter, we observed strong F42G10.2 promoter activity in pharyngeal muscles and several unspecified neurons (Fig. 7). Unlike the promoter of *kin-18*, the promoter of F42G10.2 was not active in the intestine, anus, or excretory cell. The overlapping expression pattern with *kin-18* and similarity to MEK4 suggest that the product of F42G10.2 may be an effector of KIN-18.

To test this hypothesis, a cDNA encoding F42G10.2 was cloned by RT-PCR from whole worm RNA. When expressed in HEK-293 cells, F42G10.2 can be detected as a protein of approximately 42 kDa. The immunoprecipitated kinase exhibits no autophosphorylation but is phosphorylated by recombinant TAO1(1–416) purified from Sf9 cells (Fig. 8A). We sought additional evidence to support this kinase-substrate relationship by testing for physical interactions between TAO1 and F42G10.2. We showed previously that TAO1 and TAO2 bind selectively to stress-sensitive MEKs (Hutchison et al., 1998; Chen et al., 1999). Epitope-tagged fusions of the proteins, Myc-F42G10.2 and TAO1-HA, were transfected into HEK-293 cells. Each was immunoprecipitated and probed on western blots for the presence of the other. We detected Myc-F42G10.2 in TAO1-HA immune complexes as well as the converse, indicating that the nematode MEK binds to mammalian TAO1 when the two proteins are co-expressed (Fig. 8B).

3.6. Tc1 insertion strain shows no abnormal phenotype

A strain with a Tc1 insertion in *kin-18*, *pk71::Tc1*, exhibits no abnormal phenotype. However, using PCR, we mapped the Tc1 insertion to an intron downstream of amino acid 700. By RT-PCR, we found that the Tc1 insertion is removed during splicing and full length KIN-18 is expressed (data not shown). This is not surprising as splicing to remove Tc1 from mutant pre-mRNAs allows many Tc1 insertions to be phenotypically silent (Rushforth and Anderson, 1996).

3.7. A *mek-1* null mutation does not suppress the starved phenotype

The *C. elegans* *mek-1* gene encodes a second kinase sharing about 40% sequence identity with mammalian stress-sensitive MEKs that is expressed in the pharynx and intestine (Koga et al., 2000). A null mutant *mek-1* strain is hypersensitive to starvation (Koga et al., 2000). Because mammalian TAO activates stress-sensitive MEKs, we sought to determine if a *mek-1* null mutation would suppress the growth defect observed in TAO and KIN-18 overexpression strains. However, *mek-1; myo-2::TAO1(1–416)* worms showed an even more severe defect than the *myo-2::TAO1(1–416)* worms, growing more slowly and having a smaller brood size. This indicates an exacerbation of the starved phenotype, rather than an amelioration that would have been anticipated if MEK-1 lay downstream of KIN-18. Attempts to generate *mek-1; kin-18::kin-18(1–395)* worms were unsuccessful; no viable strains were obtained. However, *mek-1/+; kin-18::kin-18(1–395)* heterozygotes were viable.

4. Discussion

TAO1 and TAO2 are recently identified protein kinases that have been placed in stress-sensitive MAPK pathways by virtue of activating the stress-responsive MEKs 3,4, and 6 in vitro and in transfected cells. These MEKs activate p38 MAP kinases, consistent with the effects of TAOs on p38 in reconstitution experiments. The four mammalian p38 MAP kinases have been associated with responses to inflammation and environmental stresses (Ono and Han, 2000). The similarity of KIN-18 to TAOs in the catalytic domain and throughout their lengths suggests that these enzymes are functional homologs. Although TAO mRNAs are most highly expressed in the mammalian brain (Hutchison et al., 1998; Chen et al., 1999), *kin-18* promoter activity is strongest in the pharynx, gut, anus, and excretory cell. This is interesting because in an intact worm, the pharyngeal lumen comes into contact with chemicals in the external environment. Therefore, *kin-18* may be part of a signaling pathway triggered by noxious agents in the external environment as they are ingested.

Expressing active truncation mutants of TAO1 or KIN-18 causes worms to exhibit a smaller size in the larval stages, retarded growth, and decreased brood size. These phenotypes can all be caused by decreased feeding and/or altered pharyngeal pumping. Although there was not a decrease in the pumping rate (data not shown), there was a subtle electrophysiological defect. The small E:R ratio in the mutants may reflect weaker or slower onset of the pharyngeal muscle action potential, which could make feeding less efficient and lead to the observed phenotype. Unfortunately, there is no direct relationship between the severity of an EPG defect and the severity of a feeding defect: some mutants with normal EPGs have

severely impaired feeding, while others with strikingly defective EPGs have only mild feeding defects. Compared with the phenotype of *myo-2::TAO1(1–416)* worms, the phenotype of the *kin-18::kin-18(1–395)* strain is more extreme. This may be a result of KIN-18 having a higher affinity than TAO1 for downstream proteins in *C. elegans*. Because *kin-18::kin-18(1–395)* was expressed from its own promoter, the more impaired phenotype may also reflect the importance of *kin-18* signaling pathways in the intestine, anus, ring neurons, or excretory cell on worm growth and feeding.

Expression of full length TAO1 in the pharynx did not produce a mutant phenotype, likely due to the low activity of this form of the kinase (Chen et al., 1999). Expression of a catalytically defective TAO1 (TAO1(D169A)) in the pharynx also did not cause a mutant phenotype. Although catalytically defective mutants of kinases often cause a dominant-inhibitory effect, the fact that TAO1 has a less pronounced effect than KIN-18 when constitutively active would be consistent with a weak inhibitory effect. Endogenous KIN-18 is still present and capable of conducting signals to its downstream effectors and TAO1(D169A) expression was limited to the pharyngeal muscle, thus not affecting signaling in cells outside the pharynx that express KIN-18. Therefore, we are unable to draw any conclusions about the *kin-18* loss of function phenotype.

Because TAO1 fits into a stress response pathway in mammals, it is intriguing that *kin-18* is expressed in pharyngeal muscle and expression of its active form leads to reduced feeding. Since the pharynx comes into contact with elements in the external environment, it seems reasonable to hypothesize that *kin-18* may be part of a signaling pathway that responds to harmful agents in the environment. In this case, *kin-18* may mediate reduced feeding to minimize ingestion of harmful agents. The idea of noxious stimuli eliciting stress responses in the pharynx has already been established; heavy metals and antifungal compounds known as captans, which are poisonous to *C. elegans*, activate stress-responsive heat shock promoters in the pharynx (Jones et al., 1996). Exposure to lead, zinc, copper, and captans induces pharyngeal expression of the heat shock protein HSP16-1 (Stringham and Candido, 1994; Jones et al., 1996). We found no induction of the HSP by active TAO1 or KIN-18 (data not shown). *mek-1* plays a role in stress response and our experiments concur with previously described results of *mek-1* null mutants being hypersensitive to starvation (Koga et al., 2000). Our results suggest that *mek-1* does not lie downstream of *kin-18* in a signaling pathway.

More than a dozen proteins activate stress-responsive MAP kinase pathways in mammals, although no clear links to physiological stimulants have been made. A search of the *C. elegans* genome shows that there is a multitude of potential MEK and MAPK homologs. Of four potential MEKs examined, we found an uncharacterized gene with an overlapping expression pattern that could be in a KIN-18-activated MAPK pathway based on biochemical studies. However, functional connections between these proteins have not been made.

Acknowledgments

We thank members of the Cobb and Avery laboratories for many helpful discussions and Dionne Ware for administrative assistance. This work was supported by grants from the National Institutes of Health (GM53032 to

MHC and HL46154 to LA). KB and MH were supported in part by an NIH Medical Scientist Training Program Grant and the Perot Family Foundation.

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Abbreviations

MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
MEK	mitogen-activated protein kinase/ extracellular signal-regulated kinase kinase
MEKK	MEK kinase
TAO	Thousand and One amino acid kinase
GFP	green fluorescent protein
ORF	open reading frame
kDa	kilodalton
PAGE	Polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
UTR	untranslated region
PCR	polymerase chain reaction
DTT	dithiothreitol
cDNA	DNA complementary to RNA
HEK	human embryonic kidney
IB	immunoblot
IP	immunoprecipitation
HSP	heat shock protein

Fig 1.

Sequence alignment of TAO1, TAO2, and KIN-18. An alignment of the sequences of TAO1, TAO2, and KIN-18 was generated using Multalin and Boxshade (Corpet, 1988). Identical residues are boxed in black and conserved residues are shaded. Within its kinase catalytic domain from amino acid position 23 to 290 (denoted by arrows), KIN-18 shares 60% sequence identity with TAOs and overall, TAO1 and KIN-18 are 40% identical.

Fig 2.

Western Blot detection of KIN-18. 7.5 or 15 μ l of N2 worm protein extracts were immunoblotted (IB) with anti-peptide antiserum S101. The band at approximately 120 kDa is KIN-18. (B) Lysates were subjected to immunoprecipitation (IP) with S103 and washed three times in a high salt buffer. Proteins were resolved on SDS/PAGE and immunoblotted with S101. The same 120 kDa protein is recognized in whole worm extracts and S103 immunoprecipitates.

Fig 3. GFP expression directed by the *kin-18* promoter. (A,B) Expression of the GFP reporter from a *kin-18::GFP* transformant is very strong in the pharynx, intestine, anus, and excretory cell. GFP expression in the corpus (co), isthmus (is), excretory cell (ec), terminal bulb (tb), intestine (int) is observed.

Fig 4.

Growth retardation of nematodes expressing active TAO1 or KIN-18 (A) Age matched worms from the *kin-18::kin-18(1-395)* (left worm), *myo-2::TAO1(1-416)* (middle worm), and the control *unc-119::GFP* (right worm) strains were grown on DA837 bacteria. Worms were synchronized in the L1 phase and this picture was taken 96 h after synchronization. (B–D) Comparison of *unc-119::GFP* (bottom worm) and *kin-18::kin-18(1-395)* (top worm) worms 72 h after being synchronized in L1. While both have developed vulvas, the *kin-18* overexpression strain contains no eggs and has a light, starved appearance. In comparison, the control worm contains eggs and is larger with darker intestinal granules. Higher magnification of *kin-18::kin-18(1-395)* worm (panel C) shows the worm has a vulva (arrow), but its gonad contains no eggs. An arrow points to an egg in the gonad of the *unc-119::GFP* worm (panel D).

Fig 5.

Nematodes expressing active kinases take longer to starve plates and have decreased brood sizes (A) 10 L3 worms of each of the indicated strains were placed individually on plates seeded with 120 μ l of DA837 bacteria. The control *unc-119::GFP* worms consumed all bacteria in 10 ± 0 days (AVG $\pm$ SEM). *myo-2::TAO1*(1-416) consumed all bacteria in 13.5 ± 0.3 days. *kin-18::kin-18*(1-395) worms consumed all bacteria in 19.5 ± 0.4 days. (B) The average brood size was determined by counting eggs laid by ten worms of each strain. The average brood sizes are as follows: *unc-119::GFP*, 225.4 ± 7.3 eggs; *myo-2::TAO1*(D169A), 222.0 ± 6.5 eggs; *myo-2::TAO1*, 205.0 ± 3.2 eggs; *myo-2::TAO1*(1-416), 164.1 ± 5.6 eggs; *kin-18::kin-18*(1-395), 91.0 ± 5.6 eggs. (Average $\pm$ SEM.)

Fig 6.

Altered EPG recordings of nematodes expressing active kinases (A) Representative EPG recordings of a single pharyngeal pump from *unc-119::GFP rol-6(d)* worms (bottom, control) and *kin-18* overexpressing worms (top). EPG recordings from the control strain could be distinguished from those of the active TAO1 and *kin-18* mutants in a blind test ($P < 0.01$ in a Students *t*-test). However, EPG recordings from *myo-2::TAO1(1-416)* and *kin-18::kin-18 (1-395)* worms could not be distinguished from each other in a blind test. For this reason, we show one pump representative of both strains. (B) EPG recordings of 15 consecutive pumps from each of eight worms of the indicated strains were analyzed and the E:R ratio for each pump was determined. Control worms had an average E:R ratio of 0.690 ± 0.014 (SEM) while *myo-2::TAO1(1-416)* and *kin-18::kin-18 (1-395)* worms had averages of 0.490 ± 0.012 and 0.456 ± 0.011 , respectively. Each was significantly different from the others in a Students *t*-test ($P < 0.01$).

Fig 7. GFP expression directed by the F42G10.2 promoter. Expression of the GFP reporter from a *F42G10.2::GFP* transformant shows promoter activity throughout the pharynx. GFP is detected in the corpus (co), isthmus (is), and terminal bulb (tb).

Fig 8.

TAO1 phosphorylates F42G10.2, and the proteins coimmunoprecipitate (A) HEK 293 cells were transfected as indicated and proteins were immunoprecipitated with a monoclonal anti-Myc antibody. They were then incubated with recombinant TAO1(1–416) and proteins were analyzed by SDS/PAGE and autoradiography (top panel). Expression of F42G10.2 was measured by immunoblotting. (B) HEK 293 cells were transfected with vector, Myc-F42G10.2, and TAO1-(HA)₃ as indicated. Proteins immunoprecipitated with the αMyc antibody were immunoblotted with the αHA antibody and αHA immunoprecipitated proteins were immunoblotted with the αMyc antibody. Expression of proteins was verified by immunoblotting cell lysates with αMyc or αHA antibodies.