Supplementary material

Methods

Strains. Nematodes were grown at 20°C under standard conditions (Brenner 1974). Wild type and *lin-15*(*n765ts*) strains were obtained from the *Caenorhabditis* Genetics Center. The mutant *crh-1(tz2)II* has a 979 bp deletion (54730-55708 of Y41C4A) corresponding to 38aa of exon VII. The *ckk-1(tm421)III* strain has a 543bp deletion (9237-9779 of C05H8) corresponding to 151 amino acids of the CKK-1 protein. Both strains were isolated in a nested PCR screen of the trimethylpsoralen/UV treated stock strains as described (Gengyo-Ando and Mitani 2000).

cDNA isolation and expression. A previously unrecognized exon of *ckk-1* that appends 75 amino acids to the predicted C-terminus was identified by 3'-RACE. We detected no enzymatic or Ca^{2+}/CaM-dependency differences between the full-length CKK-1 (1-432) and the shorter form (1-357) used in previous studies (Tokumitsu *et al*., 1999) (data not shown). Herein we use the full-length version and the revised sequence has been submitted to Genebank (Accession Number: AB016838). *C. elegans* CaM-KI (*cmk-1*) was cloned and expressed as previously described (Eto *et al.*, 1999). Point mutants (Thr179Ala and Lys52Ala) of CMK-1 were generated using standard site-directed mutagenesis techniques and sequenced.

crh-1 cDNA was cloned in a screen of a λZAP *C. elegans* embryonic cDNA library probed with a fragment amplified from yk217f1 (kindly provided from Y. Kohara at National Institute of Genetics, Mishima, Japan), which was selected for its similarity to

the C-terminal coding region of mouse CREB. Four clones were isolated and sequenced from 2 x 10^5 plaques screened; one (*crh-1*β) contained a 1262 nucleotide ORF, while the others lacked translation start sites. 5'-RACE-PCR identified three 5' splice variants (*crh-1*α, *crh-1*β, and *crh-1*γ1) all of which preserve the integrity of the KID and bZIP domains. Further analysis of ESTs (from Y. Kohara) identified a fourth variant (*crh-1*γ*2*). All sequences submitted to Genebank (Accession Numbers: *crh-1*α; AB081597, *crh-1*β AB081595, *crh-1*γ1 AB081598, and *crh-1*γ2 AB081596). The originally isolated full-length clone, *crh-1*β, was used for further experiments.

For GAL4-binding domain mammalian expression plasmids, the transactivation domain (residues 1-242) of either wild-type or Ser29Ala mutant of *crh-1*β was ligated into the pM plasmid (CLONTECH). pET16b-vectors encoding His-CRH-1β and His-CRH-1β (Ser29Ala) were created by standard PCR, subcloning, and site-directed mutagenesis techniques.

***In vitro* phosphorylation of CRH-1 by *C. elegans* CaM-kinase cascade**. Recombinant GST-CMK1 (0.94 μg) was activated at 30 °C for 20 min with a CaMKK preparation (20ng) partially purified from *ckk-1* or mock transfected COS-7 cells by CaM-Sepharose as previously described (Eto *et al*., 1999). CKK-1 alone (0.5 ng), activated CMK-1 (22ng) or mock activated (unactivated) CMK-1 (22ng) were incubated with recombinant wild type or Ser29Ala mutant of CRH-1β (0.2 μg) in 30 mM HEPES (pH 7.5), 5 mM $MgAc_2$, 1 mM DTT, 2 mM $CaCl_2$, 3 μM CaM and 100 μM of either [γ-^{32}P]-ATP or cold ATP at 30 °C for 10 min. After addition of SDS-PAGE buffer, samples were subjected to SDS-10% PAGE followed by either autoradiography or

immunoblotting using anti-phospho-CREB antibody (NEB) or anti-His-tag antibody (Sigma).

Transcriptional activation mediated by *crh-1*. COS-7 cells (6-well dishes) were transfected with 0.5 μg of reporter plasmid (pFR-5xGAL4-binding element-Luciferase, Stratagene), either pM-*crh-1*β or pM-*crh-1*β (Ser29Ala), and a combination of expression plasmids (pME18s) encoding wild type or mutant CMK-1 (0.5 μg) and/or CKK-1 (0.5 μg), or the catalytic subunit of mammalian PKA (0.5 μg). After 24 hr, cells were serum-deprived for 6 hr then stimulated with 1 μM ionomycin for 12 hr and lysed. Luciferase activities were detected by standard methods using PicaGene (Toyo Inki).

Transgenic plasmid construction. The promoter fusion gene *ckk-1*::GFP includes 2.4kb genomic sequence from C05H8 upstream from the start site inserted into pPD95.79 (from A. Fire). Similarly, *cmk-1*::GFP includes 5.4kb upstream of the *cmk-1* start site transferred into pPD95.79 GFP, fusing CMK-1(aa1-25) with GFP. The *cmk-1* promoter construct also drove expression of *cmk-1* cDNAs: pYT41.2.2-*cmk-1*::CMK-1 (wild type); pYT42.2.5-*cmk-1*::CMK-1 (1-295), pYT42.2.2-*cmk-1*::CMK-1 (1-295, T179D), pYT42.2.6-*cmk-1*::CMK-1 (1-295, T179A) and pYT41.3.1-*cmk-1*::CRH-1β wild type . p*CRE*::GFP was generated by replacing the *hsp16/2* promoter of pPD118.26 (from A. Fire) with a synthetic ds78-mer containing four tandem CRE repeats (AGCCTGACGTCAG).

Whole-mount *in situ* hybridization. *In situ* hybridization for whole-mount animals was performed by using digoxigenin-UTP-labeled riboprobes representing full-length sense or antisense *crh-1β* cDNA sequences for overnight hybridization at 60°C.

References

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