Distinct roles of the Src family kinases, SRC-1 and KIN-22, that are negatively regulated by CSK-1 in *C. elegans*

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Received 10 October 2002; revised 5 December 2002; accepted 5 December 2002

First published online 17 December 2002

Edited by Giulio Superti-Furga

Abstract To elucidate the primitive roles of the Src family kinases (SFKs), here we characterized *Caenorhabditis elegans* orthologues of SFKs (*src-1* and *kin-22*) and their regulator kinase Csk (*csk-1*). SRC-1 and KIN-22 possess the C-terminal regulatory tyrosines characteristic of SFKs, and their activities are negatively regulated by CSK-1 in a yeast expression system. The *src-1* and *csk-1* genes are co-expressed in some head neurons, the anchor cell and the tail region, while *kin-22* and *csk-1* genes are co-expressed in pharyngeal muscles and tail region. Expression of KIN-22 induced morphological defects in the pharynx, whereas expression of SRC-1 did not show any overt phenotype in adult. RNA interference of *src-1*, but not that of *kin-22*, caused a developmental arrest in early development. These results suggest that SRC-1 and KIN-22 play distinct roles under the control of CSK-1.

Key words: *csk-1*; *src-1*; *kin-22*; Tyrosine kinase; *Caenorhabditis elegans*

1. Introduction

Protein tyrosine kinases (PTKs) are present only in metazoans, and many of them, including the Src family kinases (SFKs), are highly conserved during evolution from sponge to human [1–4]. The SFKs are non-receptor type PTKs that are associated with the plasma membrane through their fatty acylated N-termini, and consist of two peptide-binding modules, Src homology 2 (SH2) and Src homology 3 (SH3) domains, and a tyrosine kinase domain [5–7]. The SFKs serve as molecular switches involved in the initiation of a variety of cellular events in the multicellular animals, including cell growth and division, cell attachment and movement, differentiation, survival, and death [7]. The phosphorylation of the regulatory tyrosine of SFK is strictly controlled by another PTK, the C-terminal Src kinase (Csk) [8,9]. Since Csk is also evolutionally well conserved, the SFK/Csk circuit is thought to be essential in controlling the functions of SFKs in multicellular animals [2,10,11].

Although substantial studies have been accomplished, signaling pathways through SFKs have not been well established. Since SFKs are involved in a wide variety of cellular events, critical points of SFK actions still remain to be defined. Furthermore, functional redundancy among nine members has hampered the analysis of their individual in vivo functions [12]. To overcome these difficulties, we have decided to utilize more primitive multicellular animals as an experimental model, and have chosen the soil nematode *Caenorhabditis elegans*. The genomic information of *C. elegans* has already pointed that there are some SFK-related kinases [3]. Recently, it was shown that one of the SFKs, *src-1*, plays an essential role in embryonic development and contributes to spindle orientation and endoderm specification in early development [13]. In this report, we have characterized another *C. elegans* SFK gene (*kin-22*) and a csk gene (*csk-1*). The results obtained in this study suggested that SRC-1 and KIN-22 play distinct roles and are negatively regulated by CSK-1 in *C. elegans*, and that *C. elegans* would provide an excellent model to study the primitive function of SFK members in vivo.

2. Materials and methods

2.1. Worm cultivation

Wild-type *C. elegans* Bristol strain (N2) and SU93 (*jcIs1*) IV were cultivated at 20°C on NGM agar plates seeded with the OP-50 strain of *Escherichia coli* [14].

2.2. Identification of csk-1, src-1 and kin-22 genes and plasmid constructions

The *csk-1* (Y48G1C.2), *src-1* (Y92H12A.1) and *kin-22* (F49B2.5) in *C. elegans* were identified by a simple module architecture research tool (SMART) search with architecture analysis of Csk and SFKs consisting of SH3, SH2 and tyrosine kinase domain [15,16]. The EST clones, yk657d3, yk117f2 and yk256f2 (obtained from Y. Kohara), corresponding to *csk-1*, *src-1* and *kin-22*, respectively, were sequenced and subcloned. To assess the gene expression, 5′-promoter sequences of the genes were introduced into the plasmid pPD95.77 (kindly provided by A. Fire) carrying DsRed2 (Clontech) or green fluorescent protein (GFP) reporter gene. To obtain *csk-1* promoter::DsRed2 fusion gene, 5′-promoter sequence (5.0 kb) was amplified by polymerase chain reaction (PCR) and subcloned into the pPD95.77 vector carrying DsRed2. For *src-1* and *kin-22* genes, 5′-promoter sequences (4.9 kb and 2.7 kb, respectively) were amplified by PCR and subcloned into the pPD95.77 vector carrying GFP. For expression of *kin-22*, *kin-22* K268M and *kin-22* Y500F, cDNAs were subcloned into the pPD49.26 vector carrying the 5′-regulatory sequence of *kin-22*.

2.3. Germline transformation

Germline transformation was carried out as described by Mello et al. [17]. To express promoter::reporter fusion gene, 50 ng/μl of *src-1*

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Abbreviations: SFK, Src family kinase; PTK, protein tyrosine kinase; Csk, C-terminal Src kinase; SH2, Src homology 2; SH3, Src homology 3; SMART, simple module architecture research tool; DIC, differential interference contrast

0014-5793/02/$22.00
doi:10.1016/S0014-5793(02)03819-X

promoter::gfp or *kin-22* promoter::gfp vector was co-injected with 50 ng/μl *csk-1* promoter::DsRed2 vector into N2 strain. Transgenic animals were visualized on a Carl Zeiss LSM 510 confocal microscope. For *kin-22* cDNA expression, 50 ng/μl plasmid was injected into N2 strain together with 50 ng/μl *kin-8* promoter::gfp as injection marker [18].

2.4. Yeast growth assay

Saccharomyces cerevisiae YPH499 strain and pESC epitope tagging vector used in this work were purchased form Stratagene. *csk-1* was subcloned in FLAG-tagged pESC vector and *src-1* and *kin-22* were subcloned in myc-tagged pESC vector. Yeast cells were kept in standard minimal medium lacking the appropriate amino acids for plasmid selection [19]. Yeasts were cultured in non-inductive medium containing dextrose at growth phase and then diluted to 0.01 at OD_{600} in medium containing galactose to induce the *csk-1* under the *gal-10* promoter or *src-1* and *kin-22* under the *gal-1* promoter. To assess the yeast growth, OD_{600} was measured after induction at indicated time. The protein extracts from yeast cell were obtained by lysing cell using the buffer-glass beads method, as described previously [20]. The lysis buffer contained 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% DOC, 1% sodium dodecyl sulfate (SDS), 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml aprotinin. Proteins were run on SDS gels and immunoblotting.

2.5. Morphological analysis of pharynx

Parental worms of *kin-22* transgenic animals were cultured on NGM plates and laid eggs for 24 h, and then we removed the parental worms. Laid eggs on NGM plates were cultured at 20°C for 4 days, and then the pharynx morphology of adult worms with injection marker was observed by differential interference contrast (DIC) microscopy (Olympus BX-60). Two independent lines of each transgenic worm were assayed.

3. Results and discussion

3.1. Identification of SRC-1, KIN-22 and CSK-1 as counterparts of mammalian c-Src, Fyn and Csk

Csk and SFKs share functional domain structures including SH3, SH2 and tyrosine kinase domain. From SMART search analysis using the architecture containing SH3, SH2 and kinase domain, some genes were detected in database [15,16]. One of them showed significant homology to mammalian Csk and the other two genes showed significant homology to mammalian SFKs. The primary structure of Y48G1C.2 that potentially encompasses the gene for the Csk counterpart (*csk-1*) was analyzed by comparison with the sequences of the EST yk657d3 clone and reverse transcription (RT)-PCR products. The putative gene product, termed CSK-1, consists of 539 amino acids and shows 54% identity in the kinase domain and 47% identity overall with mouse Csk. This *csk-1* gene product was different from the predicted open reading frame (ORF) (Y48G1C.2) in database.

Y92H12A.1 and F49B2.5 were previously identified as *src-1* and *kin-22* [13,21]. Protein products of these genes have significant homology to mammalian SFKs and contain the conserved autophosphorylation site in the kinase domain and the negative regulatory tyrosine residue in the carboxyl-terminus, both of which are hallmarks of the SFKs. SRC-1 and KIN-22 consist of 533 and 507 amino acids, respectively, and have 62 and 61% identity in the kinase domain, and 47 and 50% identity in overall mouse c-Src and Fyn, respectively (Fig. 1). There is no significant homology in the N-terminal unique domain of SRC-1, KIN-22 and CSK-1, but the amino-terminal fatty acylation signals, another characteristic of SFKs, are recognized in SRC-1 and KIN-22, but not in CSK-1 (data not shown).

To confirm that CSK-1, SRC-1 and KIN-22 serve as functional homologues of Csk, c-Src and Fyn, respectively, we characterized these kinases in yeast cells. Expression of SRC-1 or KIN-22 alone resulted in growth inhibition around 40 h after induction. The extent of growth inhibition is known to be proportional to the catalytic activity of c-Src [22]. The expression of CSK-1 alone had no effect on cell growth because CSK-1 is predicted to be highly specific for the C-terminal tyrosine of SRC-1 and KIN-22. When CSK-1 was co-expressed with SRC-1 or KIN-22, normal growth was restored. However, co-expression of CSK-1 K300M, a kinase negative mutant, could not restore normal growth. Furthermore, growth inhibition induced by the expression of SRC-1 Y528F or KIN-22 Y500F, constitutive active mutants having Phe instead of Tyr at the C-terminal regulatory site, was not affected by CSK-1 expression (Fig. 2A). These findings suggest that CSK-1 could target the regulatory tyrosine of SRC-1 and KIN-22 to inhibit their activities. To verify this possibility, the kinase activities of SRC-1 and KIN-22 in the cells were measured by immunoblot analysis using anti-phosphotyrosine antibody (Fig. 2B). The tyrosine phosphorylation levels in the cellular proteins reflect the catalytic activities of CSK-1, SRC-1 and KIN-22 because tyrosine kinases are absent from yeast. While the expression of SRC-1 or KIN-22 substantially increased the tyrosine phosphorylation level of yeast proteins, CSK-1 did not, reflecting that CSK-1 is highly specific for SFKs. When CSK-1 was co-expressed with SRC-1 or KIN-22, the tyrosine phosphorylation levels were significantly decreased as compared with the levels induced by SRC-1 or KIN-22 alone. However, co-expression of kinase-inactive CSK-1 K300M did not reduce SRC-1- or KIN-22-induced tyrosine phosphorylation, and the activities of SRC-1 Y528F and KIN-22 Y500F were not inhibited by CSK-1 at all. These results are consistent with those of growth inhibition assays, and clearly demonstrate that CSK-1 specifically

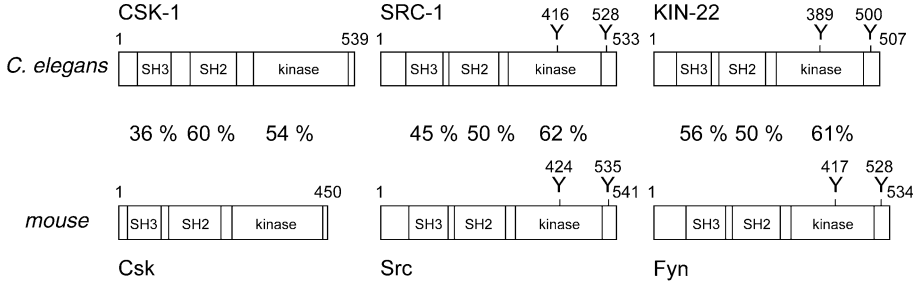

Fig. 1. Schematic structure of CSK-1, SRC-1 and KIN-22. The amino acid identity of each domain is calculated between *C. elegans* and mouse molecules. The autophosphorylated tyrosines in the kinase domains and the C-terminal regulatory tyrosines are indicated by Y. The cDNA sequence data of *csk-1* will appear in the DDBJ/EMBL/GenBank databases with the accession number(s) AB096875.

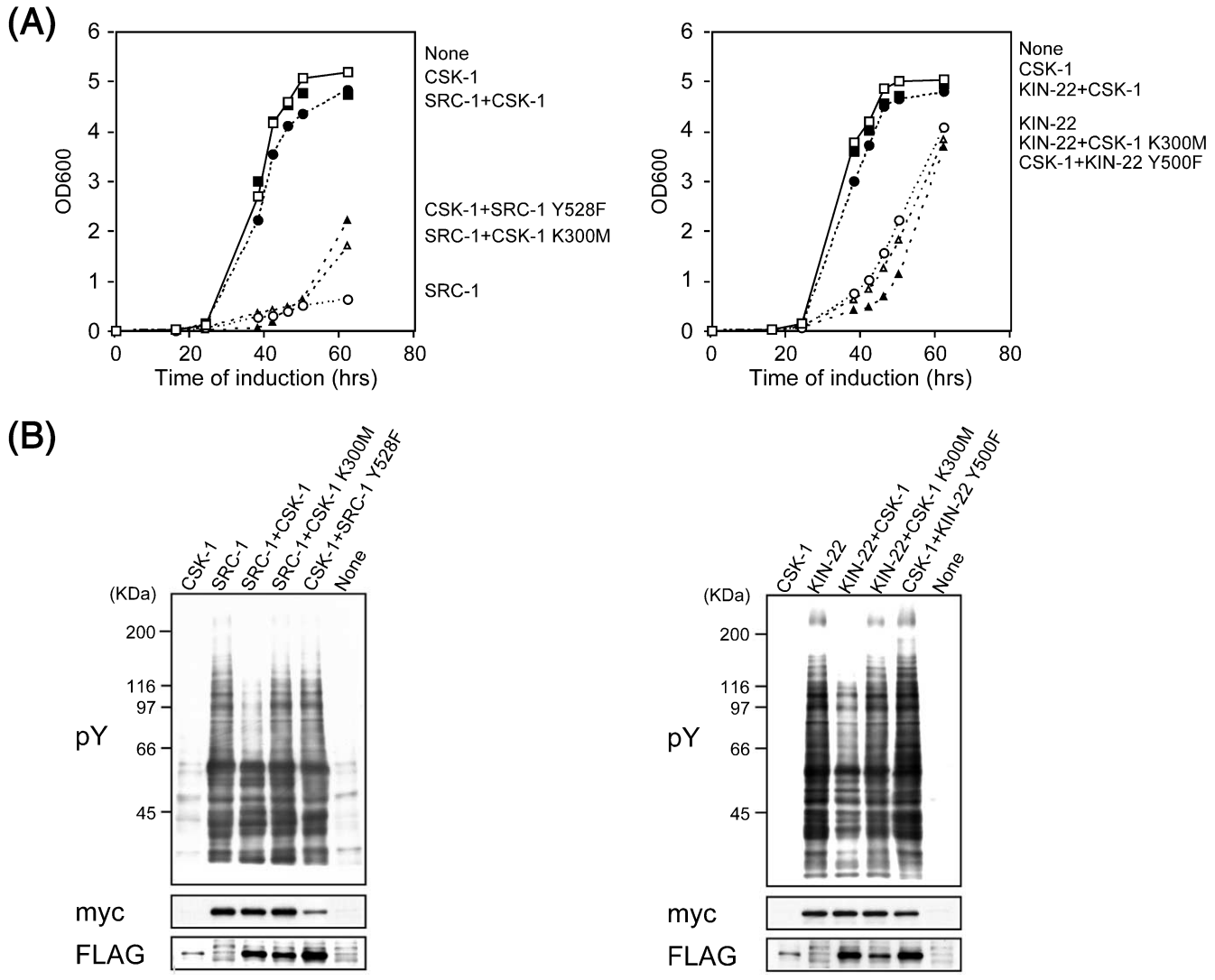

Fig. 2. *S. cerevisiae* based growth assay of the ability of CSK-1 to regulate SRC-1 and KIN-22. A: Growth curves of yeasts expressing indicated gene product; control vector (open squares), CSK-1 (closed squares), SRC-1 or KIN-22 (open circles), SRC-1 and CSK-1 or KIN-22 and CSK-1 (closed circles), SRC-1 and CSK-1 K300M or KIN-22 and CSK-1 K300M (open triangles), CSK-1 and SRC-1 Y528F or CSK-1 and KIN-22 Y500F (closed triangles). The time after induction is indicated in hours. B: Immunoblots of lysates prepared from yeasts expressing indicated gene products at 38 h post-induction. Tyrosine phosphorylation of the cellular proteins was detected with anti-phosphotyrosine antibody (4G10) (upper panel), SRC-1 or KIN-22 were detected with anti-myc antibody (9E10) (middle panel) and CSK-1 protein was detected with anti-FLAG antibody (M5) (bottom panel).

targets the C-terminal tyrosine of SRC-1 and KIN-22 to negatively regulate their activities. Recently, more primitive orthologues of SFKs and Csk have been isolated in Sponge [2]and Hydra [10]. Taking these lines of information together with our data, it is likely that SFKs have evolved along with Csk since the appearance of metazoans, and that the regulation by Csk is indispensable for SFKs to exert their functions.

3.2. Expression patterns of src-1, kin-22 and csk-1

If CSK-1 is a physiological regulator for SRC-1 and KIN-22, it should be co-expressed with SRC-1 or KIN-22 in the same cells. To address this, we assessed the tissues in which the genes were expressed by detecting reporter proteins (DsRed2 for CSK-1; GFP for SRC-1 or KIN-22) in transgenic worms. In Fig. 3A, the expressions of *csk-1* and *src-1* genes were visualized by the fluorescent images of DsRed2 and GFP, respectively. The expressions of both genes were widely overlapping, and the co-expression signals were detected in some neurons (ASE, ADF, AVA, AUA, RMDV and BAG) in the head region, the anchor cell, the vulva, the cells around the anus, the body wall muscle, pharyngeal muscles in procorpus and metacorpus and distal tip cells of gonad. Fig. 3B shows the expression patterns of the reporter proteins for *csk-1* and *kin-22* genes. These reporters are strictly co-expressed in pharyngeal muscles, the vulva and some cells around anus. These observations indicate that the expression patterns of *src-1* and *kin-22* genes are significantly different, and the expression of *csk-1* gene overlaps with the total expression patterns of *src-1* and *kin-22* genes.

3.3. Functional analysis of SRC-1 and KIN-22

To analyze the functions of SRC-1 and KIN-22 in vivo, cDNAs encoding these proteins were introduced into the N2 strain under the control of their own promoters. The intro-

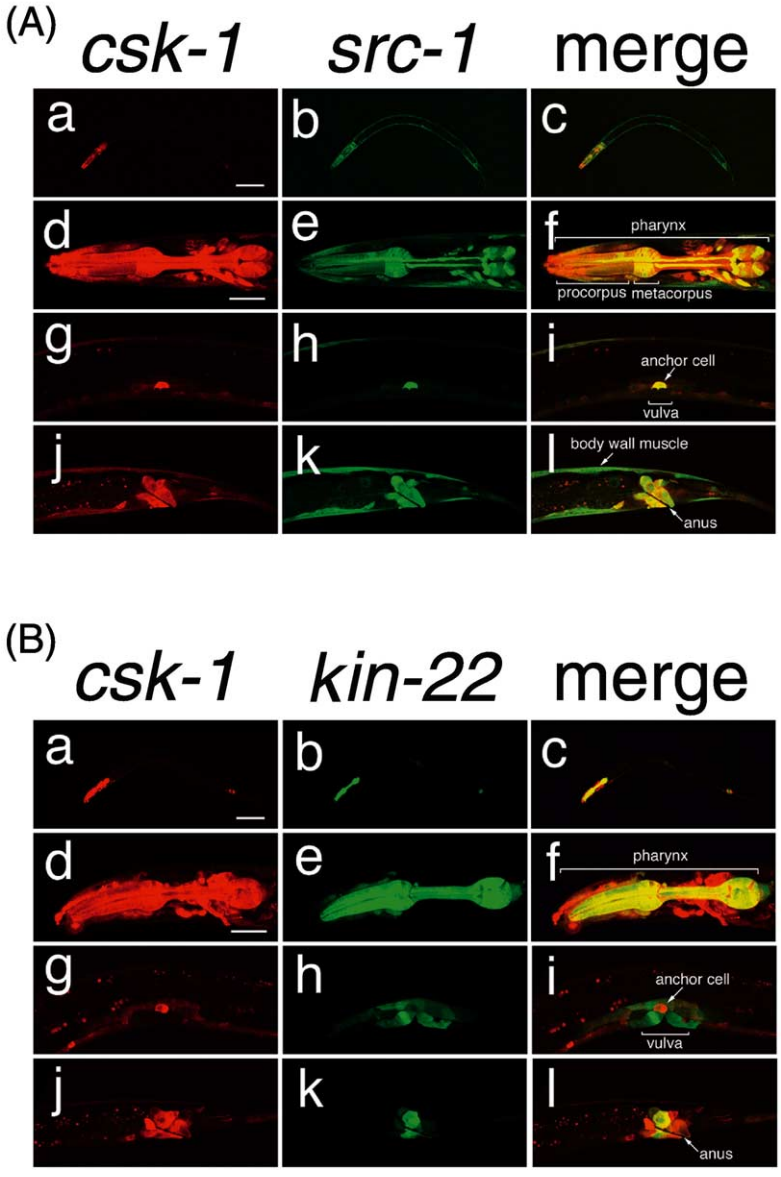

Fig. 3. A: Fluorescent images of the reporter proteins expressed in L3 stage of worms using *csk-1* promoter (DsRed2; a, d, g and j) and *src-1* promoter (GFP; b, e, h and k). Merged images are shown in c, f, i and l. Whole body images are shown in a–c. Higher magnification images of pharynx (d–f), vulva (g–i) and tail (j–l). Scale bars are 100 μm in a and 20 μm in d, respectively. B: Fluorescent images of the reporter proteins expressed in L3 stage of worms using *csk-1* promoter (DsRed2; a, d, g and j) and *kin-22* promoter (GFP; b, e, h and k). Merged images are shown in c, f, i and l. Whole body images are shown in a–c. Higher magnification images of pharynx (d–f), vulva (g–i) and tail (j–l). Scale bars are 100 μm in a and 20 μm in d, respectively.

duction of *kin-22* cDNA greatly reduced viability in the early stages of development. By the larval stage, 44.6% ($n = 121$), 30.7% ($n = 163$) and 76.3% ($n = 139$) of worms carrying the wild-type *kin-22* (*kin-22*), an inactive form of *kin-22* (*kin-22* K268M) and an active form of *kin-22* (*kin-22* Y500F), respectively, were dead. Furthermore, substantial numbers of escapers showed an obvious defect in the structure of the pharynx where the expression of *kin-22* was the most prominent (Fig. 4A–D). The expression of *kin-22* and *kin-22* Y500F caused a bending of the pharynx structure with higher incidence in the active form (line 1, 34.5%; line 2, 38.8%) than the wild type (28.1%, 25.8%). However, *kin-22* K268M induced the defect with much lower incidence (4.33%, 4.93%). These findings demonstrate that the pharynx abnormality is dependent on the kinase activity of KIN-22. The introduction of *src-1* also caused lethality by the stage of larva with the incidences being 44.8% ($n = 255$), 19.6% ($n = 301$) and 44.0% ($n = 159$) for wild-type *src-1*, an inactive form and an active form, respectively. However, there was no apparent abnormality in the escapers of any type of *src-1* transgenic worms (data not shown). These observations suggest that KIN-22 and SRC-1 play important roles during the development of *C. elegans*, and that KIN-22 could involve the morphogenesis or maintenance of the morphology of the pharynx, although its molecular mechanism remains to be studied.

Effects of loss of function of *src-1* and *kin-22* genes were examined by RNA interference analysis (*RNAi*). The *RNAi* of *src-1* induced a growth arrest of embryo at the stages between gastrulation and 2-fold stage. This embryonic lethality has recently been observed in the *src-1* deletion mutant [13], sug-

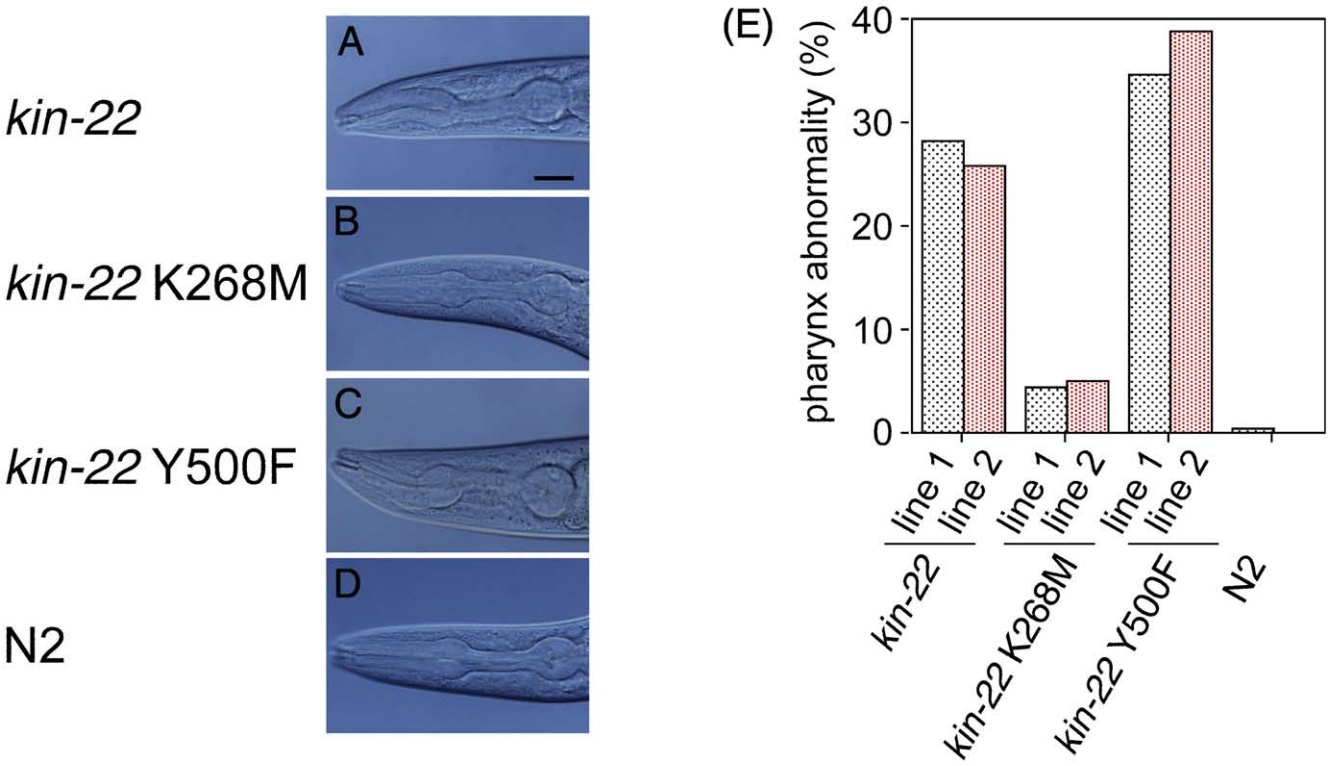

Fig. 4. Effects of expression of *kin-22*, *kin-22* K268M or *kin-22* Y500F in the wild-type background. Head regions of worms expressing indicated genes (A–C) and wild-type strain N2 (D) are observed under DIC microscopy. Incidences of the pharynx abnormality are plotted in E. Ratios of worms having pharynx abnormality to total worms expressing injection marker were calculated for the two transgenic lines (*kin-22* line 1, $n=217$; line 2, $n=240$; *kin-22* K268M line 1, $n=277$; line 2, $n=284$; *kin-22* Y500F line 1, $n=278$; line 2, $n=232$) and wild-type N2 ($n=261$). Scale bar is 20 μm in A.

gesting that *src-1* is an essential gene in embryonic development of *C. elegans*. In contrast, the *RNAi* analysis for *kin-22* gene did not show any overt phenotype (Fig. 5). These results suggested that SRC-1 and KIN-22 might play distinct functions in *C. elegans*.

In this study, we have identified *C. elegans* orthologues of mammalian SFKs and Csk. In vitro and in vivo studies using yeasts and transgenic worms, respectively, suggest that SRC-1 and KIN-22 are regulated by CSK-1 in the same manner as the mammalian system. Differential expression and functions of the SFKs in the worms suggest that the SFKs have already diverged in *C. elegans* to take their share in responsibility for important developmental processes. In addition to the abundant genetic information, the small number (only two) of SFK family members and relatively clear differences in their expression patterns and functions in *C. elegans* may be great advantages to define the primitive functions of SFKs.

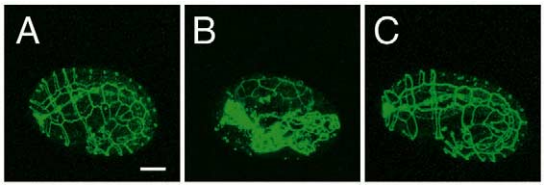

Fig. 5. Effect of *RNAi* on embryonic development of SU93 strain expressing *jam-1::gfp*. Fluorescent images of JAM-1-GFP in 1.5-fold stage of embryos are shown. The three-dimensional images were reconstituted from multiple confocal images obtained using Carl Zeiss LSM 510. A: Control SU93 strain, B: *src-1 RNAi* animal and C: *kin-22 RNAi* animal. Scale bar is 20 μm in A.

Acknowledgements: We thank J.A. Cooper for discussions and comments on the manuscript, H. Sawa for germline transformation, Yuji Kohara for EST clones, A. Coulson and J. Sulstone for physical mapping, cosmid and YAC clones, and A. Fire for the vectors. We also thank members of our laboratory for providing materials and valuable discussion. Some of the strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health Center for Research Resources. This work was supported in part by grants-in-aid from the Ministry of Education, Science, Technology, Sports and Culture of Japan and by grants from the Nagase Science and Technology Foundation, The Mitsubishi Foundation, Japan Research Foundation for Clinical Pharmacology, and The Program of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research.

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