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# Characterization of a Novel Ras-Binding Protein Ce-FLI-1 Comprising Leucine-Rich Repeats and Gelsolin-like Domains

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**Ras proteins are conserved from yeasts to mammals and implicated in regulation of the actin cytoskeleton. The *flightless-1* (*fli-1*) gene of *Drosophila melanogaster* and its homologs in *Caenorhabditis elegans* and humans encode proteins (FLI-1) comprising a fusion of a leucine-rich repeats (LRRs) domain and a gelsolin-like domain. This LRRs domain is highly homologous to those of three proteins involved in Ras-mediated signaling; *Saccharomyces cerevisiae* adenylyl cyclase, *C. elegans* SUR-8, and mammalian RSP-1. Here we report that the LRRs domain of *C. elegans* FLI-1 (Ce-FLI-1) associates directly with Ras ($K_d$ = 11 nM) and, when overexpressed, suppresses the heat shock sensitive phenotype of yeast cells bearing the activated *RAS2* gene ($RAS2^{Val\text{-}19}$). Further, the gelsolin-like domain of Ce-FLI-1 is shown to possess a $Ca^{2+}$-independent G-actin-binding activity as well as F-actin-binding and -severing activities. FLI-1 may be involved in regulation of the actin cytoskeleton through Ras.** © 1999 Academic Press

Ras proteins are small guanine nucleotide-binding proteins that cycle between the active GTP-bound and the inactive GDP-bound states (1). They are conserved from yeasts to mammals and play essential signaling roles in regulation of gene expression. Ras is implicated in regulation of the actin cytoskeleton as well through observation of the phenotypes of mammalian cells transformed by the oncogenic *ras* gene (2). In mammals, a serine/threonine kinase Raf-1 is a major effector of Ras (1). Ras associates directly with Raf-1 in a GTP-dependent manner, leading to activation of a phosphorylation cascade which ultimately activates the mitogen-activated protein kinase (MAPK). In addition to Raf-1 and its isoforms B-Raf and A-Raf, recent searches have identified a number of mammalian Ras effectors and effector candidates such as Ral guanine nucleotide dissociation stimulator (RalGDS), phosphoinositide 3-kinase, protein kinase Cζ and AF-6 (1, 3). On the other hand, in yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, Ras possesses effectors of distinct structures, adenylyl cyclase and a protein kinase Byr2, respectively (4–7).

Raf-1 and RalGDS share similar globular peptide-folding patterns, the ubiquitin superfold, in their Ras-associating domains (8–10). On the other hand, *S. cerevisiae* adenylyl cyclase associates with Ras at its leucine-rich repeats (LRRs) domain, whose structure bears no resemblance to the Ras-associating domains of Raf-1 and RalGDS (5, 11–13). The LRRs are found in a number of proteins with diverse functions and consist of a tandem repetition of amphipathic 20-28-amino acid units (14). The LRRs of ribonuclease inhibitor have been shown to assume a nonglobular, horseshoe-like structure (15). The LRRs domain of adenylyl cyclase belongs to a small subgroup bearing a distinct 23-amino acid consensus unit (PXXαXXLXXLXXLXLNXLXXα, where α is an aliphatic amino acid and X is any amino acid) (5).

Interestingly, in addition to the LRRs of adenylyl cyclase, LRRs belonging to the same subgroup are found in two putative scaffold proteins modulating Ras-dependent signaling, RSP-1 (also referred to as RSU-1) and SUR-8 (16, 17). RSP-1 was isolated on the basis of its ability to suppress Ras-transformed phenotypes of mammalian cells. In the cells overexpressing RSP-1, Ras-dependent MAPK activation is greatly enhanced, while Jun kinase activation is suppressed (18). RSP-1 is thought to enhance MAPK activation by form-

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Abbreviations used: MAPK, mitogen-activated protein kinase; RalGDS, Ral guanine nucleotide dissociation stimulator; LRRs, leucine-rich repeats; GST, glutathione *S*-transferase; MBP, maltose-binding protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin.



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ing a stable ternary complex with Ras and Raf-1, while blocking association of a Ras effector necessary for Jun kinase activation, which may account for its suppressive activity on cellular transformation. The *sur-8* gene is required for Ras-dependent vulval differentiation in nematode *Caenorhabditis elegans* (17). In contrast to the yeast adenylyl cyclase, SUR-8 associates with both GTP- and GDP-bound Ras and is thought to ensure efficient Ras-dependent Raf activation through complex formation with Ras and Raf. It is possible that other proteins containing LRRs domains belonging to the same subgroup may also interact with Ras. Here we report characterization of such a protein Ce-FLI-1, which contains an additional domain homologous to members of the actin-severing protein family such as gelsolin and villin (19).

## MATERIALS AND METHODS

*Cell strains.* *S. cerevisiae* strains KP-1 (*MATα leu2 his3 ura3 trp1 can1 ade8 ras2::URA3*), TK35-1 (*MATα leu2 his3 ura3 trp1 cyr1-2 ras2::URA3*), and TK161-R2V (*MATα leu2 his3 ura3 trp1 ade8 RAS2$^{Val-19}$*) were cultured as described previously (4, 11, 12, 20). Survival of yeast cells after heat shock treatment was examined as described (12, 20).

*cDNA cloning.* In the course of the *C. elegans* genome sequencing project, a predicted open reading frame encoding a 380-amino acids polypeptide with a striking homology to the yeast adenylyl cyclase LRRs was discovered in a cosmid clone B0303 (21). A λZAPII *C. elegans* cDNA library, a gift from Dr. Y. Kohara (National Institute of Genetics, Mishima, Japan), was screened by hybridization with a $^{32}$P-labeled oligonucleotide corresponding to the LRRs-coding sequence, and sequence determination of the two positive overlapping clones predicted a protein of 1257-amino acids (Fig. 1A). While this work was in progress, cloning of the *Drosophila melanogaster flightless-1* (*fli-1*) gene was reported (22), and we realized that the gene we cloned was identical to the *C. elegans* homolog of *fli-1* (GenBank U01183).

*GST-Ras-association assay.* By a polymerase-chain reaction using a suitable pair of oligonucleotide primers, a DNA fragment encoding amino acids 1-770 of Ce-FLI-1 was fused to that encoding an epitope tag (QPELAPEDPED, HSV-tag) and cloned into pBluescript SK(−). The resultant plasmid pBluescript-Ce-FLI-1(1–770)-HSV was used to produce the Ce-FLI-1 polypeptide carrying the HSV tag at its C-terminus, Ce-FLI-1(1–770)-HSV, by *in vitro* transcription/translation with the T7 polymerase-coupled reticulocyte lysate system (Promega). Human B-Raf cDNA was cloned into pBluescript SK(−) and used to produce untagged B-Raf protein similarly. Human Ha-Ras cDNA was cloned into pGEX-2T to yield pGEX-2T-Ha-Ras for production as a glutathione *S*-transferase (GST)-fusion protein in *Escherichia coli.* pGEX-2T-Rac1, -RhoA, -Cdc42, and -RalA were generous gifts from Dr. K. Kaibuchi (Nara Institute of Science and Technology, Nara, Japan). The assay for association of immobilized GST-fusion proteins with Ce-FLI-1(1–770)-HSV or B-Raf was carried out essentially as described (23), except that associated Ce-FLI-1(1–770)-HSV or B-Raf was detected by Western immunoblotting with anti-HSV monoclonal antibody (Novagen, Inc., Madison, WI) or anti-B-Raf polyclonal antibody C19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively.

*Expression plasmids for Ce-FLI-1.* Various regions of Ce-FLI-1 cDNA were cloned into pAD4, pAD-GST and pMal-cRI for expression as native polypeptides in yeast, as GST-fusions in yeast, and maltose-binding protein (MBP)-fusions in *E. coli*, respectively (Fig. 1A).

*Adenylyl cyclase inhibition assay.* Yeast adenylyl cyclase, overproduced in yeast TK35-1, was solubilized from the membrane fraction as described (12, 20). The post-translationally-modified form of yeast Ras2 was purified as described (12). Purification of various GST-fusion proteins of Ce-FLI-1 produced in yeast KP-1 cells was carried out by glutathione agarose chromatography as described (13). Measurement of adenylyl cyclase activity dependent on the guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS)-loaded Ras2 and of its inhibition by the GST-Ce-FLI-1 polypeptides were carried out as described previously (12).

*Actin-binding and -severing assays.* For the assay of G-actin-binding, rabbit skeletal muscle monomeric actin or bovine serum albumin (BSA) was immobilized on agarose resin as described (24). A lysate of yeast KP-1 cells harboring pAD4-Ce-FLI-1 was dialyzed against the binding buffer A (2 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol) containing either 0.2 mM $CaCl_2$ or 10 mM EGTA. After centrifugation at 100,000 × *g* for 1 h, a 200 μl aliquot was incubated with 25 μl slurry of actin-agarose for 1 h at 4°C. After washing with the same buffer, bound Ce-FLI-1 was detected by Western immunoblotting with an anti-Ce-FLI-1 antibody (see below). For assays of F-actin-binding and -severing activities, F-actin filaments were prepared as described (24) and incubated for 30 min at 25°C with either MBP-Ce-FLI-1(372–1257) or human gelsolin in the binding buffer B (2 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM ATP, 1 mM dithiothreitol). Samples were either centrifugated at 100,000 × *g* for 1 h for the co-sedimentation assay or negatively stained with 2% uranylacetate for electron microscopic observation. Human gelsolin was kindly provided by Dr. T. Sakurai (University of Tokyo, Tokyo, Japan).

*Antibody preparation.* Anti-Ce-FLI-1 antibody was raised in rabbits with MBP-Ce-FLI-1(771–1257) as an antigen. By Western immunoblotting with the anti-Ce-FLI-1 antibody, a lysate of the nematode worms gave a major band of approximately 140 kDa in size, which coincided with that of Ce-FLI-1 protein expressed in yeast cells harboring pAD4-Ce-FLI-1 (Fig. 1B).

## RESULTS

*Association with Ras.* The N-terminal region of Ce-FLI-1 contained LRRs with 17-fold repetition of a 23-amino acid consensus unit exactly corresponding to that of the yeast adenylyl cyclase LRRs (Fig. 1A). In order to test the ability of Ce-FLI-1 to associate with Ras, the N-terminal 770 amino acids containing the LRRs domain was produced as a fusion with the HSV epitope tag by *in vitro* translation. As shown in Fig. 2A, this fusion protein, Ce-FLI-1(1–770)-HSV, did associate with GST-Ha-Ras (lanes 2 and 3) but not with GST only (lane 1). Further, it did not associate with GST-Rac1, -RhoA, -Cdc42, and -RalA, supporting the specificity of binding (not shown). However, Ce-FLI-1(1–770)-HSV associated equally with both the GTP- and the GDP-bound forms of Ha-Ras (lanes 2 and 3), in contrast to a clear GTP-dependent association observed with B-Raf (lanes 5 and 6).

Association of Ce-FLI-1 with Ras was confirmed by an analysis based on inhibition of the Ras-dependent adenylyl cyclase activity (12). Purified GST-fusion protein of full-length Ce-FLI-1 and that lacking the LRRs domain, GST-Ce-FLI-1 (372–1257) (Fig. 1C), were added to the reaction mixture for measuring adenylyl cyclase activity dependent on Ras2 protein.

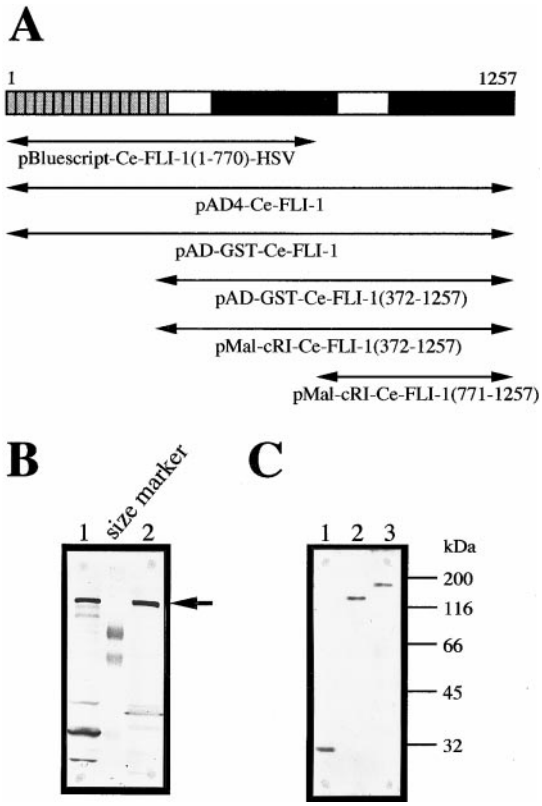


**FIG. 1.** Structure and expression of Ce-FLI-1. **(A)** Structure of Ce-FLI-1. Shaded boxes represent the LRRs domain, while filled boxes represent the gelsolin-like domain composed of a tandem duplication of a 400-amino-acid sevelin-like unit structure, as observed for gelsolin and villin (19). Amino acids expressed from various plasmids are also indicated below. **(B)** Ce-FLI-1 protein was detected by Western immunoblotting in the crude lysates of yeasts harboring pAD4-Ce-FLI-1 (30 $\mu$g protein) (lane 1), and that of the mixed stage nematodes (80 $\mu$g protein) (lane 2). The lysates were separated by SDS-PAGE (5% gel). Prestained molecular size markers (Bio-Rad) were phosphorylase b (106 kDa), BSA (80 kDa), and ovalbumin (49.5 kDa). The position of Ce-FLI-1 (approximately 140 kDa) is indicated by an arrow. **(C)** Approximately 0.2 $\mu$g each of the purified GST (lane 1), GST-Ce-FLI-1(372–1257) (lane 2), and GST-Ce-FLI-1 (lane 3) was separated by SDS-PAGE (8% gel) and stained with Coomassie brilliant blue.

A strong dose-dependent inhibition was observed by GST-Ce-FLI-1, but not by either GST-Ce-FLI-1 (372–1257) or GST (Fig. 2B). GST-Ce-FLI-1 had no effect on the $Mn^{2+}$-dependent adenylyl cyclase activity, indicating that GST-Ce-FLI-1 exerted its effect by interacting with Ras but not with adenylyl cyclase (not shown).

We extended the analysis by utilizing varying concentrations of GST-Ce-FLI-1 or Ras2 to prove the competitive nature of the inhibition as described (12). At each point of Ras2 concentration in the presence of GST-Ce-FLI-1, we obtained free Ras2 concentration available for adenylyl cyclase activation as that required for giving the same adenylyl cyclase activity in the absence of GST-Ce-FLI-1. A difference between the original and the free concentrations of Ras2 was regarded as that bound to GST-Ce-FLI-1 protein, and a reciprocal of this value was plotted against a reciprocal

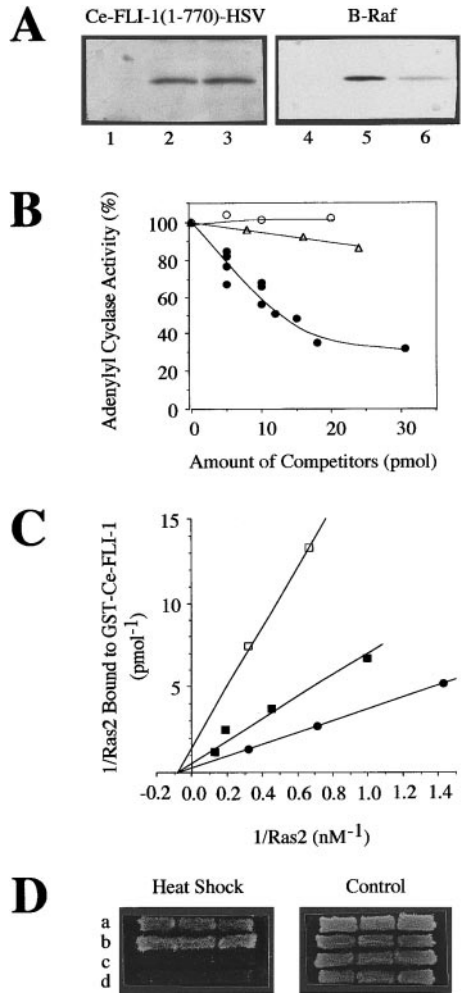


**FIG. 2.** Association of Ce-FLI-1 with Ras. **(A)** Reticulocyte lysates containing either Ce-FLI-1-HSV (lanes 1-3) or B-Raf (lanes 4-6) were incubated with either GST (lanes 1 and 4) or GST-Ha-Ras loaded with either GTP$\gamma$S (lanes 2 and 5) or GDP (lanes 3 and 6) immobilized on glutathione agarose resin. After elution of GST or GST-Ha-Ras with a buffer containing 10 mM glutathione, co-eluted Ce-FLI-1-HSV or B-Raf was detected by Western immnumoblotting. **(B)** Adenylyl cyclase activity was measured in the presence of 0.6 pmol of Ras2 with the addition of various amounts of GST-Ce-FLI-1 (●), GST-Ce-FLI-1 (372–1257) (△), and GST (○), in a reaction volume of 150 $\mu$l. **(C)** Adenylyl cyclase activities dependent on various concentrations of Ras2 were measured in the presence of various amounts of GST-Ce-FLI-1 as follows: 5 (□), 12.2 (■), and 30.6 pmol (●). The amounts of free and Ce-FLI-1-bound Ras2 were calculated as described (12). Reciprocal of bound Ras2 was plotted against a reciprocal of the free Ras2 concentration. **(D)** Yeast TK161-R2V cells were transformed with pAD4-Ce-FLI-1 (a), pAD-GST-Ce-FLI-1 (b), pAD-GST-Ce-FLI-1(372–1257) (c), and pAD-GST (d). Three independent $Leu^+$ colonies from each transformation were examined for heat shock sensitivity. Shown are two replica plates, one subjected to 55°C heat shock for 15 min and the other without the heat shock treatment after 2 days of growth at 30°C.

of the free Ras2 concentration (Fig. 2C). This gave a series of straight lines for each value of GST-Ce-FLI-1, which converged on the horizontal axis. The data indicated that GST-Ce-FLI-1 bound directly to Ras2 and competitively sequestered it from adenylyl cyclase. The $K_d$ value for Ras2 was calculated from the point of intersection with the horizontal axis and determined to be approximately 11 nM.

We have reported previously that overexpression of the Ras-associating region of Raf-1 could suppress the heat shock sensitive phenotype of yeast cells (TK161-R2V) bearing the activated *RAS2* gene ($RAS2^{Val\text{-}19}$) by competitive sequestration of $Ras2^{Val\text{-}19}$ protein from adenylyl cyclase *in vivo* (12). As shown in Fig. 2D, overexpression of Ce-FLI-1 and GST-Ce-FLI-1 proteins clearly suppressed the heat shock sensitivity of these cells (a and b), whereas GST-Ce-FLI-1(372–1257) and GST did not (c and d).

*Actin-binding and -severing activities.* Ce-FLI-1 contained an originally-unexpected C-terminal extension of about 900-amino acids bearing considerable homology to gelsolin (19) (Fig. 1A). However, amino acids involved in binding $Ca^{2+}$ (equivalents of $Glu^{97}$, $Asp^{109}$, $Gly^{114}$, and $Ala^{116}$ in gelsolin) (25) are not conserved in Ce-FLI-1. We therefore examined whether Ce-FLI-1 binds to G-actin and whether the binding activity requires $Ca^{2+}$. A crude yeast extract containing the full-length Ce-FLI-1 protein was incubated with actin-agarose resin at the condition which favored formation of monomeric actin. As shown in Fig. 3A, Ce-FLI-1 bound efficiently to actin-agarose (lanes 3 and 4), but not to BSA-agarose (lanes 1 and 2). In contrast to gelsolin, Ce-FLI-1 did not appear to require the presence of a micromolar concentration of $Ca^{2+}$ for binding actin because inclusion of 10 mM EGTA had no inhibitory effect (lane 4).

As shown in Fig. 3B, purified MBP-Ce-FLI-1(372–1257), added to F-actin, co-sedimented with F-actin during ultracentrifugation (lane 2), indicating that Ce-FLI-1 binds to F-actin as well. Strikingly, however, with the addition of increasing amount of MBP-Ce-FLI-1(372–1257), actin was found in the supernatant together with MBP-Ce-FLI-1(372–1257) (lane 3), suggesting an F-actin-severing activity of Ce-FLI-1. This activity was confirmed by the electron microscopic observation that MBP-Ce-FLI-1(372–1257) has an activity to cause fragmentation of F-actin into actin oligomers similarly to gelsolin (Fig. 3C). Hydrolysis or sequestration of ATP by the *E. coli dnaK* gene product known to be co-purified with overexpressed fusion proteins cannot accout for the observed fragmentation of F-actin, since prolonged treatment with ATP and $Mg^{2+}$ to remove the *dnaK* gene product (26) did not affect the activity of MBP-Ce-FLI-1(372–1257) (unpublished observation).

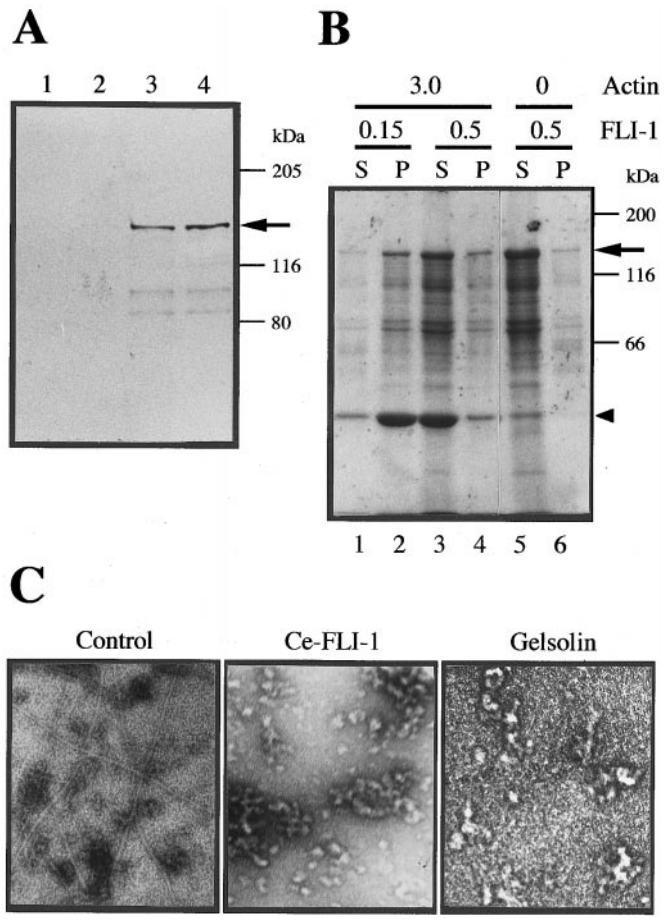


**FIG. 3.** Actin-binding and -severing activities of Ce-FLI-1. **(A)** A crude extract of yeast cells harboring pAD4-Ce-FLI-1 was incubated with either BSA-agarose (lanes 1 and 2) or actin-agarose (lanes 3 and 4) in the presence of either 0.2 mM $CaCl_2$ (lanes 1 and 3) or 10 mM EGTA (lanes 2 and 4). Bound Ce-FLI-1 (arrow) was detected by Western immunoblotting. **(B)** GST-Ce-FLI-1(372–1257) was incubated with or without F-actin and subjected to ultracentrifugation. Equivalent amounts of supernatant (S) and pellet (P) were fractionated by SDS-PAGE (8% gel) and stained with Coomassie brilliant blue. Numbers above indicate concentrations ($\mu$M) of F-actin (Actin) and GST-Ce-FLI-1(372–1257) (FLI-1). Positions of GST-Ce-FLI-1(372–1257) and actin are indicated by an arrow and an arrowhead, respectively. **(C)** Electron micrographs of F-actin filaments (8 $\mu$M) incubated in the absence (Control) or presence of 1 $\mu$M GST-Ce-FLI-1(372–1257) (Ce-FLI-1) or human gelsolin (Gelsolin).

## DISCUSSION

A putative LRRs domain strikingly homologous to that of yeast adenylyl cyclase was found during the *C. elegans* genome sequencing project (21). Since yeast adenylyl cyclase was the only known effector of Ras at that time, this prompted us to determine the full-length structure of the corresponding protein by cDNA cloning, and the protein turned out to be the *C. elegans* homolog of *D. melanogaster* FLI-1 (22). Soon after these reports, Raf-1 was shown to associate with Ras in a GTP-dependent manner (27–31), followed by similar

reports on RalGDS and its homolog RGL (32–34). Further, we have observed that the LRRs domain of yeast adenlyly cyclase also associates with Ras in a GTP-dependent manner (13). The GTP-independent association of Ce-FLI-1 with Ras did not appear meaningful until the recent discovery of SUR-8 (17). SUR-8 is almost entirely composed of LRRs which are also strikingly homologous to those of yeast adenylyl cyclase and yet associates with Ras in a GTP-independent manner. This suggests that the LRRs with the 23-amino acid consensus unit may be a conserved Ras-associating module. The nature of non-consensus residues could be the determinant of whether each LRRs exhibits GTP-dependent or -independent association with Ras.

Association of Ce-FLI-1 with Ras was confirmed by a kinetic analysis based upon competitive inhibition of Ras-dependent adenylyl cyclase activity. The obtained $K_d$ value of Ce-FLI-1 for Ras2 (11 nM) was comparable to the value (3.5 nM) of Raf-1 for Ha-Ras, that (5 nM) of B-Raf for Ha-Ras, or that (1 nM) of Byr2 for Ras2 determined by the same method (7, 12, 35). Further, the suppression of the heat shock sensitive phenotype of yeast cells bearing the $RAS2^{Val\text{-}19}$ gene by overexpression of Ce-FLI-1 strongly suggests that Ce-FLI-1 associates with Ras *in vivo*. In contrast, Liu and Yin (36) recently reported that human FLI-1 LRRs domain, produced by *in vitro* translation, failed to associate with Ras. It is possible that an aberrant folding of LRRs polypeptide takes place when human FLI-1 is translated *in vitro*. Indeed, we repeatedly encountered insolubility of the Ce-FLI-1 LRRs domain expressed in *E. coli*, yeast and Sf9 insect cells. These authors also failed to detect association of FLI-1 with Ras by the yeast two-hybrid assay, but it is known that association of yeast adenylyl cyclase LRRs domain with Ras is also only barely detectable by this assay (30). Instead of Ras, they reported that a novel protein termed FLAP associates with FLI-1 LRRs domain (36). In addition, Wilson et al. (37) reported association of a novel RNA-binding protein, TRIP, with this domain. However, functions of these novel proteins are presently unknown.

The C-terminal region of Ce-FLI-1 was highly homologous to gelsolin. Liu and Yin (36) showed that human FLI-1 binds to agarose beads carrying G-actin. However, the requirement of $Ca^{2+}$ for the binding was not examined. We have shown that Ce-FLI-1 binds G-actin in a $Ca^{2+}$-independent manner, an important difference from gelsolin. More importantly, we have demonstrated that Ce-FLI-1 possesses F-actin-binding and -severing activities, suggesting the involvement of FLI-1 in a dynamic assembly/disassembly of actin filaments. This agrees well with the observation that *fli-1* gene is required for actin organization during embryogenesis and myogenesis of *D. melanogaster* (38, 39). Consistent with this, *Ce-fli-1* mRNA (4.8 kb) is found most abundantly expressed during the embryonic stages of *C. elegans*, where a complex morphogenesis takes place (unpublished observation).

Functional relationship between the LRRs and gelsolin-like domains are not known. However, it is possible that the complex formation of the LRRs domain with Ras, or the joining of a Ras effector to this complex could serve as the regulatory switch for activities of the gelsolin-like domain. For example, protein kinase C$\zeta$, by joining the complex in a GTP-dependent manner, may phosphorylate and activate the gelsolin-like domain. A profound alteration of the cytoskeletal structure is observed in cultured mammalian cells microinjected with activated Ras protein (40–42). Although some of these activities of Ras may be mediated by other proteins like Rac1 and RhoA (2), it still remains possible that Ras itself is engaged directly in regulation of the actin cytoskeleton. Further study will provide insights into whether FLI-1 serves as a direct link between Ras and the actin cytoskeleton.

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