The Caenorhabditis elegans Sex Determination Protein FEM-1 Is a CED-3 Substrate That Associates with CED-4 and Mediates Apoptosis in Mammalian Cells*

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Shing-Leng Chan‡, Karen S. Y. Yee‡, Karen Mei Ling Tan, and Victor C. Yu§
From the Institute of Molecular and Cell Biology, 30 Medical Dr., Singapore 117609, Republic of Singapore

Sex-specific elimination of cells by apoptosis plays a role in sex determination in Caenorhabditis elegans. Recently, a mammalian pro-apoptotic protein named F1Aα has been identified. F1Aα shares extensive homology throughout the entire protein with the C. elegans protein, FEM-1, which is essential for achieving all aspects of the male phenotype in the nematode. In this report, the role of FEM-1 in apoptosis was investigated. Overexpression of FEM-1 induces caspase-dependent apoptosis in mammalian cells. FEM-1 is cleaved in vitro by the C. elegans caspase, CED-3, generating an N-terminal cleavage product that corresponds to the minimal effector domain for apoptosis. Furthermore, CED-4 associates with FEM-1 in vitro and in vivo in mammalian cells and potentiates FEM-1-mediated apoptosis. Similarly, Apaf-1, the mammalian homologue of CED-4 was found to associate with F1Aα. These data suggest that FEM-1 and F1Aα may mediate apoptosis by communicating directly with the core machinery of apoptosis.

Apoptosis is an evolutionarily conserved process that is critical for tissue homeostasis and development including sex determination in essentially all multicellular organisms (1). Genetic studies of apoptosis in the nematode Caenorhabditis elegans have identified four genes that define the core machinery of apoptosis. ced-3, ced-4, and egl-1 promote and ced-9 inhibits apoptosis (2, 3). The direct association of CED-4 with both CED-9 and pro-CED-3 was demonstrated by co-immunoprecipitation assay in mammalian cells and in vitro pull-down assays (4–6). Furthermore, CED-4 was also shown to oligomerize and facilitate the proteolytic activation of CED-3 in mammalian cells (7, 8). It has been proposed that the EGL-1 protein activates apoptosis by binding to and thereby negatively regulating CED-9 (9). The binding of EGL-1 to CED-9 was subsequently shown in the mammalian system to disrupt the association between CED-9 and CED-4 resulting in the CED-4-dependent activation of CED-3 (10).

By isolating mutations that transform the entire animal from one sex to the other, a group of genes that controls the sexual fate of C. elegans has been identified (11–14). Three fem genes, fem-1, fem-2, and fem-3 (15) are essential for male development. Loss-of-function mutations in any one of the fem genes prevent all aspects of male development and transform the animals that are genetically males into females (16, 17). The predicted product of the fem-1 gene is an intracellular protein that contains ankyrin repeats, which in many other proteins mediate specific protein-protein interaction (17). However, despite the vital role of FEM-1 in sex determination, no biochemical function has yet been ascribed to the protein.

A human homologue of FEM-1, called F1Aα or FEM1β (18, 19), has recently been identified. F1Aα is pro-apoptotic and shares high homology with FEM-1 (18). The percentage identity between the two proteins ranges from 22 to 47% depending on the regions of the proteins that are compared (Fig. 1A). The degree of homology between FEM-1 and F1Aα is comparable to that between several of the functionally conserved components of apoptosis in C. elegans and mammals. For example, the amino acid identity between CED-9 and Bcl-2 is 23% (20) and that between CED-3 and caspase-3 is 34% (21). In this study we evaluated the possible molecular functions of FEM-1 in apoptosis using a mammalian cell line as a model system.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies against the Myc epitope (A14, 9E10) and HA epitope (Y11, F7) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. NIH3T3 and L929 were originally from American Type Culture Collection (ATCC). The tumor necrosis factor-sensitive MCF7 breast carcinoma cells were provided by Dr. V. Dixit, University of Michigan. SH-SY5Y human neuroblastoma cells that are routinely used in the laboratory (22, 23) were originally provided by Dr. Wolfgang Sadee, University of California, San Francisco. Cell lines were grown according to the directions provided by suppliers. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 mg of streptomycin/ml and 100 IU of penicillin/ml, Life Technologies, Inc.). The peptide caspase inhibitor ZVAD-fmk was purchased from Enzyme System Products.

Plasmid Constructions—DNA for fem-1 was from Dr. Andrew Spence, University of Toronto, Canada; cDNAs for ced-3, ced-4, and ced-9 were from Dr. Robert Horvitz, MIT. cDNA for apaf-1 and caspase-8 were from Dr. Xiaodong Wang, Howard Hughes Medical Institute, Dallas, TX and Dr. Marcus E. Peter, German Research Center, Heidelberg, Germany, respectively. Expression plasmids for CED-3(C8) (CED-3C358S) (24), FEM-1, FEM-1-D320A, and FEM-1-D344A were generated by site-directed mutagenesis using the Transformer™ Site-Directed Mutagenesis kit (CLONTECH). DNA fragments for epitope-tagged constructs containing CED-3CS, CED-4, CED-9, Apaf-1, FEM-1D320A, FEM-1D344A, FEM-1, and its deletion mutants were obtained by polymerase chain reaction amplification using pfu polymerase (Stratagene). Appropriate restriction sites and epitope tags were included in the primers. Amplification products were inserted into the Pmx40 mammalian expression vector driven by the CMV promoter (25). All epitope tags are at the N termini. DNA fragments generated by polymerase chain reaction and the junctions of insertion were confirmed by sequencing. pEGFP-FEM-1 was constructed by releasing the BamHI/KpnI fragment from pXHAFEM-1 and inserting it into the BglII

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‡ These authors contributed equally to this work.
§ To whom correspondence should be addressed. Tel.: 65-8743740; Fax: 65-7771117; E-mail: mcyduck@imcb.nus.edu.sg.

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and KpnI sites of the pEGFPC1 vector (CLONTECH). Construction of the pXJHAF1a vector has been described (18).

Preparation of Recombinant His tagged-CED-3 (221–503) Fusion Protein—Catalytic domain of CED-3 with an N-terminal 6× His tag, His-tag-CED-3(221–503), was expressed in E. coli from the pQE30 vector (Qiagen) and purified by affinity chromatography on nickel-agarose.

In Vitro Cleavage Analysis—FEM-1 and its mutants were [35S]methionine-labeled using the TrtTr247 coupled reticulocyte lysate system (Promega). 1–2 μl of the in vitro translated product was incubated with 20 μl of recombinant CED-3 enzyme at 30 °C for 2 h. For the control reactions, the in vitro translated products were mixed with 20 μl of Ni-NTA resin slurry that had been incubated with lysate from bacteria containing the expression vector alone.

Cell Death Assays—Mammalian cells at approximately 60–70% confluence were transfected with 2 μg of expression plasmids of FEM-1 or its mutants together with 0.5 μg of pCMV-β-Gal using LipofectAMINE (Life Technologies, Inc.). At defined times after transfection, β-Gal positive cells were counted and the apoptotic cells were scored as described (18).

Nuclear Staining of EGFP-expressing Cells—SH-SY5Y cells on glass coverslips at 70% confluence were transfected with pEGFP or pEGFP-FEM-1. 24 h after transfection, the cells were fixed, rinsed with phosphate-buffered saline and then incubated for 2 min with Hoescht 33342 dye (Molecular Probes Inc.) to enable nuclear staining. The cells were subsequently visualized using a Zeiss Axiosplan microscope.

RESULTS AND DISCUSSION

Overexpression of FEM-1 Induces Caspase-dependent Apoptosis in Mammalian Cells—SH-SY5Y human neuroblastoma cells were co-transfected with expression vectors encoding full-length FEM-1 or control vector together with pCMV-β-Gal as a marker for transfected cells. At 24 h post-transfection, a significant percentage of total β-galactosidase-positive (blue) cells co-transfected with FEM-1 became shrunken and rounded, displaying morphological features typical of cells undergoing apoptosis (Fig. 1B). Nuclear condensation, another hallmark of apoptosis, was also observed in cells expressing EGFP-FEM-1, a fusion protein of FEM-1 and the green fluorescent protein (GFP) (Fig. 1C). In the round cell apoptosis assay, extended incubation up to 30 h resulted in a progressive increase in the percentage of total blue cells that exhibited the round cell morphology (Fig. 1D). Treatment with the broad spectrum caspase inhibitor, ZVAD-fmk, abrogated the apoptotic activity of FEM-1 (Fig. 1D) suggesting that FEM-1 mediates apoptosis through caspase-dependent pathways in SH-SY5Y cells. Overexpression of F1Aα in SH-SY5Y cells yielded similar results (Fig. 1D). The apoptotic effect of FEM-1 overexpression is not restricted to SH-SY5Y cells as a similar degree of apoptosis was observed when the protein was overexpressed in L929 fibrosarcoma cells (data not shown). However, FEM-1 was unable to induce apoptosis in the breast carcinoma MCF-7 and NIH3T3 cells (data not shown) suggesting that the apoptotic function of FEM-1 may be cell-type dependent. We (18) and others (19) have noted that F1Aα is a member of a gene family. It would be interesting to determine if other members of the F1A gene family may also have a role in apoptosis and whether this activity is cell type-dependent.

**FEM-1 Is a Substrate of CED-3—**F1Aα has been demonstrated to be a substrate of caspase-3 in vitro (18). Although the caspase-3 cleavage site in F1Aα, Asp-Asn-Ile-Asp 342, is not completely conserved in FEM-1 in which the corresponding site is Ala-His-Thr-Asp342 (Fig. 2B), alternate caspase cleavage site(s) may be present in FEM-1. We tested the ability of the C. elegans caspase, CED-3, to cleave FEM-1 in vitro. Incubation of 35S-labeled FEM-1 with recombinant CED-3 generated a product of approximately 37 kDa (Fig. 2A). The estimated size of the full-length FEM-1 including the N-terminal hemagglutinin (HA) epitope tag was 74 kDa; therefore, the cleavage might have occurred in the center of the protein generating two fragments of similar size. To facilitate the visualization of the cleavage products, a radiolabeled N-terminal deletion mutant of FEM-1, FEM-1(44–656), was subjected to cleavage analyses. Whereas the D320A mutant appeared to be resistant to cleavage by CED-3 (Fig. 2B), the D344A mutant was cleaved as efficiently as FEM-1, the D320A mutant appeared to be resistant to cleavage by CED-3 (Fig. 2C), suggesting that CED-3 cleaves FEM-1 at Asp 344 (Fig. 2C). Supporting the idea that CED-3 cleaved FEM-1 in the central region of the molecule.

To determine the cleavage site, the aspartic acid (Asp) residue of two potential caspase cleavage sites (Fig. 2B), Asp320 and Asp344 were independently mutated to alanine (Ala) in FEM-1. 35S-Labeled FEM-1 and its point mutants were then subjected to cleavage analyses. Whereas the D320A mutant was cleaved as efficiently as FEM-1, the D320A mutant appeared to be resistant to cleavage by CED-3 (Fig. 2C), suggesting that CED-3 cleaves FEM-1 at Asp320.

**Mapping the Effector Domain of Apoptosis in FEM-1—**As FEM-1 was cleaved by CED-3, it was of interest to determine if the cleavage products would be as active as the wild type protein in mediating apoptosis. Overexpression of the N-terminal cleavage product, FEM-1(1–320) induced apoptosis in SH-SY5Y cells (Fig. 3). The C-terminal fragment, FEM-1(321–
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The activity of FEM-1 and its mutants in SH-SY5Y cells. The cleavage analyses were systematically replaced with alanine residues in subsequent cleavage site in F1A mutant. SH-SY5Y cells were transfected with 2 bars sequence in F1A and the positions of the aspartic acid residues. The sequence (one-letter code) between amino acids 314 and 348 of FEM-1 and the corresponding sequence in F1Aα are shown. The asterisk indicates the caspase-3 cleavage site in F1Aα (18). Arrows indicate the aspartic acid residues in the tetrapeptides Glu-Leu-Leu-320-Asp and Ala-His-Thr-344-Asp that were systematically replaced with alanine residues in subsequent cleavage analyses. C, FEM-1 is cleaved by CED-3 at Asp320 in vitro. 35S-Labeled FEM-1, FEM-1D320A, or FEM-1D344A was incubated with CED-3, and the cleavage products were fractionated by SDS-PAGE and detected by autoradiography.

Apoptotic cells (%) - 0 20 40 60 80 100

FEM-1 - 320 656

**Ankyrin repeat**

FEM-1-(1–320) appeared to be more potent in inducing apoptosis than the wild type protein as cell death could be observed at an earlier (18 h) time point when FEM-1-(1–320) was overexpressed (Fig. 3). Similar to wild-type FEM-1, the apoptotic activity of FEM-1-(1–320) was inhibited by the caspase inhibitor, ZVAD-fmk (data not shown). Deletion of the C-terminal region up to the border of the ankyrin repeat cluster (FEM-1-(1–257)) rendered the molecule apoptotically inactive (Fig. 3) indicating that the region immediately distal to the ankyrin repeat cluster up to amino acid 320 is important for apoptotic activity. Deletion of the first 83 amino acids of FEM-1 as in FEM-1-(84–656), also abrogated the apoptotic activity of FEM-1 (Fig. 3). The lack of activity of the deletion mutants was not attributable to the lack of protein expression in SH-SY5Y cells. The wild type as well as the deletion mutants of FEM-1 were expressed at similar levels except for FEM-1-(1–320), which was expressed at a relatively low level (data not shown). These data suggest that the minimal effector domain of apoptosis resides in the N-terminal region of FEM-1 (amino acids 1–320).

While overexpression of wild-type FEM-1 and the D344A mutant were able to induce apoptosis in SY5Y cells to a similar degree, the cleavage-resistant D320A mutant had no apoptotic activity (Fig. 3). As deletion analysis indicated that the effector domain for apoptosis resides in the N terminus of FEM-1, these results suggest that the C-terminal domain of FEM-1, similar to F1Aα, might serve a regulatory role in suppressing its intrinsic apoptotic activity.

CED-4 Associates with FEM-1 in Vivo and in Mammalian Cells—CED-4, respectively (18, 26). As predicted, the HA-tagged F1Aα was co-immunoprecipitated with Myc-CED-3C/S, a catalytically inactive mutant of CED-3 (24), CED-4, or CED-9 in 293T cells. HA-FEM-1 was immunoprecipitated, and the associated Myc-tagged proteins were detected with anti-Myc antibody. CED-4 but not CED-3/C/S or CED-9 appeared to interact with FEM-1 (Fig. 4A, lanes 4, 2, and 6). HA-FEM-1 also co-immunoprecipitated with Myc-CED-4 in the reciprocal experiment in which the Myc-tagged proteins were immunoprecipitated (data not shown). FEM-1 expressed as a GST fusion protein was able to specifically pull down in vitro translated 35S-labeled CED-4 (Fig. 4C) suggesting that the proteins were in direct physical contact. Parallel experiments were performed with F1Aα and Apaf-1, which are the mammalian homologues of FEM-1 and CED-4, respectively (18, 26). As predicted, the HA-tagged F1Aα co-immunoprecipitated with the Myc-tagged Apaf-1 (Fig. 4B, lane 8), and the association appeared to be direct since in vitro translated 35S-labeled Apaf-1 was specifically pulled down by GST-F1Aα (Fig. 4C).

CED-4 Potentiates the Apoptotic Activity of FEM-1—The association of CED-4 with FEM-1 in the cells raised the possibility that CED-4 might have a role in modulating the function of FEM-1 or vice versa. In SH-SY5Y cells, overexpression of FEM-1 induced apoptosis in a dose-dependent and saturable manner at a defined time point (data not shown). When the amount of FEM-1 expression plasmid was reduced from 2 to 0.5 μg, no significant apoptosis was observed at the 24-h time point (Fig. 4D). Consistent with a previously reported observation (6), overexpression of CED-4 itself did not induce apoptosis in mammalian cells (Fig. 4, D and E). However, CED-4 co-expression significantly enhanced FEM-1-mediated but not caspase-8-mediated apoptosis (Fig. 4D). Furthermore, CED-4 increased the rate of FEM-1-mediated apoptosis (Fig. 4E), and the effect appeared to be specific because the rate of cell death induced by caspase-8 overexpression in these cells was unaffected by CED-4 co-expression (data not shown). The levels and kinetics of FEM-1 protein expression were not affected by CED-4 co-
observation that the dominant negative mutant of caspase-9 blocks F1Aa apoptotic activity (18) suggest that F1Aa is in the Apaf-1-caspase-9 signaling pathway.

The Potential Role of FEM-1 in Apoptosis—A recent study has linked the sex determination pathway directly to the core machinery of cell death in the hermaphrodite-specific neurons of C. elegans (27). TRA-1A, the terminal regulator in the C. elegans sex determination pathway, is found to repress the transcription of the cell death activator gene, egl-1, leading to the survival of hermaphrodite-specific neurons during hermaphrodite development. Since FEM-1 is a negative regulator of TRA-1A in the genetic pathway of sex determination, the question arises as to whether the observed apoptotic activity of FEM-1 in mammalian cells is mediated through a TRA-1A-like activity propagating a signal to the cell death pathway. However, tr-1 is known to be one of the most rapidly diverging genes when the homologues in C. elegans and Caenorhabditis briggsae are compared (28); it is questionable whether a mammalian protein similar to TRA-1 exists. Our data suggest that FEM-1 is able to communicate directly with the signaling components that constitute the core cell death machinery in C. elegans.

Despite its critical role in mediating the male phenotype, FEM-1 protein can be detected in the soma and germ cells of both sexes of C. elegans throughout development and during adulthood (29). It is therefore possible that FEM-1 might be involved in apoptosis during sexual development as well as in other developmental and/or physiological pathways.

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FIG. 4. CED-4 associates with FEM-1 and accelerates the kinetics of FEM-1-mediated apoptosis. A, CED-4 associates with FEM-1 in vivo. 293T cells were transiently transfected with expression plasmids of HA-tagged FEM-1 and the indicated Myc-tagged proteins. 30 h after transfection, HA-F1A was immunoprecipitated from the cell lysates with anti-HA antibody (Y11) as indicated by plus signs in lanes 1–6. Control experiments using rabbit IgG for immunoprecipitation are indicated by minus signs. Co-precipitating Myc-tagged proteins were detected by Western blot analysis using anti-Myc antibody (9E10) (lanes 1–6). An aliquot (10 µl) of the total extract was analyzed for protein expressions (middle panel). The immunoprecipitated HA-FEM-1 are presented in the lowest panel. B, Apaf-1 associates with F1Aa in vivo. Myc-Apaf-1 was immunoprecipitated from cell lysates and co-precipitating HA-F1A was detected by Western blot analysis. Expressions of HA-F1A and immunoprecipitated Myc-Apaf-1 are indicated in the middle and lowest panels, respectively. C, in vitro interaction. Equivalent amounts of 35S-labeled, in vitro translated CED-4 or Apaf-1 (5 × 105 cpm) were incubated with the indicated GST or GST fusion proteins immobilized on glutathione-Sepharose beads. Retained CED-4 or Apaf-1 was analyzed by SDS-PAGE and autoradiography. The same gel was Coomassie-stained, and the bands representing GST and GST fusions were aligned to show equivalency of loading (lower panel). D, CED-4 potentiates the apoptotic activity of FEM-1. SH-SY5Y cells were transfected with the indicated expression vectors together with 0.5 µg of pCMV-β-Gal. The amounts of DNA used were FEM-1, 0.5 µg, CED-4, 0.5 µg, and caspase-8, 0.125 µg. The total amount of DNA (2 µg) in each transfection was equalized by vector DNA. 24 h after transfection the cells were analyzed for apoptosis. E, CED-4 accelerates the kinetics of FEM-1-mediated apoptosis. SH-SY5Y cells were transfected as in D. At the indicated time points the cells were analyzed for apoptosis.
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