The Caenorhabditis elegans Sex-determining Protein FEM-2 and Its Human Homologue, hFEM-2, Are Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase Phosphatases That Promote Apoptosis*

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In Caenorhabditis elegans, fem-1, fem-2, and fem-3 play pivotal roles in sex determination. Recently, a mammalian homologue of the C. elegans sex-determining protein FEM-1, F1\(\alpha\), has been described. Although there is little evidence to link F1\(\alpha\) to sex determination, F1\(\alpha\) and FEM-1 both promote apoptosis in mammalian cells. Here we report the identification and characterization of a human homologue of the C. elegans sex-determining protein FEM-2, hFEM-2. Similar to FEM-2, hFEM-2 exhibited PP2C phosphatase activity and associated with FEM-3. hFEM-2 shows striking similarity (79% amino acid identity) to rat Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase phosphatase (rCaMKPase). hFEM-2 and FEM-2, but not PP2C\(\alpha\), were demonstrated to dephosphorylate CaM kinase II efficiently in vitro, suggesting that hFEM-2 and FEM-2 are specific phosphatases for CaM kinase. Furthermore, hFEM-2 and FEM-2 associated with F1\(\alpha\) and FEM-1 respectively. Overexpression of hFEM-2, FEM-2, or rCaMKPase all mediated apoptosis in mammalian cells. The catalytically active, but not the inactive, forms of hFEM-2 induced caspase-dependent apoptosis, which was blocked by Bcl-XL or a dominant negative mutant of caspase-9. Taken together, our data suggest that hFEM-2 and rCaMKPase are mammalian homologues of FEM-2 and they are evolutionarily conserved CaM kinase phosphatases that may have a role in apoptosis signaling.

Apoptosis, or programmed cell death (PCD),\(^{1}\) plays important roles in tissue homeostasis and development in essentially all multicellular organisms (1, 2). Genetic analyses of the PCD pathway in Caenorhabditis elegans have successfully identified key regulatory genes that define the core machinery of cell death. Orthologues of the apoptosis genes of C. elegans have subsequently been identified in other organisms including mammals (3, 4), suggesting that the molecular strategies that regulate the fundamental aspects of this important biological process are likely to be conserved across species.

The molecular strategies that are involved in sex determination among species, however, are thought to be quite diverse (5, 6). Indeed, genes in the sex-determining pathway are known to be rapidly diverged even between two closely related species, C. elegans and Caenorhabditis briggsae (7, 8). In C. elegans, many genes have been identified that affect sexual fate. One of the key steps in the sex determination pathway in C. elegans is regulated by three fem genes, fem-1, fem-2, and fem-3 (8, 9). Loss-of-function mutations in any one of the fem genes prevent all aspects of male development and transform both males and hermaphrodites into females (9, 10). fem-1 encodes a protein with ankyrin repeats (11), and fem-2 encodes a serine/threonine protein phosphatase of the PP2C type (12), which interacts directly with FEM-3 (13, 14), a protein without any recognizable functional motif.

F1\(\alpha\) has recently been identified as a mammalian Fas death domain-interacting protein in a yeast two-hybrid screen (15). Overexpression of F1\(\alpha\) induces apoptosis in mammalian cells, and F1\(\alpha\) is a caspase substrate (15). Interestingly, F1\(\alpha\) was found to be highly homologous throughout its entire protein sequence, to the C. elegans sex determination protein FEM-1 (15, 16). The degree of similarity between FEM-1 and F1\(\alpha\) (30% amino acid identity) is comparable with that between several of the functionally conserved components of PCD in nematodes and mammals, e.g. the amino acid identity between CED-9 and Bcl-2 is 23% (17), and that between CED-3 and caspase-3 is 34% (18).

Despite their exclusive roles in masculinizing somatic tissues in males and regulating the production of male germ cells (8, 9), FEM-1 and FEM-2 proteins are expressed throughout development in all somatic tissues at equivalent levels in both sexes (12, 19). This observation raises the question as to whether these proteins would have additional functions other than sex determination. Although there is no evidence to link F1\(\alpha\) to sex determination function, FEM-1 and its mammalian homologue, F1\(\alpha\), were found to induce apoptosis when overexpressed in mammalian cells (15, 16). In vitro experiments demonstrated that FEM-1 is cleaved by the C. elegans caspase, CED-3, demonstrating a striking parallel to its mammalian homologue, F1\(\alpha\) (16). It is possible that fem-1, in contrast to many other sex-determining genes in C. elegans, was conserved during evolution because of its function in apoptosis. The functional conservation between FEM-1 and F1\(\alpha\) in mediating apoptosis in mammalian cells raises an intriguing possibility that perhaps not only FEM-1 but a subset of genes in the C. elegans sex determination pathway may also be conserved if they have a role to play in apoptosis signaling.
To test our hypothesis, we initiated a search for putative mammalian homologues of fem genes. A search in the GenBank™ data base revealed a human cDNA that encodes a full-length protein, which we named hFEM-2, that exhibits extensive amino acid sequence similarity to FEM-2 (28% amino acid identity). hFEM-2 exhibited enzymatic characteristics of the PP2C type similar to those for FEM-2. Like FEM-2, hFEM-2 associates with FEM-3, whereas a related PP2C phosphatase, PP2Ca (20), does not.

The putative human homologue of fem-2 shares 79% amino acid identity with the rat Ca2+/-calmodulin-dependent protein kinase phosphatase (rCaMKPase) that was recently cloned from rat brain (21). CaM-dependent protein kinases are multifunctional protein kinases that control a variety of cellular functions including apoptosis (22–27). CaM kinase II is activated through autophosphorylation, whereas CaM kinase I and IV are activated through phosphorylation by upstream Ca2+/-calmodulin-dependent protein kinase kinases (28). CaM kinase phosphatase was initially purified from rat brain using a synthetic peptide corresponding to the autophosphorylation site of CaM kinase II (28). The rCaMKPase dephosphorylated and deactivated CaM kinase II activated by autophosphorylation (28), and was later shown to be able to dephosphorylate CaM kinase I and IV activated upon phosphorylation by CaM kinase kinases (28, 29). We show that FEM-2, hFEM-2, and rCaMKPase mediate caspase-dependent cell death when overexpressed in mammalian cells. We also demonstrate that FEM-2 and its human homologue can dephosphorylate autophosphorylated CaM kinase II efficiently in vitro. Hence, FEM-2 and its mammalian homologues are evolutionarily conserved CaM kinase phosphatases that may play a role in apoptosis signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Lines—**Mono- and polyclonal antibodies against the Myc epitope (9E10, A14), monoclonal antibody against the HA epitope (F7), and polyclonal antibody against poly(ADP-ribose) polymerase (PARP) (A20) were obtained from Santa Cruz Biotechnology, Inc. (HeLa cells and kidney cells, NH3T3 fibroblast cells, and Hela cells were originally from the American Type Culture Collection (ATCC).) Tumor necrosis factor-sensitive MCF-7 breast carcinoma cells were maintained as described previously (15, 16). Ca2+/-calmodulin-dependent kinase II (CaM kinase II) was obtained from Calbiochem.

**Construction of Plasmids—**Plasmids containing the cDNAs for fem-1, fem-2, and fem-3 were kindly provided by Dr. Andrew Spence (University of Toronto, Toronto, Ontario, Canada). The cDNA for rat Ca2+/-calmodulin kinase phosphatase was kindly provided by Dr. Hitoshi Fujisawa (Asahikawa Medical College, Asahikawa, Hokkaido, Japan).

cDNA fragments for hFEM-2 and hPP2Ca (20) were obtained by poly-merase chain reaction (PCR) amplification from human spleen cDNA (CLONTECH) with the Expand™ high fidelity PCR system (Roche Molecular Biochemicals) with primers incorporated with appropriate restriction sites and inserted into the pXJ40 mammalian expression vector driven by the CMV promoter (30). The constructs were sequenced to ensure that no PCR error was introduced. All epitope tags are at the NH2 termini. Point mutations were introduced using the Transformertm site-directed mutagenesis kit (CLONTECH).

**Northern Blot Analysis—**Human multiple tissue Northern blots (CLONTECH) were hybridized with a 32P-labeled probe corresponding to the NH2-terminal 295-base pair coding region of hFEM-2 using ExpressHybr™ hybridization solution (CLONTECH) according to the instructions of the manufacturer.

**Immunofluorescence Assay—**MCF-7 cells were transfected at 70% confluence on glass coverslips using Lipofectamine™ (Life Technologies, Inc.). The cDNAs for rat Ca2+/-calmodulin kinase phosphatase (rCaMKPase) that was recently cloned from rat brain (21), and human cDNA fragments for hFEM-2 and hPP2Ca (20) were obtained by polymerase chain reaction (PCR) amplification from human spleen cDNA (CLONTECH) with the Expand™ high fidelity PCR system (Roche Molecular Biochemicals) with primers incorporated with appropriate restriction sites and inserted into the pXJ40 mammalian expression vector driven by the CMV promoter (30). The constructs were sequenced to ensure that no PCR error was introduced. All epitope tags are at the NH2 termini. Point mutations were introduced using the Transformertm site-directed mutagenesis kit (CLONTECH).

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**RESULTS**

**Identification, Cloning, and Tissue mRNA Distribution of hFEM-2**—To identify mammalian homologues of fem-2 and fem-3, we searched the GenBank database for cDNAs encoding proteins with homology to these genes. No gene with significant similarity to fem-3 was found. However, the amino acid sequence predicted by analysis of an open reading frame (ORF) of a cDNA clone, kiaa0015 (GenBank accession no. D18340), derived from randomly sampled cDNA clones prepared from human immature myeloid cell line KG-1 (31), showed significant homology to FEM-2. The kiaa0015 clone contained a single long ORF encoding a protein of 454 amino acids (Fig. 1A), 16 h after transfection, the cells were fixed, rinsed with PBS, and then incubated for 2 min with Hoechst 33342 dye (Molecular Probes Inc.) to enable nuclear staining. The cells were subsequently fixed and then visualized using a Zeiss Axiosplan microscope.

**Apoptosis Assay**—Apoptosis assays were performed as described (15, 16). Briefly, HeLa cells were seeded onto glass coverslips at 70% confluence and transfected with pCMV-Myc or pCMV-β-galactosidase. Vector plasmid was supplemented to bring the total amount of plasmids for each transfection to 5 μg. Transfections were carried out with LipofectAMINE for 6 h, followed by change of medium, and VXAD was added to the fresh medium at this point where indicated. 24 h later, the cells were stained by propidium iodide (red) or 0.2% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution at 37 °C. Cells were stained by propidium iodide (red) or 0.2% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution at 37 °C. Cells were visualized by phase-contrast microscopy and blue (β-galactosidase-positive) cells were scored for apoptotic morphology. The data (mean ± S.D.) described are percentage of round blue cells as a function of total number of blue cells counted (~400–500 cells/sample) from three to five randomly chosen fields.

**PARP Cleavage Assay**—For detection of PARP cleavage, HeLa cells cultured on 100-mm dishes were transiently transfected with the various pXJ-Myc constructs (10 μg). Whole cell extracts were prepared by lysing the cells in 0.2 ml of lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM PMSF, 50 μg/ml aprotinin, and 10 μg/ml leupeptin). The extracts were fractionated on SDS-PAGE followed by Western blotting analyses using PARP-specific antibody (A20).

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ogy with FEM-2, with a relatively long amino-terminal extension flanking the catalytic domain. Over the entire length of the protein, the amino acid identity between hFEM-2 and FEM-2 is 28%. The degree of similarity is not uniform over the entire length of the protein. In the catalytic domain, the two proteins are 30% identical, whereas in the amino-terminal region, the proteins are 18% identical. The catalytic domains among different members (α, β, γ) (20, 32, 33) of PP2C family generally share high degree of sequence similarity (34); however, the catalytic domain of FEM-2 is more similar in sequence to that of hFEM-2 than to other mammalian PP2Cs.

The expression profile of hFEM-2 in human tissues was assessed by Northern blot analysis. Two transcripts, with estimated length of 6.5 and 3 kb, were detected in all adult tissues studied (Fig. 1A). The level of hFEM-2 mRNA message was variable from tissue to tissue, with the highest expression in testis (Fig. 1B). The subcellular localization of hFEM-2 was assessed in MCF-7 cells using immunocytochemistry. Myc-tagged hFEM-2 was expressed in MCF-7 cells, and the expression of the protein was detected using FITC-conjugated Myc antibody, whereas propidium iodide was used to stain the nuclei. hFEM-2 was consistently found diffusely distributed in the cytosol with no notable expression in the nucleus (Fig. 1C), suggesting that hFEM-2 is a cytosolic protein.

hFEM-2 Exhibits PP2C Phosphatase Activity toward the Peptide Substrate (RRA(pT)VA) in Vitro—As FEM-2 is known to exhibit PP2C phosphatase activity (13), we proceeded to examine the phosphatase activity of hFEM-2 in vitro. PP2C is the main enzyme subtype of the PPM family of serine-threonine phosphatases, and some of its members include mammalian PP2Cα (20), PP2Cβ (32), and PP2Cγ (33). In contrast to other protein phosphatases, the dephosphorylation activity of PP2C absolutely requires the metal cations, Mn$^{2+}$ or Mg$^{2+}$, but its activity is not sensitive to the tumor promoter okadaic acid and other inhibitors of the PPM family (34). Full-length NH$_2$-terminal Myc-tagged hFEM-2, FEM-2, or hPP2Cα were transiently overexpressed in 293T cells. The Myc-tagged proteins were immunoprecipitated using anti-Myc antibody, and the immunoprecipitates were assayed for phosphatase activity in vitro. The phosphatase activity was assayed by dephosphorylating the phosphothreonine peptide RRA(pT)VA as described under "Experimental Procedures." Immunoprecipitates of hFEM-2 protein from transiently transfected 293T cells exhibited phosphatase activity to an extent similar to that of FEM-2 and PP2Cα in the presence of 20 mM Mg$^{2+}$ or Mn$^{2+}$ (Fig. 2A, lanes 3, 4, 7, and 8). However, in the presence of 20 mM Ca$^{2+}$ or Mg$^{2+}$-free buffer, little phosphatase activity above basal level was detected (Fig. 2A, lanes 2 and 5). The dephosphorylation activity was not inhibited by okadaic acid (10 μM) (Fig. 2A, lane 6). Both hFEM-2 and FEM-2 displayed substrate concentration-dependent kinetics as the phosphatase activities increased with increasing substrate concentration (Fig. 2B). Taken together, these data suggest that hFEM-2 is a PP2C phosphatase.

hFEM-2 Is a Ca$^{2+}$/Calmodulin-dependent Protein Kinase Phosphatase—A protein initially identified as CaM kinase phosphatase has recently been purified from rat brain (28). rCaMKPase was shown to dephosphorylate autophosphorylated CaM kinase II, whereas phosphorylase kinase, mixed histones, myelin basic protein, α-casein, and phosphorylase a were not significantly dephosphorylated (28), suggesting that it is a specific phosphatase that regulates CaM kinases. While characterization of hFEM-2 was in progress, the cDNA encoding rCaMKPase was isolated (21). Interestingly, the amino acid sequence of rCaMKPase is 79% identical to that of hFEM-2 (Fig. 3A), suggesting that hFEM-2 may be the human orthologue of rCaMKPase. Similar to hFEM-2, rCaMKPase shares 27% amino acid identity with C. elegans FEM-2, suggesting that FEM-2 may also be a CaM kinase phosphatase.

Bacterially expressed rCaMKPase, hFEM-2, FEM-2, and PP2Cα GST fusion proteins were tested for their ability to dephosphorylate autophosphorylated CaM kinase II in vitro. Equivalent amounts of GST-rCaMKPase, GST-hFEM-2, and GST-FEM-2 were able to dephosphorylate $^{32}$P-labeled, autophosphorylated CaM kinase II (isoform α), in the presence of 10 μg/ml poly(Lys) (Fig. 3B, lanes 2, and 5, respectively). The activity of rCaMKPase has been shown to be greatly stimulated by poly(Lys) (21, 28). Similar to rCaMKPase, in the absence of poly(Lys), the dephosphorylation activities of GST-hFEM-2 and GST-FEM-2 were significantly reduced (Fig. 3B, lanes 4 and 7). An equivalent amount of GST-hPP2Cα, which exhibited higher phosphatase activity toward the RRA(pT)VA substrate than GST-hFEM-2 (data not shown), failed to dephosphorylate CaM kinase II in the presence (Fig. 3B, lane 10) or absence of 10 μg/ml poly(Lys) (data not shown), suggesting that rCaMKPase, hFEM-2, and FEM-2 are specific phosphatases for CaM kinases. In the presence of 25 mM EDTA, a chelator of Mg$^{2+}$, GST-FEM-2, GST-hFEM-2, and GST-rCaMKPase were unable
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Fig. 3. hFEM-2 and FEM-2 dephosphorylate CaM kinase II in vitro. A, hFEM-2 is highly homologous to rCaMKPase. The predicted amino acid sequence of hFEM-2 is aligned with that of rat CaM kinase phosphatase (21), by the Jotun Hein method of the DNASTAR program. Amino acid residues in hFEM-2 that are identical to that of rat CaM kinase phosphatase are represented by dots. The percentage identity is 79%. B, hFEM-2 and FEM-2 dephosphorylate CaM kinase II in vitro. Equivalent amounts of CaM kinase II (~10 ng), which had been pre-auto-phosphorylated with [γ-32P]ATP as described under "Experimental Procedures," were incubated at 30 °C for 20 min with ~2 μg of GST protein and the indicated GST fusion proteins under the various conditions as indicated in parentheses (lanes 2–10) or with immunoprecipitated Myc-tagged proteins as indicated (lanes 11–13). Where indicated, 25 mM EDTA or 1 mM orthovanadate was added. Aliquots of the reactions were analyzed by SDS-PAGE, followed by autoradiography. The position corresponding to the α isoform of CaM kinase II is indicated (upper panel). The gel was Coomassie-stained, and the bands representing the various GST fusion proteins were aligned to show equivalency of protein (lower panel, lanes 1–10). The lower bands represent BSA, which migrated very closely with the indicated GST fusion proteins on SDS-PAGE. BSA was used to stop the CaM kinase II autophosphorylation reaction (lower panel, lanes 2–10). Immunoprecipitates of the Myc-tagged proteins were analyzed by SDS-PAGE followed by Western blotting, probed using monoclonal anti-Myc antibody, and aligned to show equivalency of protein (lower panel, lanes 11–13).

When compared with immunoprecipitates of vector transfected cells (Fig. 3B, lane 11), immunoprecipitates of Myc-tagged hFEM-2 and FEM-2 were able to dephosphorylate CaM kinase II in vitro (Fig. 3B, lanes 12 and 13), suggesting that the proteins overexpressed in mammalian cells possess the ability to constitutively dephosphorylate CaM kinases.

hFEM-2 Specifically Associates with FEM-3—FEM-2 has been shown to interact with FEM-3 in the yeast two-hybrid system and in vitro GST pull-down assay (13). To assess whether hFEM-2 shares functional similarity with FEM-2 in this respect, 293T cells were transiently co-transfected with expression plasmids encoding HA-tagged FEM-3 (5 μg) and the indicated Myc-tagged proteins (5 μg). 5 h after the change of medium, the Myc-tagged proteins were immunoprecipitated (IP) with polyclonal anti-Myc antibody (A-14). Co-immunoprecipitated HA-tagged FEM-3 proteins were detected by immunoblot analysis using monoclonal anti-HA antibody (top panel). Expression of HA-tagged FEM-3 was determined by Western blot analysis of an aliquot (1%) of the total extract (1 ml) with monoclonal anti-HA antibody (F-7) (middle panel). After stripping the blot, immunoprecipitated Myc-tagged PP2C proteins were detected using monoclonal anti-Myc antibody (9E10), and the bands representing the different Myc-tagged proteins were aligned (lower panel). Both the blots of the top panel and the lower panel were subjected to similar exposure time (5 min). B, hFEM-2 associates with FEM-3 in vitro. Equivalent amounts of 35S-labeled, in vitro translated FEM-3 (5 × 10^6 cpm) were incubated with purified GST fusion proteins immobilized on glutathione-Sepharose beads. Retained 35S-FEM-3 protein was analyzed by SDS-PAGE and autoradiography (upper panel). The gel was Coomassie-stained, and the bands representing the various GST fusion proteins were aligned to show equivalency of loading (lower panel).

to dephosphorylate CaM kinase II (Fig. 3B, lanes 6, 3, and 9, respectively), suggesting that, like rCaMKPase (21, 28), the CaM kinase phosphatase activities of FEM-2 and hFEM-2 are dependent on Mg2+/Mn2+. In this assay, orthovanadate (1 mM), an inhibitor of many protein phosphatases, including PP-1, PP2B, and PP2C (28), had no significant effect on the dephosphorylation of CaM kinase II by GST-hFEM-2, GST-FEM-2, and GST-rCaMKPase (data not shown). These data suggest that FEM-2 and its mammalian homologues exhibit unique enzymatic properties toward CaM kinases that distinguish them from other phosphatases.
HA-tagged hFEM-2 (data not shown).

We next examined the interaction of hFEM-2 with FEM-3 in vitro. In vitro translated, 35S-labeled FEM-3 bound to immobilized GST fusion proteins of FEM-2 and hFEM-2 but not immobilized GST or GST-PP2Ca (Fig. 4B), suggesting that there is a direct association between hFEM-2 and FEM-3.

hFEM-2 Specifically Associates with F1Aα—Genetic analyses suggest that the three genes fem-1, fem-2, and fem-3 act at the same level in a mutually dependent fashion in the sex determination pathway in C. elegans (8, 9). Although FEM-2 associates with FEM-3 (13), the molecular relationships between FEM-1 and FEM-2/FEM-3 remain undefined. This prompted us to examine whether hFEM-2 and F1Aα, which are putative human homologues of FEM-2 and FEM-1, respectively, are able to associate in vivo. 293T cells were transiently co-transfected with expression plasmids encoding Myc-tagged hFEM-2, rCaMKPase, or FEM-2 and HA-tagged F1Aα. Immunoprecipitation of Myc-hFEM-2, rCaMKPase, and FEM-2 from cell extracts with anti-Myc antibody co-immunoprecipitated HA-F1Aα (Fig. 5A, left panel). Under similar conditions, Myc-hPP2Ca failed to co-immunoprecipitate F1Aα (Fig. 5A, left panel), suggesting that F1Aα interacts specifically with FEM-2 and its mammalian homologues. HA-tagged F1Aα did not form nonspecific immunoprecipitates with the anti-Myc antibody (Fig. 5A, left panel). Conversely, Myc-tagged F1Aα also co-immunoprecipitated HA-hFEM-2, but not HA-PP2Ca (data not shown). Myc-FEM-2 was also able to co-immunoprecipitate HA-FEM-1 (Fig. 5A, right panel) when overexpressed in mammalian cells, suggesting that FEM-1 and FEM-2 may function as components of a signal transduction protein complex.

We performed GST pull-down assay to test whether hFEM-2 can associate with F1Aα and FEM-1 in vitro. Both in vitro translated F1Aα and FEM-1 were found to associate with GST-hFEM-2 but not GST-hPP2Ca (Fig. 5B), suggesting direct interaction between F1Aα and FEM-1 with hFEM-2. In vitro translated FEM-1 also bound to GST-FEM-2 (data not shown). Taken together, these data suggest that FEM-2 specifically associates with FEM-1 and that the association function between hFEM-2 and F1Aα has been conserved during evolution.

hFEM-2 and FEM-2 Mediate Caspase-dependent Apoptosis in Mammalian Cells—To determine whether FEM-2 and its mammalian homologues are able to mediate apoptosis like FEM-1, we transiently transfected HeLa cells with mammalian expression plasmids encoding the various GFP-tagged constructs. 18 h after transfection, cells were fixed and stained with Hoechst dye and viewed under a fluorescence microscope. Cells that appeared apoptotic morphologically (rounded cell appearance) exhibited nuclear condensation and fragmentation, as judged by Hoechst staining (Fig. 6A). Overexpression of GFP-FEM-2 and GFP-hFEM-2 but not GFP-hPP2Ca significantly enhanced the percentage of apoptotic cells compared with GFP vector control (Fig. 6A), suggesting that apoptosis mediated by FEM-2 and hFEM-2 is likely to be specific and not a general effect of phosphatase activity from overexpressing a phosphatase in the cell.

To quantitate pro-apoptotic activity, expression constructs of hFEM-2 and its mammalian homologues (2 μg) were co-transfected with β-galactosidase (0.5 μg) in HeLa cells. 18 h after transfection, cells were stained for β-galactosidase activity to mark the transfected cells and scored for apoptotic morphology as described (15, 16). Overexpression of hFEM-2 and rCaMKPase significantly increased the number of apoptotic cells as compared with cells transfected with vector control (Fig. 6B). FEM-2, but not hPP2Ca, also mediated apoptosis in HeLa cells to an extent similar to that for the mammalian CaMKPases (Fig. 6B). Cell death mediated by hFEM-2 was efficiently blocked by 20 μM ZVAD (Fig. 6B), suggesting that caspase activation may be required for its activity. Similarly, cell death mediated by FEM-2 and rCaMKPase was also efficiently blocked by 20 μM ZVAD (data not shown).

Cleavage of PARP serves as a marker for the activation of caspases in cells undergoing apoptosis (35). The cleavage of PARP to the signature 85-kDa apoptotic fragment

![Figure 5](http://www.jbc.org/)
was observed in HeLa cells transiently transfected with hFEM-2, rCaMKPase, and FEM-2, but not vector-transfected cells (Fig. 6C).

The pro-apoptotic activity of FEM-2 and hFEM-2 was evaluated in two other mammalian cell lines, NIH3T3 and MCF-7. FEM-2 mediated apoptosis in all three cell lines, HeLa, NIH3T3, and MCF-7 (data not shown), whereas hFEM-2 mediated apoptosis in NIH3T3 cells but not MCF-7 cells (data not shown), suggesting that hFEM-2 exhibits cell type-dependent pro-apoptotic activity. The protein levels of all the transiently expressed proteins in MCF-7 cells were comparable with that in HeLa and NIH3T3 cells (data not shown); therefore, the lack of pro-apoptotic activity of hFEM-2 in MCF-7 cells is not a consequence of low protein expression. Transient overexpression of PP2Ca or FEM-3 failed to induce apoptosis in all cell lines tested (data not shown), suggesting an absence of intrinsic pro-apoptotic activity in these molecules.

Apoptosis Mediated by hFEM-2 Is Blocked by Bel-XL or Dominant Negative Caspase 9—Apoptosis signaling mediated through the Fas death receptor is thought to diverge at caspase-8, with one branch of the pathway leading directly to effector caspase activation and the other branch communicating with the mitochondria cell death pathway that are dependent on caspase-9 and Bel-XL (36, 37). In HeLa cells, the predominant pathway for Fas signaling is Bel- XL-dependent (37). To investigate possible pathways by which CaM kinase phosphatase may be using for mediating the pro-apoptotic effect in mammalian cells, blocking experiments using anti-apoptotic Bel-XL and dominant negative mutants of caspase-8 and caspase-9 were performed. Although dominant negative mutant of caspase-8, caspase-8(1-415), was able to block apoptosis mediated by transient overexpression of Fas or tumor necrosis factor receptor 1 (Ref. 38 and data not shown), it was only marginally effective in blocking apoptosis induced by overexpression of hFEM-2 in HeLa cells (Fig. 7). In contrast, Bel-XL and dominant negative mutants of caspase-9, caspase-9-DN (39, 40), were effective inhibitors of apoptosis induced by hFEM-2 overexpression (Fig. 7). These observations suggest that hFEM-2 may mediate its pro-apoptotic effect through a signaling pathway that involves caspase-9 and Bel-XL.

Mutation Analysis of hFEM-2—A study of the time course of apoptosis mediated by hFEM-2 in HeLa cells showed that significant apoptosis occurs only after 16 h (data not shown). At 6 or 10 h after transfection, cells transfected with 2 μg of hFEM-2 showed only a marginal increase in number of pro-apoptotic cells (Fig. 8A). To delineate the potential effector and regulatory domains of hFEM-2 responsible for the pro-apoptotic effect, HeLa cells were transiently transfected with expression vectors encoding Myc-tagged hFEM-2 (2 μg) or various deletion mutants (Fig. 8A, left panel). Cells transfected with hFEM-2-(94-454) or hFEM-2-(157-454) displayed efficient pro-apoptotic activity at 6 and 10 h (Fig. 8A), suggesting that the catalytic domain of hFEM-2 is required for its pro-apoptotic activity. Cells transfected with the NH2-terminal domain of hFEM-2 alone (1-157) showed no significant increase in apoptosis (Fig. 8A) even after 24 h (data not shown), suggesting that the NH2-terminal domain is inactive in apoptosis. Interestingly, the NH2-terminal deleted constructs appeared significantly more potent than wild type hFEM-2 in inducing apoptosis (Fig. 8A), suggesting that the NH2-terminal domain may have a negative regulatory function. The NH2-terminal deleted mutants and full-length hFEM-2 all exhibited similar levels of protein expression at 6 and 10 h (data not shown), supporting the argument that the difference in pro-apoptotic ability was not a consequence of variation of protein expression. NH2-terminal deleted mutants of FEM-2, however, showed pro-apoptotic activity similar to that of full-length FEM-2 (data not shown), suggesting that the negative regulatory function of the NH2-terminal domain of FEM-2 had been acquired during evolution.
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The deletion mutants were also evaluated for their ability to associate with F1Aα. Both the N157 and 157–454 (catalytic domain) mutants associated with F1Aα (data not shown). To examine whether the association of hFEM-2 and F1Aα is required for the apoptosis function, point mutants in the catalytic domain of hFEM-2 that selectively abolish the association but not the catalytic function of the hFEM-2 protein need to be identified.

The results of the deletion analysis suggest that the region containing the catalytic domain of hFEM-2 is required for its pro-apoptotic activity. Because critical amino acid residues required for catalytic activity of PP2C phosphatases have been defined, we next asked the question whether the phosphatase activity of hFEM-2 is required for mediating apoptosis. hFEM-2 mutants were generated in which Gly202 or Arg326 (Fig. 8B), which are conserved residues that have been shown to be necessary for PP2C activity (13, 41, 42), was mutated to Asp (G202D) or Ala (R326A), respectively. As expected, the abilities of these mutants to dephosphorylate the RRg(T)VA peptide were diminished to essentially background level in vitro (Fig. 8C, left panel). A control mutant was also created where the nonconserved amino acid Arg207 (Fig. 8B) was mutated to Ala (R207A). hFEM-2 (R207A) exhibited PP2C activity at a level similar to wild type hFEM-2 (Fig. 8C, left panel). The protein levels of all the point mutants were similar to wild type (Fig. 8C, lower panel). The G202D and R326A mutants that showed reduced PP2C phosphatase activity were also unable to dephosphorylate CaM kinase II in vitro (data not shown), whereas the R207A mutant dephosphorylated CaM kinase II to an extent similar to that of wild type hFEM-2 (data not shown).

hFEM-2 and the R207A mutant that retained phosphatase activity both mediated apoptosis to similar extents (Fig. 8C, right panel). However, the R326A and G202D mutants that lacked phosphatase activity failed to mediate apoptosis beyond basal level (Fig. 8C, right panel). All the point mutants of hFEM-2 were still able to associate with both FEM-3 and F1Aα (data not shown), suggesting that the mutations did not result in a disruption in the entire conformation of the protein. A corresponding point mutant of FEM-2 (R336A), which had been shown previously to lose activity as a phosphatase as well as sex determination function (13), also lost pro-apoptotic activity in HeLa cells (data not shown), suggesting that the catalytic activities of FEM-2 and its mammalian homologues are required for their ability to mediate apoptosis.

**DISCUSSION**

Our earlier observations that FEM-1 and its human homologue F1Aα are substrates of caspases and are able to mediate apoptosis have led us to hypothesize that perhaps a subset of C. elegans sex-determining genes may be involved in apoptosis signaling. Our search for mammalian homologues of other fem genes has led us to identify hFEM-2, a putative human homologue of FEM-2.

Co-conservation of sequence and function is an important principle during evolution. As a consequence, sequence-related genes often have similar functions in evolutionarily distant species. Here we show that FEM-2 and its mammalian homologues share similar functions as a PP2C phosphatase and as a specific protein partner of FEM-3. Furthermore, our characterization revealed several novel molecular functions of FEM-2 and its mammalian homologues, namely 1) they associate physically with FEM-1/F1Aα, 2) they are specific CaM kinase phosphatases, and 3) they promote apoptosis in mammalian cells.

Although the catalytic domains of hFEM-2 and FEM-2 are required for mediating apoptotic function, the NH2-terminal domain of hFEM-2 appears to negatively regulate its pro-apoptotic activity. Interestingly, the NH2-terminal domain of F1Aα, but not FEM-1, has also been observed to negatively regulate the pro-apoptotic function of F1Aα (15, 16), supporting the hypothesis that mammalian proteins are capable of more complex control than their homologues in lower organisms. The presence of a negative regulatory domain that inhibits pro-apoptotic function is not unique to hFEM-2 and F1Aα. For example, the pro-apoptotic activity of Bid, a member of the Bcl-2 family of cell death regulators, is also negatively regulated through its NH2-terminal domain (43). The NH2-terminal deletion mutant, hFEM-2(94–454), in contrast to wild type

**FIG. 8. Mutation analysis of hFEM-2.** A, pro-apoptotic activity of hFEM-2 and its deletion mutants at 6- and 10-h time points. Empty and filled bars indicate data at 6- and 10-h time points, respectively. The horizontal bars on the left represent the amino acid sequences of hFEM-2 and its deletion mutants. B, Alignment of the conserved regions in the PP2C motifs of various PP2C phosphatases. Identical amino acids are boxed and positions of amino acid residues chosen to be mutated in hFEM-2 are indicated by arrows. C, The enzymatic activity of hFEM-2 is required for its pro-apoptotic function. The left panel shows the phosphatase activity of hFEM-2 and its point mutants. 293T cells were transfected with expression vector containing hFEM-2 or its point mutants. 24 h after transfection, the cells were collected and lysed in storage buffer. The Myc-tagged proteins were immunoprecipitated using anti-Myc rabbit polyclonal antibody, and the immunoprecipitates were incubated with 100 μM phosphothreonine peptide substrate in the presence of 20 mM Mg2+ at 30 °C for 1 h. Free phosphate generated was measured spectrophotometrically according to the instructions of the manufacturer (Promega). The right panel shows the extent of apoptosis mediated by hFEM-2 and its point mutants. HeLa cells were transfected with 2 μg of the indicated expression plasmids and 0.5 μg of pCMV-β-galactosidase. Apoptosis assays were performed as described under “Experimental Procedures.” The data (mean ± S.D.) shown are percentage of round blue cells as a function of total number of blue cells. Aliquots of immunoprecipitates from 293T cells were analyzed using monoclonal anti-Myc antibody to evaluate the level of expression of various constructs. The lower panel shows protein expression levels of hFEM-2 and its point mutants. WT, wild type.
hFEM-2, was pro-apoptotic in all three cell types tested (data not shown). This observation raises the possibility that the lack of pro-apoptotic activity of wild type hFEM-2 in MCF-7 cells is because of its failure to overcome the negative regulation conferred by the NH\textsubscript{2} terminus in this particular cellular environment.

Protein phosphatases are known to regulate various cellular events such as cell growth, differentiation, and apoptosis. Dephosphorylation appears to be an important regulatory step with respect to the kinase activity of CaM kinases (22–25). Indeed, autophosphorylation of CaM kinase II at Thr\textsuperscript{286} has been shown to be up-regulated in ischemic tolerance (44), and loss of CaM kinase activity has been suggested to play a role in initiating the changes leading to ischemia-induced cell death (45). KN-93, a specific inhibitor of CaM kinase II, induces apoptosis in NIH 3T3 cells (46). CaM kinase inhibition has been shown to potentiate thapsigargin-mediated cell death in SH-SY5Y cells (47). Furthermore, CaM kinase IV has been shown to inhibit apoptosis induced by oxygen deprivation in cerebellar granule neurons (48), suggesting that inhibition of CaM kinase activity might lead to apoptosis. Because CaM kinases have been implicated in regulating apoptosis in various experimental paradigms, protein phosphatases that dephosphorylate CaM kinases may be important for regulating apoptotic events. We show here that hFEM-2, FEM-2, and rCaMK-Pase are all capable of dephosphorylating autophosphorylated CaM kinase II and mediating apoptosis in HeLa and NIH3T3 cells, suggesting that these CaM kinase phosphatases are involved in apoptosis signaling. The ability to mediate apoptosis appears to require an intact phosphate domain as point mutants in which the phosphatase activity was disrupted failed to induce apoptosis. CaM kinase kinase, the kinase that phosphorylates the CaM kinases, has been implicated in activation of PKB/Akt, a kinase in a cell survival signaling pathway (25). CaM kinase phosphatases may promote cell death by antagonizing this action of CaM kinase kinase.

FEM-2 is known genetically to be essential for the specification of male development in C. elegans (12). Its phosphatase activity is required for its sex-determining function, suggesting the involvement of a kinase in directing the sexual fate of C. elegans (13). Currently, among all the sex-determining proteins in C. elegans, no protein containing a kinase domain or exhibiting kinase activity has been described. The Ca\textsuperscript{2+}/calmodulin-dependent protein kinase cascade is conserved from Drosophila, and mammals (56). These results suggest that at least some aspects of sexual regulation have a common evolutionary origin. The precise physiological role of mammalian homologues of FEM-2 awaits further investigation in CaM kinase phosphatase deficient animals.

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REFERENCES

1. Metzstein, M. M., Stanfield, G. M., and Horvitz, H. R. (1998) Trends Genet. 14, 410–416
2. Vaux, D. L., and Korsemeyer, S. J. (1999) Cell 96, 245–254
3. Hengartner, M. O. (1999) Nature 391, 440–442
4. Horvitz, H. R. (1999) Cancer Res. 59, 1701s–1706s
5. Schaefer, A. J., and Goodfellow, P. N. (1999) Bioessays 18, 955–963
6. Marin, I., and Baker, S. B. (1998) Science 281, 1990–1994
7. Hansen, D., and Pilgrim, D. (1998) Genetics 149, 1553–1562
8. Hansen, D., and Pilgrim, D. (1999) Mech. Dev. 83, 3–15
9. Kimble, J., Edgar, L., and Hirsh, D. (1994) Dev. Biol. 165, 234–239
10. Doniach, T., and Hodgkin, J. (1991) Dev. Biol. 139, 225–235
11. Spence, A. M., Coulson, A., and Hodgkin, J. (1990) Cell 66, 981–990
12. Pilgrim, D., McGregor, A., Jackle, P., Johnson, T., and Hansen, D. (1995) Mol. Biol. Cell 6, 1159–1171
13. Chin-Sang, I. D., and Spence, A. M. (1996) Genes Dev. 10, 2314–2325
14. Ahringer, J., Rosenquist, T. A., Lawson, D. N., and Kimble, J. (1992) EMBO J. 11, 2303–2310
15. Chan, S. L., Tan, K. O., Zhang, L., Yee, K. S., Ronca, F., Chan, M. Y., and Yu, V. C. (1999) J. Biol. Chem. 274, 32461–32468
16. Chan, S. L., Yee, K. S., Tan, K. M., and Yu, V. C. (2000) J. Biol. Chem. 275, 17625–17629
17. Hengartner, M. O., and Horvitz, H. R. (1994) Cell 76, 665–676
18. Xue, D., Shaham, S., and Horvitz, H. R. (1996) Genes Dev. 10, 1073–1083
19. Gaudet, J., VanderElist, I., and Spence, A. M. (1996) Mol. Biol. Cell 7, 1107–1121
20. Mann, D. J., Campbell, D. G., McGowan, C. H., and Cohen, P. T. (1992) Biochim. Biophys. Acta 1130, 100–104
21. Kitani, T., Ishida, A., Okuno, S., Takeuchi, M., Kameshita, I., and Fuisawa, H. (1999) J. Biochem. (Tokyo) 125, 1022–1028
22. Nairn, A. C., and Picciotto, M. R. (1994) Science 265, 767–770
23. Fujisawa, H. (2001) J. Biochem. (Tokyo) 129, 193–199
24. Soderling, T. R. (2000)Curr. Opin. Neurol. 13, 375–380
25. Soderling, T. R. (1999) Trends Biochem. 24, 232–237
26. Cohen, O., Feinstein, E., and Kinuchi, A. (1997) EMBO J. 16, 998–1008
27. Wright, S. C., Schellenberger, U., Ji, L., Wang, H., and Larrick, J. W. (1997) FASEB J. 11, 843–849
28. Ishida, A., Kameshita, I., and Fuisawa, H. (1998) J. Biochem. 124, 1004–1010
29. Ishida, A., Okuno, S., Kitani, T., Kameshita, I., and Fuisawa, H. (1998) Biochim. Biophys. Res. Commun. 253, 159–163
30. Yee, K. S., and Yu, V. C. (1998) J. Biol. Chem. 273, 5364–5374
31. Nomura, N., Miyajima, N., Sano, T., Tanaka, A., Kawarabayasi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K., and Tabata, S. (1994) DNA Res. 1, 27–56
32. Marley, A. E., Kline, A., Crabtree, G., Sullivan, J. E., and Beri, R. K. (1998) FEBS Lett. 431, 121–125
33. Travis, S. M., and Welsh, M. J. (1997) FEBS Lett. 412, 415–419
34. Cohen, P. (1998) Annu. Rev. Biochem. 58, 453–508
35. Tewer, M., Quan, L. T., O'Gorek, K., Desthayere, S., Zeng, Z., Beidler, D. R., Puirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809
36. Adams, J. M., and Cory, S. (1998) Science 281, 1322–1326
37. Scaffidi, C., Fuhr, S., Srinivasan, A., Friese, C., Li, F., Tommaselli, K. J., Debatis, K. M., Krammer, P. H., and Peter, M. E. (1998) EMBO J. 17, 1675–1687
38. Boldin, M. P., Gornecharov, T. M., Goltsay, Y. V., and Wallach, D. (1996) Cell 85, 803–815
39. Pan, G., O'Rourke, K., and Dixit, V. M. (1998) J. Biol. Chem. 273, 5841–5845
40. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) Mol. Cell 1, 949–957
41. Sheen, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 975–980

\textsuperscript{2} D. Pilgrim, personal communication.
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42. Das, A. K., Helps, N. R., Cohen, P. T., and Barford, D. (1996) EMBO J. 15, 6798–6809
43. Tan, K. O., Tan, K. M., and Yu, V. C. (1999) J. Biol. Chem. 274, 23687–23690
44. Shamloo, M., Kamme, F., and Wieloch, T. (2000) Neuroscience 96, 665–674
45. Shackelford, D. A., Yeh, R. Y., Hsu, M., Buzsaki, G., and Zivin, J. A. (1995) J. Cereb. Blood Flow Metab. 15, 450–461
46. Tombes, R. M., Grant, S., Westin, E. H., and Krystal, G. (1995) Cell Growth Differ. 6, 1063–1070
47. McGinnis, K. M., Wang, K. K., and Gnegy, M. E. (2001) Neurosci. Lett. 301, 99–102
48. See, V., Boutillier, A. L., Bito, H., and Loeffler, J. P. (2001) FASEB J. 15, 134–144
49. Eto, K., Takahashi, N., Kimura, Y., Masuho, Y., Arai, K., Muramatsu, M., and Tokumitsu, H. (1999) J. Biol. Chem. 274, 22556–22562
50. Whitfield, L. S., Lovell-Badge, R., and Goodfellow, P. N. (1993) Nature 364, 713–715
51. Tucker, P. K., and Lundrigan, B. L. (1993) Nature 364, 715–717
52. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991) Nature 351, 117–121
53. de Bono, M., and Hodgkin, J. (1996) Genetics 144, 587–595
54. Kuwabara, P. E. (1996) Genetics 144, 597–607
55. Raymond, C. S., Shamu, C. E., Shen, M. M., Seifert, K. J., Hirsch, B., Hodgkin, J., and Zarkower, D. (1998) Nature 391, 691–695
56. Smith, C. A., McClive, P. J., Western, P. S., Reed, K. J., and Sinclair, A. H. (1999) Nature 402, 601–602
The Caenorhabditis elegans Sex-determining Protein FEM-2 and Its Human Homologue, hFEM-2, Are Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase Phosphatases That Promote Apoptosis
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