F1Aα, a Death Receptor-binding Protein Homologous to the Caenorhabditis elegans Sex-determining Protein, FEM-1, Is a Caspase Substrate That Mediates Apoptosis*

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Apoptosis is an evolutionarily conserved process that is critical for tissue homeostasis and development including sex determination in essentially all multicellular organisms. Here, we report the cloning of an ankyrin repeat-containing protein, termed F1Aα, in a yeast two-hybrid screen using the cytoplasmic domain of Fas (CD95/APO-1) as bait. Amino acid sequence analysis indicates that F1Aα has extensive homology to the sex-determining protein FEM-1 of the Caenorhabditis elegans, which is required for the development of all aspects of the male phenotype. F1Aα associates with the cytoplasmic domains of Fas and tumor necrosis factor receptor 1, two prototype members of the “death receptor” family. The F1Aα protein also oligomerizes. Overexpression of F1Aα induces apoptosis in mammalian cells, and co-expression of Bcl-XL or the dominant negative mutants of either FADD or caspase-9 blocks this effect. Deletion analysis revealed the center region of F1Aα, including a cluster of five ankyrin repeats to be necessary and sufficient for maximum apoptotic activity, and the N-terminal region appears to regulate negatively this activity. Furthermore, F1Aα is cleaved by a caspase-3-like protease at Asp342, and the cleavage-resistant mutant is unable to induce apoptosis upon overexpression. F1Aα is therefore a member of a growing family of death receptor-associated proteins that mediates apoptosis.

Caspases are critical mediators of apoptosis (5–8). In addition to autoactivation and activation of other caspases, caspases are thought to participate in apoptosis by disabling important cellular processes and breaking down structural components of the cell. Caspases also activate signaling molecules that upon cleavage commit the cells to apoptosis. Molecules that transmit death signals upon cleavage by caspases have been identified in various apoptotic pathways (9–13).

Some members of the tumor necrosis factor (TNF)† receptor superfamily, known as the death receptors (14–17), efficiently transmit death signals via a cytoplasmic motif called the “death domain” (18, 19). Among members of the death receptor family, the receptor-proximal events have been best characterized for Fas and TNFR1. Stimulation of these receptors results in aggregation of their intracellular death domains (20, 21), leading to the recruitment of key signaling proteins (14). The Fas receptor signals to caspase-8 through the recruitment of the adaptor protein, FADD/MORT1 (22–25), whereas TNFR1 signals to caspase-8 and caspase-2 through the TRADD-FADD (26) and TRADD-RIP-RAIDD/CRADD (27, 28) pathways, respectively. Although the precise mechanism is still not clear, it is known that formation of a death receptor-FADD-caspase 8 complex is required for the activation of caspase-8, which is an early step in one of the cascades of apoptotic events induced by Fas and TNFR1 (10, 11, 13, 29, 30).

In our effort intended to identify additional components of the death receptor signaling pathways, we identified an ankyrin-repeat containing protein, termed F1Aα, in a yeast two-hybrid screen using the cytoplasmic domain of the mouse Fas receptor (mFas) as bait. The amino acid sequence of F1Aα is highly homologous to the C. elegans protein, FEM-1, which is essential for achieving all aspects of the male phenotype in the nematode (31). F1Aα binds to mFas, TNFR1, and itself. Overexpression of F1Aα induces apoptosis in MCF7 cells that can be blocked by expression of Bcl-XL or the dominant negative mutants of either FADD or caspase-9. F1Aα is therefore a member of a growing family of death receptor-associated proteins (25, 32–35) that mediate apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—Mono- and polyclonal antibodies against the Myc epitope (9E10, A14) and polyclonal antibody against the HA

† The abbreviations used are: TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PARP, poly(ADP-ribose) polymerase; NGF, nerve growth factor; NGFR, nerve growth factor receptor; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; kb, kilobase pair; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; DN, dominant negative; HA, hemagglutinin; CMV, cytomegalovirus; Z, benzoyloxyce- bonyl; fmk, fluoromethyl ketone.

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epitope (Y11) were obtained from Santa Cruz Biotechnology. Monoclonal antibody against the HA epitope (12CA5) was purchased from Roche Molecular Biochemicals. Antibody against PARP (C2–10) was from Dr. G. Poirier, CHUL Research Center, Canada. HeLa, 293, and NIH3T3 were originally from American Type Culture Collection (ATCC). Thirty TGND-mutants of MCF7 (used in this study) were provided by Dr. V. Dixit, University of Michigan. 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 protease inhibitors YVAD and aprotinin and bovine serum were from Biozym Systems Products, CA, USA. Recombinant caspase-3, -6, -7, and 8 were purchased from Pharmingen.

**Plasmids Construction**—Plasmids containing the cDNA for TNFR1 and NGFR were from M. Chao, Cornell University Medical College and mFas from K. B. Elkon, Cornell University. DNA fragments for plasmid constructs containing mFas-IC-(166–306), mFas-FD5-(166–292), TNFR1-IC-(205–426), TNFR1-ICa15-(205–411), and NGFR-IC-(271–427) were obtained by PCR amplification using the Expand™ high fidelity polymerase chain reaction (PCR) System (Roche Molecular Biochemicals) with primers incorporated with appropriate restriction sites and epitope tags as needed into the pXJ40 mammalian expression vector driven by the CMV promoter (36). The constructs were sequenced to ensure that no PCR error was introduced. Unless otherwise stated all constructs containing mFas or TNFR1-IC-(205–426) vector driven by the CMV promoter (36). The constructs were sequenced to ensure that no PCR error was introduced. Unless otherwise stated all cDNAs were sequenced using SDS-PAGE fractionation on SDS-PAGE for visualization of the expression of proteins. The remaining cell lysates were incubated with 1 μg of polyclonal anti-HA antibody for 1 h on ice and then mixed with 20 μl of a 1:1 slurry of protein A-agarose and incubated for another 1 h at 4 °C. The agrose beads were washed once in 1 ml of lysis buffer, 2 times in 1 ml of lysis buffer containing 500 mM NaCl, and 2 times in 1 ml of lysis buffer before fractionation on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Bound proteins were visualized by autoradiography.

**Immunoprecipitation**—For co-immunoprecipitation experiments, 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum to 80% confluency. Transfection was carried out with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were incubated with the LipofectAMINE/DNA mixture for 12 h followed by change of fresh media. The cells were harvested 8 h after change of media and lysed in 1 ml of lysis buffer (50 mM HEPES (pH 7.6), 350 mM NaCl, 1% Nonidet P-40, and 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml apro tinin, and 10 μg/ml leupeptin). An aliquot (10 μl) of the cell lysates was used for visualization of the expression of proteins. The cDNA of caspase-9-DN (caspase-9-C287A) was generated by PCR error. DNA fragments generated by PCR and the junctions of DNA were made by introducing a stop codon mutation at the restriction sites, or vector control. 24 h after transfection, the cells in mono layers were washed twice with ice-cold PBS (pH 7.4) and then washed with 20 μl of a 1:1 slurry of protein A-agarose and incubated for another 1 h at 4 °C. The agrose beads were washed once in 1 ml of lysis buffer, 2 times in 1 ml of lysis buffer containing 500 mM NaCl, and 2 times in 1 ml of lysis buffer before fractionation on SDS-PAGE. Western blotting analyses were performed subsequently with procedure as described previously (39) using monoclonal anti-Myc antibody.

**Apoptosis Assays**—MCF7 cells were maintained in a 35-mm dish in RPMI media supplemented with fetal bovine serum and transfected using LipofectAMINE. Transfections were carried out in 1 ml of serum-free Dulbecco’s modified Eagle’s medium and incubated for 6 h after transfection. In serum-containing RPMI was added to allow analysis of transfection efficiency. The cells were washed once with PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde in 1× PBS for 5 min at 4 °C, and stained with a histochemical reaction mixture (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, and 1 mg/ml X-gal) at 37 °C. After 10 h, cells were washed with PBS and then washed with phase-contrast microscopy. For detection of PARP cleavage, MCF7 cells cultured on 100-mm dishes were transiently transfected, and nuclear extracts were prepared as described (39). The extracts were fractionated on SDS-PAGE followed by Western blotting analyses using PARP-specific antibody (C2–10).

**Nuclear Staining Assay**—The assay was performed with procedure as described previously (40). Briefly, cells were seeded onto glass coverslips and transfected with plasmids expressing F1Aex, its deletion mutants, or vector control. 24 h after transfection, the cells in monolayers were washed twice with ice-cold PBS (pH 7.4) and then fixed at 4 °C with absolute methanol (−20 °C). The washing step with PBS was then repeated once. To stain the nuclei, the cells were incubated for 10 min with 10 μg/ml Hoechst 33342 (Molecular Probes Inc., Eugene, OR) and then washed with PBS. The coverslips with the stained cells were mounted in 80% glycerol in PBS containing 1 mM p-phe nylendiamine and examined with a Zeiss Axioplan microscope.

**In Vitro Cleavage Reactions**—Cleavage reactions were carried out for 1 h at 37 °C. 5 × 105 cpm of in vitro translated, 35S-labeled F1As or mutants were incubated with bacterially expressed active forms of caspase-3 (0.1 μg), caspase-6 (0.2 μg), caspase-7 (0.1 μg), and caspase-8 (0.5 μg) in 20 μl of reaction buffer containing 20 mM PIPES (pH 7.2), 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 10 mM dithiothreitol. The reaction was incubated at 37 °C for 1 h followed by SDS-PAGE and autoradiography.

**RESULTS**

Cloning of HB12—A yeast two-hybrid screen was performed to isolate clones that may interact with the cytoplasmic domain of the mouse Fas receptor (mFas). The cDNA fragment encoding amino acids 166–292 of mFas (mFasFD5) was cloned in frame with the DNA binding domain of Gal4 (GRD-mFasFD5) and used to screen a human B cell library. A truncated cytoplasmic domain of Fas (mFasFD5) was used because deletion of
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A negative regulatory region at the C terminus of Fas markedly enhances its ability to mediate apoptosis (18) and to bind FADD (25). Approximately 3 million independent transformants were screened, and 17 putative positive clones were identified, of which two failed to show interaction with any of the heterologous proteins (see “Experimental Procedures”). Sequence analysis revealed that one of the clones encoded the full-length FADD/MORT1 (18, 24), whereas the other clone, HB12, contains a partial complementary DNA with an open reading frame encoding a polypeptide of 469 amino acids.

Although the HB12 clone encoded an N-terminal truncated protein, the 469 amino acid polypeptide may contain the effector domain of the full-length protein. Since HB12 interacted with the death receptors, its ability to modulate apoptosis was evaluated by overexpressing HB12 in MCF7 cells by transient transfection. MCF7 cells were transiently transfected with HB12 (10 μg), Z-VAD or DEVD (20 μM) were added to the cells 5 h after transfection. 24 h after transfection, nuclear extracts were prepared from the cells, and endogenous PARP was detected by Western blot analysis using the monoclonal anti-PARP antibody, C-2-10. D. MCF7 cells were transiently transfected with pCMV-β-galactosidase (0.5 μg) and 1.5 μg of expression vector for HB12, Z-VAD-fmk, and DEVD-fmk (20 μM) were added as described in C. Cells were fixed and stained for β-galactosidase, and 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 counted (about 400–500 cells per sample) from 3 to 5 randomly chosen fields.

**Fig. 1.** Overexpression of clone HB12 in MCF7 cells induces caspase-dependent apoptosis. A, MCF7 cells were co-transfected with pCMV-β-galactosidase and three times molar excess of expression vector containing clone HB12 or the empty vector. 24 h after transfection, the cells were fixed and incubated in a buffer containing X-gal to visualize the β-galactosidase activity. Morphological differences of cells transfected with the indicated expression vectors are shown. B, Hoechst staining of the nuclei of MCF7 cells transfected with the indicated expression vectors. C, PARP cleavage in HB12-overexpressing cells. MCF7 cells were transiently transfected with HB12 (10 μg), Z-VAD or DEVD (20 μM) were added to the cells 5 h after transfection. 24 h after transfection, nuclear extracts were prepared from the cells, and endogenous PARP was detected by Western blot analysis using the monoclonal anti-PARP antibody, C-2-10. D, MCF7 cells were transiently transfected with pCMV-β-galactosidase (0.5 μg) and 1.5 μg of expression vector for HB12, Z-VAD-fmk, and DEVD-fmk (20 μM) were added as described in C. Cells were fixed and stained for β-galactosidase, and 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 counted (about 400–500 cells per sample) from 3 to 5 randomly chosen fields.

Isolation of F1Aα—By using HB12 cDNA fragment as a probe, we screened a human hypothalamus cDNA library (CLONTECH) and obtained several cross-hybridizing cDNA clones. One of these, clone HH6.15, contained a 3.1-kb cDNA insert with a 1881-nucleotide open reading frame beginning with a translational initiation consensus sequence (42) and predicted a protein of 627 amino acids with a molecular mass of 70 kDa (Fig. 2). A corresponding murine cDNA clone was subsequently obtained by screening a mouse testis library. The amino acid sequence deduced from the murine cDNA clone is virtually identical to that of the human clone (Fig. 2A). Data base searches revealed that the predicted protein shares extensive similarity throughout the entire sequence, including the presence of six tandemly arranged ankyrin-repeats at the N terminus (Fig. 2B), with the sex-determining protein in *C. elegans* known as FEM-1 (Fig. 2A). Because of its ability to modulate apoptosis in mammalian cells we named the protein F1A for FEM-1-like protein in the apoptotic pathway.

To determine the tissue distribution of F1A mRNA, Northern analysis was performed with the HB12 cDNA as a probe. Two transcripts, 5.5 and 7.5 kb, were found to be ubiquitously distributed, with varying abundance; two additional smaller transcripts, 1.35 and 2.5 kb, were found only in the testis (Fig. 2C). In the mouse tissues, only the ubiquitously distributed 7.5-kb and the testis-specific 2.4-kb transcripts were detected (data not shown). The different transcripts may be generated by alternate splicing or derived from other related genes. Sequence analysis of several independent partial cDNA clones failed to detect the existence of splice variants or related family members. However, a search in the EST database by NCBI has revealed several human and murine cDNA clones that share high level of similarity (~30%) in amino acid sequence with F1A, suggesting F1A might be a member of a gene family. During the preparation of this manuscript, the deduced amino acid sequence of two members, Fem1a and Fem1b, of a mouse gene family was reported (43). The amino acid sequence of Fem1b is identical to that of mF1A confirming that F1A is indeed a member of a gene family. We therefore refer to our protein as F1Aα.

**F1Aα Specifically Interacts with the Death Receptors and Self-associates**—In the two-hybrid assay, clone HB12 (F1Aα-(159–627)) interacted with mFas-FD5-(166–292) and the intracellular domain of TNFR1 (TNFR1-IC-(205–426)). Radiolabeled in *vitro* translated full-length F1Aα was tested for *in vitro* binding with various glutathione S-transferase (GST) fusion proteins (Fig. 3A). F1Aα specifically associated with GST-mFas-FD5 and GST-TNFR1-IC in the assay but not with either GST or GST-FADD. In contrast to the two-hybrid results (data not shown), F1Aα interacted equally well with both GST-mFas-IC-(166–306) and GST-mFas-FD5. Parallel experiments using HB12 clone, F1Aα-(159–627), yielded identical results (data not shown). *In vitro* self-association of F1Aα was not evaluated because we were unable to obtain a reasonable yield of GST-F1Aα; however, 35S-F1Aα was found to associate with GST-F1Aα-(482–627) (data not shown).
To demonstrate these interactions in vivo, full-length F1Aα tagged with the HA epitope (HA-F1Aα) was expressed with Myc-tagged mFas-FD5, TNFR1-IC, TNFR1-ICΔ15-(205–411) or F1Aα in human embryonic kidney 293 cells. Expression of the cytoplasmic domain of the death receptors resulted in poor yields of the expressed proteins as a result of extensive cell death. To prevent this, Z-VAD-fmk at 20 μM was added to the culture media. HA-F1Aα was immunoprecipitated, and the associated Fas, TNFR1, and F1Aα were detected by Western blotting with anti-Myc antibody. These Myc-tagged proteins did not form nonspecific immunoprecipitates with the HA antibody (data not shown). The cytoplasmic domains of mFas-FD5, TNFR1-IC, and TNFR1-ICΔ15-(205–411) associated with F1Aα, whereas FADD did not. The inability of mFas-IC to interact with F1Aα in the mammalian cells and HB12 in yeast suggests that an in vivo mechanism may have restricted its accessibility to the protein. Similar to other signaling components serving members of the TNF/NGF receptor superfamily, F1Aα was able to self-associate in vivo (Fig. 3B, lane 6).

Deletion Analysis of F1Aα—To delineate the functional domain of F1Aα responsible for the apoptotic effect, MCF7 cells were transiently transfected with expression vectors encoding the HA-tagged F1Aα or various deletion mutants. Interestingly, wild type F1Aα exhibited slower kinetics in inducing apoptosis than FADD (Fig. 4A). When cells were scored for apoptosis 24 h post-transfection, F1Aα appeared to be inactive, whereas FADD was apoptotic (Fig. 4A). However, when the transfected cells were examined at 36 and 48 h post-transfection, substantial apoptotic activity was demonstrated by F1Aα. At the 48-h time point, F1Aα was as potent as FADD in inducing apoptosis in MCF7 upon overexpression (Fig. 4A). In our initial efforts to characterize the cDNA obtained from the yeast two-hybrid library, we observed a significant level of apoptosis in cells overexpressing HB12 at the 24-h time point, suggesting that HB12 was killing the cells with a different kinetics from F1Aα. Since HB12 is an N-terminal truncated form of F1Aα, it raised the possibility that the N-terminal region contains a negative regulatory domain. To facilitate the identification of the potential negative regulatory domain in F1Aα, we subjected F1Aα and its deletion mutants to apoptosis assay at both 24- and 48-h time points.
Cells transfected with an N-terminal truncated form of F1Aα, F1Aα(82–627), exhibited morphological changes characteristic of apoptosis similar to those cells transfected with expression constructs of HB12 (F1Aα-(159–627), Fig. 1) at the 24-h time point (Fig. 4B). Deletion of 40 amino acids from the N terminus (F1Aα(40–627)) was insufficient to relieve the inhibition, and the apoptotic activity of F1Aα-(40–627) was only apparent at the 48-h time point. Additional N-terminal deletions to eliminate the entire ankyrin repeat cluster as in F1Aα-(253–627) rendered the mutant inactive at both the 24- and 48-h time points. F1Aα-(82–627) appeared to be substantially more effective in inducing apoptosis than HB12 at both time points, suggesting that additional ankyrin repeats other than the two found in HB12 are required for full apoptotic activity. The absence of apoptotic function in the C-terminal region was demonstrated by the overexpression of two C-terminal mutants, F1Aα-(82–253) and F1Aα-(82–253) did not induce apoptosis (Fig. 4B, data not shown). Thus the region immediately distal to the ankyrin repeat of F1Aα is essential for apoptotic function, and the minimum effector domain is from amino acids 82 to 342. F1Aα(1–530) and F1Aα-(1–342) exhibited apoptotic activity only at the 48-h time point, providing further support for the possible regulatory role of the N-terminal region of F1Aα. C-terminally tagged constructs gave identical results (data not shown). Western blot analyses showed that all the mutants were expressed at the 24-h time point, and protein levels were generally higher among mutants that were not apoptotic (Fig. 4D).

The presence of a negative regulatory domain that regulates the potency of a pro-apoptotic molecule has been suggested in Bim (44). Bim is a member of the “BH3 domain-only” family of pro-apoptotic proteins for which splice variants have been described. The three isoforms are very similar but there are clear differences in their cytotoxicity upon overexpression. Since the short form was the most potent inducer of cell death, the regions specific to Bimshort and Bimshort were suggested to have a negative regulatory role (44).

To ensure that the apoptotic effect of F1Aα is not restricted to MCF7 cells, the effects of transient expression of F1Aα and its deletion mutants in HeLa and NIH3T3 cells were evaluated. In both cell types, F1Aα overexpression resulted in cell death that could be blocked by treatment with 20 μM Z-VAD-fmk or DEVD-fmk at the 48-h time point (Table I). Similar to that observed in MCF7 cells, the presence of the N-terminal region affected the kinetics of killing suggesting a negative regulatory role for the N-terminal region. The apoptotic activity of HB12 was also compromised in HeLa and NIH3T3 cells supporting the suggestion that a cluster of five ankyrin repeats is required for full apoptotic activity.

**F1Aα Is Specifically Cleaved by a Caspase-3-like Protease**—To explore the possibility that F1Aα is a caspase substrate, F1Aα and F1Aα-(82–627) were labeled by in vitro translation in the presence of [35S]methionine and subjected to cleavage analysis with a panel of recombinant caspases. While
caspase-3, -6, and -7 were able to cleave PARP and caspase-8 cleaved Bid (data not shown), only caspase-3 cleaved F1Aa into a ~38- and a ~32-kDa fragment (Fig. 5A). Several potential caspase cleavage sites were identified in F1Aa by sequence inspection. Cleavage at two of these, i.e. DNID\(^{342}\) and VYAD\(^{356}\), would generate fragments of the predicted sizes. To confirm the cleavage site, the aspartic acids of F1Aa at position 342 and 356 were mutated to alanine. F1Aa(D342A) was resistant to caspase-3 cleavage, whereas F1Aa(D356A) was cleaved in a similar manner as the wild type F1Aa (Fig. 5C, lanes 5 and 6). The predicted sizes of the F1Aa proteolytic fragments resulting from cleavage at Asp\(^{342}\) are consistent with what is observed: 1–342 (38 kDa) and 343–627 (32 kDa) (Fig. 5B). The apoptotic N-terminal deletion mutant F1Aa-(82–627) was also susceptible to caspase-3 cleavage, yielding cleavage products of expected sizes, 82–342 (29 kDa) and 343–627 (32 kDa) (Fig. 5A, lane 7). We were unable to detect the cleavage products of F1Aa in MCF7 and HeLa cells. Detection of cleavage product of caspases that is capable of inducing cell death has been shown to be technically challenging in transient transfection experiments (45).

Caspase inhibitors block the apoptotic effect of F1Aa, and this protein can be cleaved by caspase-3 in vitro, yielding F1Aa-(1–342) as one of the cleavage products. Furthermore, F1Aa-(1–342) was found to be apoptotically active by deletion analysis. These observations raise the possibility that proteolytic cleavage of F1Aa may be required for its apoptotic function. To test this possibility, the wild type and cleavage-resistant forms of F1Aa, F1Aa(D342A), were overexpressed in MCF7 cells. The proteins were expressed at comparable levels as verified by Western blot analysis (data not shown). F1Aa(D342A) was inactive in the apoptosis assay (Fig. 5D), whereas F1Aa and the control point mutant F1Aa(D356A), which could be cleaved by caspase-3 in vitro, were apoptotic. F1Aa(D342A) was able to associate with the death receptors such as mFas-FD5 and TNFR1-IC in vitro as indicated by immunoprecipitation assay (data not shown) suggesting that the overall conformation of the protein was still intact. Taken together these data suggest that proteolytic cleavage of F1Aa at Asp\(^{342}\) is a prerequisite for its apoptotic activity.

**Apoptotic Effect of F1Aa Is Blocked by Bcl-XL and Dominant Negative Mutants of FADD and Caspase-9—Apoptosis mediated by death receptors (Fas/CD95 and TNFR1) involves FADD recruitment of caspase-8 and its subsequent proteolytic activation (23, 25, 46). However, in a variety of cell types, apoptotic signaling in response to Fas or TNFR1 activation is regulated at least in part by a Bcl-2 and/or Bcl-XL-inhibitable step (30, 47). To establish a possible link between F1Aa and components of various apoptotic pathways, blocking experiments using the anti-apoptotic Bel-XL (48) and dominant negative mutants of several signaling molecules were performed. Dominant negative mutant of caspase-8, caspase-8-(1–415), which has been shown to block death receptor-mediated cell death (46), was only marginally effective in blocking apoptosis induced by overexpression of F1Aa in MCF7 cells (Fig. 6). In contrast, Bcl-XL and dominant negative mutants of caspase-9, caspase-9-DN (49, 50), and FADD, FADD-DN (51), were potent inhibitors of apoptosis induced by F1Aa overexpression (Fig. 6) suggesting that they might have a role in the signaling pathway of F1Aa.”

**DISCUSSION**

In this report, we describe the identification and characterization of F1Aa, a novel death receptor binding protein. F1Aa does not have a death domain; however, it has six ankyrin motifs arranged in tandem at the N terminus. Ankyrin repeat structures are protein-protein interaction domains capable of associating with diverse proteins through heterotypic interactions (52, 53). The ankyrin repeat motifs are found in F1Aa-(1–342), which apparently is important in mediating the apoptotic effect of F1Aa. However, F1Aa-(1–342) was unable to interact with the cytoplasmic domain of mFas or TNFR1 as suggested by co-immunoprecipitation experiment (data not shown). Deletion of 93 amino acid residues from the C terminus abolished the ability of F1Aa to associate with these proteins. The 145-amino acid C-terminal region of F1Aa alone is insufficient to interact with both the death receptors (data not shown) suggesting that the death receptor binding and death effector domains are separable in F1Aa similar to that reported in the FADD protein (25). Therefore, F1Aa-(1–342) is likely to interact with another protein in the apoptotic pathway. The identification of this protein partner(s) would provide further in-
sights into the molecular mechanism of F1αa action. Caspase-3, but not caspase-6, -7, and -8, was found to cleave F1αa at a specific site, Asp342, situated at the C-terminal boundary of the minimum effector domain of death in F1αa. The caspase that processed F1αa, however, may not be caspase-3 because F1αa is not as susceptible as DFF45/ICAD, which is an established substrate of caspase-3 (54), to caspase-3 digestion in vitro. Whereas DFF45/ICAD was completely cleaved, only a fraction of F1αa was cleaved under the same experimental condition (data not shown). Therefore, a caspase-3-like rather than caspase-3 activity is likely to regulate the apoptotic activity of F1αa. More than a dozen caspases have been identified in mammals (7); it is possible that caspases other than those used in the present study are able to process F1αa more efficiently. A single point mutation (D342A) in F1αa resulted in a cleavage-resistant mutant devoid of apoptotic activity suggesting the C-terminal domain of F1αa might also have a negative regulatory role. The activation of a pro-apoptotic molecule by caspase cleavage is well documented in BID, a “BH3 domain only” member of the Bcl-2 family. Upon activation of the TNFRI or Fas apoptotic signaling pathways, BID is cleaved by caspase-8 to generate a C-terminal fragment, tBID, which is a potent inducer of cytochrome c release and apoptosis. Cleavage of BID by caspase-8 therefore relieves the inhibitory effect of the N-terminal region that controls its pro-apoptotic activity (10, 11, 13, 63).

Overexpression of F1αa induced apoptosis in MCF7 cells and co-expression of Bcl-XL or the dominant negative mutants of either FADD or caspase-9 could diminish this apoptotic effect. These observations suggest that F1αa may be a component of a signaling pathway that involves FADD, caspase-9, and Bcl-XL. The dominant negative mutant of FADD may compete with F1αa for binding to the death receptor, thus diminishing its apoptotic activity. Fas signaling 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 that are caspase-9- and Bcl-XL-dependent (10, 11, 30). In MCF7 cells because of caspase-3 deficiency, the predominant pathway for Fas signaling is Bcl-XL-dependent (30). The blocking data thus suggest that F1αa most probably play a role in the Fas signaling pathway upstream of Bcl-XL and caspase-9.

F1αa shares substantial amino acid sequence homology (~30% identity) with the gene product of the C. elegans sex-determining gene, fem-1. The fem-1 gene was identified in genetic screens and is required for sex determination of male phenotype in both germ line and somatic tissues in the nematode C. elegans (55, 56). We have demonstrated the ability of F1αa to induce apoptosis, which raises the question about F1αa function in C. elegans. The current understanding of sex determination in C. elegans during development does not appear to rule out an apoptotic role for FEM-1. In C. elegans, the earliest sex-specific events that occur during embryogenesis are two sets of cell deaths, one male and the other hermaphrodite-specific (57). In the male embryo, two motor neurons called HSNs undergo apoptosis at hour 8 of embryonic development (hatching occurs at hour 13). At about the same time in the hermaphrodite embryo, four sensory neurons called CENs also die by apoptosis. The molecular pathways that control these sex-specific apoptosis events are not well understood.

Despite its exclusive role in masculinizing somatic tissues in males and regulating the production of male germ cells in both males and hermaphrodites, FEM-1 protein is expressed throughout development in all somatic tissues at equivalent levels in both sexes (58). The activity of FEM-1 is therefore thought to be controlled post-translationally. Alternatively, FEM-1 may have a role other than sex determination in C. elegans.

The relatively high degree of sequence homology between FEM-1 and F1αa is intriguing considering that two other genes flanking fem-1 in the sex determination pathway, tra-1 and tra-2, are the most highly diverged genes compared between the two Caenorhabditis species, C. elegans and Caenorhabditis briggsae (59, 60). Although the sex determination pathways in mammals and C. elegans are thought to be quite different, recent evidence suggests that certain regulatory proteins in these pathways are indeed conserved (61, 62). Whether F1αa has a role in determining the sexual fate in mammals requires further investigation.

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