FLAME-1, a Novel FADD-like Anti-apoptotic Molecule That Regulates Fas/TNFR1-induced Apoptosis*

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We identified and cloned a novel human protein that contains FADD/Mort1 death effector domain homology regions, designated FLAME-1. FLAME-1, although most similar in structure to Mch4 and Mch5, does not possess caspase activity but can interact specifically with FADD, Mch4, and Mch5. Interestingly, FLAME-1 is recruited to the Fas receptor complex and can abrogate Fas/TNF-induced apoptosis upon expression in Fas/ tumor necrosis factor-sensitive MCF-7 cells, possibly by acting as a dominant-negative inhibitor. These findings identify a novel endogenous control point that regulates Fas/TNF1-mediated apoptosis.

Several members of the caspase family of proteases (1) have been implicated as key regulators of programmed cell death or apoptosis (2, 3). The proapoptotic caspases can be divided into two groups: those with a large prodomain such as ICH-1 (caspase-2), Mch4 (caspase-10), Mch5/MACH/FLICE (caspase-8) and Mch6/ICE-Lap-6 (caspase-9) and those with a small prodomain such as CPP32/YAMA/Apopain (caspase-3), caspase-7 (Mch6/ICE-Lap-6), caspase-8 (Mch4/FADD-FDH, residues 3–80; FDH B, residues 102–177; FDH, residues 9–177), and Mch5/ICE-Lap-3 (caspase-7). Caspases with large prodomains are probably the most upstream caspases (4, 5). They are recruited by several death-signaling receptors that belong to the TNFR family (6) through interactions of their prodomain with the receptor-interacting adaptor molecules FADD/Mort1 (7, 8) or CRADD/RAIDD (5, 9). For example, the prodomains of Mch4 and Mch5 contain two tandem regions that show significant homology with the N-terminal death effector domain (DED)† of FADD (10–12). Engagement of Fas/FADD results in recruitment of FADD to the receptor complex (13), which presumably triggers activation of the caspase apoptotic pathway through interaction of its DED with the corresponding motifs in the prodomain of Mch4 and probably Mch5. CRADD presumably functions like FADD by recruiting ICH-1 to the Fas/TNFR1 complex, through interaction of its N-terminal domain with the corresponding motif in the prodomain of ICH-1 (5, 9). Thus, the prodomains of caspases function to physically link the death receptors to the downstream caspase activation pathway.

In our efforts to characterize novel apoptotic/anti-apoptotic molecules that contain FADD-DED homology regions, we have identified a human molecule designated FLAME-1 (FADD-like antiapoptotic molecule) that is structurally related to Mch4 and Mch5. We show that FLAME-1 is recruited to the Fas signaling pathway through interaction with FADD. Interestingly, FLAME-1 can block Fas/TNF1-induced apoptosis but not UV-induced apoptosis.

MATERIALS AND METHODS
cDNA Cloning—The full-length FLAME-1 cDNA was cloned from human Jurkat Uni-ZAP XR cDNA library (14) by screening the library with a partial FLAME-1 cDNA probe. The probe was amplified by PCR using FLAME-1-specific primers derived from the 3′ (GenBank accession no. aa002262) and 5′ (GenBank accession no. aa001257) sequences of human expressed sequence tag (EST) clone 427786.

Chromosomal Mapping—The human genes for Mch4 and FLAME-1 were mapped on previously described rodent-human hybrid panels (15) and on the Genebridge 4 and Stanford G3 radiation hybrid panels (Research Genetics) using specific oligonucleotide primers.

In Vitro Binding Assays—This was performed as described recently (5).

Mammalian Expression Vectors—Epitope tagging was done by cloning cDNAs in-frame into the multiple cloning sites of a modified T7-pcDNA3 and/or the Flag plasmid pFLAG-CMV-2 (IBI Kodak). All deletion and point mutants were generated by PCR and verified by sequencing. Flag-tagged Fas was constructed in pcDNA3 as described (8).

Transfection, Immunoprecipitation, and Western Analysis—293 or 293T human embryonic kidney cells were transiently transfected with the expression plasmids using the LipofectAMINE (Life Technologies, Inc.) method. Cell lysis, immunoprecipitation, and Western blotting were done as described (9).

Yeast Two-hybrid Analysis—Mch4-FDH (residues 18–189), Mch5b (α-DH A, residues 3–80; α-DH B, residues 102–177; α-DH, residues 9–177), FLAME-1-FDH (residues 1–160), and murine FADD-DED (residues 1–78) were subcloned into yeast two-hybrid vectors. Yeast two-hybrid analysis was then performed as described recently (16).

Apoptosis Assay—MCF7-FAS cells were transiently cotransfected with reporter and test plasmids at a ratio of 1:10 and assayed for apoptosis as described (5, 16). The percentage of viable cells (mean ± S.D.) under each condition was determined by measuring the number of viable blue cells compared with total blue cells.

RESULTS AND DISCUSSION

Identification and Cloning of FLAME-1—An EST (clone 427786) with statistically significant similarity to Mch5 (p < 0.001) was identified. Based on its sequence a probe was generated by PCR and used to isolate and clone the full-length†† sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AFOO9616–AFOO9620.

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The abbreviations used are: DED, death effector domain; PCR, polymerase chain reaction; kb, kilobases; CDH, caspase domain homology; GST, glutathione S-transferase; FDH, FADD-DED homology; PAGE, polyacrylamide gel electrophoresis.

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cDNA (~2 kb) from a human Jurkat cDNA library. This cDNA encodes a novel protein (designated FLAME-1) of 445 amino acids with predicted relative molecular mass of 51 kDa (Fig. 1A).

**Structural Organization of FLAME-1**—FLAME-1 is most similar to the Mch4 and Mch5 caspases (Fig. 1A). It has three distinct homology regions; there are two N-terminal tandem stretches of approximately 67–79 residues that are significantly homologous to the N-terminal DED (residues 1–79) of FADD, here referred to as FADD-DED homology A (FDH-A, residues 5–71) and B (FDH-B, residues 90–168) regions. FDH-A and FDH-B share 38 and 28% identity with the DED of FADD, respectively. The FDH regions share 28–33% identity with the corresponding regions in Mch4 and Mch5. They are followed by a stretch of 249 residues (residues 197–445) with significant homology to the caspase domain of Mch4 or Mch5 (27–31% identity), here referred to as the caspase domain homology A and B regions.

**Fig. 2. In vitro interactions of FLAME-1.** A, cleavage of FLAME-1 by caspases. In vitro translated 35S-labeled FLAME-1 (upper panel) or FLAME-1-D341A (lower panel) was incubated without (lane 1) or with 100 ng of purified recombinant caspase-2 (ICH-1), caspase-3 (CPP32), caspase-6 (Mch2), caspase-7 (Mch3), caspase-8 (Mch5), or caspase-10 (Mch4) (lanes 2–7) for 1 h at 37°C. Proteins were then analyzed by SDS-PAGE and autoradiography. Full-length FLAME-1 and its p39 and p12 fragments are indicated to the right. B–D, in vitro interactions. The indicated in vitro translated 35S-labeled proteins were precipitated with GST (B–D, lanes 1) or GST-FADD (B, lanes 2), GST-FLAME-1 (C, lanes 2), or GST-FLAME-1-CDH (residues 196–445) (D, lanes 2) fusion proteins immobilized on glutathione-Sepharose beads. The bound proteins were then analyzed by SDS-PAGE and autoradiography. Truncated proteins FADD-DD or FADD-DD contain residues 1–79 or 80–205, respectively.

mRNAs. Numbers on the right indicate kilobases. PBL, peripheral blood leukocyte. C, FLAME-1, Mch4, and Mch5 genes are localized to chromosome 2q33–34.
could be an alternatively spliced isoform of FLAME-1 mRNA.

Chromosomal Localization of FLAME-1—Chromosomal mapping linked the FLAME-1 and Mch5 genes to the D2S116 and D2S348 markers on chromosome 2q33–34 using radiation hybrid panels, in close proximity to where we had previously localized Mch4 (10) (Fig. 1C). This finding and the high degree of homology among their genes or gene products suggest that they might be descendents of a common ancestral gene through gene duplication.

FLAME-1 Is a Target of Active Caspases—Unlike Mch4 or Mch5 (4), expression of FLAME-1 in bacteria or in the baculovirus expression system did not result in its cleavage (autoactivation) or generation of a caspase-like activity as determined with the tetrapeptide substrates YVAD-AMC (where AMC is 7-amino-4-methylcoumarin) or DEVD-AMC (data not shown), suggesting that FLAME-1 might be enzymatically inactive or possess an unknown enzymatic activity. Interestingly, in vitro translated FLAME-1 can be cleaved by several purified caspases including CPP32, Mch2, Mch3, Mch4, Mch5, and ICH-1 to generate two fragments (p39 and p12) topologically equivalent to the large and small subunits of caspases (Fig. 2A). This cleavage occurs at Asp-341 in the LEVD↓G site, since a D to A mutation in this site prevents these caspases from cleaving FLAME-1. Transfection studies showed that FLAME-1 may also be a caspase substrate in vivo. Expression of a T7 epitope-tagged FLAME-1 (T7-FLAME-1) in 293 cells produced both full-length and cleaved (p39) FLAME-1 (see Fig. 3, B and E). This cleavage was not observed with the D341A mutant FLAME-1 (T7-FLAME-1-D341A, Fig. 3, C and E). Furthermore, stimulation of FLAME-1-transfected MCF7-FAS cells with anti-Fas antibody increased the amount of cleavage products, whereas addition of caspase inhibitors significantly reduced it (not shown). Thus, FLAME-1 appears to be a caspase target in apoptotic cells.

Interactions of FLAME-1—To investigate the participation of FLAME-1 in Fas/TNFFR1 apoptotic signaling pathways, in vitro and in vivo binding studies and yeast two-hybrid analysis were performed. Radiolabeled FLAME-1, Mch4, Mch5β, FADD, or mutants of these proteins were precipitated with various glutathione S-transferase (GST) fusion proteins immobilized on glutathione-Sepharose beads (Fig. 2, B–D). Mch4, Mch5β, and FLAME-1 associated specifically with GST-FADD, although the interaction of FLAME-1 with FADD was weaker than that observed with Mch4 or Mch5 (Fig. 2B). FADD, FADD-DD, Mch4, Mch5β, and Mch5β-FDH, but not FADD-DD, also associated specifically with FLAME-1β (GST-FLAME-1β) (Fig. 2C). These observations suggest that the interactions are mediated by the homologous FDH regions of these proteins. Interestingly, Mch5β but not Mch4 associated with a truncated FLAME-1 lacking its FDH regions (GST-FLAME-1-CDH), suggesting that the two proteins can also interact through their homologous CDH regions (Fig. 2D).

To demonstrate these interactions in vivo, we transiently

**TABLE I**

| DNA-binding hybrid | Activation hybrid | Liquid assay β-galactosidase activity | Miller units |
|--------------------|------------------|-------------------------------------|-------------|
| LexA-FLAME-1-FDH  | B42              | 23.2 ± 0.8                          |             |
| LexA-FLAME-1-FDH  | B42-Mch5β-FDH-A | 52.7 ± 22.6                         |             |
| LexA-FLAME-1-FDH  | B42-Mch5β-FDH-B | 728.1 ± 58.5                        |             |
| LexA-FLAME-1-FDH  | B42-Mch5β-FDH   | 300.2 ± 42.3                        |             |
| LexA-FLAME-1-FDH  | B42-Mch4-FDH    | 697.6 ± 103.6                       |             |
| LexA-FLAME-1-FDH  | B42-FADD-DD     | 324.1 ± 34.0                        |             |
| LexA-FLAME-1-FDH  | B42-FLAME-1-FDH | 1634.0 ± 297.0                      |             |
1-Fas interaction (Cotransfection of exogenous T7-FADD enhanced the FLAME-1 recruitment to Fas (not shown). These observations demonstrate that FLAME-1 can be recruited to recruit Mch4 and Mch5 to Fas (not shown). These Fas (generated by cleavage at Asp-341, also formed a complex with fragment coprecipitated with full-length FADD, Mch4, Mch5 and their isolated FDH regions (Fig. 3A). T7-FLAME-1 and its p39 fragment coprecipitated with full-length FADD, Mch4, Mch5β, or their isolated FDH regions (Fig. 3B). Full-length T7-FLAME-1 (but a negligible amount of the p39 fragment) associated with Mch5-CDH (Fig. 3B), suggesting that the entire CDH region of FLAME-1 is required for optimal interaction between these proteins. Similar results were obtained with T7-FLAME-1-D341A and T7-FLAME-1-CDH (Fig. 3C and D). No interactions were observed between T7-FLAME-1β and Flag-Mch4-CDH or Flag-Mch5-CDH (data not shown), suggesting that these proteins can only interact through their respective FDH or CDH regions. The yeast two-hybrid analysis confirmed the interactions of FLAME-1-FDH with FADD, Mch4, and Mch5 FDH regions (Table 1). This analysis also revealed that FLAME-1-FDH can also strongly interact with Mch4, and Mch5 FDH regions (Table I). This analysis also demonstrating that these proteins can only interact through their FDH regions are potent inducers of apoptosis in 293 cells (not shown), active site Cys to Ala Flag-tagged mutants were used in these experiments to investigate their interactions with FLAME-1. Consistent with the in vitro results, T7-FADD co-precipitated with full-length FLAME-1, Mch4, and Mch5 or their isolated FDH regions (Fig. 3A). T7-FLAME-1 and its p39 fragment coprecipitated with full-length FADD, Mch4, Mch5β, or their isolated FDH regions (Fig. 3B). Full-length T7-FLAME-1 (but a negligible amount of the p39 fragment) associated with Mch5-CDH (Fig. 3B), suggesting that the entire CDH region of FLAME-1 is required for optimal interaction between these proteins. Similar results were obtained with T7-FLAME-1-D341A and T7-FLAME-1-CDH (Fig. 3C and D). No interactions were observed between T7-FLAME-1β and Flag-Mch4-CDH or Flag-Mch5-CDH (data not shown), suggesting that these proteins can only interact through their respective FDH or CDH regions. The yeast two-hybrid analysis confirmed the interactions of FLAME-1-FDH with FADD, Mch4, and Mch5 FDH regions (Table 1). This analysis also revealed that FLAME-1-FDH can also strongly interact with itself (Table 1).}

**FLAME-1 Is Recruited to the Death Receptor Fas—**FADD can recruit Mch5 (MACH/FLICE) (11, 12) and possibly Mch4 (10) to the Fas/TNFR1 signaling complex. To determine whether FLAME-1 can also be recruited through FADD, coprecipitation experiments were performed in 293T cells (Fig. 3E). FLAME-1 or FLAME-1-D341A was able to form a complex with Fas (lanes 4 and 8), possibly through interaction with endogenous FADD. Cotransfection of exogenous T7-FADD enhanced the FLAME-1-Fas interaction (lanes 5 and 7). The p39 fragment, which is generated by cleavage at Asp-341, also formed a complex with Fas (lane 5). Interestingly, FLAME-1 was also able to prevent recruitment of Mch4 and Mch5 to Fas (not shown). These observations demonstrate that FLAME-1 can be recruited to Fas and may interfere with the assembly of a functional death signaling complex.

**Inhibition of Fas/TNF-induced Apoptosis by FLAME-1—**To study the functional role of FLAME-1 in Fas/TNFR1- or UV-induced apoptosis, it was transfected into MCF-7-FAS cells. FLAME-1 did not induce apoptosis in these cells (Fig. 4). However, FLAME-1 and FLAME-1β significantly blocked Fas- and TNFR1-induced apoptosis but not UV-induced apoptosis (Fig. 4). This indicates that overexpression of the FDH regions of FLAME-1 is sufficient to block Fas/TNFRI-induced apoptosis. This protective effect approached 60–65% of that observed by Bcl-xl overexpression. The isolated CDH region of FLAME-1 did not have any protective effect (not shown).

Taken together, the data presented here establish FLAME-1 as the first example of an endogenous FDH-containing protein that can act as a negative regulator of apoptosis. Recently, we demonstrated that viral FDH-containing proteins E8 and MC159 can abrogate Fas/TNFRI-mediated apoptosis (16). Both FLAME-1 and the viral proteins appear to target the Fas/FADD/caspase signaling complex by a potential dominant negative mechanism. Binding of FLAME-1, its FDH regions, or the viral proteins to the caspases Mch4 or Mch5 or the adaptor molecule FADD blocks Fas/TNFRI-induced apoptosis possibly by interfering with the assembly of a functional death receptor signaling complex. Consequently, it appears that molecules which contain FDH regions could be either proapoptotic like FADD, Mch4, or Mch5 or anti-apoptotic such as FLAME-1 and the viral proteins E8 and MC159. Since the proapoptotic and anti-apoptotic proteins might have different expression levels, their ratios could determine how a given cell or cell type responds to FasL or TNF. For example, the high expression of FLAME-1 in Fas+ immortalized testis and placenta, see Fig. 1B) could be the reason for their resistance to FasL killing.

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![Figure 4](image-url)