dFADD, a Novel Death Domain-containing Adapter Protein for the Drosophila Caspase DREDD*

Received for publication, May 24, 2000, and in revised form, July 24, 2000
Published, JBC Papers in Press, August 8, 2000.
DOI 10.1074/jbc.C00041200

Shimin Hu and Xiaolu Yang†
From the Department of Molecular and Cellular Engineering, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Apoptotic cell death occurs through activation of procaspases, the precursors of a group of aspartate-specific cysteine proteases known as caspases. Procaspase activation is mediated by death adapter proteins such as the mammalian proteins FADD and Apaf-1 and the Caenorhabditis elegans protein CED-4. These adapters bind to procaspases and facilitate oligomerization and subsequent auto-proteolytic processing of thezymogens. Here we report cloning and characterization of dFADD, a FADD homologue in Drosophila. dFADD contains a death domain that is highly homologous to the FADD death domain, and it also shares a novel domain with a Drosophila caspase DREDD, which we call death-inducing domain. dFADD binds to DREDD through the death-inducing domain and enhances the cell death activity and proteolytic processing of DREDD. dFADD and DREDD are stabilized by their interaction. The structural and functional similarities between dFADD and FADD suggest the existence of a FADD-like apoptosis pathway in Drosophila.

Apoptosis is a physiological process of cell auto-destruction that eliminates unwanted or severely damaged cells in multicellular organisms. It plays critical roles in development, maintenance of homeostasis, and host defense (1, 2). Apoptosis is executed by a proteolytic system involving caspases. Caspases are produced as latent precursors (procaspases) and during apoptosis are activated sequentially in cascades through proteolytic processing (3–5). A key step in determining cell life or death is the activation of the apical procaspase (initiator caspase) in such a caspase cascade. This activation is mediated by death adapter proteins including the mammalian apoptotic proteins FADD (6) and Apaf-1 (7) and the Caenorhabditis elegans protein CED-4 (8). An activated apical caspase processes downstream procaspases (effector caspases), which then cleave key cellular substrates to dismantle cells.

An evolutionarily conserved apoptosis pathway is initiated by cell-intrinsic developmental cues or cytotoxic reagents and is found in the nematode C. elegans and mammals (9, 10). Upon apoptosis activation, the death adapter proteins CED-4 and Apaf-1 form homo-oligomers that subsequently aggregate the CED-3 caspase and procaspase-9, respectively (11–13), through homotypic interactions mediated by the caspase recruitment domain (CARD)† (14). Mammals have also evolved an extrinsic or instructive apoptosis pathway, mediated by small death adapters such as FADD, that allows a cell to instruct another to undergo self-destruction (15, 16). This pathway is engaged by a unique group of death receptors in the tumor necrosis factor receptor superfamly (e.g. TNFR1 and Fas) and plays important roles in the regulation of immune responses and the maintenance of homeostasis. Upon binding to their trimeric ligands, these receptors recruit the adapter protein FADD via death domain (DD)-DD interaction (6). FADD then binds to procaspase-8 through another homotypic interaction involving death effector domain (DED), a motif present in the N-terminal region of both proteins (17, 18). Recruitment of procaspase-8 to the ligand-aggregated death receptors leads to its oligomerization and subsequent activation (19–21).

An Apaf-1/CED-4 homologue, Drosophila Apaf-1-related killer (DARK)/homolog of Apaf-1 and CED-4 (HAC)-1/Dapaf-1, was recently identified in the model genetic organism Drosophila (22–24). However, it remains unclear whether a FADD-like pathway also exists in Drosophila. Here we describe the identification and characterization of a Drosophila FADD homologue, dFADD, and present evidence that dFADD is an adapter that activates the Drosophila apical caspase DREDD.

MATERIALS AND METHODS

Cell Lines, Expression Vectors, and Reagents—Human embryonic kidney 293 cells, human cervical carcinoma HeLa cells, and murine fibroblast 3T3 cells were cultured in complete Dulbecco’s modified Eagle’s medium, and human breast carcinoma MCF7 cells were maintained in RPMI 1640. Expression constructs were based on pRK5 (a gift from Dr. D. Goeddel), pcDNA3 (Invitrogen), pEGFP-C1 (CLONTECH; for Aequorea victoria green fluorescence protein (GFP) fusion constructs), and pcSpeR-hs (a gift of Dr. M. Fortini; for SL2 cell expression constructs). FLAG and HA-tags were placed at the N termini of the fusion constructs. Anti-FLAG and anti-HA polyclonal antibodies (Santa Cruz) were purchased from the indicated sources.

Cloning of dFADD—A Drosophila expressed sequence tag clone (GenBank™ accession number A1294992) was found to contain an open reading frame with significant sequence homology to mammalian death domain-containing proteins. The sequence of the open reading frame was confirmed on both strands with an automated sequencer (Applied Biosystems).

Northern Blotting—A Northern blot membrane containing mRNA from different developmental stages of Drosophila embryos was hybridized with a 32P-labeled cDNA probe corresponding to the DNA sequence of the predomain (residue 1–141) of dFADD or a probe specific for ribosomal RNA RP49 as a leading control.

Transfection, Coimmunoprecipitation, and Western Analysis—Transfection was confirmed on both strands with an automated sequencer (Applied Biosystems).

The abbreviations used are: CARD, caspase recruitment domain; CED, cell death abnormal; DED, death effector domain; DD, death-inducing domain; DD, death domain; DREDD, death-related CED-4/Nedd-2-like caspase; FADD, Fas-associated death domain protein; GFP, Aequorea victoria green fluorescence protein; HA, the hemagglutinin epitope tag; mAb, monoclonal antibody.

* This work was supported by an academic development fund from the University of Pennsylvania (to X. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: 421 Curie Blvd., 610 BRBII/III, University of Pennsylvania School of Medicine, Philadelphia, PA 19104. Tel.: 215-573-6739; Fax: 215-573-8606; E-mail: xyang@mail.med.upenn.edu.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available on line at http://www.jbc.org
h after transfection, cell lysates were prepared and immunoprecipitated with anti-FLAG mAb M2 beads. The precipitates were resolved by SDS polyacrylamide gel electrophoresis and analyzed by immunoblotting with polyclonal anti-FLAG and anti-HA antibodies.

Cell Death Assay—HeLa, 3T3, and MCF7 cells were transiently transfected with each of the test plasmids plus the reporter plasmid pCMV-lacZ (1 and 0.2 μg, respectively, unless indicated otherwise). 22–24 h after transfection, cells were fixed in 0.5% glutaraldehyde and stained with X-Gal. The percentage of apoptotic cells was determined by the number of membrane-blebbing cells divided by the total number of blue cells. Data presented were obtained from representative experiments performed in duplicates, and the mean and standard deviation were calculated.

Death Domain-containing Adapter for DREDD

**RESULTS AND DISCUSSION**

Identification of a Drosophila Death Domain-containing Protein, dFADD—In a search for Drosophila death domain-containing proteins in the expressed sequence tag data base, we identified a full-length cDNA predicted to encode a protein with a death domain at its C terminus (Fig. 1). This protein has an overall structure similar to that of FADD, and its death domain is highly homologous to the FADD death domain (28% identity).
and 49% similarity) (Fig. 1B). We hence designated this first *Drosophila* death domain-containing protein dFADD.

No homology was found between the N-terminal region of dFADD and any other proteins in the database using the BLAST and SMART programs. However, a careful comparison of the dFADD sequence with the sequence of DREDD, a *Drosophila* apical caspase, revealed significant homology between the dFADD N-terminal region and a region in the DREDD prodomain (19% identity and 39% similarity) (Fig. 1C). Although DREDD was thought to contain two DED domains (26), we found no homology between DREDD prodomain and any DED-containing proteins using BLAST or SMART. The conserved domain in dFADD and DREDD is distinct from DED and CARD (two domains involved in death adapter-procaspase interaction), but its function is similar to those of DED and CARD (see below). We therefore named this novel domain DIDs (death-inducing domain). Analysis of the dFADD genomic sequence revealed that it contains a single exon on chromosome 3.

Expression of dFADD mRNA—To examine the mRNA expression of dFADD, we hybridized mRNAs sampled from different stages of *Drosophila* embryonic development with a dFADD cDNA probe. Only the 3- to 12-h embryos contained a different stages of expression of dFADD, we hybridized mRNAs sampled from different stages of embryonic development with a dFADD cDNA probe. Only the 3- to 12-h embryos contained a single 1.7-kilobase dFADD transcript (Fig. 1D). Thus, the expression of dFADD is tightly regulated during *Drosophila* development.

**dFADD Specifically Associates with DREDD**—Because death adapter proteins associate with caspases through homotypic DED-DED or CARD-CARD interaction, we investigated whether dFADD interacted with DREDD through their homologous DIDs. Co-immunoprecipitation assays confirmed that dFADD specifically associated with DREDD but not with DRONC, a CARD domain-containing *Drosophila* apical caspase (27) (Fig. 2B). The specificity of the dFADD-DREDD interaction was further underlined by the observation that dFADD did not interact with various mammalian caspases except for its weak interaction with caspase-10 (Fig. 2C). The interaction domains in dFADD and DREDD were mapped using a panel of deletion mutants (Fig. 2A). The N-terminal region of dFADD containing the DID domain was both necessary and sufficient for the interaction with DREDD (Fig. 2D). DfADD interacted strongly with the DREDD prodomain but not with DRONC nor DRAID (Fig. 2E). The specificity of the dFADD-DREDD interaction was further confirmed by the observation that DREDD interacted strongly with dFADD but weakly with DRAID. Transfection and co-immunoprecipitation assays were performed as described in Fig. 2B.

**dFADD Increases DREDD Killing**—Because DREDD is a proapoptotic protein and acts upstream of caspase-10 (28), we investigated whether dFADD increased DREDD killing. As shown in Fig. 3, dFADD increased DREDD killing in HeLa cells but not in DRONC or DRONC-DO (Fig. 3B). In HeLa cells, 0.1–0.2 μg of DREDD increased apoptosis by 40% (lane 2, Fig. 3B), whereas 0.2 μg of DRONC plus 1 μg of vector, dFADD, or DREDD-DD increased apoptosis by 10%, 15%, and 20%, respectively (Fig. 3A). The specificity of the dFADD-DREDD interaction was further confirmed by the observation that DREDD did not interact with various mammalian caspases except for its weak interaction with caspase-10 (Fig. 2C). The specificity of the dFADD-DREDD interaction was further confirmed by the observation that DREDD interacted strongly with dFADD but weakly with DRAID. Transfection and co-immunoprecipitation assays were performed as described in Fig. 2B.

**FIG. 3. dFADD enhances DREDD but not DRONC killing.** HeLa (A), 3T3 (B), or MCF7 (C) cells were transfected with indicated amounts of DREDD (A) or 0.2 μg of vector, DREDD, or DRONC (B and C) together with vector, dFADD, DREDD-N, or DREDD-DD (1 μg each). D. HeLa cells were transfected with indicated amount of DRONC (in μg) or 0.2 μg of DRONC plus 1 μg of vector, dFADD, DREDD-DD, or DREDD-N. Each transfection also included 0.1–0.2 μg of pCMV-lacZ to label transfected cells. Apoptosis was determined as described under “Materials and Methods.”

**FIG. 4. Enhancement of DREDD processing by dFADD and comparison of dFADD-DREDD and DARK-DREDD interactions.** A. dFADD enhances DREDD processing. 293 cells were transfected with 3 μg of DREDD or DRONC and 5 μg of DREDD together with p35 and CrmA. Cell extracts were immunoprecipitated with anti-FLAG mAb M2 antibody and analyzed by immunoblotting with polyclonal anti-FLAG antibody. B. self-association of DREDD. 293 cells were transfected with indicated plasmids, and co-immunoprecipitation assay was performed as in A. DREDD-C/S, the DREDD active site cysteine to serine mutation. Asterisks, nonspecific bands serving as protein-loading control. C and D. DREDD interacts strongly with dFADD but weakly with DARK. Transfection and co-immunoprecipitation assays were performed as described in Fig. 2B.

**FIG. 5. Localization and mutual stabilization of dFADD and DREDD in mammalian cells.** A. cellular localization of GFP-dFADD and GFP-DREDD. HeLa cells were transfected with 1 μg of indicated plasmids and examined under a fluorescence microscope 20–24 h after transfection. B and C, mutual stabilization of dFADD and DREDD. In B, 293 cells were transfected with the indicated amount of HA-dFADD plasmid and 1 μg of vector, DREDD, or DRONC. In C, transfection was done with 1 μg of HA-dFADD and 1 μg of vector (lane 1), DREDD-casp (lane 2), or DREDD-pro (lane 3). Cell extracts were made 22 h after transfection and fractionated into supernatant (S/N) and pellet fractions. 2% of each fraction was analyzed by SDS polyacrylamide gel electrophoresis and immunoblotting with anti-HA antibody. Asterisks, nonspecific bands serving as protein-loading control.
weakly with the caspase domain (Fig. 2E). Further mutagenesis analysis of the DREDD prodomain region revealed that the DID domain was required for the interaction with dFADD (Fig. 2F). Therefore, the DID domains mediate the interaction between DREDD and dFADD.

**dFADD Enhances DREDD-induced Apoptosis**—To examine the effect of dFADD on DREDD-induced apoptosis, we transfected Drosophila SL2 cells with dFADD and DREDD expression plasmids either alone or together. No apoptosis was observed (data not shown), consistent with previous results that ectopic expression of DREDD alone in SL2 cells did not lead to activation of the caspase (26). However, expression of DREDD in several mammalian cell lines caused significant apoptosis (Fig. 3, A, B, and C). We examined the effect of dFADD on DREDD-mediated apoptosis in these cells and found that although dFADD did not induce apoptosis, it potently enhanced DREDD-mediated apoptosis in a dose-dependent manner (Fig. 3, A, B, and C). The dFADD was both necessary and sufficient for this enhancement. dFADD did not enhance DRONC-induced apoptosis, consistent with the lack of interaction between these two proteins (Fig. 3D).

**dFADD Promotes DREDD Processing**—Initiator caspases are activated by adapter-mediated oligomerization and subsequent auto-processing. Because dFADD associated with DREDD and enhanced DREDD-induced apoptosis, we investigated whether dFADD promoted DREDD processing. Overexpression of DREDD in human 293 cells led to zymogen processing (Fig. 4A). DREDD associated with itself at high expression levels either directly or indirectly via an endogenous adapter protein (Fig. 4B), and such self-association could facilitate its auto-cleavage. The DREDD processing was not inhibited by p35 and crmA, two active site-specific caspase inhibitors that can inhibit most caspases (Fig. 4C and data not shown), and DREDD may possess unique substrate specificity. In the presence of dFADD, DREDD processing went to completion (Fig. 4A), whereas DRONC was not processed upon overexpression either in the presence or absence of dFADD (Fig. 4A). It appears that the enhancement of DREDD processing by dFADD is mediated by direct protein-protein interaction.

**Comparison of dFADD-DREDD and DARK-DREDD Interactions**—DREDD was previously reported to associate with the Drosophila Apaf-1 homologue DARK (23). Other Drosophila caspases such as DRONC were also reported to be targets for DARK (22, 24). We compared the dFADD-DREDD and DARK-DREDD interactions using co-immunoprecipitation assay and found that the former was much stronger than the latter. Unlike the dFADD-DREDD interaction that was mainly mediated by the DREDD prodomain (Fig. 2E), both the DREDD prodomain and the caspase domain interacted weakly with DARK (Fig. 4D). The strong dFADD-DREDD interaction suggests that DREDD may mainly serve as an apical caspase for dFADD.

**Cellular Localization of dFADD and Stabilization of DfADD Protein by DREDD**—To examine the cellular localization of dFADD and DREDD, we fused them to GFP. When expressed in HeLa cells, both GFP-dFADD and GFP-DREDD proteins were localized in the cytoplasm (Fig. 5A, a and c). This localization of dFADD was mediated by its N-terminal region (Fig. 5A, e and f). dFADD and DREDD appeared to stabilize each other, because expression of DREDD significantly increased fluorescence intensity in the GFP-dFADD-transfected cells (Fig. 5A, b versus a), and similarly, expression of dFADD enhanced fluorescence intensity in the GFP-DREDD-transfected cells (Fig. 5A, d versus c). We also compared the expression levels of dFADD in 293 cells in the presence and absence of DREDD. Co-transfection of dFADD with DREDD led to a higher level of dFADD in both the soluble and insoluble fractions of the cell extracts compared with transfection of FADD alone (Fig. 5B). In contrast, DRONC did not enhance dFADD protein expression (Fig. 5B). The stabilization of dFADD by DREDD was mediated by the DREDD prodomain (Fig. 5C and data not shown). Reciprocally, dFADD stabilized DRONC but not DRONC in 293 cells (data not shown). The mutual stabilization of dFADD and DREDD suggests that the expression of these two proteins may be co-regulated in vivo.

In summary, we describe here the molecular cloning and partial characterization of the first Drosophila death domain-containing protein dFADD. dFADD contains a bipartite structure highly homologous to that of the mammalian adapter FADD. It functions similarly to FADD and physically interacts with and activates the Drosophila caspase DREDD. The interaction between dFADD and DREDD stabilizes both proteins. We conclude that Drosophila also contains a FADD-like apoptotic pathway. To date, no death receptors have been identified in Drosophila, and despite the high homology between the death domains of FADD and dFADD, we did not detect interaction between dFADD and any of the mammalian death receptors (data not shown). Identification of components upstream of dFADD should help determine the regulation and function of this evolutionarily conserved apoptosis pathway.

**Acknowledgments**—We thank Lara Gardner for technical assistance, Judith Leatherman and Dr. Thomas Jongens for providing Drosophila Northern blots, and Drs. J. Abram, S. Kumar, H. Steller, and E. Alnemri for reagents. We also thank Yihong Ye and Drs. Mark Fortini and Morris Birnbaum for advice.

**REFERENCES**

1. Steller, H. (1998) Science 267, 1445–1449
2. Jacobsen, M. D., Weil, M., and Raff, M. C. (1997) Cell 88, 335–345
3. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
4. Cryns, V., and Yuan, J. (1998) Genes Dev. 12, 1551–1570
5. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
6. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
7. Zhou, H., Henzel, W. I., Liu, X., Lusch, A., and Wang, X. (1997) Cell 90, 405–413
8. Metzstein, M. M., Stanfield, G. M., and Horvitz, H. R. (1998) Trends Genet. 14, 410–416
9. Horvitz, H. R., Shaham, S., and Hengartner, M. O. (1994) Cold Spring Harbor Symp. Quant. Biol. 59, 377–385
10. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
11. Yang, X., Chang, H. Y., and Baltimore, D. (1998) Science 281, 1355–1357
12. Srinivasula, S. M., Ahmad, M., Fernandez-Alnemri, T., and Alnemri, E. S. (1998) Mol. Cell 1, 949–957
13. Zhou, H., Li, Y., Liu, X., and Wang, X. (1999) J. Biol. Chem. 274, 11549–11556
14. Hofmann, K., Bucher, P., and Tschopp, J. (1997) Trends Biochem. Sci. 22, 155–156
15. Nagata, S. (1997) Cell 88, 355–365
16. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
17. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Brezeta, J. D., Zhang, M., Genta, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
18. Boldin, M. P., Goncharov, T. M., Golstev, Y. V., and Wallach, D. (1996) Cell 85, 803–815
19. Yang, X., Chang, H. Y., and Baltimore, D. (1996) Mol. Cell 1, 319–325
20. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) J. Biol. Chem. 273, 2926–2930
21. Martin, D. A., Siegel, R. M., Zheng, L., and Lenardo, M. J. (1998) J. Biol. Chem. 273, 4345–4349
22. Zhou, L., Song, Z., Tittel, J., and Steller, H. (1999) Mol. Cell 4, 745–755
23. Rodriguez, A., Oliver, H., Zhou, H., Chen, P., Wang, X., and Abrams, J. M. (1999) Nat. Cell Biol. 1, 272–279
24. Kanuka, H., Sawamoto, K., Isohara, N., Matsuno, K., Okano, H., and Miura, M. (1999) Mol. Cell 4, 757–769
25. Hu, S., Vinzenz, C., Ni, J., Genta, R., and Dixit, V. M. (1997) J. Biol. Chem. 272, 17255–17257
26. Chen, P., Rodriguez, A., Erskine, R., Thach, T., and Abrams, J. M. (1998) Dev. Biol. 210, 202–216
27. Dorsett, L., Colussi, P. A., Quinn, L. M., Richardson, H., and Kumar, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4307–4312
28. Stanger, B. Z., Leder, O., Lee, T.-O., Kim, E., and Seed, B. (1995) Cell 81, 513–523
29. Dueval, H., and Dixit, V. M. (1997) Nature 385, 86–89
30. durfee, T., Mancini, M. A., Jones, D., Eldledge, S. J., and Lee, W. H. (1994) J. Cell Biol. 127, 609–622
31. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495–504