The pivotal discovery that Fas-associated death domain protein (FADD) interleukin-1β-converting enzyme (FLICE)/MACH was recruited to the CD95 signaling complex by virtue of its ability to bind the adapter molecule FADD established that this protease has a role in initiating the death pathway (Boldin, M. P., Gontcharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) Cell 85, 803–815; Muzio, M., Chinnaiyan, A. M., Kischkel, K. C., O’Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827). In this report, we describe the cloning and characterization of a new member of the caspase family, a homologue of FLICE/MACH, and Mch4. Since the overall architecture and function of this molecule is similar to that of FLICE, it has been designated FLICE2. Importantly, the carboxyl-terminal half of the small catalytic subunit that includes amino acids predicted to be involved in substrate binding is distinct. We show that the pro-domain of FLICE2 encodes a functional death effector domain that binds to the corresponding domain in the adapter molecule FADD. Consistent with this finding, FLICE2 is recruited to both the CD95 and p55 tumor necrosis factor receptor signaling complexes in a FADD-dependent manner. A functional role for FLICE2 is suggested by the finding that an active site mutant of FLICE2 inhibits CD95 and tumor necrosis factor receptor-mediated apoptosis. FLICE2 is therefore involved in CD95 and p55 signal transduction.

The conserved mechanisms of programmed cell death that play a fundamentally important role in tissue homeostasis, embryogenesis, and cellular defense mechanisms have only recently been subject to molecular analysis (1). Studies in Caenorhabditis elegans were important in providing a molecular framework for the cell death pathway. In particular, the discovery that the C. elegans death gene, Ced-3, possessed substantial homology to the mammalian interleukin-1β-converting enzyme (ICE)(2) was a major step forward (2). ICE is an unusual cysteine protease that processes pro-interleukin-1β to the mature cytokine by cleaving after Asp residues. Subsequently, several mammalian Ced-3 homologues have been characterized that are unable to process pro-interleukin-1β but cleave poly(ADP-ribose) polymerase (PARP), a protein known to be proteolytically processed early in apoptosis. This and other evidence suggested that these related family members played a more prominent role in apoptosis. Recently, this family of cysteine, aspartate-specific proteases has been named the caspase family to denote cysteine, aspartate-specific proteases (3). Depending upon susceptibility to tetrapeptide inhibitors, this family of proteases can be divided into CPP32-like (DEVDD-inhibitable) and ICE-like (YVAD-inhibitable) enzymes (4). Evidence is accumulating for the existence of a cascade of caspases that can potentially activate each other, thereby amplifying the death signal leading to precipitous cleavage of death substrates and rapid demise of the cell (5, 6). The caspases are activated by a number of physiological and pharmacological stimuli that induce apoptosis (7).

A number of intriguing questions remain, however, including the identity of the caspases that initiate the cascade, the mechanism of activation, and the exact sequence of events leading to activation of downstream effector caspases. The identification of FLICE/MACH as a receptor-associated caspase like protease suggested a surprisingly direct mechanism for engagement of the death pathway by the death receptors CD95 and the p55 tumor necrosis factor 1 (8, 9). Upon activation, both these receptors use their death domains to bind the corresponding domain of adapter molecules. Therefore, the death domain appears to represent a protein-protein interaction motif. The death domain containing adapter molecule FADD plays a central role as a conduit for death signals from both the CD95 and p55 receptors (10). Dominant negative versions of FADD that lack the amino-terminal segment (yet retain the death domain) effectively attenuate both CD95- and p55-induced apoptosis (11, 12). Since the amino-terminal domain of FADD appeared necessary to engage the downstream components of the death pathway, it was dubbed the death effector domain (DED) (10). The importance of this domain was underlined by the discovery that a caspase (FLICE/MACH) possessed sequences homologous to the DED within its pro-domain. Biochemical and mutagenesis studies revealed that the DED of FADD, by virtue of its ability to bind to the corresponding sequence motif in the pro-domain, recruited FLICE/MACH to the receptor signaling complex (8). These studies for the first time suggested a homophilic binding mechanism involving DEDs that allowed the death receptors to physically engage the caspases through the adapter molecule FADD.

As part of our continuing effort to characterize additional members of the caspase family, we have identified a new member, designated FLICE2, that is a close structural homologue of FLICE and very similar to Mch4 with certain important exceptions. We demonstrate for the first time that FLICE2 is a Ced-3
homologue capable of interacting with both p55 and CD95 receptors through the adapter molecule FADD. Further, a catalytically inactive form of FLICE2 inhibited both p55- and CD95-induced apoptosis, suggesting that FLICE2 can be recruited to the receptor signaling complex and participate in the propagation of the death signal.

MATERIALS AND METHODS

Oligonucleotides—The following oligonucleotides were used: T96R, GAAAGATGACACGAGTACGG; pCDMS 5’F2, AATGCTGTAACACTCTGCCC; FL2R, CTTTAAAGCTCTATGACTCGAGGT; pCDMS 3’, CACACACGAGAATACTGCTCTT; FL2#, AACAACAGA CTAAGTGTTGACTCTT; T96stop, AAGGCTCTCGAAAAGAATGAAGCTG.

Antibodies—A FLICE2 peptide, ISAQTPPRMMWRWS, that corresponds to amino acids 505–518 of the small catalytic subunit was used to immunize rabbits and obtain polyclonal antisera.

Cloning of Human FLICE2—An EST clone (GenBank accession no. T96912) was identified as a new caspase family member. This clone contained a 1.5-kilobase insert encoding sequences corresponding to the carboxy-terminal segment of caspases, stop codon, 3’-untranslated, and poly(A)-tail. Full-length sequence was obtained by two rounds of PCR using gene- and vector-specific primers. Initially, cDNA from a human MCF7 library was used as template. T96R as the gene-specific reverse primer, and pCDMS 5’F2 as the vector-specific forward primer. A second gene-specific reverse primer, FL2R, was designed, and an additional upstream sequence was obtained from a melanoma library employing FL2R as the gene-specific primer and pCDMS 3’ as the vector-specific primer. Finally, a full-length clone was obtained by PCR with the two gene-specific primers FL2# and T96stop using a thermostable proofreading polymerase (Clontech). The resulting PCR product was subcloned, sequenced, and used as template for other constructions.

Expression Vectors—All eukaryotic expression vectors were constructed in pcDNA3 (Invitrogen) by standard PCR techniques using custom-designed primers encoding epitope tags and appropriate restriction sites. FLICE2 harboring a His6 tag at its carboxy terminus was constructed in the pET23b vector (Novagen).

Northern Blot Analysis—Human multiple tissue and human cancer cell line poly(A) RNA blots were obtained from Clontech. 293, 293EBNA, Jurkat, U937, MCF7, and THP1 RNA were purified using the RNAsky kit (Qiagen) following the manufacturer’s instructions and analyzed by Northern blotting as described previously (13). The 32P-labeled FLICE2 probe encoded amino acids 229–265.

Granyme B Activation and PARP Cleavage—His6-tagged FLICE2 was generated by coupled in vitro transcription/translation using the TNT kit (Promega). The translated protein was purified as described previously (14). In vitro activation of purified FLICE2 by granyme B (gift from C. J. Froelich, Northwestern University Medical School) was performed essentially as described previously (14). Briefly, 0.3 pmol of granyme B were used to cleave 50 nmol of FLICE2. The tetrapeptide aldehyde inhibitors, YVAD-CHO and DEVD-CHO (Bachem), were performed essentially as described previously (14). Briefly, 0.3 pmol of granyme B were used to cleave 50 nmol of FLICE2. The tetrapeptide aldehyde inhibitors, YVAD-CHO and DEVD-CHO (Bachem), were added at a final concentration of 1 μmol following incubation with granyme B.

In Vitro Binding—[35S]Methionine-radiolabeled proteins obtained by coupled in vitro transcription/translation were incubated with bacterially expressed His6-tagged proteins immobilized onto Ni-NTA agarose beads. Binding reactions were performed as described previously (8). Full-length sequence was obtained by two rounds of PCR extension employing vector- and gene-specific primers. The derived open reading frame encoded a protein of 521 amino acids with a molecular mass of 59 kDa. Because of its significant homology over its entire sequence (28% identity) to FLICE/MACH (8, 9), it was designated FLICE2. Recently, however, a third FLICE homologue, Mch4, has been described (18).

Comparison of the crystal structure of CPP32 and the sequence of FLICE2 reveals complete conservation of substrate contacts for the P1 aspartate residue (19). Surprisingly, the amino acids that contact the P4 residue in the CPP32 crystal structure and the homologous residues in FLICE2 are divergent. Of the seven amino acids contacting the P4 residue, only one is conserved (Trp-457). Three are conserved substitutions (Phe-449, Glu-454, Trp-491). The remaining two contact the P1 aspartate residue (19). Surprisingly, the amino acids that contact the P4 residue in the CPP32 crystal structure and the homologous residues in FLICE2 are divergent. Of the seven amino acids contacting the P4 residue, only one is conserved (Trp-457). Three are conserved substitutions (Phe-449, Glu-454, Trp-491). The remaining two contact the P1 aspartate residue (19).
FIG. 1. A, alignment of the deduced amino acid sequence of FLICE2 with Mch4 and FLICE. Differences between FLICE2 and Mch4 are highlighted in blue. The open reading frame corresponding to EST T96912 is indicated by a black line. Amino acids boxed in red identify death effector domain residues in FADD that are conserved with the two DEDs in FLICE and FLICE2. The conserved pentapeptide QACQG is boxed in...
Fig. 2. FLICE2 binds through FADD to the death receptors CD95 and p55. A, AU1FADD or AU1FADD constructs were cotransfected with the indicated FLICE2 expression vectors into 293 cells. Cell lysates were immunoprecipitated with anti-AU1 antibodies and analyzed by immunoblotting with an antibody specific for the small catalytic subunit of FLICE2. B, the carboxyl-terminal FLAG-tagged pro-domain of FLICE2 was cotransfected with the indicated FADD constructs. FLAG-tagged MCH2, an unrelated caspase, served as a negative control. C, recombinant His6 FADD and His6 FADD-DN were immobilized onto NiNTA beads and incubated with the indicated in vitro translated 35S-labeled FLICE proteins. Bound proteins were analyzed by autoradiography and quantified by phosphorimager analysis. D and E, 293 cells were cotransfected with the indicated expression constructs. Cell lysates were prepared after 40 h, followed by immunoprecipitation with FLAG antibodies and immunoblotting as in panel A.

(21). Granzyme B, an aspartate-specific protease from cytotoxic T cells granules, is capable of activating CPP32-like caspase zymogens in vitro (8, 14, 18, 17, 22, 23). His6-tagged FLICE2 was obtained by coupled in vitro transcription/translation, purified, and activated by granzyme B. Residual granzyme B activity was neutralized by addition of anti-GraB, a specific inhibitor of granzyme B (14). FLICE2 enzymatic activity was assessed by the addition of the substrate PARP. Immunoblot analysis revealed FLICE2 to be a competent Ced-3-like protease that was capable of cleaving PARP to its signature 85-kDa apoptotic form (Fig. 1B). Additionally, PARP cleavage was inhibited by the tetrapeptide inhibitor DEVD-CHO, but not by YVAD-CHO, consistent with FLICE2 being a CPP32-like but not ICE-like protease.

FLICE2 Expression—Human tissue and cell line RNA blots were probed with a 35P-labeled cDNA specific for FLICE2 and not contained within Mch4 (Fig. 1C). A transcript of 4.4 kilobases was detected and is consistent with the size of the cloned cDNA. The tissue distribution was strikingly similar to that of FLICE (8) In particular, tissues enriched in lymphoid cells expressed a substantial amount of FLICE2 transcript. Embryonic expression was high in all tissues with the exception of the brain. A variety of transformed cell lines expressed low levels of FLICE2. K562, a chronic myelogenous leukemia line, displayed significant expression. Importantly, the cell lines used for transfections in this study including 293, 293EBNA, and MCF7 did not express detectable levels of endogenous FLICE2 transcript.

While the mRNA expression patterns are consistent with FLICE-related proteins being involved in the maturation of the lymphoid system, additional functions are likely as suggested by the high level of expression of MCH4 and FLICE in the heart (9, 18). FADD and FLICE2 mRNA expression patterns are not identical, suggesting that situations may exist where the two function independently of each other.

FLICE2 Binds the Death Adapter Molecule FADD—Death effector domains have been shown to be the protein interaction motifs that mediate the binding of FLICE to FADD (8). FLICE2, like FLICE, contains two DEDs, with the first being more conserved. To establish the in vivo function of the DEDs, FLICE2/FADD binding experiments were undertaken (Fig. 2A). Co-immunoprecipitation analysis clearly revealed the ability of FLICE2 to specifically bind full-length FADD but not FADD, which lacks a functional DED due to truncation of the first 18 amino-terminal amino acids. Conversely, FLICE2 lacking the DEDs (encoding only the catalytic subunits) did not coprecipitate with FADD. Indeed, the DED containing pro-domain of FLICE2 by itself was fully capable of binding FADD (Fig. 2B). This interaction was specific since the pro-domain of FLICE2 did not bind to FADD with a disrupted DED (ΔFADD). The unrelated caspase Mch2 served as a negative control. Notably, the catalytically inactive cysteine mutant C401S FLICE2 retained its ability to bind FADD, suggesting potential for use as a dominant negative inhibitor.

Fig. 2C shows the result of analogous binding experiments performed in vitro using purified recombinant FADD and FADD-DN that lacks the DED. Again, FLICE2 specifically bound the DED of FADD through its pro-domain. This interaction as assessed by binding of input radiolabeled protein was equivalent for both FLICE and FLICE2. The ability to reconstitute FLICE2-FADD binding in vitro using purified molecules suggested that the interaction was direct and not mediated by an intermediary molecule.

FLICE2 Is Recruited to the Death Receptors CD95 and p55—

green. An asterisk indicates the cleavage site between the large and small subunits of the catalytic domain. Based on the CPP32 crystal structure, the symbols above the alignment indicate residues involved in contacting the substrate. The corresponding amino acids in CPP32 are also indicated above the symbols. +, active site cysteine; ○, contacts with P1 residue; ▲, second tier hydrogen bonds with P1; ×, contacts with P4 where the intensity of the violet shading indicates the level of sequence divergence from CPP32. B, PARP cleavage of Granzyme B-processed FLICE2. In vitro translated His6 FLICE2 was purified and activated with granzyme B as described under “Materials and Methods.” PARP cleavage was performed in the absence or presence of the indicated tetrapeptide aldehyde inhibitors (1 μM). C, multiple human tissue and cell line mRNA blots were probed with a 32P-labeled probe specific for FLICE2.
The FLICE2-FADD interaction raised the possibility that FLICE2, like FLICE, could be recruited to the CD95 or p55 signaling complexes in a FADD-dependent manner. To directly assess if FLICE2 could be recruited to the CD95 or tumor necrosis factor receptors, FLICE2 was cotransfected with FLAG-tagged p55 or CD95 receptors (Fig. 2, panels D and E). As shown, FLICE2 bound both death receptors, and a substantial increase in binding was observed when FADD was included in the transfections (Fig. 2, panels D and E, lanes 1 and 2). This was consistent with initial binding being mediated by endogenous FADD and being enhanced by the expression of exogenous FADD. Confirming this was the finding that expression of FADD-DN, which lacks a DED and is therefore unable to bind FLICE or FLICE2, attenuated the association of FLICE2 with the death receptors (Fig. 2, panels D and E, lane 3).

Overexpression of FLICE2 Induces Apoptosis—The homology of FLICE2 with other members of the caspase family suggests that it is a protease involved in apoptosis. MCF7 or 293EBNA cells were transiently transfected with FLICE2, and recipient cells underwent morphological changes including nuclear condensation, cellular shrinkage, and membrane blebbing, all of which are hallmarks of apoptosis (Fig. 3A). The induction of apoptosis could be efficiently blocked in both cell lines by the well-characterized viral inhibitors of caspases, CrmA and p35 (20, 24–26). Importantly, the active site cysteine mutant (C401S FLICE2) inhibited killing by native FLICE2 in 293EBNA cells. This inhibition was probably due to the formation of inactive heterodimers composed of wild type and catalytically inactive molecules as suggested by the crystal structures of ICE and CPP32 (19, 27, 28).

Inhibition of CD95 and p55-induced Cell Death by the FLICE2 Active Site Mutant—293EBNA cells underwent apoptosis when transiently transfected with CD95 receptor (Fig. 3B). This autoactivation on overexpression occurred in a dose-dependent manner (lanes 1 and 3) and has been reported previously (9). Cotransfection with the active site FLICE2 cysteine mutant effectively inhibited the induction of apoptosis to the same extent as CrmA or p35 (lanes 6 and 7). Similarly, transfected cells overexpressing the p55 receptor underwent an apoptotic demise by 24 h. Again, expression of the FLICE2 active site mutant inhibited apoptosis to the same extent as p35, CrmA, and the active site mutant of FLICE (C360S FLICE). Taken together, these results are in keeping with the involvement of FLICE2 in the death pathway engaged by both CD95 and p55 (Fig. 3C). Additionally, these results are consistent with FLICE2 operating at the apex of the caspase cascade.

FLICE, the first caspase shown to be associated with p55 receptors, has similar properties. This is predictable given the conservation of functional domains between the two molecules. Both have functional death effector domains in their pro-sequences that can bind FADD, and the signature sequence surrounding the catalytic cysteine is QACQG and not QACRG as it is in the other mammalian caspases. The amino acids that are predicted to contact the P4 site, however, diverge significantly (Fig. 1A). Therefore, FLICE and FLICE2 probably have different substrate specificities. An attractive hypothesis is the notion that receptor oligomerization activates the caspase cascade by approximating the two FLICEs such that they act as substrates for each other. FLICE activation involves two aspartate specific cleavages: Asp-374 between the large and small catalytic subunits and Asp-216 between the pro-domain and large catalytic subunit. The P4 amino acids are Ile and Arg, respectively. A positively charged residue in position 4 is intriguing, given the glutamate substitution (Glu-453) in the P4 binding pocket of FLICE2 and suggests that FLICE2 may be capable of cleaving the pro-domain of FLICE.

In summary, we have shown that FLICE2 is a signaling caspase able to interact with the death receptors p55 and CD95 through the adapter molecule FADD. A dominant negative version of FLICE2 effectively inhibited apoptosis, establishing a role for this molecule in signaling from the death receptors. Future studies will investigate the possibility of whether FLICE/FLICE2 transactivation is responsible for initiating the caspase cascade.

Acknowledgments—We thank Chris Froelich for the granzyme B, Arul Chinnaiyan, Marta Muzio, Kim Orth, and Karen O’Reourke for
reagents and protocols, and Ian M. Jones for expertise in making the figures.

Note added in proof—Caspase-10/b is the name assigned to FLICE2 according to the reorganized ICE/Ced-3 protease nomenclature.

REFERENCES

1. Fraser, A., and Evan, G. (1996) Cell 85, 781–784
2. Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993) Cell 75, 641–652
3. Alnemri, E. A., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) Cell 87, 171
4. Enari, M., Talanian, R., Weng, W., and Nagata, S. (1996) Nature 380, 723–726
5. Orth, K., O'Rourke, K., Salvesen, G. S., and Dixit, V. M. (1996) J. Biol. Chem. 271, 20977–20980
6. Liu, X., Kim, C. N., Pohl, J., and Wang, X. (1996) J. Biol. Chem. 271, 803–815
7. Martin, S. J., and Green, D. R. (1995) Cell 82, 349–352
8. Muzio, M., Chinnaiyan, A. M., Kischkel, K. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Genta, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
9. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) Cell 85, 803–815
10. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
11. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Heilbrunn, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4961–4965
12. Hsu, H., Shu, H.-B., Pan, M.-P., and Goeddel, D. V. (1996) Cell 84, 299–308
13. Sarma, V., Wolf, F. W., Marks, R. M., Shows, T. B., and Dixit, V. M. (1992) J. Immunol. 149, 3302–3312
14. Quan, L. T., Tewari, M., O'Rourke, K., Dixit, V. M., Snipas, S. J., Poirier, G. G., Ray, C., Pickup, D. J., and Salvesen, G. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1972–1976
15. O'Rourke, K. M., Laherty, C. D., and Dixit, V. M. (1992) J. Biol. Chem. 267, 24921–24924
16. Vincenz, C., and Dixit, V. M. (1996) J. Biol. Chem. 271, 20029–20034
17. Duan, H., Orth, K., Chinnaiyan, A., Poirier, G., Froelich, C. J., He, W.-W., and Dixit, V. M. (1996) J. Biol. Chem. 271, 16720–16724
18. Fernandes-Alnemri, T., Armstrong, R. C., Krebs, J., Srivivasula, S. M., Wang, L., Bullrich, P., Fritz, L. C., Trapani, J. A., Tomaselli, K. J., Litwick, G., and Alnemri, E. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7464–7469
19. Rotonda, J., Nicholson, D. W., Fazil, K. M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E. P., Rasper, D. M., Ruel, R., Vaillancourt, J. P., Thornberry, N. A., and Becker, J. W. (1996) Nat. Struct. Biol. 3, 619–625
20. Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. (1997) J. Biol. Chem. in press
21. Nicholson, D. W. (1996) Nat. Biotechnol. 14, 297–301
22. Darmon, A. J., Nicholson, D. W., and Bleackley, R. C. (1995) Nature 377, 446–448
23. Gu, I., Sarnecki, M. A., Fleming, M. A., Lippke, J. A., Bleackley, R. C., and Su, M. S.-S. (1996) J. Biol. Chem. 271, 10816–10820
24. Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P., and Salvesen, G. (1994) J. Biol. Chem. 269, 19331–19337
25. Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A. H., Miller, L. K., and Wong, W. W. (1995) Science 269, 1885–1888
26. Xue, D., and Horvitz, H. R. (1995) Nature 377, 248–251
27. Wilson, K. P., Black, J. A. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) Nature 370, 270–275
28. Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Houy, W., Mankovich, J. A., McGuiness, L., Orlewicz, E., Faskind, M., Pratt, C. A., Reis, P., Summanan, A., Terranera, M., Welch, J. P., Xiong, L., Moller, A., Tracey, D. E., Kamen, R., and Wong, W. W. (1994) Cell 78, 343–352