# MOLECULAR STUDIES OF FAS SIGNALING AND PROGRAMMED CELL DEATH

by

HOWARD Y. CHANG

A.B. Biochemical Sciences

Harvard College, 1994

SUBMITTED TO THE DEPARTMENT OF BIOLOGY
IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

AT THE

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 1998



Signature of the Author: _____

Department of Biology
September, 1998

Certified by : _____

Dr. David Baltimore
Ivan Cottrell Prof. of Mol.
Biol. and Immunology
Thesis Supervisor

Accepted by: _____

Dr. Alan Grossman
Professor of Biology
Head, Graduate Committee

## Dedication

This thesis is dedicated to the memory of my grandparents, Chang Ming-Fah and Wu Bao-Dai, who instilled in me the importance of scholarship.

## Acknowledgments

As Newton once wrote, "If I have seen farther, it was by standing upon the shoulders of giants." First and foremost, I want to thank my friend, close collaborator, and partner in crimes **Xiaolu Yang**. Many of the experiments described in this thesis were done in close collaboration with Xiaolu, and his thoughtfulness, patience, ingenuity, and drive were critical in pushing our work forward. Our daily discussions of scientific directions, experimental techniques, and theoretical models formed the basis of the most rewarding research experience that I could have hoped for. I am grateful to my advisor Prof. **David Baltimore** for his mentorship, warm encouragement, and steadfast support. I have benefited richly from our discussions about science and life, and on several occasions, his blow by blow dissection of my experiments made them work in the end. By his example he propelled me to ask the deepest questions with the greatest scientific rigor. I thank my erstwhile collaborators **Hidenori Ichijo, Hideki Nishitoh,** and **Roya Khosravi-Far** for key scientific insights and fruitful collaborations. I gratefully acknowledge **Alex Hoffmann** for his help with biochemical experiments. I thank my bay neighbors **Tony Koleske** and **Bruce Horwitz** for putting up with me, and members of the **Baltimore lab** for many kind acts bestowed and helpful advice.

My classmates **Fangli Chen** and **Ken Tsai** deserve credit for helping me weather the ups and downs of graduate student life. I thank my fiancee **Anne Su** for her love and support; this work could not have been possible without her patience, affection, and understanding. I am also indebted to my **parents** for their support and encouragement. This research was supported by a grant from the **Medical Scientist Training Program** at **Harvard Medical School**.

# MOLECULAR STUDIES OF FAS SIGNALING AND PROGRAMMED CELL DEATH

by

HOWARD Y. CHANG

Submitted to the Department of Biology on September 11th, 1998
in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy in Biology

## Abstract

Fas is cell surface receptor that induces apoptosis upon activation. To understand its signaling, Daxx, a novel protein binding to the intracellular tail of Fas was identified by the yeast two hybrid screen. Daxx enhanced Fas-induced apoptosis by activating the Jun N-terminal kinase (JNK) pathway, a kinase cascade regulating gene induction previously implicated in cell death. Daxx activation of JNK was independent of the previously characterized FADD pathway downstream of Fas. Fas activation induced Daxx to interact with Apoptosis Signal-regulating Kinase 1 (ASK1), an upstream MAP kinase kinase kinase of the JNK pathway. This interaction relieved an inhibitory interaction between the $NH_2$-terminal regulatory domain and the remainder of the kinase, leading to its activation. These results complete a pathway from cell surface death receptors to intracellular kinase cascades.

Fas was previously known to recruit pro-caspase-8 and lead to its activation. Because Fas is activated by a trimeric ligand, Fas-mediated oligomerization of pro-caspase-8 may lead to its activation. In two heterologous inducible oligomerization systems, oligomerization of pro-caspase 8 led to autoproteolytic processing to generate mature caspase subunits and activated apoptosis. Other pro-caspases can also be similarly activated, suggesting that pro-caspase oligomerization of may be a general mechanism for activating initiator caspases in apoptosis. In the classic apoptosis model *Caenorhabditis*

*elegans*, the pro-caspase CED-3 is activated by CED-4, which is in turn inhibited by the Bcl-2-like protein CED-9, both via direct protein interactions. The function of these three proteins can be understood by the regulated oligomerization of CED-4. CED-4 formed homotypic oligomers in vitro and in cells, which induced CED-3 proximity and activation. CED-9 interaction with CED-4 competed with CED-4 oligomerization. Enforced oligomerization of CED-3 induced CED-3 processing and activated apoptosis. Thus, pro-caspase oligomerization is the common mechanism of control of cell surface death receptors and the evolutionarily conserved Bcl-2 pathway.

Thesis supervisor: David Baltimore

Title: Ivan Cottrell Professor of Molecular Biology and Immunology

## Table of Contents


Title Page ..... 1

Dedication ..... 2

Acknowledgments ..... 3

Abstract ..... 4

Table of Contents ..... 6

Chapter I. Introduction ..... 8

Chapter II. Identification and characterization of Daxx, a novel Fas binding protein that activates JNK and apoptosis

i. Summary ..... 21

ii. Introduction ..... 22

iii. Results ..... 25

iv. Discussion ..... 33

v. Experimental Procedures ..... 37

vi. References ..... 43

vii. Figure legends ..... 47

viii. Figures ..... 51

Chapter III. Activation of Apoptosis Signal-regulating Kinase 1 (ASK1) by the adaptor protein Daxx

i. Summary ..... 59

ii. Introduction ..... 60

iii. Results ..... 61

iv. Discussion ..... 65

v. Experimental Procedures ..... 66

vi. References ..... 69

vii. Figure legends ..... 71

viii. Figures ..... 76

Chapter IV. Autoproteolytic activation of pro-caspases by oligomerization

i. Summary..... 81

ii. Introduction..... 82

iii. Results..... 84

iv. Discussion..... 90

v. Experimental Procedures..... 93

vi. References..... 97

vii. Figure legends..... 99

viii. Figures..... 102

Chapter V. Essential role of CED-4 oligomerization in CED-3 activation and apoptosis

i. Summary..... 108

ii. Introduction..... 109

iii. Results..... 110

iv. Discussion..... 113

v. Experimental Procedures..... 114

vi. References..... 116

vii. Figure legends..... 118

viii. Figures..... 121

Chapter VI. Concluding remarks and perspectives

i. Recent developments in Fas signaling..... 126

ii. Pro-caspase oligomerization-- the death machine revealed?..... 129

iii. Concluding remarks..... 133

iv. Figure..... 142

## Chapter I. Introduction

Over the last two decades, programmed cell death has been recognized as a phenomenon of fundamental importance in development, tissue homeostasis, cancer, and many degenerative diseases (Jacobson et al., 1997; Thompson, 1995). The term programmed cell death was originally coined to describe cell death that would occur in predictable fashion and times during development (Lochshin and Williams, 1964). In a seminal paper, Kerr, Wylie and Currie used morphologic evidence to argue that programmed cell death was distinct from pathologic cell death found in acute ischemic lesions (Kerr et al., 1972). The latter, a process termed necrosis, is characterized by cellular and organelle swelling, followed by disintegration of the membranes, leakage of cellular material, and often leading to an inflammatory response. In contrast, programmed cell death, as observed during normal development or in the periphery of ischemic lesions, is characterized by cellular shrinkage and condensation, relative preservation of cellular membranes with little leakage of debris, quick phagocytosis by neighboring cells, and little accompanying inflammatory response. Kerr and colleagues named this morphologically distinct form of programmed cell death "apoptosis", a Greek word that describes leaves falling from branches, to emphasize the physiologic and orderly nature of this form of cell death (Kerr et al., 1972).

Many diverse biological processes are now believed to depend on the proper and timely induction of apoptosis. During development, apoptosis is used to remove vestigial structures and sculpt body parts. For example, cells in the tadpole tail undergo apoptosis during frog metamorphosis, and developing fingers are shaped by the selective death of cells in the interdigital webs (Jacobson et al., 1997). In the nervous system, apoptosis has an important role in matching neurons to their target tissues. An excess of neurons is generated during neuronal development; these cells are destined to die unless their axons encounter target-derived trophic factors (Levi-Montalcini, 1987). This mechanism ensures

that only neurons whose axons have properly connected to their targets survive and automatically matches the correct number of neuronal innervation for each target tissue. Apoptosis also has a critical role in the development and function of the immune system. During lymphoid development, lymphocytes are generated with random antigen specificity, including those cells that recognize self antigens. Autoreactive T cells are induced to undergo programmed cell death in the thymus, and autoreactive T and B cells in periphery are continually deleted by apoptosis in adult animals to prevent autoimmunity (Van Parijs and Abbas, 1998). Not surprisingly, dysregulation of apoptosis underlies many pathologic conditions. Tumor cells, such as those that have lost the tumor suppressor gene p53, fail to undergo apoptosis by many stimuli and are therefore able to proliferate in the hypoxic environment of tumor masses and resist chemotherapy (Symonds et al., 1994; Lowe et al., 1994; Graeber et al., 1996). In degenerative diseases, cells may be too sensitive to apoptosis and die inappropriately. In a *Drosophila* model of retinitis pigmentosa, a degenerative disease of photoreceptor cells, prevention of apoptosis allows the photoreceptors to continue to function and cures the disease (Davidson and Steller, 1998). Thus, understanding the control and execution of apoptosis promises to reveal important insights into many arenas of biology and medical science.

Because the morphologic features of programmed cell death in different organisms and in different circumstances were very similar, Kerr and colleagues proposed that apoptosis was caused by an active cell suicide program (Kerr et al., 1972). This belief was bolstered by the observations that in some instances, such as the death of neurons in the absence of nerve growth factor, apoptosis can be prevented by inhibitors of RNA or protein synthesis (Oppenheim et al., 1980). Over the last decade, genetic studies in the nematode *Caenorhabditis elegans* have paved the way for exponential advances in understanding apoptosis at the molecular level. In a series of seminal experiments, Horvitz and colleagues defined a set of genes that controls the programmed cell death of 131 cells that die during *C. elegans* hermaphrodite development (reviewed by Ellis et al., 1991;

Steller, 1995). These genes control different aspects of the apoptotic process: some are involved in specifying the cells that die, some in the execution of cell death, and some in the engulfment of the cell corpses. In particular, four genes (*egl-1*, *ced-9*, *ced-4*, and *ced-3*) comprise a genetic pathway that controls the apoptotic execution of all dying cells (Conradt and Horvitz, 1998; Steller, 1995). *egl-1*, *ced-4*, and *ced-3* are required in the dying cell for apoptosis to occur while *ced-9* inhibits apoptosis. All of these genes have mammalian homologs, some of which were independently identified to be involved in regulating cell death:

*C. elegans*


Mammalian

| | | | |
|---|---|---|---|
| Bax Bid<br>Bak Bik<br>Bad Hrk | Bcl-2<br>Bcl-xL<br>Bcl-w<br>Mcl-1<br>A-1 | Apaf-1 | Caspases<br>1 to 12 |

CED-3 is a member of a large family of cysteine proteases, termed caspases, that when activated can cleave various cellular proteins to execute apoptosis (Yuan et al., 1993). Apaf-1, a mammalian homolog of CED-4, has been identified biochemically in an in vitro system of caspase activation (Zhou et al., 1997). CED-9 is homologous to the mammalian Bcl-2 family of anti-apoptotic proteins (Hengartner and Horvitz, 1994). Bcl-2 was first identified as an oncogene that is overexpressed by the t(14;18) chromosomal translocation in follicular lymphoma. It was soon recognized that it did not confer enhanced proliferation but instead caused the cell to resist apoptosis by a variety of stimuli (Reed, 1994). EGL-1 is homologous to a growing family of mammalian pro-apoptotic proteins which can bind to and antagonize the function of anti-apoptotic Bcl-2 family members (Conradt and Horvitz, 1998). Interestingly, mammalian Bcl-2 can complement *ced-9* mutation in the worm (Vaux

et al., 1992; Hengartner and Horvitz, 1994), and worm CED-3 and CED-4 proteins can induce apoptosis in human cell lines (Chinnaiyan et al., 1997). The functional similarity of the *C. elegans* and mammalian death genes suggests that apoptosis is controlled by an evolutionarily conserved genetic program that is essential for metazoan life.

In understanding mammalian cell death pathways, one important and fruitful area of study has been cell surface receptors that induce apoptosis. In 1989, two groups independently isolated monoclonal antibodies that induced apoptosis in a variety of human tissue culture cell lines (Trauth et al., 1989; Yonehara et al., 1989). These antibodies recognized cell surface proteins, designated Fas and APO-1 respectively, which were thought to mediate the cytotoxic effect of the antibodies. Using binding to anti-Fas antibody as the screen, Nagata and colleagues were able to isolate the cDNA of human Fas by expression cloning (Itoh et al., 1991). When the APO-1 antigen was purified and sequenced, it was discovered that Fas and APO-1 were one and the same (Oehm et al., 1992). Human Fas contains 325 amino acids; it has an N-terminal signal sequence and a transmembrane region in the middle of the protein, qualifying it as a type I transmembrane protein (Itoh et al., 1991). The primary sequence of Fas immediately suggested that it was a member of the tumor necrosis factor (TNF) receptor superfamily of cytokine receptors (Itoh et al., 1991). TNFR family members include TNFR 1 and 2, CD40, CD30, CD27, the low affinity NGF receptor, and several others (reviewed by Smith et al., 1994). Many members of the TNFR superfamily are essential regulators of cell proliferation, cell death, and immune effector functions (Smith et al., 1994). For example, TNFR 1 and 2 mediate signaling by TNF-$\alpha$, a pleiotropic cytokine that is a potent inducer of inflammation, fever, and septic shock. CD40 is an obligate costimulatory receptor for B cell proliferation and immunoglobulin isotype switching; mutation of its ligand, CD40L, in humans causes X-linked hyper-IgM syndrome. The extracellular domain of Fas comprises three cysteine-rich repeats, the homology of which define the TNFR superfamily (Itoh et al., 1991). Introduction of human Fas cDNA into mouse cell lines allowed those cell lines to be killed

by apoptosis after exposure to anti-Fas antibody (Itoh et al., 1991). In the cell lines that constitutively express human Fas, apoptosis (as measured by morphological changes and cell detachment) occurred in 5 hours after exposure to anti-Fas and was enhanced by the addition of protein synthesis inhibitor cycloheximide (Itoh and Nagata, 1993). These studies confirmed that Fas indeed is a cell surface receptor capable of inducing apoptosis. The cytoplasmic domain of Fas does not contain any sequence suggestive of enzymatic activities, but it is similar to a domain in the cytoplasmic tail of TNF receptor 1 (TNFR1), which is known to transduce cytotoxic signals (Itoh and Nagata, 1993). Mutagenesis of both TNFR1 and Fas defined an approximately 60 amino acids motif in the cytoplasmic tail of both receptors, termed the "death domain", that is essential for inducing apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). More recently, several novel death receptors were identified in expression sequence tag (EST) databases by the sequence similarity of their cytoplasmic tails to death domains (Chinnaiyan et al., 1996; Golstein, 1997).

To isolate the ligand for Fas, researchers focused on a hybridoma cell line that was able to kill target cells expressing Fas but not the parental cell line lacking Fas expression (reviewed by Nagata and Golstein, 1995). Target cell killing by this hybridoma was blocked by a soluble fusion protein of the extracellular domain of Fas with immunoglobulin Fc region (Fas-Fc) but not by TNFR-Fc, suggesting that this killing resulted from cognate Fas and Fas ligand (FasL) interaction. This hybridoma cell line was repeatedly sorted by fluorescence-activated cell sorting with Fas-Fc to establish a subline, d10S16, which expressed approximately 100 fold more Fas-Fc staining and target cell cytotoxicity (Suda et al., 1993). Using d10S16 and Fas-Fc, FasL was both purified and its cDNA cloned by expression cloning. Enforced expression of FasL cDNA in COS cells, which normally do not induce Fas-dependent death, allowed them to kill specifically Fas expressing target cells (Suda et al., 1993). Conceptual translation of FasL cDNA revealed that it lacks a signal sequence but contains a single transmembrane domain in the middle of the protein, suggesting that it is a type II transmembrane protein with a carboxyl-terminal extracellular

domain. The extracellular domain of FasL contains a region of substantial homology with other ligands of the TNF superfamily, such as TNF, lymphotoxin, and CD40 ligand (Suda et al., 1993). Based on the crystal structure of TNF, the prototypical ligand of the TNFR superfamily, FasL probably exists naturally as a trimer with the three-fold axis of symmetry perpendicular to the plasma membrane (Banner et al., 1993). Soluble Fas and TNF-$\alpha$ can be released from cell surface by proteolytic cleavage by a metalloproteinase (Gearing et al., 1994). Both membrane-bound and soluble forms of FasL are active in apoptosis induction although the membrane-bound form is more potent (Tanaka et al., 1996). Northern blot analysis indicated that Fas is ubiquitously but weakly expressed in most tissues, but FasL is expressed only on activated T cells and several discrete sites (Nagata and Golstein, 1995). FasL message is rapidly induced by T cell receptor activation, and this activation is blocked by inhibitors of tyrosine kinases or the immunosuppressant cyclosporin A (an inhibitor of the calcium-dependent phosphatase calcineurin) (Nagata and Golstein, 1995).

The major clue for the in vivo function of Fas and FasL came from mapping their genomic locations. The murine Fas and FasL genes map respectively to chromosomes 19 and 1 on the locations of *lpr* and *gld*, two previously characterized spontaneous mutations that cause autoimmune disease (Nagata and Golstein, 1995). *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) are two autosomal recessive mutations that can cause severe autoimmune disease in certain genetic backgrounds (reviewed by Cohen and Eisenberg, 1991). MRL*lpr/pr* or MRL*gld/gld* mice develop severe lymphadenopathy, splenomegaly, high titers of circulating autoantibodies, nephritis, and arthritis, and usually die by 5 months of age (Cohen and Eisenberg, 1991). These symptoms are reminiscent of human systemic lupus erythrematosus and rheumatoid arthritis, prompting interesting in identifying the underlying defect in these two mice strains. MRL mice are prone to autoimmunity, and the disease is greatly accelerated by homozygous *lpr* or *gld* mutations. The lymphocytes that accumulate in the *lpr* and *gld* mice are unusual; they are polyclonal

$CD4^-CD8^-$ T cells with functional T cell receptor rearrangements, which are normally not seen outside of the thymus. Based on bone marrow transplantation and antibody-mediated depletion experiments, it is believed that these abnormal lymphocytes are derived from mature single positive $CD4^+$ or $CD8^+$ peripheral T cells (reviewed by Nagata and Golstein, 1995). Although *lpr* and *gld* are not allelic, their similar phenotypes suggested that they may act in the same pathway. Adoptive transfer of *gld* bone marrow into congenic +/+ animal recapitualted a *gld*-like disease whereas adoptive transfer of *lpr* bone marrow into +/+ animals caused graft-versus-host disease. These and other experiments suggested that *gld* may affect a cytokine and *lpr* affects its interacting receptor (Allen et al., 1990). In the *lpr* mutation, the murine Fas gene is interrupted by a transposable element, and Fas transcript level is severely depressed (Watanabe-Fukunaga et al., 1992). A weak allele of *lpr*, termed $lpr^{cg}$, is caused by a point mutation in the cytoplasmic death domain of Fas (Watanabe-Fukunaga et al., 1992). The *gld* mutation is a point mutation in the extracellular domain of FasL, which disrupts the ability of FasL to interact with Fas (Takahashi et al., 1994). As expected, *lpr* T cells are not killed by anti-Fas antibody, but *gld* cells readily killed by anti-Fas antibody.

More recently, humans with Fas mutations have also been identified. Autoimmune Lymphoproliferative Syndrome (ALPS) is a disease in children characterized by massive lymphadenopathy, peripheral accumulation of double negative $CD4^-CD8^-$ T cells, and autoantibody production leading to hepatosplenomegaly, hemolytic anemia, and thrombocytopenia. A majority of ALPS kindreds harbor heterozygous mutations in the Fas gene (Fisher et al., 1995; Rieux-Laucat et al., 1995). In addition to the phenotypic similarity to the*lpr* mutation in mice, several lines of evidence strongly support that Fas mutations are responsible for ALPS: 1) Fas mutations cosegregate with defective lymphocyte apoptosis in ALPS families and are never detected in control patients (Fisher et al., 1995; Rieux-Laucat et al., 1995); 2) the mutant Fas alleles do not induce cell death when they are overexpressed in vitro (Fisher et al., 1995); and 3) unlike the recessive

murine *lpr* mutation, the human mutant Fas alleles in fact act dominantly to inhibit Fas-induced cell death in normally Fas-sensitive cell lines (Fisher et al., 1995). Collectively, these results provide strong genetic evidence that Fas is essential for peripheral tolerance and prevention of autoimmunity in mice and humans.

The physiologic function of Fas in preventing autoimmunity is critically dependent on its ability to induce apoptosis. Following cognate T cell receptor-MHC interaction and CD28 activation, primary T cells become activated and proliferate. However, restimulation of activated T cells cause them to commit autocrine suicide, a process that is called activation-induced cell death (AICD) (reviewed by Van Parijs and Abbas, 1998). AICD is a mechanism for eliminating unwanted lymphocytes at the end of an immune response and deleting peripheral autoreactive T cells. AICD but not activation-induced proliferation is blocked in *lpr* or *gld* T cells, and AICD can also be blocked in wild type T cells by Fas-Fc, which functions as a decoy receptor for FasL (Dhein et al., 1995; Brunner et al., 1995; Ju et al., 1995). In transgenic mice bearing T cell receptors against model antigens, clonal deletion of peripheral antigen-specific lymphocytes are blocked in the *lpr* or *gld* background (Van Parijs and Abbas, 1998). These results suggest that the induction of FasL by repeated T cell activation allows cognate FasL-Fas interaction, leading to T cell apoptosis. However, FasL induction usually precedes AICD by one or two days, suggesting that additional regulatory mechanisms control T cell sensitivity to Fas killing (Nagata and Golstein, 1995). In addition to AICD, activated cytotoxic T cells use FasL as a weapon to kill infected target cells (Nagata, 1997), and excess Fas-mediated killing may underlie immune-mediated tissue destruction in fulminant hepatitis (Kondo et al., 1997). CD4+ T cells use FasL to kill autoreactive B cells in vivo (Rathmell et al., 1995); autoantibody production by B cells are in fact responsible for most of the pathologic symptoms in *lpr* and *gld* animals. Moreover, some tumors and immune privileged sites such as the testis and eyes constitutively express FasL but not Fas (Abbas, 1996). These tissues may then kill infiltrating lymphocytes by Fas-mediated apoptosis and evade the immune system. Finally, in other autoimmune

diseases such as Hashimoto's thyroiditis and type I diabetes, abnormal cytokine production is thought to induce ectopic Fas or FasL expression and lead to tissue destruction (Giordano, 1997; Chernovsky et al., 1997). Thus, better understanding of Fas signaling may lead to new therapeutic insights in treating a number of autoimmune diseases.

In this thesis, I have focused on Fas signaling as a starting point to investigate the molecular mechanisms of programmed cell death. At the time I began these studies, it was known that via an adaptor protein named FADD, Fas can recruit the zymogen form of caspase-8 and somehow activate a caspase cascade. Chapter II describes the identification of a novel Fas death domain binding protein, Daxx, that is involved in the activation of the Jun N-terminal kinase (JNK) pathway, a kinase pathway previously implicated in regulating cell death in mammalian cells. As described in Chapter III, Daxx binds to and activates a MAP kinase kinase kinase termed ASK1 (Apoptosis Signal-regulating Kinase 1), completing the connection between death receptors and the intracellular kinase cascades. In Chapter IV, oligomerization of several pro-caspases was shown to cause their autoproteolytic activation, which explains how Fas activates pro-caspase-8 and may be a general mechanism in initiating apoptosis. This hypothesis was partially confirmed by studies in Chapter V, where the regulated oligomerization of CED-4 was shown to be the mechanism of control for the *C. elegans* cell death pathway.

## References

Abbas, A. K. (1996). Die and let live: eliminating dangerous lymphocytes. Cell *84*, 655-658.

Allen, R. D., Marshall, J. D., Roths, J. B., and Sidman, C. L. (1990). Differences defined by bone marrow transplantation suggest that lpr and gld are mutations of genes encoding an interacting pair of molecules. J. Exp. Med. *172*, 1367-1375.

Banner, D. W., D'Arcy, a., Janes, W., Gentz, R., Schoenfeld, H.-J., Broger, C., Loetscher, H., and Lesslauer, W. (1993). Crystal structure of the soluble human 55 kD TNF receptor-human TNFβ complex: implication for TNF receptor activation. Cell *73*, 431-445.

Brunner, T., Mogil, R. J., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Martin, S. J., Force, W. R., Lynch, D. H., Ware, C. F., and Green, D. R. (1995). Cell-autonomous Fas(CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature *373*, 441-444.

Chernovsky, A. V., Wang, Y., Wong, F. S., Visintin, I., Flavell, R. A., Janeway, C. A., and Matis, L. A. (1997). The role of Fas in autoimmune diabetes. Cell *89*, 17-24.

Chinnaiyan, A. M., O'Rourke, K., Lane, B. R., and Dixit, V. M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science *275*, 1122-1126.

Chinnaiyan, A. M., O'Rourke, K., Yu, G.-L., Lyons, R. H., Garg, M., Duan, D. R., Xing, L., Gentz, R., Ni, J., and Dixit, V. M. (1996). Signal transduction by DR3, a death domain-containing receptor related to TNFR1 and CD95. Science *274*, 990-992.

Cohen, P. L., and Eisenberg, R. A. (1991). Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu. Rev. Immunol. *9*, 243-269.

Conradt, B., and Horvitz, H. R. (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519-529.

Davidson, F. F., and Steller, H. (1998). Blocking apoptosis prevents blindness in Drosophila retinal degeneration mutants. Nature *391*, 587-591.

Dhein, J., Walczak, H., Baumler, C., Debatin, K.-M., and Krammer, P. H. (1995). Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature *373*, 438-440.

Ellis, R. E., Yuan, J., and Horvitz, H. R. (1991). Mechanisms and functions of cell death. Ann. Rev. Cell Biol. *7*, 663-698.

Fisher, G. H., Rosenberg, F. J., Straus, S. E., Dale, J. K., Middleton, L. A., Lin, A. Y., Strober, W., Lenardo, M. J., and Puck, J. M. (1995). Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell *81*, 935-946.

Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L., et al.

(1994). Processing of tumor necrosis factor-α precursor by metalloproteinases. Nature *370*, 555-557.

Giordano, C. et al. (1997). Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Science *275*, 960-963.

Golstein, P. (1997). TRAIL and its receptors. Curr Biol. *7*, R750-R753.

Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., and Giaccia, A. J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature *379*, 88-91.

Hengartner, M. O., and Horvitz, H. R. (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell *76*, 665-676.

Itoh, N., and Nagata, S. (1993). A novel protein domain required for apoptosis. J. Biol. Chem. *268*, 10932-10937.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell *66*, 233-243.

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.

Ju, S.-T., Panka, D., Cui, H., Ettinger, R., El-Khatib, M., Sherr, D. H., Stanger, B. Z., and Marshak-Rothstein, A. (1995). Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature *373*, 444-448.

Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implication s in tissue kinetics. Br. J. Cancer *26*, 239-257.

Kondo, T., Suda, T., Fukuyama, H., Adachi, M., and Nagata, S. (1997). Essential roles of the Fas ligand in the development of hepatitis. Nature Medicine *3*, 409-413.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science *237*, 1154-1162.

Lochshin, R. A., and Williams, C. M. (1964). Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. J. Insect Physiol. *10*, 643.

Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994). p53 status and the efficacy of cancer therapy in vivo. Science *266*, 807-810.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-365.

Nagata, S., and Golstein, P. (1995). The Fas death factor. Science *267*, 1449-1456.

Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Li-Weber, M., Richards, S., Dhein, J., Trauth, B. C., et al. (1992). Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor /nerve growth factor

receptor superfamily: sequence identity with the Fas antigen. J. Biol. Chem. *267*, 10709-10715.

Oppenheim, R. W., Prevette, D., Tytell, M., and Homma, S. (1980). Naturally occuring and induced neuronal death in the chick embryo in vivo requires protein and RNA synthesis: evidence for the role cell death genes. Dev. Biol. *138*, 104-113.

Rathmell, J. C., Cooke, M. P., Ho, W. Y., Grein, J., Townsend, S. E., Davis, M. M., and Goodnow, C. C. (1995). CD95(Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. Nature *376*, 181-184.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. J. Cell. Biol. *124*, 1-6.

Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I. A. G., Debatin, K. M., Fischer, A., and de Villartay, J. P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science *268*, 1347-1349.

Smith, C. A., Farrah, T., and Goodwin, R. G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation , and death. Cell *76*, 959-962.

Steller, H. (1995). Mechanisms and genes of cellular suicide. Science *267*, 1445-1449.

Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993). Molecular cloning and expression of the Fas ligand: a novel member of the tumor necrosis factor family. Cell *75*, 1169-1178.

Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell *78*, 703-711.

Takahashi, T., Tanaka, M., Brannan, C. I., Jenkins, N. A., Copeland, N. G., Suda, T., and Nagata, S. (1994). Generalized lymphoproliferative disease in mice caused by a point mutation in the Fas ligand. Nature *76*, 969-976.

Tanaka, M., Suda, T., Haze, K., Nakamura, N., Sato, K., Kimura, F., Motoyoshi, K., Mizuki, M., Tagawa, S., Ohga, S., and al., e. (1996). Fas ligand in human serum. Nature Med. *2*, 317-322.

Tartaglia, L. A., Ayres, T. M., Wong, G. H. W., and Goeddel, D. V. (1993). A novel domain within the 55 kD TNF receptor signals cell death. Cell *74*, 845-853.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*.

Trauth, B. C., Klas, C., Peters, A. M. J., Matzku, S., Moller, P., Falk, W., Debatin, K.-M., and Krammer, P. H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science *245*, 301-305.

Van Parijs, L., and Abbas, A. K. (1998). Homeostasis and self-tolerance in the immune system: turning
lymphocytes off. Science *280*, 243-248.

Vaux, D. L., Weissman, I. L., and Kim, S. K. (1992). Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. Science *258*, 1955-1957.

Watanabe-Fukunaga, R., Brannan, C. I., Itoh, N., Yonehara, S., Copeland, N. G., Jenkins, N. A., and Nataga, S. (1992). The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J. Immunol. *148*, 1274-1279.

Yonehara, S., Ishii, A., and Yonehara, M. (1989). A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor for tumor necrosis factor. J. Exp. Med. *169*, 1747-1756.

Yuan, J., Shaham, S., Ledoux, H. M., and Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-β-converting enzyme. Cell *75*, 641-652.

Zhou, H., Henzel, W. I., Liu, X., Luschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in the cytochrome c-dependent activation of caspase-3. Cell *90*, 405-413.

# Chapter II. Identification and characterization of Daxx, a novel Fas-binding protein that activates JNK and apoptosis.


## Summary

The Fas cell surface receptor induces apoptosis upon receptor oligomerization. We have identified a novel signaling protein, termed Daxx, which binds specifically to the Fas death domain. Overexpression of Daxx enhances Fas-mediated apoptosis and activates the Jun N-terminal kinase (JNK) pathway. A C-terminal portion of Daxx interacts with the Fas death domain while a different region activates both JNK and apoptosis. The Fas-binding domain of Daxx is a dominant negative inhibitor of both Fas-induced apoptosis and JNK activation while the FADD death domain partially inhibits death but not JNK activation. The Daxx apoptotic pathway is sensitive to both Bcl-2 and dominant negative JNK pathway components and acts cooperatively with the FADD pathway. Thus, Daxx and FADD define two distinct apoptotic pathways downstream of Fas.


## My contribution to this project

This project was the result of a close collaboration of three people: postdocs Xiaolu Yang, Roya Khosravi-Far, and myself. Xiaolu carried out the Fas death domain two hybrid screen and isolated the Daxx cDNA. I carried out the protein interaction studies. Roya made the discovery that Daxx activated the JNK pathway, and Xiaolu and I performed the cell death experiments. This work has been published in the following reference:


Yang, X.*, R. Khosravi-Far*, H.Y. Chang*, and D. Baltimore (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell *89*, 1067-1076.


*these authors contributed equally.

## Introduction

Fas (also known as CD95 or APO-1) is a widely expressed cell death receptor that has a critical role in the regulation of the immune system and tissue homeostasis. Fas is activated by Fas ligand (FasL), a trimeric transmembrane protein (reviewed by Nagata, 1997). Fas is thought to have an essential role in deleting autoreactive lymphocytes and maintaining peripheral tolerance. Inherited Fas mutations in humans and mice cause a syndrome of massive lymphoproliferation and autoantibody production (reviewed by Nagata, 1997). Fas-induced apoptosis is also a major mechanism in cytotoxic T lymphocyte-mediated cytolysis and in the maintenance of immune privilege sites (reviewed by Abbas, 1996). Moreover, depending on the signal from the B cell antigen receptor, Fas may induce either apoptosis or proliferation of B cells in vivo (Rathmell et al., 1996).

Fas belongs to the tumor necrosis factor (TNF) receptor superfamily, which includes TNF receptor 1 (TNFR1), TNFR2, CD40, and the p75 low affinity NGF receptor; these receptors share characteristic cysteine-rich repeats in their extracellular domains (reviewed by Smith et al., 1994). The intracellular tails of Fas and TNFR1 share homologous death domains, an approximately eighty amino acid protein motif that is critical for signaling apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). Over the last two years, elucidation of the mechanism for Fas-mediated apoptosis has begun (reviewed by Cleveland and Ihle, 1995; Fraser and Evan, 1996). FADD, also known as MORT1, is a cytoplasmic protein that has a C-terminal death domain which interacts with Fas and an N-terminal domain that can induce cell death (Chinnaiyan et al., 1995; Boldin et al., 1995b). The N-terminus of FADD interacts with pro-caspase-8 (also named MACH or FLICE), an interleukin-1β converting enzyme (ICE) family cysteine protease (caspase) that potently induces apoptosis (Boldin et al., 1996; Muzio et al., 1996). Although the details are not yet clear, other caspases, including caspase-1 (ICE) and caspase-3 (CPP32), are sequentially activated to execute the apoptotic dissolution of the cell (Enari et al., 1996). TNFR1 also interacts with FADD via an adaptor protein termed TRADD (Hsu et al.,

1996). The emerging model from these molecular studies is that Fas, via FADD, directly engages and activates apoptotic ICE family proteases. However, this model fails to explain how Bcl-2 and other physiologic signals may modulate Fas-mediated apoptosis (Fraser and Evan, 1996). It remains possible that other signaling molecules in addition to FADD are involved in Fas-mediated apoptosis.

Fas can also activate the Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) pathway (Latinis and Koretzky, 1996; Lenczowski et. al., 1997; Goillot et. al., 1997). Analogous to the MAP kinase cascade, the prototypical JNK/SAPK pathway involves the sequential activation of the proteins MEKK1, SEK1, JNK, and c-Jun. Other targets of the JNK pathway include the transcription factors Elk-1 and ATF-2 (reviewed by Kyriakis and Avruch, 1996). This pathway was initially characterized by the ability of UV irradiation and transforming Ha-Ras to activate the AP-1 transcription factor; subsequently it was shown that TNF-$\alpha$ and other stress-activated signals may also activate this pathway. The significance of Fas-mediated JNK activation has been unclear. One hypothesis is that activation of the JNK pathway contributes to Fas-mediated apoptosis (Goillot et. al., 1997). Dominant negative constituents of the JNK pathway can block stress- and TNF-induced apoptosis in several cell lines, suggesting that activation of JNK pathway is required for these apoptotic inducers (Verheij et al., 1996). Similarly, in PC12 cells that undergo apoptosis in response to nerve growth factor withdrawal, activation of the JNK pathway in concert with the suppression of the ERK pathway is critical to induction of programmed cell death (Xia et al., 1995). Alternatively, Fas-mediated JNK activation may drive cellular proliferation via activation of the proto-oncogene c-Jun and AP-1 transcriptional activity (Rathmell et al., 1996).

Recently, Liu et. al. have demonstrated that overexpression of FADD, the established downstream signal transducer of Fas, cannot activate JNK but that two other proteins engaged by TNFR1-- RIP and TRAF2-- are responsible for JNK activation by

TNF (Liu et al., 1996). This raises the question of whether Fas also engages other proteins to activate the JNK pathway.

Herein we describe the molecular cloning and characterization of Daxx, a novel Fas binding protein. Daxx binds to the Fas death domain, yet lacks a death domain of its own. Overexpression of Daxx leads to JNK activation and potentiates Fas-induced apoptosis. The Fas binding domain of Daxx acts as a dominant negative inhibitor of Fas-induced apoptosis and JNK activation. Furthermore, using dominant negative and constitutively active forms of Daxx and FADD, we show that Fas engages two independent pathways to induce cell death: one pathway via Daxx that involves JNK activation and is blocked by Bcl-2, and a second pathway via FADD that is Bcl-2 insensitive.

## Results

### Two-hybrid Screen for Novel Fas-Interacting Proteins

To identify novel Fas interacting proteins, we performed a two-hybrid screen with the death domain of murine Fas fused to the DNA binding protein LexA (LexA-mFasDD). A plasmid library of fusions between a transcription activation domain and cDNAs from human HeLa cells was screened for interaction with LexA-mFasDD in a yeast reporter strain. One group of positive interactors, typified by clone A21, interacted strongly with FasDD. However, it interacted poorly with either Fas-*lpr*$^{cg}$, an Ile224Asn mutation in the Fas death domain which abrogates Fas signaling and causes lymphoproliferation in mice (reviewed by Nagata, 1997) or with Fas-FD8, a functionally inactive deletion mutation of the Fas death domain (Itoh and Nagata, 1993) (Figure 1B). Sequence analysis of clone A21 revealed it to encode a portion of a novel protein.

In the two-hybrid system, clone A21 also interacted with the intracellular domain of human Fas and the death domain of TNFR1, but not with the intracellular region of CD40, a closely related receptor that lacks a death domain (Figure 1B). The sequence C-terminal to the Fas death domain has been shown to inhibit the cytotoxicity of Fas death domain (Itoh and Nagata, 1993) and it also inhibits the binding of FADD to Fas (Chinnaiyan et al., 1995). The presence of this inhibitory region had no effect on the binding of clone A21 to Fas because clone A21 interacted equally well with FasDD and FasIC (Figure 1B).

We mapped the Fas interaction domain on clone A21 to its C-terminal 112 amino acids by deletion analysis (Figure 1C). This region showed no evident sequence similarity to death domains, suggesting that the interaction between clone A21 and Fas is not through a homotypic death domain association.

### Cloning of Daxx cDNA and Northern Analysis

Using clone A21 as the probe, we cloned a cross-hybridizing full length murine cDNA. Sequence analysis revealed an open reading frame able to encode a protein of 739 amino acids with a predicted molecular mass of 81.4 kDa (Figure 2A). The C-terminus of

this protein is homologous to clone A. We call this protein Daxx for Fas death domain associated protein. A database search using BLAST revealed that Daxx is a novel protein with no significant sequence similarity to any other protein. Daxx contains a region of 62 amino acids with a high content (71%) of glutamic acid and aspartic acid and contains two small proline-rich regions (Figure 2A).

To determine the tissue distribution of Daxx, we performed a Northern analysis with a Daxx C-terminal probe. A 2.6 kb transcript, consistent with the length of the open reading frame, was detected in various adult mouse tissues (Figure 2B). The expression of Daxx appeared uniform, with the exception of stronger expression in testis. Shorter hybridizing transcripts were also detected in liver, kidney, and testis. Cell lines from many tissues have been reported to support the ability of ectopically expressed Fas to induce apoptosis, suggesting that the downstream signaling mechanism is present in most tissues. Similarly, FADD is expressed ubiquitously in adult tissues (Boldin et al., 1995b; Chinnaiyan et al., 1995).

**Daxx Interacts with Fas Both in vitro and in vivo**

In the two-hybrid system, the Daxx C-terminal region interacted strongly with Fas, confirming that Daxx is the functional homolog of clone A21 (Figure 1B). We then tested the binding of full length murine Daxx protein in vitro and in mammalian cells. In vitro translated, $^{35}$S-labeled Daxx bound to immobilized glutathione S-transferase (GST) fusion proteins of Fas death domain and TNFR1 intracellular tail but not to immobilized GST, GST-CD40 intracellular tail or GST-Fas *lpr*$^{cg}$ death domain (Figure 3A). Daxx migrated with an apparent molecular weight of approximately 120 kDa on SDS-PAGE; this slower than expected migration may reflect the high content of acidic residues in Daxx. In the GST pull-down assay, $^{35}$S-Daxx bound to GST-FasDD but only very weakly to GST-TNFR1. This discrepancy with the two-hybrid result (Figure 1) may be due to the nonlinear readout of the two-hybrid system. Deletion of 162 amino acids from the C-terminus of Daxx abrogated binding to GST-FasDD while the C-terminal 112 amino acids

(DaxxC) was sufficient to bind GST-FasDD (Figure 3B), consistent with the two-hybrid results.

To determine whether Daxx interacted with Fas in mammalian cells, human embryonic kidney 293 cells were cotransfected with constructs expressing hemagglutinin-tagged Daxx (HA-Daxx) and GST-Fas intracellular tail (GST-FasIC). HA-Daxx was coprecipitated with GST-FasIC but not with GST using glutathione beads. Again, this interaction was dependent on the C-terminus of Daxx (Figure 3C). GST-FasIC was also able to coprecipitate HA-tagged Fas death domain, confirming that death domains may multimerize (data not shown). Because Fas is overexpressed and thereby is activated, we are uncertain whether Daxx binds to the inactive Fas. Collectively, these data show that the C-terminus of Daxx mediates an interaction between the Fas death domain and Daxx, and that this interaction is likely to occur directly and in vivo.

**Daxx Potentiates Fas-mediated Apoptosis**

To study the role of Daxx in Fas signaling, we chose 293 cells and HeLa cells. Both are sensitive to Fas- and TNF-mediated apoptosis, and their normally flat morphology facilitates the scoring of apoptotic cells, characterized by membrane blebbing, pyknosis, and cell body condensation (Figure 4A). Cells scored to be apoptotic by morphology also exhibited nuclear condensation and fragmentation as judged by Hoechst staining (data not shown). HeLa and 293 cells were transfected with various expression constructs and an expression construct for β-galactosidase; at defined times after the transfection, cells were stained for β-galactosidase activity to mark the transfected cells and scored for apoptotic morphology. In 293 cells, transient overexpression of Fas induced apoptosis in a dose-dependent and saturable manner (Figure 4B). Fas activation in the absence of activating ligand is due to a documented propensity of death domains to multimerize (Boldin et al., 1995a). However, the addition of activating anti-Fas antibodies, Jo2, did not increase cell death in Fas-transfected 293 cells, implying that a function downstream of receptor activation may be limiting (data not shown). Overexpression of Daxx by itself did not

induce apoptosis, but Daxx coexpression significantly enhanced Fas-mediated apoptosis (Figure 4B). Parallel experiments with TNFR1 did not show any enhancement of apoptosis by Daxx, consistent with the much lower affinity of Daxx for TNFR1 (Figure 3A).

In HeLa cells, transient transfection of Fas led to robust, dose-dependent and saturable cell death (Figure 4A, C), which was further enhanced by the addition of Jo2. As in 293 cells, overexpression of Daxx alone did not induce apoptosis in HeLa cells. In the range where apoptosis was proportional to input Fas DNA, coexpression of Daxx significantly increased Fas-mediated apoptosis (Figure 4C), suggesting that Daxx activity may be a rate limiting step downstream of receptor engagement.

In an analogous approach to assess the function of Daxx, we established murine fibroblast L929 cell lines that stably overexpressed Daxx (L/Daxx). L/Daxx cells are substantially more susceptible to Fas killing compared to vector transfected cells (L/EBB). This stimulation effect appeared to be a kinetic one: compared to L/EBB, the L/Daxx culture had greater than three-fold more apoptotic cells 24 hours after Fas transfection, but L/EBB cells caught up by 48 hours after transfection (Figure 4D). TNF-α–, TNFR1-, or FADD-mediated apoptosis was not increased in L/Daxx cells (Figure 4D and not shown). Therefore, L/Daxx cells are not generally more sensitive to apoptosis, but are specifically sensitized to the Fas signal, suggesting that Daxx is a mediator of Fas-induced killing.

**Daxx Activates the JNK/SAPK Pathway**

The lack of death domain homology or constitutive cell death activity suggests that Daxx may play a different role from previously identified death domain binding proteins in Fas signaling. Fas has been reported to activate the JNK pathway (Latinis and Koretzky, 1996; Lenczowski et. al., 1997; Goillot et. al., 1997), which is required in certain cell lines for the analogous TNF-α-induced apoptosis (Verheij et al., 1996). We therefore analyzed the ability of Fas and Daxx to activate the kinase activity of JNK and JNK-dependent transcription. In transient transfection assays in 293 cells, Fas activated JNK-1, the major

JNK activity in cells (Derijard et al., 1994). Fas-induced JNK activation was not blocked by the serpin ICE inhibitor crmA (Figure 5A), a peptide ICE inhibitor Z-VAD, or a peptide CPP32 inhibitor Z-DEVD (data not shown; Goillot et. al., 1997). FADD overexpression did not induce JNK activation (Figure 5A). Therefore, Fas activation of JNK is not secondary to FADD activity or apoptosis. Interestingly, Daxx overexpression activated JNK-1 to a level similar to that of Fas (Figure 5A). To assay endogenous JNK activation by Fas and Daxx, we used L929 cells stably expressing murine Fas (L/Fas) and the L/Daxx and L/EBB cells. In L/Fas cells, Fas-induced JNK activation was observed approximately 15 minutes after Fas ligation and reached maximal activity in about one hour (Figure 5A and data not shown). L/Daxx cells had constitutive activation of JNK activity compared to L/EBB cells (Figure 5B), and the level of JNK activation correlated with the level of Daxx overexpression in various L/Daxx cell lines (data not shown).

As an independent measure of JNK activity, we tested the ability of Fas and Daxx to stimulate signaling to SRF (Serum Response Factor). JNK can phosphorylate and activate SRF independent of the MEK/MAPK pathway, and the level of JNK activation in vivo can be assayed using a reporter gene driven by SRE-L, a derivative of SRE that specifically binds SRF (Hill et. al., 1995). In 293 cells, Fas induced SRF-dependent transcription about 4-fold, and Daxx induced it about 6-fold. TNF-$\alpha$, a known inducer of the JNK pathway, stimulated the SRF-reporter gene to a level similar to that induced by Fas or Daxx (Figure 5C).

Collectively, these data show that Daxx is an activator of the JNK pathway.

**Deletion Mutagenesis of Daxx**

To further dissect Daxx signaling, we asked which regions of Daxx were required for its three activities: Fas binding, enhancement of apoptosis, and activation of JNK. We have already determined that the C-terminal 112 amino acids of Daxx (DaxxC) are necessary and sufficient for Fas binding (Figure 3B). Significantly, Daxx mutants missing either the C-terminus (Daxx$\Delta$C) or N-terminal 500 amino acids (DaxxC501) acquired a

modest constitutive cell death activity for 293 cells in the absence of Fas (Figure 6A). HeLa cells and L929 cells were not sensitive to this activity. The result in 293 cells suggested that deletion of either end of Daxx activated a normally latent cell death activity. Because DaxxΔC is unable to bind Fas death domain (Figure 3B, C), this cell death activity is likely to be independent of Fas or other death domain proteins. Further deletions revealed that a peptide containing amino acids 501 to 625, which lies immediately N-terminal to the Fas binding domain, contained most of the cell death activity (Figure 6A).

When these Daxx mutants were tested for their ability to activate JNK, we observed that each deletion mutant maintained some level of JNK activity with the exception of DaxxC; the majority of the activity came from just the region with amino acids 501-625 (Figure 6A). This result suggests that JNK activation may be involved in Daxx-stimulated apoptosis. However, full length Daxx activates JNK but does not cause constitutive apoptosis, suggesting that full length Daxx may activate other pathways that counterbalance the apoptotic JNK signal.

**DaxxC is a Dominant Negative Inhibitor of Fas-mediated Apoptosis and JNK Activation**

Deletion mutagenesis showed that DaxxC, the C-terminal 112 amino acids of Daxx, was necessary and sufficient to bind Fas but more N-terminal domains were required to activate JNK and cell death. Thus, we tested whether DaxxC can act as a dominant negative inhibitor of endogenous Daxx by competing with its binding to Fas. We chose to use HeLa cells in these experiments because the cells have a robust response to transfected Fas (Figure 4C) and are the source of clone A21 from the two-hybrid screen. In Figure 6B, we show that expression of DaxxC gave a dose-dependent suppression of Fas-mediated apoptosis. Fas-induced c-Jun phosphorylation was also inhibited by DaxxC in HeLa cells (data not shown) and in 293 cells (Figure 6C). To address the specificity of DaxxC, we then coexpressed full-length Daxx with DaxxC and asked if this combination now reversed the dominant negative effect. If DaxxC were binding other death domain

containing proteins (e.g. FADD), coexpression of full length Daxx would further titrate FADD away from Fas and inhibit apoptosis. Instead, coexpression of Daxx with Fas and DaxxC gave a dose-dependent rescue of Fas-induced apoptosis (Figure 6B). This result argues that the only functions made deficient by DaxxC are those of intact Daxx, implying that DaxxC specifically competes with endogenous Daxx but not other proteins for binding to Fas. These results suggest that endogenous Daxx is required for Fas-induced apoptosis and JNK activation.

**Daxx and FADD Define Two Distinct Fas-mediated Signaling Pathways**

Because Daxx and FADD are both required for Fas-induced apoptosis, we assessed how these two effectors may be related to each other by a dominant negative approach. The FADD death domain, FADD(80-205), has been shown to block Fas-induced death presumably by preventing the binding of endogenous FADD (Chinnaiyan et al., 1996). We found that FADD(80-205) partially inhibited Fas-induced death (Figure 7A, lanes 1-3) but did not inhibit JNK activation (Figure 7B). Moreover, the effect of FADD(80-205) on cell death was not reversed by coexpression of excess Daxx (Figure 7A, lane 4). These results contrast with the effects of DaxxC (Figure 6) and suggest that Daxx and FADD bind independently to Fas and activate distinct pathways. Consistent with this interpretation, FADD- induced cell death is not blocked by DaxxC (Figure 7C). In addition, DaxxC plus FADD(80-205) inhibited Fas-induced cell death substantially more than saturating amounts of either dominant negative protein alone (Figure 7A, lanes 7). Thus, Daxx and FADD activate apoptosis downstream of Fas by distinct but cooperative pathways.

Fas-mediated apoptosis can be inhibited by crmA and in some cell types by Bcl-2, a negative regulator of cell death (Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995; Itoh et al., 1993; Lacronique et. al., 1996). To dissect the apoptotic pathways initiated by overexpression of Fas, Daxx, and FADD, we tested the ability of crmA, Bcl-2, SEK(AL) and TAM67 to block each apoptotic inducer. SEK(AL) and TAM67 are dominant negative inhibitors of the JNK pathway. SEK1 is the kinase that phosphorylates

and activates JNK; SEK(AL) encodes a mutant that has a single mutation at the ATP-binding site, abrogating the kinase activity (Sanchez et al., 1995). TAM67 is a variant of c-Jun in which amino acids 3-122 have been deleted. This mutant can dimerize and bind DNA but lacks a transcriptional activation domain (Brown et al., 1994). First, we tested the ability of the panel of inhibitor genes to block Fas in several cell types commonly used in apoptosis studies. We found that Fas-induced apoptosis in L929, 293, and HeLa cells can be blocked by crmA and Bcl-2-type inhibitors, but only 293 cells and L929 cells required the JNK pathway for Fas-induced apoptosis (Figure 8A). This result is consistent with the work of Liu et. al., who reported that the JNK pathway appeared dispensable for TNF-α-induced apoptosis in HeLa cells (Liu et al., 1996). Kolesnick and colleagues have reported that TNF-α-induced apoptosis is inhibited in U937 human monoblastic leukemia cells that stably express TAM67 (Verheij et al., 1996); these cells are also resistant to Fas-mediated apoptosis (data not shown). Taken together, these observations indicate that the requirement for the JNK pathway in Fas-mediated apoptosis is cell-type specific. We note that this type of dominant negative experiment gives a positive result only if the protein in question is uniquely required for a particular process. In a case where a negative result (no inhibition) is obtained, it may be that the protein in question is involved but is functionally redundant or in great excess. The continued discovery of JNK relatives makes such scenarios plausible (Gupta et al., 1996).

Next, we tested the same panel of inhibitor genes on FADD and Daxx-induced apoptosis. Since full length Daxx does not induce apoptosis by itself, we used two alternative strategies: we examined the apoptotic response of Fas plus Daxx in 293 cells (where a large fraction of the apoptotic response is Daxx-dependent, Figure 4B) and the apoptotic response of Daxx 501-625, the smallest domain that has constitutive cell death activity in 293 cells (Figure 6A). Daxx-dependent apoptosis was blocked by crmA, Bcl-2, and required the JNK pathway, which paralleled the inhibition profile of Fas in 293 cells (Figure 8B). In contrast, FADD, which can not activate JNK, was inhibited only by

crmA, but not by Bcl-2, SEK(AL), or TAM67. Similarly, Hsu et al. have shown that TRADD mediated apoptosis is not blocked by Bcl-2 (Hsu et al. 1995). Consistent with the two pathway model, the residual Fas-induced death remaining after either DaxxC or FADD(80-205) treatment are qualitatively different: the apoptosis remaining after FADD(80-205) treatment is Bcl-2 sensitive but the apoptosis remaining after DaxxC treatment is not (Figure 7A, lanes 8 and 9). Our results suggest that Fas activates two distinct cell death pathways--one via FADD that is Bcl-2 insensitive and a second one via Daxx that activates JNK and is Bcl-2 sensitive.

## Discussion

### Daxx is the Missing Link between Fas and the JNK pathway

The Fas-FADD-FLICE connection is currently the best understood model for apoptotic signal transduction (Fraser and Evan, 1996; Nagata, 1997), but its inability to explain Fas-induced JNK activation suggests that the current model is at best incomplete. We and other investigators have demonstrated that Fas can robustly activate the JNK pathway (Figure 5; Latinis and Koretzky, 1996; Lenczowski et. al., 1997; Goillot et. al., 1997). JNK activation is unlikely to be secondary to apoptosis because it is not inhibited by blocking the apoptotic caspases (Figure 5A). The fact that FADD induces cell death but not JNK activation provides powerful evidence that Fas must engage additional signaling molecules in order to activate JNK. While RIP and TRAF2 are recruited by TNFR1 to activate JNK, currently no known Fas effector can account for its JNK activation. In this study, Daxx emerges as the missing link between Fas and the JNK pathway. Daxx directly binds to the death domain of Fas, and overexpression of Daxx or its effector domain is sufficient to activate the JNK pathway. In addition, a specific dominant negative inhibitor of Daxx blocks Fas-induced JNK activation. The JNK pathway is activated by many stress stimuli (such as oxidative damage, irradiation, and ischemia-reperfusion) that culminate in apoptosis (Verheij et. al., 1996), but the signaling events upstream of the

kinase cascades are poorly understood. Daxx may have general importance in coupling other death signals to JNK, and its effector domain should be a useful probe for uncovering the molecular connections that lead to JNK activation.

It is becoming increasingly clear that activation of the JNK pathway can induce apoptosis. For example, ASK1, an activating kinase of the JNK pathway that functions in response to TNF-α is both sufficient to induce apoptosis and is required for TNF-induced cell death (Ichijo et. al., 1997) Similarly, MEKK, the upstream kinase of the classical JNK kinase cascade, induced apoptosis upon ectopic expression (Johnson et. al., 1996). Because the JNK pathway culminates in the activation of transcription factors, the JNK pathway may directly or indirectly counteract the expression of survival factors, such as NF-kB and Bcl-2. It is well known that in many cell types, Fas-mediated apoptosis is greatly enhanced by transcriptional and translational inhibitors, suggesting an important role for the modulation of gene expression during apoptosis

However, the involvement of the JNK pathway in apoptosis is commonly analyzed by dominant negative proteins and may be complicated by several factors. For example, Fas-mediated apoptosis has been reported to not involve JNK in Jurkat cells (Lenczowski et al., 1997) but to require JNK in SHEP cells (Goillot et. al., 1997). These results may reflect a cell-type dependent variation of the relative contribution of Daxx and FADD pathways. Alternatively, a negative result may reflect the functional redundancy of kinase isoforms in the MAP kinase cascades that make up the JNK and p38/Mpk2 pathways. Another possibility is that various tissue culture cell lines are originally derived from different tumors, and oncogenes, such as Ha-Ras, are known to activate the JNK pathway (Hibi et al., 1993; Derijard et al., 1994), giving different basal JNK activity in different cell lines. As the most receptor-proximal protein leading to JNK activation, Daxx may be an experimentally tractable target for dissecting the contribution of JNK signals to Fas-induced cell death. One prime example is in HeLa cells. Although dominant negative inhibitors of the JNK pathway did not inhibit Fas-induced death in HeLa cells, HeLa cells clearly

employ Daxx and the JNK pathway as shown by the ability of DaxxC to block Fas-induced JNK activation and cell death (Figure 6 and 7).

**Daxx and FADD Define Two Distinct Fas-induced Pathways to Apoptosis**

In this study, we present several lines of evidence that Daxx and FADD bind to Fas independently and activate distinct cell death signals. Although both Daxx and FADD cannot bind to the *lpr$^{cg}$* point mutant of Fas, NMR structural analysis of the Fas death domain suggest that this mutant may be unfolded and is thus uninformative for mapping the exact site of binding (Huang et. al., 1997). However, Daxx has no evident death domain and interacts with Fas death domain via a novel domain contained in the 112 amino acids of DaxxC while FADD interacts with Fas via homotypic death domain-death domain interactions. In addition, the Daxx-Fas interaction is not affected by the C-terminal 15 amino acids of Fas (Figure 1) while the FADD-Fas interaction is partially inhibited by this C-terminal "salvation domain" of Fas (Chinnaiyan et al., 1995). These differences between FADD and Daxx are consistent with a model of FADD and Daxx binding to distinct surfaces of the Fas death domain. We have produced mutations in the intracellular tail of Fas that selectively bind either Daxx or FADD (X.Y., H.Y.C., D.B., unpublished results). Moreover, dominant negative forms of Daxx and FADD (each consisting of the minimal Fas binding domains) did not inhibit the heterologous protein's biologic function even at saturating levels (Figure 7). This last result in particular suggests that Daxx and FADD bind to Fas independently in vivo.

Daxx and FADD not only appear to bind differently to Fas but transduce physiologically distinct death signals. As summarized by the model in Figure 8C, FADD directly binds FLICE and is thought to induce apoptosis by recruiting FLICE and activating a caspase cascade. However, this pathway cannot activate JNK. By contrast, Daxx transduces an apoptotic signal via JNK (Figure 8C). Because inhibition of caspases did not block JNK activation but blocked Daxx-induced cell death, the caspases are placed downstream of Daxx and JNK (Figure 8C). The Daxx pathway is also sensitive to Bcl-2

(Figure 7A and 8B). Although either the Daxx- or the FADD- induced pathway alone is sufficient to activate cell death, these two pathways probably work together in vivo. For example, in HeLa cells, both Daxx and FADD act downstream of Fas, and it is necessary to block both death signals to effectively protect the cell from Fas (Figure 7A). Why expand the number of potentially dangerous cell suicide signals if one signal is sufficient? As in the case of growth regulation, the collaboration of multiple signaling pathways allows fine-tuned regulation and creates multiple checkpoints for control. In this instance, incorporation of the Daxx pathway may allow the Fas death signal to be regulated by Bcl-2.

## Experimental Procedures

### Reagents and Cell Lines

Anti-murine Fas Jo2 antibody was the generous gift of S. Nagata (Ogasawara et al., 1993). Murine TNF-α (Genzyme), monoclonal antibody M2 against Flag epitope (Kodak/IBI), and anti-JNK-1 antibody C17 (Santa Cruz Biotech) were obtained from the indicated sources. HeLa, 293, and L929 were obtained originally from American Type Culture Collection (ATCC). To establish the L/Daxx cell line, HA-Daxx vector was cotransfected with pBabe-puro into L929 cells. Resistant cells were selected in media containing 2.5 μg/ml of puromycin and HA-Daxx expressing clones were identified by immunoblot analysis using anti-HA antibody 12CA9. The L929 cells expressing mFas (L/Fas) were established by transfecting pRc/CMV-mFas into L929 cells and subsequently selecting for resistant cells in 600 μg/ml G418. Resistant clones were then screened for Fas expression by FACScan using Jo2 antibody.

### Plasmid Construction

DNA fragments for most plasmid constructs were obtained by PCR amplification using Pfu polymerase (Stratagene) and primers incorporated with appropriate restriction sites and epitope tags as needed. The fragments for LexA and transcription activator fusions were cloned into plasmid pEG202 and pJG4-5 (Gyuris et al., 1993), respectively. The I225N mutation in LexA-*lpr*$^{cg}$ was made by PCR site-directed mutagenesis. GST constructs were made in pGEX vector (Pharmacia). In vitro translation constructs were made in pET3a (Novagen) or His6T-pRSET (A. Hoffmann) for DaxxC. Daxx(-162) was obtained by digestion of a full length Daxx construct with HindIII. For expressing proteins in mammalian cells, full length Daxx and FADD and their mutants were cloned into pEBB with a hemagglutinin (HA) epitope-tag at the 5' end. Full length murine Fas was cloned into pEBB and pRc/CMV (Invitrogen). Fas intracellular region (amino acids 165 to 306) was fused to GST in pEBG, a derivative of pEBB expressing GST. Full length murine

TNFR1 was cloned into pEBB. Each construct was confirmed by partial DNA sequence and by immunoblot analysis.

The following plasmids were described in or obtained from the indicated sources: LexA-CD40IC (G. Cheng, UCLA), LexA-Bicoid (Gyuris et al., 1993), pHD1.2 (crmA) and pBabe-Bcl-2 (J. Yuan, Harvard), Bcl-$X_L$ (R.J. Lutz, Apoptosis Inc.), TAM67 and Flag-JNK (M. Karin, UCSD), pEBG-SEK(AL) (C.J. Der, UNC), SRE-L-Luc (R. Treisman, ICRF, UK).

**Two-hybrid Screen and β–Galactosidase Assay**

The two-hybrid screen was performed essentially as described (Gyuris et al., 1993). Among 1 x $10^6$ library plasmids screened, 33 interacting clones were obtained. The cDNA inserts of the library plasmids within those colonies were isolated and grouped. Representative plasmids from each group were re-transformed back into yeast to test their interaction with different baits.

For assaying β-Galactosidase expression, each pair of DNA-binding (LexA) and activation hybrids were co-transfected together the lacZ reporter plasmid p18-34 (Gyuris et al., 1993) into EGY48 yeast cells. Filter lift assay for colony color and quantitative liquid assay were done as described (Yang et al., 1994) for three to six independent transformants.

**cDNA Cloning and Northern Blot Analysis**

The cDNA insert in clone A21 was used to screen a murine thymus cDNA libraries (Y.W.Choi, Rockefeller Univ.). Sequences of the longest cDNA clone (2.4kb) as well as the cDNA inserts of clone A21 were determined on both strands with an automated sequencer (Applied Biosystem). Sequence comparison was done by using GeneWorks program of IntelliGenetics, Inc. The Daxx amino acids 501 to 739 exhibit 57% sequence identity to clone A21.

For Northern analysis, the C-terminal 0.7 kb fragment of murine Daxx and a human β-actin cDNA (Clontech) were used to probe a mouse multiple tissue Northern blot (Clontech), according to manufacture's protocol.

**In Vitro Binding and Coprecipitation Assays**

Glutathione-S-transferase (GST) fusions were purified as described (Smith and Johnson, 1988). $^{35}$S-proteins were made with TNT Reticulocyte Lysate System (Promega). $^{35}$S-proteins were incubated with 10 μg of each GST fusion protein in 0.1 ml modified E1A buffer (Hsu et. al., 1996) with 50 mM NaCl and 10% glycerol for 1 to 2 hours, washed three times, and analyzed by SDS-PAGE and autoradiography. A fraction of the reaction mixture was analyzed by Coomassie staining to visualize GST fusion proteins.

For testing association in mammalian cells, HA-Daxx and HA-DaxxΔC were cotransfected with GST-FasIC or GST into 293 cells by calcium phosphate method. Thirty-six hours after cotransfection, cells were solubilized in E1A buffer, precipitated with glutathione beads (Molecular Probes), washed 3 times, and immunoblotted for HA using ECL (Amersham). Comparable levels of GST fusions were verified by immunoblotting for GST.

**Apoptosis Assays**

Cells were plated onto 6-well dishes the day before transfection at 2 x $10^5$ cells/well for Hela and 293 cells and 5 x$10^5$ for L/Daxx and L/EBB cells. The wells were precoated with 0.2% gelatin for 293 cells. HeLa and 293 cells were transfected by the calcium phosphate precipitation method and L/Daxx and L/EBB with lipofectamine (GIBCO-BRL). X-gal staining was done for 4 hours to overnight. The percentage of apoptotic cells was determined by the number of blue cells with apoptotic morphology divided by the total number of blue cells. Specific apoptosis was calculated as the percentage of blue cells with apoptotic morphology in each experimental condition minus the percentage of blue cells

with apoptotic morphology in pEBB vector-transfected cells. pEBB vector control transfection was always done in parallel and had about 5 percent or less apoptotic cells. At least 400 cells from 4 random fields were counted in each experiment and the data shown are the average and s.d. of at least three independent experiments.

For L929 apoptosis assay in Figure 8, L/Fas cells were seeded onto 6-well plates 2.5 x $10^5$ cells/well. The next day cells were co-transfected with 200 ng of pHook-1 plasmid (Invitrogen) and 400 ng of crmA, Bcl-xL or SEK(AL) with lipofectamine. Twenty-four hours after transfection cells were removed from the dish in 1 ml of PBS/3 mM EDTA. Magnetic beads (1.5 x $10^{6)}$ were added to the cells and incubated at 37 $^0$C for 30 min. The cells were then washed 3 times in media, counted and plated in duplicate in 96 well plates (~5000 cells/well). After culture for 14-16 hours, Fas-killing was induced using 1 μg/ml Jo2 antibody and 0.5 μg/ml actinomycin D and measured by counting the number of surviving cells in four random fields 24 hours later.

**JNK Activity and Reporter Gene Assays**

HeLa and 293 cells were transfected in 60 mm dishes with Flag-JNK plus the indicated expression plasmids by the calcium phosphate method. Approximately 24 hours after transfection cells were serum starved for 14-16 hours. Cells transfected with Fas were treated with 0.5 μg/ml of Jo2 antibody for 30 min. To test for the effect of protease inhibitors, cells were treated with 0.5 μg/ml of Jo2 and 100 μM of ICE inhibitor Z-Val-Ala-Asp-CH2F or CPP32 inhibitor Z-Asp-Glu-Val-Asp-CH2F (Enzyme Systems Products, CA) for 30 min. JNK-1 was immunoprecipitated with anti-Flag antibody, and in vitro kinase assay with 1μg of GST-cJun (1-79) was performed as previously described (Khosravi-Far et al., 1996). JNK1 kinase activity in L929 cells stably expressing Fas or Daxx was measured by immunoprecipitation of the endogenous JNK-1 using anti-JNK-1 C-17 antibody. TNFR1- and Fas-transfected cells were treated with 20 ng/ml TNF-α for 10 minutes and 0.5 μg/ml Jo2 for 30 minutes, respectively.

SRE-L reporter gene assay: 293 cells were cotransfected with 1 μg of the indicated plasmids with 1μg of SRE-L-Luc reporter construct (Hill et al., 1995). After an incubation of 24 hours, cells were switched to 0.5% FCS media for 14-16 hours. Cell lysates were prepared in 250 μl reporter lysis buffer (Promega) and 20 μl of the lysate was assayed in Luminometer with 100 μl ATP and Luciferin reagents (Promega) as described (Khosravi-Far et al., 1996).

**Acknowledgments**

We are indebted to Dr. Genhong Cheng for his help in initial phase of this project. We thank Drs. R. Brent, Y.W. Choi, C.J. Der, D.V. Goeddel, M. Karin, R.N. Kolesnick, P.H. Krammer, R.J. Lutz, S. Nagata, R. Treisman, D. Wallach, and J. Yuan their generous gifts of reagents and S.W. Fesik for examining the Daxx sequence. We thank P. Svec and Y. Li for excellent technical assistance and Drs. A.J. Koleske, B. Chen, Z. Songyang, Y. Yamanashi, I. Stancovsky and other members of Baltimore lab for valuable advice. X.Y. and R.K.-F. are fellows of the Leukemia Society of America and Irvington Research Institute, respectively. H.Y.C. is supported by the Medical Scientist Training Program at Harvard Medical School. D.B. is an American Cancer Society Research Professor. This work was supported by the NIH grant CA51462.

## References

Abbas, A. K. (1996). Die and let live: eliminating dangerous lymphocytes. Cell *84*, 655-658.

Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. Cell *85*, 803-815.

Boldin, M. P., Mett, I. L., Varfolomeev, E. E., Chumakov, I., Shemer-Avni, Y., Camonis, J. H., and Wallach, D. (1995a). Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/Apo1 prompts signalling for TNF and Fas/Apo1 effects. J. Biol. Chem. *270*, 387-391.

Boldin, M. P., Varfolomev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995b). A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J. Biol. Chem. *270*, 7795-7798.

Brown, P. H., Chen, T.K., and Birrer, M. J. (1994). Mechanism of action of a dominant-negative mutant of c-Jun. Oncogene *9*, 791-799.

Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996). FADD/MORT is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J. Biol. Chem. *271*, 4961-4965.

Cleveland, J. L., and Ihle, J. N. (1995). Contenders in Fas/TNF death signaling. Cell *81*, 479-482.

Derijard, B., Hibi, M., Wu, I.-H., Barret, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-ras that binds and phosphorylates the c-Jun activation domain. Cell *76*, 1025-1037.

Enari, M., Hug, H., and Nagata, S. (1995). Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature *375*, 78-81.

Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996). Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature *380*, 723-726.

Fraser, A., and Evan, G. (1996). A license to kill. Cell *85*, 781-784.

Goillot, E., Raingeaud, J., Ranger, A., Tepper, R.I., Davis, R.J., Harlow, E., and Sanchez, I. (1997). Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. Proc. Natl. Acad. Sci. USA *94*, 3302-3307.

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996). Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO *15*, 2760-2770.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphotase that associated with Cdk2. Cell *75*, 791-803.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV- responsive protein kinase that binds and phosphorylates the c-Jun activation domain. Genes Dev *7*, 2135-2148.

Hill, C.S., , Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell *81*, 1159-1170.

Hsu, H., Xiong, J., and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kB activation. Cell *81*, 495-504.

Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D. V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell *84*, 299-308.

Huang, B., Eberstadt, M., Olejniczak, E.T., Meadows, R.P., Fesik, S.W. (1996). NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. Nature *384*, 638-641.

Ichijo, H. , Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science *275*, 90-94.

Itoh, N., and Nagata, S. (1993). A novel protein domain required for apoptosis. J. Biol. Chem. *268*, 10932-10937.

Itoh, N., Tsujimoto, Y., and Nagata, S. (1993). Effect of bcl-2 on Fas antigen-mediated cell death. J. Immunol. *151*, 621-627.

Johnson, N. L., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Gleavy, J., Jarpe, M. B., Minden, A., Karin, M., Zon, L. I., and Johnson, G. L. (1996). Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death. J. Biol. Chem. *271*, 3299-3237.

Khosravi-Far, R., White, M. A., Westwick, J. K., Solski, P. A., Chrzanowska-Wodnicka, M., van Aelst, L., Wigler, M. H., and Der, C. J. (1996). Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathway is sufficient to cause tumorigenic transformation. Mol. Cell. Biol. *16*, 3923-3933.

Kyriakis J.M., and Avruch, J. (1996). Protein kinase cascades activated by stress and inflammatory cytokine. BioEssay *18*, 567-577.

Lacronique, V., Mignon, A., Fabre, M., Viollet, B., Rouquet, N., Molina, T., Porteu, A., Henrion, A., Bouscary, D., Varlet, P., Joulin, V., and Kahn, A. (1996). Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. Nat. Med. *2*, 80-86.

Latinis, K. M., and Koretzky, G. A. (1996). Fas ligation induces apoptosis and Jun kinase activation independently of CD45 and Lck in human T cells. Blood *87*, 871-875.

Lenczowski, J. M., Dominguez, L., Eder, A. M., King, L. B., Zacharchuk, C. M., and Ashwell, J. D. (1997). Lack of a role for Jun kinase and AP-1 in Fas-induced apoptosis. Mol. Cell. Biol. *17*, 170-181.

Liu, Z.-g., Hsu, H., Goeddel, D. V., and Karin, M. (1996). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kB activation prevents cell death. Cell *87*, 565-576.

Los, M., Van de Craen, M., Penning, L. C., Shenk, H., Westendorp, M., Baeuerle, P. A., Droge, W., Krammer, P. H., Fiers, W., and Schulze-Osthoff, K. (1995). Requirement of an ICE/CED3 protease for Fas/APO1-mediated apoptosis. Nature *375*, 81-83.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. 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). FLICE, a novel FADD-homologous ICE/CED-3 like protease, is recruited to the CD95 (Fas/Apo1) death inducing signal complex. Cell *85*, 817-827.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-365.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. (1993). Lethal effect of the anti-Fas antibody in mice. Nature *364*, 806-809.

Rathmell, J. C., Townsend, S. E., Xu, J. C., Flavell, R. A., and Goodnow, C. C. (1996). Expansion or elimination of B cells in vivo: dual roles for CD40- and Fas(CD95)-ligands modulated by the B cell antigen receptor. Cell *87*, 319-329.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Clonning: A Laboratory Manual, Second Edition Edition (Cold Spring Harbor, New York: Cold Spring Harbor Press).

Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M., Zon, L.I. (1994). Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature 372, 794-798.

Smith, C. A., Farrah, T., and Goodwin, R. G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation , and death. Cell *76*, 959-962.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in E. coli as fusions with glutathione-S-transferase. Gene *67*, 31-40.

Tartaglia, L. A., Ayres, T. M., Wong, G. H. W., and Goeddel, D. V. (1993). A novel domain within the 55 kD TNF receptor signals cell death. Cell *74*, 845-853.

Tewari, M., and Dixit, V. M. (1995). Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. J. Biol. Chem. *270*, 3255-3260.

Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. N. (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature *380*, 75-79.

Watanabe-Fukunaga, R., Brannan, C. I., Itoh, N., Yonehara, S., Copeland, N. G., Jenkins, N. A., and Nataga, S. (1992). The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J. Immunol. *148*, 1274-1279.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science *270*, 1326-1331.

Yang, X., Jiang, R., and Carlson, M. (1994). A family of proteins containing a conversed domain that mediates interation with the yeast SNF1 protein kinase complex. EMBO J. *13*, 5878-5886.

## FIGURE LEGENDS

Figure 1. Interaction of Clone A and Daxx with Fas Death Domain in Yeast

(A) Schematic representation of the cytoplasmic domain of murine Fas (Watanabe-Fukunaga et al., 1992). Boundaries of the transmembrane domain (TM), death domain (DD), and the negative regulatory domain (NR) are labeled.

(B) Protein interactions in the two-hybrid system. LexA constructs contained the indicated sequences (amino acids in parenthesis) of receptors which expressed similar level of fusion proteins in yeast. Colony color and β-galactosidase units were determined as described in Experimental Procedures. The activation hybrid Act-DaxxC501 contained amino acids 501 to 739 of Daxx.

(C) The C-terminus of clone A interacts with Fas death domain. Amino acids contained in each activation hybrid are indicated.

Figure 2. Daxx Sequence and mRNA Distribution

(A). Conceptually translated amino acid sequence of Daxx protein. The open reading frame of murine Daxx follows an in-frame stop codon and begins with a Kozak consensus sequence. The regions enriched for acidic residues and proline are underlined. The partial human cDNA sequence from A21 is shown below the mouse sequence with identical amino acids indicated by "-".

(B). Tissue distribution of Daxx. A mouse multiple tissue Northern blot was probed with a C-terminal 0.7 kb fragment of Daxx and a human β-actin cDNA .

Figure 3. Interaction of Daxx with Fas in vitro and in mammalian cells.

(A) Binding of in vitro translated $^{35}$S-Daxx to GST-fusion proteins. Positions of MW standards (in kDa) are shown at left. Coomassie stained GST fusion proteins from the same gel were aligned to show protein levels.

(B) Binding of full length and truncated $^{35}$S-Daxx to GST (lane 3, 5, 7) and GST-FasDD (lane 2, 4, 6). Daxx(-162) lacked the C-terminal 162 aa of Daxx; DaxxC corresponded to aa 628-739. Input of $^{35}$S-Daxx and $^{35}$S-Daxx(-162) proteins in binding assays are shown in lane 1 and 2, respectively. GST fusion proteins are shown on the bottom panel.

(C) Association of HA-Daxx and HA-DaxxΔC (lacking aa 626-739) with the GST fusion of Fas intracellular tail (GST-FasIC) in 293 cells (top panel). The presence of HA-Daxx and HA-DaxxΔC in extracts was verified by immunoblotting for HA (bottom panel).

Figure 4. Daxx Potentiates Fas-induced Apoptosis

(A) Normal and apoptotic 293 and HeLa cells. Cells were transiently transfected with the indicated plasmids, stained with X-gal, and examined by light microscopy. Fields were chosen to illustrate morphologic differences but not relative percentages of apoptosis. Scale bar = 50 μm.

(B) Daxx potentiates Fas-induced apoptosis in 293 cells. Indicated amounts (in μg) of pEBB-Fas and pEBB-HA-Daxx plasmids were cotransfected with 0.5 μg of pCMV-lacZ. Amounts of transfected DNA were equalized by adding vector DNA. The cells were stained with X-gal 20 hours after transfection and analyzed for apoptotic morphology as described in Experimental Procedures.

(C) Daxx potentiates Fas-induced apoptosis in HeLa cells. Transfection and specific apoptosis were done and measured as in 293 cells except that X-gal staining was done at 24 hrs after transfection.

(D) L929 cells stably overexpressing Daxx have accelerated apoptosis in response to Fas. L/EBB and L/Daxx were transfected with 1 μg of pEBB-Fas, pEBB-TNFR1, or pRK-FADD plus 0.2 μg of pCMV-lacZ. Specific apoptosis was determined as in Figure 4B at indicated time after transfection. Similar results were obtained with multiple L/Daxx lines.

Figure 5. Daxx Activates the JNK Pathway

(A) Daxx activates JNK in transient transfection. Flag-tagged JNK1 (Flag-JNK) and the indicated plasmids (1 μg each) were cotransfected into 293 cells. Top: phosphorylation of GST-cJun. Bottom: expression of Flag-JNK. The data shown are representative of four independent assays.

(B) Stable expression of Daxx constitutively activates JNK. Top: phosphorylation of GST-Jun. Bottom: expression of endogenous JNK. The data shown are representative of three independent assays.

(C) Daxx activates a JNK-dependent reporter gene. The data shown are the average and s.d. of three independent experiments in duplicate.

Figure 6. Deletion Analysis of Daxx

(A) Apoptosis and JNK activation by Daxx deletion mutants. The horizontal bars represent Daxx sequences present in deletion mutants. Apoptosis assay: 3 μg of each Daxx mutant construct was transfected into 293 cells as in Figure 4B. JNK assay: transient transfection of 1 μg of each Daxx mutant construct or pEBB vector (v) with 1 μg of Flag-JNK and in vitro JNK assay was done as in Figure 5A. Equal Flag-JNK expression was verified by immunoblotting for Flag.

(B) DaxxC inhibits Fas-induced apoptosis. HeLa cells were transfected with 0.5 μg pEBB-Fas and pCMV-lacZ and the indicated amount (in μg) of HA-Daxx and HA-DaxxC. Total amount of transfected DNA was made constant by adding pEBB. Jo2 antibody (12.5 ng/ml) was added 16 hrs later. X-gal staining was done at 24 hrs after transfection.

(C) DaxxC inhibits Fas-induced JNK activation. Transient transfection of 1 μg of each indicated plasmid with 1 μg of Flag-JNK and in vitro JNK assay were done as in Figure 5A.

Figure 7. Daxx and FADD activate distinct apoptotic pathways.

(A) Inhibition of Fas-induced apoptosis by DaxxC and FADD(80-205). HeLa cells were transfected with pEBB-Fas (0.5 μg), pCMV-lacZ (0.5 μg), and plasmids expressing the indicated genes (in μg). Jo2 (12.5 ng/ml) was added 16 hrs later; X-gal staining was done 24 hrs after transfection.

(B) FADD(80-205) fails to inhibit Fas-induced JNK activation. JNK kinase activity was assayed after transient transfection of the indicated plasmids (2 μg each) with Flag-JNK (2 μg).

(C) DaxxC does not inhibit FADD-mediated apoptosis. HeLa cells were transfected with the indicated amount (in μg) of FADD and DaxxC and assayed as Figure 4C.

Figure 8. Inhibition Profile of Daxx- and FADD- induced Apoptosis

(A) Inhibition profile of Fas-induced apoptosis in L929, 293, and HeLa cells. Transfection and apoptosis analysis in L/Fas cells were performed as described in Experimental Procedures. 293 cells were cotransfected with pEBB-Fas (2 μg) plus vector or plasmids expressing indicated genes (2 μg each) and pCMV-lacZ (0.5 μg) as in Figure 4B. HeLa cells were transfected with pEBB-Fas (1 μg) plus plasmids expressing the indicated genes (3 μg each) and pCMV-lacZ (0.5 μg); Jo2 (12.5 ng/ml) addition and X-gal staining were done as in Figure 6B.

(B) Inhibition of profile of Fas+Daxx, Daxx 501-625 and FADD in 293 cells. 293 cells were transiently transfected with Fas, Daxx, Daxx 501-625, or FADD (1 μg each) plus empty vector or plasmids expressing the indicated apoptotic inhibitor genes (3 μg each) and pCMV-lacZ (0.5 μg) as in Figure 4B.

(C) Two pathways of Fas signaling that induce cell death.

**Figure 1**

A


**Fas cytoplasmic tail**

B

| DNA-binding hybrid | Activation hybrid | Colony color | ß-Gal units |
|---|---|---|---|
| LexA-mFasIC (165-306) | A21 | Blue | 110 |
| LexA-mFasDD (192-295) | A21 | Blue | 120 |
| LexA-mFasDD (192-295) | Act-DaxxC501 | Blue | 180 |
| LexA-lpr$^{cg}$ (192-295, I224N) | A21 | Light Blue | 14 |
| LexA-mFasFD8 (192-283) | A21 | White | 7 |
| LexA-hFasIC (175-319) | A21 | Blue | 40 |
| LexA-mTNFR1DD (322-425) | A21 | Blue | 120 |
| LexA-mCD40 IC (198-285) | A21 | White | 4 |
| LexA-Bicoid | A21 | White | 2 |

C

| Activation hybrids | | | | Colony color | ß-Gal units |
|---|---|---|---|---|---|
| A21 | 1 | | 248 | Blue | 120 |
| A39 | 60 | | 248 | Blue | 120 |
| A21N | 1 | | 163 | White | 3 |
| A21C | 136 | | 248 | Blue | 110 |
| A21C1 | 170 | | 248 | White | 4 |

**Figure 2**

A

```
1    MATDDSIIVLDDDDEDEAAAQPGPSNLPPNPASTGPGPGLSQQATGLSEP
51   RVDGGSSNSGSRKCYKLDNEKLFEEFLELCKTETSDHPEVVPFLHKLQQR
101  AQSVFLASAEFCNILSRVLARSRKRPAKIYVYINELCTVLKAHSIKKKLN
151  LAPAASTTSEASGPNPPTEPPSDLTNTENTASEASRTRGSRRQIQRLEQL
201  LALYVAEIRRLQEKELDLSELDDPDSSYLQEARLKRKLIRLFGRLCELKD
251  CSSLTGRVIEQRIPYRGTRYPEVNRRIERLINKPGLDTFPDYGDVLRAVE
301  KAATRHSLGLPRQQLQLLAQDAFRDVGVRLQERRHLDLIYNFGCHLTDDY
351  RPGVDPALSDPTLARRLRENRTLAMNRLDEVISKYAMMQDKTEEGERQKR
401  RARLLGTAPQPSDPPQASSESGEGPSGMASQECPTTSKAETDDDDDDDDD
451  DDEDNEESEEEEEEEEEKEATEDEDEDLEQLQEDQGGDEEEEGGDNEGN
501  E.SPTSPSDFFHRRNSEPAEGL.RTPEGQQKRGLTETPASPPGASLDPPS
1    DK--M-SLQISNEK-L--GKQIS-SSGE--NK-RIVS-SLLSEEP-A-S-
549  TDAESSGEQLLEPLLGDESPVSQLAELEMEALPEE......RDISSPRKK
51   I----N---PE-LT-EE-------F---I----LDTPSSVET----S--Q
593  SEDSLPTILENGAAVVTSTSVNGRVSSHTWRDASPPSKRFRKEKKQLGSG
101  --EPFT-V-----GM-S---F--G--P-N-G-SG--C-KS------T---
643  LLGNSYI.KEPMAQQDSGQNTSVQPMPSPPLASVASVADSSTRVDSPSHE
151  P-----VERQRSVHEKN-KKICTL-S-PS----L-P-------------G
692  LVTSSLCSPSPSLLLQTPQAQSLRQCIYKTSVATQCDPEEIIVLSDSD 739
201  -------I---AR-S---HS-PP-PGTC-------------------- 248
```

B

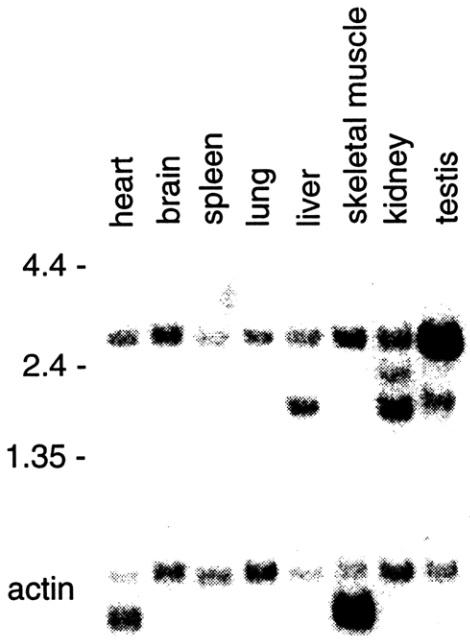
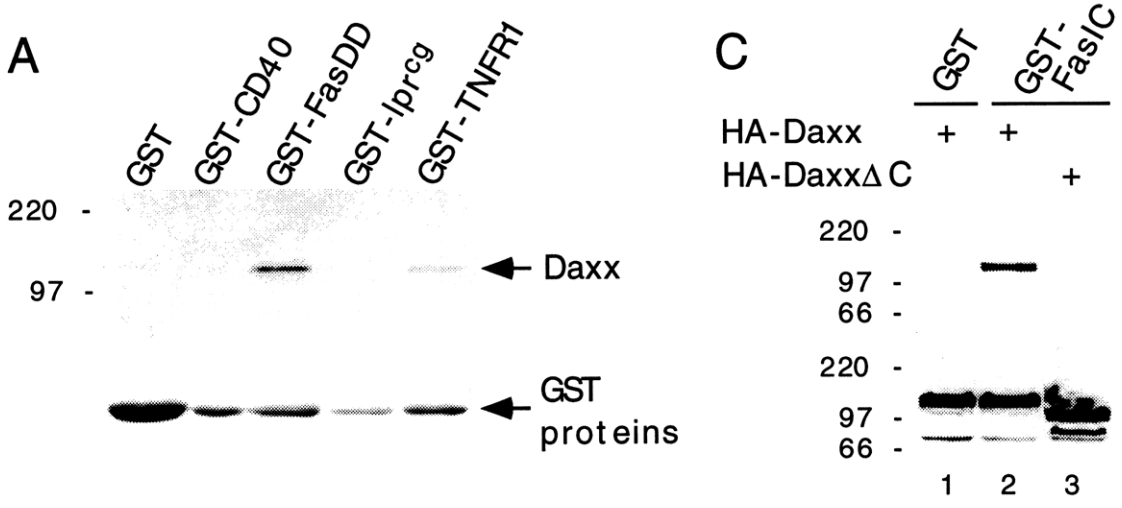
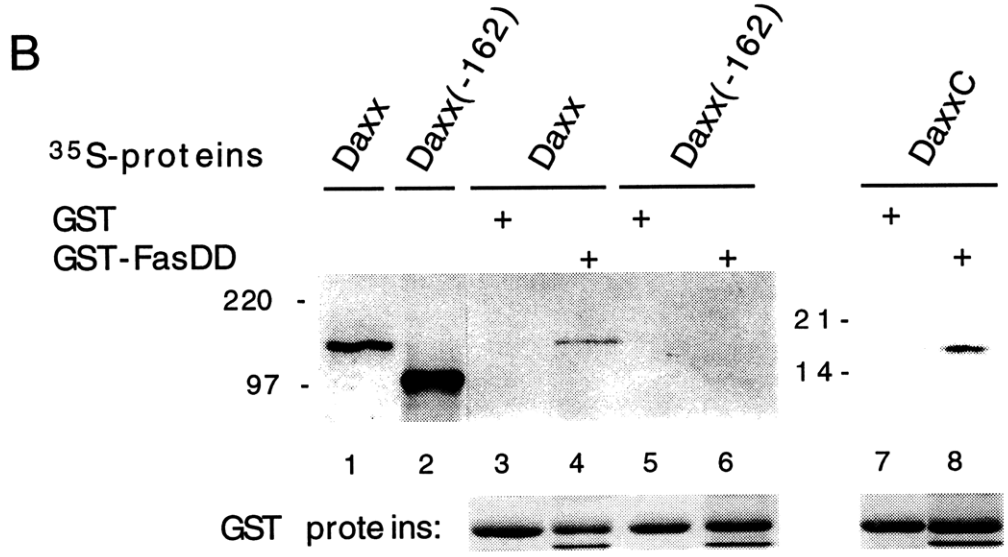


**Figure 3**


A
GST
GST-CD40
GST-FasDD
GST-lpr^cg
GST-TNFR1
220 -
97 -
Daxx
GST proteins
C
GST
GST-FasIC
HA-Daxx + +
HA-DaxxΔC +
220 -
97 -
66 -
220 -
97 -
66 -
1 2 3


B
35S-proteins
Daxx
Daxx(-162)
Daxx
Daxx(-162)
DaxxC
GST + + +
GST-FasDD + + +
220 -
97 -
21 -
14 -
1 2 3 4 5 6 7 8
GST proteins:

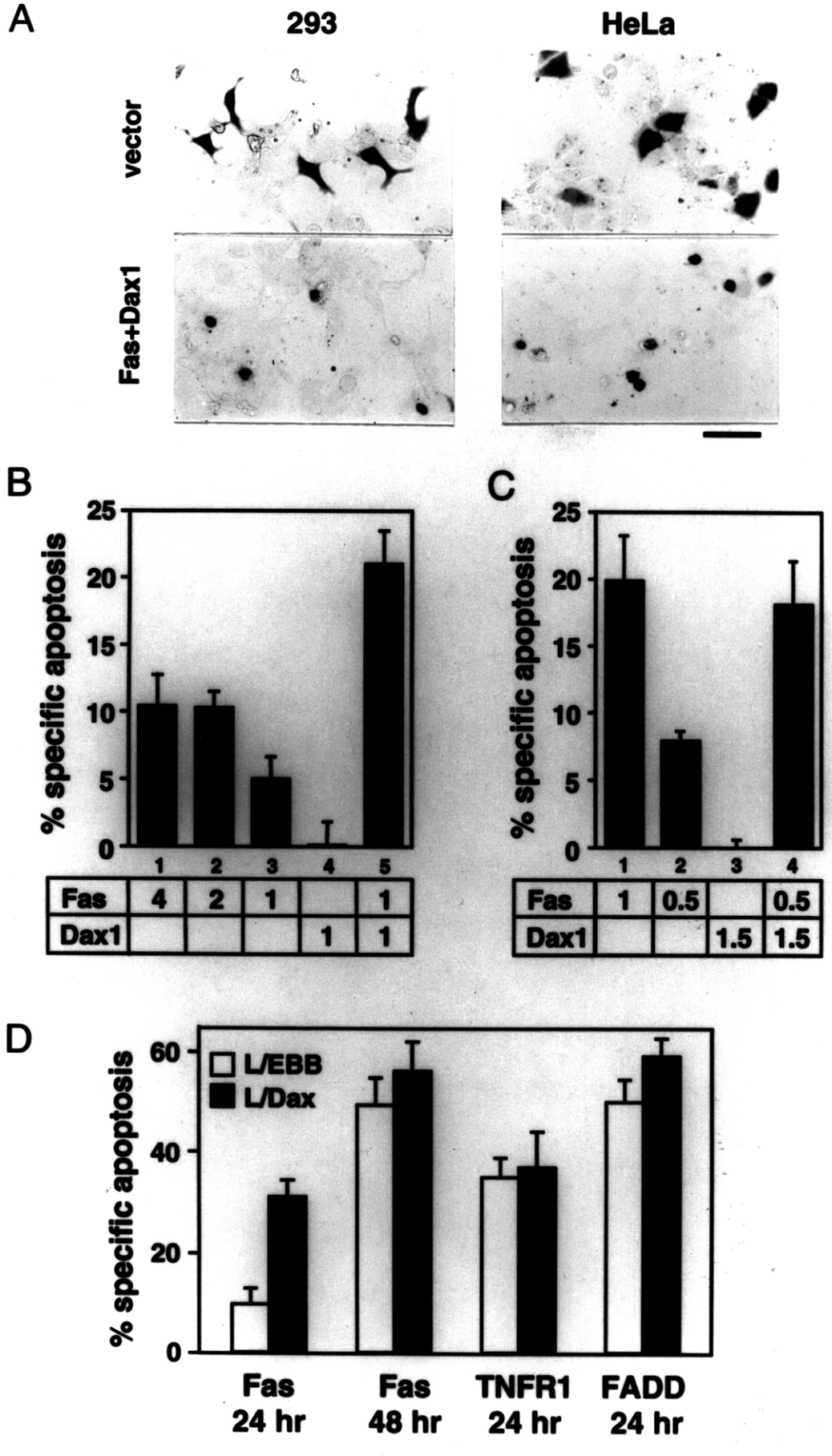
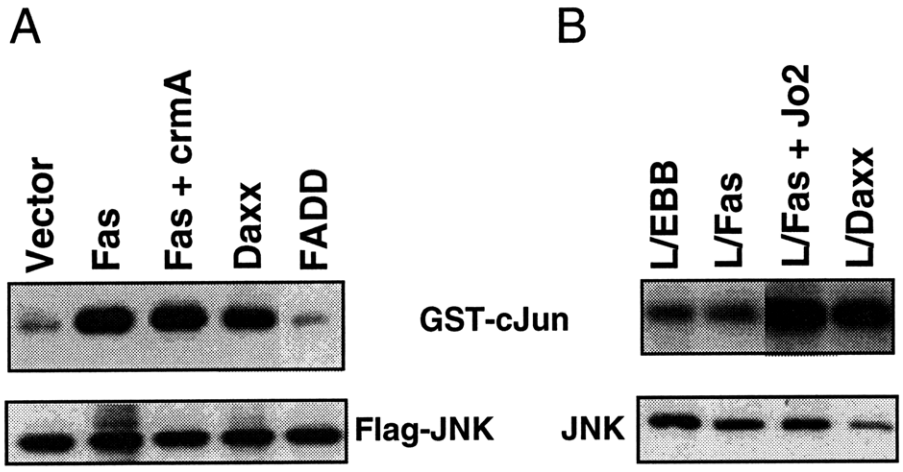

A
293
HeLa
vector
Fas+Dax1
B
% specific apoptosis
25
20
15
10
5
0
1 2 3 4 5
Fas 4 2 1 1
Dax1 1 1
C
% specific apoptosis
25
20
15
10
5
0
1 2 3 4
Fas 1 0.5 0.5
Dax1 1.5 1.5
D
% specific apoptosis
60
40
20
0
L/EBB
L/Dax
Fas 24 hr
Fas 48 hr
TNFR1 24 hr
FADD 24 hr

Figure 5


A
Vector
Fas
Fas + crmA
Daxx
FADD
GST-cJun
Flag-JNK
B
L/EBB
L/Fas
L/Fas + Jo2
L/Daxx
JNK

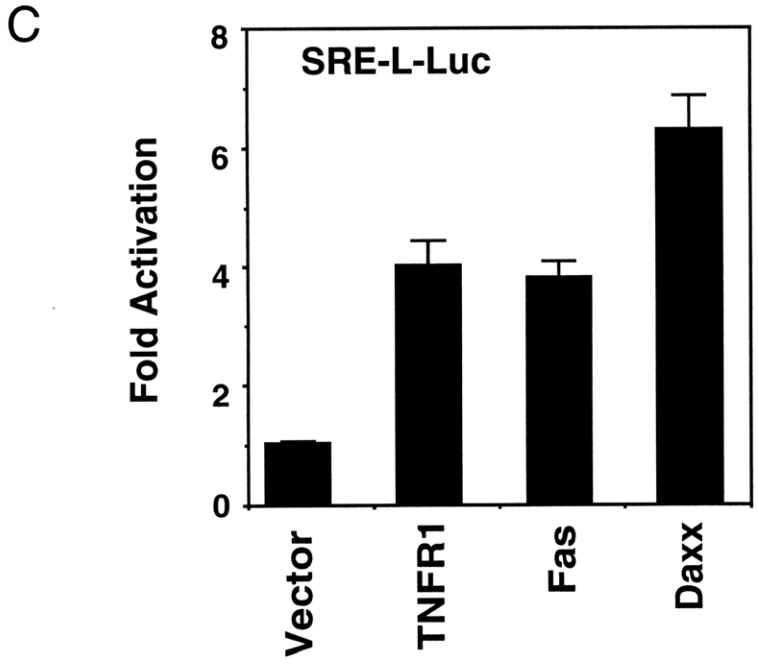

C
SRE-L-Luc
Fold Activation
8
6
4
2
0
Vector
TNFR1
Fas
Daxx

Figure 6

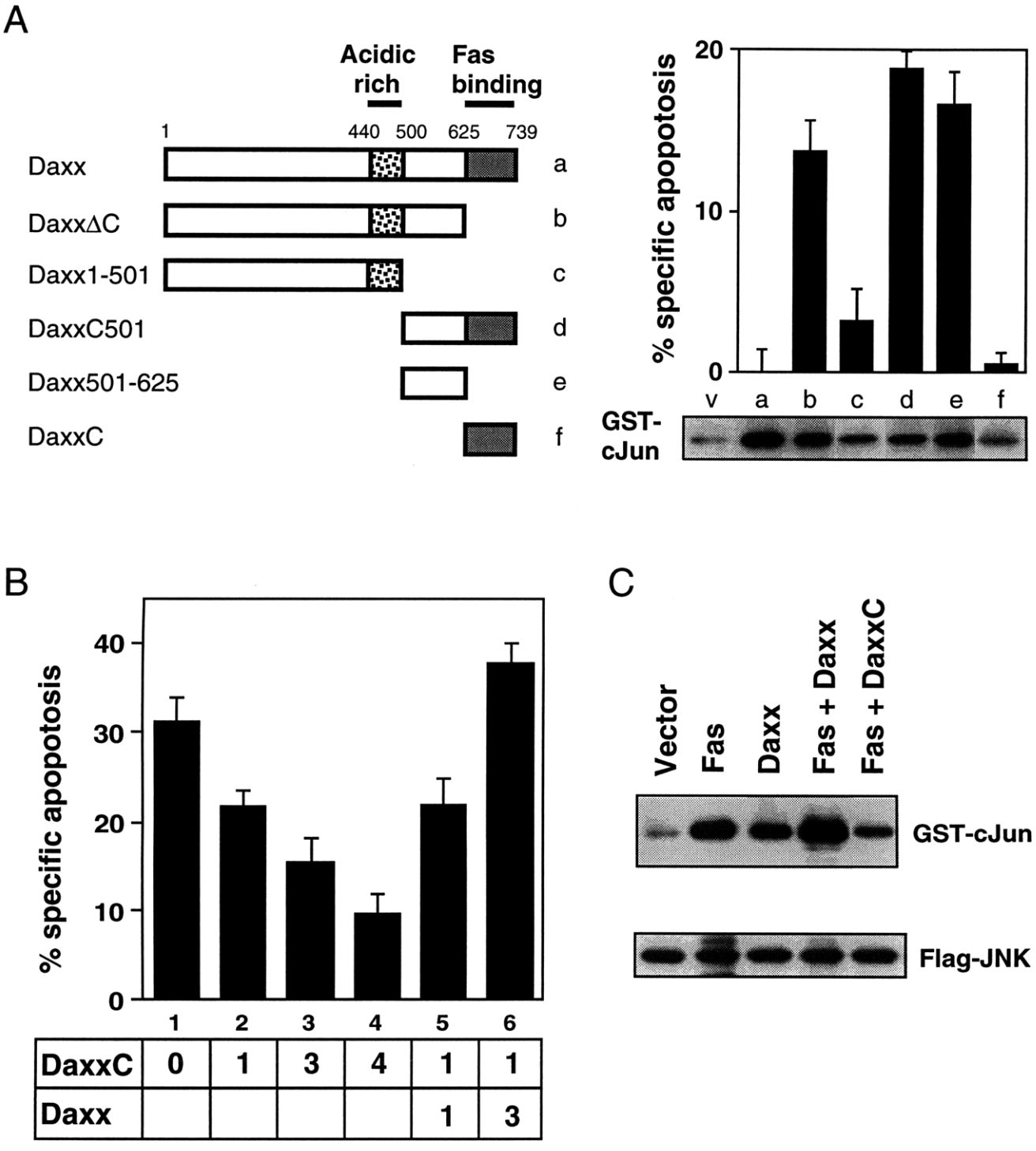
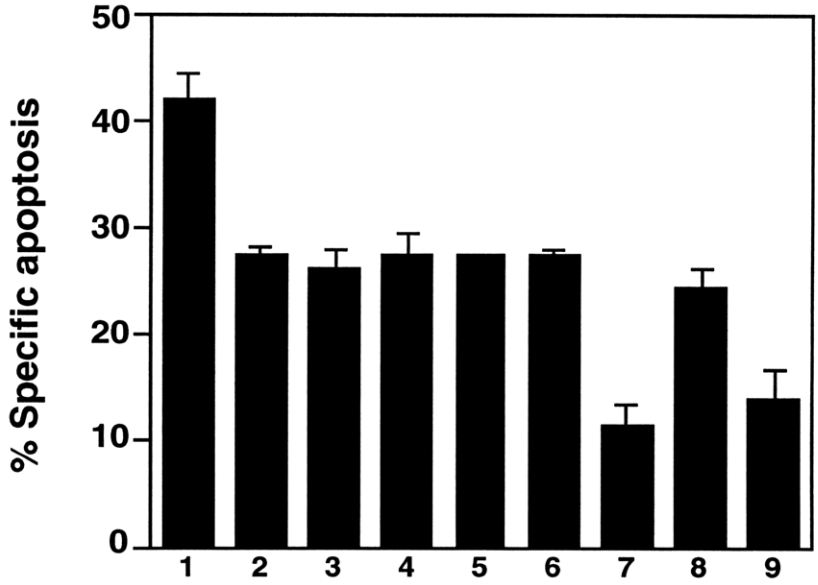

A
Acidic rich
Fas binding
1
440
500
625
739
Daxx
DaxxΔC
Daxx1-501
DaxxC501
Daxx501-625
DaxxC
a
b
c
d
e
f
% specific apopotosis
20
10
0
v
a
b
c
d
e
f
GST-cJun
B
% specific apopotosis
40
30
20
10
0
1
2
3
4
5
6
DaxxC 0 1 3 4 1 1
Daxx 1 3
C
Vector
Fas
Daxx
Fas + Daxx
Fas + DaxxC
GST-cJun
Flag-JNK

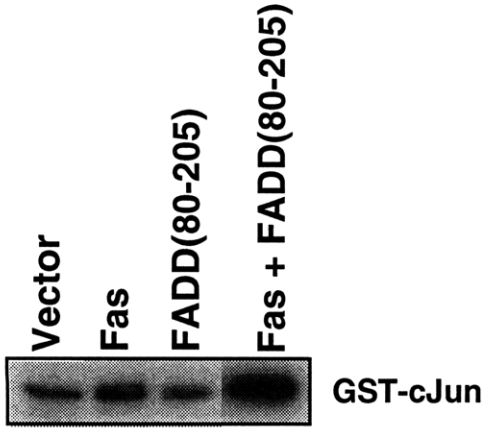
Figure 7

A

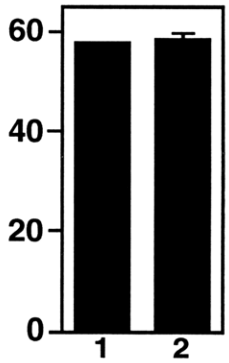



| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| FADD(80-205) | 0 | 1 | 3 | 1 | | | 1 | | 1 |
| Daxx | | | | 3 | | | | | |
| DaxxC | | | | | 1 | 3 | 1 | 1 | |
| Bcl-2 | | | | | | | | 3 | 3 |

B

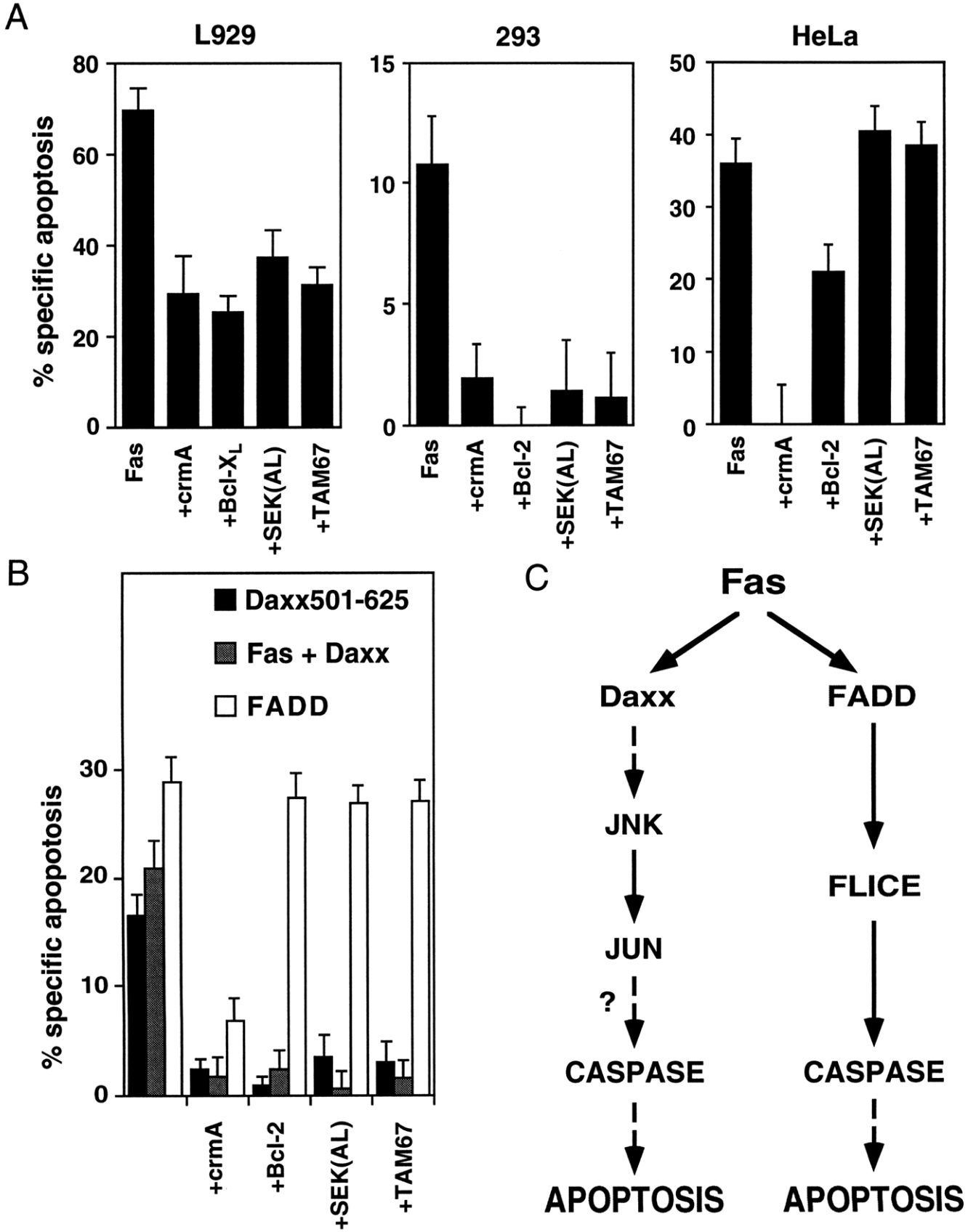



C




| | | |
|---|---|---|
| FADD | 1 | 1 |
| DaxxC | 0 | 3 |

Figure 8


A
L929
293
HeLa
% specific apoptosis
80
60
40
20
0
15
10
5
0
50
40
30
20
10
0
Fas
+crmA
+Bcl-X$_L$
+SEK(AL)
+TAM67
+Bcl-2
B
Daxx501-625
Fas + Daxx
FADD
% specific apoptosis
30
20
10
0
C
Fas
Daxx
FADD
JNK
JUN
?
FLICE
CASPASE
CASPASE
APOPTOSIS
APOPTOSIS

# Chapter III. Activation of Apoptosis Signal-regulating Kinase 1 (ASK1) by the death adaptor Daxx.


## Summary

The Fas death receptor can activate the Jun $NH_2$-terminal kinase (JNK) pathway through the receptor-associated protein Daxx. Here we show that Daxx activates the JNK kinase kinase, ASK1, and overexpression of a kinase deficient ASK1 mutant inhibited Fas- and Daxx-induced apoptosis. Fas activation induces Daxx to interact with the $NH_2$-terminal regulatory domain of ASK1, which consequently displaces an inhibitory intramolecular interaction between the $NH_2$- and COOH-termini of ASK1, activating its kinase activity. The Daxx-ASK1 connection completes a direct signaling pathway from a cell surface death receptor to kinase cascades that modulate nuclear transcription factors.


## My contribution to this project

I initiated and carried out most of the experiments in this project. I was fortunate to collaborate with Hideki Nishitoh and Hidenori Ichijo at Tokyo Medical and Dental University, who originally discovered ASK1. They performed the ASK1 kinase assays and the immunoprecipitation experiment in Figure 3B. This work is being published in the September 18, 1998 issue of *Science*:

Chang, H.Y.*, H. Nishitoh*, X. Yang, H. Ichijo,and D. Baltimore (1998) Activation of Apoptosis Signal-regulating Kinase 1 (ASK1) by the adaptor protein Daxx. Science, *in press.* *these authors contributed equally.

## Introduction

Fas is a cell surface receptor that induces apoptosis upon receptor oligomerization. Fas belongs to a growing family of homologous death receptors, which include the receptors for tumor necrosis factor-α (TNF-α) and the cytotoxic ligand TRAIL (Nagata, 1997; Golstein, 1997). Fas-induced apoptosis has a critical role in maintaining peripheral immune tolerance and tissue homeostasis (Nagata, 1997). Fas can activate two independent pathways to induce programmed cell death. One well-characterized pathway involves the adaptor protein FADD, which recruits pro-caspase-8 and activates a protease cascade (Nagata, 1997; Boldin et al., 1996). The second pathway is mediated by Daxx, which enhances Fas-induced apoptosis by activating the JNK pathway (Yang et al., 1997). The JNK pathway is a MAP kinase cascade composed of several kinases that sequentially phosphorylate and activate one another, culminating in the phosphorylation and activation of transcription factors such as c-Jun (Kyriakis and Avruch, 1996; Lange-Carter et al., 1993). The JNK pathway is also activated by inflammatory cytokines, growth factors, and environmental stress (Kyriakis and Avruch, 1996); how Daxx and other upstream signals lead to JNK activation is not well understood.

## Results

### Identification of ASK1 as a downstream target of Daxx

Because the JNK pathway is a well-known kinase cascade, we reasoned that Daxx likely activates JNK indirectly by activating a MAP kinase kinase kinase (MAP3K). Many MAP3Ks have been recently identified by molecular cloning(Yamaguchi et al., 1995; Tibbles et al., 1996; Ichijo et al., 1997); these kinases contain conserved kinase domains, the similarity of which define MAP3Ks, and distinct regulatory domains. We were attracted to a recently identified MAP3K termed ASK1 as a potential downstream target of Daxx for the following reasons. ASK1 activates both JNK and p38 subgroups of MAPKs by phosphorylating the MAP2Ks SEK1, MKK3, and MKK6 (Ichijo et al., 1997). Enforced expression of ASK1 in low serum condition potently activates apoptosis (Ichijo et al., 1997). ASK1 activity is activated by tumor necrosis factor-α (TNF-α), and dominant negative ASK1 can block TNF-α–induced death(Ichijo et al., 1997). We tested whether Daxx can activate the kinase activity of ASK1 by a coupled immunoprecipitation (IP)-kinase assay after coexpression in human embryonic kidney 293 cells (Ichijo et al., 1997). ASK1 activity was strongly activated by coexpression with Daxx (Figure 1A). Another MAP3K which can activate the same kinase cascade, TAK1 (Yamaguchi et al., 1995), was not activated by Daxx and served as a negative control. We have previously mapped a domain within Daxx, consisting of amino acids 501-625, that encodes its JNK activation and apoptotic activities (Yang et al., 1997). This same domain and fragments incorporating it, but not other parts of Daxx, also activated ASK1 activity (Figure 1B). These data implicate ASK1 as a downstream target of Daxx.

Finally, ASK1 activation by Daxx implies that ASK1 is part of the Fas signaling pathway. Endogenous ASK1 activity was activated by Fas crosslinking in Jurkat cells in a dose-dependent manner (Figure 1C). ASK1 activation occured rapidly following Fas ligation; low but detectable activation of ASK1 activity was evident as early as 5 minutes after Fas crosslinking (Figure 1C).

**Functional roles of ASK1 in Daxx and Fas signaling**

To determine the functional role of ASK1 in Daxx and Fas signaling, we tested the effect of altering ASK1 activity on the apoptotic activities of Daxx and Fas. An activated allele of Daxx, DaxxC501, can induce cell death in a Fas independent manner (Yang et al., 1997). This activity is evident in 293 cells but not in HeLa cells (Yang et al., 1997); one possible explanation is that a downstream factor is rate-limiting in HeLa cells. Expression of either ASK1 or DaxxC501 alone in HeLa cells grown in serum-containing media did not induce apoptosis, but the coexpression of ASK1 and DaxxC501 significantly induced apoptosis (Figure 2A). This synthetic lethal effect required the kinase activity of ASK1 because a conservative point mutation in the ATP binding loop of ASK1 (K709R) completely abrogated cell killing (Figure 2A). To assess the requirement of ASK1 activity in Fas- and Daxx- induced apoptosis, we constructed ASK(K709M), which has less residual kinase activity than ASK1(K709R). Coexpression of ASK(K709M) potently inhibited apoptosis by Fas and DaxxC501 in a dose-dependent manner (Figure 2B and 2C). ASK1(K709M) also inhibited the ability of DaxxC501 and Fas to activate JNK although the caspase inhibitor crmA did not (Figure 2D). Collectively, these results suggest a critical role for ASK1 activity in Fas and Daxx signaling.

**Interaction between Daxx and ASK1**

Because MAP3Ks such as Raf are known to directly interact with upstream signaling proteins, we tested for physical interaction between Daxx and ASK1 by co-immunoprecipitation from transfected 293T cells. Full length human Daxx specifically co-immuno-precipitated with ASK1 (Figure 3A), indicating that these two proteins physically interact in mammalian cells. FLAG-tagged FADD was not coprecipitated by ASK1 under the same condition. To evaluate the observed Daxx-ASK1 interaction under more physiological conditions, we examined the association of endogenous Daxx and ASK1 by

co-immunoprecipitation in L/Fas cells, a mouse fibroblast cell line expressing murine Fas (Yang et al., 1997). Daxx became associated with ASK1 following Fas ligation by an agonistic monoclonal antibody (mAb); this interaction peaked in 15 minutes and decreased thereafter (Figure 3B). The Daxx-ASK1 interaction raised the possibility that ASK1 may interact indirectly with Fas through Daxx. In L929/Fas cells, the endogenous ASK1 was specifically co-immunoprecipitated with Fas after mAb crosslinking (Figure 3C, lane 3), indicating that ASK1 does interact with Fas and therefore may be a component of the Fas receptor signaling complex. In contrast, addition of mAb to Fas after cell lysis, which immunoprecipitates monomeric Fas (Kischkel et al., 1995) , did not coprecipitate ASK1 (Figure 3C, lane 2). The Fas-ASK1 interaction is apparently mediated by Daxx because coexpression of DaxxC, the C-terminal 112 amino acid Fas-binding domain of Daxx, blocked the Fas-ASK1 interaction, presumably by competing out endogenous Daxx (Figure 3D, lane 3). The ability of DaxxC to block ASK1 recruitment to Fas may explain the documented dominant negative effects of DaxxC on both Fas-induced apoptosis and JNK activation (Yang et al., 1997). In the yeast two hybrid system, ASK1(K709R) fused to LexA DNA binding domain interacted specifically with mouse Daxx but not with mouse Fas intracellular tail fused to a transcription activation domain, suggesting that Daxx interacts directly with ASK1 and bridges ASK1 and Fas. Relative β-gal units $\pm$ standard deviation of ASK1(KR) alone are $2.4 \pm 0.2$; ASK1(KR) plus Daxx are $119 \pm 8.8$; ASK1(KR) plus Fas are $1.8 \pm 0.4$. Deletion mutagenesis showed that the $NH_2$-terminal 648 amino acids of ASK1, termed ASKN, could interact with Daxx (Figure 3E and 3F, lane 7) whereas other parts of ASK1 could not interact.

**A mechanism for ASK1 activation by Daxx**

How does the Daxx-ASK1 interaction activate ASK1 activity? The N-terminal regulatory domains of several MAP3Ks have been shown to be autoinhibitory, and N-terminal deletions oftern led to constitutitvely active kinase activities (Yamaguchi et al.,

1995). Deletion of the N-terminal 648 amino acids of ASK1 also caused the constitutive activation of ASK1 kinase activity (Saitoh et al., 1998). Purified recombinant GST-ASKN inhibited the in vitro kinase activity of ASK1 but not ASK1ΔN immunoprecipitated from cells (Fig. 4A), suggesting that one or more interacting cellular factors regulate ASKN autoinhibition. ASK1ΔN exhibited constitutive cell death activity in HeLa cells in the absence of added Daxx (Fig. 4B). Apoptosis induced by ASK1ΔN was quantitatively similar to that induced by ASK1 plus DaxxC501 and was not enhanced by coexpression with DaxxC501 (Fig. 4B). These results indicate that an activated allele of ASK1 functions as a genetic bypass of Daxx and suggests that with regard to ASK1 activation, the function of Daxx is to relieve the inhibition caused by the $NH_2$-terminal regulatory domain. We tested this model directly by in vivo interaction assays. ASKN interacted with Daxx (Fig. 4C, lane 2). It also specifically co-immunoprecipitated ASK1ΔN (Fig. 4C, lane 4), implying an intramolecular interaction in full length ASK1. Importantly, when an excess of Daxx was coexpressed with ASKN and ASK1ΔN, ASKN associated with Daxx but not ASK1ΔN (Fig. 4C, lane 6). This supports a model whereby Daxx activates ASK1 activity by displacing an inhibitory intramolecular interaction between the $NH_2$- and COOH-termini of the kinase and "opening up" the kinase into an active conformation. In support of this model, ASKN can inhibit the constitutive apoptotic activity of ASK1ΔN *in trans*, and this inhibition is fully reversed by the coexpression of Daxx (Fig. 4D).

## Discussion

The present results suggest a Fas-Daxx-ASK1 axis in activating JNK and p38 MAP kinase cascades. The mechanism by which ASK1 is activated by Daxx is similar to that described for the activation of Byr2, a MAP3K in the *S. pombe* mating pheromone pathway, by its activators Ste4 and Shk1 (Tu et al., 1997). Fas activation has been reported to activate JNK by caspase-dependent (Cahill *et al.*, 1996; Lenczowski *et al.*, 1997; Toyoshima *et al.*, 1997) and independent pathways (Yang et al., 1997; Goillot et al., 1997; Wajant *et al.*, 1998). During apoptosis, caspases can cleave and activate PAK2 and MEKK (Rudel and Bokoch, 1997; Lee et al., 1997; Cardone et al., 1997), two kinases that can activate the JNK pathway; JNK activation in this context is believed to effect morphologic changes associated with apoptosis (Rudel and Bokoch, 1997; Lee et al., 1997). The Daxx-ASK1 connection provides a mechanism for caspase-independent activation of JNK by Fas and perhaps other stimuli. In mice deficient for JNK3, hippocampal neurons are protected from apoptosis following excitotoxic injury, illustrating that in certain circumstances JNK is essential for the apoptotic program (Yang *et al.*, 1997). In this study, we have used several tumor-derived cell lines where JNK activation by the Fas-Daxx-ASK1 axis led to apoptosis. Because *FADD*-deficient embryonic fibroblasts and T cells are blocked for Fas-induced apoptosis (Yeh *et al.*, 1998; Zhang *et al.*, 1998), at least in these cells Daxx does not provide an independent death pathway. The physiologic role of the Daxx-ASK1 axis and its cell specificity in vivo remain to be addressed in future studies.

## Experimental Procedures

### Reagents and Cell lines

CH-11 anti-human Fas antibody (MBL, Nagoya Japan or gift of S. Nagata), soluble M2 anti-FLAG antibody or coupled to agarose beads (Kodak) , anti-Myc antibody coupled to agarose beads (Santa Cruz Biotech.), polyclonal anti-FLAG antibody (Santa Cruz Biotech.) were obtained from the indicates sources. Antiserum to ASK1 (DAV) was raised to the peptide sequence DAVATSGVSTLSSTVSHDSQ, amino acids 1217-1236 in human ASK1 as described (Tobiume et al., 1997). Rabbit polyclonal antibody to mouse Daxx (DSS) was raised against the peptide sequence DSSTRVDSPSHELVTSSLC (amino acids 680 to 698). L929/Fas was previously described (Yang et al., 1997).

### Plasmid Construction

pEBB-Daxx, Daxx mutants, pEBB-Fas, pCMV-FLAG-JNK1, and pJG4-5-mDaxx were as described (Yang et al., 1997). pcDNA3-ASK1 and pcDNA3-ASK1(K709R) (Ichijo et al., 1997), Myc-TAK1 (Yamaguchi et al., 1995), pRK5-crmA (Hsu et al., 1995), EG202-ASK1(K709R) (Saitoh et al., 1998), were as described. ASK1ΔN, ΔC, kinase, FLAG-ASK1, Myc-ASK1, ASK1 (K709M)-HA, and Myc-ASKN were constructed in pcDNA3 (Invitrogen) by PCR. FLAG-tagged human Daxx and hDaxxC were derived from EST clone AA085057 and constructed in pRK5 (Hsu et al., 1995) by PCR. pCI-AU1-hFas was constructed by J. Wang and M.J. Lenardo. The plasmids of GST-human MKK6 and GST SAPK3/p38γ(KN) for bacterial fusion protein were constructed in pGEX-4T-1 (Pharmacia Biotech, Inc.) by PCR.

### MAP3K IP-kinase assay

293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), glucose (4.5 μg/ml) and penicillin (100 U/ml) and transfected with Tfx-50 (Promega) according to the manufacturer's instructions. Jurkat cells were cultured in RPMI 1640

medium containing 10% FBS and antibiotics in 5% CO2 atmosphere at 37 C. GST-rat SAPK3/p38γ and ATF2 peptide (1-109) were kindly provided by M. Goedert and Z. Yao, respectively. Cells were extracted and immunoprecipitated with with anti-Myc mAb (Ab-1, Calbiochem), M2 anti-FLAG mAb (Kodak) or antiserum to ASK1 (DAV), using protein G (for Ab-1 or M2) or A (for DAV) -Sepharose. Immune complex assay were performed essentially as described (12). Phosphorylation of ATF2 peptide or GST-SAPK/p38γ was analyzed by a Fuji BAS2000 image analyzer. ASK1 or TAK1 protein was detect by immunoblotting and ECL, which in exposures less than 10 minutes did not detect $^{32}P$ radioactivity from kinase autophosphorylation. Protein levels from immunoblot were quantified by densitometry (Quantity One program: pdi, Inc.).

**Yeast Two Hybrid experiment**

EGY48 yeast strain was tranformed with EG202-ASK1(K709R), pJG4-5 vector, pJG4-5-mDaxx, or pJG-mFas(192-295), and JK101 reporter plasmids, and quantitative liquid β-galactosidase assay were performed as described (Gyuris et al., 1993).

**Co-immunoprecipitation of transfected and endogenous proteins**

2 x $10^6$ 293T cells [grown in DMEM supplemented with 10% FBS, penicillin-streptomycin (100U/ml), and glutamine (1 mM)] were plated per 60 mm dish the day before transfection. 24 hours after transfection, cells were washed once in ice-cold PBS, and lysed in 300 μl IP-lysis buffer [50 mM Hepes (pH 7.4), 1 % NP-40, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM DTT] supplemented with 1 mM PMSF and 1% aprotinin. Extract (50 μl) was diluted in IP-lysis buffer (500 μl) and immuno-precipitated with antibody reagents as described in Figure legends. In Fig. 2B, L/Fas cells were lysed in 1ml lysis buffer. Cell lysates were immunoprecipitated with antiserum to ASK1 using protein A-Sepharose. The beads were washed twice with the washing buffer, separated by SDS-PAGE, and immunoblotted with anti-Daxx antiserum (DSS).

**Acknowledgement**

We are grateful to P. Svec for technical support. We thank S. Nagata, K. Matsumoto, J. Wang, M.J. Lenardo, D.V. Goeddel, M. Goeddert, and Z. Yao for gifts of reagents. We thank A. Hoffmann for valuable advice and critical review of the manuscript. H.I. thanks K. Miyazono for valuable discussion. H.Y.C. is supported by the Medical Scientist Training Program at Harvard Medical School. H.I. is supported by Grants-in-Aid for scientific research from the Ministry of Education, Science, and Culture of Japan. X.Y. is a fellow of the Leukemia Society of America. Supported by NIH grant CA51462.

## References

Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. Cell *85*, 803-815.

Cahill, M.A., M.E. Peter, F.C. Kischkel, A.M. Chinnaiyan, V.M. Dixit, P.H. Krammer and A. Nordheim (1996). CD95 (APO-1/Fas) induces activation of SAP kinases downstream of ICE-like proteases. Oncogene *13*, 2087-2096.

Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997). The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. Cell *90*, 315-323.

Goillot, E., Raingeaud, J., Ranger, A., Tepper, R. I., Davis, R. J., Harlow, E., and Sanchez, I. (1997). Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. Proc. Natl. Acad. Sci. USA *94*, 3302-3307.

Golstein, P. (1997). TRAIL and its receptors. Curr Biol. *7*, R750-R753.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphotase that associated with Cdk2. Cell *75*, 791-803.

Hsu, H., Xiong, J., and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kB activation. Cell *81*, 495-504.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science *275*, 90-94.

Kischkel, F. C., Hellbardt, S. H., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD-95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. *14*, 5579-5588.

Kyriakis, J. M., and Avruch, J. (1996). Protein kinase cascades activated by stress and inflammatory cytokine. BioEssay *18*, 567-577.

Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993). A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science *260*, 315-319.

Lee, N., MacDonald, H., Reinhard, C., Halenbeck, R., Roulston, A., Shi, T., and Williams, L. T. (1997). Activation of hPAK65 by caspase cleavage induces some of the morphological and biochemical changes of apoptosis. Proc. Nat'l Acad. Sci. USA *94*, 13642-13647.

Lenczowski, J.M., L. Dominguez, A.M. Eder, L.B. King, C.M. Zacharchuk and J.D. Ashwell (1997). Lack of a role for Jun kinase and AP-1 in Fas-induced apoptosis. Mol. Cell. Biol. *17*, 170-181.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-365.

Rudel, T., and Bokoch, G. M. (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science *276*, 1571-1574.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. submitted.

Tibbles, L. A., Ing, Y. L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J. R., and Lassam, N. J. (1996). MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. EMBO J. *15*, 7026-7035.

Tobiume, K., Inage, T., Takeda, K., Enomoto, S., Miyazono, K., and Ichijo, H. (1997). Molecular cloning and characterization of the mouse Apoptosis Signal-regulating Kinase 1. Biochem Biophys Res Commun *239*, 905-910.

Toyoshima, F., T. Moriguchi and E. Nishida (1997). Fas induces cytoplasmic apoptotic responses and activation of the MKK7-JNK/SAPK and MKK6-p38 pathways independent of CPP32-like proteases. J Cell Biol *139*, 1005-1015.

Tu, H., Barr, M., Dong, D. L., and Wigler, M. (1997). Multiple regulatory domains on the Byr2 protein kinase. Mol. Cell. Biol. *17*, 5876-5887.

Wajant, H., F.J. Johannes, E. Haas, K. Siemienski, R. Schwenzer, G. Schubert, T. Weiss, M. Grell and P. Scheurich (1998). Dominant-negative FADD inhibits TNFR60-, Fas/Apo1- and TRAIL-R/Apo2-mediated cell death but not gene induction. Curr Biol. *8*, 113-116.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science *270*, 1326-1331.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science *270*, 2008-2011.

Yang, D.D., C.Y. Kuan, A.J. Whitmarsh, M. Rincon, T.S. Zheng, R.J. Davis, P. Rakic and R.A. Flavell (1997). Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature *389*, 865-870.

Yang, X., Khosravi-Far, R., Chang, H. Y., and Baltimore, D. (1997). Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell *89*, 1067-1076.

Yeh, W.-C., J.L. de la Pompa, M.E. McCurrach, H.-B. Shu, A.J. Elia, A. Shahinian, M. Ng, A. Wakeham, W. Khoo, K. Mitchell, W.S. El-Deiry, S.W. Lowe, D.V. Goeddel and T.W. Mak (1998). FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science *279*, 1954-1958.

Zhang, J., D. Cado, A. Chen, N.H. Kabra and A. Winoto (1998). Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature *392*, 296-300.

**Figure Legends**

Fig. 1.Daxx- and Fas-induced activation of ASK1.

(A) Daxx activates ASK1. pcDNA3-Myc-ASK1 (0.5 μg) or pCS3-Myc-TAK1 (0.5 μg) was cotransfected with pEBB-Daxx (1.5 μg) into 293 cells. ASK1 was immunoprecipitated by anti-Myc antibody. The immune complex was incubated with GST-MKK6 and GST-SAPK/p38γ, and the kinase activity was measured with the substrate ATF2(1-109) peptide. Top: phosphorylation of ATF2 after in vitro kinase (IVK) assay. Middle: immunoblotting (WB) of immunoprecipitated Myc-ASK1 and Myc-TAK1. Bottom: fold activation of ASK1 and TAK1 kinase activities. Kinase activities relative to the amount of ASK1 or TAK1 proteins were calculated, and the activities are shown as fold activation relative to the activities of ASK1 or TAK1 from Daxx-negative cells.

(B) ASK1 activation by Daxx deletion mutants. pcDNA3-FLAG-ASK1 (0.5 μg) and each Daxx mutant (1.5 μg) were cotransfected into 293 cells, and ASK1 was immuno-precipitated with anti-FLAG antibody. The immune complex was incubated with GST-MKK6, and then the kinase activity was measured with the substrate GST-SAPK/p38γ(KN). The sequences incorporated in each Daxx construct are as follows: Daxx (aa 1-739), DaxxΔC (aa 1-625), Daxx1-501 (aa 1-501), DaxxC501 (aa 501-739), Daxx501-625 (aa 501-625), DaxxC (aa 626-739). Top: phosphorylation of GST-SAPK3/p38γ(KN). Middle: expression of FLAG-ASK1. Bottom: fold activation of ASK1 kinase activities. Essentially identical results were also obtained with HeLa cells.

(C) Fas-induced activation of ASK1. 5 x $10^6$ Jurkat cells were treated with CH-11 anti-human Fas antibody (MBL, Nagoya Japan) (100ng/ml) for the indicated times (left) or with the indicated concentrations for 30 min (right). The endogenous ASK1 was immunoprecipitated with an anti-ASK1 antiserum (DAV), and the ASK1 kinase activity was measured as described in (B).

Fig. 2. Role of ASK1 in Daxx- and Fas- induced apoptosis and signaling.

(A) Synthetic lethality of ASK1 with DaxxC501. HeLa cells were transfected with 0.5 μg of pcDNA3-ASK1 or pcDNA3-ASK1(K709R) and 1.0 μg of pEBB-DaxxC501 along with 0.5 μg of pCMV-lacZ reporter by the calcium phosphate method. Total amount of transfected DNA was made constant by adding vector DNA. 24 hours after transfection the cells were stained with X-gal and scored for apoptotic morphology (Yang et al., 1997). Specific apoptosis was calculated as the percentage of apoptotic blue cells in each experimental condition minus the percentage of apoptotic blue cells (approxmiately 5 percent) in parallel vector-transfected cells. The data shown are the average and standard deviation of two to four independent experiments.

(B) Inhibition of Fas-induced apoptosis by ASK1(K709M). HeLa cells were transfected with 0.5 μg pEBB-Fas and pCMV-lacZ and the indicated amount (in μg) of ASK1(K709M). Total amount of transfected DNA was made constant by adding pEBB. Jo2 antibody (12.5 ng/ml) was added 16 hrs later. X-gal staining was done at 24 hrs after transfection. Specific apoptosis was calculated as in Figure 2A.

(C) Inhibition of DaxxC501-induced apoptosis by ASK1(K709M)(18). 2.0 μg pEBB-DaxxC501 and the indicated amount (in μg) of pcDNA3-ASK1(K709M) were cotransfected with 0.5 μg of pCMV-lacZ in 293 cells. Total amount of transfected DNA was made constant by adding vector DNA. Twenty hours after transfection the cells were stained with X-gal and specific apoptosis scored as in Figure 2A.

(D) Inhibition of DaxxC501-and Fas induced JNK activation by ASK1(K709M). An expression construct for each indicated protein (1.0 μg) was cotransfected with 1.0 μg of pCMV-FLAG-JNK1 in 293 cells. The total amount of transfected DNA was made constant by adding pEBB. Cells in lanes 7-10 were treated with anti-Fas mAb Jo2 (0.5 ug/ml) for 30 minutes before assay. JNK1 was immunoprecipitated with anti-FLAG antibody, and an in vitro kinase assay with 1μg of GST-cJun(1-79) was performed as

previously described (Yang et al., 1997). Top: phosphorylation of GST-cJun(1-79). Bottom: immunoblotting of immunoprecipitated FLAG-JNK1.

Fig. 3. Daxx interacts with ASK1.

(A) Association of Daxx and ASK1 in 293T cells. 4.0 μg of pRK5-FLAG-hDaxx, pcDNA3, or pcDNA3-Myc-ASK1 were cotransfected with 2.0 μg of pRK5-crmA in 293T cells by the calcium phosphate method. (CrmA prevents the induction of apoptosis and allows the accumulation of tranfected proteins.) After 24 hours, cells were extracted in IP-lysis buffer , immunoprecipitated with anti-Myc antibody coupled to agarose beads (Santa Cruz) for 3 hours at 4° C, and washed 3 times with 500 μl IP-lysis buffer. The IP samples as well as portions of the extracts (10% of IP input) were resolved by SDS-PAGE and immunoblotted with M2 anti-FLAG antibody (Kodak) as described (Yang et al., 1997).

(B) Fas induced interaction of Daxx and ASK1. Left panel: identification of endogenous Daxx protein in L/Fas cells. Lysate from $3x10^7$ L/Fas cells was immunoprecipitated with polyclonal anti-Daxx antiserum (DSS) in the absence or presence of blocking peptide (5 μg/ml), and immunoblotted with DSS. Right panel: $3x10^7$ L/Fas cells were treated with mAb Jo2 (IgG 100 ng/ml) (21) for the indicated times (lanes 4-7) or left untreated (lane 3). Cell lysates were immunoprecipitated with the anti-ASK1 antiserum (lanes 3-7) and immunoblotted with DSS (top) (20). Equivalent IP of ASK1 was confirmed by immunoblotting of the same membrane with anti-ASK1 antiserum (bottom).

(C) Recruitment of endogenous ASK1 to Fas in a ligand-dependent manner. 1.5 x $10^7$ L929/Fas cells were incubated in the presence or absence of mAb Jo2 (IgG 2 μg/ml) to Fas for 30 minutes at 37° C. Cells were washed once with ice-cold PBS and lysed in IP-lysis buffer. The post-nuclear supernatant was immunoprecipitated with 40 μl of protein A/G-agarose (Santa Cruz) for 3 hours at 4° C. In samples without Jo2 preincubation, 2 μg/ml of isotype-matched control antibody (lane 1) or Jo2 (lane 2) were added after cell lysis. Immunoprecipitates were washed 5 times with lysis buffer, resolved by 7.5% SDS-

PAGE and immunoblotted for ASK1 by the DAV antiserum. Positions of molecular weight standards (in kDa) are shown on the left.

(D) Requirement of Daxx for Fas-ASK1 interaction. 2.0 μg of pcDNA3-ASK1(K709R), 1.0 μg of pCI-AU1-hFas, and 4.0 μg of pRK5-hDaxxC in the indicated combinations were transfected into 293T cells along with 2.0 μg of pRK5-crmA and vector DNA as needed to equalize total DNA. Transfected cells were extracted, immunoprecipitated with anti-AU1 (Babco) and protein A/G-agarose, and immunoblotted for HA-ASK1 as in Fig. 3A.

(E) Schematic diagram of ASK1 mutants. Amino acid number of domain boundaries are indicated. pcDNA3-ΔN, ΔC, and kinase each contain a C-terminal hemagglutinin (HA) epitope-tag. pcDNA3-ASKN contains a N-terminal Myc epitope-tag.

(F) Daxx interacts with the N-terminus of ASK1. 4.0 μg of each ASK1 mutant was cotransfected with 4.0 μg of pRK5-FLAG-hDaxx and 2.0 μg of pRK5-crmA in 293T cells. Samples in each lane: ASK1 (1 and 5); ΔN (2 and 6); ΔC (3 and 7); kinase (4 and 8). 24 hours after transfection cells were extracted in IP-lysis buffer and immunoprecipitated with M2 anti-FLAG antibody coupled to agarose beads. IP samples and extract aliquots were immunoblotted by anti-HA antibody as in Figure 3A. Positions of molecular weight standards (in kDa) are shown on the right.

Figure 4. Mechanism of Daxx activation of ASK1.

(A) ASKN inhibition of ASK1 activity *in vitro*. pcDNA3-ASK1-HA or pcDNA3-ASK1ΔN-HA were transfected into 293 cells and immuno-precipitated with anti-HA antibody (12CA5) and protein A-Sepharose. Equalized input kinase activities were incubated with indicated amount of GST or GST-ASKN for 60 min at 4 $^{o}$C, and subjected to the immune complex kinase assay as described in Fig. 1B. G-ASKN, GST-ASKN.

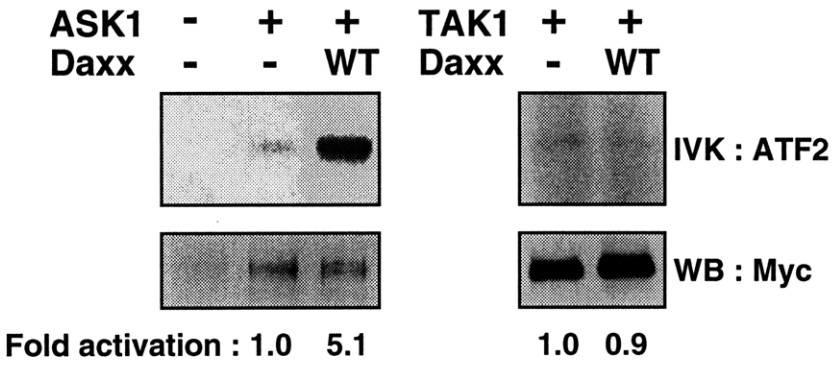
(B) Constituitive apoptotic activity of ASK1ΔN. HeLa cells were transfected with 0.5 μg of each indicated ASK1 mutant, 1.0 μg of pEBB or pEBB-DaxxC501, and 0.5 μg

pCMV-lacZ reporter. 24 hrs after transfection cells were stained with X-gal and scored for specific apoptosis as in Figure 2A.

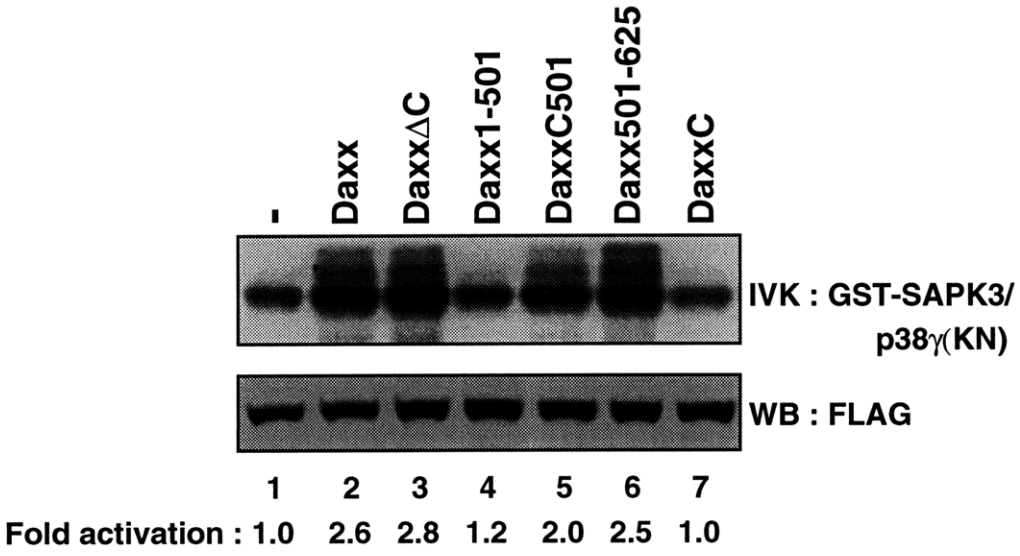
(C) Daxx releases the COOH-terminus of ASK1 from the $NH_2$-terminus of ASK1. 293T cells were transfected with pcDNA3-Myc-ASKN, pcDNA3-ASK1ΔN, and pRK5-FLAG-hDaxx as indicated along with 2.0 μg of pRK5-crmA and vector DNA as needed. 2.0 μg of each indicated DNA was transfected in lanes 1-4; in lanes 5 and 6, 0.5 μg of ASKN, 2.0 μg of ASK1ΔN, and 4.0 μg of Daxx were transfected. Transfected cells were extracted, immunoprecipitated with anti-Myc antibody coupled to agarose beads, and immunoblotted with anti-HA and anti-FLAG as in Figure 3A.

(D) 1.0 μg each of pcDNA3-ASK1ΔN, pcDNA3-ASKN, and pRK5-FLAG-hDaxx were cotransfected as indicated with 0.5 μg of pCMV-lacZ and vector DNA as needed in HeLa cells. 24 hrs after transfection cells were stained with X-gal and scored for specific apoptosis as in Figure 2A.

A


ASK1 - + +
Daxx - - WT
TAK1 + +
Daxx - WT
IVK : ATF2
WB : Myc
Fold activation : 1.0 5.1
1.0 0.9

B


-
Daxx
DaxxΔC
Daxx1-501
DaxxC501
Daxx501-625
DaxxC
IVK : GST-SAPK3/
p38γ(KN)
WB : FLAG
1 2 3 4 5 6 7
Fold activation : 1.0 2.6 2.8 1.2 2.0 2.5 1.0

C

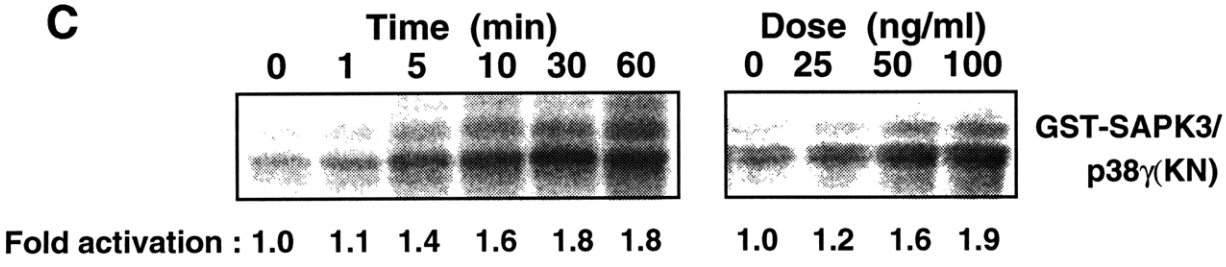

Time (min)
0 1 5 10 30 60
Dose (ng/ml)
0 25 50 100
GST-SAPK3/
p38γ(KN)
Fold activation : 1.0 1.1 1.4 1.6 1.8 1.8
1.0 1.2 1.6 1.9

Figure 2

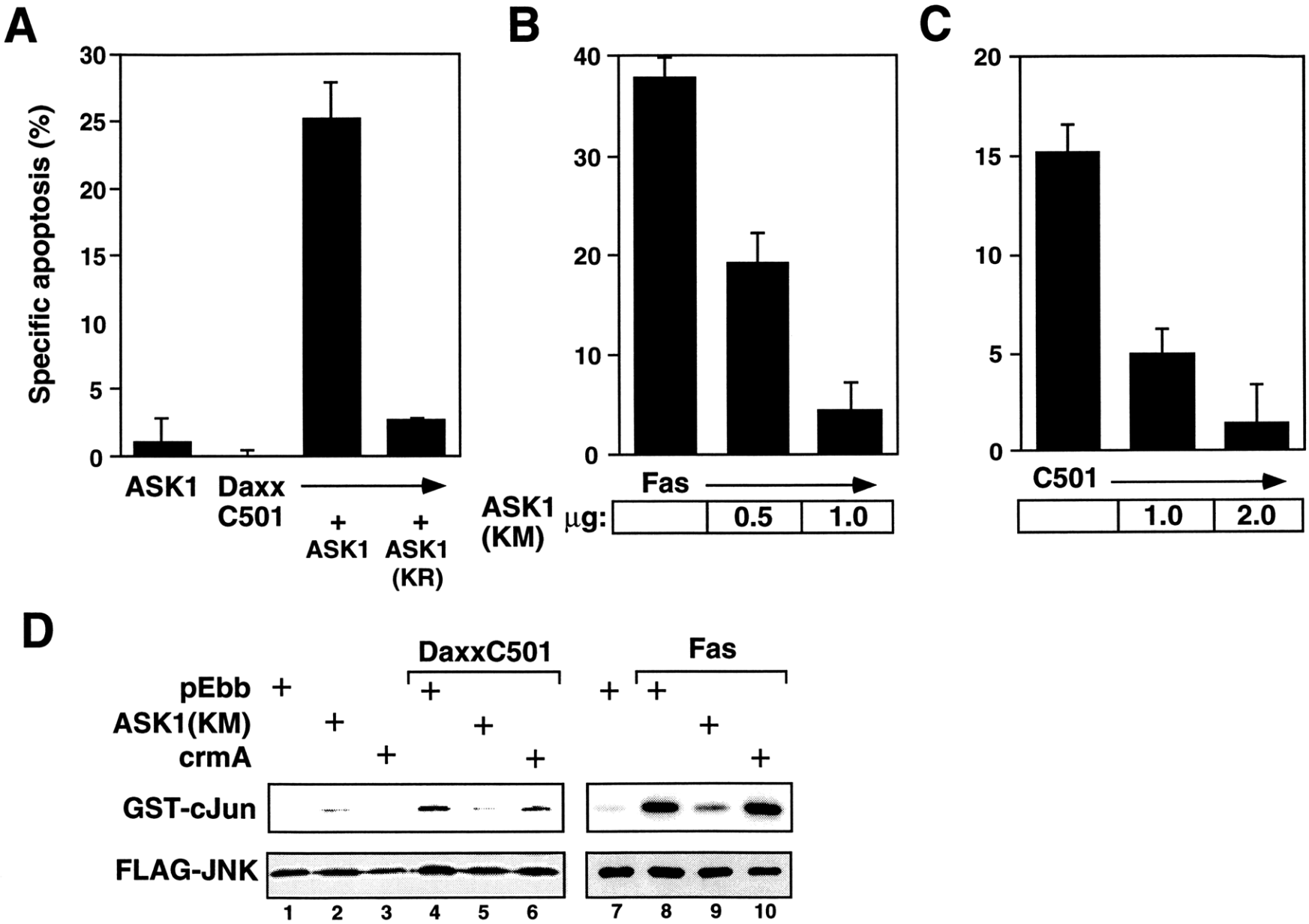

A
Specific apoptosis (%)
30
25
20
15
10
5
0
ASK1
Daxx C501
+ ASK1
+ ASK1 (KR)
B
40
30
20
10
0
Fas
ASK1 (KM) μg:
0.5
1.0
C
20
15
10
5
0
C501
1.0
2.0
D
DaxxC501
Fas
pEbb
ASK1(KM)
crmA
GST-cJun
FLAG-JNK
1 2 3 4 5 6 7 8 9 10

Figure 3

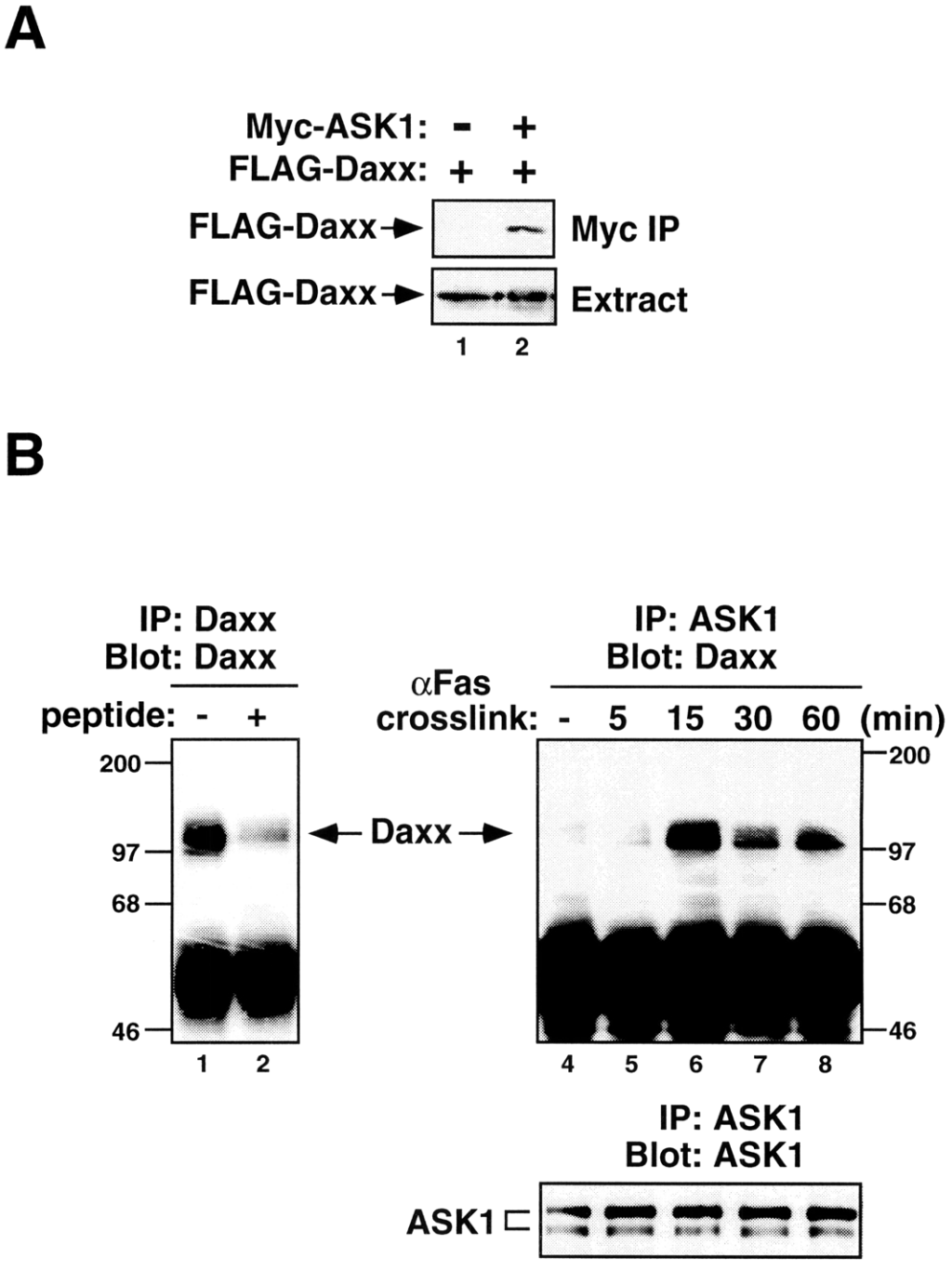
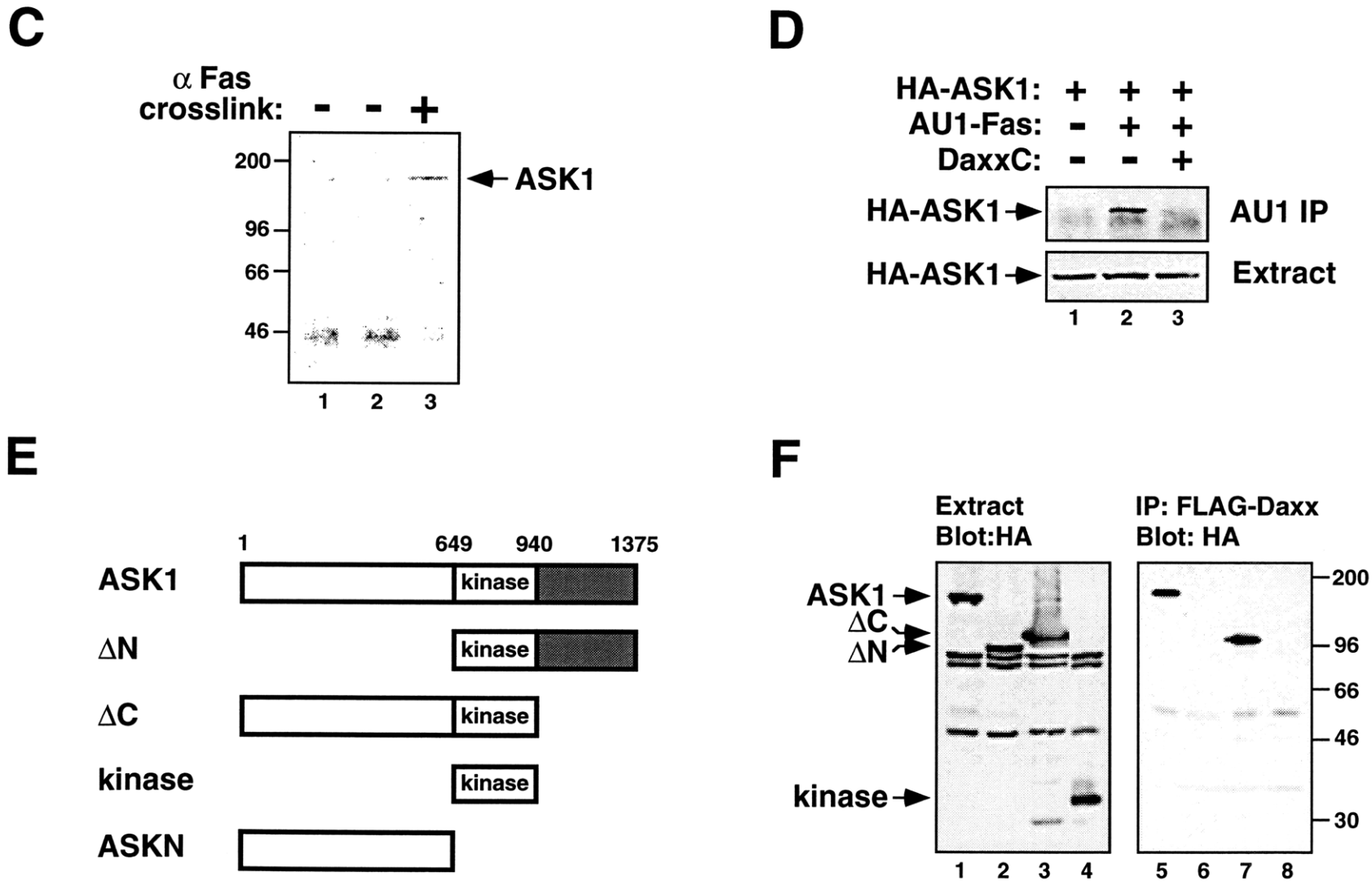
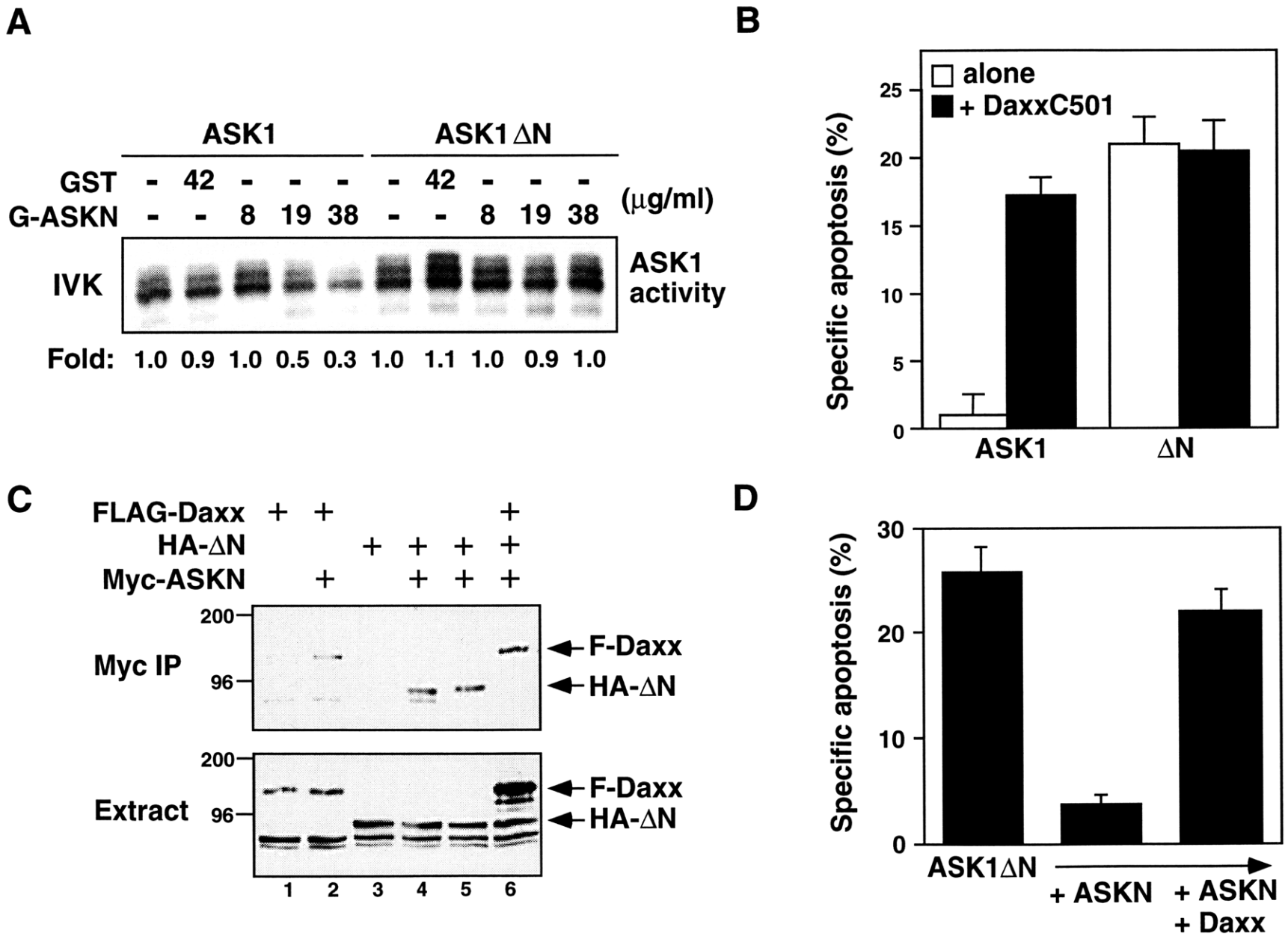

A
Myc-ASK1: - +
FLAG-Daxx: + +
FLAG-Daxx
Myc IP
FLAG-Daxx
Extract
1 2
B
IP: Daxx
Blot: Daxx
peptide: - +
200
97
68
46
1 2
Daxx
αFas
crosslink: - 5 15 30 60 (min)
IP: ASK1
Blot: Daxx
200
97
68
46
4 5 6 7 8
IP: ASK1
Blot: ASK1
ASK1

Figure 3


C
α Fas
crosslink: - - +
200
96
66
46
ASK1
1 2 3
D
HA-ASK1: + + +
AU1-Fas: - + +
DaxxC: - - +
HA-ASK1
AU1 IP
HA-ASK1
Extract
1 2 3
E
1
649
940
1375
ASK1
kinase
ΔN
kinase
ΔC
kinase
kinase
ASKN
F
Extract
Blot:HA
IP: FLAG-Daxx
Blot: HA
ASK1
ΔC
ΔN
kinase
200
96
66
46
30
1 2 3 4
5 6 7 8


A
ASK1
ASK1 ΔN
GST - 42 - - - - 42 - - -
G-ASKN - - 8 19 38 - - 8 19 38
(μg/ml)
IVK
ASK1 activity
Fold: 1.0 0.9 1.0 0.5 0.3 1.0 1.1 1.0 0.9 1.0
B
alone
+ DaxxC501
Specific apoptosis (%)
25
20
15
10
5
0
ASK1
ΔN
C
FLAG-Daxx + +
HA-ΔN + + + +
Myc-ASKN + + + +
200
96
Myc IP
F-Daxx
HA-ΔN
200
96
Extract
F-Daxx
HA-ΔN
1 2 3 4 5 6
D
Specific apoptosis (%)
30
20
10
0
ASK1ΔN
+ ASKN
+ ASKN
+ Daxx

# Chapter IV. Autoproteolytic activation of pro-caspases by oligomerization.


## Summary

Initiation of apoptosis requires the conversion of pro-caspases to mature caspases. Here we show that oligomerization of pro-caspases is sufficient to induce proteolytic generation of mature caspase subunits and activation of their cell death activity. Deletion of the protein interaction motif DED from pro-caspase-8 greatly suppresses its apoptotic activity. Cell death activity can be restored by oligomerization of pro-caspase-8 protease domains by two heterologous inducible oligomerization systems. Induced oligomerization also activates the apoptotic activity of pro-caspase-1 but not pro-caspase-3. In vitro, oligomerization leads to pro-caspase processing to form the mature caspase subunits; this processing requires the intrinsic caspase activity of zymogens and proceeds via a novel order of cleavage events.


## My contribution to this project

This project was a collaboration between myself and postdoc Xiaolu Yang. Xiaolu initiated this project, and we made the DNA constructs together. Xiaolu performed the cell death experiments and I carried out the caspase processing experiments. This work has been published in the following reference:


Yang, X., H.Y. Chang, and D. Baltimore (1998) Autoproteolytic activation of pro-caspases by oligomerization. Molecular Cell *1*, 319-325.

## Introduction

Mammalian caspases, an emerging family of cysteine proteases which cleave after aspartate, have been recognized as the central executors of the programmed cell death pathway (reviewed by Henkart, 1996; Salvesen and Dixit, 1997). Caspase-1 also processes pro-interleukin-1β and plays an important role in inflammation. Caspases are produced as inactive zymogens known as pro-caspases (Alnemri et al., 1996). Based on the crystal structures of caspase-1 and -3, a mature caspase consists of a tetramer of two large subunits of approximately 20 kDa surrounding two small subunits of approximately 10 kDa (reviewed by Henkart, 1996; Salvesen and Dixit, 1997). The protease active site includes residues from both subunits; the large subunit contains the conserved QACXG pentapeptide where C is the nucleophilic cysteine. Both the large and small subunits are generated from a single pro-caspase polypeptide by proteolytic cleavages. These cleavages separate the C-terminal protease domains from the N-terminal prodomains of various lengths and also separate the two protease subunits. These cleavages occur after critical aspartate residues which conform to the substrate recognition consensus of caspases. Mature caspases can often process their own precursors as well as other pro-caspases in vitro, suggesting that caspases may function in a cascade (Porter et al., 1997; Salvesen and Dixit, 1997).

A prime example of a caspase cascade is found in apoptosis induced by the cell death receptor Fas (reviewed by Nagata, 1997). The intracellular tail of Fas interacts with an adaptor protein termed FADD; FADD contains a death effector domain (DED), which interacts with homologous DEDs on pro-caspase-8 (FLICE/MACH/Mch5). Recruitment of pro-caspase-8 to the receptor completes the death-inducing signaling complex (DISC) and somehow activates pro-caspase-8. Active DISC formation then leads to the sequential activation of caspase-1-like and caspase-3-like activities. Caspases have been conceptually divided into initiators and executioners based on their potential roles in a cascade (Salvesen and Dixit, 1997). Caspase-3 or caspase-3-like proteases are responsible for the proteolytic

cleavage of many death substrates, which leads to the morphological changes and DNA fragmentation that are the hallmarks of apoptosis (Porter et al., 1997). Caspase-3 is therefore regarded as an executioner caspase (Salvesen and Dixit, 1997). Consistent with this idea, pro-caspase-3 has a very short prodomain, and it can be proteolytically activated by an upstream caspase (Muzio et al., 1997). In such a cascade, the key question of how an initiator caspase such as pro-caspase-8 becomes activated remains unanswered. Several mechanisms have been postulated: the removal of an inhibitor (Fraser and Evan, 1996); the presence of an inducible cofactor (such as CAP3 in DISC (Medema et al., 1997)); or processing of pro-caspases by non-caspases (Zhou and Salvesen, 1997).

Because oligomerization of Fas by ligand binding is activating, it is possible that pro-caspases are directly activated by oligomerization. When the zymogen molecules are brought into close proximity, they may display weak or transient proteolytic activity and cleave one another or adjacent molecules. Using two strategies for controlled oligomerization, we show that oligomerization is sufficient to initiate pro-caspase processing in vivo and in vitro and activate their cell death activity.

## Results

### Apoptosis mediated by pro-caspase-8 requires its DED domains

Overexpression of full length pro-caspase-8 potently induces cell death (Boldin et al., 1996; Muzio et al., 1996). DED domains can mediate homophilic interactions and may oligomerize pro-caspase-8 molecules to activate them. To test this possibility, we fused wild type pro-caspase-8 and mutants missing the DED domains to a protein motif allowing inducible oligomerization. As shown in Figure 1A, each fusion protein contained three tandem repeats of the FK506 binding protein (FKBP12), which is induced to oligomerize by addition of the divalent small chemical ligand AP1510 (Amara et al., 1997). To facilitate membrane localization and product detection, fusion proteins carried an N-terminal c-Src myristylation signal and a hemagglutinin tag (HA) and a FLAG tag at the fusion junction and the C-terminus, respectively. The C-terminal epitope tag does not interfere with p10 generation or caspase function (Xue et al., 1996). In the absence of the dimerizer, expression of Fkp3-Casp-8 in HeLa cells potently induced apoptosis (Figure 1B). The apoptotic cells showed the characteristic morphologies of membrane blebbing, pyknosis, and rounding up of the cell body. The cell death activity of Fkp3-Casp8 was blocked by the poxvirus serpin inhibitor crmA, consistent with previous results (Boldin et al., 1996; Muzio et al., 1996). In contrast to the full length pro-caspase-8 fusion, three mutants that lack the DED domains showed much reduced apoptosis (Figure 1B), indicating that the DED domains of pro-caspase-8 are required for its cell death activity.

### Activation of pro-caspase-8 by FKBP-mediated oligomerization

If the function of DED domains is to oligomerize pro-caspase-8, oligomerization of the Fkp3-caspase-8 mutants by AP1510 should restore their apoptotic activity. Fkp3-Casp8(180) caused little cell death in the absence of the dimerizer (less than 5%, Figure 1B and 2A). Addition of AP1510 caused a dose-dependent increase of apoptosis in HeLa cells expressing Fkp3-Casp8(180). Similar results were also observed when 293T cells were

used for transfection (data not shown). At an AP1510 concentration of 500 nM, more than 40% of transfected cell underwent apoptosis (Figure 2A), a level comparable to that seen in cells expressing Fkp3-Casp8. Addition of the monomeric, competitive FKBP ligand FK506 inhibited this apoptotic effect. In addition, Fkp3, which consists of only the tandem FKBP12 domains (Figure 1A), caused a dominant negative inhibition of killing induced by AP1510 (Figure 2A). These results show that oligomerization of the protease domain of pro-caspase-8 activates its killing activity. The killing by Fkp3-Casp8(180) is also inhibited by crmA. As a control, mutation of the active site cysteine (C360S) in Fkp3-Casp8(180) abolished the apoptotic activity in the presence of dimerizer. This indicates that cell death induced by oligomerization is dependent on the intrinsic caspase activity of caspase-8.

As with Fkp3-Casp8(180), the addition of AP1510 also activated the apoptotic activity of Fkp3-Casp8(206) (Figure 2B). Both constructs contain residue Asp216, which is recognized during the processing of pro-caspase-8 (Medema et al. 1997). In contrast, killing by Fkp3-Casp8(217), which lacks this cleavage recognition residue, did not increase with the addition of AP1510 (Figure 2B). This result implies that pro-caspase processing at residue 216 may be required for generating active caspase and thus for oligomerization-induced killing.

**Fas extracellular domain-mediated oligomerization of three pro-caspases**

To confirm that oligomerization activates pro-caspase-8, we used an alternative strategy of inducing oligomerization through a membrane-bound receptor. We chose the extracellular domain of murine Fas (FasEC, Figure 3A) for two reasons. First, its agonistic antibody, Jo2, is a pentameric IgM antibody which allows effective oligomerization (Ogasawara et al., 1993). Jo2 does not recognize human Fas and therefore has no toxic effect on human cell lines. Second, by making a FasEC fusion of the protease domain of pro-caspase-8 (FasEC-Casp8(182)), we can bypass several intermediaries that participate in the in vivo Fas-FADD-pro-caspase-8 connection: the death domain of Fas, FADD, and

the DED domains of pro-caspase-8. We therefore could address the function of these protein motifs. As shown in Figure 3B, addition of Jo2 did not cause cell death in human HeLa cells expressing FasEC. However, addition of Jo2 activated the apoptotic activity of FasEC-Casp8(182) in a dose-dependent fashion (Figure 3B). The level of cell death induced by FasEC-Casp8(182) was similar to that induced by wild type Fas at the maximal Jo2 concentration. Jo2-induced apoptosis through FasEC-Casp8(182) was blocked by crmA. Again, oligomerization-induced death required the intrinsic protease activity of the caspase because a catalytic cysteine to serine mutant, FasEC-Casp(182, C360S), did not respond to Jo2. These results confirm the results from the FKBP fusions and provide evidence that pro-caspase-8 can be activated by oligomerization. They also strongly suggest that with regard to the activation of pro-caspase-8, the role of the intermediary protein motifs between Fas and pro-caspase-8 is to physically link the pro-caspase-8 protease domain to the ligand binding extracellular domain of Fas.

To determine whether oligomerization-induced activation is a general property of caspases, we tested pro-caspase-1 and -3 in the FasEC fusion system. Pro-caspase-1 has a long prodomain like pro-caspase-8 but has a different substrate specificity compared to caspase-8 (Thornberry et al., 1997). The substrate specificity of caspase-3 overlaps with that of caspase-8 (Thornberry et al., 1997), but pro-caspase-3 contains a very short prodomain. Similarly to pro-caspase-8, apoptosis by a fusion of FasEC with pro-caspase-1 (FasEC-Casp1) was activated by the addition of Jo2 in a dose-dependent manner (Figure 3C). This killing required intrinsic caspase-1 protease activity because FasEC-Casp1(C284S), in which the catalytic cysteine was mutated, caused no death even in the presence of Jo2.

In contrast to pro-caspase-1 and pro-caspase-8, a FasEC fusion of pro-caspase-3, FasEC-Casp3, failed to respond to Jo2 in killing cells, even though FasEC-Casp3 caused substantial cell death by itself (Figure 3C). Similarly to the fusions of the other two proteases, the killing by FasEC-Casp3 also required its intrinsic protease activity because

FasEC-Casp3(C163S) caused no killing. Thus, FasEC-Casp3 is not activated by oligomerization but may be activated by pre-existing cellular caspases. Taken together, these results suggest that oligomerization-induced activation may be a property of initiator caspases but not of executioner caspases.

**Induction of pro-caspase processing by oligomerization**

To understand the mechanism of oligomerization-induced apoptosis, we followed the fate of pro-caspase proteins in transfected cells with and without induced oligomerization. We used 293T cells in these experiments due to their high transfection efficiency. At DNA concentrations that allow detection of transfected gene products by immunoblotting, Fkp3-Casp8(180) induced some apoptosis and was slowly processed in the absence of dimerizer (Figure 4A, lanes 1-5). This slow processing may represent the basal probability of membrane-targeted Fkp3-zymogen molecules randomly encountering one another. Processing generated two peptides containing the C-terminal FLAG-epitope: p37 (labeled ΔN) which appeared first and p10. p10 most likely corresponds to the small subunit in mature caspase-8 (Medema et al., 1997), which is derived from the C-terminus of pro-caspase-8, and p37 is an processing intermediate. In contrast, when Fkp3-Casp8(180) was expressed in the presence of AP1510, the zymogen was completely processed after only one hour of dimerizer treatment and only ΔN was detected (Figure 4A, lane 6). ΔN was completely processed after two hours of dimerizer treatment (Figure 4A, lane 7). In the presence of the dimerizer, newly synthesized zymogen was rapidly processed and accumulated as ΔN and p10 (Figure 4A, lane 8-10). This result suggests that oligomerization induces the processing of Fkp3-Casp8(180). Moreover, this processing required the intrinsic caspase activity of the zymogen. The active site C360S mutant of Fkp3-Casp8(180) did not become processed even in the presence of AP1510 (Figure 4A, lanes 11-12). This result argues against a model of activation where oligomerized pro-caspases become better substrates for a pre-existing cellular caspase and

strongly implies that pro-caspase processing is an autoproteolytic process. Similarly, deletion of one caspase recognition site in Fkp3-Casp8(217) blocks processing induced by AP1510 (Figure 4A, lanes 13-14), which explains why this mutant fails to induce apoptosis upon oligomerization.

We were surprised by our ability to detect p10 because we imagined that cell death would occur quickly once active caspase-8 was generated in the cytosol. Instead, we believe that the p10 detected in this experiment is likely to be still bound to the membrane-targeted Fkp3-zymogen oligomer. In the course of generating the mature tetrameric caspase from two zymogen molecules, one zymogen may be processed before the other, producing p20 and p10 subunits that are bound to unprocessed zymogens. This explains why p10 is detected in dimerizer-treated cells only when there is also some low level of the full length zymogen (Figure 4A, lanes 9-10). The cowpox virus serpin inhibitor crmA has been reported not to affect pro-caspase-8 processing but to inhibit mature caspase-8 activity (Medema et al., 1997; Muzio et al., 1997). When crmA was coexpressed with Fkp3-Casp8(180), processing was significantly slowed, and the processing intermediate ΔN was observed only in the presence of AP1510 (Figure 4B).

To circumvent the limitations of intracellular expression experiments, we established a cell free system where pro-caspase processing could be initiated by inducing oligomerization. The addition of AP1510 to in vitro-translated, $^{35}$S-labeled Fkp3-Casp8(206) led to the proteolytic generation of peptides corresponding to the mature caspase-8 subunits p18 and p10, and three processing intermediates p46, p37, and p20 (Figure 5A). Processing initiated by AP1510 was blocked by the addition of FK506, the monomeric ligand of FKBP that competes for AP1510 binding. It was also inhibited by the addition of the caspase inhibitor z-DEVD (Figure 5A). These results demonstrate directly that pro-caspase processing in vitro requires oligomerization and caspase activity. Fkp3-Casp8(217) served as a negative control and was incapable of being processed in the presence of AP1510 (Figure 5A). In the absence of the in vitro translated pro-caspase, the

reaction mixture contains no caspase activity as demonstrated by the lack of PARP cleavage in the presence of AP1510 (data not shown).

Based on time course experiments (Figure 5B), relative band intensities normalized by the known methionine content of the predicted peptides, comigration with known truncation mutants, and the presence of the C-terminal FLAG epitope tag, we were able to tentatively assign the identities of the proteolytic products (Figure 5B) and suggest a model of pro-caspase processing. First, the prodomain was separated from the protease domain, generating p46 and p37. p46 corresponds to the N-terminal Fkp3, and p37 corresponds to ΔN in Figure 4 and consists of the protease domain. Second, the protease domain is cleaved to generate p20 and p10. The p20 is then processed to p18. p18 and p10 are the mature subunits of caspase-8 that presumably associate to form a tetrameric enzyme. Consistent with this sequence of cleavages, deletion of the predicted first cleavage site in Fkp3-Casp8(217) completely blocked processing (Figure 4A and Figure 5A).

Taking FKBP-fused pro-caspase-8 as a model, these data collectively show that in vivo and in vitro, oligomerization can induce the autoproteolytic processing of pro-caspases.

## Discussion

A key question for understanding programmed cell death is how initiator caspases become activated. Using two inducible oligomerization systems, we show that oligomerization activates pro-caspase processing, which in turn activates their cell death activity. Pro-caspases may possess weak proteolytic activity and cleave one another when they are brought into close proximity. Alternatively, two pro-caspase molecules may form a mature caspase-like structure which cleaves other pro-caspases. Consistent with this idea, a mature caspase is a tetramer derived from two pro-caspase molecules. The pro-caspase-8 products from cell free processing suggest an order of cleavage events: the prodomain is first separated from the protease domain, followed by the separation of the two protease subunits. This order of cleavages is distinct from that observed when pro-caspase-8 is used as a substrate for active DISC (Medema et al., 1997). This difference reinforces the idea that activation of pro-caspase processing by oligomerization occurs through a distinct mechanism from that involved in processing of a pro-caspase by a mature caspase as occurs in other steps of the cascade.

Oligomerization-induced activation is likely to reflect the in vivo situation for pro-caspase-8. The processing of pro-caspase-8 in vivo is known to occurs at caspase sites (Medema et al., 1997). Oligomerization-induced processing is also inhibited by the caspase inhibitor z-DEVD (Figure 5A) and abrogated by deletion of the recognition site at amino acid 216 (Figure 4A and 5A). In addition, the processed products are in agreement with those intermediates detected in cells undergoing Fas-induced apoptosis (Medema et. al., 1997). Furthermore, by making a FasEC-caspase-8 fusion, we bypassed the intervening protein interaction motifs for the in vivo Fas-FADD-caspase-8 connection. FasEC-caspase-8 killed cells in a Jo2-dependent manner, strikingly similar to Fas-mediated cell death (Figure 3B). Therefore, the in vivo function of many proteins in the Fas receptor complex is likely to facilitate and regulate oligomerization of pro-caspase-8.

We propose that oligomerization of pro-caspases may be a general mechanism in initiating caspase cascades. Caspases have been divided into three major groups based on their substrate specificities: a caspase-8-like group, a caspase-1-like group, and a caspase-3-like group (Thornberry et al., 1997). We showed that oligomerization induces the activation of pro-caspase-8 and -1. A caspase-3-like protease, caspase-2 (Ich-1), binds to the death adaptor RAIDD via its prodomain and is recruited to TNF receptor 1 (Duan and Dixit, 1997). Membrane recruitment and oligomerization of pro-caspase-2 may then lead to autoproteolytic activation in a fashion similar to that found for pro-caspase-8. On the other hand, induced oligomerization of pro-caspase-3 did not enhance its cell death activity. Therefore, prodomain structure, rather than substrate specificity, may determine the ability of pro-caspases to be activated by oligomerization. The difference among pro-caspases-8, -1, and -3 may reinforce the conceptual division of initiator and executioner caspases and suggest that oligomerization-induced activation is a property of the initiator caspases.

Controlling the oligomerization state of pro-caspases may be a critical regulatory event in the cellular decision for life or death (Figure 6). Specific adaptor proteins that interact with the prodomains of various pro-caspases may allow distinct apoptotic stimuli to engage the death machinery by inducing pro-caspase oligomerization. A logical step in inducing pro-caspase oligomerization is to bring them near membranes; this reduces their movement from three dimensions to two dimensions and increases their local concentration. A recent study showed that pro-caspase-8 can also be recruited to the endoplasmic reticulum (ER) membrane through an ER protein, p28 Bap31 (Ng et al., 1997). Cell death effectors such as CED-4, Bcl-2 and cytochrome c may exert their effects through regulating oligomerization of pro-caspases. The *C. elegans* death effector CED-4 and its mammalian homolog Apaf-1 interacts with the zymogen form of CED-3 and caspase-9, respectively (reviewed by Hengartner, 1997; Li et al., 1997). CED-4 also associates with the cell death inhibitor CED-9, while Apaf-1 associates with the cell death

initiator cytochrome c (Hengartner, 1997). It is likely that additional regulatory strategies may also control the activity of caspases.

Oligomerization-induced activation of pro-caspases is reminiscent of the activation of receptor tyrosine kinases. Oligomerization of receptor tyrosine kinases leads to intermolecular cross-phosphorylation, which can increase their kinase activity and enhance their interaction with cellular proteins (Ullrich and Schlessinger, 1990). In a similar fashion, autoproteolytic processing of pro-caspases produces mature caspases that possess greater enzymatic activity. Oligomerization-induced autoproteolysis is a well established mechanism in activating the complement protease cascade. Our data show that a similar mechanism is used to initiate an intracellular protease cascade for programmed cell death and provide an additional example of oligomerization in activating signal transduction pathways.

## Experimental Procedures

### Reagents

The dimerizer AP1510 was the generous gift of Dr. M. Gilman (Ariad Pharmaceutical, Inc.). FK506 was the generous gift of Dr. S. Schreiber (Harvard University). Anti-murine Fas IgM Jo2 (Pharmingen, CA), anti-FLAG mAb M2 (Kodak), rabbit polyclonal anti-FLAG (Santa Cruz), and Z-Asp-Glu-Val-Asp (z-DEVD, Enzyme System Products, CA) were obtained from the indicated sources.

### Expression Plasmids

FKBP and FasEC fusions of caspases were constructed in pRK5 (Generous gift of Dr. D.V. Goeddel). Plasmids for FKBP fusions: first, a fragment containing three tandem repeats of FKBP12 with a N-terminally fused c-Src myristylation signal and a C-terminally-fused HA tag, was amplified by polymerase chain reaction (PCR) from pMF3E (Spencer et al., 1993), digested with EcoRI and BamHI, and cloned into pRK5, yielding pFkp3-HA. Second, DNA fragments containing full length and deletion mutants of human pro-caspase-8 with a C-terminal FLAG tag were digested with BamHI and HindIII and cloned into pFkp3-HA. pFkp3 was made by the deleting the SalI/XhoI fragment of Caspase-8(180) in pFkp3-Casp8(180). Murine Fas EC (residues 1 to 189) fusions were made by three-way ligation: For FasEC-Casp1, a BamHI/NcoI fragment of FasEC was ligated to a NcoI/SalI fragment of murine caspase-1 in pRK5. For FasEC-Casp3 and FasEC-Casp8(182), a BamHI/EcoRI fragment of FasEC was ligated with a EcoRI/SalI fragment of human caspase-3 or human caspase-8 residues 182 to 479 in pRK5. Active site C to S mutants were made by PCR mutagenesis and assembled in the same way as the corresponding wild type constructs. Each construct was confirmed by partial DNA sequencing and immunoblotting or in vitro translation. pRK-crmA (Hsu et al., 1995) and pEBB-mFas (Yang et al., 1997) were previously described.

**Cell Death Assay**

The cell death assay was performed essentially as previously described (Yang et al., 1997). 1.5x $10^5$ HeLa cells/well was transfected with the calcium phosphate precipitation method. For each transfection, 0.25 μg of a β-galactosidase reporter plasmid pCMV-lacZ was included and the total amount of DNA was adjusted to 1.25 μg with the vector plasmid pRK5. AP1510 or FK506 was added 6 hours after transfection as indicated and cells were stained with X-gal 16 hour after transfection. For Fas-EC fusions, Jo2 antibody was added as indicated 10 hours after transfection and cells were stained with X-gal 18 hours after transfection. The percentage of apoptosis was determined by the number of blue cells with apoptotic morphology divided by the total number of blue cells. Specific apoptosis was calculated as the percentage of apoptosis in each transfection minus the percentage of apoptosis in the vector transfected cells that was not treated with either AP1510 or Jo2. The vector control was always included in each experiment and was less than 5%. Data shown are the averages and standard deviations of two to four independent experiments. For each experiment, 200 or more blue cells were counted from randomly chosen fields.

**Pro-caspase processing in transfected cells**

7.5 x $10^5$ 293T cells plated on 35 mm dishes were transfected with 1 μg of the indicated expression plasmids by the calcium phosphate method. Eleven hours after transfection, the medium from parallel transfections was exchanged for medium containing either vehicle or 1 μM AP1510. At the indicated time points, the transfected cells were washed once with ice cold PBS and extracted in 100 μl IP-lysis buffer (Hsu et al., 1995). Protein concentrations in extracts were equalized by Bradford assay and analyzed by immunoblot for FLAG with ECL (Amersham).

**Cell free processing of pro-caspase-8**

In vitro transcription and translation of the indicated constructs with $^{35}$S-labeled methionine were carried out with the TNT Reticulocyte Lysate System (Promega). The reaction mixture contains 1 μl of the in vitro translation product and the indicated drugs dissolved in 3 μl of CED3 reaction buffer (Xue et al., 1996). Final drug concentrations of 100 nM of AP1510 or FK506 and 10 μM of z-DEVD were used. The reaction mixture was incubated in 30 °C for 4 hours, and the reaction stopped by the addition of SDS sample buffer. Reaction products were visualized by SDS-PAGE and autoradiography.

**Acknowledgment**

We thank Y.H. Li and P. Svec for excellent technical support. We are grateful to M. Gilman for the generous gift of AP1510. We thank Drs. E.S. Alnemri, D.V. Goeddel, M.J. Lenardo, J. Liu, Z. Luo, S.L. Schreiber, Z. Song, D. Xue, and J. Yuan for reagents and advice. We thank Dr. A. Hoffmann for help with in vitro translation, A. J. Koleske, B. Horwitz, Z. Songyang and other members of the Baltimore lab for valuable advice. X.Y. is a fellow of the Leukemia Society of America, and H.Y.C. is supported by the Medical Scientist Training Program at Harvard Medical School. D.B. is an American Cancer Society Research Professor. This work was supported by the NIH grant CA51462.

**References**

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan , J. (1996) Human ICE/CED-3 protease nomenclature. Cell *87*, 171.

Amara, J. F., Clackson, T., Rivera, V. M., Guo, T., Keenan, T., Natesan, S., Pollock, R., Yang, W., Courage, N. L., Holt, D. A., and Gilman, M. (1997). A versatile synthetic dimerizer for the regulation of protein-protein interactions. Proc. Natl. Acad. Sci. U S A 1997 *94*, 10618-10623.

Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. Cell *85*, 803-815.

Duan, H., and Dixit, V. M. (1997). RAIDD is a new 'death' adaptor molecule. Nature *385*, 86-89.

Porter, A. G., Ng, P., and Janicke, R. (1997). Death substrates come alive. BioEssays *19*, 501-507.

Fraser, A., and Evan, G. (1996). A license to kill. Cell *85*, 781-784

Hengartner, M. O. (1997). Apoptosis. CED-4 is a stranger no more. Nature *388*, 714-715.

Henkart, P. A. (1996). ICE family proteases: mediators of all apoptotic cell death? Immunity *4*, 195-201.

Hsu, H., Xiong, J., and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kB activation. Cell *81*, 495-504.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascades. Cell, *91*, 479-489.

Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H., and Peter, M. E. (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J *16*, 2794-2804.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. 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). FLICE, a novel FADD-homologous ICE/CED-3 like protease, is recruited to the CD95 (Fas/Apo1) death inducing signal complex. Cell *85*, 817-827.

Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997). FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J. Biol. Chem. *272*, 2952-2956.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-365.

Ng, F. W. H., Nguyen, M., Kwan, T., Branton, P. E., Nicholson, D. W., Cromlish, J. A., and Shore, G. C. (1997). p28 bap31, a bcl-2/Bcl-XL- and procaspase-8 associated protein in the endoplasmic reticulum. J Cell Biol *139*, 327-338.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. (1993). Lethal effect of the anti-Fas antibody in mice. Nature *364*, 806-809.

Salvesen G. S. and Dixit, V. M. (1997) Caspases: intracellular signaling by proteolysis. Cell *91*, 443-446.

Spencer, D. M., Wandless, T. J., Schreiber, S. L., and Crabtree, G. R. (1993). Controlling signal transduction with synthetic ligands. Science *262*, 1019-1024.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., Nicholson, D.W. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J. Biol. Chem. *272*,17907-17911

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203-212.

Xue, D., Shaham, S., and Horvitz, H. R. (1996). The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Dev. *10*, 1073-1083.

Yang, X., Khosravi-Far, R., Chang, H.Y., and Baltimore, D. (1997). Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell *89*, 1067-1076.

Zhou, Q. and Salvesen, G.S. (1997). Activation of pro-caspase-7 by serine proteases includes a non-canonical specificity. Biochem. J. *324*, 361-364.

**Figure Legends**

Figure 1. Apoptotic activity of FKBP fusions of full length and mutant pro-caspase-8.

(A) Schematic diagram of the FKBP fusions of pro-caspase-8. Fkp: FK506 binding protein FKBP12. DED: death effector domain. p18 and p12: subunits that form caspase-8. D: aspartic acids at the cleavage sites for the generation of p18 and p12. "*": the active site cysteine to serine mutation. c-Src myristylation signal (M) and HA and FLAG tags are also indicated.

(B) Apoptosis induced by pro-caspase-8 fusion proteins. HeLa cells were transfected with 0.125 μg of each Fkp-Casp8 plasmid and 0.25 μg of pRK-crmA as indicated. Percentage of specific apoptosis were determined as described in the Experimental Procedures.

Figure 2. Oligomerization of the protease domain of pro-caspase-8 induces apoptosis.

(A) Caspase-8(180)-induced apoptosis requires oligomerization and intrinsic protease activity. HeLa cells were transfected with 0.125 μg of Fkp3, Fkp3-Casp8(180), or Fkp3-Casp8(180, C360S), together with 0.25 μg of pRK-crmA or 0.125 μg of Fkp3 as indicated. Treatment of AP1510 (concentration indicated) and FK506 (50 nM) were done for 10 hours as described in the Experimental Procedures.

(B) Pro-caspase processing is required for oligomerization-induced apoptosis. HeLa cells were transfected with 0.125 μg of Fkp3, Fkp3-Casp8(206), or Fkp3-Casp8(217). AP1510 treatment was done as described in A.

Figure 3. Apoptotic activity of pro-caspase fusions with the Fas extracellular domain .

(A) Schematic diagram of pro-caspase-1, -3, and -8 fusions with the murine Fas extracellular domain. The fusion constructs contained either pro-caspase-8(amino acids 182 to 479), full length murine pro-caspase-1, or full length human pro-caspase-3 – as well as the corresponding catalytic Cys to Ser mutations (not shown) – fused to the extracellular and transmembrane domain of murine Fas (FasEC). The leader peptide (L) and

transmembrane domain (TM) of murine Fas, FLAG tag, and the large and small subunits of each caspase are indicated.

(B) FasEC-mediated oligomerization of pro-caspase-8 activates its apoptotic activity. HeLa cells were transfected with FasEC (25 ng), FasEC-Casp8(182) (25 ng), FasEC-Casp8(182, C360S) (125 ng), or pEBB-mFas (250 ng), together with pRK-crmA (250 ng) as indicated. Jo2 treatment and X-gal staining are as described in the Experimental Procedures.

(C) FasEC-mediated oligomerization activates the apoptotic activity of pro-caspase-1 but not pro-caspase-3. Experiments were done as described above. The amount of plasmids used for each transfection was 12.5 ng for FasEC and FasEC-Casp1, 125 ng for FasEC-Casp1(C284S), FasEC-Casp3, and FasEC-Casp3(C163S), and 250 ng for pRK-crmA.

Figure 4. Pro-caspase processing in transfected cells.

(A) Pro-caspase processing induced by oligomerization. One μg of the indicated Fkp3-fusion expression constructs was transfected in 293T cells. Vehicle or 1 μM AP1510 was added 11 hours after transfection, and cell extracts were made after the indicated times and immunoblotted for FLAG.

(B) Processing in the presence of crmA. One μg of Fkp3-Casp8(180) was cotransfected with 1 μg of pRK5-crmA into 293T cells. Drug treatment, cell extracts, and FLAG immunoblot were performed as in A.

Figure 5. Pro-caspase processing in a cell free system.

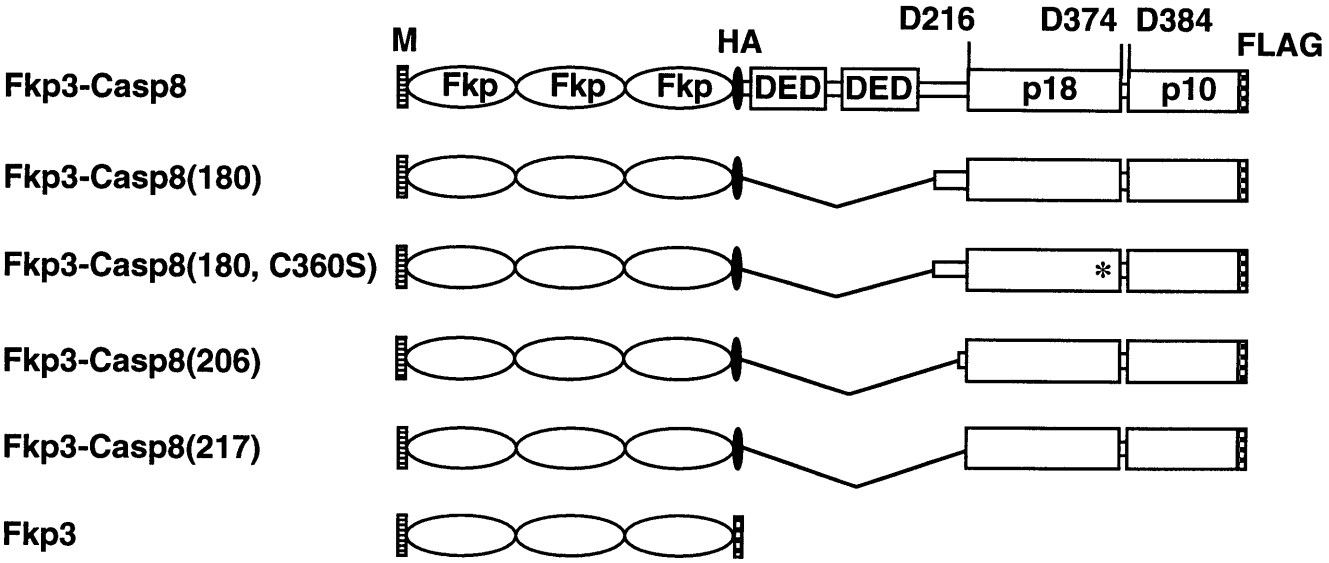
(A) Pro-caspase processing induced by oligomerization. The processing reaction was carried out with in vitro-translated, $^{35}$S-labeled FKBP-fusions as described in Experimental Procedures and visualized by SDS-PAGE and autoradiography.

(B) Time course of pro-caspase processing. The deduced domain structure of the indicated bands is shown on the right.

Figure 6. Model of pro-caspase activation in apoptosis.

Figure 1

A


M
HA
D216
D374
D384
FLAG
Fkp3-Casp8
Fkp
Fkp
Fkp
DED
DED
p18
p10
Fkp3-Casp8(180)
Fkp3-Casp8(180, C360S)
*
Fkp3-Casp8(206)
Fkp3-Casp8(217)
Fkp3

B

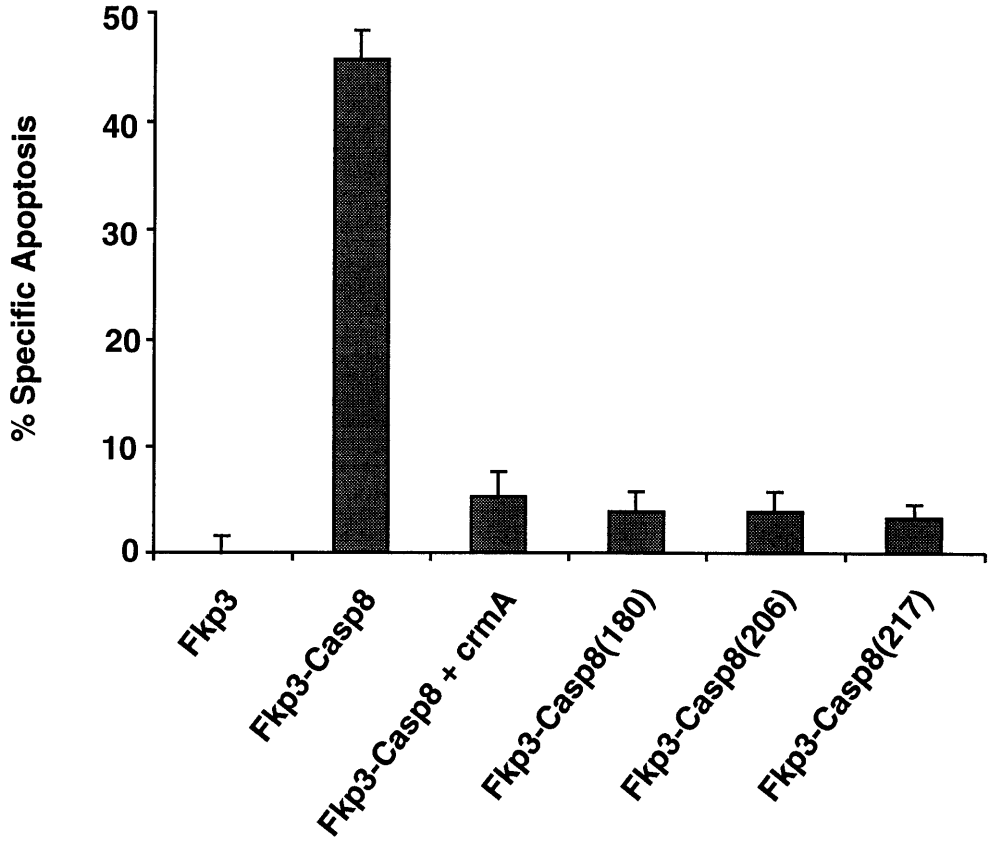

% Specific Apoptosis
50
40
30
20
10
0
Fkp3
Fkp3-Casp8
Fkp3-Casp8 + crmA
Fkp3-Casp8(180)
Fkp3-Casp8(206)
Fkp3-Casp8(217)

Figure 2

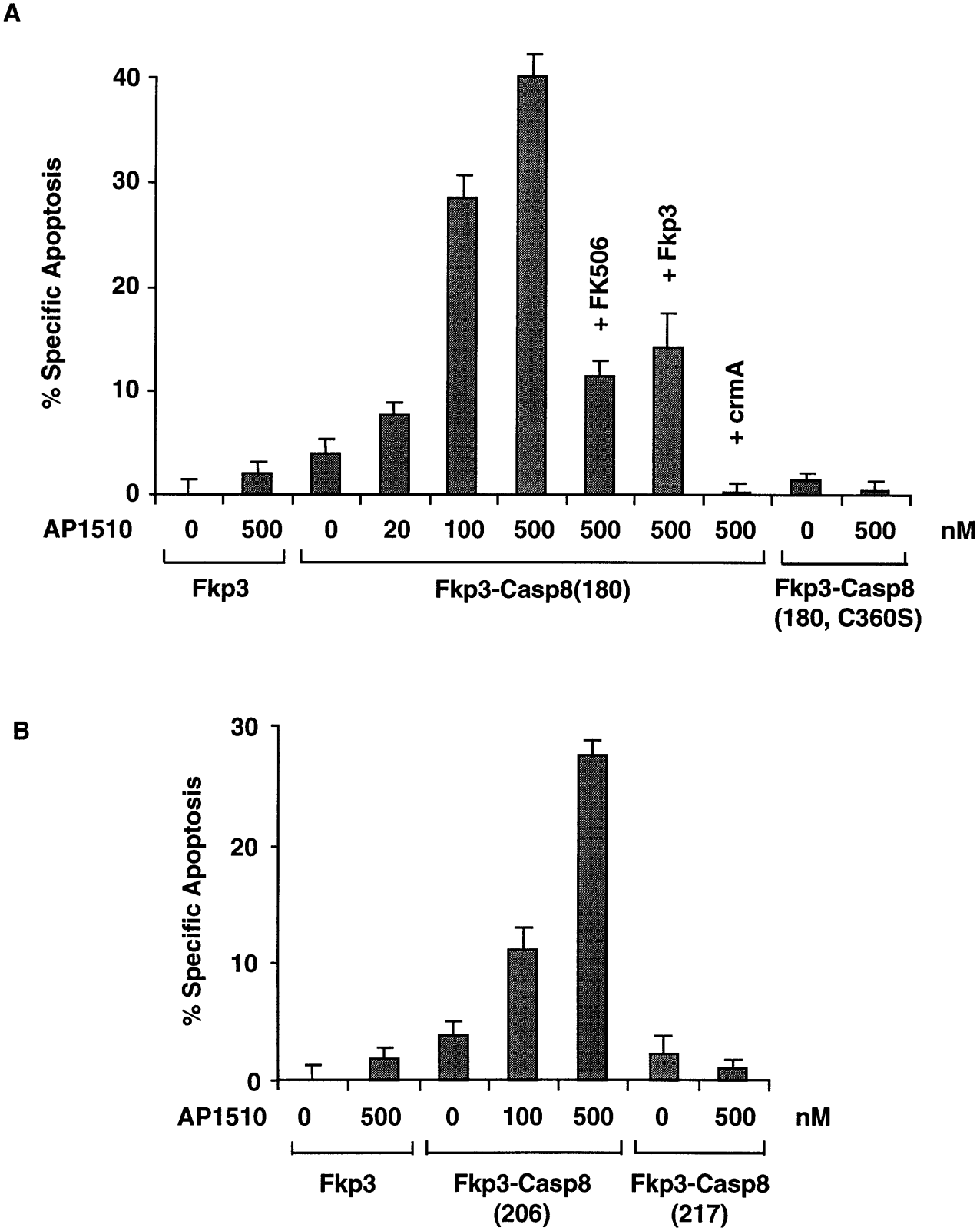

A
% Specific Apoptosis
40
30
20
10
0
+ FK506
+ Fkp3
+ crmA
AP1510
0
500
0
20
100
500
500
500
500
0
500
nM
Fkp3
Fkp3-Casp8(180)
Fkp3-Casp8
(180, C360S)
B
% Specific Apoptosis
30
20
10
0
AP1510
0
500
0
100
500
0
500
nM
Fkp3
Fkp3-Casp8
(206)
Fkp3-Casp8
(217)

Figure 3

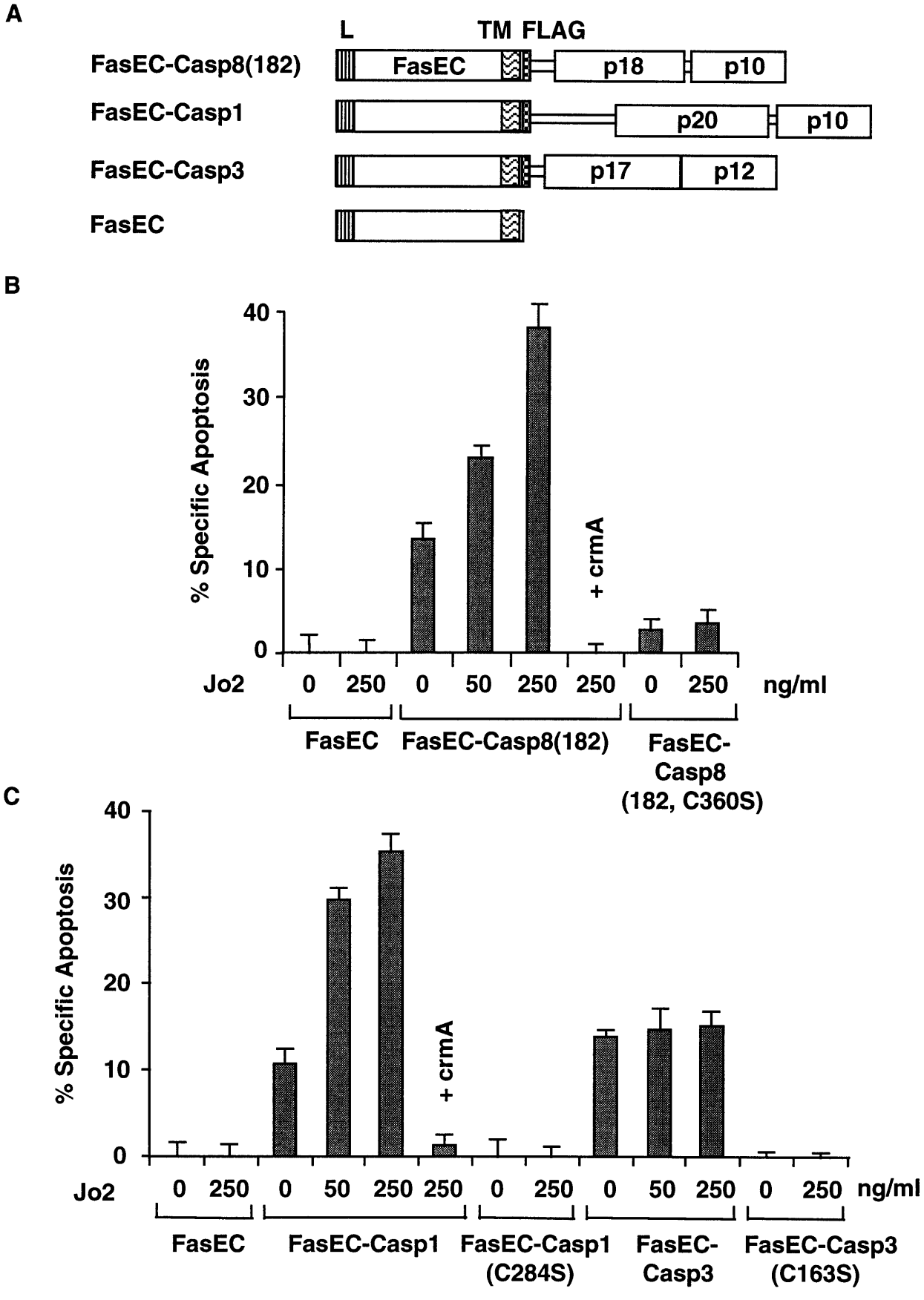

A
L
TM
FLAG
FasEC-Casp8(182)
FasEC
p18
p10
FasEC-Casp1
p20
p10
FasEC-Casp3
p17
p12
FasEC
B
% Specific Apoptosis
40
30
20
10
0
+ crmA
Jo2
0
250
0
50
250
250
0
250
ng/ml
FasEC
FasEC-Casp8(182)
FasEC-Casp8 (182, C360S)
C
% Specific Apoptosis
40
30
20
10
0
+ crmA
Jo2
0
250
0
50
250
250
0
250
0
50
250
0
250
ng/ml
FasEC
FasEC-Casp1
FasEC-Casp1 (C284S)
FasEC-Casp3
FasEC-Casp3 (C163S)

Figure 4

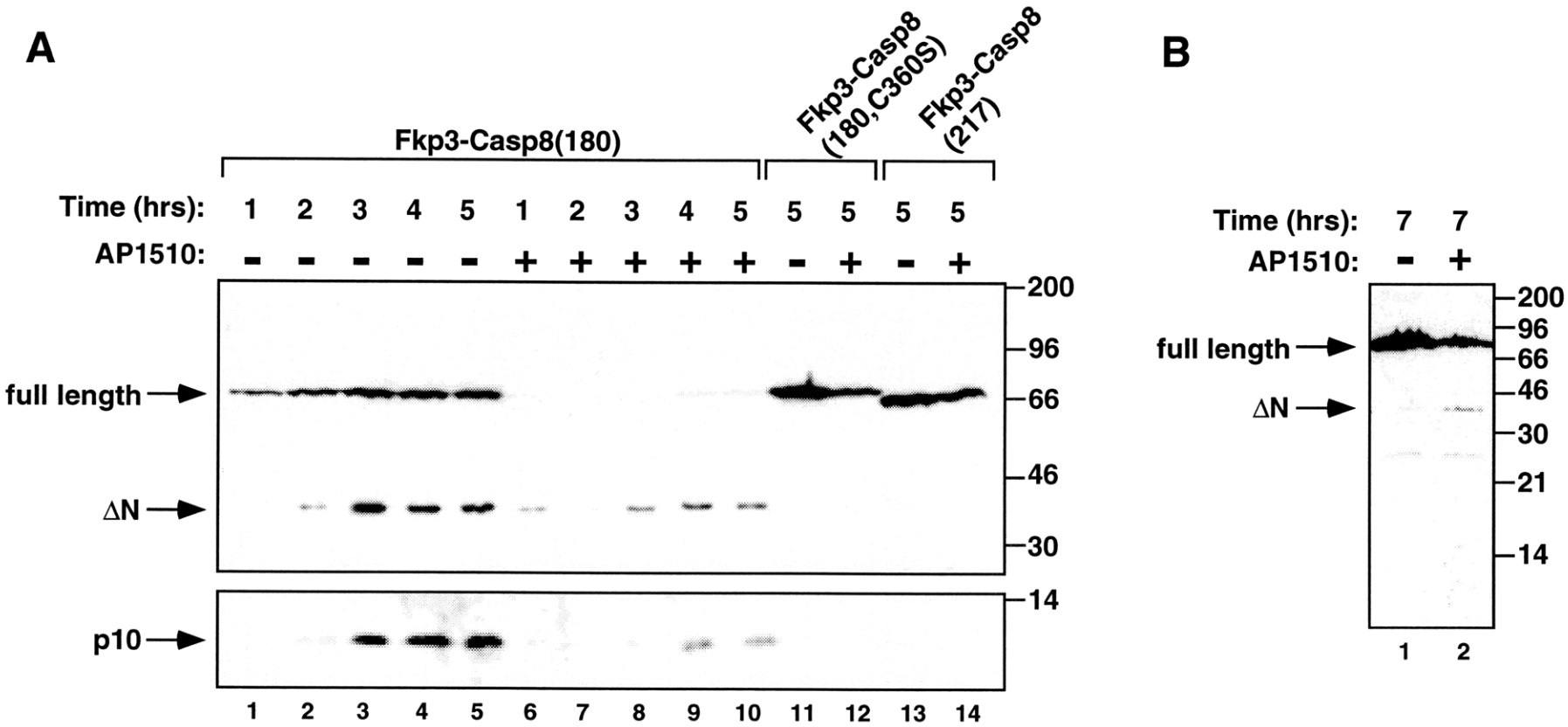
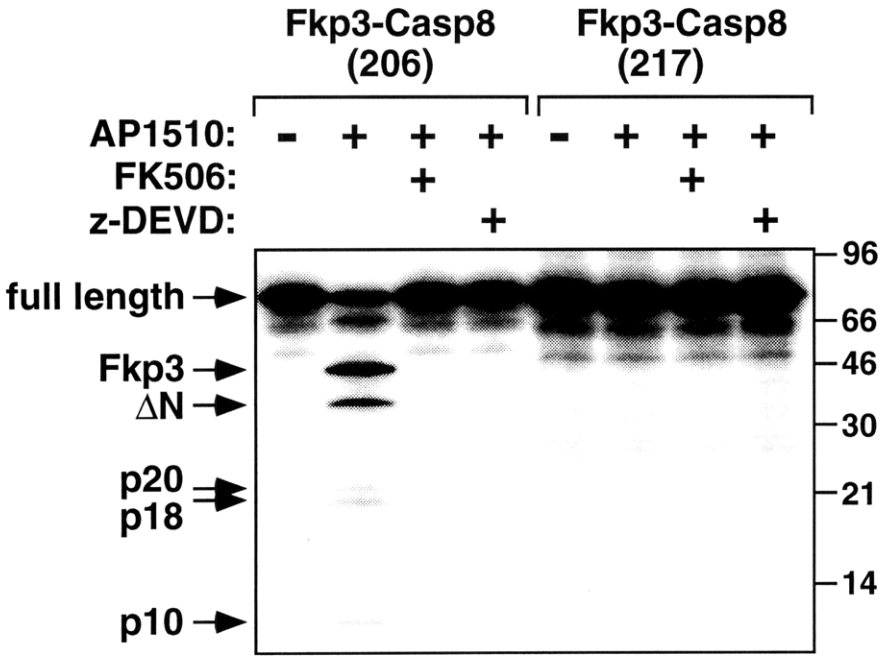

A
Fkp3-Casp8(180)
Fkp3-Casp8 (180,C360S)
Fkp3-Casp8 (217)
Time (hrs): 1 2 3 4 5 1 2 3 4 5 5 5 5 5
AP1510: - - - - - + + + + + - + - +
full length
ΔN
p10
200
96
66
46
30
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
Time (hrs): 7 7
AP1510: - +
full length
ΔN
200
96
66
46
30
21
14
1 2

Figure 5

A


Fkp3-Casp8
(206)
Fkp3-Casp8
(217)
AP1510: - + + + - + + +
FK506: + +
z-DEVD: + +
full length
Fkp3
ΔN
p20
p18
p10
96
66
46
30
21
14

B

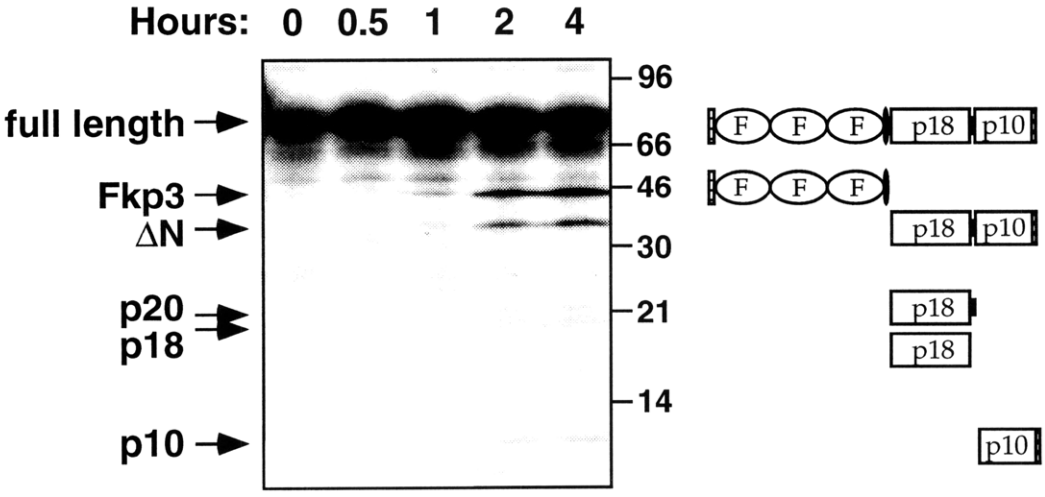
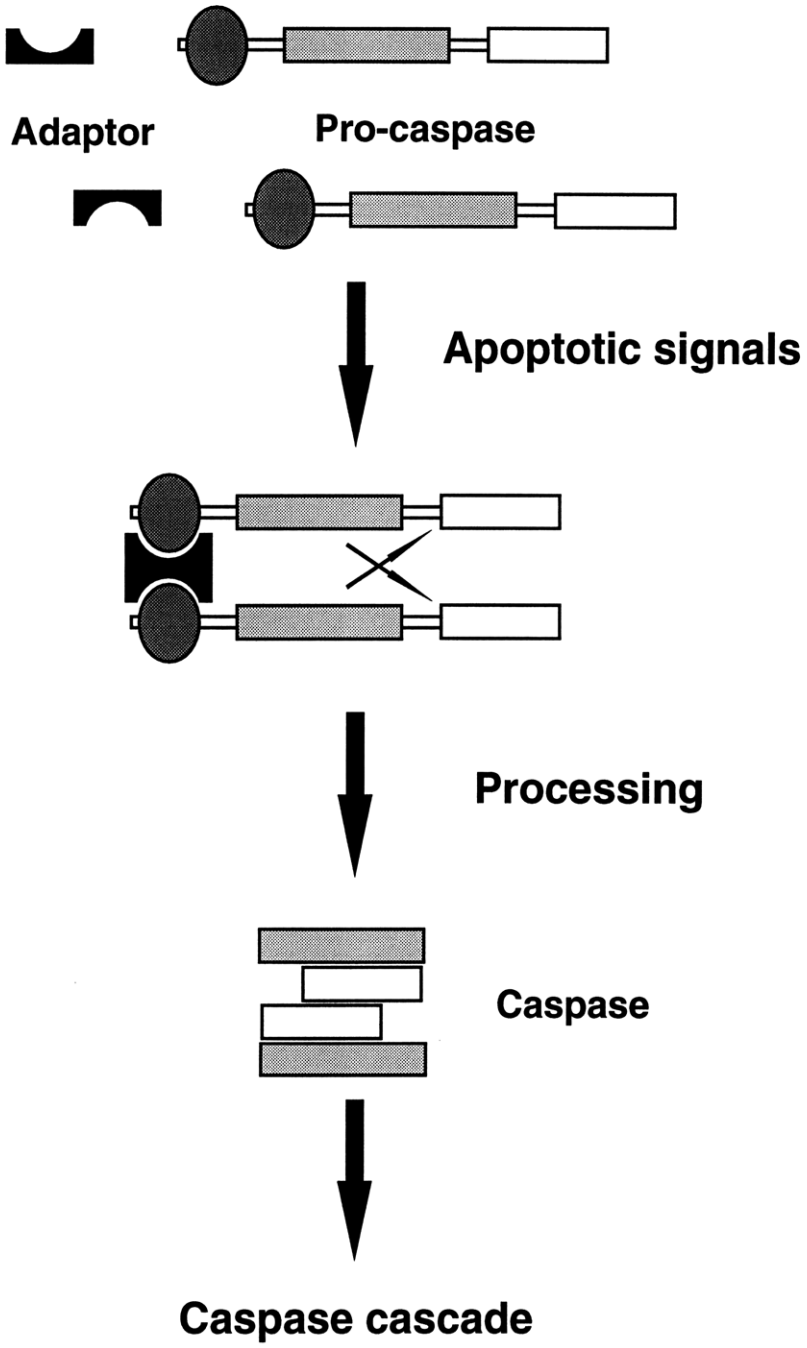

Hours: 0 0.5 1 2 4
full length
Fkp3
ΔN
p20
p18
p10
96
66
46
30
21
14
F F F p18 p10
F F F
p18 p10
p18
p18
p10

Figure 6


Adaptor
Pro-caspase
Apoptotic signals
Processing
Caspase
Caspase cascade

# Chapter V. Essential role of CED-4 oligomerization in CED-3 activation and apoptosis.


## Summary

Control of the activation of apoptosis is important both in development and in protection against cancer. In the classic genetic model *Caenorhabditis elegans*, the pro-apoptotic protein CED-4 activates the CED-3 caspase and is inhibited by the Bcl-2-like protein CED-9. Both processes are mediated by protein-protein interaction. Here facilitating proximity of CED-3 zymogen molecules was shown to induce caspase activation and cell death. CED-4 protein oligomerized in cells and in vitro. This oligomerization induced CED-3 proximity and competed with CED-4:CED-9 interaction. Mutations that abolished CED-4 oligomerization inactivated its ability to activate CED-3. Thus, the mechanism of control is that CED-3 in CED-3:CED-4 complexes is activated by CED-4 oligomerization, which is inhibited by binding of CED-9 to CED-4.


## My contribution to this project

This project was another close collaboration between myself and Xiaolu Yang which we initiated together. I designed and performed the experiements on CED-3 processing, CED-4 oligomerization domain mapping, and the effects of CED-4 oligomerization on CED-9 and CED-3 interaction. Xiaolu set up the HeLa cell death assay for evaluating CED-4 function and performed the cell death experiments. This work has been published in the following reference:


Yang, X., H.Y. Chang, and D. Baltimore (1998) Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. Science *281*, 1355-1357.

## Introduction

Apoptosis, a process of cell suicide critical for development and tissue homeostasis of multicellular organisms, is controlled by an evolutionarily conserved program (Steller, 1995; Horvitz *et al.*, 1994). Genetic studies in the nematode *Caenorhabditis elegans* have identified three core components of the death machine (Horvitz *et al.*, 1994). CED-4 activates the apoptotic protease CED-3 while CED-9 inhibits CED-4 function, these interactions involving direct protein-protein contact in a ternary protein complex termed the apoptosome (Chinnaiyan et al., 1997; Wu et al., 1997; Spector et al., 1997; Hengartner, 1997). How CED-4:CED-3 interaction activates cell death while CED-9:CED-4 interaction inhibits cell death is unknown. CED-3 is homologous to a family of mammalian cysteine protease (caspase) zymogens that, when activated, cleave various cellular proteins to execute apoptosis (Yuan et al., 1993). CED-9 is homologous to the mammalian Bcl-2 family of anti-apoptotic proteins (Hengartner and Horvitz, 1994). Apaf-1, a mammalian homologue of CED-4 that activates pro-caspase-9, has also been identified (Zhou et al., 1997).

## Results

### Activation of CED-3-dependent apoptosis and zymogen processing by artificial oligomerization

Caspase zymogens that link to surface receptors are autoproteolytically converted to mature enzymes by the induced proximity of their protease domains (Yang et al., 1998; Muzio et al., 1998; Steller, 1998). To test whether CED-3 can be activated in a similar way, we fused the CED-3 protease domain to three copies of the FK506 binding protein (Fkp) (Figure 1A). Expression of this fusion protein, Fkp3-CED-3(205), in HeLa cells caused minimal cell death. However, oligomerization of the fusion protein by a dimeric ligand for Fkp, AP1510 (Amara et al., 1997), induced apoptosis in a dose-dependent and saturable manner while addition of the monomeric, competitive Fkp ligand FK506 partially inhibited the effect (Figure 1B). In a cell-free system, AP1510 induced in vitro-translated, $^{35}$S-labeled Fkp3-CED-3(205) fusion protein to be cleaved, generating the mature CED-3 subunits, p17 and p15 (Figure 1C, lane 2). CED-3 processing was inhibited by FK506 or the caspase inhibitor z-DEVD (Figure 1C, lanes 3, 4), indicating that oligomerization and caspase activity are required for zymogen processing. These results strongly imply that CED-3 can be activated by the induced proximity provided by drug-mediated protein aggregation.

### CED-4 protein forms oligomers in cells and in vitro

Because CED-4 interacts with CED-3, we reasoned that CED-4 could induce CED-3 proximity and activation if CED-4 had the ability for homotypic oligomerization. This possibility was examined by co-immunoprecipitation assays in transfected 293T cells expressing two differentially epitope-tagged CED-4. Myc-CED-4 specifically co-immunoprecipitated FLAG-CED-4, suggesting that CED-4 protein can oligomerize in cells (Figure 2A, B). Deletion analysis revealed that the CED-4 region consisting of amino acids 171 to 435 and mutants incorporating it, but not other parts of CED-4, interacted with full length CED-4 (Figure 2A, B). The oligomerization domain of CED-4 is distinct from the

N-terminal CED-3 interaction domain (Chinnaiyan et al., 1997), smaller than the sequence required for CED-9 interaction (Ottilie et al., 1997), and is encompassed in a domain that is homologous to mammalian Apaf-1 (Zhou et al., 1997) (Figure 2A). Deletion or mutation (K165R) of the putative ATP-binding P-loop (Chinnaiyan et al., 1997), had no effect on CED-4 oligomerization. CED-4L, an anti-apoptotic splice variant of CED-4 (Shaham and Horvitz, 1996), was also able to bind CED-4. In vitro translated, $^{35}$S-labeled CED-4 specifically bound to purified recombinant GST-CED-4 fusion protein. Equal amounts of either in vitro-translated CED-3 or CED-4 bound with comparable efficiency to GST-CED-4, suggesting a similar affinity of the two interactions (Figure 2C). The CED-4 oligomerization is probably direct or at a minimum involves proteins found in reticulocyte lysates. Finally, the Apaf-1 region (amino acids 1 to 465) that is homologous to CED-4 and sufficient to activate pro-caspase-9 (Hu et al., 1998) was also capable of homotypic oligomerization (Figure 2D).

**Effect of CED-4 oligomerization on interaction with CED-3 and CED-9**

To examine whether homotypic CED-4 interactions could affect the trimeric CED-3:CED-4:CED-9 complex, we added the three components along with a truncated CED-4 (171-549), which binds to CED-4 but not CED-9 or CED-3. Myc-tagged CED-4 provided the handle for immunoprecipitation. CED-4(171-549) competed out CED-9 but not CED-3 from the complex with Myc-CED-4 (Figure 3, lanes 2, 3). CED-4(171-549) also competed away a mammalian CED-9 homolog, Bcl-$x_L$ (Chinnaiyan et al., 1997; Hu et al., 1998), from the CED-3:CED-4:Bcl-$x_L$ complex without affecting the CED-4:CED-3 interaction (Figure 3, lanes 5 and 6). These results argue that CED-9 or Bcl-$x_L$ binding to CED-4 is mutually exclusive with CED-4 oligomerization, and that CED-4 can simultaneously associate with itself and CED-3. Furthermore, Myc-CED-3 specifically co-immunoprecipitated with FLAG-CED-3 only in the presence of CED-4 (Figure 3B), demonstrating that CED-4 can induce the proximity of CED-3 molecules.

**Role of CED-4 oligomerization in apoptosis induction**

To assess the functional role of CED-4 oligomerization, we mutated amino acids within the CED-4 oligomerization domain that are conserved among CED-4, Apaf-1, and related plant *R* genes (van der Biezen and Jones, 1998). Each CED-4 mutant (mut 1-5, Figure 4A) expressed stable full length protein but failed to oligomerize (Figure 4B). Mutation 1 (D250A, D251A) was previously shown to be a loss-of-function mutant but still able to bind CED-3 (Chinnaiyan et al., 1997). Similarly, mutations 2 through 5 also retained their ability to bind CED-3 (data not shown), consistent with previous domain mapping experiments (Chinnaiyan et al., 1997). We tested the pro-apoptotic activity of these CED-4 mutants in a transient transfection system in HeLa cells that accurately reflected the in vivo function of the apoptosome components. Expression of CED-3 or CED-4 alone had no cytotoxicity in HeLa cells (Figure 4C, lanes 2, 5), but their coexpression led to robust apoptosis in over 60% cells in a CED-4 concentration-dependent manner (Figure 4C, lanes 6-8). This apoptotic effect required the intrinsic caspase activity of CED-3 (Figure 4C, lane 4). Coexpression of anti-apoptotic proteins CED-9 or Bcl-$x_L$ completely inhibited the ability of CED-4 to activate CED-3-dependent death (Figure 4C, lanes 9, 10). All five CED-4 mutants showed no ability to activate CED-3-dependent death (Figure 4C, lanes 11-15). The strong correlation between the loss of CED-4 oligomerization and the loss of the ability to activate CED-3 indicates that CED-4 oligomerization may be essential for its pro-apoptotic function.

## Discussion

The present study suggests CED-4 oligomerization as a unifying mechanism in apoptosome function. Previous work demonstrated that CED-9 or Bcl-$x_L$, upon binding pro-apoptotic, BH3-containing ligands such as Bax, is released from the apoptosome (Chinnaiyan et al., 1997). EGL-1, a *C. elegans* BH3-containing ligand functioning upstream of CED-9, is also thought to displace CED-9 from CED-4 (Conradt and Horvitz, 1998). We propose that release from CED-9 allows CED-4 to oligomerize, bringing the associated CED-3 proteins into close proximity and facilitating subsequent autoproteolytic activation (Figure 5). Mammalian Apaf-1 can also oligomerize and may function in a similar way to activate pro-caspase-9. In this model, the activation of CED-9 binding activity of a pro-apoptotic protein upstream of the apoptosome (EGL-1 in Figure 5) is thus a key event and requires further investigation. Additional regulators, such as dATP and cytochrome c (Liu et al., 1996), may control apoptosome function through similar or distinct mechanisms.

## Experimental Procedures

### Plasmid Construction

pFkp3-CED-3(205), containing three copies of Fkp fused to CED-3 amino acids 205 to 503, was made in pRK5 as described (Yang et al, 1998). CED-3, CED-3(C358S), CED-4, CED-4 mutants, CED-9, Bcl-$x_L$, and Apaf-1(1-465) were each fused with a C-terminal FLAG epitope tag in pRK5. CED-3(C358S) was also fused to a C-terminal AU1 epitope tag in pRK5. Myc-CED4 and Myc-CED-4 (K165R) were described (Chinnaiyan et al., 1997). Myc-Apaf-1(1-465) and Myc-CED-3(C358S) was made in pcDNA3.1(-)/MycHis (Invitrogen) with a C-terminal Myc-tag. Authenticity of each construct was confirmed by DNA sequencing.

### Co-immunoprecipitation Assay

2 x $10^6$ 293T cells [grown in DMEM supplemented with 10% FBS, penicillin-streptomycin (100U/ml), and glutamine (1 mM)] were plated per 60 mm dish the day before transfection. 24 hours after transfection by the calcium phosphate method, cells were lysed in 300 µl IP-lysis buffer [50 mM Hepes (pH 7.4), 1 % NP-40, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM DTT] supplemented with 1 mM PMSF and 1% aprotinin. 100 µl extracts were diluted 1:1 in IP-lysis buffer and immunoprecipitated with antibody for 3 hours at 4 °C, washed with 600 µl buffer and resolved by SDS-PAGE.

**Acknowledgement**

We are grateful to F. Chen and H.R. Horvitz for gift of reagents and valuable discussions. We thank P. Svec and A. Darbinian for technical support. We thank V.M. Dixit, M. Gilman, S.L. Schreiber, and X. Wang for gifts of reagents. X.Y. is a fellow of the Leukemia Society of America. H.Y.C. is supported by the Medical Scientist Training Program at Harvard Medical School. Supported by NIH grant CA51462.

## References

Amara, J. F., Clackson, T., Rivera, V. M., Guo, T., Keenan, T., Natesan, S., Pollock, R., Yang, W., Courage, N. L., Holt, D. A., and Gilman, M. (1997). A versatile synthetic dimerizer for the regulation of protein-protein interactions. Proc Natl Acad Sci U.S.A. *94*, 10618-10623.

Chinnaiyan, A. M., Chaudhary, D., O'Rourke, K., Koonin, E. V., and Dixit, V. M. (1997). Role of CED-4 in the activation of CED-3. Nature *388*, 728-729.

Chinnaiyan, A. M., O'Rourke, K., Lane, B. R., and Dixit, V. M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science *275*, 1122-1126.

Conradt, B., and Horvitz, H. R. (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519-529.

Hengartner, M. O. (1997). CED-4 is a stranger no more. Nature *388*, 714-715.

Hengartner, M. O., and Horvitz, H. R. (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell *76*, 665-676.

Horvitz, H. R., Shaham, S., and Hengartner, M. O. (1994). The genetics of programmed cell death in the nematode Caenorhabditis elegans. Cold Spring Harbor Symp. Quant. Biol. *LIX*, 377-385.

Hu, Y., Benedict, M. A., Wu, d., Inohara, N., and Nunez, G. (1998). Bcl-$x_L$ interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc. Natl Acad. Sci. U.S.A. *95*, 4386-4391.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998). An induced proximity model of caspase-8 activation. J. Biol. Chem. *273*, 2926-2930.

Ottilie, S., Wang, Y., Banks, S., Chang, J., Vigna, N. J., Weeks, S., Armstrong, R. C., Fritz, L. C., and Oltersdorf, T. (1997). Mutational analysis of the interacting cell death regulators CED-9 and CED-4. Cell Death Differ. *4*, 526-533.

Shaham, S., and Horvitz, H. R. (1996). Alternatively spliced C. elegans ced-4 RNA encodes a novel cell death inhibitor. Cell *86*, 201-208.

Spector, M. S., Desnoyers, S., Hoeppner, D. J., and Hengartner, M. O. (1997). Interactions between the C. elegans cell death regulators CED-9 and CED-4. Nature *385*, 653-656.

Steller, H. (1998). Artificial death switches: induction of apoptosis by chemically induced caspase multimerization. Proc. Natl Acad. Sci. USA *95*, 5421-5422.

Steller, H. (1995). Mechanisms and genes of cellular suicide. Science *267*, 1445-1449.

van der Biezen, E. A., and Jones, J. D. G. (1998). The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Curr. Biol. *8*, R226-R227.

Wu, D., Wallen, H. D., and Nunez, G. (1997). Interaction and regulation of the subcellular localization of CED-4 and CED-9. Science *275*, 1126-1129.

Yang, X., Chang, H. Y., and Baltimore, D. (1998). Autoproteolytic activation of pro-caspases by oligomerization. Molecular Cell *1*, 319-325.

Yang, X., Khosravi-Far, R., Chang, H. Y., and Baltimore, D. (1997). Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell *89*, 1067-1076.

Yuan, J., Shaham, S., Ledoux, H. M., and Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-β-converting enzyme. Cell *75*, 641-652.

Zhou, H., Henzel, W. I., Liu, X., Luschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in the cytochrome c-dependent activation of caspase-3. Cell *90*, 405-413.

**Figure Legends**

Figure 1. Activation of the CED-3 protease by induced proximity.

(**A**) Schematic representation of CED-3 protein and Fkp fusion of the CED-3 protease domain. "p17" and "p15" indicate domains that form the mature CED-3 protein. D, aspartic acids at the cleavage sites for the generation of mature CED-3. M, c-Src myristylation signal. HA and FLAG are epitope tags.

(**B**) Oligomerization of Fkp-CED-3(205) enhanced its cell death activity. HeLa cells were transiently transfected with 0.15 μg of Fkp3 or Fkp3-CED3(205) plasmid together with pCMV-lacZ (0.25 μg). Twelve hours after transfection, AP1510 (Ariad Pharmaceuticals) at indicated final concentration and FK506 (gift of S.L. Schreiber) at 50 nM were added to the cultures. Cells were stained for β-galactosidase expression 8 hours later and scored for specific apoptosis (Yang *et al.*, 1997). Data (mean ± SD) were from at least three experiments, and in each experiment more than three-hundred blue cells were counted.

(**C**) CED-3 processing induced by oligomerization. $^{35}$S-methionine-labeled Fkp3-CED-3(205) was produced by coupled in vitro transcription and translation with TNT Reticulocyte Lysate System (Promega) for 30 minutes. Processing reaction was carried out with final drug concentrations of 500 nM AP1510, 200 nM FK506 or 1 μM Z-Asp-Glu-Val-Asp (z-DEVD, Enzyme System Products, CA) as described (Yang *et al.*, 1998). Reaction products were resolved by SDS-PAGE and detected by autoradiography. The bottom panel was exposed twice as long as the top panel. Molecular weight standards (in kDa) are shown on the right.

Figure 2. Homotypic CED-4 oligomerization in cells and in vitro.

(**A**) Summary of CED-4 oligomerization data. Amino acid boundaries of deletion mutants, CED-4 sequence that is similar to Apaf-1 (shaded box) (Zhou *et al.*, 1997), the nucleotide binding P-loop (solid vertical line) (Chinnaiyan *et al.*, 1997), and previously identified caspase activation and recruitment domain (CARD) (van der Biezen and Jones, 1998), and

CED-9 interaction domain are indicated (Ottilie *et al.*, 1997). CED-4L contains a 24 amino acid insert after amino acid 212 (Shaham and Horvitz, 1996). Each CED-4 mutant was FLAG-tagged at the C-terminus and tested for interaction with Myc-CED-4 by coimmunoprecipitation as illustrated in Fig. 2B.

(**B**) Representative data examining CED-4 self association. 293T cells were cotransfected with 2 μg each of vector (lanes 1, 5) or Myc-CED-4 (lanes 2-4, 6-8) and FLAG-CED-4 (Lanes 1, 2, 5, 6), FLAG-CED-4(171-435) (lanes 3, 7), or FLAG-CED-4(171-366) (lanes 4, 8). Immunoprecipitation (IP) with anti-Myc antibody conjugated to agarose beads (Santa Cruz Biotech.) was performed as described (24). The bound proteins (right panel) or 5% of input IP extract (left panel) were resolved by SDS-PAGE and detected by immunoblotting with polyclonal FLAG antibody (Santa Cruz). Molecular weight standards (in kDa) are indicated on the right.

(**C**) Interaction of GST-CED-4 with in vitro translated CED-3 and CED-4. $^{35}$S-labeled CED-3 and CED-4 proteins were made with the TNT Reticulocyte Lysate System. The translation product (2 μl) was incubated with 1 μg of immobilized glutathione-*S*-transferase (GST) (lane 1 and 3) or GST-CED-4 fusion protein (lane 2 and 4) in 100 μl IP-lysis buffer for 2 hours at 4 $^{o}$C. After three washes with 500 μl buffer, the bound $^{35}$S-CED-3 or CED-4 proteins were resolved by SDS-PAGE and detected by autoradiography.

(**D**) Oligomerization of Apaf-1(1-465). Human 293T cells were transfected with 2.5 μg of FLAG-Apaf-1(1-465) plus 2.5 μg of vector (lane 1) or Myc-Apaf-1(1-465) (lane 2). Top: Cell lysates were immunoprecipitated with anti-Myc beads and immunoblotted with anti-FLAG antibody as described in (B). Bottom: expression of the FLAG-Apaf-1(1-465) in the extracts.

Figure 3. (**A**) Effect of CED-4 oligomerization on the ternary CED-9:CED-4:CED-3 complex. FLAG-CED3(C358S), FLAG-CED-9 or FLAG-Bcl-$x_L$, and FLAG- or Myc-CED-4 (1 μg each) were cotransfected with pRK vector or FLAG-CED-4(171-549) (2 μg each) in 293T cells in the indicated combinations. (**B**) CED-4 mediates CED-3:CED-3 interaction. 293T cells were cotransfected with Myc-CED-3(C358S) (2 μg), FLAG-CED-3(C358S) (1 μg), and FLAG-CED-4 (2 μg) in the indicated combinations. Top: cell lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-FLAG as in Fig. 2B. Bottom: immunoblot of 5% (A) or 7.5% (B) of IP input. Molecular weight standards (in kDa) are shown on the left.

Figure 4. Role of CED-4 oligomerization in CED-4 function.

(**A**) Summary of CED-4 point mutants. Amino acid substitutions; CED-4 sequence that is similar to Apaf-1 (shaded box) (Zhou *et al.*, 1997); motifs conserved among CED-4, Apaf-1, and plant *R* gene products (black boxes) (van der Biezen and Jones, 1998); and the CED-4 oligomerization domain (dashed line) are indicated.

(**B**) Interaction profile of CED-4 mutants. Each FLAG-CED-4 mutant was cotransfected with Myc-CED-4 (2 μg each) in 293T cells. Top: cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG as in Fig. 2B. Bottom: immunoblot of 5% of input IP extract.

(**C**) Pro-apoptotic activity of CED-4 mutants. HeLa cells were transfected with indicated amount of CED-4 or CED-4 mutants, 50 ng of vector, CED-3(C358S), or CED-3, 1 μg of CED-9 or Bcl-$x_L$ as indicated, and 0.25 μg of pCMV-lacZ. Cells were stained 16 hours after transfection and scored for specific apoptosis (Yang *et al.*, 1997). Data shown (mean ± SD) are from at least three independent experiments and in each experiments more than 300 blue cells were counted.

Figure 5. A mechanistic model of CED-3 activation.

Figure 1

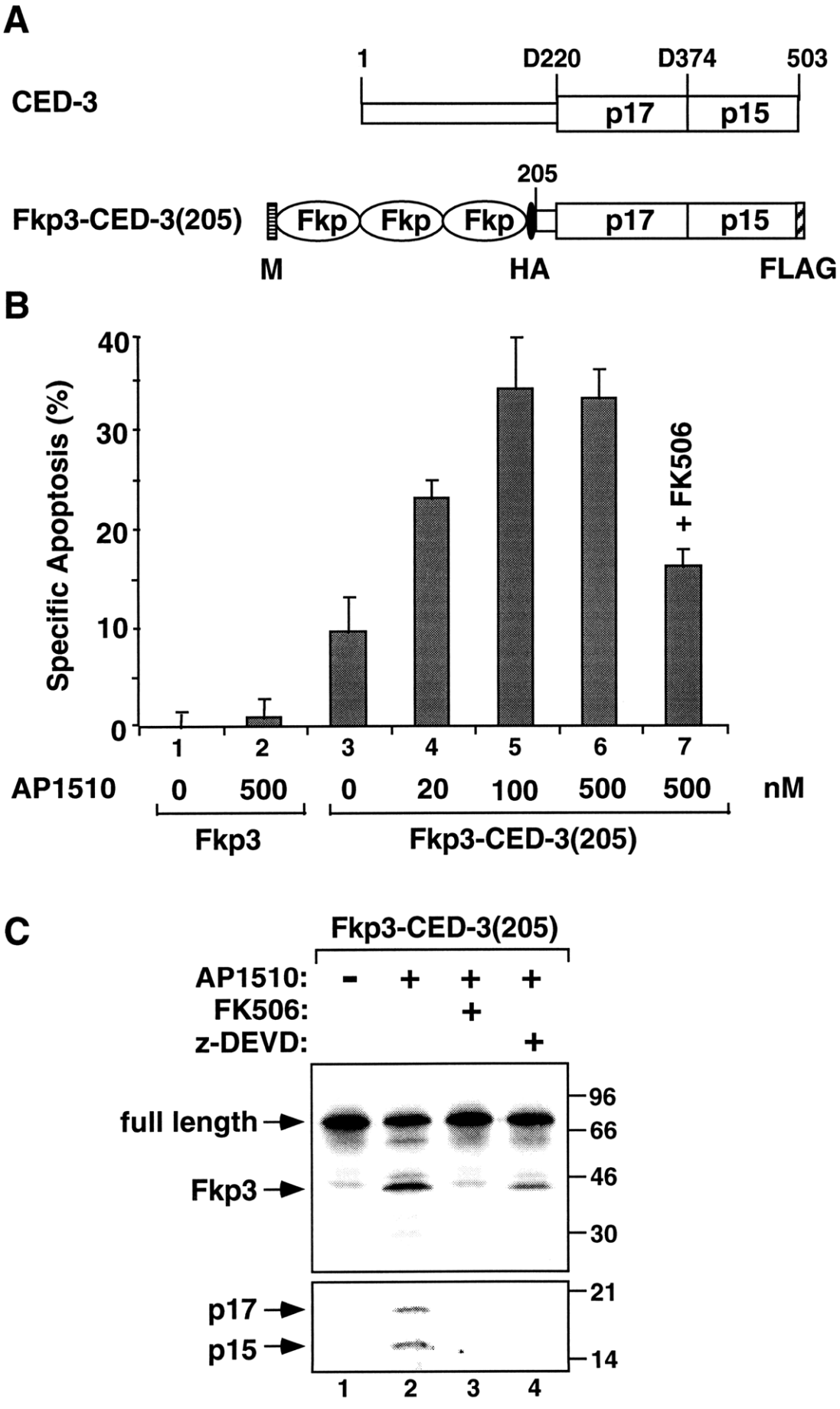

A
CED-3
1
D220
D374
503
p17
p15
Fkp3-CED-3(205)
205
Fkp
Fkp
Fkp
p17
p15
M
HA
FLAG
B
Specific Apoptosis (%)
40
30
20
10
0
+ FK506
1
2
3
4
5
6
7
AP1510
0
500
0
20
100
500
500
nM
Fkp3
Fkp3-CED-3(205)
C
Fkp3-CED-3(205)
AP1510:
-
+
+
+
FK506:
+
z-DEVD:
+
full length
Fkp3
96
66
46
30
p17
p15
21
14
1
2
3
4

Figure 2

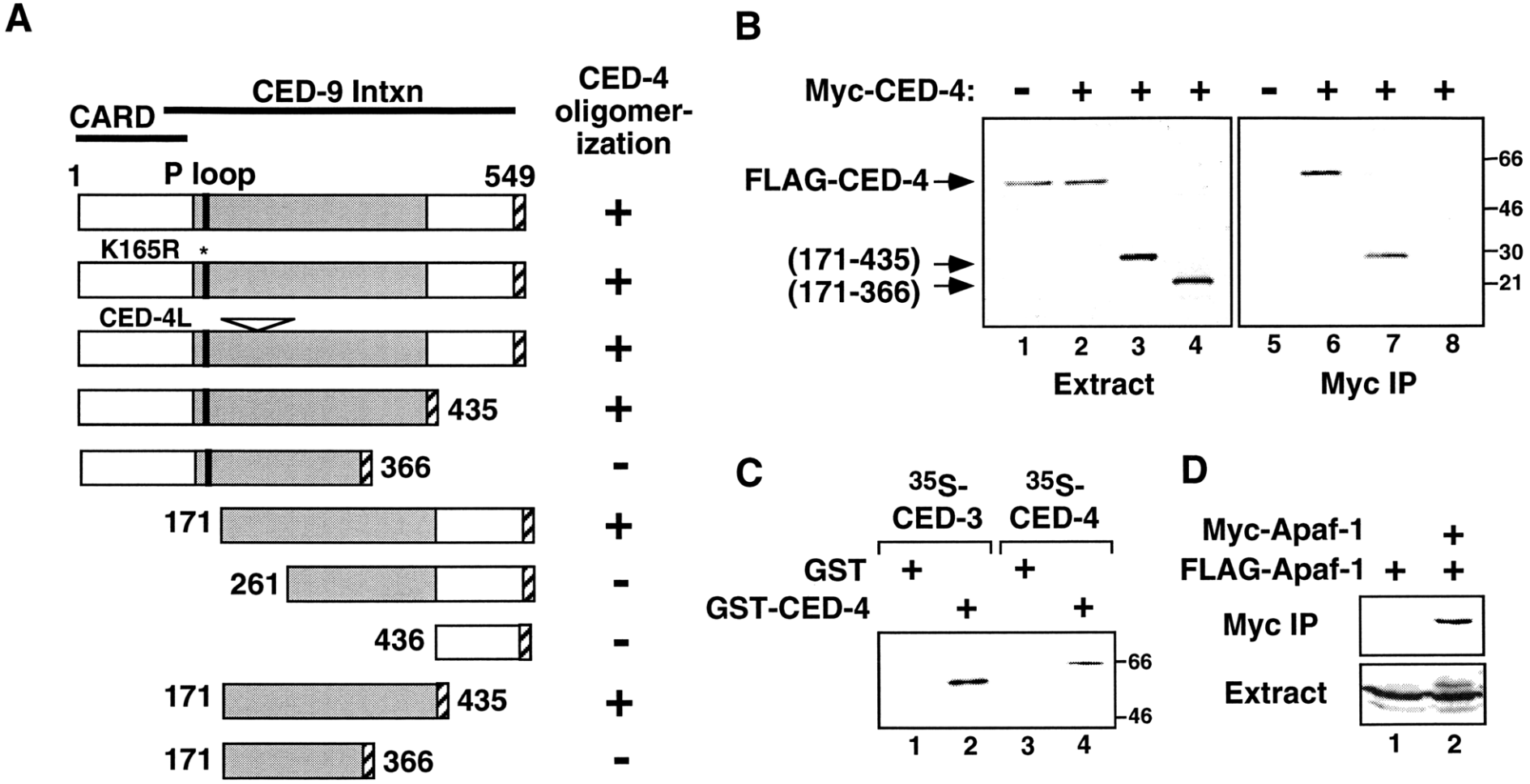

A
CARD
CED-9 Intxn
CED-4 oligomer-ization
1
P loop
549
K165R
*
CED-4L
435
366
171
261
436
171
435
171
366
+
+
+
+
-
+
-
-
+
-
B
Myc-CED-4:
- + + + - + + +
FLAG-CED-4
(171-435)
(171-366)
66
46
30
21
1 2 3 4 5 6 7 8
Extract
Myc IP
C
35S-CED-3
35S-CED-4
GST + +
GST-CED-4 + +
66
46
1 2 3 4
D
Myc-Apaf-1 +
FLAG-Apaf-1 + +
Myc IP
Extract
1 2

Figure 3

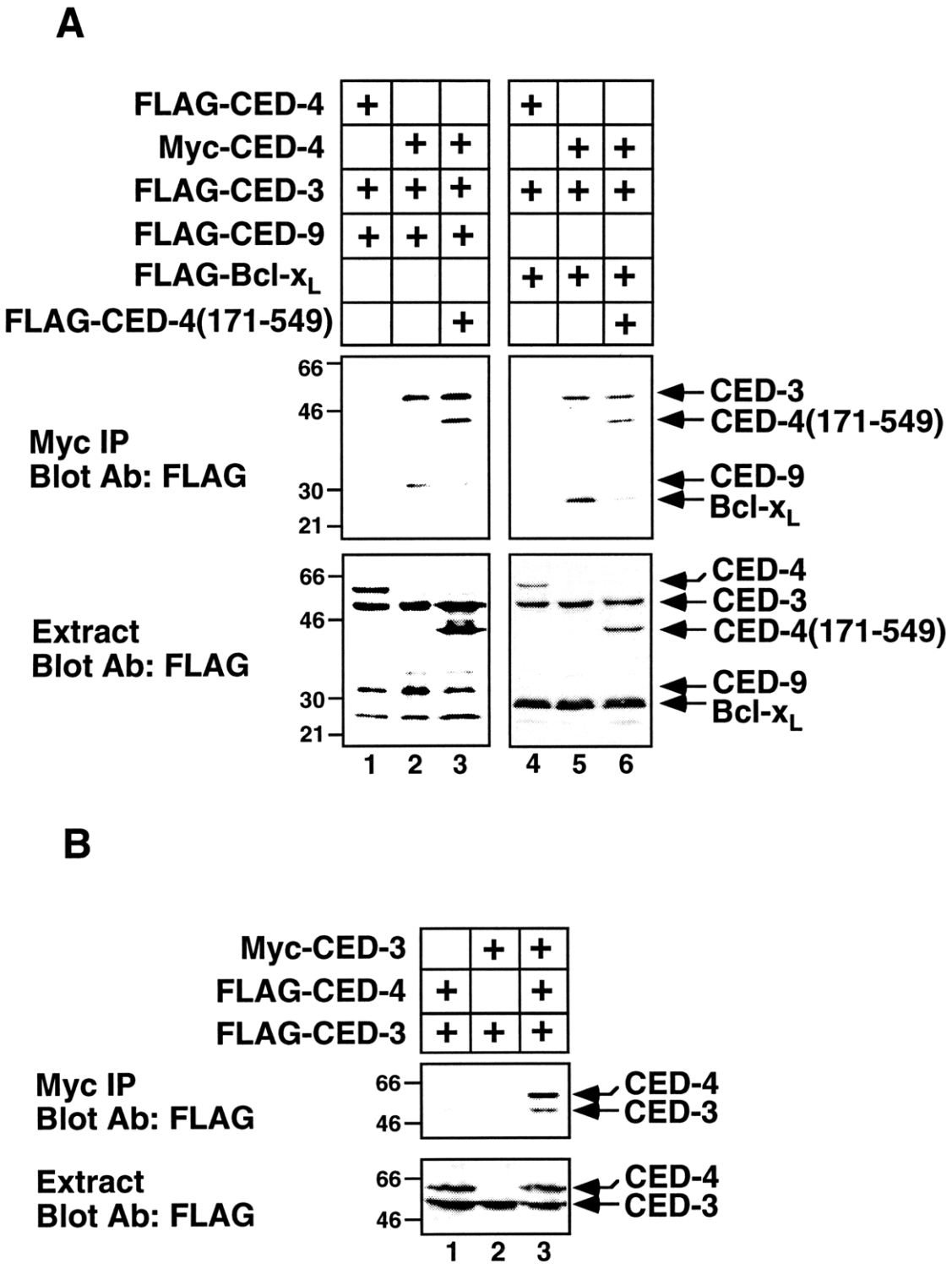
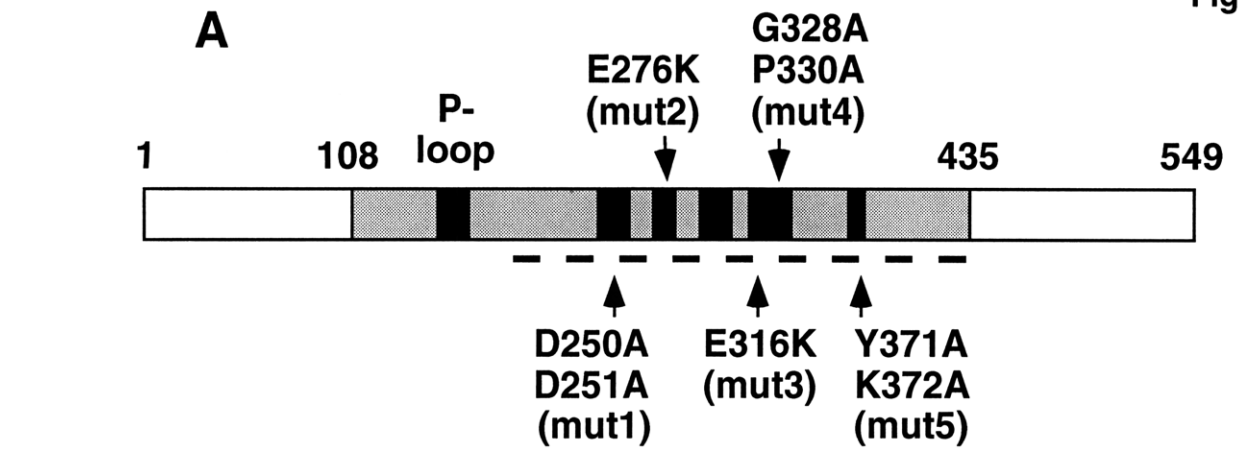
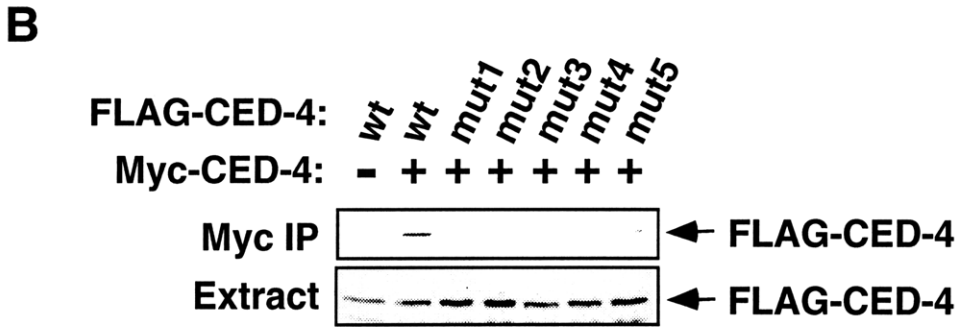

A
FLAG-CED-4
Myc-CED-4
FLAG-CED-3
FLAG-CED-9
FLAG-Bcl-xL
FLAG-CED-4(171-549)
Myc IP
Blot Ab: FLAG
66
46
30
21
CED-3
CED-4(171-549)
CED-9
Bcl-xL
Extract
Blot Ab: FLAG
CED-4
CED-3
CED-4(171-549)
CED-9
Bcl-xL
1 2 3 4 5 6
B
Myc-CED-3
FLAG-CED-4
FLAG-CED-3
Myc IP
Blot Ab: FLAG
66
46
CED-4
CED-3
Extract
Blot Ab: FLAG
CED-4
CED-3
1 2 3

Figure 4


A
G328A
P330A
(mut4)
E276K
(mut2)
P-
loop
1
108
435
549
D250A
D251A
(mut1)
E316K
(mut3)
Y371A
K372A
(mut5)


B
FLAG-CED-4:
wt
wt
mut1
mut2
mut3
mut4
mut5
Myc-CED-4:
- + + + + + +
Myc IP
FLAG-CED-4
Extract
FLAG-CED-4

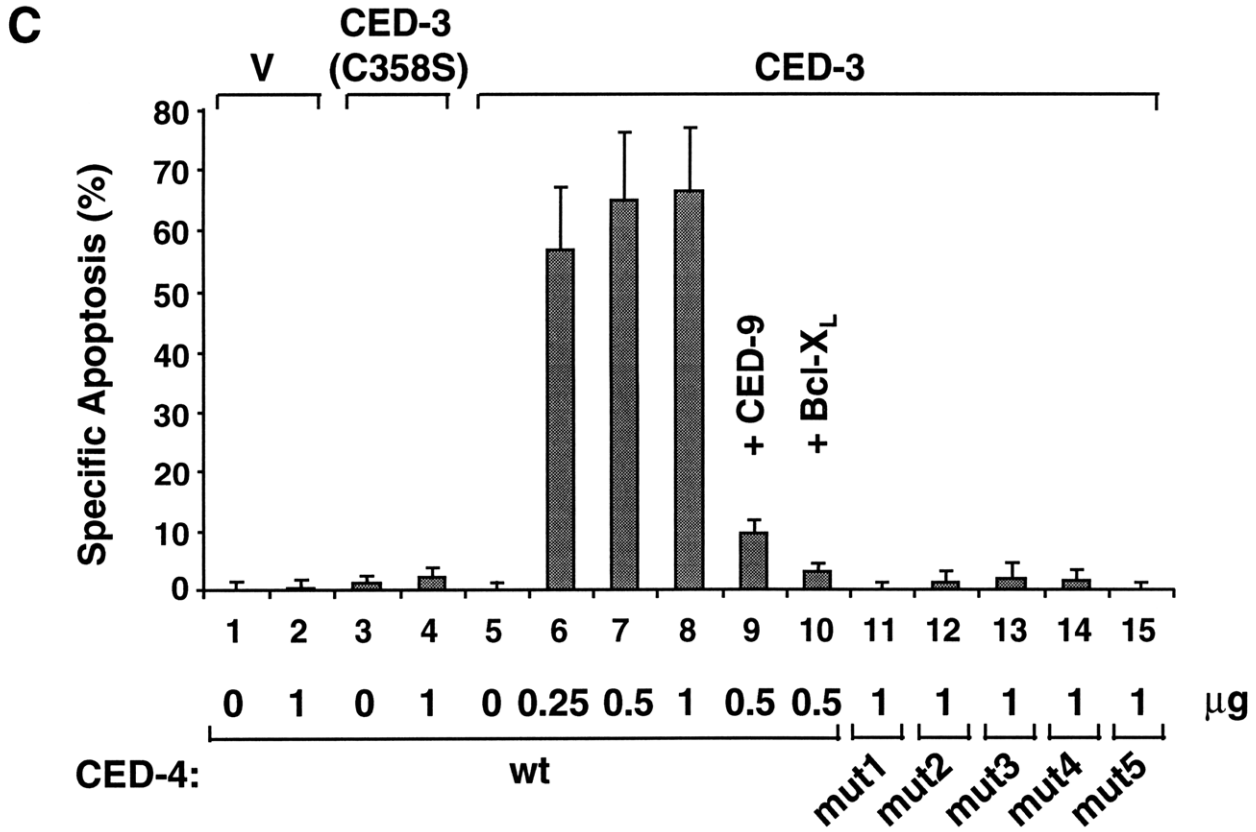
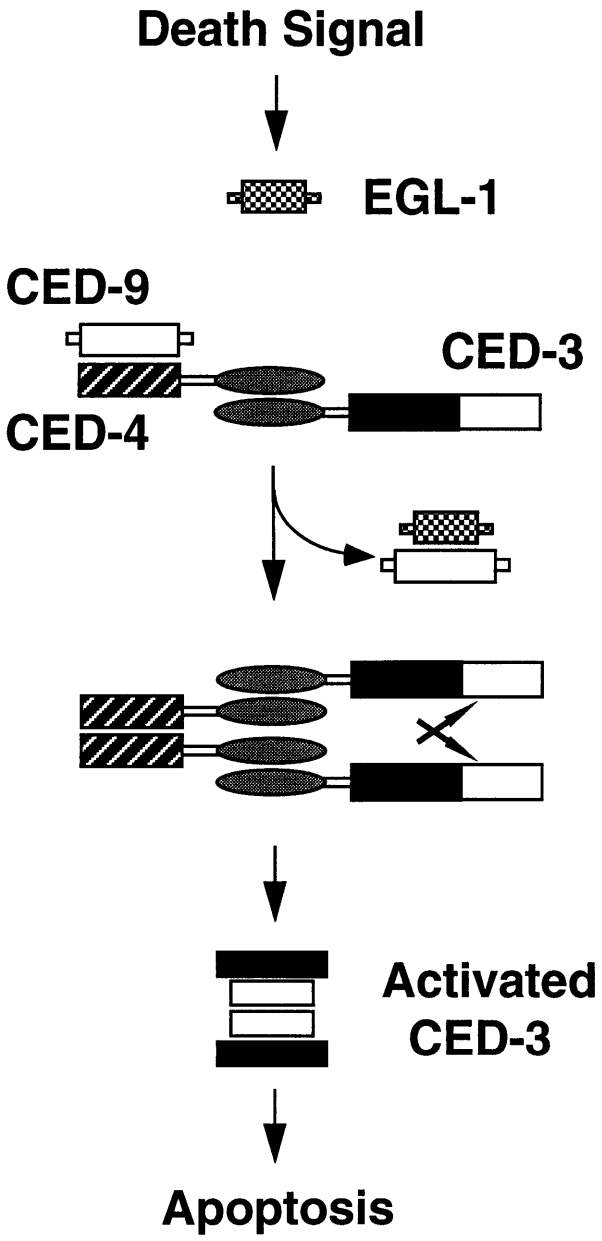

C
V
CED-3
(C358S)
CED-3
Specific Apoptosis (%)
80
70
60
50
40
30
20
10
0
+ CED-9
+ Bcl-X$_L$
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0 1 0 1 0 0.25 0.5 1 0.5 0.5 1 1 1 1 1
μg
CED-4:
wt
mut1
mut2
mut3
mut4
mut5

Figure 5



# Chapter VI. Concluding remarks and perspectives

## Recent developments in Fas signaling

Over the last several years, rapid progress has been made in understanding signaling by Fas and other cell surface death receptors. Following the discovery that pro-caspase-8 is recruited to the Fas signaling complex, several viral and cellular pro-caspase-8 like proteins, termed FLIPs (FLICE-like inhibitory protein, also named CASH, Casper, FLAME, I-FLICE, or MHIT), have been identified (Wallach et al., 1998). These proteins contain FADD interacting DED motifs but not functional protease domains, and they are potent inhibitors of Fas and TNF-induced cell death in tissue culture cells and in primary lymphocytes (Irmler et al., 1997; Refaeli et al., 1998). In particular, c-FLIP expression is downregulated in T cells following autocrine IL-2 stimulation (Refaeli et al., 1998), which is a key regulatory step in activation-induced cell death (Lenardo, 1991). The *in vivo* role of the Fas-FADD-pro-caspase-8 axis has also been bolstered by the generation of mice deficient for FADD or overexpressing the FADD death domain (Newton et al., 1998; Walsh et al., 1998; Yeh et al., 1998; Zhang et al., 1998; Zornig et al., 1998). FADD-deficient mice are embryonic lethal due to caridac defects (Yeh et al., 1998). FADD-/- ES cells are resistant to Fas, TNF-$\alpha$, and some but not all death receptors (Yeh et al., 1998). Similarly, caspase-8 deficient embryos are prenatally lethal, and Casp8-/- fibroblasts are resistant to death induction by Fas, TNF-$\alpha$, and the death receptor family member DR3 (Varfolomeev et al., 1998). Surprisingly, T cells that are FADD-/- or transgenically expressing FADD death domain are blocked in activation-induced proliferation (Zhang et al., 1998; Newton et al., 1998; Walsh et al., 1998; Zornig et al., 1998), and B cells do not develop in RAG2-/- animals reconstituted with FADD-deficient ES cells (Zhang et al., 1998). Because these defects are more severe and distinct from the phenotypes of Fas or TNF receptor deficient animals, FADD is believed to have roles other than death receptor signaling. Unfortunately, because FADD-/- T cells could not go through the activation

program, it is not possible to test the role of the FADD axis in the natural context of activation-induced cell death. Similarly, the role of FADD in B cell apoptosis, the defect of which is required for autoimmune disease in *lpr* animals (Shlomchik et al., 1994), could not be tested. To address these questions, I have isolated a Fas death domain mutant that has a selective defect in binding FADD. By reconstituting cells from *lpr* mice with mutant Fas, it should be possible to address the role of the Fas-FADD axis in many cell types and in the natural context of lymphocyte activation (H.Y. Chang, L. van Parijs, and D. Baltimore, unpublished observations).

In an effort to uncover other signaling pathways from Fas, we isolated Daxx and provided experiments to indicate its involvement in the activation of JNK. Fas activation induced Daxx to interact and activate ASK1, a upstream MAP3K of JNK and p38 kinase pathways. Cells overexpressing the death domain of FADD or are deficient in caspase-8 have no defect in JNK activation in response to Fas or TNF-α despite the lack of apoptosis induction, supporting the notion that caspase-independent signaling pathways exist (Yang et al., 1997; Wajant et al., 1998; Varfolomeev et al., 1998). In the TNF receptor 1 signaling complex, the adaptor protein TRAF2 mediates NF-κB and JNK activation. TNF-α treatment induces TRAF2 to interact with and activate ASK1, which is responsible for JNK but not NF-κB activation by TNF-α (Nishitoh et al., in press). Therefore, ASK1 appears to be a common upstream mediator of JNK activation by death receptors.

However, many questions remain regarding the role of JNK and other kinase pathways in death receptor signaling. First and foremost is the physiologic role of JNK activation. The role of JNK activation in cell death has been controversial, with contradictory evidence indicating that it is required for apoptosis, that it is not required, and that it may be anti-apoptotic (as discussed by (Swat et al., 1998) and references therein). These divergent results may be due to the fact that JNK ultimately controls a transcriptional program, and AP-1, in combination with other transcriptional factors that are active in different cell types and under different stimuli, may activate different transcriptional

programs that affect proliferation, death, and differentiation. Because JNK activation may lead to different outcomes under different circumstances, it is important to focus one's attention on physiologic models. Mice deficient in JNK3, a brain-specific isoform of JNK, have defects in AP-1-dependent trancription and are protected from neuronal apoptosis following excitotoxic injury (Yang et al., 1997). These results are the strongest evidence to date for a functional role of the JNK pathway in apoptosis. In animals deficient for SEK1, a ubiquitous MAP2K upstream of JNK, lymphocyte development is normal but JNK activation is not universally blocked(Swat et al., 1998). In particular, stimulation by CD40, a TNF-receptor superfamily that signals through TRAF2, still activated JNK in SEK1-/- cells (Swat et al., 1998). This may be due to partial redundancy of SEK1 with MKK7, a newly discovered MAP2K of the JNK pathway (Tournier et al., 1997). Fas killing was unimpaired in SEK1-/- thymocytes and B cells (Swat et al., 1998) . Interestingly, aged RAG2-/-, SEK1-/- chimera develop a severe polyclonal lymphoproliferative syndrome, suggesting that SEK1 is required for proper lymphocyte homeostasis *in vivo* (Swat et al., 1998) . Animals deficient in Daxx or that express a Fas mutant unable to bind Daxx may be helpful in addressing the physiologic role of the JNK pathway in death receptor signaling.

The second pressing question is the mechanism by which JNK activation may lead to apoptosis. Two major hypotheses have been forwarded. First, several groups have suggested that Fas ligand can be induced by AP-1(Faris et al., 1998; Kasibhatla et al., 1998), and *gld* cells are more resistant to apoptosis induced by some stress signals that activate JNK (Reap et al., 1997). These results suggest that JNK activation may potentiate Fas ligand expression, which can then kill cells in an autocrine or paracrine manner. Secondly, JNK has been identifed as a kinase that phosphorylates an inactivation loop in Bcl-2 (Maundrell et al., 1997). Bcl-2 family members contain a variable length loop between conserved BH4 and BH3 domains, and phosphorylation of this loop appears to inactivate the function of several Bcl-2 family members (Chang et al., 1997). This

hypothesis fits in nicely with recent studies addressing the role of Bcl-2 in death receptor-induced apoptosis. Theoreteically, once caspase-8 becomes activated, it should be able to process and activate downstream caspases to carry out apoptosis. However, recent studies showed that the caspase cascade is amplified by the Bcl-2 regulatable mitochondrial apoptosome(Scaffidi et al., 1998; Kuwana et al., 1998). Caspase-8 activity can induce cytochrome c release and Apaf-1 activation, and in some cells, caspase-9 activation via Apaf-1 is believed to be the rate limiting step in the pathway to effector caspase activation (Scaffidi et al., 1998; Kuwana et al., 1998). This observation may explain why in some cells Fas can be inhibited by Bcl-2 and potentially regulated by JNK. Whether these mechanisms or other yet unidentified connections really explain the role of JNK remain to be determined by future studies.

**Pro-caspase oligomerization- the death machine revealed?**

Starting from the insight that cell surface death receptors recruit pro-caspase molecules, experiments in Chapter 4 and 5 showed that oligomerization of pro-caspases appears to be a fundamental and unifying mechanism in initiating programmed cell death. Caspase zymogens have weak proteolytic activity (approximately 100 fold less than the mature enzyme) (Muzio et al., 1998). However, once pro-caspases are brought into proximity near one another, they can cleave each other in *trans* to generate mature caspases and intiate a proteolytic cascade(Yang et al., 1998; Muzio et al., 1998). Adaptor proteins that activate pro-caspases, such as the Fas-FADD module or CED-4, impart regulated oligomerization; one end of the adaptor protein binds to pro-caspases while the other end of the protein facilitates oligomerization. For example, the extracellular domain of Fas binds to the trimeric Fas ligand on activated T cells, and the intracellular death domain of Fas, via FADD, recruits pro-caspase-8. Similarly, the death adaptor CED-4 or its mammalian homolog Apaf-1 has a bipartite structure: a $NH_2$-terminal domain that allows binding to CED-3 or caspase-9, respectively, and an evolutionarily conserved COOH-

terminal domain that has the intrinsic ability to form homotypic oligomers. Pro-caspase-9 binding to Apaf-1 is thought to occur via homotypic interactions of the caspase activation and recruitment domains (CARD) on the $NH_2$-termini of Apaf-1 and pro-caspase-9 (Li et al., 1997). In CED-4:CED-3 binding, Chaudhary et al. have suggested a more complicated model : the P-loop motif binds to the prodomain of CED-3 whereas sequences before and after the P-loop within the $NH_2$-terminus of CED-4 bind to the protease domain of CED-3 (Chaudhary et al., 1998). In healthy cells, induction of apoptosis by CED-4 or Apaf-1 is held in abeyance by the binding of the anti-apoptotic CED-9 or Bcl-2 family proteins. The CED-9 binding site on CED-4 overlaps with the CED-4 oligomerization domain, and CED-9:CED-4 interaction competes with CED-4 oligomerization. However, upon CED-9 binding to BH3 domain containing proteins such as EGL-1, CED-9 is displaced from CED-4, allowing CED-4:CED-3 complex to oligomerize and CED-3 zymogen to initate autoproteolytic activation. Consistent with this model, Alnemri and colleagues have used a biochemical system with purified components to show that Apaf-1 can cause two pro-caspase-9 molecules to cleave each other (Srinivasula et al., 1998). Thus, pro-caspase oligomerization provides a simple and satisfying framework for understanding apoptosis induction by cell surface death receptors and the mitochondrial apoptosome.

In mammalian and worm cells, additional mechanisms exist to control programmed cell death. Apaf-1 was originally identified as a required cofactor in an *in vitro* extract system of caspase activation; this system also required cytochrome c, an electron transport protein from the intermembrane space of mitocondria, and dATP (Liu et al., 1996). Subsequently, Wang and colleagues provided evidence that cytochrome c and dATP are involved in regulating the ability of Apaf-1 to activate pro-caspase-9 (Li et al., 1997). In addition to a CARD domain and a CED-4 like domain, Apaf-1 contains multiple WD-40 repeats in its C-terminus (Zhou et al., 1997). The WD-40 repeats apparenly block the ability of the N-terminal CARD domain to bind pro-caspase-9 (Zhou et al., 1997). It is believed that upon cytochrome C binding to the WD-40 repeat, the intramolecular inhibition

is relieved and Apaf-1 becomes competent to bind and activate pro-caspase-9. Supporting this model, an Apaf-1 mutant missing the WD-40 repeats no longer requires cytochrome c or dATP to activate pro-caspase-9 (Hu et al., 1998; Srinivasula et al., 1998). Cytochrome c release from the mitochondria has been found to be a general feature of apoptosis in mammalian cells, and microinjection of cytochrome c into mammalian cytosol rapidly induces apoptosis (Reed, 1997). However, CED-4 does not contain WD-40 repeats nor is it known to be regulated by cytochrome c. It remains to be determined if cytochrome c release is an evolutionarily conserved mechanism in regulating apoptosis.

The issue of cytochrome c release highlights the complexity in the mechanisms of action of mammalin Bcl-2 family of anti-apoptotic proteins. In apoptotic cells, release of cytochrom c occurs before the loss of mitochondrial membrane potential , and its cytosolic translocation can be blocked by overexpression of Bcl-2 or Bcl-$x_L$ (Yang et al., 1997; Kluck et al., 1997; Vander Heiden et al., 1997). As discussed above, cytosolic translocation of cytochrome c activates Apaf-1 and pro-caspase-9, but even in the absence of caspase activation, cytochrome c translocation will disrupt mitochondrial electron transport and be lethal to the cell. Vander Heiden et al. have observed physical disruption of the outer membrane but not of the inner membrane in mitochondria of apoptotic cells by electron microscopy (Vander Heiden et al., 1997). They suggest that early in apoptosis, mitochondria lose osmotic balance and because the mitochondrial matrix is more dense, swelling of the larger inner mitochondrial membrane bursts the outer mitochondrial membrane and allows cytochrome c leakage into the cytoplasm . Bcl-$x_L$ overexpression prevents inner mitochondrial membrane hyper-polarization and swelling, suggesting a mechanism for blocking cytochrome c release (Vander Heiden et al., 1997). The crystal structure of Bcl-$x_L$ is reminiscent of bacterial pore forming proteins such as colicin (Muchmore et al., 1996), and purified Bcl-$x_L$ can form channels in synthetic lipid membranes (Minn et al., 1997). These experiments suggest that Bcl-2 proteins are mitochondrial protectors, perhaps by forming channels that maintain osmotic balance in the

mitochondria. However, Bcl-$x_L$ resides in the outer mitochondrial membrane; therefore how it can relieve inner mitochondrial membrane hyperpolarization is still unclear (Reed, 1997). Also, Bcl-2 and Bcl-$x_L$ are distributed on the endoplasmic reticulum and nuclear envelope. How these proteins affect other organelles is presently unclear.

In *C. elegans*, loss of function mutations in *ced-3* or *ced-4* completely rescues the lethality of *ced-9* loss of function mutants (reviewed by Horvitz et al., 1994). In the *ced-3 ced-9* double mutant animals, cells that normally die during development live, differentiate into distinct cell types, and can be shown to function normally (Horvitz et al., 1994). These results indicate that the only function of CED-9 is to inhibit CED-3 activity, and that CED-9 has no other essential role such as mitochondrial protection. However, it should be noted that the programmed cell death during *C. elegans* development is specified by lineage, and the cells doomed to die are not damaged or stressed in any way. In mammalian cells, caspase inhibitors block the morphologic appearance of apoptosis in response to oxidative stress or chemotherapy drugs, but the cells still eventually die by necrosis (reviewed by Reed, 1997). Cells overexpressing Bcl-2 or Bcl-$x_L$ are not killed by these challenges. Caspase inhibitors also do not block cytochrome c release by these stimuli although Bcl-2 and Bcl-$x_L$ do (Vander Heiden et al., 1997). On the other hand, Bcl-2 still inhibits apoptosis after the release of cytochrome c into the cytosol (Rosse et al., 1998). Collectively, these published reports suggest that the Bcl-2 family of anti-apoptotic proteins function at two levels: firstly, they maintain mitochondrial function, which prevents the release of mitochondrial factors that trigger apoptosis and necrosis; and secondly, they function downstream of mitochondrial factors to prevent caspase activation, perhaps by complexing with CED-4-like adapter proteins and preventing their oligomerization.

In addition to cytochrome c, dATP or ATP is also required for Apaf-1 to activate pro-caspase-9 (Liu et al., 1996; Li et al., 1997). What may be the mechanistic role of dATP in regulating CED-4-like proteins? CED-4, Apaf-1, and proteins encoded by the homologous plant *R* genes all contain a conserved nucleotide binding P-loop (Chinnaiyan

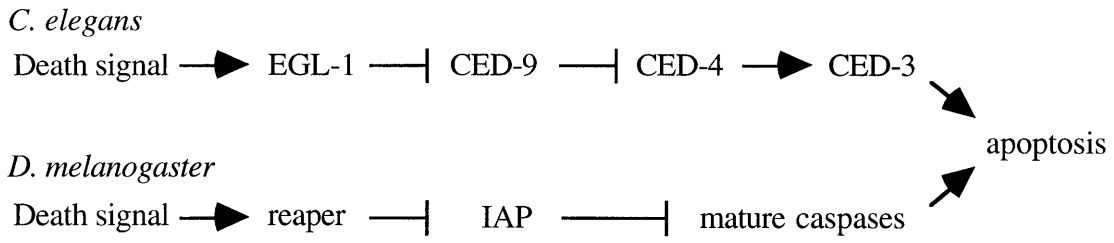
et al., 1997; van der Biezen and Jones, 1998). A single point mutation within the P-loop (lys to arg) has been reported to abrogate the ability of CED-4 to induce apoptosis or activate CED-3 processing (Chinnaiyan et al., 1997), but it does not affect the ability of CED-4 to oligomerize. Dixit and colleagues have suggested that this may be accounted for by the failure of P-loop mutants to bind to the prodomain of CED-3 (Chaudhary et al., 1998). CED-4L, an anti-apoptotic splice variant of CED-4 that contains a 24 amino acid insert within the P-loop, was reported to have the same defect (Chaudhary et al., 1998). However, the equivalent K to R mutation in an Apaf-1 fragment missing the WD-40 repeats did not affect its ability to induce pro-caspase-9 processing (Srinivasula et al., 1998). It should be noted that the latter experiment was done with purified recombinant components *in vitro* whereas experiments by Dixit and colleagues were performed with immunoprecipitated protein from transfected mammalian cells. Therefore it is possible that dATP binding is not required for the basic biochemical function of CED-4 (i.e. inducing CED-3 proximity), but it is involved in the efficiency of the process *in vivo*. For example, dATP binding may be involved in regulating the turnover of CED-3 molecules bound to CED-4. Once CED-4 induces CED-3 proximity and activation, one imagines that the CED-3 prodomain which remains bound to CED-4 needs to be released to allow CED-4 to activate other CED-3 zymogens. If and how CED-3 prodomain can be released from CED-4 should be investigated in future studies.

**Concluding Remarks**

So far, the above discussion has focused on the control of the activation of pro-caspases. However, it is now clear that there are also mechanisms to control caspases after they have become activated. The *Drosophila melanogaster* cell death genes may provide an important opportunity to understand a new regulatory paradigm of apoptosis. The IAP family of proteins was first characterized as the cellular homolog of the baculovirus-encoded Inhibitor of Apoptosis Proteins. NIAP, the first mammalian homolog identified,

was discovered as a frequently deleted gene in the spinal muscular atrophy locus (Roy et al., 1995), suggesting an obvious pathophysiologic mechanism for this neurodegenerative disease. c-IAP1 and cIAP-2 were found to be components of the TNF receptor signaling complex (Rothe et al., 1995; Uren et al., 1996), and an X-linked IAP (XIAP) was also characterized (Liston et al., 1996; Duckett et al., 1996). Survivin, the most recently discovered family member, was noted as a frequently overexpressed gene in lymphomas (Ambrosini et al., 1997). In *Drophila,* mutation of the IAP homolog *thread* leads to increased apoptosis, suggestng a physiologic role for IAP proteins in blocking apoptosis (Hay et al., 1995). An important mechanistic insight was made by Reed and colleagues, who demonstrated that certain IAPs directly bind to mature caspases and are potent inhibitors ($K_i$ of nM range) of caspase activity (Deveraux et al., 1997; Deveraux et al., 1998).

How may IAP proteins be integrated into the cellular control of apoptosis? In *Drosophila*, induction of apoptosis requires the *H99* locus (White et al., 1994), which encodes three proteins: Reaper, HID, and GRIM (White et al., 1996; Grether et al., 1995; Chen et al., 1996). In particular, Reaper is transcriptionally induced in cells that are destined to die (White et al., 1994). Induction of apoptosis by all three proteins was inhibited by IAP coexpression in lepidopteran SF-21 cells (Vucic et al., 1997; Vucic et al., 1998), and apoptosis by Reaper or HID overexpression was blocked by coexpression of *Drosophila* IAP in fly eyes (Hay et al., 1995). Recently, all three proteins were found to be capable of binding IAP proteins through a similar N-terminal motif (Vucic et al., 1997; Vucic et al., 1998). This finding raises the possiblity that the *Drosophila* death proteins may act by displacing IAP from caspases, consequently allowing caspases to commence apoptotic dissolution of the cell (Vucic et al., 1998). This model of the fly death pathway needs to be rigorously tested with appropirate combinations of the *Drosophila* proteins. Nontheless, this speculative model of the *Drosophila* death pathway shares an interesting theme with the *C. elegans* pathway as outlined below:




In both organisms, apoptosis is ready to occur but is held in check by inhibitory proteins--CED-9 in worms and IAP in flies. In response to death signals, antagonists of the inhibitor are induced, and apoptosis is allowed to proceed. The key controlling point of these death pathways is the induction of the antagonist proteins. In *C. elegans*, EGL-1 (named for the egg laying defective phenotype due to inappropriate hermaphrodite-specific neuron cell death) needs to be induced in a sex-specific and lineage specific manner (Conradt and Horvitz, 1998). Similarly in *Drosophila*, Reaper needs to be induced in cells that are destined to die in development or cells that have been lethally irradiated (White et al., 1994). *Drosophila* cells presumably have additional regulatory mechanisms to convert pro-caspases to mature caspases. At the present, no CED-4 or Bcl-2-like proteins have been found yet in the fly, and no homologs of the Drosophila death genes have been identified in mammalian cells. Interestingly, Fas can induce apoptosis and activate caspases in a *Drosophila* cell line (Kondo et al., 1997), suggesting that death receptor signaling pathways may be conserved in flies. In mammalian cells and probably invertebrate cells, multiple regulatory pathways, including Bcl-2 and IAP families of antiapoptotic proteins, exist and may represent parallel regulatory pathways that coordinately controling caspase activity (Figure 1). Bcl-2 and IAP proteins offer dual protection against cell death: Bcl-2 proteins can prevent inadvertent pro-caspase activation while IAP proteins raise the threshold of caspase activity needed to initiate the death program. Both forms of protection are essential and nonredundant from the point of view of the organism because mutations in even a single member of the anti-apoptotic pantheon (e.g. Bcl-2 or NIAP) leads to excessive programmed cell death.

The above model potentially explains the old observation that many instances of developmental apoptosis can be blocked by inhibitors of RNA and protein synthesis, yet prolonged incubation with these drugs also caused death. Inhibition of protein synthesis prevents the production of EGL-1 or reaper. However, with prolonged incubation, CED-9 and IAP may have shorter half lives than caspases and start to degrade, allowing apoptosis to proceed. Why should the death pathway be constructed in such a way that the cell has to constantly prevent itself from dying? In a multicellular organism, such a trigger for self destruction may be a clever strategy to limit damage whenever a group of cells can be traumatized. For example, if a virus infects a cell and shuts down host synthesis, this cell can automatically initiate apoptosis and prevent further viral spread. Perhaps not coincidentally, the plant *R* genes, which are homolgous to CED-4 and Apaf-1, were first isolated as mutants that could not resist viral spread. It is possible that programmed cell death first evolved to resist viruses and was only later co-opted to control development, tissue homeostasis, and tumor suppression.

## References

Ambrosini, G., Adida, C., and Altieri, D. C. (1997). A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat. Med. *3*, 917-921.

Chang, B. S., Minn, A. J., Muchmore, S. W., Fesik, S. W., and Thompson, C. B. (1997). Identification of a novel regulatory domain in Bcl-$x_L$ and Bcl-2. EMBO J. *16*, 968-977.

Chaudhary, D., O'Rourke, K., Chinnaiyan, A. M., and Dixit, V. M. (1998). The death inhibitory molecules CED-9 and CED-4L use a common mechanism to inhibit the CED-3 death protease. J. Biol. Chem. *273*, 17708-17712.

Chen, P., Nordstrom, W., Gish, B., and Abrams, J. M. (1996). grim, a novel cell death gene in Drosophila. Genes Dev. *10*, 1773-1782.

Chinnaiyan, A. M., Chaudhary, D., O'Rourke, K., Koonin, E. V., and Dixit, V. M. (1997). Role of CED-4 in the activation of CED-3. Nature *388*, 728-729.

Conradt, B., and Horvitz, H. R. (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519-529.

Deveraux, Q. L., Roy, N., Stennicke, H. R., Van Arsdale, T., Zhou, Q., Srinivasula, S. M., Alnemri, E. S., Salvesen, G. S., and Reed, J. C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J. *17*, 2215-2223.

Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. Nature *388*, 300-304.

Duckett, C. S., Nava, V. E., Dedrich, R. W., Clem, R. J., Van Dongen, L., Gilfillan, M. C., Shiels, H., Hardwick, J. M., and Thompson, C. B. (1996). A conserved family of cellular genes related to teh baculovirus iap gene and encoding apoptosis inhibitors. EMBO J. *15*, 2685-2694.

Faris, M., Kokot, N., Latinis, K., Kasibhatla, S., Green, D. R., Koretsky, G. A., and Nel, A. (1998). The c-Jun N-terminal kinase cascade play a role in stress-induced apoptosis in Jurkat cells by upregulating Fas ligand expression. J. Immunol. *160*, 134-144.

Grether, M. E., Abrams, J. M., Agapite, J., White, K., and Steller, H. (1995). The head involution defective gene of Drosophila melanogaster functions in programmed cell death. Genes Dev. *9*, 1694-1708.

Hay, B. A., Wassarman, D. A., and Rubin, G. M. (1995). Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell *83*, 1253-1262.

Horvitz, H. R., Shaham, S., and Hengartner, M. O. (1994). The genetics of programmed cell death in the nematode Caenorhabditis elegans. Cold Spring Harbor Symp. Quant. Biol. *LIX*, 377-385.

Hu, Y., Benedict, M. A., Wu, d., Inohara, N., and Nunez, G. (1998). Bcl-$x_L$ interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc. Natl Acad. Sci. U.S.A. *95*, 4386-4391.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190-195.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A., and Green, D. R. (1998). DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the action of NF-kB and AP-1. Mol. Cell *1*, 543-551.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science *275*, 1132-1136.

Kondo, T., Yokokura, T., and Nagata, S. (1997). Activation of distinct caspase-like proteases by Fas and reaper in Drosophila cells. Proc. Natl Acad. Sci. U.S.A. *94*, 11951-11956.

Kuwana, T., Smith, J. J., Muzio, M., Dixit, V., Newmeyer, D. D., and Kornbluth, S. (1998). Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome c. J. Biol. Chem. *273*, 16589-16594.

Lenardo, M. J. (1991). Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. Nature *353*, 858-861.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome C and dATP-dependent formationi of Apaf-1/Caspase-9 complex initates an apoptotic protease cascade. Cell *91*, 479-489.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A., and Korneluk, R. G. (1996). Suppression of apoptosis in mammalian cells by NIAP and a related family of IAPs. Nature *379*, 349-353.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.

Maundrell, K., Antonsson, B., Magnenat, E., Camps, M., Muda, M., Chabert, C., Gillieron, C., Boschert, U., Vial-Knecht, E., Martinou, J. C., and Arkinstall, S. (1997). Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J. Biol. Chem. *272*, 25238-25242.

Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M., and Thompson, C. B. (1997). Bcl-$x_L$ forms an ion channel in synthetic lipid membranes. Nature *385*, 353-357.

Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, B. C., Wong, S. L., et al. (1996). X-ray and

NMR structure of human Bcl-$x_L$, an inhibitor of programmed cell death. Nature *1996*, 335-341.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998). An induced proximity model of caspase-8 activation. J. Biol. Chem. *273*, 2926-2930.

Newton, K., Harris, A. W., Bath, M. L., Smith, K. G. S., and Strasser, A. (1998). A dominant interfering mutant of FADD/Mort1 enhances deletion of autoreactive lymphocytes and inhibits proliferation of mature T lymphocytes. EMBO J. *17*, 706-718.

Reap, E. A., Roof, K., Maynor, K., Borrero, M., Booker, J., and Cohen, P. L. (1997). Radiation and stress-induced apoptosis: a role for Fas/Fas ligand interactions. Proc. Nat'l Acad. Sci. USA *94*, 5750-5755.

Reed, J. C. (1997). Cytochrome c: can't live with it-can't live without it. Cell *91*, 559-562.

Refaeli, Y., Van Parijs, L., London, C. A., Tschopp, J., and Abbas, A. K. (1998). Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. Immunity *8*, 615-623.

Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, L., Jansen, B., and Borner, C. (1998). Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature *391*, 496-499.

Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995). The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitors of apoptosis proteins. Cell *83*, 1243-1252.

Roy, N., Madhadevan, M. S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R., Baird, S., Besner-Johnson, A., Lefebvre, c., Kang, X., Salik, M., Aubry, H., Tamai, K., Guan, x., Ioannou, P., Crawford, T. O., de Jong, P. J., Surh, L., Ikeda, J., Korneluk, R. G., and MacKenzie, A. (1995). The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy . Cell *80*, 167-178.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J. *17*, 1675-1687.

Shlomchik, M. J., Madaio, M. P., Ni, D., Trounstein, M., and Huszar, D. (1994). The role of B cells in lpr/lpr-induced autoimmunity. J. Exp. Med. *180*, 1295-1306.

Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol. Cell *1*, 949-957.

Swat, W., Fujikawa, K., Ganiatsas, S., Yang, D., Xavier, R. J., Harris, N. L., Davidson, L., Ferrini, R., Davis, R. J., Labow, M. A., Flavell, R. A., Zon, L. I., and Alt, F. W. (1998). SEK1/MKK4 is required for maintenance of a normal peripheral lymphoid compartment but not for lymphocyte development. Immunity *8*, 625-634.

Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997). Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. Proc. Natl Acad. Sci. U.S.A. *94*, 7337-7342.

Uren, A. G., Pakusch, M., Hawkins, C. J., Puls, K., and Vaux, D. L. (1996). Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated proteins. Proc. Natl Acad. Sci. USA *93*, 4974-4978.

van der Biezen, E. A., and Jones, J. D. G. (1998). The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Curr. Biol. *8*, R226-R227.

Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. (1997). Bcl-$x_L$ regulates the membrane potential and volume homeostasis of mitochondria. Cell *91*, 627-637.

Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Holtmann, H., Lonai, P., and Wallach, D. (1998). Targeted disruption of the mouse caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity *9*, 267-276.

Vucic, D., Kaiser, W. J., and Miller, L. K. (1997). Inhibition of Reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). Proc. Natl Acad. Sci. USA *94*, 10183-10188.

Vucic, D., Kaiser, W. J., and Miller, L. K. (1998). Inhibitor of Apoptosis Proteins physically interact with and block apoptosis induced by Drosophila proteins HID and GRIM. Mol. Cell. Biol. *18*, 3300-3309.

Wajant, H., Johannes, F. J., Haas, E., Siemienski, K., Schwenzer, R., Schubert, G., Weiss, T., Grell, M., and Scheurich, P. (1998). Dominant-negative FADD inhibits TNFR60-, Fas/Apo1- and TRAIL-R/Apo2-mediated cell death but not gene induction. Curr Biol. *8*, 113-116.

Wallach, D., Kovalenko, A. V., Varfolomeev, E. E., and Boldin, M. P. (1998). Death-inducing functions of ligands of the tumor necrosis factor family: a Sanhedrin verdict. Curr. Opin. Immunol. *10*, 279-288.

Walsh, C. M., Wen, B. G., Chinnaiyan, A. M., O'Rourke, K., Dixit, V. M., and Hedrick, S. M. (1998). A role for FADD in T cell activation and development. Immunity *8*, 439-449.

White, K., Grether, M. E., J.M., A., Young, L., Farrell, K., and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. Science *264*, 677-683.

White, K., Tahaoglu, E., and Steller, H. (1996). Cell killing by the Drosophila gene reaper. Science *271*, 805-807.

Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997). Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature *389*, 865-870.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science *275*, 1129-1132.

Yang, X., Chang, H. Y., and Baltimore, D. (1998). Autoproteolytic activation of pro-caspases by oligomerization. Molecular Cell *1*, 319-325.

Yang, X., Khosravi-Far, R., Chang, H. Y., and Baltimore, D. (1997). Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell *89*, 1067-1076.

Yeh, W.-C., de la Pompa, J. L., McCurrach, M. E., Shu, H.-B., Elia, A. J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Deiry, W. S., Lowe, S. W., Goeddel, D. V., and Mak, T. W. (1998). FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science *279*, 1954-1958.

Zhang, J., Cado, D., Chen, A., Kabra, N. H., and Winoto, A. (1998). Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature *392*, 296-300.

Zhou, H., Henzel, W. I., Liu, X., Luschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in the cytochrome c-dependent activation of caspase-3. Cell *90*, 405-413.

Zornig, M., Huber, A.-O., and Evan, G. (1998). p53-dependent impairment of T-cell proliferation in FADD dominant-negative transgenic mice. Current Biology *8*, 467-470.

Fig. 1. Convergent regulation of mammalian cell death pathways