Mitogen-activated Protein Kinase Kinase Antagonized Fas-associated Death Domain Protein–mediated Apoptosis by Induced FLICE-inhibitory Protein Expression

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Summary

Fas and Fas-associated death domain (FADD) play a critical role in the homeostasis of different cell types. The regulation of Fas and FADD-mediated cell death is pivotal to many physiological functions. The activation of T lymphocytes by concanavalin A (Con A) inhibited Fas-mediated cell death. We identified that among the several activation signals downstream of Con A stimulation, mitogen-activated protein (MAP) kinase kinase (MKK) was the major kinase pathway that antagonized Fas-triggered cell death. MKK1 suppressed FADD- but not caspase-3–induced apoptosis, indicating that antagonism occurred early along the Fas-initiated apoptotic cascade. We further demonstrated that activation of MKK1 led to expression of FLIP, a specific inhibitor of FADD. MKK1 inhibition of FADD-induced cell death was abrogated if induction of FLIP was prevented, indicating that FLIP mediates MKK1 suppression of FADD-mediated apoptosis. Our results illustrate a general mechanism by which activation of MAP kinase attenuates apoptotic signals initiated by death receptors in normal and transformed cells.

Key words: mitogen-activated protein kinase kinase • Fas-associated death domain protein • Fas • FLIP • apoptosis

Fas (APO-1, CD95) is a 45-kD transmembrane protein (1) which transmits apoptotic signals upon engagement by Fas ligand or by specific anti-Fas antibodies. The death pathway initiated from Fas involves a series of death-induced molecules (1). Fas-associated death domain protein (FADD; MORT1) is directly recruited to Fas upon Fas engagement (2, 3). FADD then binds caspase-8 (FADD-like IL-1β-converting enzyme [FLICE] or MACH; references 4–6), followed by eventual activation of caspase-3 (CPP32 [7, 8]). FADD is also recruited to TNFR1 by TNFR1-associated death domain protein (TRADD) adapter, and transmits the death signal downstream of TNF binding (9). More recently, FADD was identified as the common mediator for apoptosis triggered by death receptor (DR)3 and DR5 (10–14). In addition, FADD is essential for embryo development (14, 15).

Fas-initiated apoptosis is regulated by a few molecules, including Bcl-2, Bcl-XL, crmA, and inhibitor of apoptosis protein (IAP) (1). Of these regulatory molecules, one that interferes most upstream in the Fas-mediated death cascade is FLIP (FLAME-1, I-FLICE, CASH, casper), a homologue of FLICE (16–20). Both the short and long form of FLIP (FLIP_S and FLIP_L) interact with FADD and FLICE. Overexpression of FLIP suppresses Fas- and TNF–induced apoptosis (16–18, 20). Increased expression of FLIP is found in Fas ligand–resistant melanoma cell lines and in melanoma tumors (16). A viral homologue of FLIP also prevents DR–induced apoptosis (21).

Fas-induced apoptosis in T lymphocytes is known to be antagonized by activation through TCRs (22–24). In search of an activation signal that interferes with Fas-triggered apoptosis, we identified mitogen-activated protein kinase (MAPK) as the major kinase pathway which inhibits FADD-mediated cell death. We also demonstrated that MAPK kinase (MKK) abrogated Fas-initiated apoptosis through the induction of FLIP expression. Our results reveal the molecular mechanism by which activation of MAPK, mostly through tyrosine kinase receptors and G

*Abbreviations used in this paper: DR, death receptor; FADD, Fas-associated death domain protein; FLICE, FADD-like ICE; FLIP, FLICE-inhibitory protein; ICE, IL-1β-converting enzyme; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; nt, nucleotide(s); PI, propidium iodide; RT, reverse transcription.
protein-coupled receptors, attenuates apoptotic signals initiated by DRs in normal and transformed cells.

Materials and Methods

Reagents and Cell Lines. TPA, A23187, and Con A were purchased from Sigma Chemical Co. (St. Louis, MO). PD 098059, SB 203580, wortmannin, rapamycin, and calphostin c were purchased from Calbiochem Corp. (San Diego, CA). Cytochalasin D (Cayman Chemical, Ann Arbor, MI). The constitutively active form of MKK1, a gift of Sandoz Pharmaceutical Co. (Taipei, Taiwan). Anti-Fas antibody CH-11 (25) was purchased from Upsalta Biotechnology, Inc. (Lake Placid, NY). Anti-mouse Fas antibody Jo2 was obtained from PharMingen (San Diego, CA), and the kinase activity of the immune complex was determined as described previously (26). Cyclosporin A was a gift of Sandoz Pharmaceutical Co. (Taipei, Taiwan). Anti–cDNA synthesis by using oligo-dT as primer (31). 1/10 of the cDNA synthesized was then amplified by using the following primers: human (h)FLIP-V 5' GTC TAT TCT GTG GA; murine (m)FLIP-V 5' ACT ATG TGG TGT CAG AGG GCC AG; hFLIP-V 3'; murine (m)FLIP-V 3'; murine (m)FLIP-V 5'; GTC ACA TGA CAT AAC CCA GAT TGT; and mFLIP-V 3'; GTA CAC ACT GCT CCT CAC AGC.

Transfection, Immunoblot, and MAPK Assay. Jurkat T cells and activated splenic T cells were transfected with the DEAE-dextran method (32). For 293T cells, transfection was performed using the calcium phosphate method. Immunoblot was performed according to the method described previously (32). MAPK activity was determined by cotransfection of green fluorescence protein expression vector pGreen Lantern-1 (GIBCO BRL [34]). Treated cells were examined using a fluorescence microscope (Nikon Inc., Melville, NY).

Results

Inhibition of Fas-mediated A poptosis in Jurkat T cells by Con A divination was mediated by MKK. Anti-Fas antibody CH-11 (25) induced extensive cell death in Jurkat cells (Fig. 1 A). Activation of Jurkat cells by Con A reduced Fas-activated cell death from 75 to 20% (Fig. 1 A). The suppression of cell death was not due to activation-induced secretion of IL-2, as IL-2 (50 U/ml) by itself did not prevent Fas-activated apoptosis (not shown). The inhibition of Fas-triggered apoptosis must be due to the activation signal downstream of TCR engagement. We tested several signaling pathways that may be involved in the antagonism of Fas-induced cell death by using specific inhibitors for each pathway. The addition of PD 098059, a selective inhibitor of MKK (35), effectively abrogated the preventive effect of Con A (Fig. 1 A). Only a weak or minimal reversal effect was observed from inhibition of other signaling molecules on Con A-activated Jurkat T cells. The inhibitors tested included calphostin c, cyclosporin A, SB 203580, wortmannin, and rapamycin. Protein kinase C, phosphatidylinositol-3-kinase, and protein kinase B have been implicated to protect against Fas-mediated apoptosis (36, 37), yet MKK was the major kinase activated by Con A stimulation that antagonized Fas-initiated cell death. A role of MAPK in the suppression of Fas-induced T cell death by lectin has also been recently reported (23). The antiapoptotic activity of MKK was further confirmed by transient expression of active MKK1 (29) in Jurkat cells. Transfection of the vector alone (pcDNA3) did not interfere with Fas-induced apoptosis. The induction of MAPK (see below) by MKK1 prevented apoptosis of Jurkat cells triggered by anti-Fas antibody (Fig. 1 B). In contrast, the expression of active MKK3b (30), which led to activation of p38 MAPK (Fig. 1 B), did not increase resistance to Fas-induced apoptosis in Jurkat cells. Neither MKK1 nor MKK3b alone induces cell death of Jurkat cells (Fig. 1 B).

MAPK A divation Suppressed FADD-induced A poptosis but not C aspase-3-induced Cell D ath. In next attempted to identify the stage along the Fas-initiated apoptotic pathway that was antagonized by MKK. Jurkat cells were transfected with FADD and caspase-3 (CPP32) together with the vector or active MKK1 (Fig. 2 A). The cell death induced by FADD was significantly prevented by cotransfection with active MKK1. In contrast, overexpression of active MKK1 did not prevent cell death triggered by caspase-3 (Fig. 2 A). The inhibition by MKK was the early stage of the Fas-
mediated apoptotic pathway. Because only a small fraction of Jurkat cells (<10%) were transfected with the gene of interest in the transient assay, we further examined the preventive effect of MAPK in 293T cells. Tests using green fluorescence protein indicated a transfection efficiency >50%. The high efficiency of transfection was demonstrated by the MAPK activation in 293T cells transfected with active MKK1 (Fig. 2 C), in which prominent MAPK activity was detected 6 h after transfection. The activation of MAPK then subsided, but was still higher than that of the untransfected cells 12 and 24 h after transfection. Transfection of FADD and caspase-3 also led to extensive cell death of 293T cells as measured by PI staining (Fig. 2 B). The coexpression of active MKK1 in 293T cells suppressed the cell death induced by FADD. Similar to that observed in Jurkat cells, overexpression of active MKK1 did not rescue the apoptosis induced by caspase-3. MAPK activation interfered with the Fas-initiated apoptotic cascade at the stage before the activation of caspase-3.

MAPK Activation Induced FLIP Expression. The antagonism of FADD-triggered cell death by active MKK1 resembled the inhibitory activity of FLIP. Both the short form and long form of human FLIP (hFLIPs and hFLIP1) interact with FADD and FLICE, and suppress FADD-induced cell death (16–18, 20). We used primers to detect the expression of both long form and short form (hFLIP1/L and hFLIP1) long form only (hFLIP1/L). FLIP expression in Jurkat cells was induced by T cell activation from stimulation with Con A (Fig. 3 A). The expression profiles of hFLIP1/L and hFLIP1 were similar. The optimum induction of FLIP mRNA was 4 h after Con A activation. The induction of hFLIP1/L was suppressed in the presence of MKK inhibitor PD 098059, suggesting that MKK was required for FLIP expression. We further checked if activation of MKK1 would lead to induction of FLIP. The en-
at 24 h. Vector alone did not lead to any increase in
The induction was visible 6 h after transfection and peaked
However, costimulation with Con A largely prevented
mocytes. Con A by itself promoted immature T cell death.
cific because Jo2 did not promote any death in
death of
express a high level of Fas (38), were stimulated with im-
Fas-initiated apoptosis. MRL
examined whether activation of normal T cells antagonized
may be distinct from those in normal T cells, we further
Expression of FLIP in Con A–treated splenic T cells (Fig. 5
A). Fas-mediated apoptosis in activated splenic T cells
was prevented by Con A stimulation in a PD 098059–sen-
tive manner (Fig. 5 A). M KK was also essential for the
induction of FLIP in Con A–treated splenic T cells (Fig. 5
B). Therefore, M KK and FLIP antagonized Fas-induced
apoptosis in normal T cells as well as in T lymphomas.
A antagonism of FLIP Induction by Antisense Construct Abro-
gated the Protective Effect of M KK. We next examined
whether the antiapoptotic effect of M KK was indeed medi-
atend the 5’ half of FLIP cDNA (−74 to +615 nt) in anti-
sense orientation. The expression of HA–FLIP protein level
was suppressed by 50% when cotransfected with an equal
amount of the FLIP antisense into 293T cells (not shown).
The effectiveness of the FLIP antisense construct was fur-
ther confirmed by inhibition of the FLIP-mediated antiap-
optotic effect (Fig. 6 A). The FLIP antisense construct
alone did not induce cell death in 293T cells. The coex-
pression of the FLIP antisense construct with active M KK
in Jurkat cells abrogated the suppression of Fas-induced
apoptosis (Fig. 6 B). The FLIP antisense construct by itself
did not affect the viability of Jurkat cells. Similarly, FADD-
triggered cell death of 293T cells was no longer prevented
by M KK when FLIP antisense was cotransfected (Fig. 6 C).
Blockage of FLIP expression by the antisense construct
eliminated the antagonistic effect of M KK in Jurkat and
293T cells. This was also the case for normal T lympho-

dogenous FLIP mRNA in 293T cells was weak but clearly
detectable. Transfection of 293T cells with active M KK
significantly increased the expression of hFLIP (38, Fig. 3 B).
The induction was visible 6 h after transfection and peaked
at 24 h. Vector alone did not lead to any increase in
hFLIP mRNA 24 h after transfection. Both the endoge-
ous and the induced expression of FLIP were largely sup-
pressed in the presence of PD 098059 (Fig. 3 B), sup-
porting their dependence on M KK.

A antagonism of Fas-mediated apoptosis in normal T cells by
Con A Activation Was Mediated by MAPK and FLIP.
Because signaling in lymphoma and in embryonic kidney cells
may be distinct from those in normal T cells, we further
examined whether activation of normal T cells antagonized
Fas-initiated apoptosis. MRL+/+/+ thymocytes, known to
express a high level of Fas (38), were stimulated with im-
mobilized anti-mouse Fas antibody Jo2. Significant cell
death of +/- thymocytes was detected 4 h after Fas en-
gagement (Fig. 4 A). The apoptosis observed was Fas-spe-
cific because Jo2 did not promote any death in lpr/lpr thy-
mocytes. Con A by itself promoted immature T cell death.
However, costimulation with Con A largely prevented
Fas-mediated apoptosis in thymocytes (Fig. 4 A). The in-
hibitory effect of Con A was abrogated in the presence of
PD 098059 (10 μM). Similar to that observed in Jurkat
cells, Con A activation led to FLIP expression in mouse thymocytes (Fig. 4 B). The FLIP induction in thymocytes
was significantly earlier than Jurkat cells after Con A activa-
tion. The sensitivity to PD 098059 indicated that Con A–
duced FLIP expression in thymocytes was also dependent
on M KK.

In addition to thymocytes, we used mature T lympho-
cytes isolated from spleen. Fas expression in splenic T cells
was induced by TPA/A23187, and T cells were then cul-
tured in the presence of IL-2. Treatment of preactivated
splenic T cells with Jo2 led to extensive cell death (Fig.
5 A). Fas-mediated apoptosis in activated splenic T cells
was prevented by Con A stimulation in a PD 098059-sen-
titive manner (Fig. 5 A). M KK was also essential for the
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B). Therefore, M KK and FLIP antagonized Fas-induced
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Blockage of FLIP expression by the antisense construct
eliminated the antagonistic effect of M KK in Jurkat and
293T cells. This was also the case for normal T lympho-

Figure 3. FLIP expression was induced by activated M KK. (A)
Jurkat T cells were activated with Con A in the absence or
presence of PD 098059, and total RNA was isolated 2, 4, and
6 h after activation. (B) 293T cells were transfected with active
M KK1 or pcDNA3 (vector) in the absence or presence of PD
098059, and RNA was prepared at the indicated time points. 2 μg
of total RNA was used for cDNA synthesis by using oligo-
dT as primer. 1/10 of the cDNA
were resolved on an agarose gel for comparison.

Figure 4. Antagonism of Fas-induced apoptosis in normal thy-
mocytes by Con A activation was mediated by M KK. (A) Freshly
isolated thymocytes from 8-wk-old MRL+/+ and MRL+/lpr mice
were stimulated with immobilized anti-Fas antibody Jo2 (coated at 5
μg/ml) and/or Con A (4 μg/ml) in the absence or presence of PD
098059 (PD, 10 μM) for 4 h. Cell death was determined by Annexin
V (Clontech, Palo Alto, CA) staining. Data shown are thymocytes
from +/- mice except Jo2 (lpr). The result is the average of duplicates 
± SD. Experiments were repeated twice with identical results. (B)
Induction of FLIP was M KK–dependent in thymocytes. +/- thymocytes
were activated with Con A in the absence or presence of 10 μM PD
098059, and total RNA was isolated L 2, and 3 h after activation. FLIP
transcript was determined as described in the legend to Fig. 3 except
primers for mFLIP were used during PCR analysis.
cytes. Even though the transfection efficiency in preactivated splenic T lymphocytes was much lower than Jurkat cells, transient transfection of active MKK1 conferred resistance of preactivated splenic T lymphocytes to Jo2-induced apoptosis (Fig. 6D). The presence of FLIP antisense removed the protective effect of MKK1 in normal T cells. Therefore, the interference of Fas- and FADD-induced apoptosis by MKK was likely mediated by FLIP in different types of cells.

**Discussion**

In this study, we illustrated that MAPK activation effectively prevents Fas- and FADD-initiated cell death. In addition, the death-inhibitory activity of MAPK is likely due to the induced expression of FLIP. MKK inhibitor PD 098059 suppressed the induction of FLIP in T cells by Con A (Figs. 3A, 4B, and 5B), and the protective effect of Con A was abrogated (Figs. 1A, 4A, and 5A). FLIP mRNA induction reached its optimum 4 h after Con A stimulation, but decreased thereafter. The transient induction of FLIP mRNA may seem incompatible with the prolonged suppression of Fas- and FADD-triggered cell death 24 h after induction. The prolonged antiapoptotic effect of FLIP may be due to its location, as demonstrated by both FLIP and viral FLIP being incorporated in the Fas-containing death-inducing signaling complex (16, 21). Transient expression of FLIP may generate enough FLIP protein to suppress the activity of the death-inducing signaling complex. This was further supported by the fact that the antisense construct of FLIP, which inhibited the expression of FLIP, eliminated most of the antagonistic effect of MKK1 (Fig. 6, B–D). Induction of FLIP may thus account for most of the antiapoptotic activity of active MKK1. Notably, the central role of FLIP in the regulation of Fas-induced apoptosis in T cells is supported by increasing evidence. Toso, a recently identified specific inhibitor of Fas-mediated apo-

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**Figure 5.** Inhibition of Fas-induced apoptosis in preactivated splenic T cells by Con A activation was mediated by MKK. (A) +/+ splenic T cells were purified and activated with TPA/A23187 for 24 h. The cells were then washed and incubated in IL-2 (10 U/ml) for another 3 d. The viable cells were recovered by Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) and stimulated with anti-Fas antibody Jo2 (coated at 5 μg/ml) and/or Con A (4 μg/ml) in the absence or presence of PD 098059 (PD, 20 μM) for 16 h. For Con A stimulation, 4 × 10^4 glutaraldehye-fixed TA3 (reference 27) were added in each well. DNA content was determined by staining with 20 μg/ml PI as described in the legend to Fig. 1. The result is the average of duplicates ± SD. Experiments were repeated twice with identical results. (B) Activated splenic T cells were stimulated with Con A–fixed TA3 in the absence or presence of 20 μg/ml PD 098059, and total RNA was isolated 2 and 4 h after activation. FLIP transcript was determined as described in the legend to Fig. 4.

**Figure 6.** Antagonism of Fas/FADD-induced apoptosis was abrogated by inhibition of FLIP induction. (A) Expression of FLIP inhibited FADD-induced apoptosis, which was abrogated by the FLIP antisense. 293T cells were transfected with 2 μg of FADD, 2 μg of HA-pcDNA3-FLIP (FLIP), with or without 5 μg of pcDNA3-antisense-FLIP (FLIP-anti). The fraction of cells with sub-G1 DNA content was determined 24 h later and used as percent cell death. (B) Coadministration of the FLIP antisense blocked the preventive effect of MKK1 on Fas-induced apoptosis in Jurkat cells. Jurkat T cells were transfected with pGreen Lantern-1 and/or MKK1, in the presence or absence of 5 μg pcDNA3-antisense-FLIP. 30 h later, half of the transfected cells were treated with CH11 (black bars) and the other half were untreated (white bars). Viability of cells was determined after another 15 h by the cells expressing green fluorescence in a 5-mm² area (reference 34). N umbers are averages of five independent measurements. (C) Coadministration of FLIP antisense reversed the antagonist effect of MKK1 on FADD-induced death. 293T cells were transfected with FADD, MKK1, and/or pcDNA3-antisense-FLIP. Percent cell death represents the fraction of cells with sub-G1 DNA content. (D) FLIP antisense abrogated the protective effect of MKK1 on Fas-initiated apoptosis in normal T cells. Preactivated splenic T cells were transfected with pGreen Lantern-1, vector (Control), or MKK1, in the presence or absence of 5 μg pcDNA3-antisense-FLIP by the DEAE-dextran method. Viability was determined as described in B except Jo2 was used to induce cell death.
tosis, acts through induction of FLIP expression (39). Part of the mechanism by which IL-2 enhances Fas-mediated T cell apoptosis is mediated by an inhibition of FLIP expression (40).

Recent studies indicate that apoptotic signal transmitted by Fas engagement is enhanced by TCR signals in T hybridomas and in mature T cells (41, 42). Similarly, we have observed that anti-CD3 alone failed to protect Fas-induced apoptosis of activated splenic T cells (not shown). Our observation that Con A activation led to inhibition of Fas-initiated cell death in T cells (Figs. 1, 4, and 5) suggests that costimulation signals are required for effective antagonism of FADD-mediated apoptosis. This is consistent with a report on the protection against Fas-induced apoptosis by superantigen and CD28 in mature human T cells (24).

FADD is the apoptotic mediator of several DRs, including Fas, TNFR1, DR3, and DR5 (2, 3, 9–14). Hence, FADD is activated by death signals through the binding of the Fas ligand, TNF, and TNF-related apoptosis–inducing ligand (TRAIL), whereas MAPK is activated by engagement of tyrosine kinase receptor, G protein-coupled receptor, and protooncogenes (43, 44). The activation of MAPK is essential for cell proliferation and mitogenesis, yet its role in antiapoptosis is less definite. MAPK has been shown to inhibit apoptosis induced by nerve growth factor deprivation in PC12 cells (45). MAPK has also been demonstrated to be involved in the inhibition of ceramide-induced apoptosis by sphingosine 1-phosphate (46) and in the suppression of cardiac myocyte apoptosis by cardiotrophin 1 (47). Because of the diverse biological targets of MAPK, the mechanism of MAPK in the antagonism of apoptosis remains mostly elusive. This study reveals a novel mechanism by which MAPK antagonizes FADD-mediated death through the induction of a FADD inhibitor. Therefore, the antagonism of FADD-initiated apoptosis by M KK represents an interesting cross-talk between two distinct signaling pathways, one apoptotic and the other mitogenic. Given the range of physiological functions and pathological consequences mediated by DRs, the antagonism of FADD by M KK illustrates how the early stage of DR-induced apoptosis can be interrupted. Interestingly, the Fas-activated apoptotic process is accompanied by an early cleavage of R af-1, the activator of M KK (48). In this manner, the antiapoptotic M KK pathway is inhibited during the progression of the death pathway. Further molecular characterization of the antagonism between FADD and M KK will help efforts to place DR-mediated apoptosis under the appropriate control.

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