Mind bomb 1 regulation of cFLIP interactions

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Zhang L, Gallagher PJ. Mind bomb 1 regulation of cFLIP interactions. Am J Physiol Cell Physiol 297: C1275–C1283, 2009. First published August 26, 2009; doi:10.1152/ajpcell.00214.2009.—Mind bomb 1 (Mib1) is a multidomain E3 ligase that directs ubiquitination of the Notch ligands Delta and Jagged to promote their endocytosis. Here we examine Notch-independent functions of Mib1 and find that its activities are linked to the initiation of the extrinsic cell death pathway. Expression of Mib1 induces a spontaneous, caspase-dependent cell death. Consistent with this, depletion of endogenous Mib1 decreases tumor-necrosis factor (TNF)-induced cell death. Mib1 was found to bind to cellular Fas-associated death domain (FADD)-like IL-1b converting enzyme (FLICE)-like inhibitory proteins (cFLIP-L and cFLIP-S), whereas only cFLIP-S can inhibit Mib1-induced cell death. The interaction between Mib1 and cFLIP decreases the association of caspase-8 with cFLIP, which activates caspase-8 and induces cell death. Collectively, these results suggest that in addition to a central role in Notch signaling, Mib1 has an important role in regulating the extrinsic cell death pathway.

MATERIALS AND METHODS

Reagents. Anti-Flag M2 antibody, anti-vinculin antibody, z-Val-Ala-Ala-Asp(OCH3) fluoromethylketone (z-VAD-fmk), z-IETD-fmk, z-Leu-Glu(OMe)-His-Asp(OMe) fluoromethylketone (z-LEHD-fmk), trypan blue solution (0.4%), and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Anti-poly-ADP-ribose polymerase (PARP), anti-tumor necrosis factor (TNF) receptor 1-associated death domain (TRADD), anti-Omni probe (D-8), anti-IκB-α, and anti-Lamin A/C were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8, anti-caspase-9, and anti-FADD were from Cell Signaling (Danvers, MA). Anti-p65 was from StressGen (Ann Arbor, MI). Anti-GFP was from BD Biosciences/Clontech (Mountain View, CA). Anti-Mib1 (previously named anti-DIP1) was as described (20). Fugene 6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). DharnaFect-1 small interfering RNA (siRNA) transfection reagent was from Dharmacon (Lafayette, CO).

Plasmids. The construction of p3xFlag-Mib1 wt has been described previously (20). Mib1 RING mutant constructs were made using site-directed mutagenesis kit (Stratagene) and with mutated oligonucleotide primers corresponding to mutation sites. CrmA, DN-FADD, and TRADD constructs were kindly provided by Dr. Maureen A. Harrington (Indiana University) (38). FADD construct was kindly provided by Dr. Preet M. Chaudhary (University of Pittsburgh Medical Center). The Mib2 expression plasmid was kindly provided by Dr. Young-Yun Kong (Pohang University, South Korea). pCDNA3.1/Hisc-TRADDΔ195–312 and TRADDΔ301–312 were generated by polymerase chain reaction (PCR). cFLIP-L and cFLIP-S constructs were kindly provided by Dr. Shi-Yong Sun (Emory University), and pcDNA3.HisB-cFLIP-L and cFLIP-S were generated by PCR.

Cell culture and transient transfection. Human embryonic kidney (HEK)293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient transfection was carried out using equal amounts of total plasmid DNA (adjusted with the corresponding empty vectors) together with Fugene 6 transfection reagent according to the manufacturer’s guidelines. siRNAs for TRADD were synthesized by Dharmacon using the published sequence (target sequence 5′-CUGGCC-GAGCUGGAGAUG-3′) (39). siRNA for Mib1 was used as previously described (20, 43). Nontargeting siRNAs were purchased from Dharmacon and used as negative control. DharnaFect 1 was used for the siRNA transfection.

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Western blotting and immunoprecipitation. Western blotting and immunoprecipitation (IP) were performed as described previously (43).

Nuclear protein extraction. Nuclear protein from HEK293 cells was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) following the manufacturer’s instructions.

Trypan blue exclusion assay. Mib1-induced cell death was also evaluated using the trypan blue exclusion assay. After the cells were transfected with Mib1 for 36 h, both adherent and nonadherent cells were harvested, washed with PBS, and resuspended in PBS. After being mixed with equal volume of trypan blue solution (0.4%), the cells were counted using a hemacytometer. Blue cells are dead cells stained by trypan blue. Each experiment was repeated at least three times. Cell death was represented by the mean percentage of blue cells per total cells.

Reporter gene assays. PGL4 nuclear factor (NF)-κB reporter construct was from Promega. Transfection was carried out with FuGENE6 transfection reagent (Roche) according to the manufacturer’s guidelines. The level of promoter activity was evaluated by measurement of the firefly luciferase activity relative to the internal control TK-Renilla luciferase activity using the Dual Luciferase Assay System essentially as described by the manufacturer (Promega). A minimum of six independent transfections was performed.

Fig. 1. Mind bomb 1 (Mib1) specifically induces caspase-dependent cell death that is dependent on its RING3 E3 ubiquitin ligase activity. Human embryonic kidney (HEK)293 cells were transfected with the indicated amounts of plasmids encoding Flag-tagged Mib1 (pCDNA4to-Flag-Mib1) (A), green fluorescent protein (GFP)-tagged Mib2 (B), or empty vector. At 36 h posttransfection, cells were lysed and Western blotting was carried out to detect the levels of Mib1 or Mib2 expression and poly-ADP-ribose polymerase (PARP) cleavage using anti-Mib1, anti-GFP, or PARP antibodies. Vinculin was used as loading control.

C: schematic drawing of the domains of Mib1 and location of RING domain mutations. Amino acid changes are indicated at the right side of the schematic.

D: detection of Mib1 autoubiquitination activity in wild-type (WT) or mutant (R1m, R2m, R3m, R1R2m, R1R2R3m) Mib1 constructs. HEK293 cells were transfected with different Mib1 constructs and a plasmid vector encoding HA-Ubiquitin (UB). CrmA was cotransfected to attenuate caspase activation induced by Mib1 expression. Mib1 was immunoprecipitated from cell lysates using an anti-Flag antibody, and the relative levels of Mib1 or UB were detected by Western blotting using anti-Flag or anti-HA antibodies. IP, immunoprecipitate; CE, cell extract.

E: HEK293 cells were transfected with plasmids for expression of either Mib1 with a single mutation in a RING domain [RING1 mutant (R1m), RING2 mutant (R2m), or RING3 mutant (R3m)], with combination mutations (R1R2m, R1R2R3m) or WT Mib1. Cells were lysed and analyzed by Western blotting to evaluate the relative levels of PARP cleavage as a measure of caspase activation.

F: HEK293 cells were transfected with the indicated Mib1 plasmids or empty vector. At 36 h posttransfection, adherent and nonadherent cells were harvested and stained with trypan blue solution and counted using a hemacytometer.
for each replicate, and all assays were replicated at least twice. Results are reported as means ± SE.

RESULTS

Mib1 induces a caspase-dependent apoptosis that requires Mib1RING3 E3 ubiquitin ligase activity. Previous studies have demonstrated that Mib1 is an E3 ubiquitin ligase that targets the Notch ligands Delta and Jagged (1, 9, 19, 21, 42), as well as DAPK (20, 43) for proteasome-mediated degradation. Subsequently, it was noted that ectopic expression of this E3 ligase reduced cellular viability (20). To confirm that ectopic expression of Mib1 induces apoptosis, we expressed Mib1 in HEK293 and COS-1 cells (data not shown) and used Western blotting to detect cleavage of the caspase target PARP. The results of these experiments (Fig. 1A) revealed that ectopic expression of Mib1 in HEK293 cells for 36 h induced a caspase-mediated apoptosis as evidenced by cleavage of PARP. In control experiments, expression of another highly related E3 ligase Mib2 did not alter PARP cleavage, suggesting that induction of apoptosis is a function of Mib1 (Fig. 1B).

To determine whether the E3 ligase activity of Mib1 is responsible for induction of apoptosis, the conserved cysteine residues residing within each of three mouse Mib1 RING fingers were mutated both singly and in combination using site-directed mutagenesis (Fig. 1C). Mutation of residues C834S/H836N within the most NH2-terminal RING domain (RING1; R1m) and mutation of residue C890S within the middle RING domain (RING2; R2m) either singly or in combination (R1mR2m) had no effect on Mib1 autoubiquitination, whereas mutation of C985S, which resides in COOH-terminal RING3 domain, strongly attenuated Mib1 autoubiquitination (Fig. 1D). This set of experiments confirmed that the RING3 domain is directly responsible for the autoubiquitinination of Mib1. To determine the importance of the E3 ligase activity in Mib1-induced cell apoptosis, the Mib1 RING mutants were expressed in cells to examine their effect on cell apoptosis. As shown in Fig. 1, E and F, the expression of either wild-type Mib1 or the RING1 or RING2 mutant in HEK293 cells induces cell death as evidenced by PARP cleavage and the trypan blue exclusion assay. In contrast, expression of the single RING3 mutant (R3m) or the triple mutant RING1/RING2/RING3 (R1R2R3m) did not induce cell death. This result suggests that the E3 ligase activity of RING3 in Mib1 is necessary for the induction of cell death.

To further confirm that exogenous expression of Mib1 activated a caspase-dependent cell death, HEK293 cells were transfected with Mib1 constructs, and 12 h later cells were treated with either vehicle (DMSO) or concentrations of the pan-caspase inhibitor z-VAD-fmk ranging from 1 to 40 μM. As shown in Fig. 2A, treatment of cells with z-VAD-fmk antagonized Mib1-induced cell death as evidenced by a dose-dependent attenuation of PARP cleavage. These Western blotting data also reveal that Mib1 expression activates caspase-8 as determined by the appearance of the caspase-8 cleavage products (p43/41) in the absence of z-VAD-fmk (Fig. 2A). This suggests that Mib1 may activate the extrinsic cell death pathway upstream of caspase-8 in absence of ligand activated death receptor signaling. In the extrinsic cell death pathway, caspase-8 can either directly activate caspase-3 or cleave the pro-apoptotic BH3-only Bcl-2 family protein BID to form tBID, which activates the intrinsic death cascade leading to activation of caspase-9 (12, 25, 27, 35). With the use of this paradigm, it is expected that if expression of Mib1 activates the extrinsic cell death pathway, then caspase-9 will also be activated as part of the Bid amplification loop. To discriminate between these possibilities, cells were transfected with an expression vector for Mib1, and treated with either the caspase-8-like inhibitor z-IETD-fmk, a caspase-8-like inhibitor (A); z-IETD-fmk, a caspase-8-like inhibitor (B); or z-LEHD-fmk, a caspase-9-like inhibitor (C). Cell lysates were prepared and analyzed by Western blotting to detect Mib1 expression, PARP, caspase-8, or caspase-9 activation using anti-Mib1, anti-PARP, anti-caspase-8, or anti-caspase-9 antibodies.

Fig. 2. Expression of Mib1 activates caspase-dependent cell death. HEK293 cells were transfected with pCDNA4to-Flag-Mib1 (2 μg), a vector for expression of Mib1, and 12 h later the transfected cells were treated for 24 h with the indicated concentrations of z-VAD-fmk, a pan-caspase inhibitor (A); z-IETD-fmk, a caspase-8-like inhibitor (B); or z-LEHD-fmk, a caspase-9-like inhibitor (C). Cell lysates were prepared and analyzed by Western blotting to detect Mib1 expression, PARP, caspase-8, or caspase-9 activation using anti-Mib1, anti-PARP, anti-caspase-8, or anti-caspase-9 antibodies.
Mib1 activates the extrinsic cell death pathway without affecting NF-κB pathway. As caspase-8 is a critical component of the death receptor apoptotic pathway, the involvement of Mib1 in TNF signaling pathway was examined. The effect of Mib1 on TNF-induced cell death in HEK293 cells (Fig. 3A) and HeLa cells (Fig. 3B) was determined by depleting endogenous Mib1 using a specific siRNA. Cell lysates were examined by Western blotting to determine relative levels of PARP cleavage and caspase-8 activation in Mib1-depleted or control siRNA-treated cells. In addition, trypan blue staining was used to distinguish apoptotic from nonapoptotic HEK293 (Fig. 3C) or HeLa (Fig. 3D) cells following treatment with either Mib1 or control siRNAs (36h) and TNF treatment for either 16 h (HEK293) or 4 h (HeLa). These results show that depletion of Mib1 attenuated TNF-induced activation of caspase-8 and the cleavage of a caspase substrate PARP. Consistent with inhibition of caspase activation, TNF-induced cell death was also attenuated.

To determine whether Mib1 altered activation of the NF-κB arm of the TNF-signaling pathway, HEK293 cells were transfected with an expression vector for Mib1 or empty vector. After 36 h the cells were treated with TNF for up to 60 min and then prepared for Western blotting analysis to detect IκB-α (Fig. 3E). These results showed that profile of TNF-induced degradation of IκB-α was not significantly altered by ectopic expression of Mib1. In a parallel experiment, TNF-treated and control cells were fractionated and nuclear fractions were purified and examined by Western blotting to detect the p65 subunit of NF-κB (Fig. 3F). The results of this experiment show that the p65 subunit of NF-κB is efficiently translocated into the nucleus in response to TNF, and nuclear trafficking of NF-κB is not altered by ectopic expression of Mib1. Finally, to confirm that Mib1 does not influence NF-κB transcriptional activity, an NF-κB gene reporter assay was carried out. As shown in Fig. 3G, the luciferase activity of an NF-κB reporter was significantly increased after TNF treatment for 5 h. In
addition, there was no significant difference in NF-κB reporter activity whether Mib1 was overexpressed or depleted by siRNA. Together these results suggest that Mib1 activities contribute to the apoptotic but not the survival arm of the extrinsic cell death-signaling pathway.

**Mib1 impacts the extrinsic cell death pathway downstream of TRADD**. To determine whether or not Mib1 acts upstream of caspase-8, HEK293 cells were cotransfected Mib1 and either a dominant negative (DN) FADD (DN-FADD; residues 80–205) (38) or dominant negative TRADD (DN-TRADDΔ301–312 and DN-TRADDΔ195–312) (17). DN-FADD lacks a death effector domain and blocks recruitment of caspase-8 and FADD in the TNFR signaling pathway. TRADDΔ301–312 and TRADDΔ195–312 are DN mutants of TRADD, which lack a portion of their death domain and cannot interact with TNFR1 to induce cell death (17). The results of these experiments show that expression of DN-FADD abolished Mib1-induced cell death as evidenced by lack of PARP cleavage (Fig. 4A). In contrast, the expression of either of the two DN-TRADDS (TRADDΔ301–312 or TRADDΔ195–312) had no effect on Mib1-induced cell death (Fig. 4B). Similarly, depletion of endogenous TRADD with a specific siRNA had no effect on Mib1-induced cell death (Fig. 4C). These data suggest that Mib1 acts downstream of TRADD and upstream of FADD.

cFLIP-S inhibits Mib1-induced cell death, whereas Mib1 induces FLIP-L cleavage by caspases. In the extrinsic cell death pathway, the cellular FLICE-inhibitory proteins (cFLIP) can bind to and modulate the activation of caspase-8 and cell death (22, 23). cFLIP proteins are expressed as a long (cFLIP-L) and a short (cFLIP-S) splice variants that can bind to FADD through their death effector domains to inhibit caspase-8 activation and cell death (22, 23). As our results show that expression of a DN-FADD (Fig. 4) or treating cells with a caspase-8 inhibitor (Fig. 2) prevent Mib1-induced cell death, we examined the role of cFLIP in Mib1-induced cell death. As shown in Fig. 5A, expression of cFLIP-S prevented Mib1-induced cell death, whereas expression of cFLIP-L had no effect. Since activated caspase-8 can cleave cFLIP-L, three different caspase inhibitors were used to determine whether they could inhibit Mib1-induced cFLIP-L cleavage.

As shown in Fig. 5B, both z-VAD-fmk and z-IETD-fmk attenuated cFLIP-L cleavage. These results suggest that z-VAD-fmk (pan-Caspase inhibitor) and z-IETD-fmk (caspase-8-like inhibitor) can inhibit FLIP-L degradation, whereas z-LEHD-fmk, a caspase-9-like inhibitor, had little effect. It has been reported that cFLIP can also be degraded by proteasome pathway (7), and we asked whether Mib1-induced cFLIP-L degradation was occurring through the proteasome pathway by using proteasome inhibitors MG132 and lactacystin. As shown in Fig. 5, C and D, the degradation of cFLIP-L was not attenuated in the presence of either proteasome inhibitor or by the γ-secretase inhibitor DAPT. In addition, these studies suggested that the E3 ligase activity of Mib1 appears to be important for cleavage of FLIP1 as the level of FLIP-L expression was unaltered by expression of the RING 3 mutant of Mib1(Mib1-R3) (Fig. 5D). These findings demonstrate that cFLIP-L degradation was likely caused by Mib1-induced caspase-8 activation.

**Mib1 interacts with cFLIP-L and cFLIP-S and antagonizes the interaction between cFLIP and caspase-8**. Since cFLIP inhibits cell death by binding to caspase-8 (22, 23) and cFLIP-S antagonizes Mib1-induced cell death, co-immunoprecipitation was carried out to see whether Mib1 interacts with cFLIP. For this experiment, HEK293 cells were cotransfected with vectors for expression of Flag-tagged Mib1, Flag-tagged Mib1 RING3M, or an empty control vector together with cFLIP. As shown in Fig. 6A, either Flag-tagged Mib1 or Mib1RING3M co-immunoprecipitated with cFLIP-L and cFLIP-S. Immunoprecipitation of caspase-8 (Fig. 6, B and C) revealed that the interaction between cFLIP and caspase-8 was signifi-
cantly decreased upon expression of wild-type Mib1. In contrast, expression of the E3 ligase-deficient mutant of Mib1 (Mib1 RING3M) had no effect on the level of cFLIP-L or cFLIP-S that co-immunoprecipitates with caspase-8. Since the expression level of cFLIP-L was reduced by the presence of Mib1, we tested the effect of Mib1 on caspase-8 and cFLIP-L interactions in the presence of z-VAD-fmk. As shown in Fig. 6B, culturing cells in the presence of the pan-caspase inhibitor z-VAD-fmk restored cFLIP-L expression levels, suggesting that a caspase is responsible for cFLIP-L degradation. This result is consistent with published studies (15, 23) showing that caspase-8 targets cFLIP-L for cleavage and also with the ability of the caspase-8-like inhibitor z-IETD-fmk to restore cFLIP-L expression (Fig. 5B). Together this series of experiments suggests that Mib1 activates the cell death-signaling pathway by decreasing the association of cFLIP and caspase-8 and by enhancing the cleavage of cFLIP-L by caspase-8.

**DISCUSSION**

Mib1 is a complex, multidomain E3 ligase that has received a great deal of attention for its role in regulating Notch signaling (1, 9, 19, 21, 24). In addition to its role in Notch signaling, our previous studies have shown that Mib1 (previously referred to as DIP1) induces DAPK ubiquitination and proteasomal degradation (20), suggesting that Mib1 has additional roles not associated with the Notch signaling pathway. In the current study, we have extended these observations to further define the underlying signaling pathway through which mammalian Mib1 regulates cell death.

Mib1 activates the extrinsic death receptor cell death pathway. Ectopic expression of Mib1 in cultured mammalian cells results in a dose-dependent activation of a caspase-dependent cell death that could be inhibited by caspase-inhibitors (Fig. 2) but not by proteasome inhibitors (Fig. 5). Depletion of Mib1 using an siRNA also attenuated TNF-induced cell death (Fig. 3), suggesting that Mib1 expression is able to activate the extrinsic death receptor pathway in absence of a death receptor ligand. Consistent with this is the finding that expression of DN-FADD, but not DN-TRADD (Fig. 4), can block Mib1-induced activation of the apoptotic cascade, and this finding also supports a role for Mib1 in activation of the extrinsic death receptor signaling cascade downstream of TRADD and upstream of FADD.

Mib1 induces cell death by decreasing the association of cFLIP and caspase-8. Upon activation of death receptors, caspase-8 is recruited to the receptor through its interaction with FADD. FADD nucleates the assembly of the death-inducing signaling complex (4, 5, 10, 31) to result in autoactivation of caspase-8 activity. The association of cFLIP-L or cFLIP-S with caspase-8 blocks caspase-8 activation (22, 23), so a decrease in this inhibitory binding would be expected to enhance caspase-8 activation and promote cell death. Our results show that expression of Mib1 results in a decrease in cFLIP-L expression, and several caspase inhibitors can antagonize this decrease. Although the specificity of these peptide caspase inhibitors has been questioned, especially when used in high concentrations (2, 3, 16, 28, 36), our dose-dependent results are consistent with the suggestion that a caspase has a
role in Mib1-induced cell death. As previous studies have shown, both cFLIP-L and cFLIP-S associate with and regulate activation of caspase-8, and based on these studies, it is likely that Mib1 indirectly alters activation of caspase-8 through cFLIP binding. Our results suggest that the association of Mib1 with c-FLIP prevents the inhibitory association of c-FLIP with caspase-8. This in turn allows activation of caspase-8 and subsequent cell death. Interestingly, Mib1-induced caspase-8 activation triggers a decrease in the expression levels of cFLIP-L but not cFLIP-S (Fig. 5A). Consistent with this, it has been reported that activated caspase-8 can cleave cFLIP-L leading to decreased cFLIP-L levels (15, 23). Since there is no caspase-8 cleavage site within cFLIP-S, activated caspase-8 cannot directly regulate the level of cFLIP-S. The finding that cFLIP-S prevents Mib1-induced cell death but not cFLIP-L, suggests that it is the disruption of interactions between cFLIP-S and caspase-8 by Mib1 that is an important first step to trigger cell death. As the cFLIP-L level is significantly decreased upon Mib1 expression and a reported caspase-8 inhibitor z-IETD-fmk (Fig. 5) can prevent this decrease, our results are consistent with a model in which Mib1 disruption of cFLIP-S binding to caspase 8 activates cell death, and degradation of cFLIP-L further promotes cell death.

RING3 E3-ligase activity of Mib1 is required for Mib1-induced cell death. The finding that mutation of all three RING finger domains (R1R2R3M) of Mib1 or a single mutation of RING3 (R3M) can block Mib1-induced cell death (Fig. 1) suggests that Mib1 E3 ligase is important for activation of the extrinsic apoptotic signaling pathway. The effects of Mib1 on cell death induction are specific for Mib1 as ectopic expression of the highly related E3 ligase Mib2 did not induce cell death in cultured cells. Together these results show that E3 ligase activity of RING3 is of critical importance for regulating a Notch-independent function of Mib1, the induction of the extrinsic death receptor-signaling pathway.

Although other studies have examined the potential importance of each of the three RING finger motifs present in Mib1 (9, 42), these studies have utilized only zebrafish Mib1 in a heterologous culture systems, and none of these studies have determined the importance of the RING finger domains in Notch-independent signaling functions. Our current studies now demonstrate that the most COOH-terminal RING domain RING3 of the mouse Mib1 protein is essential for promoting Mib1-induced cell death. These studies also confirm that, like the zebrafish homologue, the E3 ligase activity of the RING3 domain is required for autoubiquitination (9, 42). Our data show that the proteasome inhibitors MG-132 or lactacystin do not inhibit Mib1-induced cell death or rescue cFLIP expression levels. This finding suggests that proteasomal degradation of cFLIP-L or cFLIP-S is not mediated by Mib1 ubiquitination of cFLIP. One unexplained finding of these studies is that both the wild-type Mib1 and mutant Mib1 R3M can interact with cFLIP, although the mutant Mib1 R3M is unable to block cell death. This suggests that the E3 ligase activity of Mib1 is essential for Mib1-induced cell death. One possible explanation is that an unidentified target for ubiquitination by Mib1 is not ubiquitinated by the mutant Mib1 lacking E3 ligase activity. If this target protein is an inhibitor of cFLIP and caspase-8 binding, then the lack of ubiquitination and degradation of the target inhibitor would prevent activation of cell death. Alternatively, it is also possible that mutation of the

Fig. 6. Mib1 decreases the association of cFLIP and caspase-8. A: HEK293 cells were cotransfected with cFLIP-L or cFLIP-S and either empty vector or Mib1 as indicated. At 36 h posttransfection, cells were lysed and Mib1 was immunoprecipitated with anti-Flag agarose beads. HEK cells were cotransfected with cFLIP-L (B) or cFLIP-S (C) and either empty vector, wild-type (wt), or mutant (R3M) Mib1 as indicated. The pan-caspase inhibitor z-VAD-fmk (10 μM) was added following transfection (B). At 36 h posttransfection, caspase-8 was immunoprecipitated with anti-caspase-8 antibody, and the immunoprecipitates were analyzed by Western blotting to detect the relative levels of caspase-8, cFLIP-L, or cFLIP-S as indicated. *Nonspecific band.
RING fingers in Mib1 alters the structure of Mib1 such that it can still bind cFLIP but cannot mediate an interaction between cFLIP and caspase-8.

In summary, these studies indicate Mib1, an important E3 ligase in Notch signaling pathway, also can activate the extrinsic apoptotic pathway. Although the Notch signaling paradigm does not involve death receptor or apoptotic signaling, there have been some reports that Notch may regulate the cell death of different cells (6, 26, 30, 33, 40, 44). Also several lines of evidence suggest that alterations of Notch signaling or expression contribute to tumorigenesis (11, 13, 14, 29, 30), suggesting a potential role in cell survival. The results of these studies suggest that Mib1 can activate the extrinsic signaling pathway. In this signaling pathway, Mib1 interaction with cFLIP prevents an inhibitory association of caspase-8 with cFLIP-L and cFLIP-S. This interaction promotes activation of caspase-8 and initiation of cell death.

REFERENCES


