An antiapoptotic protein, c-FLIP_L, directly binds to MKK7 and inhibits the JNK pathway

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Inhibition of NF- κ B activation increases susceptibility to tumor necrosis factor (TNF) α -induced cell death, concurrent with caspases and prolonged c-Jun N-terminal kinase (JNK) activation, and reactive oxygen species (ROS) accumulation. However, the detailed mechanisms are unclear. Here we show that cellular FLICE-inhibitory protein (c-FLIP) is rapidly lost in NF- κ B activation-deficient, but not wild-type fibroblasts upon TNF α stimulation, indicating that NF- κ B normally maintains the cellular levels of c-FLIP. The ectopic expression of the long form of c-FLIP (c-FLIP_L) inhibits TNF α -induced prolonged JNK activation and ROS accumulation in NF- κ B activation-deficient fibroblasts. Conversely, TNF α induces prolonged JNK activation and ROS accumulation in c-Flip^{-/-} fibroblasts. Moreover, c-FLIP_L directly interacts with a JNK activator, MAP kinase kinase (MKK)7, in a TNF α -dependent manner and inhibits the interactions of MKK7 with MAP/ERK kinase kinase 1, apoptosis-signal-regulating kinase 1, and TGF β -activated kinase 1. This stimuli-dependent interaction of c-FLIP_L with MKK7 might selectively suppress the prolonged phase of JNK activation. Taken that ROS promote JNK activation and activation of the JNK pathway may promote ROS accumulation, c-FLIP_L might block this positive feedback loop, thereby suppressing ROS accumulation.

The EMBO Journal (2006) **25**, 5549–5559. doi:10.1038/sj.emboj.7601423; Published online 16 November 2006

Subject Categories: signal transduction; differentiation & death

Keywords: c-FLIP; c-Jun N-terminal kinase; NF- κ B; reactive oxygen species; tumor necrosis factor

Introduction

NF- κ B is a collective term of dimeric transcriptional factors that belong to the Rel family of proteins, and regulates expression of various inflammatory cytokines, chemokines,

and adhesion molecules (Ghosh and Karin, 2002). NF- κ B is activated by various inflammatory cytokines and cellular stress including tumor necrosis factor (TNF) α , interleukin-1 (IL-1), UV, and γ -irradiation. Moreover, NF- κ B, especially the RelA-containing complex, inhibits cell death induced by TNF α , Fas ligand, TRAIL, and genotoxic stress. Currently, the antiapoptotic functions of NF- κ B are supported to be mainly mediated by the upregulation of antiapoptotic genes such as *cellular FLICE-inhibitory protein (c-Flip)*, the members of the *Bcl-2* family, and *X-chromosome-linked inhibitor of apoptosis (Xiap)* (Karin and Lin, 2002). However, the detailed molecular mechanisms are not completely understood.

The regulation of cell death and survival is also controlled in part by another signaling cascade activated by the mitogen-activated protein kinase (MAPK) following cellular stress or cytokine signaling (Davis, 2000; Kyriakis and Avruch, 2001). In mammals, the MAPK cascades are composed of three distinct signaling modules, the c-Jun N-terminal kinase (JNK), the p38MAPK, and the extracellular signal-regulated kinase (ERK) cascades. Each MAPK is activated by sequential protein phosphorylation through a MAPK module. In the case of the JNK cascade, the MAP kinase kinase kinases (MAPKKKs) include apoptosis-signal-regulating kinase (ASK)1, MAP/ERK kinase kinases (MEKKs), MTK1 (also known as MEKK4), and TGF β -activated kinase (TAK)1. These MAPKKKs activate MAP kinase kinase (MKK)4 and/or MKK7, which in turn activate JNKs (Davis, 2000; Kyriakis and Avruch, 2001). Cytokines and growth factors including TNF α and IL-1 induce rapid (within 10 min) yet transient MAPK activation, cellular stress, such as UV- or γ -irradiation, induces prolonged MAPK activation. Several lines of evidence suggest that transient MAPK activation is associated with gene expression, proliferation, and differentiation, whereas prolonged MAPK activation promotes cell death in a cell-type- and stimuli-dependent manner (Chen *et al*, 1996; Guo *et al*, 1998).

In the past, the contributions of the NF- κ B and JNK pathways have been discussed independently. However, two recent studies have revealed that inhibition of NF- κ B activation induces prolonged JNK activation that promotes TNF α -induced cell death (De Smaele *et al*, 2001; Tang *et al*, 2001). Later studies have shown that TNF α -induced accumulation of reactive oxygen species (ROS) is enhanced in NF- κ B activation-deficient cells including RelA^{-/-}, I κ B kinase β ^{-/-}, and TNF receptor-associated factor (traf)2^{-/-}traf5^{-/-} murine embryonic fibroblasts (MEFs) (Sakon *et al*, 2003; Pham *et al*, 2004; Ventura *et al*, 2004; Kamata *et al*, 2005). Notably, this TNF α -induced ROS accumulation and prolonged JNK activation are completely suppressed by antioxidants, but not caspase inhibitors, indicating that TNF α -induced ROS accumulation is caspase-independent in NF- κ B activation-deficient MEFs. Furthermore, expression of antioxidant enzyme genes including *manganese-dependent superoxide dismutase (Mnsod)* and *ferritin heavy chain (fhc)* is upregulated by TNF α in an NF- κ B-dependent manner, and the ectopic

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Received: 8 July 2006; accepted: 4 October 2006; published online: 16 November 2006

expression of these genes inhibits TNF α -induced ROS accumulation in NF- κ B-deficient MEFs (Pham *et al*, 2004; Kamata *et al*, 2005). Although these studies have revealed that the caspase-independent pathway plays a crucial role in ROS accumulation, a recent study has shown that activated caspase-3 cleaves the p75 subunit of complex I of the mitochondrial electron transport chain, which would impair the function of complex I, resulting in ROS accumulation (Ricci *et al*, 2004). In addition, Giorgio *et al* (2005) have shown that proapoptotic signals induce the release of p66^{Shc} from a putative inhibitory complex and the released p66^{Shc} then oxidizes reduced cytochrome *c*, thereby generating ROS. Therefore, the mechanisms whereby TNF α induces ROS accumulation are still controversial (Papa *et al*, 2004b; Luo *et al*, 2005; Nakano *et al*, 2006).

c-FLIP, also designated as CASH or Casper, was first identified as a molecule that interacts with FADD or is structurally related to procaspase-8 (Goltsev *et al*, 1997; Irmeler *et al*, 1997; Shu *et al*, 1997). *c-Flip* encodes two splicing variants, long form of c-FLIP (c-FLIP_L) and short form of c-FLIP (c-FLIP_S). While c-FLIP_S only contains two N-terminal death effector domains (DEDs), c-FLIP_L consists of the N-terminal DEDs and a C-terminal caspase-like domain that does not possess enzymatic activity. Many studies have shown that both c-FLIP_L and c-FLIP_S inhibit death receptor-induced apoptosis by binding to and inhibiting caspase-8 activation via DED–DED interaction (Thome and Tschopp, 2001). Consistently, *c-Flip*^{−/−} cells showed an increased susceptibility to TNF α - and Fas ligand-induced cell death (Yeh *et al*, 2000). Moreover, recent studies have shown that c-FLIP and viral FLIP are also involved in the Wnt signaling pathways (Naito *et al*, 2004; Nakagiri *et al*, 2005).

In the present study, we have shown that c-FLIP_L is rapidly degraded after TNF α stimulation, and the ectopic expression of c-FLIP_L inhibits the TNF α -induced ROS accumulation and prolonged JNK activation in NF- κ B activation-deficient MEFs. Conversely, TNF α induces prolonged JNK and ERK activation, and ROS accumulation in *c-Flip*^{−/−} cells. We have also shown that c-FLIP_L binds to MKK7 and MEK1 in a TNF α -dependent manner, resulting in suppression of the MKK7–JNK and MEK1–ERK pathways. Taken that ROS promote JNK activation and activation of the JNK pathway may induce ROS accumulation, c-FLIP_L might block this positive feedback loop, thereby suppressing ROS accumulation. Collectively, c-FLIP_L is essential for suppression of TNF α -induced prolonged JNK activation and ROS accumulation.

Results

NF- κ B protects signal-dependent degradation of c-FLIP_L

To explore signaling intermediate(s) that link the TNF receptor to ROS accumulation, we first compared protein expression levels of downstream signaling molecules of the TNF receptor in wild-type (WT), *RelA*^{−/−}, and *traf2*^{−/−}*traf5*^{−/−} MEFs before and after TNF α stimulation. Among various signaling molecules investigated, we found that TNF α stimulation induced a rapid and progressive loss of c-FLIP_L in both *RelA*^{−/−} and *traf2*^{−/−}*traf5*^{−/−} MEFs, concurrent with the appearance of a degradation fragment of 43 kDa (Figure 1A). Notably, MEFs appeared to express 55 kDa c-FLIP_L, but not 26 kDa c-FLIPs. A similar degradation fragment was also observed in WT MEFs after TNF α stimulation,

while the levels of c-FLIP_L were not apparently reduced. When pretreated with a protein synthesis inhibitor, cycloheximide (CHX), c-FLIP_L was progressively lost also in WT MEFs (Figure 1B). These results indicate that TNF α induces rapid degradation of c-FLIP in both WT and NF- κ B activation-deficient MEFs, but the expression levels of c-FLIP_L are maintained in WT MEFs by NF- κ B-dependent *de novo* synthesis. It has been shown that the processing of c-FLIP_L to the p43 fragment is mediated by caspase-8 (Shu *et al*, 1997; Kataoka and Tschopp, 2004), and c-FLIP levels are also controlled in part by the ubiquitin/proteasome pathway (Kreuz *et al*, 2001; Micheau *et al*, 2001). Consistently, the TNF α -induced degradation of c-FLIP_L at early time points was partially inhibited by z-VAD-fmk or a proteasome inhibitor, MG132, in *RelA*^{−/−} and *traf2*^{−/−}*traf5*^{−/−} MEFs (Supplementary Figure S1). Therefore, the TNF α -induced rapid degradation of c-FLIP_L may be at least partly mediated by caspases and the ubiquitin/proteasome pathway.

c-FLIP_L inhibits TNF α -induced ROS accumulation and prolonged JNK activation

Consistent with our previous study (Sakon *et al*, 2003), TNF α -induced ROS accumulation and prolonged JNK activation are suppressed by an antioxidant, butylated hydroxyanisole (BHA), but not a broad caspase inhibitor, z-VAD-fmk in *RelA*^{−/−} and *traf2*^{−/−}*traf5*^{−/−} MEFs (Supplementary Figure S2A). This indicates that ROS accumulation and prolonged JNK activation are induced in a caspase-independent manner in these cells. To test directly whether the degradation of c-FLIP_L is responsible for the TNF α -induced ROS accumulation and prolonged JNK activation, we ectopically expressed c-FLIP_L in *RelA*^{−/−} and *traf2*^{−/−}*traf5*^{−/−} MEFs. Expression of c-FLIP_L almost completely inhibited TNF α -induced ROS accumulation in *RelA*^{−/−} and *traf2*^{−/−}*traf5*^{−/−} MEFs (Figure 1C), suggesting that c-FLIP_L might also suppress the caspase-independent pathway leading to ROS accumulation. TNF α -induced JNK activation at 10 min was not different between WT and *RelA*^{−/−} MEFs, but this early phase JNK activation was severely impaired in *traf2*^{−/−}*traf5*^{−/−} MEFs (Supplementary Figure S2B). Intriguingly, c-FLIP_L specifically inhibited the sustained (at 120 min), but not early (at 10 min), phase of JNK activation in *RelA*^{−/−} and *traf2*^{−/−}*traf5*^{−/−} MEFs (Figure 1D). Moreover, expression of c-FLIP_L substantially increased cell viability after TNF α stimulation in *RelA*^{−/−} and *traf2*^{−/−}*traf5*^{−/−} MEFs (Figure 1E). Taken that TNF α induces caspase-dependent and -independent cell death in NF- κ B activation-deficient cells (Sakon *et al*, 2003), these results indicate that ectopic expression of c-FLIP_L is sufficient for suppression of TNF α -induced ROS accumulation, prolonged JNK activation, and cell death through suppression of the caspase-dependent and -independent pathways in NF- κ B activation-deficient cells.

TNF α induces ROS accumulation and prolonged JNK activation in *c-Flip*^{−/−} MEFs

To test directly whether c-FLIP_L is essential for suppression of JNK activation and ROS accumulation, we first investigated the time course of caspase-3 activities after TNF α stimulation in *c-Flip*^{−/−} MEFs. TNF α did not induce activation of caspase-3 in WT MEFs, unless the cells were stimulated with TNF α in the presence of CHX (Figure 2A). In sharp contrast, TNF α stimulation induced a robust activation of caspase-3 at 1 h

Figure 1 TNFα induces rapid degradation of c-FLIP_L and ectopic expression of c-FLIP_L inhibits TNFα-induced accumulation of ROS, prolonged JNK activation, and cell death in *RelA*^{-/-} and *traf2*^{-/-} *traf5*^{-/-} MEFs. (A) WT, *RelA*^{-/-}, and *traf2*^{-/-} *traf5*^{-/-} MEFs were stimulated with TNFα (10 ng/ml) for the indicated times, then the lysates were analyzed by immunoblotting with anti-c-FLIP (upper panel) and anti-tubulin (lower panel) antibodies. p55 and p43 indicate full and degraded form of c-FLIP_L, respectively. (B) WT MEFs were stimulated with TNFα (10 ng/ml) in the presence of CHX (10 μg/ml) for the indicated times. Immunoblotting was performed as in (A). The asterisks indicate nonspecific bands. (C) Transfection of c-FLIP_L inhibits TNFα-induced ROS accumulation in *RelA*^{-/-} and *traf2*^{-/-} *traf5*^{-/-} MEFs. Total cell lysates from mock (M) or respective transfectants (T) were immunoblotted with anti-Flag antibody. The transfectants were unstimulated (thin line) or stimulated (bold line) with TNFα (10 ng/ml) for 4 h, and then the cells were labeled with CM-H₂DCFDA (1 μM) and analyzed by flow cytometry. (D) c-FLIP_L inhibits prolonged, but not early JNK activation in *RelA*^{-/-} and *traf2*^{-/-} *traf5*^{-/-} MEFs. Mock- or c-FLIP_L-transfected *RelA*^{-/-} and *traf2*^{-/-} *traf5*^{-/-} MEFs were stimulated with TNFα (10 ng/ml) for the indicated times, and then the lysates were immunoblotted with anti-phospho-JNK (upper panel) and anti-total JNK (lower panel) antibodies. (E) c-FLIP_L inhibits TNFα-induced cell death in *RelA*^{-/-} and *traf2*^{-/-} *traf5*^{-/-} MEFs. Mock- or c-FLIP_L-transfected *RelA*^{-/-} and *traf2*^{-/-} *traf5*^{-/-} MEFs were stimulated with the indicated amounts of TNFα for 16 h. Cell viability was determined by WST assay. Results are presented as the mean of triplicate samples and represent three independent experiments with similar results. Standard errors are within 5%. **P* < 0.05 compared to mock transfectant.

and then continued at 2 h, which was completely suppressed in the presence of z-VAD-fmk or z-VAD-fmk plus BHA, but not BHA in *c-Flip*^{-/-} MEFs (Figure 2A). We next tested whether JNK activation is prolonged in *c-Flip*^{-/-} MEFs. While TNFα stimulation only induced transient JNK activation in WT MEFs, the sustained phase of JNK activation gradually appeared at 1 h after stimulation in *c-Flip*^{-/-} MEFs, which was completely suppressed by z-VAD-fmk (Figure 2B). However, JNK activation continued at 2 h even in the presence of z-VAD-fmk, and this activation was substantially inhibited by the further addition of BHA. These results suggest that the caspase-dependent pathway plays a dominant role in JNK activation at 1 h. This is then followed by the caspase-independent ROS-inducing pathway leading to JNK activation at 2 h. Similarly, TNFα-induced ERK activation was prolonged in *c-Flip*^{-/-} MEFs. Phosphorylation of JNK and ERK in TNFα plus BHA-treated cells at 2 h appeared to decrease compared to TNFα plus zVAD-fmk-treated cells. Taken that BHA alone inhibited neither caspase activation nor ROS accumulation at 2 h (Figure 2A and C), these decreases might not be due to direct inhibitory effect of BHA on JNK and ERK activation, but rather due to massive

cell death of TNFα plus BHA-treated *c-Flip*^{-/-} MEFs (data not shown).

We tested whether TNFα induces ROS accumulation in *c-Flip*^{-/-} MEFs. ROS slightly accumulated at 1 h, and then progressively accumulated at 2 h after stimulation in *c-Flip*^{-/-} MEFs (Figure 2C). In contrast to NF-κB activation-deficient cells (Supplementary Figure S2A), TNFα-induced ROS accumulation was significantly suppressed by z-VAD-fmk at 2 h after stimulation. However, a substantial amount of ROS still accumulated in *c-Flip*^{-/-} MEFs at 2 and 4 h even in the presence of z-VAD-fmk (Figure 2C). We could not evaluate ROS accumulation in TNFα- or TNFα plus BHA-stimulated *c-Flip*^{-/-} MEFs at 4 h due to cell death. Consistent with an inhibitory effect on TNFα-induced JNK activation, BHA plus z-VAD-fmk almost completely suppressed TNFα-induced ROS accumulation at 2 and 4 h after stimulation. Together, these results suggest that both caspase-dependent and -independent pathways may play crucial roles in TNFα-induced prolonged JNK activation and ROS accumulation in *c-Flip*^{-/-} MEFs. The reason why BHA could not inhibit caspase-dependent ROS accumulation is currently unknown. Similarly, another antioxidant, N-acetyl cysteine, could not inhibit

Figure 2 TNFα induces ROS accumulation and prolonged JNK activation in *c-Flip*^{-/-} MEFs. (A) WT MEFs and *c-Flip*^{-/-} MEFs were stimulated with TNFα (10 ng/ml) in the absence or presence of CHX (10 μg/ml), zVAD-fmk (zVAD) (50 μM), BHA (100 μM), or zVAD-fmk (50 μM) plus BHA (100 μM) for the indicated times. Caspase-3 activities were measured by using fluorogenic substrates. Results are presented as mean ± s.e.'s of triplicate samples. Caspase activities were not detected in zVAD-fmk- or zVAD-fmk plus BHA-treated cells. (B) WT and *c-Flip*^{-/-} MEFs were stimulated as in (A), and then the lysates were analyzed as in Figure 1D. zB indicates zVAD-fmk (50 μM) plus BHA (100 μM) treatment. (C) WT and *c-Flip*^{-/-} MEFs were unstimulated (thin line) or stimulated (bold line) with TNFα (10 ng/ml) as in (A) for the indicated times, accumulated ROS were analyzed as in Figure 1C. NE (not examined) indicates that ROS accumulation was not analyzed due to massive cell death. (D) *c-Flip*^{-/-} MEFs were stimulated with the indicated amounts of TNFα in the absence or presence of zVAD-fmk (zVAD) (50 μM), BHA (100 μM), or zVAD-fmk (50 μM) plus BHA (100 μM) for 14 h. Cell viability was determined by WST assay as in Figure 1E. **P* < 0.05 compared to TNFα plus zVAD-fmk-treated cells.

TNFα-induced ROS accumulation in *c-Flip*^{-/-} MEFs (data not shown). Consistent with these results, zVAD-fmk plus BHA treatment more efficiently increased cell viability of *c-Flip*^{-/-} MEFs than zVAD-fmk alone (Figure 2D). Collectively, c-FLIP is essential for suppression of TNFα-induced ROS accumulation, prolonged JNK activation, and cell death.

We also tested whether deficiency of c-FLIP could affect the duration of the JNK pathway induced by other stimuli. Consistent with a previous study (Chen *et al*, 1996), anisomycin and UV induced prolonged JNK activation even in WT MEFs (Supplementary Figure S3A and B). Similarly, these stimuli induced prolonged JNK activation in *c-Flip*^{-/-} MEFs.

In contrast, IL-1 induced transient JNK activation in both WT and *c-Flip*^{-/-} MEFs (Supplementary Figure S3C), suggesting that c-FLIP-dependent suppression of the JNK pathway is specific to TNFα-induced JNK activation.

TNFα-induced NF-κB activation and expression of *fbc* and *Mnsod* genes are not different between WT and *c-Flip*^{-/-} MEFs

Previous studies have shown that NF-κB-dependent upregulation of antioxidant enzyme genes including *fbc* and *Mnsod* are responsible for elimination of ROS (Pham *et al*, 2004; Kamata *et al*, 2005). Therefore, TNFα induces ROS accumulation in NF-κB activation-deficient cells. On the other hand,

several studies have shown that c-FLIP_L activates NF- κ B under particular conditions (Kataoka *et al*, 2000; Golks *et al*, 2006). To test the possibility that accumulation of ROS is due to impaired upregulation of *fhc* and/or *Mnsod* genes in *c-Flip*^{-/-} MEFs, we first investigated whether TNF α -induced I κ B α degradation is impaired in *c-Flip*^{-/-} MEFs. Consistent with a previous study (Yeh *et al*, 2000), TNF α -induced degradation of I κ B α was not impaired in *c-Flip*^{-/-} MEFs (Figure 3A). Moreover, TNF α -induced upregulation of *fhc* and *Mnsod* mRNAs were not significantly different up to 120 min after TNF α stimulation in WT and *c-Flip*^{-/-} MEFs (Figure 3B). We could not investigate the induction of mRNAs of these genes at later time points after 120 min, as almost all *c-Flip*^{-/-} MEFs died.

To further explore the mechanism underlying c-FLIP-dependent suppression of the ERK and JNK pathways, we examined whether TNF α -induced MEK1 phosphorylation is prolonged in *c-Flip*^{-/-} MEFs. Phosphorylation of endogenous MEK1 was detected at 10 min upon TNF α stimulation in both WT and *c-Flip*^{-/-} MEFs. Notably, phosphorylation of MEK1 reappeared at 60 min and persisted up to 120 min in *c-Flip*^{-/-} MEFs (Figure 3C), whereas phosphorylation of MEK1 disappeared at 60 min in WT MEFs. The decrease in the size of phosphorylated MEK1 at 120 min in *c-Flip*^{-/-} MEFs might be due to a degradation of MEK1. However, at least under our experimental conditions, we could not detect phosphorylation of endogenous MKK7 upon TNF α stimulation possibly

due to a poor sensitivity of the antibody to recognize phosphorylated MKK7 (data not shown). Collectively, these results indicate that TNF α induces prolonged ERK and JNK activation through activation of MEK1 and possibly MKK7 in *c-Flip*^{-/-} MEFs, which are not suppressed by NF- κ B-inducible genes other than *c-Flip*.

c-FLIP_L inhibits JNK and ERK activation

Taken that TNF α -induced ROS accumulation is abolished in *Jnk1*^{-/-}*Jnk2*^{-/-} (*Jnk*^{-/-}) MEFs (Ventura *et al*, 2004) and the above results together, we surmised that c-FLIP_L might directly inhibit the JNK pathway. Thus, we first tested whether c-FLIP_L inhibits phosphorylation of MAPKs triggered by MAPKKs including MEKK1, ASK1, and TAK1, which have been implicated in TNF α -induced MAPK activation (Nishitoh *et al*, 1998; Yuasa *et al*, 1998; Sato *et al*, 2005). We transfected HEK293 cells with HA-tagged MAPKs along with a constitutively active form of MEKK1 (MEKK1 Δ N) or ASK1 in the presence or absence of c-FLIP_L. Expression of c-FLIP_L substantially inhibited the MEKK1 Δ N-induced JNK and ERK phosphorylation (Figure 4A and B). c-FLIP_L also inhibited the ASK1-induced JNK, but not p38 phosphorylation (Figure 4C and D). Collectively, c-FLIP_L preferentially inhibits the JNK and ERK, but not p38 pathways. As we could not detect the interaction of c-FLIP_L with JNK, ERK, or p38 in co-immunoprecipitation experiments (data not shown), we speculated that c-FLIP_L inhibits the JNK and ERK pathways by suppressing their activators such as MAPKKs or MAPKKKs. Therefore, we next tested whether c-FLIP_L inhibits MEKK1 Δ N- and ASK1-triggered phosphorylation of MAPKKs in their activation loop motifs. c-FLIP_L substantially inhibited the MEKK1 Δ N- and ASK1-induced phosphorylation of MKK7 (Figure 4E and F). Similarly, c-FLIP_L moderately inhibited the MEKK1 Δ N-induced MEK1 and MKK4 phosphorylation (Figure 4G and H). In contrast, c-FLIP_L did not inhibit the ASK1-induced MKK6 phosphorylation (Figure 4I). Moreover, c-FLIP_L inhibited the TAK1-induced MKK7, MKK4, and JNK, but inhibitory effect of c-FLIP_L on p38 phosphorylation was marginal. Similarly, c-FLIP_L inhibited Tpl-2-induced JNK and ERK activation (Supplementary Figure S4). These results indicate that c-FLIP_L selectively inhibits the MKK7-JNK, MKK4-JNK, and MEK1-ERK, but not MKK6-p38 pathways.

c-FLIP_L binds to MKK7 and MEK1 in a TNF α -dependent manner, and disrupts their interactions with MEKK1, ASK1, and TAK1

The inhibition of the MEKK1-induced MEK1, MKK4, and MKK7 phosphorylation by c-FLIP_L prompted us to investigate whether c-FLIP_L interacts with MAPKKs. Intriguingly, c-FLIP_L interacted with MEK1 and MKK7, and their interactions were significantly enhanced in the presence of MEKK1 Δ N (Figure 5A and D). These results suggest that the MEKK1-induced phosphorylation and/or conformational changes of MEK1 and MKK7 might enhance their binding to c-FLIP_L. Similarly, MKK4 weakly interacted with c-FLIP_L, but MKK6 did not (Figure 5B and C). We tested whether c-FLIP_L could interact with MKK7 and MEK1 under more physiological conditions. We first used *RelA*^{-/-} MEFs stably expressing Flag-c-FLIP_L. Interactions of c-FLIP_L with endogenous MKK7 and MEK1 were undetectable in unstimulated cells, but their interactions were weakly induced at 5 min and increased up to 2 h after TNF α stimulation (Figure 5E). Notably, the

Figure 3 TNF α -induced NF- κ B activation and expression of *fhc* and *Mnsod* mRNAs are not different between WT and *c-Flip*^{-/-} MEFs. (A) TNF α induces degradation of I κ B α in WT and *c-Flip*^{-/-} MEFs. WT and *c-Flip*^{-/-} MEFs were stimulated with TNF α (10 ng/ml) for the indicated times, then the lysates were analyzed by immunoblotting with anti-I κ B α (upper panel) and anti-tubulin (lower panel) antibodies. (B) Induction of *fhc* and *Mnsod* mRNAs upon TNF α stimulation. WT or *c-Flip*^{-/-} MEFs were stimulated with TNF α as in (A), and then total RNAs were extracted. Expression of the indicated genes was analyzed by real-time PCR. mRNA levels of the indicated genes were compared as a ratio to the unstimulated time points. The data are shown as mean $\pm$ s.e.'s of triplicates samples. (C) TNF α induces prolonged MEK1 activation in *c-Flip*^{-/-} MEFs. MEFs were stimulated as in (A), and the lysates were analyzed by immunoblotting with anti-phospho MEK1 (upper panel) and anti-total MEK1 (lower panel) antibodies. The asterisks indicate the degraded form of MEK1.

Figure 4 c-FLIP_L inhibits the MKK7-JNK, MKK4-JNK, and MEK1-ERK pathways. (A–I) HEK293 cells were transfected with the indicated expression vectors. After immunoprecipitation (IP) with anti-HA antibody, the phosphorylation of MAPKs or MAPKKs was analyzed by immunoblotting with the indicated phospho-specific antibodies (top panels). Expression levels of the transfected proteins in the immunoprecipitates (IP) (second panels) and the total lysates (TL) (third and bottom panels) were analyzed by immunoblotting with anti-HA, anti-Myc, and anti-Flag antibodies.

interactions of MKK7 or MEK1 with Flag-c-FLIP_L were specific, as anti-Flag, but not control antibody, specifically co-immunoprecipitated MKK7 and MEK1. More importantly, the interaction of endogenous c-FLIP_L with endogenous MKK7 was induced upon TNF α stimulation in HEK293 cells (Figure 5F). Together, c-FLIP_L physically interacts with MKK7 and MEK1 in a TNF α -dependent manner.

We next investigated the mechanism whereby c-FLIP_L inhibits the MKK7-JNK and MEK1-ERK pathways. One possible scenario is that the binding of c-FLIP_L to MKK7 or MEK1 might disrupt the interaction of MKK7 or MEK1 with MEKK1, thereby suppressing the MEKK1-induced MKK7 or MEK1 activation. A previous study has shown that once MEKK1 binds to and phosphorylates MKK4, MEKK1 rapidly dissociates with the phosphorylated MKK4 (Xia *et al*, 1998). Consistently, we could not detect the interaction of the WT MKK7 with MEKK1 in co-immunoprecipitation experiments (data not shown). As we previously observed the stable interaction of a kinase-inactive mutant MKK7 (MKK7-KM) with MEKK1 in co-transfection experiments (Takekawa *et al*, 2005), we tested whether c-FLIP_L inhibits the interaction of MKK7-KM with MEKK1. Transfection of c-FLIP_L inhibited the interaction of MKK7-KM with MEKK1ΔN in a dose-dependent manner (Figure 5G). Similarly, c-FLIP_L inhibited the interaction of MEK1-KM with MEKK1ΔN (Figure 5H). Collectively, c-FLIP_L inhibits the MKK7-JNK and MEK1-ERK pathways by disrupting the interactions of MKK7 or MEK1 with MEKK1. We also found that c-FLIP_L inhibited the interaction of MKK7-KM with ASK1 or TAK1 (Supplementary Figure S4). We could not detect the interaction of c-FLIP_L with ASK1 or MEKK1 (data not shown).

The C-terminal caspase-like domain of c-FLIP_L is responsible for the binding to MKK7

We determined the functional domain of c-FLIP_L that binds to and suppresses the MKK7-JNK pathway. We constructed expression vectors for c-FLIPs and c-FLIPΔN lacking the N-terminal DEDs. We investigated whether these mutants interact with MKK7. While both c-FLIP_L and c-FLIPΔN efficiently interacted with MKK7, c-FLIPs did not (Figure 6B). Consistently, expression of c-FLIPΔN, but not c-FLIPs inhibited the MEKK1ΔN-induced MKK7 and JNK phosphorylation (Figure 6C and D), indicating that the binding of c-FLIP_L to MKK7 is tightly correlated with the inhibitory effect of c-FLIP_L on the MKK7-JNK pathway. To further delineate the binding region of c-FLIP_L to MKK7, we constructed a series of deletion mutants of c-FLIP_L and tested their ability to interact with MKK7 (Figure 6A). Notably c-FLIP_LΔC(–220) interacted with MKK7, but could not inhibit the MKK7-JNK pathway (Figure 6E–G). Taken that c-FLIP_LΔC(–153) bound to and inhibited MKK7 activation, the residues 203–260 are sufficient for the binding of c-FLIP_L to MKK7, but additional residues 261–327 are required for inhibition of the MKK7-JNK pathway. Consistent with this notion, the residues 261–327 was required to inhibit the interaction of MEKK1 with MKK7, as c-FLIP_LΔC(–220) lost the ability to inhibit the binding of MEKK1 to MKK7 (Figure 6H).

Reconstitution of c-FLIP_L, but not c-FLIPs, completely inhibits TNF α -induced ROS accumulation, JNK activation, and cell death in c-Flip^{–/–} MEFs

To show directly a role for the C-terminal caspase-like domain of c-FLIP_L in suppression of the JNK pathway under more

Figure 5 Interactions of c-FLIP_L with MEK1 and MKK7 are induced in a TNF α -dependent, and c-FLIP_L disrupts their interactions with MEKK1. (A–D) HEK293 cells were transfected with the indicated expression vectors. After immunoprecipitation (IP) with anti-Flag antibody, co-immunoprecipitated MAPKKs were detected by immunoblotting with anti-HA antibody (top panels). Expression levels of the transfected proteins in the immunoprecipitates (IP) (second panels) and the total lysates (TL) (third and bottom panels) were analyzed by immunoblotting with anti-Flag, anti-HA, and anti-Myc antibodies. (E) *RelA*^{−/−} MEFs stably expressing Flag-c-FLIP_L were stimulated with TNF α (10 ng/ml) for the indicated times. After immunoprecipitation with control (C) or anti-Flag (F) antibody, co-immunoprecipitated MKK7 and MEK1 were detected by immunoblotting with anti-MKK7 and anti-MEK1 antibodies, respectively. Expression levels of Flag-c-FLIP_L in the immunoprecipitates (IP), MKK7 and MEK1 in the total lysates (TL) were analyzed by immunoblotting with the indicated antibodies. (F) HEK293 cells were stimulated with TNF α (10 ng/ml) for the indicated times. After immunoprecipitation with control (C) or anti-c-FLIP antibodies, co-immunoprecipitated MKK7 was detected by immunoblotting with anti-MKK7 antibody (top panel). Expression levels of c-FLIP_L and MKK7 in the total lysates (TL) (second and bottom panels) were analyzed by immunoblotting with the indicated antibodies. (G, H) HEK293 cells were transfected with the indicated expression vectors. After immunoprecipitation (IP) with anti-Myc antibody, co-immunoprecipitated MKK7-KM or MEK1-KM were detected by immunoblotting with anti-HA antibody (top panels). Expression levels of the transfected proteins were analyzed as in (A).

physiological conditions, we reconstituted *c-Flip*^{−/−} MEFs with c-FLIP_L or c-FLIPs. While TNF α could not induce prolonged JNK activation in c-FLIP_L transfectant, JNK activation gradually increased at 2 h and continued at 4 h after TNF α stimulation in c-FLIPs transfectant (Figure 7A). Similarly, TNF α -induced ROS accumulation was induced in c-FLIPs, but not c-FLIP_L transfectants (Figure 7B). Reconstitution of c-FLIP_L or c-FLIPs substantially increased cell viability of these transfectants compared to mock transfectant; however, c-FLIP_L more efficiently protected cells from TNF α -induced cell death (Figure 7C). Together, the C-terminal caspase-like domain is required for c-FLIP-dependent survival signals through suppression of the JNK pathway and ROS accumulation.

Discussion

In the present study, we have demonstrated an essential role for c-FLIP_L in suppression of TNF α -induced ROS accumulation and prolonged JNK activation using NF- κ B activation-deficient and *c-Flip*^{−/−} MEFs. c-FLIP_L and c-FLIPs have been shown to bind directly to FADD and caspase-8 via DEDs, thereby inhibiting death receptor-mediated apoptosis (Goltsev *et al*, 1997; Irmeler *et al*, 1997; Shu *et al*, 1997; Yeh

et al, 2000). In addition to this well-known antiapoptotic function of c-FLIP, our present study has revealed that c-FLIP_L, but not c-FLIPs, directly interacts with MKK7 and downregulates JNK activation. c-FLIP_L might inhibit the JNK pathway through suppression of the interactions of MKK7 with MAPKKs, including MEKK1, ASK1, and TAK1 (Figure 5 and Supplementary Figure S4). Together, c-FLIP_L has two distinct functions, one is inhibition of the caspase pathway via the N-terminal DED, and the other is suppression of the JNK pathway via the C-terminal caspase-like domain.

Although our present study has revealed that c-FLIP_L-dependent suppression of the JNK pathway is to disrupt the interaction of MKK7 with MAPKKs, it is also likely that c-FLIP_L might suppress the MKK7-induced JNK activation. Consistent with this possibility, we observed that c-FLIP_L inhibited JNK activation induced by a constitutive active form MKK7, MKK7-3E, in which two serines and one threonine at the activation loop motif were mutated to glutamic acids (Holtmann *et al*, 1999) (Supplementary Figure S5). Moreover, our preliminary study showed that the binding domain of MKK7 to c-FLIP_L was mapped to the N-terminal region of the MKK7 (data not shown), which has been shown to be responsible for the interaction with JNK (Tanoue *et al*,

Figure 6 An intimate correlation between binding of c-FLIP_L to MKK7 and inhibition of the MKK7–JNK pathway. **(A)** Schematic diagrams of deletion mutants of c-FLIP_L, and the summary of their binding and inhibition experiments. **(B, E)** HEK293 cells were transfected with the indicated expression vectors. After immunoprecipitation (IP) with anti-Flag antibody, co-immunoprecipitated MKK7 was detected by immunoblotting with anti-HA antibody (top panels). Expression levels of the transfected proteins were analyzed as in Figure 5A. **(C, D, F, G)** HEK293 cells were transfected with the indicated expression vectors. After immunoprecipitation (IP) with anti-HA antibody, the phosphorylation of MKK7 (C, F) or JNK (D, G) was analyzed by immunoblotting with anti-phospho-MKK7 or anti-phospho-JNK antibodies (top panels). Expression levels of the transfected proteins were analyzed as in Figure 4A. **(H)** HEK293 cells were transfected with the indicated expression vectors. After immunoprecipitation (IP) with anti-Myc antibody, co-immunoprecipitated MKK7-KM was detected by immunoblotting with anti-HA antibody (top panels). Expression levels of the transfected proteins were analyzed as in Figure 5A.

2000). This suggests that c-FLIP_L disrupts the binding of MKK7 to JNK. Further study will be required to address this possibility. Taken that the interaction of c-FLIP_L with MKK7 is induced in a TNF α -dependent manner (Figure 5E and F), c-FLIP_L might selectively downregulate the prolonged phase, but not the early phase JNK activation (Figure 1D). Taken that the interactions of c-FLIP_L with MKK7 or MEK1 were weakly detected at 5 min after stimulation (Figure 5E and F), these early and weak interactions might not be sufficient for inhibiting MKK7 and MEK1 activation. Consistently, TNF α -induced JNK and ERK activation at 10 min were not different between WT and *c-Flip*^{-/-} MEFs, whereas JNK and ERK activation were enhanced at 1 and 2 h after TNF α stimulation in *c-Flip*^{-/-} MEFs compared to WT MEFs (Figure 2B).

Previous studies have shown that impaired upregulation of antioxidant enzyme genes including *fhc* and *Mnsod* is

responsible for TNF α -induced ROS accumulation in NF- κ B activation-deficient cells (Pham *et al*, 2004; Kamata *et al*, 2005). Then, accumulated ROS inactivate MKPs, resulting in prolonged JNK activation (Kamata *et al*, 2005). However, this scenario does not appear to be the case of *c-Flip*^{-/-} MEFs, as expression of *fhc* and *Mnsod* mRNAs were not significantly different between WT and *c-Flip*^{-/-} MEFs up to 2 h after TNF α stimulation (Figure 3B). Taken that MKPs dephosphorylate and inactivate MAPKs, but not MAPKKs, these results suggest that, in addition to ROS-dependent inactivation of MKPs, another mechanism might operate to induce prolonged MEK1 and possibly MKK7 activation in *c-Flip*^{-/-} MEFs. Consistent with this notion, c-FLIP_L inhibited activation of MEK1 and MKK7 (Figure 4 and Supplementary Figure S4).

A previous study has shown that GADD45 β , which is induced in an NF- κ B-dependent manner, binds to MKK7 and inhibits MKK7's kinase activity as competitive inhibition

Figure 7 c-FLIP_L more efficiently suppresses TNFα-induced ROS accumulation, prolonged JNK activation, and cell death than c-FLIPs. (A) c-FLIP_L, but not c-FLIPs, completely inhibits TNFα-induced prolonged JNK activation in *c-Flip*^{-/-} MEFs. Total cell lysates from mock, c-FLIPs, or c-FLIP_L transfectants were immunoblotted with anti-c-FLIP antibody. c-FLIPs and c-FLIP_L transfectants were stimulated with TNFα (10 ng/ml) for the indicated times, and then the lysates were analyzed as in Figure 1D. (B) c-FLIP_L, but not c-FLIPs, completely inhibits TNFα-induced ROS accumulation in *c-Flip*^{-/-} MEFs. The transfectants were unstimulated (thin line) or stimulated (bold line) with TNFα (10 ng/ml) for 2 h (for mock transfectant) or 8 h (for c-FLIP transfectants), then accumulated ROS were analyzed as in Figure 1C. (C) c-FLIP_L, but not c-FLIPs, completely inhibits TNFα-induced cell death in *c-Flip*^{-/-} MEFs. Mock or c-FLIP transfectants were stimulated with the indicated amounts of TNFα for 16 h. Cell viability was determined by WST assay as in Figure 1E. **P* < 0.05 compared to c-FLIPs transfectant.

of ATP (Papa *et al*, 2004a). Taken that both c-FLIP_L and GADD45β bind to MKK7, it is interesting to test whether c-FLIP_L could affect the interaction of GADD45β with MKK7. However, under our experimental conditions, we could not detect a substantial interaction of GADD45β with MKK7 in HEK293 cells (data not shown). One possible explanation to explain this discrepancy is that the interaction of GADD45β with MKK7 might take place in a cell-type-specific manner. Consistently, GADD45β-dependent JNK inhibition is predominant in T cells, but not MEFs (Amanullah *et al*, 2003; Papa *et al*, 2004a). Therefore, the relation between the interactions of MKK7 with GADD45β and c-FLIP_L is currently unknown and will be investigated in the future study. Collectively, c-FLIP_L and GADD45β might cooperatively or independently inhibit the JNK pathway in a context- and cell type-dependent manner (Figure 8A).

Chang *et al* (2006) have recently reported that JNK phosphorylates and activates a ubiquitin ligase, ITCH, that subsequently interacts with the caspase-like domain of c-FLIP_L and promotes degradation of c-FLIP_L. In this respect, the fact that c-FLIP_L, but not c-FLIPs, binds to and inhibits JNK activation is particularly interesting (Figure 6). Under normal conditions, expression levels of c-FLIP_L are sufficient for inhibition

Figure 8 NF-κB-dependent survival signals. (A) A model for c-FLIP_L-dependent suppression of the prolonged activation of the MKK7–JNK pathway. Upon TNFα stimulation, MEKK1, ASK1, and TAK1 phosphorylate and activate MKK7, which in turn activates JNK. MKPs subsequently dephosphorylate JNK, resulting in termination of the activation of JNK. In addition to this mechanism, the interaction of c-FLIP_L with MKK7 is induced in a TNFα-dependent manner. This interaction inhibits the prolonged activation of the MKK7–JNK pathway by disrupting the interactions of MKK7 with MAPKKs. Thus, c-FLIP_L selectively inhibits the prolonged, but not early phase JNK activation. (B) NF-κB upregulates antioxidants and antiapoptotic proteins including MnSOD, FHC, GADD45β, XIAP, and c-FLIP_L. Antiapoptotic proteins and antioxidants inhibit the caspase pathway and ROS accumulation, respectively. c-FLIP_L plays a crucial role for suppression of the caspase-dependent and -independent pathways leading to JNK activation and ROS accumulation. In contrast, the prolonged JNK activation promotes degradation of c-FLIP_L through activation of a ubiquitin ligase, ITCH. The delicate balance of these pathways could determine cell survival or cell death, including apoptosis and necrosis. The black and red lines indicate the positive and negative signals, respectively.

of the caspase cascade and the JNK pathway. However, once the expression levels of c-FLIP_L are downregulated due to various pathological conditions, JNK activation is gradually induced. This prolonged JNK activation further promotes degradation of c-FLIP_L in an ITCH-dependent manner (Chang *et al*, 2006). This vicious cycle along with downregulation of antioxidants and other antiapoptotic proteins ultimately induces caspase activation and/or ROS accumulation, resulting in cell death through apoptosis or necrosis (Figure 8B).

Given that c-FLIP_L does not appear to have an antioxidant function, but directly binds to MKK7 and inhibits JNK activation (Figures 4 and 5), c-FLIP_L might inhibit ROS accumulation through suppression of the JNK pathway. Consistently, a previous study has shown that TNFα-induced ROS accumulation is abolished in *Jnk*^{-/-} MEFs (Ventura *et al*, 2004). Taken that ROS promote JNK activation, activation of the JNK pathway may induce ROS accumulation, which in turn amplifies JNK activation. c-FLIP_L might block this posi-

tive feedback loop, thereby suppressing ROS accumulation in NF- κ B activation-deficient cells. To address the possibility, it is essential to generate *c-Flip*^{-/-} *Jnk*^{-/-} mice. However, we cannot formally exclude the possibility that c-FLIP_L directly suppresses ROS accumulation in an undetermined mechanism. In addition to this mechanism, c-FLIP also suppresses caspase-dependent ROS accumulation (Figure 2C). The caspase-dependent ROS accumulation was observed in *c-Flip*^{-/-} MEFs and c-FLIP-knockdown tumor cell lines, such as HeLa, A549, and HCT116 cells, but not NF- κ B activation-deficient cells (Figure 2C, Supplementary Figure S2A, and data not shown). One possible explanation is that under these conditions, in which c-FLIP is lost before stimulation, caspase activation is rapidly induced, resulting in caspase-dependent ROS accumulation along with caspase-independent ROS accumulation (Figure 2A and C). In contrast, in NF- κ B activation-deficient cells, expression levels of c-FLIP_L decrease upon TNF α stimulation (Figure 1). Under these conditions, caspase activation is gradually and weakly induced (Supplementary Figure S2C), and this gradually and weakly increased caspase activation is not sufficient for promoting ROS accumulation. Therefore, synergistic interplay between the JNK pathway and ROS accumulation may play a dominant role in ROS accumulation (Ventura *et al*, 2004). Taken that BHA, but not z-VAD-fmk, almost completely inhibited TNF α -induced ROS accumulation in NF- κ B activation-deficient cells (Sakon *et al*, 2003) (Supplementary Figure S2A), BHA may specifically inhibit caspase-independent ROS accumulation. Further study will be required to elucidate the interplay between caspase-dependent and -independent ROS accumulation.

Upregulation of c-FLIP_L has been shown to be correlated with resistance to Fas-induced apoptosis *in vitro* in certain tumor cell lines and also associated with autoimmune diseases (Thome and Tschopp, 2001). One of the strategies to treat these pathological conditions is to block the c-FLIP_L-mediated suppression of caspase activation, thereby promoting apoptosis of tumor cells and autoreactive T cells. In addition, blocking the suppressive function of c-FLIP_L on JNK activation might be an alternative strategy to treat

these diseases, resulting in prolonged JNK activation-induced cell death. In this respect, the interaction of c-FLIP_L with MKK7 might be an attractive target to develop new drugs to treat various diseases such as cancers and autoimmune diseases.

Materials and methods

Plasmids

pcDNA3-HA-MEK1, pcDNA3-HA-MEK1-KM, and pcDNA3-HA-MKK6 were described previously (Takekawa *et al*, 2005). pCR-HA-MKK4, pCR-HA-MKK7, and pCR-HA-JNK2 were generated by cloning PCR-amplified products from pCR-MKK4, pCR-MKK7, and pET-SAPK α (JNK2) (provided by E Nishida) as templates into pCR-HA vector, respectively. pCR-HA-MKK7-KM (K165M) was generated by using an *in vitro* mutagenesis kit (Stratagene). pCR-HA-p38 and pCR-HA-ERK2 were generated by cloning RT-PCR products of p38 and ERK2 into pCR-HA vector, respectively. pcDNA3-Myc-TAK1, pcDNA3-Myc-TAB1, pcDNA3-Myc-ASK1, and pcDNA3-Myc-Tlp-2 were provided by K Matsumoto, H Ichijo, and S Ley. An expression vector for a constitutively active MEKK1 (pcDNA3-Myc-MEKK1 Δ N) was constructed by deleting N-terminal regulatory region of MEKK1 (1–1119) by PCR from pSRHis-MEKK1 (provided by S Ohno). pCR-Flag-c-FLIP_L and pCR-Flag-c-FLIPs were generated by cloning PCR-amplified products from pcDNA3-CASH α and pcDNA3-CASH β (provided by D Wallach) into pCR-Flag vector, respectively. pCR-Flag-c-FLIP Δ N was constructed by PCR to delete the N-terminal DEDs. A series of deletion mutants of c-FLIP_L was generated by PCR to delete the indicated amino acids from the C-terminus.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank D Wallach, E Nishida, H Ichijo, K Matsumoto, S Ohno, S Ley, Y Gotoh, H Nishina, T Kitamura, M Kracht, P Holland, GP Nolan, and T Doi for providing reagents, and *RelA*^{-/-} MEFs. This work was supported in part by Grants-in-Aid for 21st Century COE Research and Scientific Research (B) from Japan Society for the Promotion of Science, Japan, Takeda Science Foundation, and by a Grant from Human Frontier Science Program (HFSF). AN is a Research Fellow of the Japan Society for the Promotion of Science.

Competing financial interests

The authors declare that they have no competing financial interests.

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