

Caspase-8- and JNK-dependent AP-1 activation is required for Fas ligand-induced IL-8 production

メタデータ	言語: eng 出版者: 公開日: 2017-10-05 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	https://doi.org/10.24517/00027363

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Caspase-8 and JNK-dependent AP-1 activation is required for Fas ligand-induced IL-8 production

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Running title: Mechanisms of Fas Ligand-Induced AP-1 activation

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Abbreviations: AP-1, activator protein-1; ASK1, activate apoptosis signaling kinase 1; DD, death domain; DED, death effector domain; DMSO, dimethyl sulfoxide; DN, dominant-negative mutant; ERK, extracellular signal-regulated kinase; FasL, Fas ligand; FADD, Fas-associated DD protein; fmk, fluoromethylketone; I κ B α -SR, inhibitor of κ B α super repressor mutant; IL-8, interleukin 8; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK1, MEK kinase 1; NF- κ B, nuclear factor- κ B; PMA, phorbol 12-myristate 13-acetate; RLU, relative luciferase unit; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-induced ligand; TRE, O-tetradecanoylphorbol 13-acetate-responsive element

Keywords; Fas ligand, AP-1, MAP kinase, caspase-8, IL-8 Subdivision: Immunology

Summary

Despite a dogma that apoptosis does not induce inflammation, Fas ligand (FasL), a well-known death factor, possesses pro-inflammatory activity. For example, FasL induces nuclear factor κ B (NF- κ B) activity and interleukin 8 (IL-8)-production by engagement of Fas in human cells. Here, we found that a dominant negative mutant of c-Jun, a component of the activator protein-1 (AP-1) transcription factor, inhibits FasL-induced AP-1 activity and IL-8 production in HEK293 cells. Selective inhibition of AP-1 did not affect NF- κ B activation and vice-versa, indicating that their activations were not sequential events. The FasL-induced AP-1 activation could be inhibited by deleting or introducing the *lymphoproliferation (lpr)*-type point mutation into the Fas death domain (DD), knocking down the Fas-associated DD protein (FADD), abrogating caspase-8 expression with small interfering RNAs (siRNAs), or using inhibitors for pan-caspase and caspase-8 but not caspase-1 or caspase-3. Furthermore, wild-type, but not a catalytically inactive mutant, of caspase-8 reconstituted the FasL-induced AP-1 activation in caspase-8-deficient cells. Fas ligand induced the phosphorylation of two of the three major mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) but not p38 MAPK. Unexpectedly, an inhibitor for JNK but not for MAPK/ERK kinase inhibited the FasL-induced AP-1 activation and IL-8 production. These results demonstrate that FasL-induced AP-1 activation is required for optimal IL-8 production, and this process is mediated by FADD, caspase-8, and JNK.

Introduction

FasL, a member of the tumor necrosis factor family, induces apoptosis in a variety of cells that express Fas. The signal transduction pathway of FasL-induced apoptosis has been extensively studied and is well understood: Upon being bound with FasL, Fas recruits several cytoplasmic molecules including an adaptor protein FADD (through intracellular DD of Fas), and upstream caspases such as caspase-8 and -10 (through death effector domain (DED) of FADD), forming death-inducing signaling complex [1-4]. Depending on the cell type, the upstream caspases activated in this complex in turn initiate the activation cascade of caspases or proteolytically activate Bid, a member of the proapoptotic Bcl-2 family [5,6].

The apoptotic function of FasL plays important roles in immune function and regulation, such as cytotoxic T lymphocyte- and natural killer cell-mediated cytotoxicity, and prevention of autoimmune lymphoproliferative disease. On the other hand, recent reports indicated that FasL also possesses non-apoptotic functions, such as the induction of cell proliferation and gene expression [7-11]. The ERK and p38 MAPKs as well as NF- κ B are reported to be involved in the FasL-induced gene expression. FasL also induces the activation of JNK [12-14]; however, it has been believed that the JNK activation is involved in apoptosis rather than gene expression.

One of the prominent activities of FasL is to induce inflammation *in vivo* [15-18], and this inflammatory activity of FasL seems to play deleterious roles in inflammatory diseases [19-21]. Consistent with its inflammatory activity, FasL induces various pro-inflammatory cytokines *in vivo* and *in vitro* [22], by converting inactive precursors of cytokines such as pro-IL-1 β and pro-IL-18 into their active forms [18,23], or by enhancing the expression of cytokine genes [10,24,25].

It has been reported that some normal and transformed cell lines produce IL-8, a chemokine for neutrophils, upon Fas ligation by an anti-Fas monoclonal antibody (mAb) or FasL [26-32]. To clarify how FasL induces cytokine gene expression, we have investigated the molecular mechanism of the FasL-induced IL-8 production in the untransformed human embryonic kidney cell line, HEK293. Conveniently using this cell line, we can exclude side effect of cell death, because this cell line does not show any detectable apoptosis after FasL treatment. Using this system, we recently discovered that caspase-8-mediated cell-autonomous NF- κ B activation is crucial for this response [10]. In addition, we found that FasL induces AP-1 activity, and that the AP-1 site in the minimal essential promoter of the *IL8* gene is required for the maximum FasL-induced expression of a luciferase gene under the control of this promoter. However, it remained to be answered whether AP-1 activation is required for the actual IL-8 production in response to FasL stimulation and how FasL induces AP-1 activity. In this study, we sought to elucidate these points and found that AP-1 activation is a crucial event for FasL-induced IL-8 production, and that the FasL-induced AP-1 activation depends on JNK activation, rather than on ERK or p38.

Results

FasL induces IL-8 production through AP-1 activation

Consistent with our previous report [10], we found that FasL stimulation induced AP-1 transcriptional activity in HEK293 cells without any evidence of apoptosis, using luciferase reporter constructs under the control of two tandem sites of a classical AP-1 binding sequence, 12-O-tetradecanoylphorbol 13-acetate-responsive element (TRE), (Fig. 1A). The over-expression of Fas also induced AP-1 activity, and FasL stimulation further enhanced this activity, confirming that the FasL-induced AP-1 activation was mediated by Fas.

The c-Jun and c-Fos proto-oncoproteins are the major components of the AP-1 complex [33,34]. The truncated form of c-Jun, consisting of its C-terminal region (amino acids 123-334), works as a specific inhibitor for c-Jun and c-Fos [35]. We therefore used this dominant-negative mutant (DN) to investigate the role of AP-1 in FasL-induced IL-8 production. As expected, the transient expression of c-Jun-DN inhibited the FasL-induced as well as the 4 β -phorbol 12-myristate 13-acetate (PMA)-induced AP-1 activation (Fig. 1B). Importantly, c-Jun-DN also inhibited the FasL- and PMA-induced activation of the minimal essential promoter of the IL-8 gene (Fig. 1C). Moreover, the IL-8 production induced by FasL stimulation was reduced by the expression of c-Jun-DN as effectively as by the inhibitor of NF- κ B α super repressor mutant (I κ B α -SR), which blocks NF- κ B activation (Fig. 1D). After transient transfection, the cell numbers of all experimental groups were comparable (data not shown). These results indicate that AP-1 activation is important for the actual cytokine production elicited by FasL stimulation.

Activations of AP-1 and NF- κ B induced by FasL occur independently

Because a cross-talk could take place between signal transduction pathways leading to AP-1 and NF- κ B activation [36-39], we investigated whether inhibiting one pathway affected the activity of the other. Although c-Jun-DN blocked the AP-1 activation induced by FasL (Fig. 1B), it did not affect the NF- κ B activation elicited by FasL (Fig. 2A), suggesting that AP-1 activation is not required for FasL-induced NF- κ B activation. Conversely, the transient expression of I κ B α -SR, which dramatically reduced the FasL-induced NF- κ B activation (Fig. 2B and [10]), caused little or no reduction of the FasL-induced AP-1 activation, indicating that the NF- κ B activation was not required for the FasL-induced AP-1 activation. Thus, the activations of AP-1 and NF- κ B by FasL are independent of each other.

The Fas-DD, FADD, and Caspase-8 are essential for FasL-induced AP-1 activation.

To clarify which cytoplasmic region of the Fas receptor was responsible for the FasL-induced AP-1 activation, we expressed various Fas mutants in HEK293 cells (Fig. 3A). As we described previously, comparable expression levels of wild-type Fas and its mutants in the HEK293 transfectants was confirmed by fluorescent antibody-staining of the cell-surface Fas followed by flow cytometry ([10] and data not shown). Consistent with Fig. 1A, overexpression of wild-type Fas induced AP-1 activity, and FasL stimulation enhanced it. Deletion of the C-terminal 15 amino acids of Fas up-regulates Fas's ability to induce apoptosis [40], but it did not affect its capacity to induce AP-1 activation. On the other hand, further deletion of Fas up to part of the DD (FD7 and FD2) or the *lpr* complementing generalized lymphoproliferative disease (*lpr*^{cg})-type point mutation (Val238 to Asn) in the DD (FP1), which abolishes Fas's apoptosis-inducing capacity [40], also abrogated its ability to activate AP-1. Furthermore, exogenous expression of the FD7, FD2, or FP1 mutant inhibited the FasL-induced AP-1 activation. These results indicate that the C-terminal 15 amino acids of Fas are dispensable, but the DD of Fas is indispensable for its ability to activate AP-1.

We then addressed the requirement for FADD and caspase-8, which are essential to the induction of apoptosis upon Fas ligation [41,42], in FasL-induced AP-1 activation. We sought to reduce the endogenous expression of FADD or caspase-8 in HEK293 cells using siRNAs. As shown in Fig. 3B, the FADD- or caspase-8-targeting siRNA effectively suppressed the endogenous expression of these proteins in HEK293 cells. The siRNA for FADD or caspase-8, but not a control siRNA, inhibited the FasL-induced AP-1 activation (Fig. 3C). In contrast, none of these siRNAs inhibited PMA-induced AP-1 activation. These results indicate that FADD and caspase-8 are essential for FasL-induced AP-1 activation.

FasL-induced AP-1 activation requires the catalytic activity of caspase-8.

We next investigated whether the catalytic activity of caspase-8 is required for FasL-induced AP-1 activation. The caspase-8 activation in HEK293 cells was detected by FasL stimulation or Fas over-expression and this activity was comparable with AP-1 activity in Fig. 1A (data not shown). The pan-caspase inhibitor Z-VAD-fluoromethylketone (fmk) or a caspase-8-specific inhibitor, Z-IETD-fmk, inhibited the FasL-induced AP-1 activation, whereas Z-DEVD-fmk, Z-YVAD-fmk, or Z-AAD-fmk (inhibitors for caspase-3, caspase-1, and granzyme B, respectively) showed no effect (Fig. 4A). In contrast, none of the caspase inhibitors had a significant effect on the PMA-induced AP-1 activation (data not shown).

To confirm the requirement for the catalytic activity of caspase-8 in FasL-induced AP-1 activation, we next used a subline of the HEK293 cell line, 293-K, which expresses caspase-8 at a level at least 10 times lower than that of HEK293 cells based on Western blot analyses (Fig. 4B and [43]). Strikingly, the 293-K cells did not show AP-1 activation upon FasL stimulation even when exogenous human Fas was introduced by transient transfection (Fig. 4C). When the wild-type caspase-8, but not a catalytically inactive mutant or the DEDs, was exogenously expressed in 293-K cells, the cells became responsive to FasL stimulation, in terms of AP-1 activation (Fig. 4D). Consistent with this, the reconstituted FasL-induced AP-1 activation was abrogated by pretreatment with Z-VAD-fmk. These results indicate that the catalytic activity of caspase-8 is required for FasL-induced AP-1 activation.

The JNK signaling pathway is required for FasL-induced AP-1 activation and IL-8 production.

FasL activates three major MAPK pathways under certain conditions [44,45]. Western blot analyses using a pair of phosphorylated form-specific and pan-specific antibodies against each of the three major types of MAPKs, ERK1/2, JNK1/2, and p38, showed that ERK and JNK, but not p38, were activated upon FasL stimulation in HEK293 cells (Fig. 5A). To determine the contribution of MAPK activation to the FasL-induced AP-1 activation, we examined the effect of MAPK and MAPK kinase inhibitors. Although strong activation of ERK1/2 was observed upon FasL stimulation, the MAPK/ERK kinase (MEK) 1/2 inhibitor PD98059, which inhibited the FasL-induced phosphorylation of ERK (data not shown), had no effect on the FasL-induced AP-1 activation. However, treatment with the JNK inhibitor SP600125 abrogated the FasL-induced AP-1 activation (Fig. 5B). On the other hand, the PMA-induced AP-1 activation was inhibited by PD98059 but not by SP600125. Two different p38 inhibitors (SB202190 and SB203580) showed no inhibitory effect on either the FasL- or PMA-induced AP-1 activation. These results suggest that different stimulators use distinct MAPK pathways to activate AP-1, and that JNK but not ERK or p38 contributes to the FasL-induced AP-1 activation. Consistent with this, among the MAPK or MEK inhibitors used here, only SP600125 inhibited the FasL-induced IL-8 production (Fig. 5C), suggesting that JNK activation was required for the FasL-induced IL-8 production. Since catalytic activity of caspase-8 is required for FasL-induced AP-1 activation (Fig. 4), we next examined the effect of caspase inhibitors for FasL-induced JNK activation. As shown in Fig. 5D, FasL-induced JNK activation was abrogated by pretreatment of cells with pan-caspase inhibitor (Z-VAD-fmk) and caspase-8 inhibitor (Z-IETD-fmk) but not by caspase-1 inhibitor (Z-YVAD-fmk), suggesting that the catalytic activity of caspase-8 was important for FasL-induced JNK activation.

Discussion

In this study, we demonstrated that AP-1 activation is required for optimal IL-8 production upon FasL stimulation in HEK293 cells, and that JNK activation is required for the FasL-induced AP-1 activation and IL-8 production. Although FasL also induces the activation of another major transcription factor NF- κ B [9,10,30,31], and both the NF- κ B and AP-1 activation induced by FasL require FADD and caspase-8 ([10] and this study), these responses occur independently. Consistent with this, it has been reported that tumor necrosis factor-related apoptosis-induced ligand (TRAIL) receptors signal the activation of NF- κ B and JNK through distinct pathways [46], although whether or not caspase-8 is required for TRAIL-induced JNK activation depends on the cell type.

Fas signaling has been reported to activate JNK [12,13,47]. Most of these reports focus on the link between Fas-mediated JNK activation and apoptosis, although the role of JNK activation in Fas-triggered apoptosis remains controversial. It has also been reported that Fas engagement induces gene expression through ERK or p38 activation [11,31]. However, our data presented here showed that the JNK activation, but not the ERK activation, induced by FasL is important for inflammatory chemokine production in HEK293 cells, pointing to a previously undescribed role of JNK activity downstream of Fas. Recently, several reports have shown that FasL possesses inflammatory activity [15-18]. We and other groups reported that FasL induces the expression of many inflammatory cytokine genes, which is thought to be one of the molecular mechanisms of FasL-induced inflammation [22,24,25]. It has also been suggested that FasL is very inefficient in inducing apoptosis and instead activates non-apoptotic responses in certain tumor cells [48,49]. For example, several recent studies revealed that FasL induces the expression of a number of potential survival genes and genes that are known to regulate increased motility and invasiveness in tumor cells [11]. Therefore the FasL-induced gene expression may play an important role in FasL's non-apoptotic response, and further characterization of the JNK function linked to gene expression downstream of Fas will help us understand the role of the Fas-FasL system in inflammation and/or tumorigenesis.

It is not yet known which components of the Fas downstream signaling cascade lead to the activation of JNK. The intracytoplasmic DD of Fas recruits several adaptor molecules to activate downstream signal transducers. One of these, DAXX was reported to activate apoptosis signaling kinase 1 (ASK1) and subsequently JNK kinase and JNK [50,51]. However, recent reports suggested that DAXX plays no physiological role in FasL-induced JNK signaling [52,53]. The requirement for FADD and caspase activity in FasL-induced JNK activation has also been controversial. Although it was reported that dominant negative form of FADD does not block JNK activation by Fas stimulation in HeLa cells [54], cross-linked Fas was unable to activate JNK and p38 in FADD-deficient Jurkat cell lines [55]. In terms of caspase activity, Fas-mediated JNK activation was reported to be sensitive to caspase inhibitors in Jurkat and SKW6.4 (B lymphoblast) cells [12,56], but not in a neuroblastoma cell line [13]. Importantly, our data in this study clearly demonstrate that FADD, caspase-8, and the catalytic activity of caspase-8 are critical for FasL-induced AP-1 activation at least in HEK293 cells. It has been demonstrated that caspase activated by Fas engagement cleaves MEK kinase 1 (MEKK1), an upstream regulator of JNK, and that a caspase inhibitor attenuates Fas-mediated JNK activation [57]. However, there is currently no direct evidence that Fas induces JNK activation through MEKK1 cleavage. Moreover, the proteolysis of MEKK1 is reported to be dependent on caspase-3 activation [57]. Thus, unknown caspase-8 substrate(s) that can activate the AP-1 signaling pathway downstream of Fas may exist in HEK293. We are currently searching for such candidates.

Materials and methods

Reagents

Recombinant soluble mouse FasL (previously termed WX1) [58] was prepared and purified as described previously [19]. Z-VAD-fmk, Z-IETD-fmk, Z-YVAD-fmk, Z-AAD-fmk, and the MAPK inhibitor set (PD98059, SB202190, SB203580, and SB202474) were purchased from Calbiochem (La Jolla, CA). JNK inhibitor SP600125 was purchased from Alexis Biochemicals (San Diego, CA). PMA was purchased from Sigma (St. Louis, MO). To stimulate cells, FasL and PMA were used at 1000 units·ml⁻¹ and 500 pg·ml⁻¹, respectively, unless otherwise described.

Plasmids

To generate pEF-caspase-8DED, a cDNA encoding caspase-8 was partially digested by *EcoRI*, and a cDNA fragment consisting of the DEDs was cloned into pEF-BOS. To generate a plasmid expressing c-Jun-DN (pEF-FLAG-c-Jun-DN), a PCR-amplified cDNA encoding the C-terminal region of mouse c-Jun (amino acids 123-334) was cloned into pCMV-Tag2B vector (Stratagene, La Jolla, CA) and then the FLAG-tagged cDNA was subcloned into pEF-BOS. Other plasmids used in this study were described previously [10,43].

Cell Lines

HEK293 and 293T cell lines and the subline of HEK293, 293-K that expresses caspase-8 at a very low level were described previously [10,43].

Reporter Assays

Cells (5×10^4) were transfected with one of the firefly luciferase reporter plasmids (2xTRE-Luc and -133-Luc, 100 ng; NF- κ B-Luc, 50 ng) and pRL-TK (10 or 20 ng as an internal control) using the Lipofectamine PLUS or Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), or linear polyethyleneimine (molecular weight 25, 000, Polysciences Inc, Warrington, PA). In some experiments, the cells were cotransfected with one of the tester plasmid described above. The total amount of transfected DNA per culture was kept constant within an experiment using empty vector. Cells were harvested about 24 h after transfection, and the luciferase activity was determined as described previously [10]. Firefly luciferase activity was normalized to the Renilla luciferase activity. To calculate relative luciferase units (RLU), the normalized firefly luciferase activity of an experimental group was divided by the normalized firefly luciferase activity of a control group in which the cells were transfected with luciferase constructs and control vector only and cultured without a stimulus.

Measurement of IL-8

The amount of IL-8 in the culture supernatant was determined using an ELISA kit (PharMingen, San Diego, CA).

Western blotting

Western Blotting was carried out as previously described [59] except that phospho-MAPK family and MAPK family antibody sampler kits (Cell Signaling, Beverly, MA) were used in this study. To detect phosphorylated forms of MAPKs, whole cell lysates were prepared using ice-cold lysis buffer (50 mM HEPES-OH, pH7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1% NP-40, 0.5% deoxycholate, 20 mM NaF, 1 mM EDTA, 20 mM β -glycerophosphate, 0.5 mM

dithiothreitol, 0.1mM Na₃VO₄, 1 mM p-amidinophenyl methanesulfonyl fluoride, 10 µg·ml⁻¹ leupeptin, 1 µg·ml⁻¹ pepstatin).

SiRNAs

The siRNAs used in this study were described previously [10]. Cells were transfected with double-stranded siRNAs with or without various plasmids using the Lipofectamine 2000 reagent (Invitrogen). In some experiments, cells were simultaneously transfected with reporter plasmids. Cells were harvested 48 h after siRNA transfection, and subjected to a luciferase assay or to western blotting as described above.

Acknowledgements

We thank Prof. K. Yoshioka for stimulating discussions and valuable contributions, and Ms H. Kushiyama for secretarial and technical assistance.

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Figure legends

Fig. 1. FasL induces IL-8 production through AP-1 activation. (A) HEK293 cells were transiently transfected with 2xTRE-Luc, pRL-TK and 200 ng of an expression plasmid for human Fas or a control vector, and cultured for 17 h. The cells were then cultured with or without FasL for 7 h. The AP-1 activity was expressed by RLU as described in Material and methods. (B and C) HEK293 cells were transiently transfected with 2xTRE-Luc (B) or IL-8 promoter-Luc (-133-luc) (C), pRL-TK and 50 ng of an expression plasmid for c-Jun-DN or a control vector. The transfectants were treated with or without FasL or PMA as indicated, and AP-1 (B) and IL-8 promoter (C) activities were expressed by RLU. (D) HEK293 cells were transiently transfected with 200 ng of an expression plasmid for c-Jun-DN, for I κ B α -SR, or a control vector. The transfectants were treated with FasL as described in (A). The amount of IL-8 in the culture supernatant was determined by ELISA.

Fig. 2. Activation of AP-1 and NF- κ B induced by FasL occur independently. (A) HEK293 cells were transiently transfected with pNF- κ B-luc, pRL-TK and 50 ng of an expression plasmid for c-Jun-DN or a control vector. Transfectants were stimulated with FasL, or left unstimulated during the last 7 h of the 24 h culture. The NF- κ B activity was expressed by RLU. (B) HEK293 cells were transiently transfected with pNF- κ B-luc or 2xTRE-Luc, pRL-TK, and 25 ng of an expression plasmid for I κ B α -SR or a control vector, and cultured for 16h. The cells were then stimulated with FasL for 7 h. AP-1 and NF- κ B activities were expressed by RLU.

Fig. 3. The Fas-DD, FADD, and caspase-8 are critical for the FasL-induced AP-1 activation. (A) HEK293 cells were transfected with 2xTRE-Luc, pRL-TK, and 100 ng of an expression plasmid for human Fas or its deletion or point mutants as shown in the schema, and cultured for 17 h. The cells were then stimulated with FasL for 7 h. The AP-1 activity was expressed by RLU. FP1 has a point mutation (V238N) corresponding to the *lpr^{es}* mutation of mouse Fas. (B) HEK293 cells were transiently transfected with 20 or 50 nM FADD-targeting, or 20 nM caspase-8-targeting siRNA. Whole-cell extracts were prepared 48 h after transfection, and the endogenous protein levels of FADD, caspase-8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were monitored by Western blotting. (C) HEK293 cells were transiently transfected with 50 nM FADD-, or caspase-8-targeting siRNA, or the reverse sequence of FADD-targeting siRNA (DDAF, as a negative control), and 2xTRE-Luc and pRL-TK, and cultured for 36 h. The cells were then stimulated with FasL or PMA for 12 h. The AP-1 activity was expressed by RLU.

Fig. 4. Catalytic activity of caspase-8 is essential for FasL-induced AP-1 activation. (A) HEK293 cells were transiently transfected with 2xTRE-Luc and pRL-TK, and cultured for 16 h. The cells were then pretreated with the indicated inhibitors (20 μ M) or DMSO (0.1 %) for 1 h, and further stimulated with FasL for 7 h. The AP-1 activity was expressed by RLU. Z-VAD, pan-caspase inhibitor; Z-IETD, caspase-8 inhibitor; Z-DEVD, caspase-3 inhibitor; Z-YVAD, caspase-1 inhibitor; Z-AAD, granzyme B inhibitor. (B) Whole-cell extracts prepared from HEK293 and 293-K cells were subjected to Western blotting using an anti-caspase-8 mAb or an anti-GAPDH mAb that was used to ensure equal protein loading. (C) HEK293 or 293-K cells were transiently transfected with 2xTRE-Luc, pRL-TK, and an expression plasmid for human Fas (100 ng), and cultured for 16 h. The cells were then cultured with or without FasL for 9 h. The AP-1 activity was expressed by RLU. (D) 293-K cells were transiently transfected with 2xTRE-Luc, pRL-TK, expression plasmids for wild-type (wt-casp-8, 1 ng), C/S mutant

(mut-casp-8, 1 ng), or DEDs (casp-8-DED, 0.1 ng) of caspase-8B, or an empty vector (1 ng), and an expression plasmid for human Fas (100 ng), and cultured for 6 h. Cells were then pretreated with Z-VAD-fmk (20 μ M) or DMSO for 16 h, and further stimulated with FasL for 9 h. The AP-1 activity was expressed by RLU.

Fig. 5. JNK activation is required for the FasL-induced AP-1 activation and IL-8 production. (A) HEK293 cells were stimulated with FasL (2000 units·ml⁻¹), or PMA (50 ng·ml⁻¹) for the indicated periods. The whole-cell lysates were assayed by Western blotting using antibodies against phosphorylated or entire JNK1/2, ERK1/2, or p38. The whole cell lysates after UV treatment (500 J·ml⁻²) were used as a positive control for phosphorylated JNK1/2 and p38. (B) HEK293 cells were transiently transfected with 2xTRE-Luc and pRL-TK, and cultured for 15 h. The cells were then pretreated with the indicated inhibitors (10 μ M) or DMSO (0.05 %) for 1 h, and further stimulated with FasL or PMA for 12 h. The AP-1 activity was expressed by RLU. (C) HEK293 cells were pretreated with the indicated inhibitors (10 μ M) or DMSO (0.05 %) for 1 h, and then stimulated with FasL for 12h. The amount of IL-8 in the culture supernatant was determined by ELISA. SP600125, JNK inhibitor; PD98059, MEK inhibitor; SB202190 and SB203580, p38 inhibitor; SB202474, control substance. (D) HEK293 cells were pretreated with the indicated inhibitors (20 μ M) or DMSO (0.1 %) for 1 h and further stimulated with FasL for 6 h. The whole-cell lysates were assayed by Western blotting using antibodies against phosphorylated or entire JNK1/2.

Fig.1

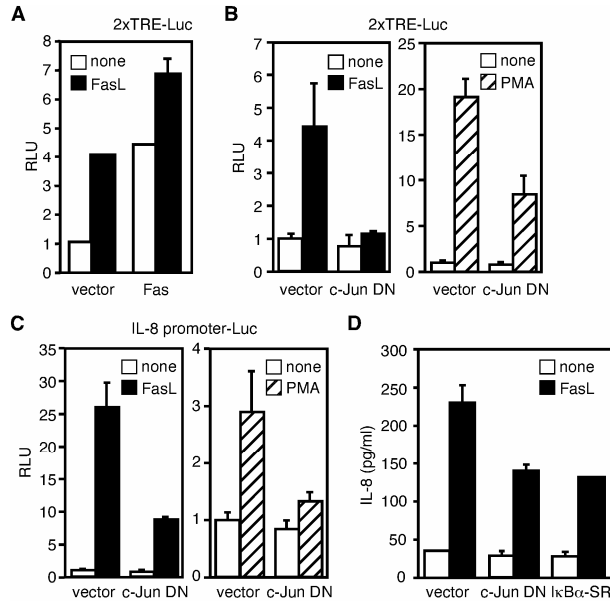


Fig.2

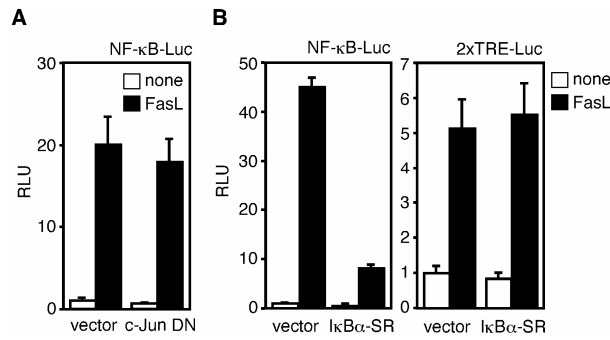


Fig.3

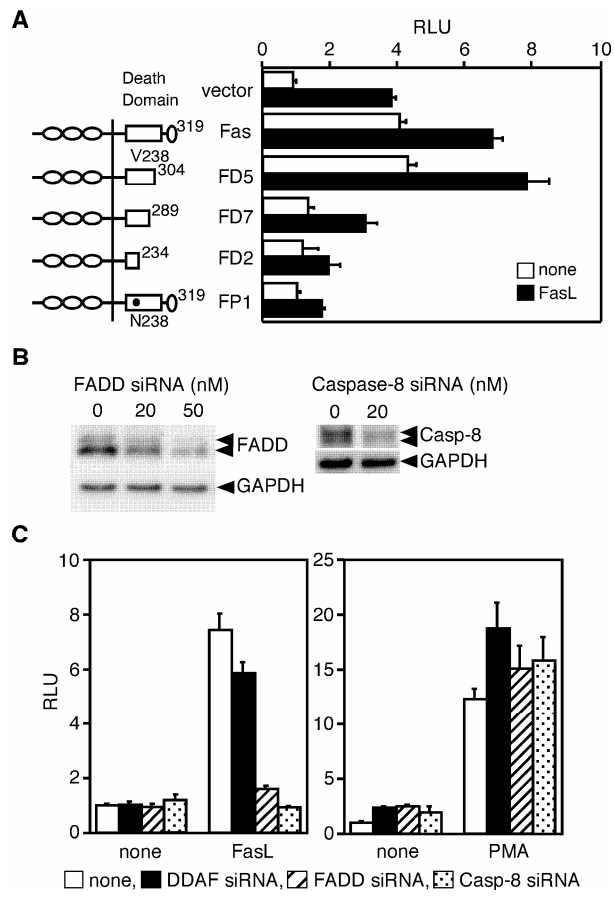


Fig.4

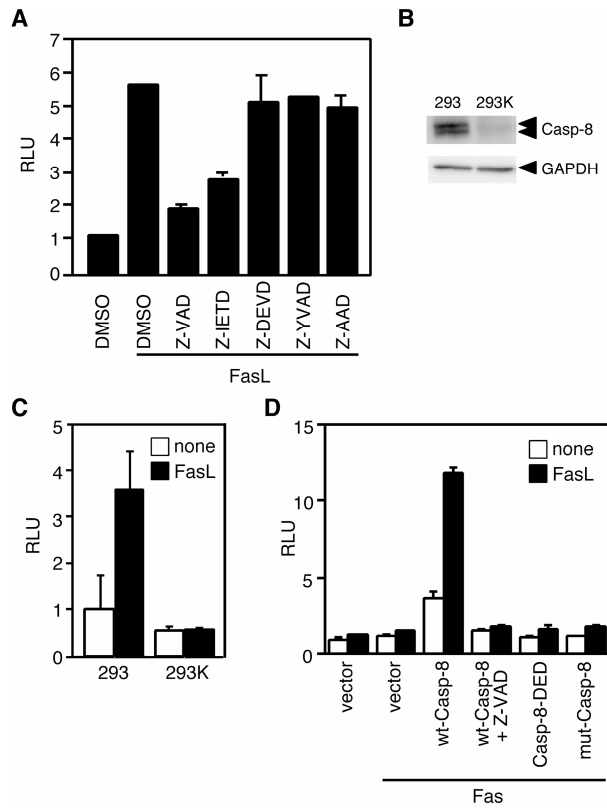


Fig. 5

