Regulation of FasL by NF-κB and AP-1 in Fas-dependent Thymineless Death of Human Colon Carcinoma Cells*

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Cell death due to thymine (dTth) deficiency, associated with the cytotoxic action of 5-fluorouracil in colon cancer, is regulated in thymidylate synthase-deficient (TS−) human colon carcinoma cells via the Fas (CD95, APO-1) death receptor. This was demonstrated by inhibiting the loss in clonogenicity of TS− cells by anti-FasL and in enhanced survival of TS− clones selected for resistance to Fas-mediated apoptosis, following dTth deprivation. During thymineless stress in TS− cells, Fas ligand (FasL) is expressed, and its promoter (hFasLP(r) is activated. Transactivation of hFasLP(r), dependent upon dTth deficiency, was inhibited following mutation of the binding sites for NF-κB or AP-1 and by preventing NF-κB or AP-1 activation, which inhibited expression of FasL and enhanced clonogenic survival in stable transformants expressing IκBαM or DN-MEKK, respectively. These results demonstrate the crucial roles for NF-κB and AP-1 in the regulation of FasL in Fas-mediated thymineless death of colon carcinoma cells.

Cell death due to dTth deficiency (thymineless death) is the mechanism of cell killing associated with 5-fluorouracil (FUra) combined with leucovorin (LV) in colon cancer and remains the most effective therapy for this disease. We have selected a human colon carcinoma cell line deficient in thymidylate synthase (TS) that allows events downstream of dTTP depletion and DNA damage to be addressed in an unambiguous manner. In our previous studies we found that inhibition of FasL binding prevented apoptosis and preserved clonogenicity in these cells when deprived of dTth, and we demonstrated the critical role of the Fas (CD95, APO-1) death receptor in the induction of cell death induced by thymineless stress (1). In TS− cells apoptosis is induced concomitantly with the expression of FasL. Similarly, in Jurkat T leukemia cells, apoptosis induced by certain chemotherapeutic drugs depends upon the expression of FasL (2, 3), analogous to activation-induced apoptosis in T lymphocytes (4), which requires FASL transcription and expression. However, signals involved in DNA damage recognition and the ultimate signaling pathway(s) leading to activation of FasL and induction of apoptosis in TS− cells via the Fas death receptor remain to be elucidated.

The response of cells to cytotoxic stress and DNA damage may depend upon the cell type, together with the type and extent of DNA damage. In addition to the activation of p53, others have reported in a variety of different systems that activation of other transcription factors including NF-κB (3–9) and AP-1 (3, 10) is involved in the induction of apoptosis following drug-induced DNA damage. κB elements are found in promoter regions of genes that are crucial for acute or immune phase responses, including FASL (3). AP-1 is a sequence-specific transcriptional activator composed of members of the Jun and Fos families (reviewed in Ref. 11) and is activated via the JNK/SAPK signaling pathway. An AP-1 binding domain has also been identified in the promoter region of FASL (3). Kasibhatla et al. (3) demonstrated in Jurkat T cells that activation of NF-κB and AP-1 and their transactivation of FASL regulated VP-16- and VM-26-induced apoptosis via the expression of FasL. In addition, others (12) have demonstrated cross-coupling between NF-κB and AP-1 to produce potentiated biological function. In this study we determined that NF-κB and AP-1 are involved downstream of DNA damage in TS− cells in the transcriptional regulation of FASL in Fas-mediated thymineless death of colon carcinoma cells. Furthermore, we demonstrated that both of these transcription factors were activated following treatment of colon carcinoma cells with FUra/LV, which correlated with up-regulated expression of Fasl in these cells.

**EXPERIMENTAL PROCEDURES**

**Cell Line—A TS− mutant clone selected from parental GC/c1 human colon carcinoma cells, which is deficient in TS mRNA and protein and auxotrophic for dTth, has been well characterized (1, 13–15).** The relationship between up-regulated expression of FasL and induction of thymineless death has been described (1). Furthermore, up-regulated expression of FasL in GC/c1 cells in response to FUra/LV (1) and the induction of thymineless death and apoptosis (13) have also been reported.

**Selection of CH-11-resistant Clones—TS− cells were selected for resistance to Fas-mediated apoptosis by treatment with the cytolytic anti-Fas mAb CH-11 (MBL International Corp.). Cells (100 × 10⁶) were mutagenized with EMS (300 μg/ml) in T-175 flasks for 22 h. Cells were cultured for a further 48 h in the absence of EMS and subsequently selected for 72 h in 200 ng/ml CH-11. Following growth for a further 7
days in the absence of CH-11, surviving cells were again mutagenized with EMS and selected in CH-11 (200 ng/ml) for a second time. Surviving colonies were allowed to grow for 2 weeks and were subsequently ring-cloned, placed in T-12.5 flasks in 2 ml of dFBS-containing medium with dThd (20 μM), and the colonies were expanded. Three colonies were selected for further evaluation.

**Clonalogenic Survival**—TS− cells were plated at a density of 3,000 cells/well in 6-well plates (Falcon). Cells were also treated with anti-Fasl (NOK-1 mAb; Pharmingen; 100 ng/ml) or a mouse IgG1 isotype-matched control mAb (Pharmingen; 100 ng/ml) at the time of plating. Following overnight attachment, cells were washed with 2 ml of Hanks’ balanced salt solution (at 37°C) and subsequently deprived of dThd by refeeding with dThd-free medium containing the respective Abs. At various times for up to 7 days, cells were rescued with dThd (20 μM) added to individual wells, and clonogenic survival was determined 11 days after dThd restoration (1). Alternatively, TS− cells and CH-11-resistant clones were plated as described above and following overnight attachment were treated with CH-11 (200 ng/ml) for 72 h in the presence of dThd (20 μM), followed 11 days later by analysis of clonogenic survival.

**Reporter Constructs**—Production and use of reporter constructs involving the 5′ promoter region for Fasl have been reported (3). Briefly, an approximately 8-kilobase HindIII fragment containing the 5′ promoter region from a genomic clone of human FASL was subcloned into a eukaryotic expression vector HsLuc carrying a luciferase reporter gene downstream of the cloned segment to generate the wild-type reporter construct for Fasl, hFaslPr. By using PCR, truncations of the FASL promoter region were obtained and subcloned into the HsLuc vector 5′ of the luciferase reporter gene. Mutations were introduced into the binding domains of NF-κB and AP-1 within the FASL promoter region using the Gene Editor kit (Promega) or the Transformer site-directed mutagenesis kit (CLONTECH) and subsequently sequenced, as reported (3). This generated hFaslPr/NF-κB and hFaslPr/AP-1, respectively. Conditioned expression vectors for IκBαM (16) and DN-MEKK (17) were employed as previously reported (3). Wild-type NF-κB (2xκB-luc; Ref. 18) and AP-1 (TRE-luc; Ref. 19) luciferase reporter constructs were as previously reported. To control for transfection efficiency, pSV-βgal was obtained from Promega.

**Promoter Studies and Transient Transfections**—To determine the optimal time for examination of transactivation of the wild-type Fasl reporter construct, hFaslPr, following dThd withdrawal, TS− cells were plated, in triplicate, at a density of 5 × 104 cells in T-75 flasks (Costar) in foetal bovine serum-free RPMI 1640 medium containing 10% dFBS, 80 nm 5-methyltetrahydrofolate, and 20 μM dThd. After overnight attachment, the cells were washed in 5 ml of serum-free Opti-MEM medium (Life Technologies, Inc.) and transfected simultaneously with hFaslPr (10 μg) and pSV-βgal (5 μg) in the presence of LipofectAMINE (30 μg; Life Technologies, Inc.) in Opti-MEM medium (10 ml) containing 20 μM dThd, for 18 h. After a subsequent wash in Opti-MEM medium (5 ml), cells were allowed to grow in foetal bovine serum-free RPMI 1640 containing 10% dFBS and 80 nm 5-methyltetrahydrofolate, either in the absence or in the presence of 20 μM dThd. Cells were harvested by trypsinization at time 0, 48, and 72 h, washed twice in PBS, and subsequently stored at −80°C prior to assay for luciferase or β-galactosidase activities.

To elucidate whether NF-κB or AP-1 were activated under conditions of thymineless stress, 2xκB-luc (10 μg) or TRE-luc (10 μg) were transfected with pSV-βgal (5 μg) for 18 h and subsequently exposed for 48 h to thymineless or dThd-replete conditions prior to harvest. In addition, activation of NF-κB or AP-1 was similarly examined following transient transfection of 2xκB-luc or TRE-luc and pSV-βgal in GC3/c1 cells for 18 h and treatment with FUra (1 μM), LV (1 μM) for 48 h either in the absence or in the presence of dThd (20 μM). To elucidate the influence of mutations within the proven binding domains of NF-κB and AP-1, hFaslPr/NF-κB and hFaslPr/AP-1 were activated under conditions of thymineless stress, 2xκB-luc (10 μg) or TRE-luc (10 μg) were transfected with pSV-βgal (5 μg) and various quantities of IκBαM (0–5 μg) or DN-MEKK (0–5 μg) in the presence of the empty vector HsLucEV (0–5 μg) to standardize the DNA concentration. Following transfection, cells were exposed for 48 h either in the absence or presence of dThd (20 μM) and subsequently harvested as described above.

To assay for luciferase activity, cell pellets were resuspended in 100 μl of lysis buffer (Promega), and 40 μl was subsequently assayed for luciferase activity using the Promega luciferase assay system, according to the manufacturer’s directions. pSV-βgal activity was assayed as reported previously (15). Luciferase/β-galactosidase ratios were subsequently calculated to correct for any differences in transfection efficiency (15).

**Electromobility Shift Assays**—Nuclear extracts were prepared, and DNA binding reactions were carried out for 30 min at room temperature in a buffer containing 80 mM Tris (pH 8.0), 20 mM MgCl2, 2 mM dithiothreitol, 0.4 mM EDTA, 400 mM KCl, 2 mM CaCl2, 45% glycerol, 10 μg of nuclear extract, 32P-labeled probe (90,000 cpm), and 1 μg of poly(dI-dC). The probes used were double-stranded synthetic oligonucleotides representing NF-κB and AP-1 sites from the human FASL promoter, as described previously (3). Oligonucleotides were labeled with T4 polynucleotide kinase (Life Technologies, Inc.) and [γ-32P]ATP (2,500 Ci/mmol; Amersham Pharmacia Biotech). Unlabeled radionucleotide was removed from labeled strands using a Qiagen nucleotide removal kit, and samples were analyzed on a 5% non-denaturing TBE gel in 0.5% TBE.

**Selection of Stable Transfectants**—Vectors for the constitutive expression of IκBαM (16) and DN-MEKK (19) were as described previously (3). TS− cells were plated at a density of 10 × 106 cells/T75 flask in NUNC peel-top flasks and transfected overnight (18 h) under serum-free conditions in DMEM with LipofectAMINE (Life Technologies, Inc.). IκBαM or DN-MEKK were cotransfected at a concentration of 20 μg of pCneo (5 μg). Cells were subsequently washed with Hanks’ and fed with RPMI 1640 containing 20% dFBS and dThd (20 μM), 5-methyltetrahydrofolate (80 μM). Following 72 h of growth, fresh media containing 75 μg/ml G418 were added. At increments of 72 h, the G418 concentration was increased to 150 μg/ml and then to 300 μg/ml. Surviving colonies were allowed to grow for 2 weeks, ring-cloned, and then placed in T12.5 flasks in 2 ml of media containing 300 μg/ml G418. Two clones were used for subsequent studies.

**Determination of Fasl Expression by RT-PCR**—Fasl expression was determined by semi-quantitative RT-PCR as described previously (1). Measurement of Apoptosis—TS− cells and clones stably transfected with IκBαM or DN-MEKK were plated at a density of 400,000 cells/well in 6-well plates. Following overnight attachment, dThd was withdrawn for periods up to 96 h. Both the floating cells and attached cells were pooled following trypsinization, fixed in 70% ethanol, and stored at −20°C prior to analysis. Apoptotic cells were detected as a sub-G1 fraction following propidium iodiide staining and analysis using a Becton Dickinson FACScan.

**RESULTS**

**Fas-mediated Regulation of Thymineless Death**—To demonstrate the crucial role of the Fas death receptor in the regulation of thymineless death, TS− cells were treated with anti-Fasl (NOK-1 mAb) or an IgG1 isotype-matched control, and
the effect on clonogenic survival was determined. By blocking Fas/FasL interactions, 100% clonogenic survival was main-
tained at 5 days, in contrast to <5% in untreated or IgG1-
treated cells (Fig. 1). Furthermore, TS
Clones selected for
resistance to CH-11 and hence Fas-mediated apoptosis were
also examined for their sensitivity to the induction of thymine-
less stress. 

**Activation of NF-κB and AP-1**—Transactivation of the wt
reporter constructs for NF-κB (2x\(\kappa B\)-luc) or AP-1 (TRE-luc)
were determined following their transient transfection simul-
taneously with pSV-\(\beta\)gal at 48 h in dThd-depleted or dThd-replete conditions (Fig. 2). This time had been predetermined
to allow maximal transactivation of hFasLPr under thymine-
less conditions, when the expression of FasL was first detect-
able (data not shown). Both 2x\(\kappa B\)-luc and TRE-luc reporters
were activated, dependent upon thymineless stress, whereas
minimal luciferase activity was detected when cells were cul-
tured in the presence of dThd.

**Importance of NF-κB and AP-1 in Transactivation of FasL**—
The dThd dependence of hFasLPr transactivation was exam-
ined in TS
cells at 48 h following transfection (Fig. 3A). In the
absence of dThd, there was significant activation of hFasLPr,
whereas in the presence of dThd, virtually no transactivation
was observed. To determine whether NF-κB may play a direct
role in the up-regulated expression of FasL, hFasLPr/NF-κB,
containing a mutation in the NF-κB-binding site, was co-trans-
fected with pSV-\(\beta\)gal, and the activation of this reporter was
examined at 48 h following dThd withdrawal (Fig. 3B). Under
these conditions a significant reduction in luciferase activity
and of the luciferase/\(\beta\)-galactosidase ratio was demonstrated,
resulting in a >10-fold reduction in transactivation of the re-
porter. Similarly, following transfection of hFasLPr/AP-1,
transactivation of this reporter was inhibited by >16-fold (Fig.
3C). These data were confirmed by EMSA. As demonstrated in
Fig. 4A, the induction of thymineless stress resulted in rapid
activation of NF-κB binding activity at 48 h that was competed
out by increasing concentrations of unlabeled oligonucleotide.
Similarly, withdrawal of dThd significantly enhanced AP-1
binding activity under these conditions (Fig. 4B). To explore
further the role of NF-κB and AP-1 in the direct activation of
FasL in TS
 cells in response to thymineless stress, cells were
transiently co-transfected with IκB
M that effectively blocks
activation of NF-κB, or DN-MEKK that effectively inhibits
JNK activity and subsequently the activation of AP-1, at dif-
ferent concentrations of DNA, and the influence on transacti-
vation of hFasLPr was examined (Fig. 5). Both IκB
M (Fig. 5A)
and DN-MEKK (Fig. 5B) induced a concentration-dependent
inhibition of the luciferase/\(\beta\)-galactosidase ratio.

**Clonogenic Survival, FasL Expression, and Induction of Ap-
optosis in Stable TS
Transfectants Expressing IκB
M or DN-
MEKK**—To elucidate the influence of IκB
M or DN-MEKK on
the survival of TS
 cells and the expression of FasL under
conditions of thymineless stress, stable clones expressing both
inhibitors were isolated. Both clones expressing IκB
M dem-
onstrated enhanced clonogenic survival compared with the
pCIneo control and TS
 cells following dThd deprivation (Fig.
6A). Similarly, under conditions of thymineless stress, inhibi-
tion of the activation of AP-1 by DN-MEKK enhanced the

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**Fig. 2. Activation of NF-κB and AP-1 in TS
 cells following thymineless stress.** NF-κB wt and AP-1 wt constructs were transiently co-transfected with pSV-\(\beta\)gal followed by exposure for 48 h to dThd-deficient or dThd-replete conditions. Data represent the mean ± S.D. of three determinations at each point. The map shows the locations of the binding sites for NF-κB and AP-1 in the FasL promoter.
Regulation of FasL by NF-κB and AP-1

We demonstrated previously in TS cells that thymineless stress-induced apoptosis correlated with up-regulated expression of FasL. First detected at 48 h after the initiation of dThd withdrawal and further elevated for up to 120 h (1). The crucial role of the Fas death receptor in the regulation of thymineless death has been demonstrated in two separate experiments. The first, which involved the blocking of Fas/FasL interactions using anti-FasL, completely prevented cell death induced by dThd deprivation, thereby allowing 100% cell survival in contrast to <5% in the absence of the inhibitory Ab. Second, the selection of TS clones resistant to CH-11-induced and hence Fas-mediated apoptosis were also resistant to the induction of thymineless death under conditions of thymineless stress.

If NF-κB and AP-1 are involved in the transcriptional regulation of FasL, then their expression should be up-regulated in response to thymineless stress in TS cells. This was found to be the case. Furthermore, mutations in the binding sites of NF-κB and AP-1 in hFasLPr, or transient co-transfection of IκBα or DN-MEKK to inhibit the activation of NF-κB and AP-1, respectively, resulted in inhibition of the transcriptional activation of hFasLPr under conditions of thymineless stress. EMSA analyses further confirmed these data, demonstrating rapid activation of both NF-κB and AP-1 binding activities, dependent upon dThd deprivation. Taken together, the data demonstrated direct transcriptional regulation of FASL by both NF-κB and AP-1 under conditions of thymineless stress and are similar to data derived in Jurkat T cells (3). Here, Kasibhatla et al. (3) proved that the introduction of mutations in the NF-κB and AP-1 binding domains within the FASL promoter region abrogated drug-induced FASL promoter activity, when cells were treated with the topoisomerase II inhibitors VP-16 or VM-26. In addition, apoptosis induced by these agents was inhibited by the chimeric protein Fas-Fc, demonstrating that transcriptional activation of FasL was involved in the mechanism of apoptosis induction.

Anticancer drugs, including VP-16 (3, 7, 9), VM-26 (3), actinomycin-D (9), anthracyclines (5, 6, 9), topoisomerase I inhibitors (9), and ara-C (7, 8), have been reported to activate NF-κB, and inhibition of this activation can inhibit apoptosis (3, 7). Reports have also indicated that activation of NF-κB has an antiapoptotic function with regard to both tumor necrosis factor-α- and chemotherapy-induced apoptosis including VP-16 and CPT-11 (16, 20, 21). Furthermore, NF-κB can function as both a proapoptotic or antiapoptotic regulatory factor within a single cell type, dependent upon the stimulus (22). Thus, phorbol 12-myristate 13-acetate plus ionomycin activated NF-κB, which up-regulated FasL expression and induced apoptosis. In contrast, inhibition of NF-κB conferred a 10-fold increase in glucocorticoid-mediated apoptosis. These results may be explained by the function of different genes under the regulation of NF-κB. Thus, DNA-damaging agents or stress stimuli that signal via Fas appear dependent upon transcriptional activation of FASL by NF-κB for the induction of apoptosis. In cells in which

FIG. 3. Mutations in the binding sites of NF-κB and AP-1 in hFasLPr inhibit transactivation. HfAsLPr, hFasLPr NF-κB, or hFasLPr/ AP-1 were co-transfected in TS cells with βgal prior to maintenance of the cells for 48 h either in the absence or in the presence of dThd (20 μM), as described under “Experimental Procedures.” Data are the means ± S.D. of three determinations for each condition.

FIG. 4. EMSA demonstrating activation of NF-κB and AP-1 binding activity 48 h after the initiation of thymineless stress. Binding activities for both 32P-labeled oligonucleotides were competed out using increasing concentrations of unlabeled oligonucleotide as described under “Experimental Procedures.”
NF-κB was inactive, activation of TRAF1, TRAF2, and the inhibitor of apoptosis (IAP) proteins c-IAP1 and c-IAP2 were all required to suppress tumor necrosis factor-induced apoptosis, whereas c-IAP1 and c-IAP2 were sufficient to suppress VP-16-induced apoptosis (23). NF-κB has also induced expression of the Bcl-2 homologue A1/Bfl-1 to suppress VP-16-induced apoptosis by inhibiting the release of cytochrome c from the mitochondria and subsequent caspase-3 activation (21). Thus, in signaling thymineless death via Fas, NF-κB has a proapoptotic role in colon carcinoma cells.

**FIG. 5.** IκBαM and DN-MEKK inhibit transactivation of hFasLPr. The influence of IκBαM or DN-MEKK on transactivation of hFasLPr was determined 48 h after culture of the cells either in the absence or presence of dThd (20 μM). Cells were co-transfected for 18 h with HFasLPr, pSV-bgal, and varied quantities of IκBαM, DN-MEKK, or the empty vector HsLucEV as described under "Experimental Procedures." Data represent the mean ± S.D. of three determinations for each condition.

**FIG. 6.** Stable expression of IκBαM or DN-MEKK enhances clonogenic survival of TS· cells during thymineless stress. TS· cells were transfected with pCIneo (TS· pCIneo), IκBαM, or DN-MEKK, and stable transformants were selected as described under "Experimental Procedures." Thymidine was withdrawn at time 0 and added to cultures at 24-h intervals from 72 to 120 h following dThd deprivation. Clonogenic survival was determined 11 days after dThd rescue as described under "Experimental Procedures." Data are the mean ± S.D. of three determinations at each time point.

**FIG. 7.** Delayed expression of FasL in stable transfectants expressing IκBαM and DN-MEKK. Expression of FasL was determined by RT-PCR (35 cycles) following dThd deprivation in TS·, TS· pCIneo, and two clones each stably expressing IκBαM or DN-MEKK. β-Actin (25 cycles) expression was analyzed as a control. Data were derived according to the procedures described previously (5).

**FIG. 8.** Reduced apoptosis in stable transfectants expressing IκBαM and DN-MEKK. Following dThd deprivation for periods of up to 96 h, apoptotic cells were detected as a sub-G1 fraction following propidium iodide staining and analysis using a Becton Dickinson FACScan.
VP-16 (3), VM-26 (3), and ara-C (10) have also activated AP-1. Further implication for the involvement of AP-1 in the induction of apoptosis comes from studies of the involvement of the JNK/SAPK in apoptosis induction. The JNK, members of the mitogen-activated protein kinase family, phosphorylate the stimulatory sites of c-Jun (11), and it is well established that AP-1 is induced via activation of the JNK/SAPK pathway (18) and that activation of JNK by cytotoxic drugs can lead to apoptosis (24, 25). However, the pathways responsible for NF-κB activation are considerably less clear and may or may not involve the JNK/SAPK pathway (3, 26). Certain reports have linked these signaling events induced by DNA-damaging drugs to Fas signaling and hence Fas-dependent apoptosis (2, 3). In other systems, the association is less clear, and NF-κB activation can also act independently of the cytotoxic function of Fas (27). However, in Jurkat T leukemia cells, VP-16- and VM-26-induced apoptosis involved activation of JNK/SAPK, NF-κB, and AP-1, and apoptosis was inhibited by Fas-Fc, suggesting that Fas signaling pathways are involved in the induction of apoptosis by these agents (3). Also in the Jurkat cell line, expression of DA-MEKK induced apoptosis, prolonged JNK activation, and enhanced FasL mRNA expression and FasL promoter activation, and inhibition of Fas/FasL interactions by Fas-Fc prevented DA-MEKK-induced apoptosis (24). Of interest was that treatment with ionizing radiation or anisomycin also produced these effects, although inhibition of Fas/FasL interactions inhibited anisomycin- but not radiation-induced apoptosis, suggesting that not all stress stimuli involving DNA damage use the same cell death pathway (24). However in TS cells, data demonstrate that NF-κB and AP-1 are both activated in thymineless stress-induced signaling in TS- cells and transcriptionally regulate FasL in Fas-dependent thymineless death of these cells. Furthermore, stable expression of either IkBαM or DN-MEKK delayed the loss in clonogenic survival, reduced the percentages of cells undergoing apoptosis, and up-regulated expression of FasL in response to thymineless stress.

We demonstrated previously that activation of FasL occurs during thymineless death induced in wt GC/c1 human colon carcinoma cells following treatment with FUra combined with LV to induce a thymineless state (1), temporally correlating with the induction of apoptosis. Furthermore, we demonstrated that a Fas-dependent component exists in FUra/LV-induced cell death in colon carcinoma cells (28). We have now demonstrated in this study that transcriptional regulation of FASL by NF-κB and AP-1 also occurs in GC/c1 cells following treatment with FUra/LV, similar to the regulation that occurs in TS- cells following dThd deprivation. Therefore, when DNA damage-induced apoptosis is mediated via Fas, NF-κB and in addition AP-1 may play important roles in the regulation of FasL and the induction of cell death.

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