Regulation of Fas-Ligand Expression during Activation-induced Cell Death in T Lymphocytes via Nuclear Factor κB

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T cell receptor engagement activates transcription factors important for cytokine gene regulation. Additionally, this signaling pathway also leads to activation-induced apoptosis in T lymphocytes that is dependent on FasL transcription and expression. Here we demonstrate that nuclear factor κB (NF-κB), which is involved in the transcriptional regulation of many cytokine genes expressed in activated lymphocytes, also plays a role in T cell activation-induced FasL expression. Inhibition of NF-κB activity in a T cell hybridoma leads to decreased FasL expression and apoptosis upon T cell receptor stimulation. We identified the NF-κB site in the FasL promoter that contributes to such regulation. Co-expression of p65 (Rel A) with the FasL promoter enhances its activity, and co-expression of IκB dramatically inhibited the inducible promoter activity. In contrast, the transcription factor AP-1 is not required for activation-induced FasL promoter activity. These results define a role for NF-κB in mediating FasL expression during T cell activation.

Activation-induced cell death (AICD) is a major mechanism to maintain immune homeostasis. AICD occurs in mature T lymphocytes to limit antigen-specific responses. Upon clearing antigens and/or pathogens from the host, activated T cells are deleted via the activation of apoptosis, which we and others have shown to be dependent upon Fas ligand (FasL, CD95 ligand) expression and ligation of its receptor, Fas (CD95) (1–5). However, despite our understanding of the importance of FasL in controlling homeostasis, little is known about its transcriptional regulation.

FasL expression and apoptosis upon T cell receptor ligation is then translocated into the nucleus as active heterodimers consisting of Rel A (p65), Rel B, and c-Rel, which regulate gene expression.

Recently, we identified a role for NF-κB and another transcription factor, AP-1, in the regulation of stress-induced FasL expression (16). In the present study, we sought to determine whether NF-κB and AP-1 regulate FasL expression in lymphocytes activated through the T cell receptor.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—Human leukemic Jurkat cells (ATCC) and Jurkat cells stably transfected with SV40 large T antigen were used in this study. The T cell hybridoma A1.1 has been described previously (2). All cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM l-glutamine, and 100 units/ml each penicillin and streptomycin (complete medium). Phorbol myristate acetate (PMA) was purchased from Sigma, and ionomycin was purchased from Calbiochem. Mouse anti-human CD3 (OKT3) antibody and hamster anti-mouse CD3ε (145–2C11) were purified from culture supernatants by protein A affinity chromatography. Anti-p65(RelA) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Induction of Apoptosis—All experiments were performed in 96-well plates and in triplicates samples, with cells resuspended at 0.5–1 × 10^6/ml in complete medium. For T cell receptor stimulation, 96-well plates were precoated with anti-CD3 antibody (2C11) in 50 mM Tris, pH 9.0. PMA and ionomycin were added at concentrations of 50 ng/ml and 0.5 μg/ml, respectively.

Reverse Transcription (RT)-PCR for FasL Expression—The expression of Fas-L was determined by RT of total RNA followed by reverse PCR analysis (RT-PCR) as described previously (16). Briefly, cDNAs were synthesized by extension of 4T primers with 200 units of SuperScript II reverse transcriptase (Life Technologies, Inc.) in a mixture containing 1 μg of total RNA digested by RNase-free DNase (2 units/μg of RNA) (Ambion) for 15 min at 37 °C. PCR of the cDNA was performed in a final volume of 50 μl, containing all four dNTPs, 2 mM MgCl₂, 2.5 units of AmpliTaq (Life Technologies, Inc.), and each primer at 0.2 μM using the geneAmp 2400 PCR system (Perkin-Elmer). Amplification of β-actin served as control for sample loading and integrity. The following primers were designed to discriminate between the amplification of cDNA (low size PCR products) and contaminating genomic cDNA (high size PCR products):

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‡ The abbreviations used are: AICD, activation-induced cell death; IL-2, interleukin 2; TCR, T cell receptor; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; PMA, phorbol myristate acetate; RT-PCR, reverse transcription-polymerase chain reaction; kbp, kilobase pair(s); bp, base pair(s); hFasL, human FasL; DN-MEKK, dominant negative mitogen-activated protein kinase protein.
hFasL forward: TAAAACGGTTTGTGGGGCC.
hFasL reverse: CTACAGCTCCTTTTTTCAGGG.
β-Actin forward: TGACGGGCTACCCACATGGCCATCT.
β-Actin reverse: CTAAGACATTTCGCTGACGAATGGAGG.

Expression Vectors and Transient Transfections—Cloning of the 1.2-kb FasL promoter and generation of the truncated version was described before (16). A 1.2- and a 0.9-kb fragment of the hFasL promoter were subsequently used in the experiments described here. Jurkat T cells containing stably transfected SV40 large T antigen were electroporated as described previously (17). Briefly, 1.5–2 × 10⁷ cells were washed twice with serum-free RPMI 1640 medium, resuspended in 500 µl of the same medium, and transferred to 4-mm gap electroporation cuvettes (Bio-Rad). 20–60 µg of FasL reporter alone or with Sra p65, IκB, BeBm, or DN MEKK expression vectors (18, 19) were added to the cells and mixed well. Electroporation was carried out at 250 V and 960 microfarads in a Bio-Rad Gene Pulse II.

Luciferase Assays—Briefly, different hFasL promoter constructs were transfected with or without co-expression vectors. pCMV β-galactosidase was used to normalize the transfection efficiencies in the various co-transfections. Forty h post-transfection, cells were activated with PMA and ionomycin and incubated for another 12–18 h. Cells were harvested, washed three times with PBS, and lysed in 100 µl of the lysis buffer. Cell debris was removed by centrifugation, and the supernatant was used in the luciferase assay using a Monolight 2010 luminometer (16).

Electromobility Shift Assays—DNA binding reactions were carried out for 20 min at 4 °C in a buffer containing 50 mM Hepes, pH 7.8, 20 mM MgCl₂, 0.5 mM EDTA, 20 mM spermidine, 500 µM dithiothreitol, 75% glycerol, and 10⁵ counts/min labeled probe. The probes used were double-stranded synthetic oligonucleotides (Retrogen, San Diego, CA) consisting of the NF-κB site from the human FasL promoter, 5′-AAGCGTGGCCACATGAAACTCCCTGCCATCGTA-CAAAAA-3′, and the consensus NF-κB oligonucleotide from Santa Cruz Biotechnology, Inc. Each strand was labeled separately with T4 polynucleotide kinase (Life Technologies, Inc.) and [γ-³²P]ATP (5000 Ci/mmol), and the strands were then slowly allowed to renature. The samples were analyzed on a 4% nondenaturing acrylamide gel in 0.5% Tris-borate buffer. In some experiments, nuclear extracts from Jurkat cells were made as described (16). Binding reactions contained 5–10 µg of nuclear extract, ³²P-labeled probe (25,000 cpm), and 2 µg of poly(dI:dC) in the binding buffer.

RESULTS

Inhibition of NF-κB Diminishes FasL Expression and T Cell Receptor-mediated Apoptosis—It has been suggested that NF-κB is required for activation-induced cell death in T cells. Recently, it was shown that antioxidants inhibit activation-induced cell death by blocking NF-κB activation, and thereby blocking FasL expression (20). Similarly, we have recently shown that NF-κB is required for stress-induced FasL expression and apoptosis in T cells (16). In contrast, previous studies by ourselves and others have suggested that NF-κB can act as an inhibitor of apoptosis induced by TNF (21–24). We therefore sought to determine whether inhibition of NF-κB can inhibit activation-induced cell death in T cells.

To examine this possibility, we employed a recently described inhibitor of NF-κB composed of a cell-permeable peptide carrying the nuclear localization signal of the NF-κB p50 subunit (25). This peptide has been shown to specifically inhibit the nuclear translocation of the NF-κB complexes (26). We studied the effect of the NF-κB inhibitory peptide on the peptide in preventing activation-induced cell death in a T cell hybridoma. The T cell hybridoma A1.1 undergoes expression of FasL and apoptosis when cross-linked with anti-T cell receptor antibodies (21). We observed a significant decrease in apoptosis in the presence of the NF-κB inhibitory peptide and not the control peptide (Fig. 1A). We then assessed FasL expression by semi-quantitative RT-PCR in these cells and observed a corresponding inhibition in the level of FasL mRNA (Fig. 1B). Together, these results suggest that NF-κB may be an important mediator of activation-induced FasL expression and subsequent cell death.

Characterization of the 1.2-kb FasL Promoter for TCR-mediated Signaling Events—Activation-induced cell death is mediated by regulation of Fas/FasL expression. T cell receptor cross-linking and pharmacologic agents mimicking these signals also induce FasL expression (27). There are potential binding sites for several transcription factors in the 1.2-kb promoter region of Fas ligand (Fig. 2A). To understand the results obtained in Fig. 1 and to dissect the molecular mechanisms underlying T cell receptor-mediated FasL expression, we used a 1.2-kb FasL promoter (16) and examined whether this reporter is activated upon TCR ligation. As shown in Fig. 2B, the 1.2-kb FasL promoter was activated upon cross-linking of the TCR receptor with anti-CD3 antibody as well as upon stimulation with the pharmacologic agents PMA and ionomycin. Similar results were reported in studies using a 486-bp FasL reporter construct (10). To identify the regulatory domains that mediate this inducible transcription of the 1.2-kb FasL promoter, we then tested the effect of a 0.9-kb FasL reporter (Fig. 2A) that has its 5′ enhancer truncated. In the experiment shown in Fig. 2C, we observed that when T cells were activated with PMA and ionomycin, FasL reporter activity of the 0.9-kb promoter was significantly reduced versus that of the 1.2-kb promoter.

T Cell Activation Induces Binding of NF-κB to Its Cognate DNA—To further examine the possible role of NF-κB in AICD, we studied the activation-dependent characteristics of a NF-κB reporter construct carrying two copies of the consensus κB

![Inhibition of NF-κB blocks activation-induced cell death and FasL expression. A, A1.1 T hybridoma cells were activated by the T cell receptor in the presence of the κB inhibitory peptide (●) or the control peptide (○) for 12–16 h, and cell death was assessed by propidium iodide uptake on FACS. Apoptotic cells were also analyzed by staining with acridine orange and ethidium bromide and examining the morphology by fluorescent microscopy. B, RT-PCR analysis of total mRNA isolated from A1.1 cells activated as described above in the presence of the κB inhibitory peptide or the control peptide.](image-url)
motif (28) and found that NF-κB is activated upon stimulation with PMA and ionomycin (Fig. 3A). A mutation in the site abrogated this activity (Fig. 3A). We have previously shown that the distal NF-κB site binds to NF-κB proteins from nuclear extracts treated with the DNA-damaging agent, etoposide (16). After treatment with PMA and ionomycin, we then examined nuclear extracts for NF-κB binding activity by an electromobility shift assay, using the distal NF-κB site from the FasL promoter (16). As shown in Fig. 3B, T cell activation induced NF-κB binding in an activation-dependent manner. The binding to this site was competed off by the wild type consensus NF-κB motif (GGGGACTTTCCC) (29) and by an anti-p65 antibody (30) but not by control serum (Fig. 3B). The inhibition of complex formation by anti-p65 antibody also suggests that p65 is part of the complex induced by PMA and ionomycin treatment.

**Inhibition of NF-κB Prevents FasL Reporter Activity**—Earlier studies using chemical activators of cAMP signaling (31) and reactive oxygen intermediates have suggested that NF-κB could be involved in activation-induced cell death (20, 32). The experiments in Fig. 1 suggested that NF-κB is required for optimal activation-induced cell death and FasL expression. We therefore investigated the possibility that NF-κB directly regulates T cell activation-mediated FasL transcription. Based on the dependence of NF-κB activation on the effective degradation of IκB, we first took the approach of inhibiting NF-κB activation using IκB or a nondegradable mutant IκBαM (21). We therefore co-transfected Jurkat cells with the FasL reporter construct together with either wild type IκB or the nondegradable mutant IκBαM (21). As shown in Fig. 4A, either form of IκB effectively blocked the basal activity as well as activation of the FasL promoter with PMA and ionomycin.

**AP-1** Is Not Critical for TCR-mediated FasL Promoter Activation—Recently, Latinis et al. (10) showed that NF-AT is an important transcription factor required for activation-induced FasL expression. Similarly, we identified AP-1 and NF-κB sites required for stress-induced FasL expression (16). T cell receptor ligation activates the early genes fos and jun that heterodimerize and bind to the AP-1 site to regulate IL-2 and other cytokine gene transcription (34). Phosphorylation of c-Jun by c-Jun N-terminal kinases (JNKSAPK) is required for AP-1-dependent promoter activity (34). Inhibiting c-Jun phosphoryla-
tion by a DN-MEKK (K432M) also down-regulates AP-1-dependent reporter activity (35). To study the effects of DN-MEKK on FasL reporter activity, we cotransfected the FasL reporter with a DN-MEKK expression vector and observed that DN-MEKK had no effect on TCR-mediated FasL reporter activity (Fig. 6A). In contrast and as shown earlier (16), DN-MEKK inhibited stress-induced FasL reporter activity (Fig. 6A). We then investigated whether the distal AP-1 binding site (16) was required for TCR-mediated FasL transcription. As shown in Fig. 6B, a mutation in the AP-1 site did not alter FasL reporter activity in response to T cell receptor signaling, thus suggesting the involvement of a different signaling mechanism.

We have previously observed that this mutation abolishes the response of the promoter to DNA-damaging agents (16).

**DISCUSSION**

TCR engagement and activation often leads to growth and differentiation as well as apoptosis in T cells (6). Some of the proximal signaling events lead to activation of AP-1, NF-κB, as well as the NF-AT family of transcription factors (9), which are known to activate cytokine gene transcription. A substantial amount of work in the past several years has helped our understanding of this complex cytokine gene regulation. There is also evidence that transcription can play a critical role in mechanisms involved in some forms of apoptosis, such as activation-induced cell death in T cells.

Activation-induced cell death is primarily mediated by Fas/FasL in mature T lymphocytes (2, 3). Previously, Latinis et al. (10) showed that NF-AT regulates activation-induced FasL promoter activity. Similarly, we showed recently that the transcription factors AP-1 and NF-κB regulate DNA damage and other stress-induced FasL expression (16). Here, we have provided evidence that NF-κB also plays a role in T cell activation-mediated FasL expression. We found that the regulation of the 1.2-kb FasL reporter was dependent on NF-κB and that inhibiting NF-κB translocation into the nucleus also inhibited FasL expression and apoptosis in T cells.

NF-κB was originally identified as a constitutively expressed protein that can form a complex with a 10-bp site in the immunoglobulin *κ* light chain enhancer (36). It was soon found to also take part in T cell activation, regulating IL-2 and IL-2α
NF-κB Regulates TCR-mediated FasL Expression

After 12–16 h.

Treated with PMA and ionomycin, and reporter activity was determined.

Construct containing the mutation in the AP-1 site as shown. Cells were treated with PMA and ionomycin, and reporter activity was determined after 12–16 h. Results shown are representative of three independent experiments. B, Jurkat cells are representative of three independent experiments. NF-kB also prevents DNA damage and other stress-induced apoptosis in Jurkat cells (16). Taken together with the results described in this paper, NF-κB seems to serve multiple functional roles under different conditions.

Apoptosis mediated by Fas often does not involve activation of NF-κB (40). On the other hand, TRAMP (TNF-related apoptosis-inducing protein)/DR3/WSL-1 and TRAIL receptors (TNF-related apoptosis-inducing ligand) induce apoptosis and also activate NF-κB (41–44). Like FasL, TRAIL is also induced upon lymphocyte activation, and its expression is inhibited by cyclosporin A, suggesting a role for calcineurin and NF-AT (45). Whether NF-κB also regulates TRAIL expression is not known. It is possible that in T cells, some of the early signals may define the de novo gene expression.

Our present study also raises some interesting questions about the complex regulation of Fas ligand. A 0.9 kb fragment of the FasL reporter had low activity upon stimulation (Fig. 1C), whereas a 486-bp reporter was shown to be sufficient for reporter activity (10). One possibility is that there could be repressor elements in the Fas ligand promoter that may act as regulators of this death-promoting gene. A recent study on human FasL promoter has suggested the existence of a repressor element between -453 to 373 in the promoter (46). This study did not, however, characterize the region between -473 and -2365 in the promoter. Our study suggests the possibility of a repressor element between -900 and -486 bp in this promoter, and there could also be another between the 1.2 kb and the 2.3 kb of the enhancer. We are currently analyzing the promoter region for any such repressor elements. It is also interesting that NF-AT alone is sufficient to drive a 486-bp FasL promoter (10), whereas in the context of a 1.2-kb promoter, a mutation in the NF-AT site did not completely inhibit the promoter activity. In contrast, a mutation in the NF-κB site had a profound effect on activation-induced Fas ligand promoter activity (Fig. 4B), suggesting that NF-κB is critical for FasL expression.

So far, NF-κB, AP-1, and NF-AT are among the transcription factors shown to be involved in some forms of apoptosis, of which NF-κB seems to possess quite distinct characteristics. Understanding the characteristics of this molecule with regard to how this differential specificity is achieved and how different signaling events converge on the same regulatory factor would also shed light on any “switch mechanism” a cell might have to regulate different cellular functions. We studied the importance of the previously identified AP-1 site (16) in T cell activation-mediated FasL expression and found that AP-1 was dispensable for this form of induction but was required for induction by stress (16). Our studies indicate that the preference of different transcription factors to regulate gene transcription varies with the mode of cell activation. The transcription factors AP-1 and NF-κB are involved in stress-induced FasL expression, whereas NF-AT and NF-κB appear to cooperate in T cell receptor-mediated FasL expression.

Fig. 6. AP-1 is not obligatory for activation-induced FasL promoter activity. A. Jurkat cells were transfected with hFasLPr (1.2 kb) and DN-MEKK1 (K432M). Cells were activated with PMA and ionomycin, and reporter activity was measured after 12–16 h. Results shown are representative of three independent experiments. B, Jurkat cells were transfected with the 1.2-kb FasL promoter reporter construct or a construct containing the mutation in the AP-1 site as shown. Cells were treated with PMA and ionomycin, and reporter activity was determined after 12–16 h.

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NF-κB Regulates TCR-mediated FasL Expression

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