Transcriptional Regulation of the Human FasL Promoter-Enhancer Region*

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The human FasL enhancer region was cloned and functionally characterized in transformed and primary T cells. Within the 2.3 kilobase pairs of the FasL untranslated region, the distal 3' 300-base pair portion contains a single transcription initiation site and confers basal and inducible transcriptional activity. Stimuli that increase \([\text{Ca}^{2+}]_i\), such as CD3 cross-linking or ionomycin, but not activation of protein kinase C, were found to induce FasL enhancer transcription in a cyclosporin-sensitive manner. Moreover, calcineurin and NFAT, but not AP1, were identified as necessary and sufficient effectors in driving FasL transcription through an NFAT cis-acting motif (GGAAA). Additional modes of T cell activation such as CD4 cross-linking were also found to induce NFAT binding to the FasL enhancer region and to functionally transactivate its transcription. These results indicate that the induction of FasL gene transcription in T cells after CD3 or CD4 activation is selectively mediated by calcineurin and NFAT.

The study of the molecular mechanisms regulating cell death by apoptosis has become a central theme in cell biology. A family of receptors and their corresponding ligands are being identified and shown to induce cell death by apoptosis, namely those of the tumor necrosis factor receptor family (reviewed in Refs. 1 and 2). Among them, Fas and its ligand FasL play a major role in a variety of immunological processes such as the control of peripheral T cell homeostasis and cytotoxicity by effector lymphocytes (3–5). Abnormal lymphocyte accumulation, immune tolerance, enhanced deletion of peripheral lymphocytes, autoimmunity, maintenance of immune privileged sites, and participation in allograft rejection are the basis for the potential relevance of Fas/FasL in clinical circumstances such as lymphoproliferative syndromes, CD4 T cell depletion present in AIDS, systemic lupus erythematosus, and transplantation (6–14). In addition, Fas/FasL may participate in other nonimmune processes such as tissue damage induced by soft and solid tumors and demyelinating diseases, including multiple sclerosis (15–17).

For the apoptosis of a Fas-bearing cell to occur, two necessary events are required. One is the induction of susceptibility to Fas-dependent apoptosis, and the second is the necessary step of encounter with the FasL (18–23). The cloning of the murine FasL gene demonstrated that its transcription was present in a constitutive fashion in a variety of tissues, specially in testis and in lymphoid organs, and that, in addition, its expression could be induced after splenocyte activation (24, 25). Although initial emphasis was placed to demonstrate that cells of lymphoid lineage could express functional FasL upon their activation and thus result in the so-called activation-induced lymphocyte death (19–23), other immune and nonimmune cells have been shown to constitutively express FasL, including macrophages, dendritic cells, and neutrophils, as well as a variety of cells present in immune privileged sites such as the testis and eye (26–31). Despite the immunological relevance of FasL, its wide cell and tissue distribution, and the apparent differences of its expression in a cell-specific manner, little knowledge is available as to how FasL expression is regulated. Although gene transcription and post-translational modifications such as its processing from a membrane into a soluble form by matrix metalloproteinases have been identified as levels of potential regulation (19–24, 32, 33), detailed molecular mechanisms governing these two processes are lacking.

The studies addressing the transcription of FasL have focused on mRNA analysis in a variety of primary and transformed lymphocytes (19–24). Altogether, such studies demonstrate absent FasL mRNA levels under resting conditions, but its rapid and transient induction by a variety of stimuli. Combination of pharmacological agents such as PMA1 and ionomycin, cross-linking of CD3, CD4, FcγRI, or lectins have all been shown to transiently increase FasL mRNA (19–24, 34), suggesting the regulation of the FasL gene by specific transcription factors. The cloning of the murine and human FasL gene included a 40-base pair sequence upstream from the translation codon in which putative cis-acting factors were noted (25). However, a functional characterization of the presumed enhancer promoter of this gene contained within this or more upstream portions has not been performed. To understand how T cell activation and other stimuli such as HIV infection induce and regulate FasL at the transcriptional level, we have cloned the enhancer-promoter region of this gene. A single transcription initiation site, a transcriptionally active enhancer region, and different putative negative regulatory regions were identified within a 2.3-kilobase pair portion of the 5'-untranslated genome. In addition, the phosphatase calcineurin and the transcription factor NFAT1(c2) were found to mediate, in an FK506-dependent fashion, the induction of FasL gene transcription induced by \([\text{Ca}^{2+}]_i\), after T cell activation by CD3. Finally, the HIV-related T cell activation by CD4, but not HIV-tat, was found to induce NFAT binding to the FasL enhancer.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF035584.

1 The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; WT, wild type; EMSA, electrophoretic mobility shift assay; IL, interleukin; CN, calcineurin; TCR, T cell receptor; LTR, long terminal repeat.

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MATERIALS AND METHODS

Cell Lines and Reagents—The Jurkat T cell line was purchased from ATCC and cultured in RPMI medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (HyClone), glutamine, and antibiotics. In experiments requiring CD4 activation, high CD4-expressing Jurkat cells were selected using flow cytometry to result in bulk Jurkat cultures with high CD4 expression levels. Purified CD3 T cells were prepared from MLR costs obtained from healthy blood donors as described previously (35–37). Peripheral blood lymphocytes were isolated by Ficoll-Hypaque (Pharmacia) density gradient followed by monocyte depletion through plastic adherence. Then CD3 T cells were purified by neuraminidase-treated sheep red blood cell (SRBC) rosetting. Purity of CD3 T cells were verified by flow cytometry, routinely obtaining >95% pure preparations. The phorbol ester PMA was purchased from Sigma and stored at −30 °C. Ionomycin was purchased from Calbiochem and stored at −20 °C. Dextran-DEAE was purchased from Pharmacia. FK506 was a kind gift of Dr. E. O’Neill, Merck Research, Rahway, NJ. Anti-CD3 antibodies were a kind gift of R. Abraham (Mayo Clinic, Rochester, MN). Anti-CD4 antibodies (Leu 3a) and IgG, isotype control were purchased from Becton Dickinson. Goat anti-mouse antibodies were obtained from Biosearch International. Anti-NFAT(c2) antibodies were a kind gift of Dr. Anjana Rao (Harvard University).

Cloning of Transcription Start Site and the 5′-Untranslated FasL Region—Both the transcription start site and promoter-enhancer region were identified using commercial kits (Marathon™ CDNA amplification kit and Human Promoter Finder™ DNA walking kit, respectively) purchased from CLONTECH. The Marathon™ CDNA amplification kit supplied the reagents and protocol to generate a CDNA library from adult T cells for subsequent PCR reactions and cDNA library was generated from HIV-infected U937 cells. Sense primers for the PCR were supplied with the kit, and gene-specific antisense primers used were 5′-GCC CAG GGA CAG CTT GCA CTG GCC TGG AC-3′ and 5′-TGC TGT CCA CCC AGT AGT TCA T-3′ in a nested PCR. The final PCR reaction was directly cloned into pCRII using the TA cloning kit (Invitrogen). Automated sequencing was performed on the cDNA or molecular biology core.

The 5′-untranslated region was cloned using the Promoter Finder™ DNA walking kit. The kit contained five genomic DNA libraries that were digested with various enzymes followed by the addition of adapter sequences to the DNA fragment ends. PCR reactions were performed using sense primers provided with the kit and gene-specific antisense primers. The unique portion of the promoter was being sequenced in a series of oligonucleotides and stepping down the sequence. This was done in both directions using the following sense oligonucleotides (5′ → 3′): TGT ATC TTA TGG TAG TAT TGG, TAC CAT TTT ACT GGG TGA TTT GGG, GAT AAA GTC TGA CCC GC, AAG TGA TAG TAA AAG TCC TCC, TTC TAA TTT AAT TGG CCA GC, TCC CTC ATG CCT GTA ATT CC, ATT GTG AAA TAC AAA GCA GC, and TTT CTT GTA CCA GCA GCT GC, with the following antisense oligonucleotides (5′ → 3′): CT TAT GAT TTT GTC GCT TCC CA, ACT CAC TTT GCA GCT GAA GC, GCT ACA GCA GAA TGC TGA GT, GAT CAG AGG CTT TGA ACC ART, TGG CAT CAA GAC ATC CTT CC, CAA GTA GGT GCT AAA CAT GTC C, and ACT CTT TCT GTC GGT AAC TTC. Sequencing was performed by our molecular biology core facility. For placement into the expression vector pGL2 Basic (Promega), another PCR reaction using the antisense primer 5′-GCC CAG CTG CAG CTT GGG CAG CTT GGT CATG AGT CA-3′ with a HindIII site was performed.

Plasmids and Oligonucleotides—The luciferase reporter gene pGL2 was purchased from Promega. The TK-β-galactosidase reporter gene was purchased from CLONTECH. The gene expressing a constitutively active calcineurin (1CAM1) cloned downstream from an SV40 expression vector, the HIV-1 Tat expression vector, and the HIV-1-TR-luciferase expression vectors have all been previously described by our group (35–37). The NFA1-T(c2) expression vector (pEFTAG-NFA1-c) was a kind gift of Dr. A. Rao. Oligonucleotides used in EMSA include: wild type (WT) FasL, 5′-AAA TTG TGG GGG GAA ACT TCT ACG GGG-3′; NFA1-mut, 5′-AAA TTG TGG GCT TTA ACT TCT ACG GGG-3′; Oligonucleotides used to introduce mutations by PCR-based techniques of NFA1-mut were the one described above and used to substitute NFAT site in the oligonucleotide used in EMSA. For the SP1 mutation, the final PCR reaction was directly cloned into PCRII using the TA cloning kit (Invitrogen). Automated sequencing was performed on the cDNA or molecular biology core.

In transfection experiments, the “background” level for luciferase or β-galactosidase was evaluated using extracts from cells transfected with DNA plasmid lacking luciferase or β-galactosidase reporter genes, respectively. In the experiments included in this report, the basal luciferase or β-galactosidase activities transcribed from luciferase or β-galactosidase reporter genes were in all cases significantly higher than the background levels. Results are therefore expressed as relative light units, which were calculated as the ratio of luciferase to β-galactosidase activities within each sample. Transfections were performed in duplicate, and the mean and standard deviation were calculated for each experiment, which was at least performed three times. For CD4 activation of Jurkat T cells, aliquots of transfected cells were incubated at 37 °C, treated with 5 μg of IgG, isotype control or anti-CD3 antibodies for 30 min at 4 °C. Thereafter, cells were transferred to 24-well plates precoated with goat anti-mouse antibodies and incubated at 2 × 10^6 cells/ml at 37 °C for an additional 6 h.

Cell Stimulation and EMSA—CD3 T cells (2 × 10^5 cells/ml) were incubated with 1 μM of anti-CD4 (Leu 3a) or isotype control at 4 °C for 1 h. Thereafter, cells were transferred to goat anti-mouse-coated 24-well plates at 2 × 10^5 cells/ml/well. As positive control, CD3+ T cells treated with 0.5 μM ionomycin were used. Nuclear extracts from CD3+ T or Jurkat T cells were obtained as described previously (36, 37). Briefly, cells were washed and resuspended in Buffer A (10 mM Heps, 1.5 mM MgCl_2, and 10 mM KCl in the presence of protease inhibitors and dithiothreitol (100 μM) and lysed by the addition of Nonidet P-40 to a final concentration of 0.1%. Nuclei were pelleted and washed in Buffer A, and nuclear protein was extracted in Buffer C (20 mM Heps, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_2, and 0.2 mM EDTA). After pelleting the nuclear debris, the supernatant was removed and diluted with equal volume of Buffer D (20 mM Heps, 20% glycerol, 50 mM KCl, and 0.2 mM EDTA). Protein concentration was calculated using the Bradford method (Bio-Rad). For each binding reaction, 3 μg of nuclear extract was incubated in NFAT binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 0.1% Nonidet P-40, and 1 mM dithiothreitol), with antibody or cold competing oligonucleotide for 15 min, followed by the addition of 3 μg of poly(dI-dC) for 10 min. ^32P-End-labeled oligonucleotide was then added and incubated for 10 min. The resulting DNA-protein complexes were analyzed by electrophoresis in 5% polyacrylamide gels.

RESULTS

The Basal Transcriptional Activity of the Enhancer-Promoter Region of FasL Is Mediated through the 3′ 200-Basic Pair Region—We first investigated whether the 2.3-kilobase pair genomic region located 5′ upstream of the FasL translation initiation site contained the transcription initiation site. Using a commercially available technique, a major transcription initiation site was repeatedly identified from different clones to originate from nucleotide −181 with respect to the first ATG (Fig. 1). The 2.3-kilobase pair region and downstream 5′ deletions were next performed and cloned upstream of a luciferase reporter gene (pGL2). The corresponding FasL-luc reporter constructs and their relationship to the transcription initiation site and genomic sequence are outlined in Fig. 2A. Transfection of these constructs into Jurkat T cells demonstrated that all the constructs yielded detectable luciferase activity beyond the background activity of parental reporter gene. Of interest, differences in the basal transcriptional activity was observed between constructs. In repeated experiments, FasL-luc 1 and 3 provide a lower activity than 2 or 4, despite controlling for potential differences in the length and relative DNA concentration in per cell of each individual construct. Hence, suggesting the presence of potential negative regulatory regions located within −2365 and −454 or within −373 and −318 (Figs. 1 and 2).

The Inducible Transcriptional Activity of FasL Is Mediated through a Region Contained within −318 and −237 and Is Induced by Extracellular Stimuli That Trigger Increased [Ca^{2+}], and Is Inhibited by FK506—To identify regulatory domains that mediate the presumed inducible transcription of the enhancer-promoter region of FasL, a series of extracellular stimuli were tested based on the presence of cis-acting sequences identified in the genomic sequence displayed in Fig. 1.
Stimuli such as tumor necrosis factor, interferon-γ, or forskolin did not induce the transcriptional activity of FasL-luc reporter constructs, whereas such stimuli induced the transcription of reporter luc genes containing concatamers of the corresponding cis-acting sequences in the same cell type (data not shown). Only ionomycin induced transcriptional activity of the FasL enhancer region contained within FasL-luc constructs (Fig. 3A), suggesting that an ionomycin-responsive element is located within the minimal region present in FasL-luc 4. To verify the ionomycin-dependent induction of FasL transcription and to determine whether protein kinase C activation by PMA synergized with ionomycin, Jurkat T cells were transfected with FasL-luc 4 construct and stimulated or not with PMA and ionomycin, separately or in combination. As observed in Fig. 3A (right panel), the combination of PMA and ionomycin exerted effects similar to those exerted by ionomycin alone, suggesting that the inducibility of the FasL enhancer region is ionomycin-dependent and does not require protein kinase C activation. The functional effects of the combination of these two stimuli were verified using an IL2 enhancer-promoter luciferase constructs in Jurkat T cells (data not shown).

Because previous studies in Jurkat T cells have indicated that FasL mRNA can be induced after CD3 cross-linking (18–23), we investigated whether such increases in FasL mRNA could be secondary to the up-regulation of the FasL-enhancer region by transcription factors presumably induced after CD3 activation. For this, Jurkat T cells were transfected with the different FasL-luc constructs and incubated with soluble anti-CD3 or IgG isotype matched control. Similarly to that observed for ionomycin treatment, CD3 stimulation

**Fig. 1.** Genome sequence of the 5′-untranslated region of human FasL gene. The number of the left column pertains to the nucleotide location of the first in each lane with respect to the (+1) site which corresponds to the ATG codon. Each arrow underlying the corresponding sequence is numbered to highlight the different constructs used in subsequent reporter assays. The transcription initiation site (TIS) is shown, and its nucleotide location is included. The arrow indicated with an asterisk corresponds to the 3′ end of each of the six FasL constructs and through which the cloning in the pGL2 plasmid was performed. Putative cis-acting motifs are highlighted: a, SP1/Ets; b, NFAT; c, NF-κB-like. RLU, relative light units.
Calcineurin and NFAT Regulate the Inducible Transcription of FasL Gene—Based on the indication of the dependence of FasL transcription on \([\text{Ca}^{2+}]\), and its inhibition by FK506, we first investigated the role of the phosphatase calcineurin (CN), a second messenger protein that is activated by \([\text{Ca}^{2+}]\), and inhibited by FK506 and cyclosporin (38–40). To test this, we used a constitutively active form of CN that has been previously shown by our group and others to target specific cis-acting sequences present in the IL2 promoter/enhancer region. The transcription factor NFAT plays a key role in transcription of different immune relevant genes, and is activated by extracellular stimuli that increase \([\text{Ca}^{2+}]\), which in turn activates CN (reviewed in Ref. 41). To test the role of NFAT, we employed an expression vector of NFAT that was separately co-transfected with three FasL constructs (constructs 1, 4, and 5), followed by stimulation of the transfected cells with ionomycin. The expression of NFAT1(c2) induced minimal transcription from the FasL-luc constructs. However, the addition of ionomycin enhanced FasL transcription in those cell groups transfected with NFAT1(c2) (Fig. 4B). As before, the observed effects of NFAT and ionomycin were dependent upon the presence of genomic DNA sequence continued within construct 4.

Next, we determined whether the observed effects of CN and NFAT could be reversed by the presence of FK506. For this, Jurkat T cells were transfected with cDNAs of NFAT, CN, or both, together with FasL-luc 4 construct, followed by cell treatment with FK506 and ionomycin. As shown in Fig. 4C, FK506 reversed the transcription of FasL that was triggered by CN or by NFAT and/or ionomycin, indicating that CN and its target transcription factor (NFAT) are responsible for the ionomycin- or CD3-mediated activation of FasL transcription.

The Effects of CN and NFAT Are Mediated by a Distinct NFAT DNA Binding Motif—Because NFAT has generally been identified as a transcription factor that interacts with related (e.g. NF-xB) or unrelated ones (e.g. AP1) to drive transcription from a variety of immune genes (reviewed in Ref. 41), we sought to investigate whether NFAT alone was sufficient to drive FasL transcription or if it required the presence of additional ones. The putative sequence(s) present within FasL construct 4 contain an NFAT binding site (GGAAA), which in turn acti-
Jurkat T cells followed by FK506 pretreatment (10 μM) were transfected in Jurkat T cells as in Fig. 2, followed by treatment with CN (1 μg/ml, striped bar), or stimulated with PMA (20 ng/ml, gray bar), or their combination (black bar). Standard deviations are shown on the top of each bar. B, the FasL-luc plasmids were transfected in Jurkat T cells as in Fig. 2, followed by treatment with OKT3 (1 μg/ml, striped bars). C, FasL-luc 4 plasmid was transfected in Jurkat T cells followed by FK506 pretreatment (10 μg/ml) for 1 h and stimulation with ionomycin (1 μg/ml) or OKT3 (1 μg/ml). FasL enhancer beyond that observed by the combination of NFAT and ionomycin (data not shown). The functionality of each expression vector was separately tested using the corresponding enhancer promoter regions previously shown to be responsive to these different transcription factors. These negative results suggest that NFAT alone is sufficient to trigger FasL transcription.

To confirm that the effects of NFAT are mediated through a bona fide NFAT binding site, a series of mutations were introduced in the Fas-luc 4 construct, which, based on all the previous experiments, contains the minimal sequences responsible for NFAT-mediated transactivation. The transcriptional activity of the mutated Fas-luc constructs was compared with that of the native (WT) Fas-luc construct in transfection experiments using NFAT and CN as effector plasmids and ionomycin as the stimulus. As shown in a representative experiment displayed in Fig. 5A, NFAT and ionomycin did not induce the transactivation of an NFAT mutant (NFATmut) Fas-luc construct while still inducing the transactivation of the WT or an SP1/Ets mutant Fas-luc construct. Using the WT and NFATmut Fas-luc constructs, we further confirmed that the effects of the constitutively active CN are also mediated through the GGAAA cis-acting motif (Fig. 5B).

Using EMSA, we next investigated whether the functional relevance of the putative NFAT motif present in the FasL enhancer region was confirmed as an NFAT DNA binding site. In addition to Jurkat T cells, primary purified CD3 T cells were used to extend the potential relevance of this NFAT motif into a more immune relevant cell. Nuclear extracts from unstimulated or ionomycin stimulated Jurkat and primary T cells were incubated with labeled oligonucleotide corresponding to the region containing the NFAT binding site, or carrying a mutation of the GGAA motif. In addition, competition of protein-DNA binding using excess of unlabeled WT oligonucleotide or anti-NFAT antibodies were used to document the specificity of the protein binding to such DNA motif. Representative results, shown in Fig. 6, indicate that the protein complex binding the GGAAAA motif is NFAT. In addition to anti-Ets antibodies, anti-p50, p65, c-Rel, c-Fos, c-Jun, or SP1 antibodies did not result in modification of the DNA binding characteristics of the NFAT complex (data not shown), suggesting that it is NFAT alone, in the absence of combination to other transcription factors, that binds this prototype DNA motif.

CD4 Cross-linking But Not HIV-tat Induces the Transcription of the FasL Enhancer Region—Although the above experiments support the relevance of NFAT and related upstream signal transduction pathways for the induction of FasL transcription under immune related circumstances, such as T cell activation via the T cell receptor, we extended the study of NFAT and its DNA binding motif present in the FasL enhancer...
to other forms of T cell activation that have been associated to pathological conditions such as HIV infection (reviewed in Ref. 9). Previous reports have indicated that CD4 cross-linking in T cells induces a transient up-regulation of FasL mRNA, which is further enhanced by the presence of HIV-tat (42). With the availability of the FasL enhancer, we questioned whether these two HIV-related stimuli (CD4 activation and HIV-tat) would target the transcriptional regulation of FasL. First, we studied whether in primary resting T cells, activation of the CD4 receptor would trigger translocation of NFAT and, if so, whether it would specifically bind to the cis-acting motif present in the FasL enhancer. The labeled oligonucleotide encompassing the GGAAA motif was incubated with nuclear extracts from IgG- or anti-CD4 antibodies (previously shown to bind to the same CD4 epitope as HIV gp120 (Leu 3a)) (43), induced the specific binding of an NFAT site (NFAT) or calcineurin (CN) followed or not by ionomycin stimulation (iono, 1 µg/ml).

We next investigated whether HIV-tat, previously shown to synergize with CD4 cross-linking in increasing FasL mRNA levels and to induce the transcription of a variety of cellular

![Image](https://example.com/image1)

**Fig. 6.** The NFAT cis-acting sequence of the FasL enhancer-promoter binds NFAT. EMSA of nuclear extracts from Jurkat (A) or purified CD8 T cells (B) stimulated or not with ionomycin (iono, 1 µg/ml) for 30 min. Nuclear extracts were incubated with an anti-NFAT, or anti-Ets supershifting antibodies (ab) or a 10-fold excess of an unlabeled wild type NFAT oligonucleotide or a mutant NFAT oligonucleotide (NFATmut). The complex binding the NFAT cis-acting DNA sequence are indicated with an arrow.

**DISCUSSION**

The studies presented here provide an initial characterization of the genomic sequence and the transcriptional regulation of the human FasL enhancer-promoter region. In addition, they identify the transcription factor NFAT as significant and necessary in the transactivation of FasL gene transcription in lymphocyte cell lines after their activation by stimuli that trigger [Ca2+]i. Such observations explain and identify the molecular target hinted by a large body of previous information that had identified T cell activation-initiated signaling and its reversibility by calcineurin inhibitors like cyclosporin A as the principal way of indicating FasL expression. Finally, we demonstrate that CD4 activation but not HIV-tat are HIV-related stimuli whereby HIV infection may target the induction and up-regulation of FasL expression.
The most upstream region of 5′-untranslated genomic DNA sequence (2.3 kilobase pairs) identified in this study was not found to be necessary for the induction of FasL gene transcription by CD3 activation, ionomycin, or calcineurin. Whether “inhibitors” of the induction of FasL mRNA by T cell activation other than cyclosporin, such as cis-retinoic acid or dexamethasone (23), target the region located 5′ upstream from the enhancer remains to be studied. Another potential role of this large 5′ genomic DNA region is its participation in driving the constitutive level of FasL transcription, such as that observed by our group in differentiated macrophages (26, 27), although it cannot be excluded that such constitutive transcription may be also regulated within the 3′ downstream 300-base pair region. Additional features of the cloning of this portion of the FasL gene was the identification of a single transcription initiation site, which was surprisingly located significantly downstream from a putative TATA box, and the suggestion that negative regulatory regions may be present along the enhancer region and the 5′ upstream region. Further studies need to characterize in further detail such potential repressor sites, as the control of FasL expression through the interference with its transcription may be of future therapeutic value.

The observation that phorbol esters such as PMA do not increase FasL gene transcription was unexpected. The majority of studies demonstrating FasL mRNA induction in T cell lines have employed the combination of PMA and ionomycin, presumably to mimic T cell receptor-initiated signaling. The rationale of the combination of these two agents also stems from the well established necessary coordination between NFAT and members of the AP1 family for an effective transcription of the gene for IL2 (reviewed in Ref. 41), and as recently shown, of the gene for IL4 (44). In addition, NFAT has been identified to participate in a variety of other immune relevant genes including IL13, granulocyte/macrophage colony-stimulating factor, and tumor necrosis factor (41, 45–47), in which other transcription factors and proximal DNA cis-acting motifs is required, in addition to NFAT for optimal transcription (reviewed in Ref. 41). The fact in the FasL enhancer-promoter region identified in this study, no proximal or functional AP1 or other transcription factors DNA binding sites were identified to be required to enhance NFAT-driven transcription highlight the uniqueness of this enhancer-promoter and the relevance of NFAT as an independent and sufficient element in driving FasL gene transcription. A recent study, while also demonstrating that NFAT participates in the activation of FasL by TCR, indicated an additional effect of PMA and ionomycin (48); based on our results, such observation may be due to a post-enhancer mechanism mediated by PMA such as RNA stability.

From the perspective of the immune activation of T lymphocytes, it is interesting that TCR/CD3 cross-linking will result in the activation of transcription factors, such as NFAT, that target not only the gene for IL2 but also the FasL gene. The functional relevance of potentially jointly inducing FasL and IL2 transcription in the outcome of the early phases of the immune response is intriguing. Whether co-activation signals such as B7-CD28 interactions regulate (e.g. down-modulate) FasL transcription to maximize T cell proliferation after TCR/CD3 activation or whether transcription factors other than NFAT that are triggered after TCR/CD3 activation down-regulate FasL transcription needs further study. Finally, the molecular tools and antibodies used in this study have enabled to determine that NFAT1(c2) regulates and binds to the NFAT prototype DNA cis-acting motif. This is also in accordance with a recent observation in an NFAT(c2) knock-out mouse, in which FasL mRNA transcripts were absent and in which lymphoproliferation was observed (49).

The studies presented here not only indicate that NFAT mediates the CD3-dependent induction of FasL transcription, but also provide evidence that an alternative, and aberrant form of T cell activation such as that initiated solely by CD4 cross-linking induces the nuclear translocation of NFAT that binds the NFAT motif in the FasL enhancer region. This may explain how CD4 activation results in de novo induction of FasL mRNA in T cells (42). The single cross-linking of CD4 is relevant and restricted to HIV pathogenesis, as it has been postulated that HIV gp120 or defective virions provide a “negative” signal to the T lymphocyte via CD4 to either result in its death or make it susceptible to apoptosis by, e.g., Fas/FasL interactions (reviewed in Ref. 6). The fact that FasL transcription is induced by HIV infection (50) and that interference of Fas/FasL interactions, or treatment with cyclosporin A, inhibits HIV-induced apoptosis in in vitro models (26, 27, 43, 51), highlights the potential relevance of NFAT and of its inhibition in the regulation of HIV-dependent CD4 T cell apoptosis. Another HIV-related mechanism previously identified to potentially lead to apoptosis of CD4 T cells is the effect of the soluble protein HIV-tat. Although tat has been shown to enhance and increase the level of FasL mRNA induced solely by CD4 activation in primary T cells (42), tat alone did not induce de novo FasL mRNA, and as shown here, did not directly increase FasL gene transcription.

In summary, the cloning of a large region of the 5′-untranslated region of the FasL gene has identified specific regulatory regions and demonstrated that calcineurin and its target transcription factors are necessary and sufficient to induce FasL gene transcription. In addition, these findings suggest that the
ascribed role of cyclosporin A and related compounds as "immunosuppressive" agents needs to be extended by their potential role as anti-apoptotic agents. Finally, the observation that the NFAT cis-acting motif in the FasL gene is a target of CD4 activation may also be of value in pursuing further studies addressing specific aspects of HIV pathogenesis.

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