Constitutive Fas Ligand Gene Transcription in Sertoli Cells Is Regulated By Sp1*

(Received for publication, October 1, 1998, and in revised form, December 14, 1998)

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The transcriptional regulation of the Fas ligand (FasL) gene in Sertoli cells was investigated, as these cells are known to have constitutive expression of FasL and hence maintain an "immune privileged" environment within the testicle. Using the Sertoli cell line TM4, it was demonstrated that a gene segment of the 5'-untranslated region located between −318 and −237 relative to the translation start site is required for constitutive FasL transcription. Deletion and mutation analysis demonstrate that an Sp1 rather than an NFAT or NFKB-like DNA binding motif present within this region is necessary and sufficient for constitutive FasL gene transcription. Nuclear extracts of Sertoli cells contain Sp1 and Sp3 that specifically binds to the Sp1 motif present in the FasL gene, and overexpression of Sp1 but not Sp3 leads to a further increase of transcription from the FasL promoter-enhancer region. The data presented demonstrate that constitutive FasL gene transcription in Sertoli cells is regulated by Sp1. In addition, it is shown that basal FasL expression in Jurkat T cells is also controlled by Sp1 and this is in contrast to induced FasL expression, which is NFAT-dependent.

Fas ligand (FasL) is a Type II trans-membrane protein that belongs to the tumor necrosis factor family of cytokines and induces apoptosis in cells expressing the Fas receptor (Fas, CD95, APO-1) (for reviews, see Refs. 1–4). The Fas/FasL system was first identified in T cells (5, 6) where it plays a key role in eliminating T cell populations following antigenic stimulation and clonal proliferation. T cell activation leads to expression of both Fas and FasL, allowing contact-induced death via apoptosis (5, 7). The regulation of FasL gene expression in T cells is currently being elucidated (8–12). More recently, it has become apparent that the Fas/FasL system is functional in a variety of other cell types including many in which expression of these proteins is constitutive rather than inducible (summarized in Ref. 4). Of particular interest is the observation that FasL is constitutively expressed by cells in "immune privileged" sites such as testicle (13), eye (14), placenta (15, 16), and brain (17) (also see Ref. 18). In addition, several malignant cell lines and primary malignancies have now been shown to express FasL (4, 17, 19–26), and it is postulated that this is important for invasive growth, as the tumors are rendered immune privileged.

Recent cloning of the FasL enhancer-promoter region has enabled investigation of the transcriptional regulation of FasL, with initial studies focusing on the inducible regulation of FasL transcript following T cell activation (8, 10, 11). In T cells, Ca2+-dependent signals, via calcineurin, lead to an increase in the nuclear concentration of the transcription factor (NFAT) which binds to the promoter/enhancer region of the FasL gene and increases its transcription (8, 11). However, it remains unknown what regulates basal non-inducible FasL gene transcription, specifically in cells known to constitutively express high levels of FasL and maintain an immune-privileged environment.

In this study, we have investigated the transcriptional regulation of FasL in a Sertoli cell line (TM4) that is known to have constitutive FasL expression (10), and compared it to Jurkat T cells in which FasL gene transcription can be induced by specific stimuli. Results from this study demonstrate that a segment of the 5'-untranslated region of the FasL gene located between −318 and −237 relative to the translation initiation site is important for constitutive basal FasL gene transcription, and that an Sp1 but not an NFAT or NFKB consensus DNA-binding site within this region is necessary and sufficient for transcription in both Sertoli and Jurkat T cells. In addition, nuclear extracts of Sertoli cells contain high levels of Sp1 and Sp3 that specifically bind to the GGGCG consensus sequence present in the FasL gene. Overexpression of Sp1 but not Sp3 leads to increased transcription from the FasL promoter region through this specific motif in both Sertoli and Jurkat T cells. This data demonstrates that the basal constitutive FasL transcription is mainly dependent on Sp1 in these different cell types demonstrating that the control of inducible and constitutive FasL gene transcription are mediated by separate transcription factors.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—The testicular murine Sertoli cell line (TM4) was purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium/F-12 medium (Bio-wittaker) supplemented with 5% horse serum (Biovitwaker), 2.5% fetal bovine serum (Intergen), 1% glutamine, 1% streptomycin, and 1% penicillin. The Jurkat T cell line was purchased from ATCC and cultured in RPMI medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Intergen), glutamine, and antibiotics as above. Anti-Sp1(PEP2X), anti-Sp3(2D-20)-GX, anti-NFATc-K-18X, and anti-STAT3(c-20)-X antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-HA antibodies were purchased from Zymed Laboratories Inc. and unconjugated anti-rat IgG(H + L) antibodies were purchased from Pierce.

**Plasmids and Oligonucleotides**—Plasmids containing FasL promoter deletion mutants were cloned upstream of the firefly luciferase reporter gene (FasL-Luc-0 through FasL-Luc-6) and have been previously described (8). The luciferase reporter gene, pGL2-basic (pGL2-B), was purchased from Promega. Point mutations targeted three separate

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1 The abbreviations used are: FasL, Fas ligand; NFAT, nuclear factor of activated T cells; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; Luc, firefly luciferase gene; NF-B, nuclear factor κB; STAT, signal transducer and activator of transcription; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase.
The image contains a single page of a scientific document discussing the regulation of the Fas ligand (FasL) promoter. The text describes experiments involving the use of antibodies, electrophoretic mobility shift assays (EMSA), Western blots, and luciferase assays. The document also includes figures showing the constructs used in transfections and EMSAs.

**Fig. 1.** Fas ligand promoter sequence showing the mutated forms used in transfections and EMSAs. A segment of the wild-type FasL promoter sequence is shown in the top line, with three transcription factor consensus sequences (Sp1, NFAT, NFKB) and their mutated forms shown below. The point mutations are underlined in each sequence. The Sp1, NFAT, and NFKB mutations are present in the oligonucleotides used in EMSA experiments (each oligonucleotide spans approximately 288 to 283) and the FasL-Luc-3 deletion constructs used in transfections.

| Sequence       | Mutation |
|----------------|----------|
| Wild type      | AAATTGTCGGCGGAAACATTTCAGGG |
| Sp1            | -288     |
| ΔSp1           | GGGCCGGGTTCGG |
| NFAT           | -276     |
| ΔNFAT          | GGAAAGGG |
| NFKB           | -275     |
| ΔNFKB          | GAAACTTCC |
| ΔNFAT-ΔNFKB    | GAACATAAT |
| ΔSp1-ΔNFAT-ΔNFKB | GGGAACTTAACCC |
| ΔSp1-ΔNFAT-ΔNFKB | GTTICCTAACCC |

**Fig. 2.** Fas ligand promoter deletion constructs used in transfections. 5' deletions of the FasL promoter/enhancer DNA sequence were cloned upstream of the luciferase (Luc) reporter gene in pGL2-B. Numbers indicate nucleotide locations with respect to the translational start site of the FasL gene. An arrow indicates the transcription initiation site. A black box indicates the location of DNA binding motifs for three transcription factors: Sp1, NFAT, NFKB. Plasmid designations are given at the right.

**Transfections**—Gene transfections in Sertoli cells were done in duplicate using LipofectAMINE (Life Technologies, Inc.) as per the manufacturer's instructions. Sertoli cells were plated in 6-well tissue culture dishes at 0.9 x 10^5 cells per well, grown for 48 h prior to the addition of DNA suspension, and incubated for 24 h following gene transfection. In all cases, the total amount of DNA in each transfection was kept equal by the addition of control DNA (pcDNA3) where appropriate. Luciferase (Luc) and β-galactosidase (β-gal) activity were read using the Dual-Light™ chemiluminescence system following the manufacturer's instructions (Tropix, Inc.). Briefly, the cells were washed twice in phosphate-buffered saline and total cell extracts prepared in 250 μl of lysis buffer. Luciferase activity of 5 μl of extract was read using a luminometer (Lumat LB9501). The solutions were left at room temperature for 1 h before the β-gal activity was read using the same lumimeter. All experiments were repeated at least twice. Reporter gene activity is reported as relative light units which results from the ratio of luciferase units to β-gal units for each data point. Error bars on transfection data represent the standard deviations of duplicate samples. Controls performed in all transfection experiments included transfection of control DNA (pcDNA3) alone, and the empty luciferase reporter vector (pGL2-B) with the TK-β-gal reporter plasmid. In each control case, the luciferase activity was detectable, minimal, and significantly below all other data points.

Gene transfections in Jurkat T cells were done using FuGene™6 Transfection Reagent (Boehringer Mannheim) as per the manufacturer’s instructions. DNA-FuGene mixtures were incubated for 48 h with cells washed in phosphate-buffered saline and split equally for luciferase and chloramphenicol acetyltransferase (CAT) assays. Luciferase activity was determined using the “Luciferase Assay System” (Promega) following the manufacturer's instructions. CAT activity was determined using an enzyme-linked immunosorbent assay (Boehringer Mannheim) following the manufacturer's instructions. For Jurkat T cells, relative light units were calculated by normalizing the luciferase with the CAT units.
RESULTS

Constitutive Activity of the FasL Enhancer-promoter Region in Sertoli Cells Is Mediated Through an 80-Base Pair 3' Region—To determine which segment(s) of the 5'-untranslated region of the FasL gene is important for transcription in Sertoli cells, these were transfected with the FasL gene promoter constructs outlined in Fig. 2, and activity from the luciferase reporter gene measured. The results shown in Fig. 3 indicate that transcription from the FasL-Luc-0 construct was detectable, but strong and increasing transcriptional activity was detected from FasL-Luc 1 to 3, with maximal activity provided by the FasL-Luc-3 reporter gene. The transcription activity then dropped sharply, and continued to decrease from FasL-Luc-4 through FasL-Luc-6. These results indicate that an approximate 80-base pair segment of the 5'-untranslated region of the FasL gene located between −318 and −237 relative to the translation initiation site is involved in promoting transcription in TM4 Sertoli cells. This region, specifically that between −280 to −267 base pairs, contains three DNA motifs known to bind the transcription factors Sp1 (GGCGCG), NFAT (GGAAA), and NFKB (GAAACTTCC) (see Fig. 1). It should be noted that the NFKB sequence is an imperfect consensus sequence, and will be referred to as NFKB-like. Although construct FasL-Luc-0 contains the stretch of three transcription factor-binding sites, the transcriptional activity of this reporter gene was barely above background, possibly suggesting the presence of a DNA sequence upstream from −463 to which transcription factors with repressor activity bind.

An Sp1 DNA Binding Motif Is Required for Effective Constitutive Transcription from the FasL Promoter in Sertoli Cells—Using the FasL-Luc-3 construct (Fig. 2), point mutations were introduced into each of three transcription factor DNA binding motifs, as shown in Fig. 1. These constructs were then transfected into Sertoli cells, and transcription from the FasL gene promoter measured by quantifying the activity of the luciferase reporter genes. The results shown in Fig. 4 demonstrate that point mutations introduced into the Sp1 binding motif resulted in the most important reduction of basal transcription from the FasL gene. A less significant reduction was observed when the NFAT or NFKB sites were separately mutated (Fig. 4). Because of the small but detectable drop of the basal FasL gene transcription when the NFKB-like motif was mutated, we investigated whether the combination of mutation of the Sp1, NFAT, and NFKB-like motifs would be additive. As shown in Fig. 4, the combined mutation of these cis-acting motifs decreases only moderately the basal FasL gene transcription beyond that observed with the single Sp1 mutation. This data
suggests that the Sp1 cis-acting motif alone is dominant in conferring the basal FasL transcription in Sertoli cells. Nevertheless, and because of the reported interaction between Sp1 and NFKB (30), we intended to further confirm or exclude the role of the NFKB-like motif as one that may confer basal FasL gene transcription independently from Sp1. For this, we tested the role of an IKBα expression vector (in which Ser 32/36 have been mutated to alanine) which has previously been shown to inhibit both basal and inducible NFKB activity (28). Sertoli cells were transfected with the FasL-Luc-3 wt or DNFKB construct together or not with the IKBα 32/36A expression vector and the TK-β-gal expression vector to normalize for transfection efficiency. As shown in Fig. 5A, the expression of IKBα 32/36A does not interfere with the basal activity of the FasL-Luc-3 wt or DNFKB. However, in the same transfection experiment in Sertoli cells, IKBα 32/36A interfered with both the basal and inducible (tumor necrosis factor) transcriptional activity of a NFKB-Luc reporter gene (Fig. 5B). Altogether, this data implies that the Sp1 cis-acting motif controls the basal activity of FasL in Sertoli cells.

Sp1 Present in Nuclear Extracts of Sertoli Cells Binds the Sp1 DNA Binding Motif of the FasL Promoter—After demonstrating that the Sp1 DNA motif is required to convey basal transcription from the FasL promoter, we wanted to identify
the composition of nuclear proteins that interact with this cis-acting motif. Using EMSA (Fig. 6A, lanes 1 and 5), we observed that nuclear extracts from Sertoli cells contain three protein complexes that bind an oligonucleotide which contains the Sp1, NFAT, and NFKB DNA binding motifs and encompasses –288 to –263 base pairs relative to the translation initiation site (Fig. 1). These DNA binding complexes were further analyzed using specific antibodies directed to Sp1, Sp3, NFAT, NFKB, and STAT-3. The results shown in Fig. 5A demonstrate that the slower migrating protein complex (a) contains Sp1, while the two faster migrating complexes (b and c) contain Sp3. We did not demonstrate what constitutes the difference in mobility seen between complexes b and c. None of the complexes contained NFAT, STAT-3, or NFKB (NFKB; data not shown).

To confirm that Sp1 and related family members constitute the major portion of nuclear protein binding to the oligonucleotide encompassing bases –288 to –263 relative to the FasL translation start site, Sertoli cell nuclear extracts were incubated with three separate oligonucleotides, each containing point mutations in one of the three transcription factor DNA binding motifs: ΔSp1, ΔNFAT, and ΔNFKB (see Fig. 1). When the oligonucleotides are used in EMSA (Fig. 6B), only mutations introduced into the Sp1 cis-acting motif eliminated DNA binding of the protein complex containing Sp proteins (Fig. 6B, panel 1). In addition, incubation with an excess of unlabeled oligonucleotide containing mutations in either NFAT of NFKB competed away all DNA binding activity of the protein complexes bound to the 32P-labeled wild type –288 to –263 FasL oligonucleotide. An excess of unlabeled oligonucleotide containing mutations in the Sp1 site did not compete with the DNA binding of the protein complex (Fig. 6B, panel 2). These results indicate that the nuclear protein complex present in Sertoli cells, which binds to the putative Sp1 cis-acting sequence, contains at least Sp1 and Sp3.

Overexpression of Sp1 but Not Sp3 in Sertoli Cells Increases Transcription from the FasL—After showing that the Sp1 cis-acting DNA motif is necessary for the constitutive transcription of the FasL promoter in Sertoli cells, and that Sp1 and Sp3 present in nuclear extracts are major components of the nuclear protein complex that binds to this motif, we next investigated whether overexpression of Sp1 or Sp3 would modify the transcriptional regulation of the FasL promoter. Expression of Sp1 and Sp3 in Sertoli cells was first verified by performing transient transfections with Sp1 and Sp3 expression vectors in Sertoli cells followed by analysis of the transfected cell lysates in immunoblotting assays with anti-Sp1 or Sp3 antibodies. As shown in Fig. 7A, significant levels of both Sp1 and Sp3 can be detected. Fig. 7B shows the effect of Sp1 and Sp3 overexpression on the transcriptional activity of the FasL promoter in Sertoli cells. Cells were transfected with the FasL-Luc-3 or FasL-Luc-3-ΔSp1 constructs plus either the Sp1 or Sp3 expression vector, or both. The results demonstrate that overexpression of Sp1 alone leads to increased transcription from the wild-type FasL promoter, but not from the FasL-Luc promoter containing the mutated Sp1 cis-acting sequence. Overexpression of Sp3 alone has essentially no transcriptional effects on the wild type or mutated FasL promoter, and its co-expression with Sp1 does not modify the observed effects of Sp1 alone. These results demonstrate that Sp1 alone is sufficient to enhance the basal transcription from the FasL promoter through the Sp1 cis-acting sequence in Sertoli cells.

To investigate the universality of the cis-acting Sp1 site in controlling the basal transcriptional activity of the FasL gene, we studied an unrelated cell line. Jurkat T cells were co-transfected with the FasL-Luc-3 wt or FasL-Luc-3-ΔSp1 reporter constructs together or not with Sp1 or Sp3 expression vectors and a TK-CAT to normalize for transfection efficiency. As shown in Fig. 8, the basal transcriptional activity from the FasL promoter is decreased by mutations in the Sp1 DNA binding motif (columns 1 and 4). In addition, overexpression of Sp1 substantially increases transcription (column 2), while overexpression of Sp3 increases transcription to a lesser degree (column 3).
DISCUSSION

We have demonstrated that an Sp1 consensus sequence is necessary for basal transcription from the FasL promoter, and that Sp1 alone is sufficient to drive transcription from the promoter in vivo. The consensus sequence for Sp1 (GGGCGG) has been identified in the promoter regions of a variety of genes although its role in gene regulation is not understood (31). The site is known to bind Sp1, an approximately 100 kDa, zinc-requiring transcription factor, and binding results in increased transcription of the associated gene (32–34). Sp1 is essential for development in mice, as Sp1−/− embryos have retarded growth and die early in gestation (35). Some studies have suggested that Sp1 is involved in cell differentiation (36) as it has been found in highest levels in hematopoetic stem cells, fetal cells, and spermatids (35). Others have speculated that it is a major transcription factor for housekeeping genes, as the Sp1 consensus sequence is commonly found in the promoter region of these genes (37). However, recent studies and our own data indicate that many mammalian gene types are controlled by Sp1, including genes for structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors, and others (31, 37). Our report adds the FasL gene to this growing list of relevant genes regulated by Sp1.

In Sertoli cells, Sp1 alone is sufficient to induce constitutive transcription from the FasL promoter, while in several other systems studied, Sp1 acts co-operatively with other transcription factors such as AP-1 (38), E2F (39, 40), EGR1 (41, 42), and STAT 1 (43). In the human immunodeficiency virus-long terminal repeat promoter region, Sp1 has been shown to interact with an NFκB site and controls basal promoter activity (30). Although an imperfect NFκB site is present in the FasL promoter, its significance for gene regulation in Sertoli cells is unclear. Our data show that although mutations in this site slightly decreased transcription from the FasL promoter, it is not due to inhibition of NFκB binding to the site, and there appears to be no significant functional interaction between Sp1 and NFκB.

We have shown that Sp3 is present in the protein complexes bound to the Sp1 cis-acting motif in the FasL gene promoter, but it does not appear to directly affect basal transcription in Sertoli or Jurkat T cells. In other studies, Sp3 has been shown to function as either an inhibitor of gene transcription (it can compete with Sp1 for binding to the consensus sequence (44–47), or an activator (47). Sp3 activity is, therefore, dependent on both the promoter characteristics and cellular context. It will be interesting to understand the upstream regulation of Sp1, and preliminary investigations have been done using other gene systems. The data indicates that dysfunctions of Sp1 inhibitors result in increased Sp1 activity and increased gene expression. Inhibitors identified include Sp1 inhibitor (Sp1-I), which binds and inactivates Sp1 (48–50), G10BP, which competes with Sp1 for binding to GC-rich sequences in some promoters (44, 50, 51), and p107, which is a protein related to the retinoblastoma protein that also binds and inac-
activates Sp1 (52). In addition, methylation of the Sp1 consensus site can inhibit Sp1 binding (53). Inactivation of Sp3 and Sp1-I can occur by the retinoblastoma protein (48–50), and Sp3 may also be inhibited by IGF-1 in some systems (45).

Inducible FasL expression within T lymphocytes has been well characterized and plays a major role in maintaining peripherical homeostasis of T cells by limiting the expansion of proliferating cells during a normal immune response (5, 6). This process is mediated by the transcription factor, NFAT, as previously shown by our group and others (8, 10). Inducible FasL also functions as a cytoytic effector molecule for cytotoxic T lymphocytes and NK cells (54, 55). However, the significance of constitutive FasL expression by both immune and non-immune cells is less well understood. It is known that constitutive FasL expression by cells in immune-privileged sites and transplanted grafts will protect these tissues from elimination by the immune surveillance system (56, 57). The constitutive FasL expressed in tissues not considered to be immune privileged may play a role in the normal homeostasis of these tissues also (58, 59). Similarly, the constitutive expression of FasL by macrophages may be involved in controlling T cell homeostasis and play a role in human immunodeficiency virus pathogenesis. Another area in which the role of constitutive FasL expression is currently being addressed is that of malignancy. Multiple types of malignancies constitutively express FasL (17, 22, 23). We have shown here that both the constitutive expression of FasL in Sertoli cells and basal FasL transcription in T cell is controlled by Sp1. Thus, it is tempting to propose that constitutive expression of FasL in general may be controlled by Sp1, while inducible transcription may be at least partially controlled by NFAT. This would be consistent with prior data showing NFAT to be critical for the inducible expression of many other cytokine genes (60). Future investigations of FasL gene transcription in additional cell types should clarify this issue.

Acknowledgments—We acknowledge the assistance given by all members of the Paya laboratory and the excellent editorial assistance of Teresa Hoff.

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