Structure/Function Analysis of the Murine CD95L Promoter Reveals the Identification of a Novel Transcriptional Repressor and Functional CD28 Response Element

Scott A. Crist, Thomas S. Griffith, and Timothy L. Ratliff

From the Department of Urology and the Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, Iowa 52242

CD28 costimulation, an important second signal for antigen-mediated T cell activation, is known to enhance expression of several genes important for the regulation of CD4+ T cell effector function including interleukin-2 and CD154. Previous studies demonstrate CD28-mediated enhancement of the transcription and expression of Fas ligand (CD95L) in T cell lines, suggesting a regulatory link between CD28 and CD95L expression. These results served as the basis for structure/function analysis of the CD95L promoter to elucidate the mechanism for CD28-mediated enhancement of CD95L. In this report, we describe a novel response element, located at -210 to -201 bp upstream of the transcription start site, that confers CD28 responsiveness to the CD95L gene. This response element is homologous to the CD28 response element (CD28RE) previously identified in the IL-2 promoter and bears structural similarities to a newly identified CD28RE in the CD154 promoter. We further demonstrate that CD28-mediated enhancement of promoter activity correlates with enhanced expression of CD95L mRNA, cell surface expression of CD95L protein, and increased apoptosis of CD95+ target cells. These results demonstrate a direct transcriptional regulatory role for CD28 in CD95L-mediated functional activity in CD4+ T cells. Mutational analysis of the CD95L promoter also reveals a novel transcriptional repressor element located -60 bp 5' of the CD28RE. The repressor element bears sequence homology to an activator protein-1 element, constitutively binds c-Fos but not c-Jun, and is activation-independent.

Ligation of receptors on the surface of T cells induces a series of signaling events culminating in specific gene expression via the activation and translocation of specific transcription factors to the nucleus. Ligation of the T cell receptor (TCR)1 is known to activate several transacting factors that mediate cytokine induction and proliferation in activated T cells. NF-κB and members of the activator protein-1 (AP-1) and nuclear factor of the activated T cell (NF-AT) families of transcription factors are dramatically activated by TCR signaling, including the NF-κB, Rel and AP1 family of proteins (19, 20). However, the transcription complex only binds the CD28RE after CD28 costimulation (17, 18). The multiprotein complex that binds to the CD28RE is composed of several transcription factors ubiquitously activated by TCR signaling, including the NF-κB, Rel and AP1 family of proteins (19, 20). However, the transcription complex only binds the CD28RE after CD28 costimulation (3, 21). Whereas the identified CD28REs in CD28-responsive genes other than IL-2 have some sequence homology with the IL-2 CD28RE, no specific motif has yet been defined. Instead, the response elements in other CD28-responsive genes have been defined empirically, through structure/function analysis of the promoter regions.

Fas ligand (CD95L), a type II transmembrane protein expressed by activated T cells, is a potent mediator of apoptosis in cells that express its cognate receptor CD95 (22, 23). CD95L is the primary mediator of cytolytic activity in CD4+ T cells and
therefore plays a critical role in effector function of CD4+ T cells (14, 23–33). Previous work by our laboratory has demonstrated CD28-dependent enhancement of transcriptional activity in CD4+ T cells isolated from CD95L promoter/luciferase reporter transgenic mice (16). This observation, when combined with other published studies demonstrating enhanced CD95L-dependent cytotoxic activity of T cell lines after co-stimulation via CD28, suggests a regulatory link between CD28 co-stimulation and CD95L expression (34, 35).

The focus of this study was to identify the role and mechanism of CD28-mediated enhanced CD95L expression in CD4+ T cells. Through the use of deletion/mutation mapping of the murine CD95L promoter, we identified a novel response element that conferred CD28 responsiveness to the CD95L gene. This response element is homologous to the CD28RE in the IL-2 promoter and bears structural similarities to a newly identified CD28RE in the CD154 promoter (9, 18). Moreover, the IL-2 promoter and bears structural similarities to a newly identified CD95L promoter, we identified a novel response element in CD4+ T cells. In addition, during the course of our studies, a response element with sequence homology to an AP-1 binding element was demonstrated to attenuate transcriptional activity of the CD95L promoter, suggesting a dual role for the AP-1 family of transcription factors in the modulation of CD95L.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—The T cell hybridoma line A1.1 and the L1210 and L1210-Fas cell lines have been previously described (33, 34). The cell lines were maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum, 10 mM glutamine, and 100 μg/ml penicillin/streptomycin at 37° C and 5% CO2. The antibodies to CD3 (145-2C11) and murine CD95L (mFL3) were obtained from Pharmingen (San Diego, CA). The agonistic CD28 antibody (clone 37.51) was obtained from e-Bioscience (San Diego, CA).

**Plasmid Constructions**—Genomic DNA was isolated from A1.1 cells using DNAeasy kit (Qiagen, Valencia, CA). A 720-bp PCR fragment corresponding to the promoter region of CD95L (−689 to +65) was generated using the upstream primer 5′-GATCCTAGTTTCTACTGGTACCCAGAG-3′ and the downstream primer 5′-GCCACCCAGCCAGGAGGATTG-3′ in a PCR using platinum Pf1 high fidelity polymerase (Invitrogen) and 50 ng of A1.1 genomic DNA template. The resultant 720-bp fragment was gel-purified, an A overhang was generated by treatment with T4 polynucleotide kinase (New England Biolabs) in 1 mM dithiothreitol, and ligated into the Topo-PCR2.1 T/A cloning vector (Invitrogen) and dATP for 15 min, and cloned into the Topo-PCR2.1 T/A cloning vector (Invitrogen) and sequenced for orientation and fidelity. This plasmid (PCR2.1−689pCD95L) served as the insert for the subsequent plasmid constructions. To make the −689 to +65 luciferase reporter plasmid, the 720-bp insert was excised with SacI and XhoI and ligated into the SacI/XhoI site of pG5-3 Basic luciferase vector (Promega, Madison, WI).

To make the 5′ truncation mutants, shorter fragments were generated using new upstream primers, and the common downstream primer was used to make the −689 to +65 insert. For the −253 to +65 fragment, the upstream primer was 5′-CGAGAACCTGGTGCAGAACATT- TCTGGGC-3′. The −321 to +65 fragment, the upstream primer was 5′-CGAGAACCTGGTGCAGAACATT- TCTGGGC-3′. The −138 to +65 fragment was produced by using the upstream primer 5′-GGTTCCTGCATCT- CACATGCTGCATCACTGC-3′, and the downstream primer 5′-GGTTCCTGCATCT- CACATGCTGCATCACTGC-3′. The samples were added to the polylinker site of Topo PCR2.1 T/A vector and sequenced for orientation and fidelity. Luciferase reporter constructs were produced by excising the various fragments with SacI and XhoI and then subcloning the fragments into the polylinker site of pG5-3 Basic.

Site-directed mutations to putative elements in the CD95L promoter region of the reporter constructs were produced using the PCR-ligation-PCR method previously described (36). Briefly, two fragments flanking the region to be deleted or mutated were produced using specific primers and Pfu polymerase (Invitrogen). The fragments generated were gel-purified, phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA), mixed, and ligated with T4 ligase (Invitrogen). Finally, full-length fragments containing the internal deletions or mutations were amplified using the 5′ and 3′ distal primers. The fragment was subcloned into Topo 2.1 PCR, sequenced for fidelity and orientation, and then subcloned into pG5-3 Basic.

**Transfections/Luciferase Assays**—A1.1 cells (104) were transfected using DEAE-dextran with 10 μg of the indicated reporter plasmid and 1 μg of the pCMV-Gal control vector. Twenty-four hours following transfection, the cells were replaced in duplicate on uncoated 6-well plates or plates coated with either anti-CD3 (5 μg/ml) alone or with anti-CD28 (10 μg/ml) for 4 h at 37° C. Following incubation, cells were harvested by repeated washing with PBS and centrifuged, and extracts were prepared by lysing cells in 50 μl of reporter lysis buffer (Promega). Luciferase activity was determined by mixing 40 μl of 100 μl of luciferase substrate (Promega) and immediately reading the sample in a Monolight 2010 luminometer (BD Biosciences, Franklin Lakes, NJ). β-Galactosidase activity was determined by mixing 20 μl of extract with galactolite β-galactosidase substrate (Tropix, Bedford, MA), incubating for 1 h at 25° C, adding 300 μl of galactolite accelerator (Tropix), and reading the sample in the luminometer. Luciferase activity was normalized by dividing the mean luciferase RLU by the mean β-galactosidase RLU. The normalized luciferase RLU from the stimulated samples were divided by the normalized RLU of the untreated sample, and values were expressed as “normalized -fold induction.”

**Preparation of Nuclear Extracts**—After stimulation on the antibody-coated plates, cells were washed by washing with cold PBS and centrifuged at 1100 rpm for 5 min to pellet. All subsequent steps were done on ice. The cell pellet was resuspended in 200 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KC1, and 0.5 mM dithiothreitol) and lysed by passing through a 28-gauge needle four times. The nuclei were then pelleted by centrifugation for 10 s, and the supernatant was aspirated. The crude nuclear preparation was then extracted by adding 120 μl of buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 420 mM KC1, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride (added fresh just before use) and incubating for 15 min on ice. 120 μl of buffer D was then added (20 mM HEPES, pH 7.9, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.5 mM sodium orthovanadate), and the sample was sedimented. The double-stranded oligonucleotides were 5′-end-labeled by mixing 1 pmol of double-stranded oligonucleotide with 10 μl of E. coli T4 polynucleotide kinase (Novagen) in a 1× PNK buffer and incubating for 1 h at 37° C. After incubation, the end-labeled double-stranded oligonucleotides were purified from free ATP by passing over a NICK column (Amersham Biosciences). A total of 7 μl of nuclear extract corresponding to 5–10 μg of protein was mixed with 1 μl of radiolabeled oligonucleotide (−50,000 cpm) in a reaction mix containing 1× binding buffer (10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, 1% (v/v) glycerol) and 1 μg of poly(dI- dC) (Amersham Biosciences) as a nonspecific inhibitor in a final volume of 10 μl. The samples were then incubated at 4°C over several bands (until no further supershift was observed) and the resulting double-stranded oligonucleotides were subjected to electrophoresis.
used as a DNA template for amplification by PCR. For detection of murine CD95L, the forward and reverse primers were 5'-CTT-GGGCTCTCCAGGGTACGT-3' and 5'-CTCTCCATCTGGCACACCCCATC-3', respectively. The PCR products generated from the CD95L primers were normalized against PCR products generated from murine β-actin forward (5'-TCATGAAGTGGTGACCTGGACATCCCGTAAAG-3') and reverse (5'-CCCTGCTGCTGTCGACTGGAGG-3'), after electrophoresis on a 1.5% agarose gel and visualization of the PCR product with UV light. The band intensity of PCR products was determined using an EpiChemi digital image analysis system (UVP, Upland, CA).

Flow Cytometry—Following stimulation, 10⁶ A1.1 cells were harvested by repeated washing with cold PBS and centrifuged, and the pellet was resuspended in 50 µl of fluorescence-activated cell sorting wash (PBS, 1% fetal calf serum, 0.05% sodium azide), containing 1 µl of biotinylated anti-murine CD95L antibody MFL3 (Pharmingen) or 1 µl of biotinylated isotype control antibody, and incubated on ice for 20 min. Cells were washed twice with fluorescence-activated cell sorting cell sorting wash and resuspended in 50 µl of fluorescence-activated cell sorting wash and streptavidin-phycocerythrin (Sigma) for 10 min on ice. Cells were washed three times and resuspended in 300 µl of PBS containing 3% paraformaldehyde. Samples were read on a Becton-Dickinson FACScan (San Jose, CA), and data were analyzed using CellQuest software (Becton-Dickinson).

Cytotoxicity Assays—The functional activity of CD95L expressed by antibody-stimulated A1.1 and primary CD4+ T cells was determined by the ability of stimulated, costimulated, or nonstimulated cells to induce apoptosis in Fas+ L1210 target cells. Briefly, L1210 and L1210-Fas cells were labeled with 200 µCi/ml 51Cr (1 mCi/ml; Amersham Biosciences) for 1.5 h at 37 °C and washed three times in complete medium. A1.1 cells or recently activated primary T cells were incubated for 4 h on flat bottom 96-well microtiter plates (BD Biosciences) coated with anti-CD3 antibody, anti-CD28 antibody, both, or neither before the addition of the radiolabeled targeted cells. The plates were incubated at 37 °C for 16 h before the supernatants were harvested using Skatron supernatant harvesting frames (Sterling, VA) and counted on a Cobra II gamma counter (Packard Instrument Co.). The percentage of specific release was calculated as follows: 100 × (cpm of experimental – cpm of spontaneous release)/cpm of maximum release – cpm of spontaneous release). The results are expressed as the mean ± S.E. of triplicate wells per assay and are representative of at least four similar analyses.

Preparation of Primary T Cells—Single cell suspensions of splenocytes were obtained from D011.10 TCR transgenic mice following red blood cell lysis. CD4+ T cells were enriched to greater than 96% CD4+ as determined by flow cytometry using a negative selection/magnetic bead CD4+ T cell isolation kit from Miltenyi Biotech (Sunnyvale, CA). The CD4+ T cells were stimulated and expanded for 2 weeks in coculture with a 10-fold excess of irradiated splenocytes (30 grays) obtained from BALB/c mice that were pulsed with OVA323-332 peptide (Research Genetics, Huntsville, AL). The medium was supplemented with 100 units/ml recombinant murine IL-2 (R&D Systems, Minneapolis, MN). Following co-culture, dead cells were removed by Ficoll density centrifugation, and the remaining viable cells were rested by placing them back in culture for 24 h in complete medium containing 100 units/ml IL-2. For the cytotoxicity assays, the recently activated, rested T cells were harvested and replated on antibody-coated plates as described above.

RESULTS

Activity of the CD95L Promoter Is Induced by TCR Ligation and Enhanced by CD28 Costimulation—To define the CD28-responsive sequence elements within the CD95L promoter, we utilized deletion/mutation mapping of the 5′ regulatory region of murine CD95L using reporter constructs and transient transfection. The readily transfectable A1.1 T cell hybridoma served as an in vitro cell model for this objective. This line has been used extensively in studying the regulation of CD95L expression, mutational analyses were initiated to determine whether a response element sensitive to CD28 was contained within the −689 bp region of the CD95L promoter. Initially, A1.1 cells were transfected with a luciferase construct encoding
the minimal inducible promoter region (~256 to +45 bp) of the murine IL-2 gene to serve as both a control for CD28-mediated enhancement of promoter activity and a comparison of relative promoter activities following activation. As shown in Fig. 1B, TCR-mediated signaling induced a 4-5-fold increase in luciferase activity over unstimulated cells. Costimulation through CD28 further enhanced CD95L transcriptional activity an additional 4-5-fold over the activity observed with TCR stimulation alone. As expected, CD28 costimulation greatly increased transcriptional activity of the minimal IL-2 reporter construct (35-fold) versus stimulation only through the TCR (Fig. 1B). These data show that the ~689 region is sufficient for TCR and CD28-mediated activation and served as a basis for mutational analysis.

**The ~210 to ~201 Region of the CD95L Promoter Is a Functional CD28RE**—To identify the CD28-responsive element within the 689 bp region, a series of luciferase constructs containing serial 5’ truncations to the ~689 bp sequence were generated (Fig. 2A). The truncation mutant reporter constructs p324 (~324 to +65), p253 (~253 to +65), p201 (~201 to +65 bp), p168 (~168 to +65), and p135 (~135 to +65), along with the p689 construct, were assessed for transcriptional activity following activation. Whereas differences in the relative activity of the various constructs were noted, a comparison of normalized -fold induction of the αCD3-stimulated and the αCD3- and αCD28-co-stimulated constructs demonstrated that truncation of the CD95L promoter from ~253 to ~201 bp abrogated CD28-mediated transcriptional enhancement of the CD95L promoter (Fig. 2B). These results suggest that sequence upstream of ~201 bp confers CD28 responsiveness.

A motif search of the ~253 to ~201 bp region of the CD95L promoter revealed a 10-bp sequence located from ~210 to ~201 with homology to the CD28RE of the IL-2 promoter (murine CD95L (GGAACTTCCA) versus murine IL-2 CD28RE (AA-GAAATTCG); the conserved sequence is underlined). Based on this homology, the functionality of this region as a CD28RE in the CD95L promoter was determined. Site-directed mutagenesis was used to generate constructs in p253 with mutations generated in the ~210 to ~201 region. In one variant (p253mut1), the ~210 to ~201 region was deleted. The second variant was constructed with the sequence of the same region randomized (p253mut2). The p253, p253mut1, and p253mut2 constructs were transfected, and cells were stimulated as previously described. As predicted, both mutations to the ~210 to ~201 region of p253 abrogated the CD28-mediated enhancement of transcriptional activity (Fig. 3A), demonstrating that the ~210 to ~201 bp region confers CD28 responsiveness to the p253 reporter construct.

Because of the differences in the relative transcriptional activity between the full-length p689 and the p253 constructs, we wanted to confirm the importance of the ~210 to ~201 region in the context of the longer upstream sequence. To this end, the ~210 to ~201 bp region in p689 was deleted (p698mut1) and tested for CD28 responsiveness. Similar reduction in CD28 transcriptional enhancement was observed in p698mut1 as observed in the shorter p253mut1 and p253mut2 constructs (Fig. 3B). Thus, these data identify the ~210 to ~201 region as critical for CD28-mediated enhancement of transcription of the CD95L gene.

**CD28 Costimulation Enhances mRNA, Protein, and CD95L-Mediated Lytic Activity in Both Primary CD4+ T Cells and A1.1 T Cell Hybridoma Cells**—To determine whether the increased transcriptional activity observed with CD28 stimulation correlated with enhanced CD95L-mediated function, we first determined whether CD28 costimulation increased steady state levels of CD95L mRNA and cell surface protein expression. CD95L mRNA expression by A1.1 T cell hybridoma cells was assessed using semi-quantitative reverse transcriptase-PCR after TCR stimulation with or without CD28 costimulation using agonistic anti-CD3 and anti-CD28 antibodies, respectively. The strength of TCR-mediated stimulation was titrated using various amounts of soluble anti-CD3 antibody. Fig. 4A demonstrates that CD28 costimulation enhances CD95L mRNA steady state levels over TCR stimulation alone. Greater CD28-mediated enhancement was observed with suboptimal TCR signaling via lower levels of anti-CD3 (3.6-fold enhancement with 0.1 μg/ml anti-CD3 versus 2.3–2.7-fold enhancement at higher levels of anti-CD3), suggesting that costimulation is required for optimal activation. To determine whether enhanced steady state levels of mRNA correlates with enhanced cell surface expression of CD95L protein, A1.1 cells were stimulated on antibody-coated plates as before, harvested, stained with an antibody specific for murine CD95L, and subjected to flow cytometry. As indicated in Fig. 4B, an increase in the number of cells staining positive for surface CD95L expression is seen in A1.1 cells costimulated with anti-CD28 over TCR stimulation alone, demonstrating CD28-mediated enhancement of cell surface CD95L protein expression correlates with enhanced levels of mRNA.

Based on these results, we next determined whether en-
Fig. 3. The −210 to −201 region of the CD95L promoter is required for CD28-mediated enhancement of transcriptional activity. A1.1 cells were transiently transfected with the p253 luciferase construct containing a deletion to the −210 to −201 region (p253mut1) or random −210 to −201 sequence (p253mut2) (A) or the p689 construct containing a deletion of the −210 to −201 region (p689mut1) (B). The transfected cells were left unstimulated (NS) or stimulated on immobilized anti-CD3 antibody only (αCD3) or both anti-CD3 and anti-CD28 antibodies (αCD3/αCD28) for 4 h. The luciferase activity, normalized to β-galactosidase activity of the stimulated cells, was compared with the normalized activity of the transfected but not stimulated cells and expressed as normalized-fold induction. The bars represent the S.E. of duplicate transfections. These data are representative of at least three independent experiments.

Fig. 4. CD28 costimulation enhances CD95L mRNA and protein. A, expression of CD95L mRNA was determined by reverse transcriptase-PCR of total RNA isolated from A1.1 cells stimulated with the indicated amounts of immobilized anti-CD3 (αCD3) and/or anti-CD28 (αCD28) for 4 h with murine CD95L-specific primers. Band intensity was normalized to the β-actin product generated using specific primers to murine β-actin. The PCR products were resolved on a 1.5% agarose gel. Bands were visualized by staining with ethidium bromide, and optical density was determined using densitometry. Relative induction was determined by comparing the normalized CD95L band intensity (CD95L intensity/β-actin intensity) of the anti-CD3-stimulated cells with that of the cells stimulated with both anti-CD3 and anti-CD28. Data shown are representative of two independent experiments. B, A1.1 cells were left unstimulated (NS) or stimulated either immobilized anti-CD3 antibodies (αCD3) or both anti-CD3 and anti-CD28 antibodies (αCD3/αCD28) for 4 h. Cell surface expression of CD95L was determined by flow cytometry. A histogram of the fluorescence of unstimulated A1.1 cells (NS, gray-filled histogram), anti-CD3-stimulated A1.1 cells (αCD3, thin black line), and both anti-CD3 and anti-CD28-stimulated A1.1 cells (αCD3/αCD28, thick black line) is shown. The data shown are representative of four independent experiments.
where truncations to the promoter region increased inducible transcriptional activity, seemingly through the removal of upstream undefined inhibitory elements (9, 14). Our data show that stimulated cells transfected with the p253 construct had 20–30-fold higher inducible transcriptional activity than the p324 construct, although the relative level of CD28-mediated enhancement of activity is similar. These data suggest the presence of a transcriptional repressor element within the /H11002 to /H11002 bp region. To further localize this element, additional 5’ truncations were made to the /H11032 bp region. The truncation mutant reporter constructs p309 (/H11002 to /H11001 bp), p298 (/H11002 to /H11001 bp), and p276 (/H11002 to /H11001 bp), along with the p324 and p253 constructs, were assessed for transcriptional activity following activation (Fig. 6). Fig. 6B shows that a 25-bp region between /H11002 and /H11002 strongly attenuated transcriptional activity of the CD95L promoter. A motif search reveals the /H11002 to /H11002 bp region bears sequence homology to an AP-1 binding element. To assess whether repressor function is dependent on the putative AP-1 element, the activation-dependent transcriptional activities of the p276, p276mut (GGACTCAGG to GAGCTAATG; mutated bases underlined), and p253 constructs were compared. Fig. 6C shows that mutation to the putative AP-1 element abrogated repressor activity in the p276 construct. Further, mutation to the AP-1 element also relieved transcriptional repression in the context of the /H11002 region (Fig. 6C), suggesting that transcriptional repression of CD95L is dependent on an intact AP-1 element in the /H11002 bp region.

To verify that the /H11002 to /H11002 bp region is a binding site for AP-1 family proteins, electrophoretic mobility shift assay and supershifting were performed using nuclear extracts prepared from unstimulated and aCD3- and aCD3/28-activated A1.1
Costimulatory signals play a critical role in T cell fate including proliferation, effector cell function, and cell death, specifically activation-induced cell death (14, 22–24, 26, 28, 29, 32, 33, 39). The association of cytokines, such as IL-2, to each of these possible aspects of T cell physiology is clearly established, making IL-2 prototypical of costimulation-regulated genes. CD28 is the most widely studied costimulatory molecule expressed by the T cell. For this reason, numerous studies have investigated the mechanism of CD28-mediated regulation of IL-2 (3, 6, 9). The observation that CD28 costimulation enhanced the expression of IL-2 mRNA in the presence of translational inhibitors such as cyclohexamide suggested that the biochemical events associated with CD28 ligation were acting directly at the level of transcriptional regulation and by an “immediate early” pathway (9). Specifically, preformed factors capable of modulating transcription were being activated, translocating to the nucleus and binding to specific elements in the regulatory region of the IL-2 gene (3, 6, 8, 9). By establishing this direct regulatory pathway, a model of costimulation-mediated regulation of gene programs associated with T cell fate was established. Subsequently, the expression of other genes has been linked to the CD28 response element defined for IL-2, including CD154 (17, 18). We present evidence in the current study that CD95L, a molecule that plays a critical role T cell fate, follows a similar model of regulation.

Previous studies established a critical role for CD95L in T cell homeostasis via its role in activation-induced cell death, as a mediator of cytotoxic activity in murine models of human disease such as experimental autoimmune encephalomyelitis, and in peripheral tolerance (30, 31, 34, 35, 40). The majority of the previous studies investigating CD95L regulation focused on TCR-mediated induction of transcriptional activity, because TCR signaling alone is sufficient to induce mRNA, protein, and CD95-dependent cytolytic activity including activation-induced cell death. These studies established direct regulatory pathways linking TCR-mediated signaling and activation of nuclear factors that mediate transcription of CD95L such as nuclear factor of activated T cells, the Fos/Jun dimers, and NF-κB (12, 15, 19, 20, 38, 41–44). The results from the current study are in agreement with these data, demonstrating that signaling via anti-CD3 induces transcriptional activity and expression of mRNA, protein, and CD95-dependent apoptosis over unstimulated A1.1 cells. Although earlier studies showed that CD28 costimulation enhanced CD95L-dependent cytotoxicity in T cell lines, a regulatory link between CD28 stimulation and CD95L expression was not established (30, 31, 34, 35, 40). Norian et al. (16) were the first to show CD28-mediated enhancement of CD95L transcriptional activity in CD4+ T cells. Subsequently, studies investigating inducible gene programs associated with T cell costimulation utilized gene expression arrays to identify CD95L among hundreds of other CD28 costimulation-responsive genes (45, 46). Whereas these studies have identified CD95L as CD28-responsive, the precise mechanism for CD28-mediated coregulation was not defined.

The current study demonstrates that CD28-mediated transcriptional control of the murine CD95L promoter is mediated
via a short element located −200 bp upstream of the transcription start site and is homologous with the CD28RE of the IL-2 promoter. Although functionally similar to the CD28RE of the IL-2 gene with respect to the ability to enhance gene expression, the current study demonstrates that deletion or mutation of the CD28RE of the CD95L promoter diminishes both CD28-mediated enhancement and TCR-inducible transcriptional activity. These data are in contrast with previous studies for IL-2 gene expression showing that mutation of the CD28RE in the IL-2 promoter abrogated CD28-mediated enhancement but had no effect on TCR-mediated transcriptional activity (9). Similar to CD95L, however, loss in TCR-inducible activity following mutation of the functional CD28RE also was observed for the CD154 gene (17, 18). These data suggest that although CD28-mediated transcriptional regulation via homologous CD28REs of different genes is similar, the role of these elements in modulating overall transcriptional activity is gene-specific and contextual within the promoter regions of the different CD28-responsive genes. Although the present study did not directly assess whether functional CD28REs are present in the human CD95L promoter region, a motif search of the human CD95L promoter reveals several sequences bearing homology to the core sequence of the CD28RE in the murine CD95L promoter (GAAanTTC) located within 350 bp upstream from the transcription start site. Previous studies investigating costimulation-mediated enhancement of transcription of other human genes, including IL-2, IL-3, and granulocyte-macrophage colony-stimulating factor, have demonstrated that the functional CD28REs of the aforementioned genes are localized to a few hundred base pairs proximal to the transcription start sites (6, 9). Deletional/mutational analysis, however, is required to confirm CD28RE function in the human CD95L gene.

The present study also identified and partially characterized a novel repressor element within the murine CD95L promoter. Several groups investigating CD95L regulation in human T cells have reported that specific promoter regions diminish activation-dependent transcription (13, 14). Previous studies have shown that deletions of the −2365 to −454 bp, the −900 to −486 bp, and the −1204 to −860 bp regions of the human CD95L gene enhance basal and inducible transcriptional activity (13, 14, 47). Taken together, these previous studies suggest the presence of multiple elements capable of attenuating both basal and inducible transcriptional activity of the human CD95L promoter. Our current data demonstrate that the −270 to −260 bp repressor element constitutively binds c-Fos, a member of the AP-1 family of proteins previously shown to be associated with transcriptional activation of CD95L, IL-2, and other cytokines in the activated T cell. The ability of c-Fos to repress activation-dependent transcriptional activity via the binding of AP-1 elements in the promoters of several genes has been previously described (48–51). The observation that c-Fos was constitutively bound to the AP-1 element and that the reporter constructs possessing a functional repressor AP-1 element were not hindered in the ability to be transactivated by TCR and CD28-mediated co-activation is novel. A motif search of the human CD95L promoter reveals several putative AP-1 elements located within each of the regions shown to repress basal and inducible transcriptional activity. Whether the multiple putative AP-1 elements located throughout the human CD95L promoter region mediate transcriptional repression will have to be determined empirically. These data suggest the possibility that AP-1 elements within the murine CD95L promoter and perhaps other gene promoters play roles as not only positive and negative transcriptional regulators but also as constitutive transcriptional “attenuators.” Further analysis of other repressor elements in AP-1-responsive genes such as IL-2 is required to verify this hypothesis.

In summary, the present study identified two mechanisms for regulating CD95L, a gene that plays a key role in CD4+ T cell effector function and cell fate. First, we identified a functional CD28RE in the murine CD95L promoter that suggests a direct regulatory mechanism for co-stimulation-mediated induction of CD95L. Similar to studies investigating CD28-mediated regulation of IL-2 expression, the current study demonstrated for the first time CD28-mediated transcriptional regulation of CD95L correlated with increased steady state levels of mRNA, protein, and CD95L-mediated lytic activity. The CD28RE identified in the promoter of the CD95L shares both sequence homology and CD28 functional activity with the CD28REs of IL-2, CD154, and other cytokine genes by playing a major role in TCR-mediated transcriptional regulation. By identifying a direct mechanism of CD28 coregulation of CD95L in this study, CD95L is now added to a growing list of costimulation-regulated or enhanced genes that play major roles in T cell function. The present study also identified and characterized a transcriptional repressor element in the CD95L promoter that binds the AP-1 family protein c-Fos. Whereas several groups have reported transcriptional repressors in the CD95L gene, the distal AP-1 element appears to attenuate transcriptional activity rather than block TCR- or CD28-mediated transcriptional activation, suggesting a novel role for AP-1-mediated regulation. Further studies are required to determine whether this novel function of AP-1-mediated regulation plays a role in the transcriptional regulation of IL-2 or other TCR-mediated cytokine genes.

REFERENCES

1. Coueronniere, N., Villalba, M., Englund, N., and Altman, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3394–3399
2. Villalba, M., Kasibhatla, S., Genestier, L., Mahboubi, A., Green, D. R., and Altman, A. (1999) J. Immunol. 163, 5813–5819
3. Appleman, L. J., Berezovskaya, A., Grass, I., and Boussiotis, V. A. (2000) J. Immunol. 164, 144–151
4. Lenschow, D. J., Walunas, T. L., and Bluestone, J. A. (1996) Annu. Rev. Immunol. 14, 253–258
5. Michel, F., Attal-Bonnetoy, G., Mangino, G., Mise-Omata, S., and Acuto, O. (2001) Immunity 15, 935–945
6. Magwire, S. B., Harhaj, E. W., and Sun, S. C. (1997) Mol. Cell. Biol. 17, 2605–2614
7. Michel, F., Mangino, G., Attal-Bonnetoy, G., Tuosto, L., Alcover, A., Roumier, A., Olive, D., and Acuto, O. (2000) J. Immunol. 165, 3820–3829
8. Nguyen, T., Kalden, J. R., and Manger, B. (2000) Eur. J. Immunol. 30, 1632–1637
9. Fraser, J. D., Irving, B. A., Crabtree, G. R., and Weiss, A. (1991) Science 251, 313–316
10. Harhaj, E. W., and Sun, S. C. (1998) J. Biol. Chem. 273, 25185–25190
11. Harhaj, E. W., Magwire, S. B., Good, L., and Sun, S. C. (1996) Mol. Cell. Biol. 16, 7676–7680
12. Parra, M., Kokot, N., Latinis, R., Kasibhatla, S., Green, D. R., Koretzky, G. A., and Nel, A. (1998) J. Immunol. 160, 134–144
13. Holz-Heppelmann, C. J., Algerciras, A., Badley, A. D., and Paya, C. V. (1998) J. Biol. Chem. 273, 4416–4423
14. Kasibhatla, S., Genestier, L., and Green, D. R. (1999) J. Biol. Chem. 274, 987–992
15. Norian, L. A., Latinis, K. M., and Koretzky, G. A. (1998) J. Immunol. 161, 1078–1082
16. Norian, L. A., Latinis, K. M., Eliason, S. L., Lyson, K., Yang, C., Ratliff, T., and Koretzky, G. A. (2000) J. Immunol. 164, 4471–4480
17. Lindgren, H., Axenro, K., and Leandersson, T. (2001) J. Immunol. 166, 4578–4585
18. Parra, E., Mustelin, T., Dohlsten, M., and Mercola, D. (2001) J. Immunol. 166, 2437–2443
19. Matsui, K., Xiao, S., Fine, A., and Ju, S. T. (2000) J. Immunol. 164, 3002–3008
20. Matsui, K., Fine, A., Zhu, B., Marshak-Rothstein, A., and Ju, S. T. (1998) J. Immunol. 161, 3469–3473
21. Xiao, S., Matsui, K., Fine, A., Zhu, B., Marshak-Rothstein, A., Wiedom, R. L., and Ju, S. T. (1999) Eur. J. Immunol. 29, 3456–3465
22. Brunner, T., Yoo, N. J., Laface, D. W., False, C., and Green, D. R. (1996) Int. Immunol. 8, 1017–1026
23. el Khatib, M., Stanger, B. Z., Dogan, H., Cui, H., and Ju, S. T. (1998) Cell. Immunol. 163, 237–244
24. Brunner, T., Yoo, N. J., Griffith, T. S., Ferguson, T. A., and Green, D. R. (1996) Behring Inst. Mitt. 161–174
25. Wang, J. K., Ju, S. T., and Marshak-Rothstein, A. (2000) Eur. J. Immunol. 30, 931–937
26. Ettlinger, R., Panka, D. J., Wang, J. K., Stanger, B. Z., Ju, S. T., and Marshak-Rothstein, A. (1995) J. Immunol. 154, 4302–4308
27. Ju, S. T., Panka, D. J., Cui, H., Ettinger, R., el Khatib, M., Sherr, D. H., Stanger, B. Z., and Marshak-Rothstein, A. (1995) *Nature* **373**, 444–448

28. Kirchhoff, S., Muller, W. W., Krueger, A., Schmitz, I., and Krammer, P. H. (2000) *J. Immunol.* **165**, 6293–6300

29. Kirchhoff, S., Muller, W. W., Li-Weber, M., and Krammer, P. H. (2000) *Eur. J. Immunol.* **30**, 2765–2774

30. Sabelko-Downes, K. A., and Russell, J. H. (2000) *Curr. Opin. Immunol.* **12**, 330–335

31. Sabelko-Downes, K. A., Russell, J. H., and Cross, A. H. (1999) *J. Neuroimmunol.* **100**, 42–52

32. Stalder, T., Hahn, S., and Erb, P. (1994) *J. Immunol.* **152**, 1127–1133

33. Rouvier, E., Luciani, M. F., and Golstein, P. (1993) *J. Exp. Med.* **177**, 195–200

34. Thilenius, A. B., Sabelko-Downes, K. A., and Russell, J. H. (1999) *J. Immunol.* **162**, 643–650

35. Wang, R., Rogers, A. M., Ratliff, T. L., and Russell, J. H. (1996) *J. Immunol.* **157**, 2961–2968

36. Ali, S. A., and Steinacker, A. (1995) *BioTechniques* **18**, 746–750

37. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995) *Science* **270**, 1189–1192

38. Farris, M., Latinis, K., Kempinski, S. J., Koretzky, G. A., and Nel, A. (1998) *Cell. Biol. Cell. Biol.* **18**, 5414–5424

39. Wang, J. K., Zhu, B., Ju, S. T., Tschopp, J., and Marshak-Rothstein, A. (1997) *Cell. Immunol.* **179**, 153–164

40. Sabelko-Downes, K. A., Cross, A. H., and Russell, J. H. (1999) *J. Exp. Med.* **189**, 1195–1205

41. Latinis, K. M., Norian, L. A., Eliason, S. L., and Koretzky, G. A. (1997) *J. Biol. Chem.* **272**, 31427–31434

42. Latinis, K. M., Carr, L. L., Peterson, E. J., Norian, L. A., Eliason, S. L., and Koretzky, G. A. (1997) *J. Immunol.* **158**, 4602–4611

43. Zhang, J., Miranda, K., Ma, B. Y., and Fine, A. (2000) *Biochim. Biophys. Acta* **1490**, 291–301

44. Zhang, J., Ma, B., Marshak-Rothstein, A., and Fine, A. (1999) *J. Biol. Chem.* **274**, 26537–26542

45. Diedhiu, M., Alizadeh, A. A., Rando, O. J., Liu, C. L., Stankunas, K., Betstein, D., Crabtree, G. R., and Brown, P. O. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11796–11801

46. Riley, J. L., Mao, M., Kobayashi, S., Biery, M., Burchard, J., Cavet, G., Gregson, B. P., June, C. H., and Linsley, P. S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11790–11795

47. Li-Weber, M., Laur, O., Hekele, A., Coy, J., Walczak, H., and Krammer, P. H. (1998) *Eur. J. Immunol.* **28**, 2373–2383

48. Shea-Eaton, W., Sandhoff, T. W., Lopez, D., Hales, D. B., and McLean, M. P. (2002) *Mol. Cell. Endocrinol.* **188**, 161–170

49. Li, L., Chambard, J. C., Karin, M., and Olson, E. N. (1992) *Genes Dev.* **6**, 676–689

50. Gurney, A. L., Park, E. A., Giral, M., Liu, J., and Hanson, R. W. (1992) *J. Biol. Chem.* **267**, 18133–18139

51. Bruder, J. M., Spaulding, A. J., and Wierman, M. E. (1996) *Mol. Endocrinol.* **10**, 35–44
Structure/Function Analysis of the Murine CD95L Promoter Reveals the
Identification of a Novel Transcriptional Repressor and Functional CD28 Response
Element

Scott A. Crist, Thomas S. Griffith and Timothy L. Ratliff

J. Biol. Chem. 2003, 278:35950-35958.
doi: 10.1074/jbc.M306220200 originally published online July 10, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306220200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 33 of which can be accessed free at
http://www.jbc.org/content/278/38/35950.full.html#ref-list-1