Viral IFN-Regulatory Factors Inhibit Activation-Induced Cell Death Via Two Positive Regulatory IFN-Regulatory Factor 1-Dependent Domains in the CD95 Ligand Promoter

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Viral IFN-Regulatory Factors Inhibit Activation-Induced Cell Death Via Two Positive Regulatory IFN-Regulatory Factor 1-Dependent Domains in the CD95 Ligand Promoter

Sabine Kirchhoff,* Thorsten Sebens,* Sven Baumann,* Andreas Krueger,* Rainer Zawatzky,§ Min Li-Weber,* Edgar Meinl,‡ Frank Neipel,§ Bernhard Fleckenstein,§ and Peter H. Krammer*‡

The CD95 (also called APO-1/Fas) system plays a major role in the induction of apoptosis in lymphoid and nonlymphoid tissues. The CD95 ligand (CD95L) is induced in response to a variety of signals, including IFN-γ and TCR/CD3 stimulation. Here we report the identification of two positive regulatory IFN-regulatory factor-dependent domains (PRIDDs) in the CD95L promoter and its 5′ untranslated region, respectively. EMSAs demonstrate specific binding of IFN-γ-induced IFN-regulatory factor 1 (IRF-1) to the PRIDD sequences. Ectopic IRF-1 expression induces CD95L promoter activity. Furthermore, we demonstrate that PRIDDs play an important role in TCR/CD3-mediated CD95L induction. Most interestingly, viral IRFs of human herpes virus 8 (HHV8) totally abolish IRF-1-mediated and strongly reduce TCR/CD3-mediated CD95L induction. We demonstrate here for the first time that viral IRFs inhibit activation-induced cell death. Thus, these results demonstrate an important mechanism of HHV8 to modulate the immune response by down-regulation of CD95L expression. Inhibition of CD95-dependent T cell function might contribute to the immune escape of HHV8. The Journal of Immunology, 2002, 168: 1226–1234.

Abbreviations used in this paper: CD95L, CD95 (APO-1/Fas) ligand; AICD, activation-induced cell death; Cat, chloramphenicol acetyltransferase; Egr, early growth response protein; GFP, green fluorescence protein; HHV, human herpes virus; IRF, IFN-regulatory factor; ISRE, IFN-stimulated responsive element; PRIDD, positive, regulatory, IRF-dependent domain; UTR, untranslated region; vIRF, viral IRF.

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apoptosis is a form of cell death involved in many physiological and pathological processes. It is induced in response to a variety of extracellular signals. Among these are stimulation of cells with IFN, UV, stress, depletion of growth factors, or triggering of death receptors of the TNF receptor superfamily. CD95 (APO-1/Fas), a type I transmembrane protein, is a member of the TNF receptor superfamily expressed in various tissues, including T cells, colonic epithelial cells, endothelial cells, and hepatocytes (1, 2). In contrast, expression of its cognate ligand (CD95L),3 a type II transmembrane protein, is tightly regulated. Deregulation of CD95L results in several diseases, such as autoimmune or uncontrolled lymphoproliferation (3–5). CD95L induces apoptosis in CD95-bearing cells via formation of a death-inducing signaling complex and initiation of a signaling cascade of caspases, finally leading to apoptotic cell death (1, 6). In T cells CD95L apoptosis is triggered during the down-phase of an immune response and was termed activation-induced cell death (AICD). This process plays an important role in the maintenance of peripheral lymphocyte homeostasis (7–11).

The transcriptional regulation of CD95L upon T cell activation has been studied over the last couple of years. Several binding sites for NF-AT (12–15), NF-κB (16, 17), and Egr (early growth response protein) (18, 19) have been identified in the CD95L promoter. TGF-β was described to inhibit CD95L expression via down-regulation of c-Myc and, thereby, to block AICD (20). Accordingly, overexpression of c-Myc results in induction of CD95L (21). The forkhead transcription factor was described as a silencer of CD95L expression (22). Recently, the contribution of IRF-1 in CD95L induction upon TCR/CD3 triggering has also been demonstrated (23).

In addition to TCR/CD3 stimulation of T cells, apoptosis is induced upon IFN-γ treatment in certain cell types. In microglia CD95 and CD95L are simultaneously up-regulated by IFN-γ (24). In neuroblastoma cells IFN-γ induces apoptosis via induction of the CD95L (25). In addition, CD95L expression is up-regulated in squamous cell carcinoma (26) and in Tera-2 embryonal carcinoma cells (27). The molecular mechanisms of IFN-γ-mediated CD95L induction are still elusive. Stimulation of the IFN-γ receptor leads to phosphorylation of STAT1 via activation of the Janus family tyrosine kinases, JAK1 and JAK2. Tyrosine-phosphorylated STAT1 assembles to form a homodimer, which then translocates into the nucleus and binds to a specific DNA sequence motif, termed the IFN-γ activation site to induce a variety of genes (28, 29). Among these genes is IFN-regulatory factor 1 (IRF-1). IRF-1 belongs to a family of transcription factors termed the IRF family. Originally identified as a transcription factor that binds to the human IFN-β gene promoter (30), it is now clear that IRF-1 has a remarkable functional diversity; it is involved in proliferation, blocks oncosogenesis, and is able to induce apoptosis (31–34). Furthermore, IRF-1 is a regulator of the immune response. It is involved in the function of multiple stages of the Th1 limb of the immune response (35–37); in addition, IRF-1 regulates the IL-15...
gene and is therefore essential for the development of NK cells (38). Studies in IRF-1−/− mice demonstrated that IRF-1 plays a crucial role in IFN-induced anti-viral and anti-bacterial responses (35, 39). Also, DNA damage-induced apoptosis in mitogenically activated mature T cells is dependent on IRF-1 (40).

Viral members of the IRF family (vIRFs) encoded by human herpesvirus 8 (HHV8) have also been identified (41). HHV8 is thought to be the viral agent involved in Kaposi’s sarcoma and primary effusion lymphoma. The genome of HHV8 contains a cluster of open reading frames encoding proteins with homology to the cellular transcription factors (41). Viral IRF1 and vIRF2 have been shown to interact with several IRFs, including IRF-1, and to inhibit IRF/IFN-mediated transcriptional activation (42, 43). At least two additional IRF-homologous genes were recently identified, namely K10.5 and K10, which are encoded by alternative splicing. Interestingly, according to two groups, K10.5/vIRF3 is identified, namely K10, which are encoded by alternative splicing.

In this study we demonstrate the involvement of IRF-1 in CD95L expression. We identified two novel positive regulatory IRF-1-dependent domains (PRIDDs) in the human CD95L promoter. Our results show that ectopic expression of IRF-1 leads to induction of CD95L, which can be totally abolished in the presence of mutated PRIDDs. In addition, PRIDDs are necessary for TCR/CD3 induction of the CD95L. Moreover, we found that vIRF1 and vIRF2 of HHV8 inhibit IRF-1-induced CD95L induction and repress AICD via down-regulation of TCR/CD3-mediated CD95L expression. These data suggest that 1) IRF-1-mediated induction of the CD95L promoter might contribute to the activity of IRF-1 as a tumor suppressor-like protein; and 2) vIRFs of HHV8 act as modulators of the immune system by repressing AICD via modulation of TCR/CD3-mediated induction of the CD95L.

Materials and Methods

Cell cultures and reagents

The human T lymphoblastoid cell line Jurkat was cultured in IMEM supplemented with 10% FCS, antibiotics (100 μg/ml), and HEPES (10 mM). HeLa, LTK−, and C243 (46) cells were grown in DMEM supplemented with 10% FCS, glutamine, and antibiotics. HaCat, an immortalized human keratinocyte cell line, was obtained from Prof. N. Fusenig (German Cancer Research Center, Heidelberg, Germany) and cultured in DMEM/F-12 (Life Technologies/BRL, Eggenstein, Germany). The hybridoma secreting OKT3 (anti-CD3 mAb) was provided by G. Moldenhauer (German Cancer Research Center) and cultured in RPMI supplemented with 10% FCS and antibiotics. Human peripheral T cells were prepared as described previously (7). For activation, resting T cells were cultured at 2 × 10^6 cells/ml with 1 μg/ml PHA (Sigma, Taufkirchen, Germany) for 16 h. T cells were then washed three times and cultured in the presence of IL-2 (30 U/ml) for additional 5 days. The anti-IRF-1 and IRF-2 polyclonal Abs were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). IFN-γ was purchased from Biomol (Hamburg, Germany).

Plasmid constructs

Vector constructions were conducted by standard procedures (48). The coding regions of vIRF1 and vIRF2 were isolated by PCR amplification from λ phage DNA, clone CB47-1 (48), using specific primer pairs and subsequent insertion in pcDNA3 (Invitrogen, Groningen, The Netherlands). Mutations in the PRIDD sequences in the −1204/+100 construct were introduced using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The primers for the mutagenesis reaction (MWG Biotech, Ebersberg, Germany) were mPRIDD1 (sense, 5′-cagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagagag...
IFN-γ induces CD95L expression. LTK−, C243, HeLa, HaCaT, and Jurkat cells were transiently transfected with a reporter construct containing the 1204 bp 5′ of the CD95L transcription start and 100 bp of the 5′ UTR in front of firefly luciferase (−1204/+100). Cells were stimulated with IFN-γ (500 U/ml) for the last 12 h. Reporter gene activity was determined 48 h after transfection. The relative induction levels, given as fold induction with respect to the untreated control culture, are indicated. One representative experiment of five is shown.

Results

IFN-γ induces CD95L promoter activity

To test whether IFN-γ induces CD95L expression via modulation of its promoter activity, we transfected a reporter construct containing a 1204-bp fragment 5′ of the transcription start site and the 100 bp of the 5′UTR (−1204/+100) of the CD95L promoter for IRF-1 inducibility. Deletion of the promoter sequence to −165 did not significantly influence IRF-1-mediated induction. Deletion of the promoter sequence up to −88 partially decreased IRF-1-mediated CD95L promoter induction in HeLa and Jurkat cells (Fig. 2B). Therefore, the sequence between −165 to −88 contains an IRF-1-responsive element, which is in agreement with the recently published data of Chow et al. (23). However, in the cell lines investigated here, this sequence contributed only partially to IRF-1-mediated induction of the promoter. Further deletion of the promoter construct from −88 to −62 completely inhibited IRF-1-mediated induction in all cell lines investigated. From these findings we conclude that at least one additional positive regulatory IRF-1-dependent domain (PRIDD) is contained in the −88/+100 fragment of the CD95L promoter.

Two novel PRIDDs in the CD95L promoter

To identify the PRIDD sequence we performed EMSAs using putative IRF-1 binding sites derived from the −88/+100 CD95L promoter construct as probes and in vitro translated IRF-1. Luciferase was used as a negative control. Two IRF-1 binding sites were found: one sequence located between −77 and −55 upstream of the transcription start site (PRIDDI), and an additional sequence in the 5′UTR between +51 and +71 (PRIDDII). An ISRE was used as a positive control for IRF-1 binding, and the sequence located
in the 5'UTR between +26/+45 was used as a negative control. As indicated in Fig. 3A, IRF-1 was able to bind the PRIDDI and PRIDDII sequences. IRF-1 recognition sequences were located in the CD95L promoter and 5'UTR, respectively. Binding to the +51/+71 sequence was much weaker compared with that to the −77/−55 sequence. The typical ISRE sequence showed a much stronger binding of IRF-1 compared with the PRIDDs of the CD95L promoter. The random sequence spanning from +26/+45 of the CD95L 5'UTR was not recognized by IRF-1. In contrast, two different complexes of IRF-1 and the ISRE were formed (Fig. 3A, IRF-1 I and IRF-1 II), probably due to oligomers of IRF-1. Only one IRF-1 complex was detectable with the PRIDD sequences, which probably reflects binding of the IRF-1 monomer. Also the supershift with an Ab directed against IRF-1 was different. In case of the typical ISRE sequence there were two supershifted complexes (Fig. 3A, scI and scII) resulting in slower migration. In contrast, binding of IRF-1 to the PRIDD sequences was blocked in the presence of the anti-IRF-1 Ab (Fig. 3A). An isotype-identical Ab directed against IRF-2 did not influence binding of IRF-1; the labeled oligonucleotides with or without anti-IRF-1 Ab in the presence or absence of in vitro translated proteins did not result in any signal (data not shown). We designated the two newly identified IRF-1 recognition sequences of the CD95L promoter PRIDDI for the −77/−55 sequence and PRIDDII for the +51/+71 sequence. Both PRIDDDs were able to competitively inhibit binding of IRF-1 to the ISRE. Competition with PRIDDI was more efficient than with PRIDDII (Fig. 3B, compare lanes 6–8 with lanes 9–11), supporting the previous finding that binding of IRF-1 to PRIDDI was not as strong as that to PRIDDII. Binding of IRF-1 to the ISRE was much stronger than that to the PRIDDs (Fig. 3B, compare lanes 3–5 and lanes 6–11). The unlabeled control sequence containing the sequence (+26/+45) did not compete for binding to the PRIDDDs (Fig. 3B, lanes 12 and 13). To test whether binding of IRF-1 to the PRIDDDs occurs under physiological conditions we stimulated Jurkat and HeLa cells with IFN-γ for 18 h and determined IRF-1 binding to the PRIDDDs. In both cell lines an induced band appeared upon IFN-γ treatment using PRIDDI as a probe (compare Fig. 3C, lanes 4 and 6, and lanes 8 and 9). In untreated Jurkat cells low amounts of complex formation were detectable. Addition of anti-IRF-1 Abs interfered with the IFN-γ-induced complex (compare Fig. 3C, lanes 6 and 7, and lanes 9 and 11). The complex consisting of cellular IRF-1 and PRIDDI migrated as fast as the one obtained with the in vitro translated material (compare Fig. 3C, lanes 2 and 6). IFN-γ treatment also induced IRF-1 binding to the PRIDDI sequence in Jurkat and HeLa cells (Fig. 3D, compare lanes 3 and 4, and lanes 5 and 6).

**Mutated PRIDDI and PRIDDII reduce CD95L induction**

To analyze the importance of the PRIDD sequences in the CD95L promoter we introduced point mutations in PRIDDI and PRIDDII to generate nonfunctional IRF-1 binding motifs. We first tested the mutated sequences in competition experiments for IRF-1 binding using the respective wild-type oligonucleotide as the probe. The wild-type sequences, but not the mutated PRIDDD sequences, influenced binding of IRF-1 to the wild-type oligonucleotide (Fig. 3E).

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**FIGURE 3.** Two novel IRF-1 binding elements in the CD95L promoter. A, EMSAs were performed with in vitro translated luciferase (Luc) or IRF-1 in the absence or presence of an Ab directed against IRF-1 (αIRF-1), using a typical ISRE, the −77/−55 (PRIDDI) sequence of the human CD95L promoter, or the +51/+71 (PRIDDII) or +24/+46 sequence from the 5'UTR. The arrows indicate migration of the two IRF-1 complexes, I and II. Asterisks indicate the supershifted complexes (scI and scII). B, Competition experiments with the indicated oligonucleotides using in vitro translated IRF-1 or luciferase (Luc) as a control with an ISRE sequence as a radiolabeled probe. Oligonucleotides were used in 20-, 50-, or 100-fold molar excess as competitors. The arrows indicate migration of the two IRF-1 complexes, I and II. C, Nuclear extracts of HeLa or Jurkat cells, which were treated with IFN-γ (18 h, 500 U/ml), or in vitro translated proteins (i.v., lanes 1–3) were used for EMSAs with the PRIDDI element of the CD95 ligand promoter in the absence or the presence of an Ab directed against IRF-1 (αIRF-1). D, EMSAs were performed as described in A with the PRIDDI sequence as radiolabeled probe. Control, unstimulated cells; IRF-1, in vitro (i.v.) translated IRF-1; Luc, in vitro translated luciferase.
FIGURE 4. Mutation of PRIDD and PRIDDI reduces IRF-1 and TCR-mediated CD95L induction. A. EMSAs were performed with in vitro translated IRF-1 using the PRIDD or PRIDDI sequences as radiolabeled probe. Competition experiments were performed with the indicated amounts of mutated or wild-type PRIDD or PRIDDI, respectively. B. HeLa and Jurkat cells were transiently transfected with a reporter construct containing an oligomerized wild-type mutated PRIDD (muPRIDDI) or PRIDDI (muPRIDDII) in front of a truncated promoter, either together with an expression plasmid coding for IRF-1 or with an empty vector. C. Jurkat cells transfected with reporter constructs containing either multimerized wild-type (wt) or mutated (mu) PRIDD sequences were stimulated via TCR/CD3 16 h before cell harvest. D. HeLa and Jurkat cells were transiently transfected with a reporter construct containing either the wild-type 1204/+100 (wt) or the −1204/+100 construct containing mutated PRIDD (muPRIDDI) or PRIDDI (muPRIDDII) alone or in combination (muPRIDDI/II) together with an expression construct coding for IRF-1 or an empty vector. E. Jurkat cells were transiently transfected as described in D and were stimulated via TCR/CD3 for 12 h. Luciferase activity was always determined 48 h after transfection. The relative induction levels given with respect to the untreated control culture are indicated. One representative experiment of three is shown. F. EMSAs were performed using nuclear extracts of 6-day activated or TCR/CD3-restimulated primary human T cells using the PRIDD or PRIDDI sequence as a radioactive probe. The arrow indicates IRF-1 binding to the PRIDD sequences.
A). To test whether the mutated PRIDDs indeed resemble non-functional elements in vivo we generated luciferase reporter constructs containing oligomers of wild-type or mutated PRIDD sequences and compared IRF-1-mediated induction after transient transfection. As indicated in Fig. 4B, oligomerized wild-type PRIDDI was induced by IRF-1 15-fold in HeLa and 4-fold in Jurkat cells, whereas oligomerized PRIDDII was only induced 2-fold in HeLa and Jurkat cells. In contrast, the mutated oligonucleotides were not induced by IRF-1 (Fig. 4B). Interestingly, both PRIDDs were also induced via TCR/CD3 in Jurkat cells; PRIDDI was induced 15-fold, and PRIDDII 3-fold via TCR/CD3 stimulation (Fig. 4C). To test the contribution of the PRIDDs to IRF-1- and TCR/CD3-mediated CD95L induction, the two PRIDD sequences were mutated either individually or in combination in the context of the −1204/+100 CD95L promoter construct. Mutation of PRIDDI alone resulted in 50% decrease in Jurkat cells and a 95% decrease in HeLa cells of IRF-1-mediated CD95L promoter activity (Fig. 4D). In contrast, mutation of PRIDDII reduced IRF-1-mediated induction only moderately (20% in Jurkat and 70% HeLa cells). Disruption of both, PRIDDI and PRIDDII abolished IRF-1-mediated induction in HeLa cells completely. However, in Jurkat cells, IRF-1-mediated induction was still detectable, however at reduced levels compared with the wild-type sequences. Mutation of PRIDDI or PRIDDII also strongly inhibited TCR/CD3-mediated CD95L promoter induction. Mutation of both PRIDDs decreased the induction of the CD95L in an additive manner (Fig. 4E). These results demonstrate that 1) both PRIDD sequences are involved in IRF-1 as well as in TCR/CD3-mediated induction of the CD95L promoter; 2) PRIDDII contributes to a much lesser extent to IRF-1 as well as TCR/CD3-mediated CD95L induction; and 3) an additional PRIDD sequence contributes to IRF-1-mediated CD95L induction in Jurkat cells. Since the PRIDDs are involved in TCR/CD3-mediated CD95L induction in Jurkat cells (23), we asked whether IRF-1 binds to the PRIDDs in activated primary human T cells. We performed EMSAs with nuclear extracts of primary 6-day-activated T cells with or without TCR/CD3 restimulation using the PRIDD sequences as a probe. As demonstrated in Fig. 4F by a supershift with an Ab directed against IRF-1, binding of IRF-1 was detectable in primary activated T cells. In TCR/CD3-stimulated T cells, IRF-1 binding to the PRIDD sequences was no more detectable, but another not yet identified band became visible in the case of PRIDDI. These results suggest that PRIDDs are also important for TCR/CD3-mediated CD95L expression in activated primary human T cells. However, IRF-1 binding is only detectable in activated T cells and is replaced by another as yet unidentified factor after TCR/CD3 restimulation.
Viral IRFs of HHV8 inhibit induction of the CD95L promoter

It was shown recently that vIRFs of HHV8 counteract IFN/IRF-1-mediated gene activation (52, 53). Since we could demonstrate that PRIDDs play an important role in IRF-1 as well as in TCR/CD3-mediated induction of the CD95L promoter we wanted to know whether vIRFs repress induction of the CD95L promoter. To investigate this question we performed cotransfection experiments using the −1204/+100 reporter construct together with an expression plasmid encoding IRF-1 in the absence or the presence of expression vectors coding for vIRF1 or vIRF2 of HHV8. As demonstrated in Fig. 5A cotransfection of either vIRF1 or vIRF2 together with IRF-1 nearly completely inhibited IRF-1-mediated induction of the CD95L promoter in HeLa as well as in Jurkat cells. TCR/CD3-mediated CD95L induction in Jurkat cells was decreased up to 7.5- and 10-fold in the presence of either vIRF1 or vIRF2, respectively, compared with 17-fold in the vector control (Fig. 5B). To investigate whether vIRFs act via the PRIDDs in case of TCR/CD3-mediated induction, we transfected the −1204/+100 reporter construct containing either mutated PRIDDI or PRIDDII alone or in combination in the absence or the presence of expression vectors coding for vIRF1 or vIRF2. As mentioned above, mutation of either PRIDDI or PRIDDII significantly reduced TCR/CD3-mediated induction of the CD95L. The presence of vIRFs further reduced the induction; however, induction of the construct containing both mutated PRIDD sequences was not further repressed (Fig. 5C). The presence of vIRFs also prevented IRF-1- as well as TCR/CD3-mediated induction of reporter constructs containing oligomerized PRIDDs (data not shown). We conclude from these results that vIRFs down-regulate CD95L expression via modulation of PRIDD activity. To test the anti-apoptotic activity of vIRFs we transfected Jurkat cells with expression constructs coding for vIRFs together with a GFP expression plasmid as a transfection marker. Twenty-four hours after transfection Jurkat cells were sorted for GFP expression and stimulated via TCR/CD3 for an additional 36 h, and AICD was determined in GFP-positive cells. As demonstrated in Fig. 5D, both vIRFs strongly inhibited TCR/CD3-mediated cell death. The antagonistic CD95L Ab NOK1 inhibited TCR/CD3-mediated apoptosis in control transfected cells, but no further inhibition was observed in vIRF-transfected cells. Therefore, we conclude that vIRF1 and vIRF2 of HHV8 inhibit AICD by down-regulation of TCR/CD3-mediated CD95L expression.

Discussion

Results from the current study demonstrate that the tumor susceptibility factor IRF-1 is able to induce the CD95L promoter in several murine and human cell lines. We have identified two PRIDDs in the CD95L gene. In vitro translated IRF-1 protein binds to both sequences. Treatment of different cells with IFN-γ induces IRF-1 binding to these sequences, as demonstrated by anti-IRF-1 Abs, which specifically inhibit binding of IRF-1 to PRIDDs. It was shown previously that IRF-1−/− cells can be transformed by an activated c-Ha-ras oncogene. Furthermore, ectopic expression of IRF-1 suppressed c-myc or jupB-induced cell transformation (31, 32). Therefore, IRF-1-mediated induction of apoptosis via CD95L expression might contribute to its tumor suppressor function.

Mutation of either PRIDDI or PRIDDII in the context of the CD95L promoter strongly inhibits IRF-1-mediated CD95L induction; PRIDDII contributes significantly more to induction, which is due to most likely stronger binding of IRF-1 to this sequence, as indicated by EMSAs performed with the PRIDDI or PRIDDII sequence in competition experiments and with in vitro translated IRF-1 (Fig. 3). Binding of IRF-1 to PRIDDs is different from IRF-1 binding to the ISRE, since addition of anti-IRF-1 Abs results in stronger retardation of the IRF-1/ISRE complex, whereas binding to the PRIDD sequences was abolished under these conditions. These results indicate that binding of IRF-1 to PRIDDs requires different domains or conformations of IRF-1, which may be masked in the presence of anti-IRF-1 Abs. Mutation of both PRIDDs prevents IRF-1-mediated CD95L induction. In activated human primary T cells, IRF-1 binding to the PRIDD sequences was also detectable. We detected a faint band of CD95L mRNA and low amounts of CD95L protein in activated human primary T cells (data not shown). The question arises of whether IRF-1 binding contributes to this low CD95L expression. Alternatively, since IRF-1 contains a repressor domain in the N-terminus, it might act as a silencer under these conditions, as described previously for a forkhead transcription factor (22, 54).

Following TCR/CD3 re-stimulation, binding of IRF-1 to PRIDDs is no more detectable, but is replaced by another as yet uncharacterized protein (Fig. 4, PRIDDI). We tested possible binding of other members of the IRF family (IRF-2, IRF-4, IRF-8) in supershift experiments, but these IRF family members did not bind to the PRIDD sequences. However, the PRIDD sequences play an important role in TCR/CD3-mediated induction of the CD95L promoter, since mutation of the PRIDD motifs strongly interfered with induction. The contribution of IRF-1 to TCR/CD3-mediated induction in Jurkat cells is in line with a recent report by Chow and co-workers (23) showing that expression of antisense IRF-1 strongly reduced PMA/ionomycin induction of CD95L in Jurkat cells. A putative IRF-1 binding site 5′ of the NF-AT and Egr-3 recognition sequence, which is located between 165 and 88 bp 5′ of the transcription start site according to our nomenclature, was described. In our hands deletion up to −88 led to a slight decrease in IRF-1-mediated CD95L induction, which is more prominent in Jurkat cells. However, deletion up to −62 completely inhibited IRF-1-mediated CD95L induction (Fig. 2). Therefore, we conclude that PRIDDI, which is located between −88 to −62 is an essential cis-acting element in IRF-1-mediated promoter activation. A promoter construct containing only PRIDDI, which is located in the 5′UTR (−62/+100, Fig. 2) was not sufficient for IRF-1-mediated gene induction. Mutation of the PRIDDI sequence in the presence of the wild-type PRIDDII sequence contained in the −1204/+100 promoter construct still allowed IRF-1-mediated induction. These results indicate that IRF-1 acts in cooperation with another factor that binds a sequence located between −62 and −1204 of the CD95L transcription start site. We observed that mutations of both PRIDDI and PRIDDII totally inhibited IRF-1-mediated induction in HeLa cells, but still allowed a weak induction in Jurkat cells (Fig. 2). Most likely, the IRF-1 recognition site identified by Chow and co-workers (23) contributes to the induction in Jurkat cells. Interestingly, deletion of the CD95L promoter from −88 to −62 nearly completely inhibited PMA/ionomycin-mediated induction, which also suggests the involvement of PRIDDI in TCR/CD3-mediated induction (57). In summary, PRIDD sequences are involved in IRF-1-mediated CD95L induction. Therefore, one can speculate that pathways leading to IRF-1 binding to the PRIDDs also modulate CD95L induction. However, IFN-γ-mediated CD95L induction is hardly detectable in Jurkat cells. Therefore, it most likely depends on the cell type whether the induced amount of IRF-1 is sufficient for CD95L expression. In addition, the composition of the transcription factors in one particular cell type upon stimulation might also play a role in the activity of IRF-1 on the CD95L promoter.

Many other transcription factors, such as NF-AT, Egr, NF-κB, and c-Myc have been found to be involved in CD95L induction. Mutation of the recognition sequence of one of these transcription factors strongly reduces CD95L induction (12–19, 58). Therefore,
it is reasonable to assume that these transcription factors act synergistically. These factors may form a large complex, an enhancerosome, resembling the scenario described for the IFN-β promoter (56–59). If one factor is missing, the conformation of the enhancerosome is disturbed, and induction is strongly reduced.

Most interestingly, vIRF1 and vIRF2 of HHV8 completely inhibited IRF-1-mediated and reduced TCR/CD3-mediated induction of the CD95L, which resulted in strong reduction of AICD (Fig. 5). One oncogenic mechanism of the HHV8 virus might be to prevent T cell-mediated apoptosis of tumor cells by inhibition of CD95L expression in activated T cells. The inhibition of CD95L expression by HHV-8 might be linked to a potentially proinflammatory effect of the CD95L, namely triggering of neutrophil inflammation or induction of maturation of dendritic cells (60, 61).

Interestingly, HHV8 also expresses the anti-apoptotic protein, vFLIP, which protects cells from CD95-mediated apoptosis (62). Thus, several viral proteins exist that inhibit the CD95 pathway and might contribute to immune escape of HHV8. Our results strongly suggest that vIRFs act as modulators of the immune response via CD95. The biological function of vIRFs in the context of the immune response has to be worked out in more detail. Also, the molecular functions of vIRF-mediated transcriptional inhibition are not fully understood. It has been reported that vIRF1 inhibits p300/CBP activity, which might be a possible explanation for the inhibition of CD95L promoter activity (63). In addition, in vitro translated vIRF1 reduced protein binding to PRIDDs, but could not bind by itself (data not shown). This result suggests that vIRF1 prevents CD95L expression by inhibition of IRF-1 binding to PRIDDs. In contrast, vIRF2 did not modulate protein binding to PRIDDs. Viral IRF2-mediated inhibition of NF-κB was described previously (43). Since NF-κB is involved in CD95L induction upon TCR/CD3 stimulation (17), and IRF-1 can act synergistically with NF-κB (64, 65), this can be a possible explanation for vIRF2-mediated repression of CD95L induction. In summary, the interplay between the transcription factors that seem to be involved in the regulation of CD95L expression under physiological conditions remains to be clarified.

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