Active Transcription of the Human FASL/CD95L/TNFSF6 Promoter Region in T Lymphocytes Involves Chromatin Remodeling

ROLE OF DNA METHYLATION AND PROTEIN ACETYLATION SUGGEST DISTINCT MECHANISMS OF TRANSCRIPTIONAL REPRESSION*

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Rény Castellano1, Bérengère Vire1, Marjorie Pion1, Vincent Quivy2, Daniel Olive3, Ivan Hirsch2, Carine Van Lint3, and Yves Collette4,5

From the 1INSERM UMR599, Centre de Recherche en Cancérologie de Marseille, Université de la Méditerranée, 13009 Marseille, France, 2INSERM U372, Unite de Pathogenie des Infections a Lentivirus, Parc Scientifique et Technologique de Luminy, 13009 Marseille, France, and 4Université Libre de Bruxelles, Institut de Biologie et de Médecine Moléculaires, Laboratoire de Virologie Moléculaire, 6041 Gosselies, Belgium

Fas ligand (FasL/CD95L/TNFSF6), a member of the tumor necrosis factor family, initiates apoptosis in lymphoid and nonlymphoid tissues by binding to its receptor Fas (CD95/TNFRSF6). Although the transcriptional control of TNFSF6 gene expression is subjected to intense study, the role of its chromatin organization and accessibility to the transcriptional machinery is not known. Here, we determined the chromatin organization of TNFSF6 gene 5′ regulatory regions. Using the indirect end-labeling technique, a unique region named HSS1 and encompassing nucleotides -189 to +185 according to the transcriptional start site, was identified throughout a 20-kilobase nucleosomal DNA domain surrounding the promoter. The HSS1 region displayed hypersensitivity to in vivo DNase I digestion in TNFSF6-expressing cells only, including upon T cell activation. Hypersensitivity to micrococcal nuclease digestion and to specific restriction enzyme digestion suggested the precise positioning of two nucleosomes across the transcription start site and minimal promoter region, likely interfering with TNFSF6 active transcription in T lymphocytes. Indeed, HSS1 hypersensitivity to nuclease digestion strictly correlated with TNFSF6 transcription, including in primary and leukemia T cells. HSS1 chromatin remodeling preceded detectable TNFSF6 mRNA accumulation and was blocked by cycloheximide that also prevented TNFSF6 transcription. However, DNA methylation levels of the TNFSF6 HSS1 region did not correlate with transcriptional activation. Induction of global protein acetylation by treatment with histone deacetylase inhibitors was not accompanied by HSS1 chromatin remodeling and/or TNFSF6 transcription. We conclude that chromatin remodeling is a primary event in the activation of TNFSF6 expression in primary and leukemia T cells and that mechanisms independent of protein deacetylation and of DNA methylation of the TNFSF6 promoter region are involved in the repression of TNFSF6 gene expression.

The TNFSF family related members are type II transmembrane proteins. Besides of their role in many important biological processes such as development, organogenesis, immunity, cell death, and survival, TNFSFs are implicated in various acquired or genetic human diseases ranging from septic shock to autoimmune disorders, allograft rejection, viral infections, and cancer (1). Hence, various therapeutic approaches aim to target these molecules, and there has been much interest toward the elucidation of the molecular mechanisms controlling their expression. TNFSF family member expression is contextually dependent on the nature, differentiation, activation, and cell cycle status of producing tissues. The regulated expression of the TNFSF genes implicates not only transcriptional but also post-transcriptional and post-translational mechanisms, together ensuring appropriate spatiotemporal expression. Escape of this tightly controlled expression has been described in various pathological conditions, including viral infections or neoplasms.

TNFSF6 (FasL/CD95L) is a key player in T lymphocyte homeostasis and cytolytic effector functions through the aggregation of its apoptosis inducing receptor, TNFRSF6 (Fas/CD95). Defective expression or function of either TNF6 or TNFRSF6 gene results in lymphoproliferation and autoimmunity in mice (for review, see Ref. 2) and human (3–5) and has been implicated in immune escape in the course of viral infection or tumor progression (6–10). Hence, tightly controlled expression of TNFSF6 is required for proper immune functions. Whereas TNFSF6 is largely distributed in various tissues, initial studies described a more restricted expression of TNFSF6 confined to immune cells, with the exception of Sertoli cells and epithelial cells of the anterior chamber of the eye (11–13). In resting T lymphocytes, TNFSF6 mRNA is absent or very low but is rapidly and transiently up-regulated upon T cell activation (11, 14). Subsequent studies have emphasized further the transcriptional control of TNFSF6 expression and have identified, cloned, and characterized the human and mouse TNFSF6 promoter-enhancer regions, NF-AT, but also NF-κB, Sp1, Egr-2 and -3, AP-1, Ets-1, and c-Myc transcription factors were shown to participate to TNFSF6 transcription (15–28). Indeed, induced expression of TNFSF6 at the cell surface of activated T lymphocytes is inhibited by cyclosporin A, an inhibitor of the calcineurin phosphatase implicated in the regulation of
NF-AT transcription factor activity and expression (29). The generation of transgenic mice in which the TNFSF6 promoter sequence controls the expression of a reporter gene evidenced a similar pattern of expression of the transgene and the endogenous gene in primary T lymphocytes, underscoring the role of these 5’-regulatory regions in TNFSF6 expression (30). Post-translational control of TNFSF6 expression has also been described. The TNFSF6 polypeptide can be sequestered as submembrane vesicles, which are rapidly released to the cell membrane by re-stimulated T lymphocytes (31, 32), and membrane-associated TNFSF6 can be processed into a soluble form by matrix metalloproteinases (33, 34). Thus, TNFSF6 surface expression is thought to be primarily controlled at the transcriptional level.

Gene transcription in eukaryotic cells is controlled by protein complexes, including general and tissue-specific transcription factors, coregulators, chromatin-remodeling complexes, and complexes responsible for signal-specific histone modifications (35). Because genomic DNA is packaged into chromatin, generally a repressive structure for transcriptional activation, transcription in the context of chromatin requires remodeling processes to reconfigure the chromatin, so that the transcription complexes have access to promoters of target genes (36). Histone acetyltransferases and histone deacetylases are chromatin-modifying enzymes that tightly cooperate with chromatin-remodeling enzymes to regulate accessibility of the template to DNA binding factors and RNA polymerase II (37). Besides histone acetylation, a variety of other histone and nonhistone protein modifications (phosphorylation, methylation, sumoylation, and ubiquitination) as well as modification of DNA itself by methylation regulates transcription initiation (38–41). orchestration of the events required for transcriptional activation is promoter-specific (42–44). Chromatin remodeling plays an important role in the regulation of immune functions by controlling the expression of a growing number of key cytokine genes (45). For example, during T lymphocyte ontogeny, the differentiation of naïve T lymphocytes into Th1- or Th2-type cytokine-producing cells is accompanied by specific and organized remodeling of the IFNβ and IL-4/IL-5/IL-13 chromatin loci, respectively, characterized by DNase I hypersensitive sites (46–48). The specific and localized remodeling of nucleosomes in the vicinity of the IL-12 p40 and IFNβ promoter regions contribute to their rapid transcription after cell stimulation (for review, see Ref. 45). Herein, we considered a possible role for chromatin structure in the regulation of TNFSF6 gene expression.

**MATERIALS AND METHODS**

**Cells, Culture Conditions, and T Cell Stimulation—**All cell culture experiments were performed at 37 °C in 5% CO₂, humidified atmosphere. The JA16 cells (49), a clone of the Jurkat T cell line and the human NK cell lines NK92, and NKL were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. HeLa cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 4% horse serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Chronic lymphocytic leukemia patients were separated on Ficoll-Hypaque gradients (50). T lymphocytes and the Jurkat T cell line were stimulated with 1 and 20 ng/ml PMA (Saint Louis, MO) and 1 and 2 μg/ml ionomycin (Calbiochem), respectively. Where indicated, the following drugs were used: cyclosporin A (CSA, 50 ng/ml, Calbiochem), cycloheximide (CHX, 20 μM, Sigma), GF109 (1 μM), trichostatin A (TSA, 500 nM, Sigma), valproic acid (5 mM, Sigma), nicotinamide (10 mM, Sigma), and sirtinol (25 μM, Calbiochem). The monoclonal antibody 289 (kindly provided by Alessandro Moretta) recognizes the CD3ζ chain of the T cell receptor complex and was immobilized on plastic culture dishes for T cell receptor stimulation. The anti-human CD28 monocolonal antibody 248 has already been described (51).

**Nuclease Digestion of Purified Nuclei—**Exponentially growing cells were harvested by centrifugation at 1600 rpm (Jouan CR4.11) for 10 min at 4 °C and washed twice with ice-cold phosphate-buffered saline. All subsequent operations were performed in ice with pre-cooled buffers. Cells were counted and resuspended at 25 × 10⁶ cells/ml in buffer A (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.3 M sucrose) and incubated on ice for 10 min, an equal volume of buffer B, 0.2% Nonidet P-40 was added, and the cells were incubated for another 10 min and resuspended at 10⁷ nuclei/ml in 1 of the 3 buffers depending on the nuclease used for digestion: buffer A for DNase I, buffer B supplemented with 10 mM CaCl₂ for micrococcal nuclease, or the specific restriction enzyme buffer for each restriction enzyme. Nuclei were digested for 10 min on ice with DNase I, for 20 min at 22 °C for micrococcal nuclease, and 20 min at the temperature according the manufacturer’s protocol for restriction enzymes. Digestion reactions were stopped by adding 1 volume of 2X proteinase K buffer (100 mM Tris, pH 7.5, 200 mM NaCl, 2 mM EDTA, 1% SDS) and mixing. Samples were solubilized for 1 h at 55 °C, proteinase K was added at 400 μg/ml, and digestion was allowed to continue overnight at 55 °C. RNase A was added at a final concentration of 50 μg/ml for 1 h at 37 °C. Samples were extracted 3 times with phenol and 2 times with chloroform/isoamyl alcohol (24:1) and precipitated with ethanol. DNA was resuspended in sterile water, and its concentration was estimated by measuring the absorbance at 260 nm.

**Southern Blotting—**Purified DNA (30 μg for DNase I and micrococcal nuclease and 15 μg for restriction enzyme) was digested with KpnI, and the fragments generated were separated by electrophoresis in 0.8–2% agarose gels in Tris borate buffer. Each size marker was generated by digesting genomic DNA (10 μg) with two restriction enzymes, KpnI and another enzyme chosen to generate a fragment of defined size and location in the region under study. Three of these markers were mixed together and co-electrophoresed with the samples. Agarose gels were incubated 2 × 30 min in denaturing solution (1.5 mM NaCl, 0.5 mM NaOH) and 2 × 30 min in neutralizing solution (1.5 mM NaCl, 1 mM Tris pH 7.2) and transferred overnight by capillarity in 20X SSC (1X SSC = 0.15 M NaCl and 0.015 M sodium citrate; Invitrogen) to nylon membranes (Hybond-N+, Amersham Biosciences). DNA was cross-linked to nylon membranes by exposure to UV light (UV Stratalinker 1800, Stratagene), washed for 20 min in 2X SSPE, and prehybridized for 2 h at 68 °C in hybridization buffer (6X SSC, 1% SDS, 1X Denhart’s, 0.1 mg/ml sonicated salmon sperm DNA). The probe was synthesized by 25 cycles (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min) of polymerase chain reaction using 100 ng of Jurkat T cell genomic DNA as a template and the primers IEL3AFASL (5’-GTCTACACCGAGATGCAACAGC-3’) and 5’-GTACCTCATGCTGCTTGG-3’,). The amplified products were separated on a 2% agarose gel and purified by using the NucleoSpin Plasmid kit procedure (Macherey-Nagel). DNA fragments were labeled by the random primer reaction (66) and purified on a G-50 Sephadex column. Denatured DNA probe was added to the...
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RESULTS

DNAse I Accessibility of the TNFSF6 5’ Regulatory Regions in TNFSF6-expressing Cells—The presence of DNAse I-hypersensitive sites (HSS) in the 20-kilobase TNFSF6 5’ regulatory region was assessed by indirect end-labeling and Southern blotting using a probe mapping 3’ to the TNFSF6 exon 2 (Fig. 1A). To identify regulatory elements associated with TNFSF6 gene active transcription, we compared DNAse I hypersensitivity patterns in differentially expressing cells. In cells that do not actively transcribe TNFSF6 such as HeLa cells, a strong and specific HSS (called HSS1) appeared in a DNase I-hypersensitive region (Fig. 1B). Both unstimulated Jurkat and primary T lymphocytes displayed a similar closed chromatin structure even at high DNAse I enzyme concentrations that correlated with the lack of TNFSF6 transcription and expression (Fig. 1, B–D). In contrast, a strong and specific HSS (called HSS1) appeared in a DNAse I-dose-dependent manner in both Jurkat and primary T lymphocytes stimulated by a combination of phorbol ester and calcium ionophore (P+I), a powerful mitogenic stimuli-inducing active transcription of TNFSF6 (Fig. 1B). The P+I-induced accessibility of HSS1 was further confirmed in numerous T cell lines (CEM, SupT1, and the J6 and JA16 Jurkat T cell lines) and primary T lymphocytes obtained from different donors (data not shown). Based on previous observations that NK cells constitutively express TNSF6 protein (54), we determined the presence of HSS1 in NK cell lines. HSS1 was detected in both the NKL and NK92 cell lines (Fig. 1B), in agreement with active TNFSF6 transcription and expression in these two cell lines (Fig. 1, C and D). Using the molecular weight markers, we mapped HSS1 between the BamHI and BglII markers—a region previously shown to mimic TNFSF6 gene transcriptional activity (Fig. 1A) (55).

Analysis of TNFSF6-HSS1 Chromatin Accessibility to Micrococcal Nuclease (MNase) and Restriction Enzymes—We next sought to map TNFSF6-HSS1 more precisely and to determine the nucleosomal organization within the TNFSF6 5’ regulatory region. MNase preferentially digests nucleosome-free DNA and linker DNA and can, thus, be used to show nucleosome remodeling. Digestion of a nucleosomal array using limited amounts of MNase will produce a ladder of DNA fragments corresponding to multiples of the nucleosome particle (~160 bp). In nuclei isolated from Jurkat cells and digested with increasing amounts of MNase. A discrete band pattern (a–h in Fig. 2A) was revealed in unstimulated Jurkat cells, a result consistent with precise nucleosome positioning. Digestion of naked genomic DNA in vitro
showed a quite similar, although not identical pattern of digestion to that obtained with in vivo digestion of chromatin, supporting the hypothesis that the pattern observed in vivo reflects TNFSF6 gene nucleosomal organization (Fig. 2A). In P+I-stimulated Jurkat cells, a unique change in MNase accessibility was found in the 20-kilobase DNA region under study (Fig. 2, A and B). This MNase-hypersensitive site mapped to HSS1.

Next, nuclei from Jurkat cells and primary T lymphocytes were digested by different restriction endonucleases (REs) that cleave DNA within the TNFSF6 region of interest. The efficiency of chromatin digestion by a given RE depends on several variables, including DNA accessibility. As described for DNase I hypersensitivity analysis, and nuclei were prepared, digested with RE cutting at 11 and 7 distinct sites for the Jurkat and primary T lymphocytes preparations, respectively, followed by Southern blotting (Fig. 2, C and D). The efficiency of digestion at each site was plotted as a function of the position of the corresponding restriction site in the genome (Fig. 2D). One bimodal peak was identified corresponding to the HSS1 region.

Because digestion by several REs located outside of this region was either unchanged or decreased upon P+I stimulation, the increased digestion most likely reflects a true increase in chromatin accessibility. In addition, for a given RE (for example BglII, in Fig. 2C, left panel), some restriction sites appeared to be cut more efficiently after the P+I stimulation (site 1), whereas others remained unaffected by this treatment (sites 2 and 3). Similar results were obtained using primary T lymphocytes (data not shown).

Based on the chromatin structure analysis of the TNFSF6 locus by means of DNase I, MNase, and RE, we established a tentative map of positioned nucleosomes within the TNFSF6 regulatory regions (Fig. 2D). In unstimulated Jurkat cells, the HSS1 region can accommodate two distinct nucleosomes linked by internucleosomal MNase-susceptible DNA (site d in Fig. 2D). Upon P+I stimulation, the relative HSSI region becomes susceptible to nuclease digestion, indicating remodeling of the two corresponding nucleosomes (slashed in Fig. 2D). In addition to the transcription and translation initiation sites, this region contains multiple binding sites for transcription factors (55), suggesting that
the presence and the remodeling of the two nucleosomes may play a critical role in TNFSF6 transcriptional regulation.

HSS1 Chromatin Remodeling in Lymphocytic Leukemia Cells—HSS1 accessibility was documented further in a physiopathological context using CLL since altered TNFSF6 expression was reported in these patients (56). To minimize the quantity of biological material required, HSS1 accessibility was investigated using in vivo BglII restriction, as described in Fig. 2C. Restriction of CLL cells yielded a similar restriction pattern as that observed in Jurkat cells, with increased restriction within HSS1 region upon P+I stimulation as compared with other regions that remained unchanged after P+I treatment (Fig. 3A). Increased HSS1 accessibility in P+I-treated CLL cells correlated with TNFSF6 mRNA (Fig. 3B). Collectively, these results support further the correlation between TNFSF6 expression and HSS1 accessibility.

Kinetics of TNFSF6-HSS1 Chromatin and TNFSF6 Gene Transcription—The kinetics of HSS1 chromatin remodeling was next monitored in parallel to TNFSF6 mRNA accumulation. Jurkat cells were stimulated with P+I for variable periods of time followed by DNase I hypersensitivity analysis. An increase in HSS1 DNase I accessibility was observed as early as 30 min after T cell activation (Fig. 4A). TNFSF6 mRNA accumulation, as detected by semiquantitative RT-PCR, increased between 1 and 2 h after T cell activation (Fig. 4B). Hence, HSS1 chromatin remodeling preceded detectable TNFSF6 mRNA accumulation (Fig. 4C), suggesting that HSS1 chromatin remodeling is a primary event in the transcriptional activation of TNFSF6 gene expression.

Requirements for TNFSF6-HSS1 Chromatin Remodeling—To precisely define the conditions leading to HSS1 remodeling and to determine whether these events are sufficient to induce gene transcription, we determined HSS1 DNase I accessibility in different settings of T cell activation. A minimal TNFSF6 promoter construct fused to the luciferase reporter gene was used in parallel to monitor for the transcriptional activity induced by these stimuli. Each of the investigated signaling pathways induced HSS1 DNase I accessibility (Fig. 5A) as well as transcriptional activity of the luciferase reporter construct (Fig. 5B), with the exception of the phorbol ester PMA, which induced HSS1 DNase I accessibility but not the luciferase reporter construct (Fig. 5, A and B).

Conversely, the P+I-induced transcriptional activity of TNFSF6 promoter was inhibited by CsA or GF109 (data not shown [57]), two drug inhibitors indirectly preventing NF-AT and NF-κB nuclear translocation, respectively, yet neither CsA nor GF109 impacted on P+I-induced HSS1 accessibility (Fig. 5C). Cycloheximide, which inhibits de novo protein synthesis, blocked both P+I-induced HSS1 accessibility (Fig. 5C) and endogenous TNFSF6 transcription but not the transcriptional activity of the transfected TNFSF6 luciferase-based reporter construct as assessed by RT-PCR analysis (Fig. 5D [58]). Together with the early P+I-induced HSS1 accessibility shown in Fig. 4, these results suggest the involvement of neo-synthesized, immediate-early gene products in HSS1 remodeling. Altogether, our results indicate that in Jurkat T cells, HSS1 accessibility requires specific T cell activation signals that precede, and most likely are required for TNFSF6 transcription. However, active
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FIGURE 3. TNFSF6-HSS1 accessibility in lymphocytic leukemia cells. Nuclei from three independent unstimulated and P-I-stimulated chronic lymphocytic leukemia samples (CLL1–3) were digested in vivo with BglII followed by indirect end-labeling analysis as described in Fig. 2C. B, PCR amplification was performed using the same samples, with primers specific for TNFSF6 (34 cycles) or β-actin (25 cycles). The efficiency of cutting at the site marked by an empty triangle in panel A was assessed by densitometric scanning of the band that resulted from the digestion. The efficiency is indicated as a chromatin remodeling index obtained as the ratio of values obtained for stimulated nuclei and those obtained for unstimulated nuclei. M, molecular weight markers.

and efficient TNFSF6 gene transcription further requires additional induced "signals" such as the NF-AT or NF-kB transcription factors.

Methylation Levels of TNFSF6-HSS1 Do Not Correlate with TNFSF6 Transcription—The differential accessibility of the TNFSF6 regulatory region HSS1 to nucleases in lymphocytes compared with HeLa cells led us to investigate the DNA methylation profile of HSS1 by sodium bisulfite DNA sequencing (Fig. 6). CpG methylation levels were high in both cell lines. Furthermore, no change of the methylation profile was found in the TNFSF6 regulatory region HSS1 in P-I-stimulated Jurkat cells (Fig. 6). These results show that the CpG methylation levels and patterns in the TNFSF6 regulatory region HSS1 do not correlate with the transcriptional activity of the TNFSF6 promoter.

Role of Histone Deacetylation in TNFSF6-HSS1 Chromatin Remodeling and TNFSF6 Transcription—Because recruitment of histone deacetylases leads to transcriptional repression of many genes, we next sought to determine the effect of histone deacetylase inhibitors on TNFSF6 HSS1 remodeling and transcription. Treatment of Jurkat cells with trichostatin A (TSA), a potent nanomolar inhibitor of class I and II histone deacetylases, induced a marked increase of acetylated histones as verified by Western blotting (Fig. 7A). However, a similar TSA treatment did not induce HSS1 chromatin remodeling, as determined by DNase I hypersensitivity analysis in Jurkat cells (Fig. 7B). In agreement with the lack of HSS1 remodeling, TSA treatment also failed to up-regulate TNFSF6 transcription in Jurkat cells, as determined either by RT-PCR analysis (Fig. 7C) or by luciferase reporter gene assays (Fig. 7D). In these experiments TSA potently up-regulated IFNγ mRNA levels (Fig. 7C) and promoter activity (Fig. 7D) as previously described. Similar results were observed in CLL despite basal mRNA levels (Fig. 7E) and in freshly isolated peripheral blood lymphocytes either using TSA or valproic acid, a class I selective histone deacetylase inhibitor (Fig. 7F). Selective inhibitors of the third class of deacetylases (class III), also named sirtuins, were also tested (nicotinamide and sirtinol) yet no impact on TNFSF6 transcription could be detected (Fig. 7G). We, thus, conclude from these results that TNFSF6 HSS1 chromatin remodeling can proceed independently of deacetylase activity and that TNFSF6 transcription cannot be induced by deacetylase inhibition.

DISCUSSION

TNFSF6 can exert either pro- or anti-inflammatory functions, but the effect largely depends on the context and level of expression rather than on TNFSF6 itself (10). Several checkpoints of TNFSF6 gene expression regulation have been described, including transcription control (57, 58), alternative splicing (59), degradation, and cleavage by metalloproteinases, each contributing to differential TNFSF6 cell surface expression (2, 32). Moreover, the expression level of a decoy receptor, TNFRSF6B (DcR3, TR6), plays a regulatory role in TNFSF6-induced signaling (60). In this study we propose a new TNFSF6 expression regulatory level involving chromatin accessibility of the TNFSF6 promoter region. This conclusion is based on several observations. First, we show that in cells displaying inactive and/or inefficient TNFSF6 gene transcription, TNFSF6 5' regulatory regions displayed a poorly accessible chromatin configuration, as assessed by DNase I, specific restriction enzymes, and MNase restriction over the 20-kilobase DNA region we analyzed. By comparison, a similar study performed on the IL-4 locus identified a complex pattern of 5 clustered DNase I-HSS within a comparable 19-ki-
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FIGURE 6. CpG methylation pattern of the HSS1 and exon 1 region of TNFSF6 in HeLa and Jurkat cell lines. The arrow in HSS1 region denotes the start site of transcription (nucleotide +1). Positions of CpG dinucleotides are shown by open circles and noted with respect to nt +1. Open circles, nonmethylated CpG residues; closed circles, methylated CpG residues. P+I, stimulation with PMA and ionomycin. Sodium bisulfite DNA sequences of independent clones are presented.

Third, active TNFSF6 transcription strictly correlated with HSS1 chromatin remodeling that, conversely, did not depend on active and/or efficient gene transcription. In lymphoid T cell lines, agonists inducing TNFSF6 transcription also induced HSS1 chromatin remodeling, whereas agonists inducing HSS1 chromatin remodeling, such as PMA, did not induce TNFSF6 transcription. Drugs preventing HSS1 chromatin remodeling, such as CHX, also blocked TNFSF6 transcription. Fourth, HSS1 chromatin remodeling appears to precede TNFSF6 gene transcription as evidenced by RT-PCR analysis. Run-on experiments will be required to assess this point definitively. However, collectively, these results are in strong support of a localized chromatin remodeling in the TNFSF6 proximal promoter region, allowing or contributing to active and/or efficient gene transcription.

In an attempt to begin the characterization of the molecular basis for TNFSF6 HSS1 chromatin accessibility to the transcriptional machinery, we determined the impact of drugs acting on TNFSF6 transcription and/or known for their effect on chromatin structure and evaluated the DNA methylation status of the TNFSF6 HSS1 region.

Recently it has been shown that nucleosomes positioned across the transcriptional control regions of different genes, including IL-12p35 (61), IL-12p40 (62, 63), IL-2 (64), IFNβ (42, 65), and HIV-1 long terminal repeat (66), are remodeled upon cell activation. Together with TNFSF6, these genes share a common low (or undetectable) basal transcription rate coupled to a high transcription rate in response to activating factors. It has been proposed that precisely positioned nucleosomes within the promoter region of these genes repress the access of the transcriptional machinery to DNA. Thus, a common feature of several inducible transcriptional control regions of different genes, including TNFSF6, is the selective remodeling in the chromatin structure in the proximal promoter region, allowing or contributing to active and/or efficient gene transcription.

In addition, we have shown recently that nucleosomes positioned across the transcriptional control regions of different genes, including IL-12p35 (61), IL-12p40 (62, 63), IL-2 (64), IFNβ (42, 65), and HIV-1 long terminal repeat (66), are remodeled upon cell activation. Together with TNFSF6, these genes share a common low (or undetectable) basal transcription rate coupled to a high transcription rate in response to activating factors. It has been proposed that precisely positioned nucleosomes within the promoter region of these genes repress the access of the transcriptional machinery to DNA. Thus, a common feature of several inducible regulatory regions studied to date, including the TNFSF6 proximal promoter, is the selective remodeling in the chromatin structure in the promoter region upon cell activation. The steps leading to chromatin remodeling and preinitiation complex assembly differ significantly depending upon the gene and its biological context. Studies performed on the IFNβ gene have identified the assembly of an enhanceosome (containing NF-κB, IRF-1 and -3, and ATF2) instructing a recruitment program of chromatin modifiers/remodelers and general transcription factors to the promoter (42, 65). A precisely positioned nucleosome blocks the core promoter in unstimulated cells, and the ordered recruit-
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A/ Anti-Ac H4:
- TSA: +
- P+1:

B/ DNase I:
- - + - +
- M:

C/ - P+1 TSA

D/ Fold induction

E/ Jurkat CLL4 CLL5

F/ - P+1 TSA VPA

G/ - P+1 Nam Sir TSA

FIGURE 7. Impact of deacetylase inhibitors on TNFSF6-HSS1 chromatin remodeling and TNFSF6 transcription. A, the Jurkat T cells were treated or not for 22 h with P+1 either in the presence or absence of TSA (300 nM) followed by anti-acetylated (Anti-Ac) H4 immunoblotting. B, Jurkat T cells were either left untreated or stimulated for 6 h by P+1 or by TSA as indicated. Subsequently, nuclei were analyzed by DNase I digestion followed by indirect end-labeling and KpnI restriction in vitro. Endogenous TNFSF6 mRNA was determined by RT-PCR after treatment of Jurkat T cells (C and G), CLL cells (E), or isolated peripheral blood lymphocytes (F) with TSA (inhibitor of class I histone deacetylase, 300 nM), valproic acid (VPA, class II inhibitor, 5 mM), nicotinamide (Nam, class III inhibitor, 10 mM), sirtinol (Sir, class III inhibitor, 25 μM), or by P+1. The IFNβ mRNA was used as a control. LTR, long terminal repeat. D, the Jurkat T cells were transfected by either pGL3basic- pTNFSF6 (nt –486 and +1) or pIFN (nt –538 and +63) luciferase reporter gene constructs and treated with either TSA (300 nM) or P+1 followed by lysis and determination of luciferase activity. Results are presented as fold induction obtained by dividing the luciferase values obtained for treated cells by values determined for untreated cells.

ment of chromatin modifiers/remodelers instructed by the induced enhanceosome promotes its sliding to a downstream position, allowing active IFNβ gene transcription (42, 65). The combined use of DNase I, MNase, and restriction endonucleases delineates nucleosomes positioned upstream and downstream of the TNFSF6 transcription start site (nt –181 according to the translation start site) in unactivated T cell lines, hence likely overlapping a previously identified NF-κB binding site (67) but leaving nucleosome-free the TNFSF6 transcription start site and the immediate proximal promoter containing binding sites for various transcription factors, including NF-AT, Egr, and Sp1. Although both GF109 and CsA blocked TNFSF6 transcription, HSS1 chromatin region was still remodeled upon T cell stimulation in the presence of these drugs. These results strongly support the hypothesis that transcription factors downstream of protein kinase C and calcineurin, such as NF-κB and NF-AT, are not required for HSS1 chromatin remodeling despite their requirement for TNFSF6 gene transcription. However, chromatin immunoprecipitation experiments using specific antibodies against these transcription factors will be required to confirm in vivo this conclusion. Blocking of both TNFSF6 chromatin remodeling and gene transcription by CHX together with the fast kinetic of chromatin remodeling (<30 min) rather implicated the involvement of neo-synthesized immediate-early factors in these events. The identification of these factors will be important to further dissect the molecular events controlling TNFSF6 gene expression.

DNA methylation at the CpG dinucleotide is well known as a mechanism mediating transcriptional repression. Notably, a recent study evidenced a small region in the promoter-enhancer of the interleukin-2 gene that demethylates in T lymphocytes after activation and remains demethylated thereafter (68). We, thus, examined the CpG methylation pattern of the TNFSF6 promoter by sodium bisulfite genomic DNA sequencing in both TNFSF6-transcribing (activated Jurkat T cells) and -non-transcribing cells (HeLa and unstimulated Jurkat T cells). Although the HSS1 and the exon 1 DNA regions showed strong methylation of the CpG dinucleotides, methylation per se did not correlate with HSS1 chromatin accessibility, since the methylation pattern was indistinguishable in the different TNFSF6 transcribing and non-transcribing cells. This result does not exclude a differential recruitment of chromatin modifiers in TNFSF6-transcribing versus -non-transcribing conditions independently of the DNA methylation levels. Because recruitment of histone deacetylases leads to transcriptional repression, either dependent on or independent of DNA methylation (38, 69), we used inhibitors of this enzymatic activity to examine whether histone deacetylases play roles in TNFSF6 chromatin accessibility and transcriptional regulation. The use of distinct inhibitors yielded similar
results; deacetylase inhibition yielded no significant HSS1 chromatin remodeling and undetectable TNFSF6 transcription despite efficient chromatin remodeling and/or induced transcription of control genes. Because three distinct classes of human histone deacetylases have been described (70, 71), inhibitors acting on class I (valproic acid), class I and II ( TSA), or class III (nicotinamide and sirtinol) were used in our study (72). Of note, despite their enzymatic activity on histone substrates in vitro, recent experimental evidence suggests that class III deacetylases target nonhistone proteins for deacetylation (73). It is possible that, because three distinct classes of human histone deacetylases have been described (70, 71), inhibitors acting on class I (valproic acid), class I and II (TSA), or class III (nicotinamide and sirtinol) were used in our study (72). Of note, despite their enzymatic activity on histone substrates in vitro, recent experimental evidence suggests that class III deacetylases target nonhistone proteins for deacetylation (73). These observations are in agreement with other studies reporting the lack of effect of deacetylase inhibitors on TNFSF6 transcription in normal or preleukemia cells from mice or from CD34+ cells from normal human donors, yet TNFSF6 transcription is significantly increased in a mouse model of acute promyelocytic leukemia and some normal myeloid leukemias (76). On the other hand, deacetylase inhibitors can induce repression of activated TNFSF6 transcription in Jurkat cells, and overexpression of histone acetyltransferases does not impact on TNFSF6 transcriptional activity (77).

Thus, we conclude from these results that TNFSF6 HSS1 chromatin remodeling can proceed independently of deacetylase activity and that TNFSF6 transcription cannot be induced by deacetylase inhibition.

TNFSF6 is a frequent target for inactivation during onogenesis, and TNF-related apoptosis plays a crucial role not only in the biology and response of malignant, but also autoimmune and viral diseases. Altered expression of TNFSF6 or TNFSF6 in tumor or infected cells enabled them to counterattack the immune system (78) and/or resist chemotherapy, likewise by epigenetic silencing (79). In a recent study published during the preparation of our manuscript, deacetylase inhibitors were shown to increase expression of TNFSF6 and TNFSF10, another pro-apoptotic member of the TNFSF family, in blasts from individuals with myelocytic leukemia but not in normal, CD34+ hematopoietic cells (76). In mice with promyelocytic leukemia valproic acid was also reported to up-regulate TNFSF6 expression in leukemia cells but not in preleukemia or in normal cells. This effect correlates with initiation of the caspase cascade and with induction of cellular apoptosis. B cell chronic lymphocytic leukemia is the most frequent leukemia in the adult life of humans in western countries. Yet cells from chronic lymphocytic leukemia patients examined in the present work displayed a similar pattern of HSS1 chromatin accessibility as that observed in normal T cells (Fig. 3). It might, therefore, be interesting to search for altered chromatin remodeling of the TNFSF6 promoter region in such pathological conditions associated with altered expression of TNFSF6 that would, thus, provide a novel therapeutic target. The identification of the mechanisms involved in the herein-described TNFSF6 HSS1 chromatin remodeling would help to clarify that issue.

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