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# Negative Regulation of CD95 Ligand Gene Expression by Vitamin D<sub>3</sub> in T Lymphocytes<sup>1</sup>

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Fas (APO-1/CD95) and its ligand (FasL/CD95L) are cell surface proteins whose interaction activates apoptosis of Fas-expressing targets. In T lymphocytes, the Fas/FasL system regulates activation-induced cell death, a fundamental mechanism for negative selection of immature T cells in the thymus and for maintenance of peripheral tolerance. Aberrant expression of Fas and FasL has also been implicated in diseases in which the lymphocyte homeostasis is compromised, and several studies have described the pathogenic functions of Fas and FasL in vivo, particularly in the induction/regulation of organ-specific autoimmune diseases. The 1,25(OH)<sub>2</sub>D<sub>3</sub> is a secosteroid hormone that activates the nuclear receptor vitamin D<sub>3</sub> receptor (VDR), whose immunosuppressive activities have been well studied in different models of autoimmune disease and in experimental organ transplantation. We and others have recently described the molecular mechanisms responsible for the negative regulation of the *IFN-γ* and *IL-12* genes by 1,25(OH)<sub>2</sub>D<sub>3</sub> in activated T lymphocytes and macrophages/dendritic cells. In this study, we describe the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the activation of the *fasL* gene in T lymphocytes. We show that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits activation-induced cell death, *fasL* mRNA expression, and that 1,25(OH)<sub>2</sub>D<sub>3</sub>-activated VDR represses *fasL* promoter activity by a mechanism dependent on the presence of a functional VDR DNA-binding domain and ligand-dependent transcriptional activation domain (AF-2). Moreover, we identified a minimal region of the promoter containing the transcription start site and a noncanonical c-Myc-binding element, which mediates this repression. These results place FasL as a novel target for the immunoregulatory activities of 1,25(OH)<sub>2</sub>D<sub>3</sub>, and confirm the interest for a possible pharmacological use of this molecule and its derivatives. *The Journal of Immunology*, 2002, 168: 1154–1166.

**F**as ligand (also called CD95L or FasL<sup>3</sup>), a type II transmembrane protein that induces programmed cell death (apoptosis) by binding to its receptor Fas (also called CD95/Apo-1), is an important molecule implicated in the activation-induced cell death (AICD) of T lymphocytes, the maintenance of immunologically privileged anatomical sites, and the CTL and NK cell-mediated cytotoxicity against a variety of tumor and virus-infected cells (1–3).

Moreover, aberrant expression of Fas and FasL has been involved in diseases in which the peripheral lymphocyte ho-

meostasis is compromised. Mutations of the genes encoding Fas or FasL (the mutations are termed *lpr* and *gld*, respectively) give rise to fatal autoimmunity and lymphadenopathy, and individuals bearing heterozygous mutations in the *fas* or the *fasL* gene develop severe autoimmune lymphoproliferative syndromes (4, 5).

Recently, a number of studies have significantly improved the knowledge of the pathophysiological role of Fas and FasL in vivo, particularly in the induction and regulation of several organ-specific autoimmune diseases. In this regard, an improperly regulated Fas/FasL system could become a serious danger for the organism, leading to selective destruction of target cells within a tissue. This process has been well documented in animal models of experimental allergic encephalomyelitis (EAE), or autoimmune diabetes, in which the initial specific cell damage is mediated by tissue-infiltrating FasL<sup>+</sup> T lymphocytes (6, 7).

Although FasL has been extensively studied for its role in delivering apoptotic signals through its receptor Fas on sensitive target cells, experimental evidence has also revealed a second role as a reverse signaling activating receptor in T cells. In fact, similarly to the previously described dual-direction signaling for different TNF superfamily of ligand-receptor pairs, such as CD27L, CD30L, OX40L, and CD40L (8, 9), triggering of FasL is required for CTLs to achieve an optimal proliferation. In this context, a further level of activation and fine regulation during an immune response is also given by the ability of the activated Fas receptor to induce phenotypic and functional maturation of dendritic cells (DC), secretion of proinflammatory cytokines such as IL-1β and TNF-α, and preferential T cell polarization into a Th1 phenotype (10).

The 1,25(OH)<sub>2</sub>D<sub>3</sub> (Calcitriol, the biologically active metabolite of vitamin D<sub>3</sub>) is a secosteroid hormone that binds and activates

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<sup>3</sup> Abbreviations used in this paper: FasL, Fas ligand; AICD, activation-induced cell death; CBP, CREB-binding protein; CsA, cyclosporin A; DBD, DNA-binding domain; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EGR, early growth response; GR, glucocorticoid receptor; LBD, ligand-binding domain; LTR, long-terminal repeat; PI, propidium iodide; RAR, retinoic acid receptor; RSV, Rous sarcoma virus; RXR, retinoid X receptor; VDR, vitamin D<sub>3</sub> receptor; VDRE, VDR-responsive element.

the nuclear vitamin D<sub>3</sub> receptor (VDR), belonging to the superfamily of steroid and thyroid hormone receptors (11, 12). The classical functions of 1,25(OH)<sub>2</sub>D<sub>3</sub> include regulation of calcium absorption in the intestine, maintenance of mineral homeostasis in the kidney, and regulation of bone remodeling (11, 12). Nevertheless, 1,25(OH)<sub>2</sub>D<sub>3</sub> also functions as a regulator of the hemopoietic system, as it modulates lymphocyte activation and proliferation, induces the differentiation of promyelocytes into monocytes, and inhibits secretion of several cytokines in T cells (13, 14). Furthermore, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits differentiation, activation, and survival of DC, thus contributing to decrease DC-dependent T cell activation and to suppress immune response (15, 16).

The immunosuppressive activity of 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogues has been studied in different models of autoimmune diseases and in experimental organ transplantation (17). Administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> can greatly reduce the severity of EAE, an animal model of the multiple sclerosis (MS) (18–20), prevent systemic lupus erythematosus in *lpr/lpr* mice (21), and ameliorate autoimmune destruction of syngeneic islet grafts in spontaneously diabetic nonobese diabetic mice in combination with cyclosporin A (CsA) (22).

We and others have recently described the molecular mechanisms responsible for the negative regulation of the IFN- $\gamma$  and IL-12 genes, by 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR in activated T lymphocytes and macrophages/DC (23, 24).

In this study, we describe the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the activation of the *fasL* gene in T lymphocytes and the regulatory action of the 1,25(OH)<sub>2</sub>D<sub>3</sub>-activated VDR on the human *fasL* promoter. We show that 1,25(OH)<sub>2</sub>D<sub>3</sub> suppresses AICD and *fasL* mRNA expression in the activated 2B4.11 T cell hybridoma. We also demonstrate that the PMA plus ionomycin-stimulated *fasL* promoter activity is inhibited by 1,25(OH)<sub>2</sub>D<sub>3</sub> in 2B4.11 cells, and in Jurkat cells cotransfected with a human VDR expression vector. The inhibition is dependent on the presence of an intact VDR DNA-binding domain (DBD) and ligand-dependent transcriptional activation domain (AF-2), and the cotransfection of retinoid X receptor (RXR) enhances this effect. Moreover, by progressive deletions of the *fasL* promoter, we delineated a minimal region containing the transcription start site and a noncanonical c-Myc-binding element that mediates the repression (25). The data presented in this work place FasL as a novel target for the different immunoregulatory activities of 1,25(OH)<sub>2</sub>D<sub>3</sub>. The physiological and pharmacological implications of these observations are discussed.

## Materials and Methods

### Cell lines and reagents

Jurkat cells, 2B4.11 murine T hybridoma cells, and P815-Fas mastocytoma cells were maintained as described previously (23). PMA and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO). The 1,25(OH)<sub>2</sub>D<sub>3</sub> and CsA were purchased from Biomol (Plymouth Meeting, PA).

### Assessment of cell viability and apoptosis

For the induction of apoptosis, 2B4.11 cells ( $5 \times 10^5$ /ml) were cultured in 24-well plates. Triplicate samples were stimulated with 20 ng/ml PMA and 0.5  $\mu$ g/ml ionomycin in the absence or in the presence of the indicated amount of 1,25(OH)<sub>2</sub>D<sub>3</sub> or 200 ng/ml CsA, for 24 h in complete medium. Cells were harvested and viability was assessed by addition of 5  $\mu$ g/ml propidium iodide (PI; Sigma-Aldrich) and immediate analysis by a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Dead cells were quantified as those taking up the dye. Apoptosis assay was performed by annexin V staining of the translocated phosphatidylserine, from the inner side of the plasma membrane to the outer layer during the early stages of apoptosis (26). Triplicate samples of 2B4.11 cells ( $5 \times 10^5$ /ml) were cultured in 24-well plates and stimulated with 20 ng/ml PMA and 0.5  $\mu$ g/ml ionomycin in the absence or in the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> or 200 ng/ml CsA, for 5 h in complete medium. Cells were then stained using an Annexin V<sup>FITC</sup> kit (Bender MedSystems, Vienna,

Austria) following the manufacturer's instructions and immediately analyzed by a FACScan flow cytometer (BD Biosciences).

### Northern blot analysis

Total RNA was extracted from 2B4.11 hybridoma T cells by TRIzol (Life Technologies, Grand Island, NY). The cells were untreated or stimulated with 20 ng/ml PMA and 0.5  $\mu$ g/ml ionomycin in the absence or in the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> for 5 h. Equal amounts of RNA (15  $\mu$ g/lane) were fractionated on a 1.5% agarose-formaldehyde gel. The specific mRNA was detected by hybridization of S&S Nytran membranes (Schleicher & Schuell, Keene, NJ) with a <sup>32</sup>P-labeled cDNA probe for murine FasL. The RNA-containing membranes were prehybridized for 20 min and hybridized for 2 h at 65°C with the QuikHyb Hybridization Solution (Stratagene, La Jolla, CA). The membranes were then washed twice in 2 $\times$  SSC containing 0.1% SDS and twice in 0.1 $\times$  SSC containing 0.1% SDS at 60°C (20 min each time), and exposed to X-Omat AR films (Eastman Kodak, Rochester, NY) at -70°C with intensifying screens. The probe for murine FasL was generated by RT-PCR according to standard methods, on total RNA extracted from 2B4.11 cells activated for 3 h with PMA plus ionomycin. Primers for amplification: FasL forward, 5'-CAGCTCTTC CACCTGCAGAAGG-3'; FasL reverse, 5'-AGATTCCTCAAAATTGATCA GAGAGAG-3'.

### Cytotoxicity assay

Fas-transfected P815 mastocytoma cells ( $10^6$  cells; kindly provided by R. De Maria, ISS, Rome, Italy) were labeled with 0.2 mCi of <sup>51</sup>Cr (Amersham, Arlington Heights, IL) at 37°C. A total of  $2 \times 10^3$  target cells was cocultured with 2B4.11 effector cells in 200  $\mu$ l of complete medium in U-bottom 96-well plates for 5 h. The 2B4.11 effector cells were previously activated with 20 ng/ml PMA and 0.5  $\mu$ g/ml ionomycin in the absence or in the presence of 20 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or 200 ng/ml CsA, for 4 h in complete medium. Cells were then harvested, washed twice in complete medium, and cocultured with <sup>51</sup>Cr-labeled target cells. After 4 h, 100  $\mu$ l of supernatant was removed from each well and counted in a gamma counter for <sup>51</sup>Cr release determination. Maximal and spontaneous release was determined by incubating <sup>51</sup>Cr-labeled target cells with 1% Nonidet P-40 or medium alone, respectively. Percent specific killing was calculated as 100% (experimental <sup>51</sup>Cr release - spontaneous <sup>51</sup>Cr release)/(maximal <sup>51</sup>Cr release - spontaneous <sup>51</sup>Cr release). All determinations were made in triplicate, and E:T ratios ranged from 80:1 to 20:1, as indicated.

### Plasmid constructions

The human FasL promoter luciferase reporter pFasL-486, the distal and proximal NF-AT-binding mutants (DNF-AT-Dist. and DNF-AT-Prox.), the RE3/FLRE-binding mutant, the 3XNF-AT-distal, and the 3XRE3/FLRE triplicated copies constructs of the indicated FasL promoter enhancers were kindly provided by G. A. Koretzky (Department of Internal Medicine, Iowa City, IA). To prepare the human FasL promoter  $\beta$ -Gal reporter pFasL-486Gal, the appropriate FasL promoter fragment was subcloned into the *Hind*III site of the promoterless pEQ3  $\beta$ -Gal reporter vector. The different deletions of the human FasL promoter -453 FasL(pGL2), -373 FasL(pGL2), -318 FasL(pGL2), -237 FasL(pGL2), and -195 FasL(pGL2) were kindly provided by C. V. Paya (Mayo Clinic, Rochester, MN). The CMV- $\beta$ -Gal expression vector pEQ176 was kindly provided by C. B. Wilson (Department of Pediatrics and Immunology, University of Washington, Seattle, WA). To prepare the Rous sarcoma virus (RSV)-luc expression vector, a *Hind*III-*Xba*I fragment containing the complete luciferase-coding region was digested from the pGL3 basic luciferase vector (Promega, Madison, WI) and cloned into the pRcRSV expression vector (Invitrogen, San Diego, CA). To prepare the RSV-Gal expression vector, a *Sal*I-*Hind*III fragment containing the RSV-LTR (long-terminal repeat) was digested from the pREP7 expression vector (Invitrogen) and cloned into the promoterless pEQ3  $\beta$ -Gal reporter vector. Expression vectors for wild-type human VDR pCMV-hVDR, and VDR DBD mutants pCMV-hVDR (EGG>GSV), pCMV-hVDR(R50G), and the pCMV-VDRF (VDRF; residues 14–114 of the VDR DBD) were kindly provided by L. Freedman (Memorial Sloan-Kettering Cancer Center, New York, NY). Expression vectors for wild-type human VDR pSG5-VDR and for VDR AF-2 domain point mutants pSG5-VDR(L417>A) and pSG5-VDR(E420>A) were kindly provided by M. R. Haussler (Department of Biochemistry, University of Arizona, Tucson, AZ). The expression vector for human VDR helix 3 domain point mutant K246A, pSG5VDR(K246>A), was kindly provided by A. Aranda (Universidad Autonoma de Madrid, Madrid, Spain). Expression vectors for wild-type human and murine RXR $\alpha$  and for C-terminal deletion mutants of murine RXR $\alpha$  (dominant-negative mutants: mRXR $\alpha$ T1 truncated at aa 448, and RXR $\alpha$ T2 truncated at aa 454) were kindly provided by P. Chambon (Centre National de la Recherche Scientifique-Institut National de la Sante et de la Recherche

Medicale-Université Louis Pasteur, Illkirch Cedex, France). The CMV and RSV-driven expression vectors for human c-Myc (pcDNA3cMyc) and c-Rel (pRSV-Rel) were provided by G. Zupi (Regina Elena Cancer Institute, Rome, Italy) and H. A. Young (National Institutes of Health, Frederick, MD), respectively.

### DNA transfections

Transfections of Jurkat cells were conducted by the DEAE-dextran method, as already described (23). To decrease variations in the experiments due to different transfection efficiency, cells were transfected in single batches that were then separated into different drug treatment groups. A CMV- $\beta$ -Gal or RSV-Gal (for luciferase assays) or RSV-luc (for  $\beta$ -galactosidase assays) expression vector was cotransfected to normalize DNA uptake. After 24 h, cells were treated with different combinations of stimuli and, after additional 24 h, cells were harvested and protein extracts were prepared for the  $\beta$ -galactosidase and luciferase assays, by four cycles of rapid freezing and thawing, followed by centrifugation at 14,000 rpm (4°C) for 15 min. Protein concentration was quantified by the bicinchoninic acid method (Pierce, Rockford, IL). Luciferase activity was read using the luciferase assay system (Promega, Madison, WI) following the manufacturer's instructions.  $\beta$ -galactosidase activity was determined spectrophotometrically at 570 nm by the hydrolysis of chlorophenol red  $\beta$ -D-galactopyranoside, as described previously (23). The 2B4.11 murine T hybridoma cells were transfected as described for Jurkat cells, using 150  $\mu$ g/ml DEAE-dextran. After 24 h, cells were treated with different combinations of stimuli for additional 8 h and then processed as described above.

### EMSA

Nuclear proteins were prepared as described previously (23). Protein concentration of extracts was determined by the bicinchoninic acid method (Pierce). The nuclear proteins (10  $\mu$ g) were incubated with radiolabeled DNA probes in a 20- $\mu$ l reaction mixture containing 20 mM of Tris (pH 7.5), 60 mM of KCl, 2 mM of EDTA, 0.5 mM of DTT, 1–2  $\mu$ g of poly(dI-dC) or poly(dG-dC), and 4% Ficoll. In some cases, the indicated amount of double-strand oligomer was added as a cold competitor, and the mixture was incubated at room temperature for 10 min before adding the DNA probe. Nucleoprotein complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels in 0.5 $\times$  Tris-borate-EDTA buffer at 12 V/cm for 2 h at room temperature. Dried gels were exposed to Kodak XAR-5 film (Eastman Kodak) at –70°C with intensifying screens. Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA synthesizer (Applied Biosystems, Foster City, CA; model 392). Complementary strands were denatured at 90°C for 5 min and annealed at room temperature. The double-strand probes were end labeled using Klenow fragment (Life Technologies) and [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham). Unincorporated [ $\alpha$ -<sup>32</sup>P]dCTP was removed by chromatography through a G-25 spin column (Boehringer Mannheim, Mannheim, Germany). Approximately 3  $\times$  10<sup>4</sup> cpm of labeled DNA was used in a standard EMSA reaction. In supershift analysis, the Ab was added to the binding reaction and the mixture was incubated for 30 min at room temperature before adding the labeled DNA probe. The Ab against VDR (clone 9A7, a rat mAb) was purchased from Biomol. An Ab against c-Jun (rabbit polyclonal corresponding to the conserved residues 247–263 of the protein; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a nonspecific Ab. The following double-strand oligomers were used as specific labeled probes or cold competitors: FasL-NF- $\kappa$ B prox. (human *FasL* gene, –162 to –135 bp), 5'-AGACAGAGGTGTTTCCCTTAGCTATGGA-3'; FasL-MycRE (human *FasL* gene, –139 to –110 bp), 5'-ATGAACTCTATAA GAGAGATCCAGCTTG-3'; consensus Myc, 5'-gacCCCCACCAGT GGTGCCTGA-3'; human VDRE (VDR-responsive element) (OC3, 4) (human osteocalcin gene), 5'-agctTCGCGGGTGAACGGGGGCA GAGCA-3'; mVDRE (mouse osteopontin gene), 5'-agctCACAAGGT TCACGAGGTTACAGTCCG-3'; NF- $\kappa$ B (HIV-1 LTR, –107 to –91 bp), 5'-gatACAAGGGACTTCCGCT-3'; OCT (human histone H2b, –60 to –35), 5'-agCTCTTCACTTATTTGCATAAGCGAT-3', was used as a nonspecific competitor.

## Results

### 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits AICD in 2B4.11 murine T cell hybridoma

In T lymphocytes, *fasL* mRNA expression is induced by TCR-mediated activation or by stimuli such as phorbol ester plus a Ca<sup>2+</sup> ionophore that bypass TCR signaling (27). Recent studies with the *fasL* promoter have identified several enhancer elements that cooperate in the transcription of this gene in activated T cells. Among these, NF-AT, early growth response (EGR) 2/3, and c-Myc play

an absolutely necessary role in this process (25, 28–33). In T lymphocytes, induction of *fasL* mRNA is inhibited by a number of immunomodulators such as CsA that inhibits the activation of the NF-AT and EGR-2/3 transcription factors, or TGF- $\beta$ 1 that inhibits c-Myc expression (30, 31, 34). In addition, ligand-mediated activation of different nuclear hormone receptors such as glucocorticoid receptor (GR) or retinoic acid receptors (RAR/RXR), which prevent AICD, also inhibits up-regulation of FasL in T lymphocytes (35–38).

To investigate whether 1,25(OH)<sub>2</sub>D<sub>3</sub> could interfere with the AICD and *fasL* gene expression in activated T lymphocytes, 2B4.11 T cells were activated with PMA plus ionomycin in the presence or absence of increasing concentrations of hormone, and cell viability and apoptosis were measured by PI uptake or annexin V/PI staining, followed by FACS analysis. As shown in Fig. 1A, activation of 2B4.11 T cells by the combination of PMA plus ionomycin for 24 h resulted in significant (~50%) cell death, which was almost completely inhibited by the presence of CsA (used in these experiments as a control for effective repression). The AICD of 2B4.11 cells significantly decreased in the presence of increasing concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> in a dose-dependent fashion (Fig. 1B); apoptosis assays performed by annexin V staining of early stage apoptotic cells confirmed inhibition of AICD, with a ~50% reduction after 5 h PMA plus ionomycin activation in the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 1C, panel c). Treatment of 2B4.11 cells with 1,25(OH)<sub>2</sub>D<sub>3</sub> alone did not alter cell viability or growth in our assays (data not shown).

### 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits *fasL* gene expression and FasL-mediated target lysis by activated 2B4.11 T cells

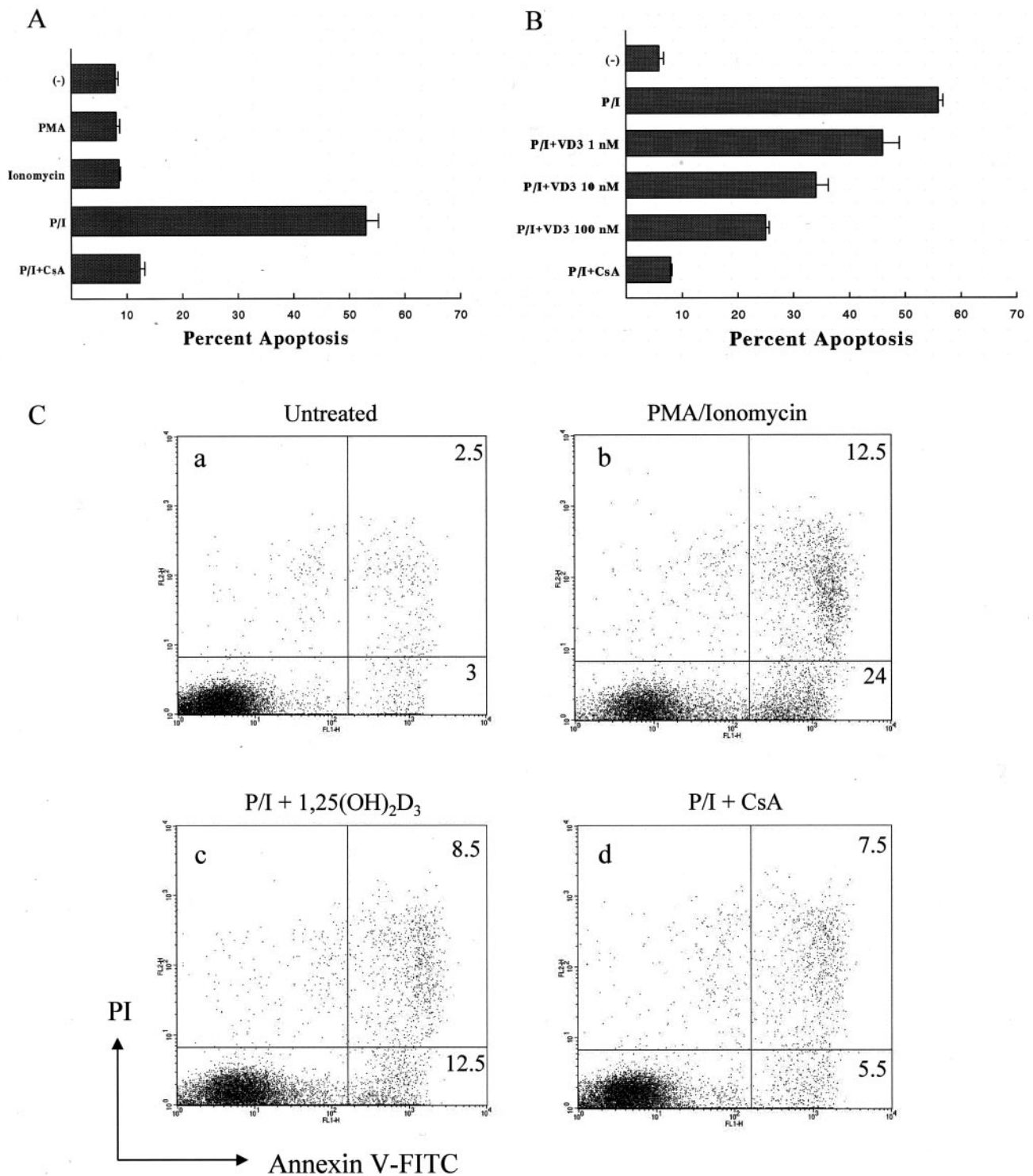
Previous studies have demonstrated that AICD in 2B4.11 T cell hybridoma proceeds via expression of FasL and subsequent Fas/FasL interaction (39). To determine whether one of the mechanisms of 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition of AICD could be direct interference with *fasL* gene expression, total RNA was isolated from 2B4.11 cells at 5 h after activation in the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> and analyzed for *fasL* mRNA expression by Northern blot assay. As shown in Fig. 2A, *fasL* mRNA was induced by PMA plus ionomycin stimulation, and inhibited by the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub>, indicating that FasL is a molecular target of this hormone.

To further verify the functional consequences of 1,25(OH)<sub>2</sub>D<sub>3</sub> on FasL expression, a cytotoxicity assay using <sup>51</sup>Cr-labeled Fas-transfected P815 target cells was performed. As shown in Fig. 2B, activation of 2B4.11 cells in the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly decreased specific killing, when compared with the cytotoxicity of 2B4.11 cells activated in the absence of hormone. The effect of CsA (used in these experiments as a control for effective repression) is also shown in the figure.

Thus, AICD, activation-induced *fasL* mRNA expression, and cytotoxicity of Fas-transfected targets are inhibited by 1,25(OH)<sub>2</sub>D<sub>3</sub> in T cells, as previously shown for other modulators such as glucocorticoids and retinoids (35–38).

### 1,25(OH)<sub>2</sub>D<sub>3</sub> modulates *fasL* promoter activity in activated T cells

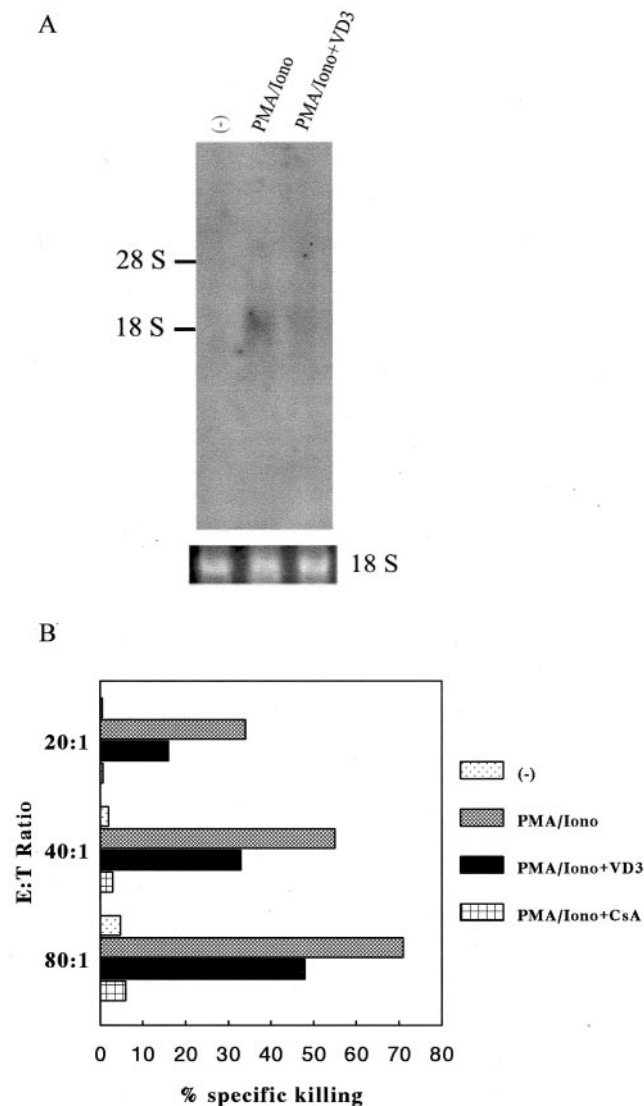
Ligand-activated nuclear hormone receptors, such as GR and RAR/RXRs, act as powerful immunomodulators and repress different cytokine genes through negative interference with a number of transcriptional factors important for specific enhancer activity (40–44). In this regard, a number of experimental data have clearly shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> can repress transcription and expression of several cytokines by directly modulating the activity,



**FIGURE 1.** The 1,25(OH)<sub>2</sub>D<sub>3</sub> represses AICD in 2B4.11 T cell hybridoma. *A* and *B*,  $5 \times 10^5$ /ml 2B4.11 hybridoma cells were stimulated in triplicate with 20 ng/ml PMA and/or 0.5  $\mu$ g/ml ionomycin in the absence or in the presence of 200 ng/ml CsA, or the indicated concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Twenty-four hours after stimulation, cells were harvested and viability was assessed by PI uptake and immediate analysis by a flow cytometer. Results are expressed as percentage of apoptosis, and represent the mean value ( $\bar{X} \pm$  SE) from at least four experiments. *C*, 2B4.11 hybridoma cells were stimulated as described above for 5 h, in the absence or in the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> or 200 ng/ml CsA. The cells stained with Annexin-V<sup>FITC</sup> single positive (*lower right*) and PI/Annexin V<sup>FITC</sup> double positive (*upper right*) are early and late phase apoptotic cells, respectively. *a*, Unstimulated cells. *b*, Five-hour PMA plus ionomycin. *c*, Five-hour PMA plus ionomycin plus 20 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>. *d*, 5-h PMA plus ionomycin plus 200 ng/ml CsA. Percentage of single- and double-positive cells is displayed in each panel (*lower right* and *upper right* quadrants). The experiment shown in the figure is representative of four independent experiments, all displaying similar results.

DNA binding, and/or the expression of different transcription factors such as NF-AT, NF- $\kappa$ B, or c-Myc in activated T lymphocytes (23, 24, 45–49). To determine whether one of the mechanisms

involved in 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition of the *fasL* gene activation could be direct interference with the transcriptional activity of its promoter, transient transfection assays were performed in



**FIGURE 2.** Effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the *fasL* mRNA expression and FasL-mediated target lysis. **A**, Northern blot analysis of total mRNA, obtained from 2B4.11 hybridoma cells untreated (-) and stimulated with 20 ng/ml PMA and 0.5 μg/ml ionomycin for 5 h, as described in Fig. 1, in the absence or in the presence of 20 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>). The experiment shown in the figure is representative of three independent experiments, all displaying similar results. **B**, <sup>51</sup>Cr-labeled P815-Fas target cells were incubated for 5 h with 2B4.11 effector cells previously activated as indicated in the figure, to induce Fas/FasL-dependent lysis. One experiment representative of three is shown.

2B4.11 and Jurkat T cells. As shown in Fig. 3A, PMA plus ionomycin treatment of 2B4.11 cells induces activation of a human *fasL* promoter fragment consisting of 486 bp immediately 5' of the translational start site (50), and the presence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly represses (~40–50%) the promoter activity. On the contrary, 1,25(OH)<sub>2</sub>D<sub>3</sub> does not repress activation of the *fasL* promoter in Jurkat cells (Fig. 3B), indicating that the Jurkat cells used in this study are resistant to 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (23). These data are substantiated by the expression level of VDR in these cells, as detected in EMSA. A specific binding of complexes containing VDR to a consensus VDRE (mVDRE (mouse osteopontin gene)) was detected only in nuclear extracts of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated 2B4.11 cells, but not in Jurkat cells (Fig. 3, C and D), indicating that expression of VDR correlates with re-

pression of FasL by 1,25(OH)<sub>2</sub>D<sub>3</sub>. Similar results were confirmed by Western blot assay (data not shown).

Thus, activation-induced *fasL* promoter activity is inhibited by 1,25(OH)<sub>2</sub>D<sub>3</sub> in T cells.

To better study the role of VDR on the repression of the *fasL* promoter, Jurkat cells were used as a convenient cell system for transfection assays, and in agreement with the data described above, cotransfection of a human VDR expression vector was required for significant 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition of the *fasL* promoter in Jurkat cells (Fig. 4A).

Vitamin D<sub>3</sub> receptor heterodimerizes with the 9-*cis* retinoic acid receptor (called RXR), and formation of these heterodimers results in increased nuclear receptor binding in vitro and cooperation for gene activation in vivo (51). As shown in Fig. 4B, cotransfection of an RXR expression vector together with VDR further enhances the repression observed in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Cotransfection of the RXR alone is ineffective either in the presence or in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub>. These data indicate that RXR cooperates in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition of the *fasL* promoter.

In agreement with the cell viability assay, promoter repression is regulated in a dose-dependent manner with detectable inhibition at 0.1 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub>, and cotransfection of RXR further magnifies this effect (Fig. 5, A and B).

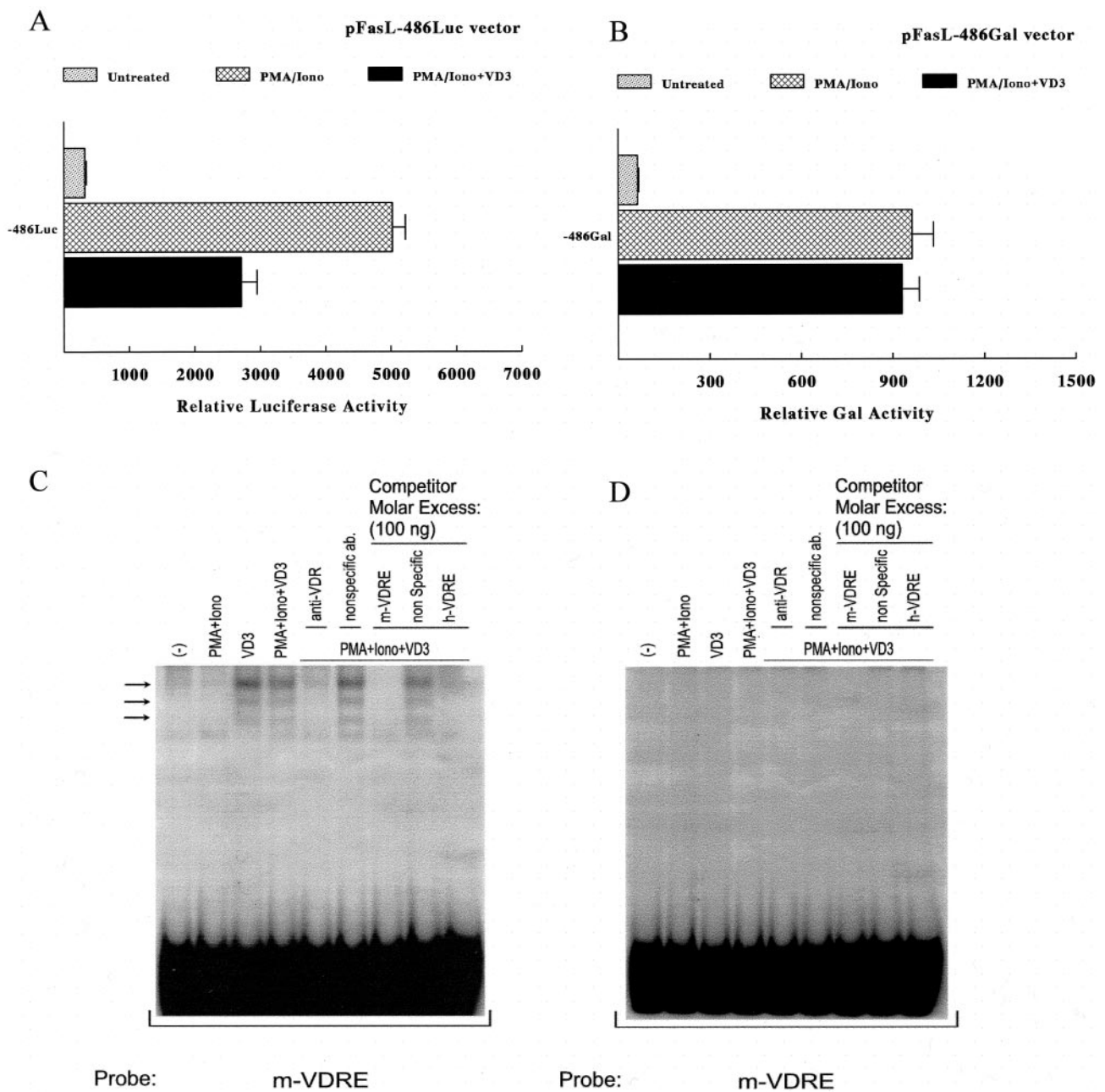
#### *Different VDR regulatory domains are involved in 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated fasL promoter repression: VDR DBD*

Transcriptional repression by ligand-activated nuclear hormone receptors may act through competition interference with DNA-binding factors to overlapping sequences, or by interference through direct protein-protein interaction (44, 52, 53). Moreover, several members of the nuclear receptor family, including GR, RAR/RXR, thyroid hormone receptor (T<sub>3</sub>R), and VDR, interact with a number of coregulators (e.g., CREB-binding protein (CBP)/p300, SRC-1, etc.) that are required for efficient transcriptional regulation (54, 55). In this regard, competition for limiting amounts of shared coregulators may partially account for the repressive action exerted by ligand-activated nuclear receptors on several promoters (55).

To investigate the relevance of the ligand-activated VDR-DNA interaction(s) in 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated *fasL* promoter repression, mutants of the human VDR DBD within its first zinc module that abrogate specific binding to DNA (56) were used in cotransfection assay. As shown in Fig. 6A, the repression mediated by two different DBD mutants (VDR EGG>GSV and VDR-R50>G) is greatly reduced in comparison with the VDR wild type, indicating that binding to DNA represents an important factor in this mechanism. A third mutant, VDRF (VDR-DBD alone; residues 14–114), which lacks the ligand-binding domain (LBD) and binds selectively and with high affinity to a positive vitamin D<sub>3</sub> response element independently of hormone (56), was also tested in cotransfection. Interestingly, repression of the *fasL* promoter was not observed either in the absence or in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 6B and data not shown), suggesting that DNA binding by itself is not sufficient for transcriptional inhibition and that other requirements are needed.

#### *Point mutations of the VDR that abrogate ligand-dependent interactions with transcriptional coactivators interfere with transrepression of the fasL promoter*

Nuclear receptors display a modular structure with an N-terminal region containing an activation function AF-1 (A/B domain), a region C containing the DBD, a hinge region D, and the C-terminal

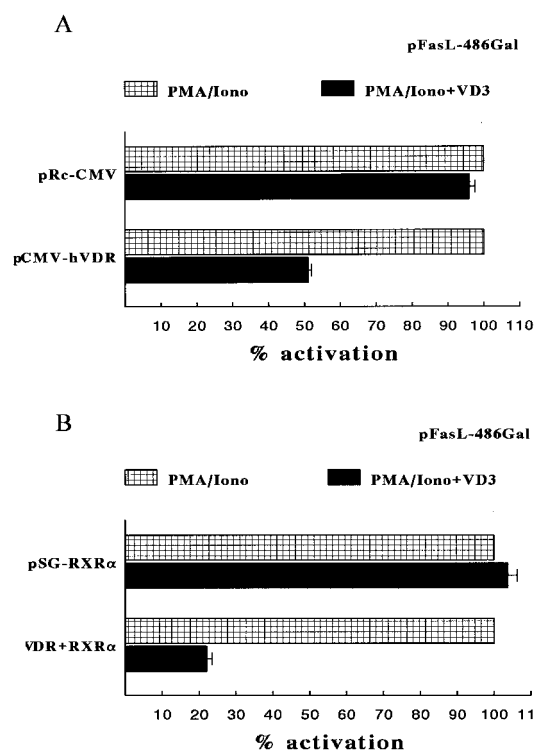


**FIGURE 3.** The  $1,25(\text{OH})_2\text{D}_3$  represses *fasL* promoter activation. *A* and *B*, 2B4.11 and Jurkat T cells were cotransfected with  $15 \mu\text{g}$  of pFasL-486Luc or pFasL-486Gal, plus  $3 \mu\text{g}$  of pEQ176 CMV- $\beta$ -Gal expression vector or RSV-luc expression vector, respectively, as described in *Materials and Methods*. Twenty-four hours after transfection, cells were stimulated with  $20 \text{ ng/ml}$  PMA and  $0.5 \mu\text{g/ml}$  ionomycin in the absence or in the presence of  $20 \text{ nM}$  of  $1,25(\text{OH})_2\text{D}_3$ . After 24 h, cells were harvested and protein extracts were prepared for the luciferase and  $\beta$ -galactosidase assays. Results are expressed as relative luciferase or  $\beta$ -galactosidase activity normalized to protein concentration as well as to  $\beta$ -galactosidase or luciferase activity produced off the internal control plasmid, and represent the mean value ( $X \pm \text{SE}$ ) from at least four experiments. *C* and *D*, EMSA was performed using the  $^{32}\text{P}$ -labeled murine VDRE oligonucleotide as a probe in the presence of nuclear extracts ( $10 \mu\text{g}$ ), from unstimulated (-) or PMA/ionomycin-treated 2B4.11 and Jurkat cells (4 h) in the absence or in the presence of  $20 \text{ nM}$  of  $1,25(\text{OH})_2\text{D}_3$ . Where indicated,  $100 \text{ ng}$  specific or nonspecific cold competitor, purified anti-VDR, or a nonspecific Ab was added to the reaction mixture to confirm specificity. Arrows represent the DNA binding of VDRE-specific complexes.

E/F region containing the LBD and the dimerization domain (57, 58). Mutational analysis of the E domain has identified a second activation-function AF-2 domain, which is critical for proper ligand-dependent activation by nuclear receptors (58). It has been proposed that upon specific ligand binding, nuclear receptors may reconfigure particular surface features of the LBD. A conformational change in helix 12, together with other changes such as bending of helix 3 in the LBD, creates a surface that allows an efficient recruitment of coactivator proteins that participate to the efficient assembly of the transcriptional apparatus (59). They in-

clude related family members such as SRC-1/NC0A1, TIF-2/GRIP-1, and ACTR/pCIP. Moreover, ligand-activated transcription by nuclear hormone receptors appears to require the CBP/p300 coactivator, which interacts directly with and is a required component common to the coactivator complexes of several nuclear receptors and general transcription factors (54, 55, 59).

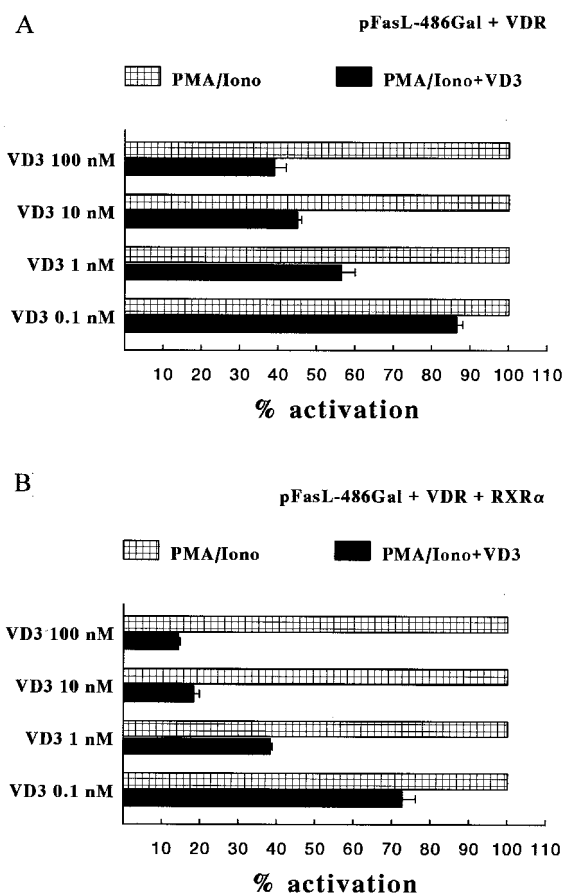
To investigate whether the observed  $1,25(\text{OH})_2\text{D}_3$ /VDR-mediated inhibition of the *fasL* promoter was due to a possible interference between ligand-activated recruitment or sequestration of shared transcriptional coactivators by VDR, different point mutants of



**FIGURE 4.** RXR cooperates in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition of the *fasL* promoter. *A*, Jurkat T cells were cotransfected with 15 μg pFasL-486Gal reporter vector plus 4 μg of pCMV-hVDR (or pRcCMV) expression vector and 3 μg of RSV-luc. Results are expressed as percentage of activation, relative to the control PMA plus ionomycin-activatable β-galactosidase expression in each cotransfection series, in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>) (considered here as 100%), and represent the mean value (X ± SE) from at least four experiments. *B*, Jurkat T cells were cotransfected with 15 μg of pFasL-486Gal reporter vector plus 3 μg of the indicated expression vector(s) (pCMV-hVDR and/or pSG-hRXRα) and 3 μg of RSV-luc. Results are shown as described in *A*. The mean relative β-galactosidase activity of PMA plus ionomycin-activated cultures in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub>, in order of appearance in the figure, was as follows: 760 and 1000 (*A*); 785 and 630 (*B*).

the human VDR within its AF-2 domain or a point mutant in the helix 3 of the LBD that abrogate normal recruitment of specific coactivators (60, 61) were used in cotransfection assay. As shown in Fig. 7, *A* and *B*, the repression mediated by these different mutants (VDR-L417>A, VDR-E420>A, and VDR-K246>A) is reduced in comparison with the VDR wild type. Although these VDR mutants are transcriptionally inactive, they maintain a correct conformation, the same ability to form heterodimers with RXR, and can bind the ligand and the DNA with affinities similar to the VDR wild type (60), indicating that competition for limiting amounts of shared coactivators might be one of the mechanisms involved in *fasL* promoter repression.

Ligand-activated VDR heterodimerizes with RXR, which also possesses an AF-2 domain involved in transcriptional activation. In this regard, C-terminally truncated RXRs function as dominant-negative partners in VDR-mediated transcription (62). It has been proposed that 1,25(OH)<sub>2</sub>D<sub>3</sub>-occupied VDR-RXR heterodimers can elicit maximal transcriptional stimulation via AF-2s of both receptors, and allosteric modulation of RXR-AF-2 domain by 1,25(OH)<sub>2</sub>D<sub>3</sub> and consequent AF-2s cooperation have been suggested (63). To investigate the possible role of the RXR AF-2 domain in 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated *fasL* promoter repression, different C-terminal (AF-2) deletion mutants of RXRα were used in cotransfection assay. As shown in Fig. 7C and in agreement with



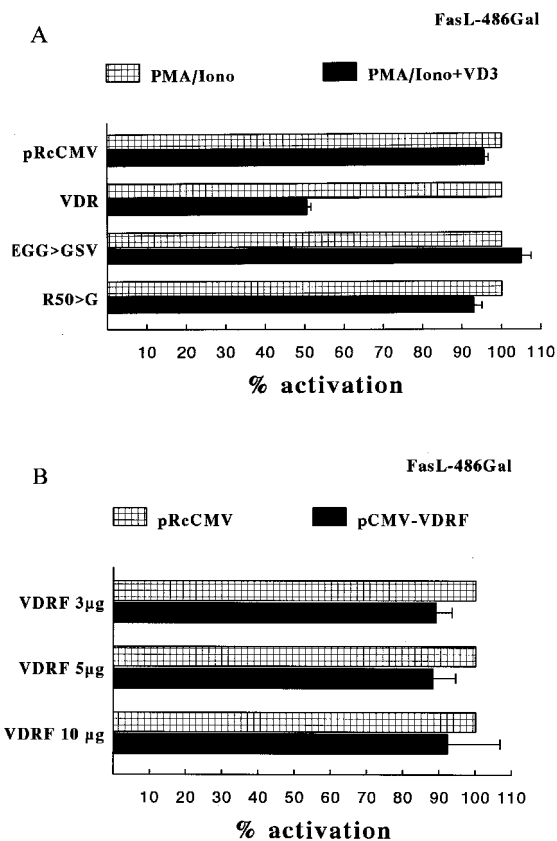
**FIGURE 5.** The 1,25(OH)<sub>2</sub>D<sub>3</sub> represses *fasL* promoter activation in a dose-dependent manner. *A*, Jurkat T cells were cotransfected with 15 μg of pFasL-486Gal reporter vector plus 4 μg of pCMV-hVDR expression vector and 3 μg of RSV-luc, as described above. To decrease variations in transfection efficiency, cells were transfected in single batches, which were then separated into different drug treatment groups. Twenty-four hours after transfection, cells were stimulated with 20 ng/ml PMA and 0.5 μg/ml ionomycin in the absence or in the presence of the indicated concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Results are shown as described above. *B*, Jurkat T cells were cotransfected with 15 μg of pFasL-486Gal reporter vector plus 3 μg of pCMV-hVDR and pSG-hRXRα expression vector, and 3 μg of RSV-luc, as described above. Results are shown as described in *A*. The mean relative β-galactosidase activity of PMA plus ionomycin-activated cultures in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> was: 540 (*A*) and 510 (*B*).

the data shown in Fig. 4B, cotransfection of RXR together with VDR enhances *fasL* promoter repression in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Cotransfection of the RXR-T454 or RXR-T448 AF-2 deletions (64) potentiated repression to a similar extent, indicating that RXR cooperates in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition of the *fasL* promoter with a mechanism independent of its AF-2 domain, and that only the AF-2 domain of VDR plays a functional role in this mechanism.

#### *Progressive deletions of the fasL promoter delineate a minimal region for 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition*

To investigate the possible presence of *fasL* promoter region(s) involved in the repression mediated by 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR, we analyzed the activity of internal mutations and progressive deletions of the *fasL* promoter by transient transfection assay, in the presence of a VDR expression vector. Transfection of *fasL* promoter constructs bearing internal mutations that abrogate binding of critical transactivators such as ΔNF-AT-Dist. (for NF-AT) or

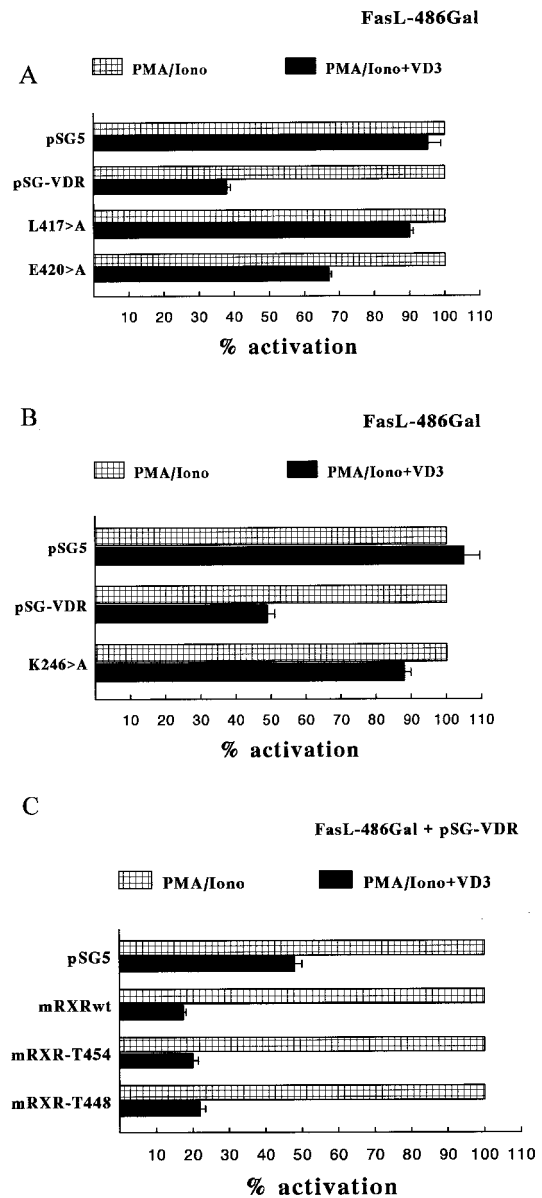




**FIGURE 6.** Mutations of the VDR DBD relieve repression of the *fasL* promoter. *A*, Jurkat T cells were cotransfected with 15  $\mu$ g of pFasL-486Gal reporter vector plus 4  $\mu$ g of the indicated VDR expression vector (or pRcCMV empty vector) and 3  $\mu$ g of RSV-luc, as described above. Percentage of activation relative to the controls in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> represents the mean value ( $X \pm$  SE) from at least four experiments. The mean relative  $\beta$ -galactosidase activity of PMA plus ionomycin-activated cultures in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub>, in order of appearance in the figure, was as follows: 800, 1040, 1035, and 992. *B*, Jurkat T cells were cotransfected with 15  $\mu$ g of pFasL-486Gal reporter vector plus the indicated amount of pCMV-VDRF (or pRcCMV empty vector) and 3  $\mu$ g of RSV-luc, as described above. Percentage of activation, relative to the controls cotransfected with the empty vector, represents the mean value ( $X \pm$  SE) from at least four experiments. The mean relative  $\beta$ -galactosidase activity of PMA plus ionomycin-activated controls cotransfected with the empty vector, in order of appearance in the figure, was as follows: 730, 760, and 620.

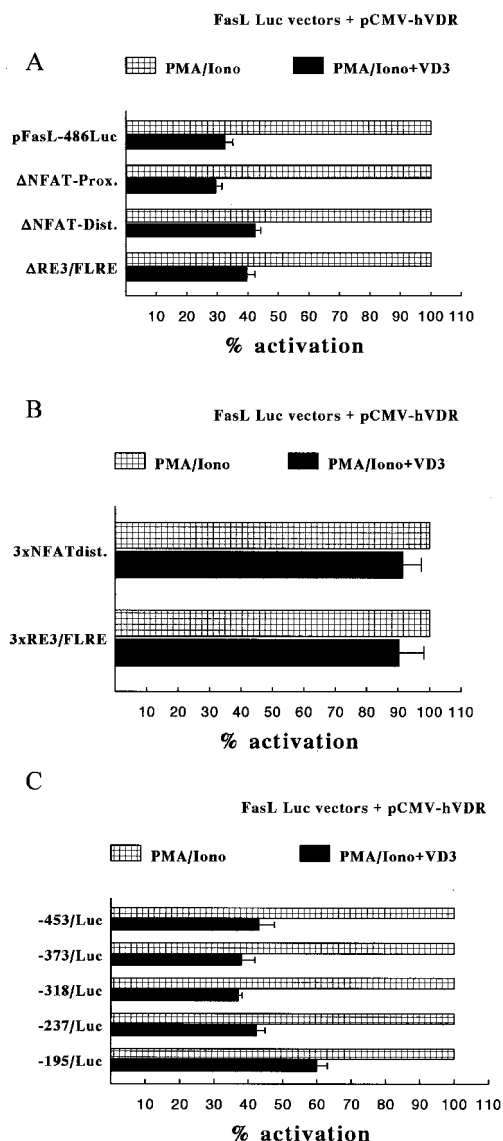
$\Delta$ RE3/FLRE (for EGR-3) considerably decreased the inducible activation following stimulation with PMA plus ionomycin in our experiments, as already described (28, 29, 50). However, the specific repression of residual promoter activity by 1,25(OH)<sub>2</sub>D<sub>3</sub> was not significantly altered in comparison with the wild-type *fasL* promoter vector (Fig. 8A). In this context, the activation of triplicated copies constructs of the NF-AT-Dist. or the RE3/FLRE binding sites (28, 29) was not inhibited by 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 8B), indicating that these enhancer elements are not direct targets for the 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated repression, at least in these experimental conditions.

By using progressive deletions, we could delineate a minimal promoter fragment spanning nucleotides from -195 bp immediately 5' of the translational start site, which is still sensitive to the 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment in activated Jurkat cells, although to a lesser extent than the fragment from -453 bp 5' of the translational start site (Fig. 8C).



**FIGURE 7.** Mutations of the VDR that abrogate its interaction with transcriptional coactivators relieve repression of the *fasL* promoter. *A* and *B*, Jurkat T cells were cotransfected with 15  $\mu$ g of pFasL-486Gal reporter vector plus 4  $\mu$ g of the indicated VDR expression vector (or pSG5 empty vector) and 3  $\mu$ g of RSV-luc, as described above. Percentage of activation relative to the controls in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> represents the mean value ( $X \pm$  SE) from at least four experiments. *C*, Jurkat T cells were cotransfected with 15  $\mu$ g of pFasL-486Gal reporter vector plus 3  $\mu$ g of pCMV-hVDR expression vector, 3  $\mu$ g of the indicated pSG-mRXR $\alpha$  expression vector (or pSG5 empty vector), and 3  $\mu$ g of RSV-luc, as described above. Percentage of activation relative to the controls in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> represents the mean value ( $X \pm$  SE) from at least four experiments. Results are shown as described in *A*. The mean relative  $\beta$ -galactosidase activity of PMA plus ionomycin-activated cultures in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> was as follows: 900, 1050, 1220, and 1380 (*A*); 800, 1200, and 1178 (*B*); 1250, 1180, 1230, and 1300 (*C*).

Several studies have recently shown that the promoter fragment from -195 to +1 bp of the *fasL* promoter contains a major transcription initiation site at -181 bp from the first ATG (32), a NF- $\kappa$ B/SP-1 binding site located at -155 bp from the first ATG (65), and a noncanonical c-Myc-binding element localized in a position originally described as a putative TATA-box sequence (25).



**FIGURE 8.** A minimal region of the *fasL* promoter is involved in 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition. **A**, Jurkat T cells were cotransfected with 15 μg of wild-type FasL-Luc reporter (pFasL-486) or its internal mutations in the Proximal-NF-AT, Distal-NF-AT, and RE3/FLRE element, plus 4 μg of pCMV-hVDR expression vector and 4 μg of pEQ176 CMV-β-Gal expression vector, as described above. Percentage of activation relative to the controls in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> represents the mean value (X ± SE) from at least four experiments. **B**, Jurkat T cells were cotransfected with 15 μg of triplicated copies constructs of the Distal-NF-AT or the RE3/FLRE binding sites, plus 4 μg of pCMV-hVDR expression vector and 4 μg of pEQ176 CMV-β-Gal expression vector, as described above. Percentage of activation relative to the controls in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> represents the mean value (X ± SE) from at least three experiments. **C**, Jurkat T cells were cotransfected with 15 μg of the indicated *fasL* promoter deletions, plus 4 μg of pCMV-hVDR expression vector and 4 μg of pEQ176 CMV-β-Gal expression vector, as described above. Percentage of activation relative to the controls in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> represents the mean value (X ± SE) from at least four experiments. The mean relative luciferase activity of PMA plus ionomycin-activated cultures in the absence of 1,25(OH)<sub>2</sub>D<sub>3</sub> was as follows: 58,000, 57,000, 4,200, and 14,000 (A); 30,000 and 5,000 (B); 33,000, 50,000, 59,000, 26,000, and 9,200 (C).

Because 1,25(OH)<sub>2</sub>D<sub>3</sub> can modulate the activity and/or the expression of transcription factors such as NF-κB or c-Myc (24, 45, 49), we investigated whether 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR might interfere with the

function of these transcription factors as regulators of the *fasL* promoter. To this purpose, nuclear extracts from 2B4.11 cells activated (4 h) in the presence or in the absence of 20 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub> were analyzed with <sup>32</sup>P-labeled probes encompassing the FasL-MycRE (human *FasL* gene, -139 to -110 bp) (25) and the FasL-NF-κB prox. (human *FasL* gene, -162 to -135 bp) (65).

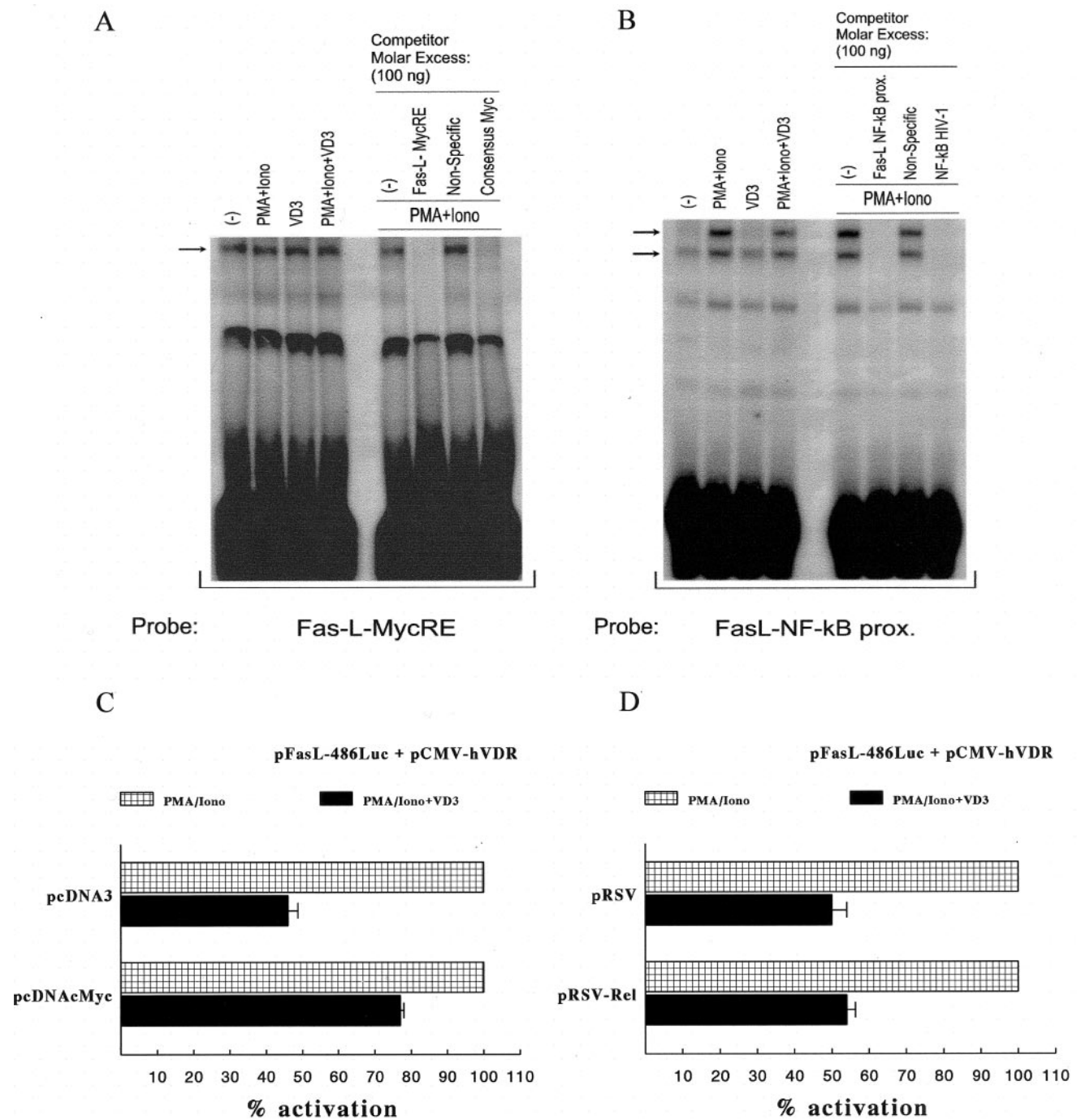
The EMSA analysis shown in Fig. 9, A and B, indicates that constitutive and inducible specific DNA-binding complexes are detected, and competed by unlabeled oligonucleotides containing a canonical E-box/c-Myc-binding sequence (consensus Myc) or a consensus NF-κB-binding sequence (NF-κB-HIV-1 LTR), respectively. In these conditions, treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> does not modify the constitutive or the inducible c-Myc and NF-κB-binding activity to these regulatory elements. In contrast, overexpression of c-Myc, but not c-Rel, gave a significant relief (≈30%) of the 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated repression in Jurkat cells (Fig. 9, C and D), indicating that 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR may limit the transcriptional function of c-Myc in the context of the whole *fasL* promoter. Repression was only partially relieved by c-Myc overexpression also when the amount of c-Myc expression vector cotransfected was increased (data not shown), suggesting that additional inhibitory mechanism(s) may take place during activation. Overexpression of p65/RelA and/or p50/NF-κB1 NF-κB family members gave results similar to c-Rel (data not shown).

## Discussion

In the last few years, a great deal of attention has focused on the role of Fas receptor and its ligand FasL, in the maintenance of lymphocyte homeostasis, and regulation of immune responses (1–3). Although Fas-mediated activation-induced programmed cell death represents an important mechanism to ensure self-tolerance through the elimination of activated autoreactive lymphocytes, the Fas/FasL system may also contribute to the pathogenesis of several organ-specific autoimmune diseases, through effector cells that cause direct tissue damage via activation of the Fas apoptotic pathway (7, 66). In this context, activated self-reactive T cells kill Fas<sup>+</sup> target cells by direct lysis, and cause cell damage in other surrounding Fas<sup>+</sup> bystander cells. In this way, infiltrating T cells may kill several MHC-negative targets (e.g., pancreatic β cells or oligodendrocytes in the nervous system) and expand local damage (7). Thus, it is becoming increasingly evident that an improperly regulated Fas-FasL system could represent a serious danger for the organism, and particularly during the onset of autoimmune diseases (7).

Several reports have shown that activation of the *fasL* gene and programmed cell death can be specifically inhibited by ligands that activate nuclear hormone receptors such as corticosteroids or retinoids in T lymphocytes (35–38). In this regard, gene regulation by ligand-activated nuclear receptors represents an important subject of immunopharmacology, and several studies have demonstrated that different hormones or specific ligands, such as retinoids, corticosteroids, peroxisome proliferator-activated receptor activators, or vitamin D<sub>3</sub>, are able to exert profound regulatory effects on the physiology of the immune system and during inflammatory reactions (23, 24, 40–49).

Previous work from other laboratories and our group has identified several cytokine genes as direct target of 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated repression in activated T lymphocytes, and has contributed to explain the molecular basis of the immunosuppressive effects of this hormone. *IL-2*, *GM-CSF*, and *IFN-γ* represent important genes repressed by 1,25(OH)<sub>2</sub>D<sub>3</sub> (23, 46–48, 56). Moreover, a direct inhibition of IL-12 production by 1,25(OH)<sub>2</sub>D<sub>3</sub> has been described in activated macrophages and DC, with a repressive effect induced by VDR/RXR on IL-12 p40 expression and



**FIGURE 9.** The  $1,25(\text{OH})_2\text{D}_3$  does not modify FasL c-Myc and NF- $\kappa\text{B}$  DNA-binding activity in 2B4.11 cells. *A* and *B*, EMSA was performed using the indicated  $^{32}\text{P}$ -labeled oligonucleotides as a probe in the presence of nuclear extracts (10  $\mu\text{g}$ ) from unstimulated (-) or PMA/ionomycin-treated 2B4.11 cells (4 h), in the absence or in the presence of 20 nM of  $1,25(\text{OH})_2\text{D}_3$ . Where indicated, 100 ng specific or nonspecific cold competitor was added to the reaction mixture to confirm specificity. Arrows represent the DNA binding of NF- $\kappa\text{B}$ - and c-Myc-specific complexes. Overexpression of c-Myc partially relieves the  $1,25(\text{OH})_2\text{D}_3$ -mediated inhibition of the *fasL* promoter. *C* and *D*, Jurkat T cells were cotransfected with 15  $\mu\text{g}$  of pFasL-486-Luc reporter plus 4  $\mu\text{g}$  of pCMV-hVDR expression vector, 4  $\mu\text{g}$  of pEQ176 CMV- $\beta$ -Gal (for c-Myc overexpression) or pRSV- $\beta$ -Gal (for c-Rel overexpression), and 5  $\mu\text{g}$  of pcDNAMyc or pRSV-Rel, as described above. As control empty vectors, the same amount of pcDNA3 or pS-RSPA was used for c-Myc and c-Rel overexpression, respectively. Percentage of activation relative to the controls in the absence of  $1,25(\text{OH})_2\text{D}_3$  represents the mean value ( $X \pm \text{SE}$ ) from at least four experiments. The mean relative luciferase activity of PMA plus ionomycin-activated cultures in the absence of  $1,25(\text{OH})_2\text{D}_3$  was as follows: 21,000 and 22,000 (*C*); 22,000 and 12,500 (*D*).

promoter activation (24). Furthermore,  $1,25(\text{OH})_2\text{D}_3$  can also block APC-dependent T cell activation by inhibiting optimal differentiation, activation, and survival of DC (15, 16).

In this study, we propose a novel molecular target of the  $1,25(\text{OH})_2\text{D}_3$  action in T cells, FasL.

The results shown in the present study indicate that *fasL* gene expression is repressed by  $1,25(\text{OH})_2\text{D}_3$  in activated T lymphocytes. Activation-induced apoptosis in 2B4.11 T cells is significantly inhibited by  $1,25(\text{OH})_2\text{D}_3$ , as demonstrated by PI uptake and Annexin-V/PI staining, followed by FACS analysis. The inhibition correlated

with decreased levels of *fasL* mRNA expression, as demonstrated by Northern blot analysis. Moreover, by EMSA and transient transfection experiments in 2B4.11 and Jurkat T cells, we have demonstrated a direct inhibitory action of the 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR on the *fasL* promoter activation. Our data indicate that formation of VDR-RXR heterodimers and a functional VDR-DBD are two important requirements for the repression of the *fasL* promoter by 1,25(OH)<sub>2</sub>D<sub>3</sub>. However (Fig. 6B), DNA binding alone is not sufficient for transcriptional inhibition, and different point mutations within the VDR AF-2 domain (helix 12) or a point mutant in the helix 3 of the LBD that abrogate recruitment of transcriptional coactivators (60) indicate that competition for limiting amounts of specific common coactivators is also involved in *fasL* promoter repression.

Interestingly, transfection of *fasL* promoter internal mutations that abrogate binding of critical transactivators, such as ΔNF-AT-Dist. and ΔRE3/FLRE (28, 29), did not significantly alter the specific repression of residual promoter activity (Fig. 8A). Moreover, the activation of triplicated copies constructs of the FasL-NF-AT-Dist. or the FasL-RE3/FLRE binding sites (28, 29) was not altered by 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 8B), suggesting that these enhancer elements are not direct targets for the 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated repression, at least in our experimental conditions. By using progressive deletions, we could identify a minimal promoter fragment spanning nucleotides from -195 bp immediately 5' of the translational start site that is still repressed by 1,25(OH)<sub>2</sub>D<sub>3</sub>. This promoter fragment contains a major transcription initiation site at -181 bp from the first ATG (32), a NF-κB/SP-1 binding site located at -155 bp from the first ATG (65), and a novel noncanonical c-Myc-binding element that overlaps a position originally described as a putative TATA-box sequence (25).

The involvement of NF-κB and c-Myc in *fasL* gene regulation has been extensively studied in the last few years. c-Myc has been shown to contribute to cell death in AICD of T cell hybridomas (2), to directly activate the *fasL* promoter (2, 25, 33), and represents a specific target for the TGF-β1-mediated inhibition of FasL expression and AICD in T cells (34). Differently, although NF-κB-dependent regulation of *fasL* gene and promoter activity has been described in different models (2, 65), recent experimental observations obtained using inhibitor of κB (IκB)-kinase γ-deficient T cells have shown that NF-κB-signaling defects have no effect on mitogen-stimulated expression of *fasL* mRNA or activation of the *fasL* promoter (67).

In activated T lymphocytes, both NF-κB and c-Myc are important targets for the inhibitory activity of 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR (45, 49), and in our cotransfection assays overexpression of c-Myc, but not c-Rel, could significantly relieve the 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated repression, indicating that 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR may interfere with the transcriptional function of c-Myc and alter optimal activation of the *fasL* promoter. The inhibition was only partially relieved, even when we used increased amounts of c-Myc expression vector cotransfected (data not shown), suggesting that additional inhibitory mechanism(s) may take place in this system.

Much evidence has indicated that nuclear receptors, including VDR, recruit specific coactivator proteins necessary for efficient transcriptional regulation. They include related family members such as SRC-1/NCoA1, TIF-2/GRIP-1, and ACTR/pCIP (54, 59). Furthermore, ligand-activated transcription by nuclear hormone receptors appears to require the CBP/p300 coactivator, an essential component in the formation of active complexes with several nuclear receptors and general transcription factors (54, 55, 59). These observations have suggested that nuclear receptors (e.g., GR or RAR/RXRs) might compete for limiting cellular pools of common transcriptional cofactors and/or adapter-integrators, normally re-

quired for an optimal activity of specific transcription factors in a promoter (44, 53–55, 59).

Our transfection experiments indicate that *fasL* promoter repression mediated by VDR mutants that abrogate normal recruitment of specific coactivators (VDR-L417>A, VDR-E420>A, and VDR-K246>A) (60) is greatly reduced in comparison with the VDR wild type (Fig. 7), and that competition for limiting amounts of common coactivators might be involved in this model.

In this regard, the observation that 1,25(OH)<sub>2</sub>D<sub>3</sub> does not modify c-Myc-binding activity to the FasL-MycRE regulatory element in 2B4.11 cells, and that overexpression of c-Myc may partially relieve 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated repression may suggest a mechanism of competition for a specific factor.

Additional experiments will be necessary to identify the limiting factor(s) that might be sequestered in activated T lymphocytes through this mechanism, and that coregulates *fasL* promoter activity.

On the contrary, overexpression of C-terminally (AF-2 domain) truncated RXR (RXR-T454 and RXR-T448) increased repression, as observed with the wild-type RXR, suggesting that RXR cooperates with ligand-bound VDR through a mechanism independent of its AF-2 domain function. An augmented nuclear import (68) and heterodimer formation (51) that enhance specific nuclear receptor binding to the DNA might explain these data. In this regard, cotransfection of VDR-DBD mutants (VDR EGG>GSV and VDR-R50>G) indicated that direct binding on specific *fasL* promoter sequences might be involved. A sequence homology search did not reveal the presence of putative VDR-binding consensus (VDREs) in the *fasL* promoter, yet several reports have shown that VDR can bind with different affinities to rather degenerate consensus sequences, as shown for the IL-2, GM-CSF, and IFN-γ promoters (23, 47, 48, 56). Additional experiments will be necessary to verify this hypothesis in the *fasL* promoter and in particular at the level of the segment -195 bp immediately 5' of the translational start site.

The 1,25(OH)<sub>2</sub>D<sub>3</sub> and a number of related nonhypercalcemic analogues have been recently evaluated as a promising category of immunosuppressive molecules that might provide new therapeutic tools for several chronic inflammatory autoimmune diseases (18–22, 69). An impairment of APC functions and a powerful repression of the Th1-type immune response by 1,25(OH)<sub>2</sub>D<sub>3</sub> (also in combination with other immunomodulators such as CsA or corticosteroids) seem to play a major role in these treatments.

The 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated regulation of FasL might cooperate with the 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated inhibition of Th1 development and pathogenesis of autoimmune disorders as observed in EAE, in which inhibition of cytokines such as IL-12 and IFN-γ and systemic increase of protective Th2 cytokines such as IL-4 or TGF-β have been described, and correlates with the prevention and/or amelioration of these diseases (11, 17–20). In addition, the observation that production of IFN-γ during the progression of inflammation may generate metabolically active 1,25(OH)<sub>2</sub>D<sub>3</sub> (from the precursor 25-hydroxyvitamin D<sub>3</sub>) in local macrophages also suggests a paracrine role of 1,25(OH)<sub>2</sub>D<sub>3</sub> in a negative feedback loop (13, 14).

These observations, and the recent notion that reverse signaling through FasL is required for CTLs to achieve optimal proliferation (8, 9), together with the ability of the activated Fas receptor to induce phenotypical and functional maturation of DC, and a preferential T cell polarization toward a Th1 phenotype (10), suggest that modulation of the *fasL* gene in T lymphocytes may represent an interesting tool for the therapy of several autoimmune disorders.

In summary, these data extend our knowledge of the complex effects mediated by 1,25(OH)<sub>2</sub>D<sub>3</sub> as a paracrine and pharmacological regulator of the inflammatory and immune responses, and propose FasL as a novel molecular target for the immunosuppressive action of 1,25(OH)<sub>2</sub>D<sub>3</sub> in T lymphocytes.

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