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Stat1-Vitamin D Receptor Interactions Antagonize 1,25-Dihydroxyvitamin D Transcriptional Activity and Enhance Stat1-Mediated Transcription

Marcos Vidal,[1] Chilakamarti V. Ramana,[2] and Adriana S. Dusso[1]*

Renal Division, Washington University School of Medicine, St. Louis, Missouri 63110,[1] and Department of Molecular Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195[2]

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The cytokine gamma interferon (IFN-γ) and the calcitropic steroid hormone 1,25-dihydroxyvitamin D (1,25D) are activators of macrophage immune function. In sarcoidosis, tuberculosis, and several granulomatoses, IFN-γ induces 1,25D synthesis by macrophages and inhibits 1,25D induction of 24-hydroxylase, a key enzyme in 1,25D inactivation, causing high levels of 1,25D in serum and hypercalcemia. This study delineates IFN-γ–1,25D cross talk in human monocytes-macrophages. Nuclear accumulation of Stat1 and vitamin D receptor (VDR) by IFN-γ and 1,25D promotes protein-protein interactions between Stat1 and the DNA binding domain of the VDR. This prevents VDR-retinoid X receptor (RXR) binding to the vitamin D-responsive element, thus diverting the VDR from its normal genomic target on the 24-hydroxylase promoter and antagonizing 1,25D–VDR transactivation of this gene. In contrast, 1,25D enhances IFN-γ action. Stat1-VDR interactions, by preventing Stat1 deactivation by tyrosine dephosphorylation, cooperate with IFN-γ/Stat1-induced transcription. This novel 1,25D–IFN-γ cross talk explains the pathogenesis of abnormal 1,25D homeostasis in granulomatous processes and provides new insights into 1,25D immunomodulatory properties.

1,25-Dihydroxyvitamin D (1,25D), the hormonal form of vitamin D, is a potent regulator of calcium homeostasis (8). In healthy individuals, to maintain calcium concentrations within the physiological range, 1,25D tightly controls its own levels in serum (26) by dual mechanisms: suppressing its own synthesis by renal and extrarenal 1α-hydroxylase and inducing 24-hydroxylase, the key enzyme in 1,25D metabolic inactivation (27).

In contrast, in sarcoidosis, tuberculosis, several granulomatoses, and rheumatoid arthritis, abnormal 1,25D homeostasis is the cause of hypercalcemia (1, 18, 19, 34). In these patients, high levels of 1,25D in serum result from excessive 1,25D synthesis by the disease-activated macrophage (2, 3, 28) and loss of the capacity of 1,25D to regulate its own synthesis and degradation (16, 42). The demonstration of a direct correlation between pleural levels of gamma interferon (IFN-γ) and 1,25D (2) suggested the involvement of the cytokine in the abnormalities in 1,25D homeostasis. In fact, exposure of normal monocytes, pulmonary alveolar macrophages, or the monocytic cell line THP-1 to IFN-γ markedly enhances macrophage 1,25D production and antagonizes 1,25D regulation of 1α- and 24-hydroxylases (16). Clearly, in vivo and in vitro, IFN-γ impairs 1,25D control of its own synthesis and catabolism.

These studies addressed the mechanisms mediating the antagonistic effects of IFN-γ on 1,25D regulation of its own homeostasis. Since the mechanism for 1,25D-suppression of 1α-hydroxylase gene transcription is poorly understood (37), we focused on the effects of IFN-γ on 1,25D enhancement of its own catabolism. 1,25D induction of 24-hydroxylase expression is a typical 1,25D genomic action, mediated by ligand-activated vitamin D receptor (VDR) as a transcription factor (6, 12). 1,25D binding to the VDR induces a conformational change in the VDR molecule that activates the VDR to translocate to the nucleus and heterodimerize with the retinoid X receptor (RXR). The VDR-RXR complex then binds to specific DNA sequences, known as vitamin D-responsive elements (VDREs), in the 24-hydroxylase promoter (11) and recruits nuclear receptor coactivator molecules to induce transcription (8). Studies of the human monocytic cell line THP-1 demonstrated that IFN-γ may directly impair 1,25D induction of 24-hydroxylase gene transcription (16). IFN-γ inhibited 1,25D induction of 24-hydroxylase mRNA, an effect that did not result from defective binding of 1,25D to VDR or reduced stability of the 24-hydroxylase mRNA, suggesting the existence of interactions between the very distinct signaling pathways of IFN-γ and 1,25D.

In the case of IFN-γ, most responses to the cytokine involve the activation of the latent cytosolic protein Stat1 (15, 45). IFN-γ binding to its cell membrane receptor induces rapid assembly of a complete IFN-γ receptor complex with Jak1 and Jak2 enzymes, which phosphorylate one another and then phosphorylate the receptor. Receptor phosphorylation results in the formation of Stat1 docking sites. Upon phosphorylation at tyrosine 701, Stat1 homodimerizes, translocates to the nucleus, and binds DNA at specific IFN-γ activation sequences (GAS), where it either activates or represses transcription (23). Maximal transcriptional activity by active Stat1 homodimers also requires Stat1 phosphorylation at serine 727 (17, 52) and recruitment to the transcription initiation complex of the CBP-p300 family of coactivators (54).

The present study delineates the mechanism and functional relevance of the interactions between the distinct signaling pathways for the steroid hormone 1,25D and the cytokine IFN-γ. IFN-γ antagonizes 1,25D-VDR transcriptional activa-

* Corresponding author. Mailing address: Renal Division, Washington University School of Medicine, 660 S. Euclid, St. Louis, MO 63110. Phone: (314) 362-8243. Fax: (314) 362-8237. E-mail: adusso@im.wustl.edu.

tion of 24-hydroxylase. Direct protein-protein interactions between activated Stat1 and the DNA binding domain (DBD) of the VDR impair VDR-RXR binding to human 24-hydroxylase VDRE. In contrast, 1,25D enhances IFN-γ action. Stat1-VDR interactions prolong Stat1 activation, thus enhancing IFN-γ–Stat1 transcriptional regulation of IFN-γ-responsive genes.

MATERIALS AND METHODS

Plasmids and antibodies. 8×GAS-Luc was described previously (9, 22). Human 24-hydroxylase (−1262, +99)–chloramphenicol acetyltransferase (CAT) and monoclonal mouse anti-VDR VG1 were from H. F. DeLuca (University of Wisconsin–-Madison). pSG5VDR, GST-VDR (4–427), 4× rat osteocalcin VDRE-Luc, and anti-VDR antibody 9A7 were obtained from Paul MacDonald (Case Western Reserve University, Cleveland, Ohio). GST-VDR (4–133) and GST-VDR (89–427) were provided by Mark Haussler (University of Arizona—Tucson). pET-Stat1 was obtained from Focco Van Decker (Cleveland Clinic). TGL-IP10 was obtained from Richard Ransohoff (Cleveland Clinic). LMX1B expression plasmid was obtained from Michael Rauchman (Washington University, St. Louis, Mo.). Anti-N terminus Stat1 and anti-pY701 Stat1 were purchased from Signal Transduction and New England Biolabs, respectively.

Cell culture. THP-1 cells were grown in suspension in RPMI 1640 containing 10% fetal bovine serum. Differentiated THP-1 cells were induced to acquire a macrophage phenotype by exposure to 160 nM phorbol 12-myristate 13-acetate (Sigma, St. Louis, Mo.) for 24 h. 2fTGH, U3A, and reconstituted U3A cells (from George Stark, Cleveland Clinic) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For all cell types, treatment with 1,25D (a gift from Milan Uskokovick, Hoffman-La Roche, Nutley, N.J.) and IFN-γ (Endogen) was conducted in serum-free medium containing 1% fatty-acid-free albumin. Incubations were done at 37°C in humidified 95% air–5% CO_2.

Transfection and reporter assays. Cells were transiently transfected by using Superfect reagent (Qiagen) according to the manufacturer's specifications. *Renilla* luciferase or β-galactosidase (Promega) was cotransfected as an internal standard. Firefly and *Renilla* luciferase, CAT, and β-galactosidase activities were all measured using Promega's kits.

Immunoprecipitation and immunoblot analysis. THP-1, 2fTGH, or U3A cells were either untreated or stimulated with IFN-γ (1,000 IU/ml), 1,25D (50 nM), or both for 4 h. Cell extracts were prepared as described previously (30). Whole-cell extracts (600 μg or 1 mg) were immunoprecipitated with anti-VDR 9A7 or nonspecific immunoglobulin G (IgG). The immune complexes were collected on Staph-A beads (Sigma) coated with goat anti-rat antibody (Pierce) and washed four times in lysis buffer (200 mM Tris-HCl [pH 7.4], 2 mM EDTA, 0.5% Nonidet P-40, 0.3 mM sodium orthovanadate, 50 mM sodium fluoride, 0.5 mM dithiothreitol, and a cocktail of protease inhibitors [Boehringer Mannheim]). Proteins were resolved on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the anti-Stat1 antibody followed by anti-mouse IgG coupled to horseradish peroxidase (Bio-Rad). The transfers were analyzed by using the Supersignal chemiluminescent reagent (Pierce).

EMSAs. Nuclear extracts or recombinant proteins were prepared, and electrophoretic mobility shift assays (EMSAs) were performed as described by Chen and DeLuca (11) or by Kotanides and Reich (29) for VDRE and GAS, respectively. The probes used were double-stranded oligonucleotides containing the 24-hydroxylase promoter-proximal VDRE sequence, 5′-ATGGAGTCAGCGA GGTGAGCGAGGGCGTCC-3′ (wild type) and 5′-ATGGAGAGTGCGAGG AGTGCGAGGAAATCC-3′ (mutant), or the high-affinity Stat binding site, SIE (m67), described by Vignais et al. (50).

GST pull-down. The constructs for bacterial expression of glutathione *S*-transferase (GST) fusion proteins were transformed in *Escherichia coli* DH5α (Gibco-BRL). The proteins were induced and purified as described previously (13) and bound to glutathione-agarose beads (Sigma). In vitro-transcribed and -translated Stat1 and luciferase were synthesized and labeled with [^{35}S]methionine (Redivue; Amersham Pharmacia) utilizing T7 TNT Reticulocyte Lysate Master Mix (Promega) according to the manufacturer's instructions and incubated with the GST beads. The beads were washed four times with binding buffer (20 mM Tris-HCl [pH 7.6], 50 mM NaCl, 0.2% Nonidet P-40, 1 mM dithiothreitol, and a cocktail of protease inhibitors [Boehringer Mannheim]) and two times with 50 mM Tris-HCl (pH 8.0) and eluted in 10 mM reduced glutathione–50 mM Tris-HCl, pH 8.0. The eluted fraction was resolved by SDS-polyacrylamide gel electrophoresis and analyzed using a PhosphorImager (Molecular Dynamics).

Immunofluorescence. 2fTGH or U3A cells, seeded on coverslips, were transfected with VDR and either untreated or exposed for 30 min to 1,25D (50 nM) or IFN-γ (1,000 IU/ml). The cells were fixed in methanol, blocked for 1 h in 10% goat serum–1% bovine serum albumin (BSA)–0.02% NaN_3 in phosphate-buffered saline, washed once with Tris-buffered saline (TBS), and incubated overnight with anti-VDR VG1 (1:50 dilution in 1% BSA in TBS). The coverslips were washed three times in TBS, incubated with anti-mouse fluorescein isothiocyanate-conjugated antibody for 1 h in the dark followed by three washes with TBS, and mounted on slides. Images were obtained from a fluorescent confocal microscope (MRC 1024; Bio-Rad).

RNase protection assays. Total RNA was prepared using the Trizol (Tel-Test) method according to the manufacturer's instructions. ^{32}P-labeled riboprobes (IFN-γ-inducible protein 10 [IP-10] from Pharmingen; glyceraldehyde-3-phosphate dehydrogenase [GAPDH] from Ambion) were generated by in vitro transcription with T7 RNA polymerase (Promega). mRNAs for IP-10 and GAPDH were measured according to the manufacturer's instructions for RiboQuant (Pharmingen). Protected fragments were separated by electrophoresis on a 4.5% polyacrylamide–urea gel.

RESULTS

IFN-γ antagonizes 1,25D transcriptional activation of 24-hydroxylase. To test whether IFN-γ antagonism on 1,25D induction of 24-hydroxylase was at the transcriptional level, the human fibrosarcoma cell line 2fTGH or the Stat1-null, 2fTGH derived-U3A cells were transfected with a CAT reporter plasmid driven by the human 24-hydroxylase promoter (−1262 to +99). A β-galactosidase expression plasmid was cotransfected to normalize for transfection efficiency. In 2fTGH cells, 1,25D treatment (50 nM 1,25D) strongly induced transcription by this promoter (Fig. 1A) (11). IFN-γ (1,000 U/ml) had no effect on basal transcriptional activity; however, simultaneous treatment with 1,25D and IFN-γ reduced the transcriptional response to 1,25D by threefold. In Stat1-null U3A cells, there was no difference in transcriptional activity between cells treated with 1,25D alone and those exposed to 1,25D plus IFN-γ. These findings indicate that IFN-γ impairs 1,25D transcriptional activation of 24-hydroxylase through a Stat1-mediated mechanism.

VDRE is sufficient for IFN-γ antagonism. A similar set of experiments was conducted with 2fTGH and U3A cells transiently transfected with a firefly luciferase reporter construct driven by an artificial promoter containing four copies of rat osteocalcin VDRE. As shown in Fig. 1B, this artificial promoter recapitulated the transcriptional regulation of the full-length human 24-hydroxylase promoter in the induction by 1,25D in both 2fTGH and U3A cells. Also, the antagonism on gene transcription by simultaneous treatment with IFN-γ and 1,25D persisted in 2fTGH but not U3A cells. This experiment suggests that the presence of positive consensus DR3 VDREs in a promoter is sufficient for IFN-γ antagonism on 1,25D-VDR transcriptional activation.

IFN-γ reduces VDR/RXR binding to VDRE. IFN-γ treatment inhibited 1,25D induction of 24-hydroxylase gene transcription but did not change the basal activity of the promoter (Fig. 1A), 1,25D binding to the VDR (16), or nuclear VDR levels (data not shown). Since IFN-γ antagonism persisted in a VDRE-driven artificial promoter (Fig. 1B), the effects of the cytokine on VDR-RXR binding to VDRE were next tested. EMSAs were conducted using the proximal VDRE of the human 24-hydroxylase promoter as a probe and nuclear extracts from THP-1 cells (Fig. 1C). 1,25D treatment induced a retarded band (compare lanes 2 and 3), which corresponds to

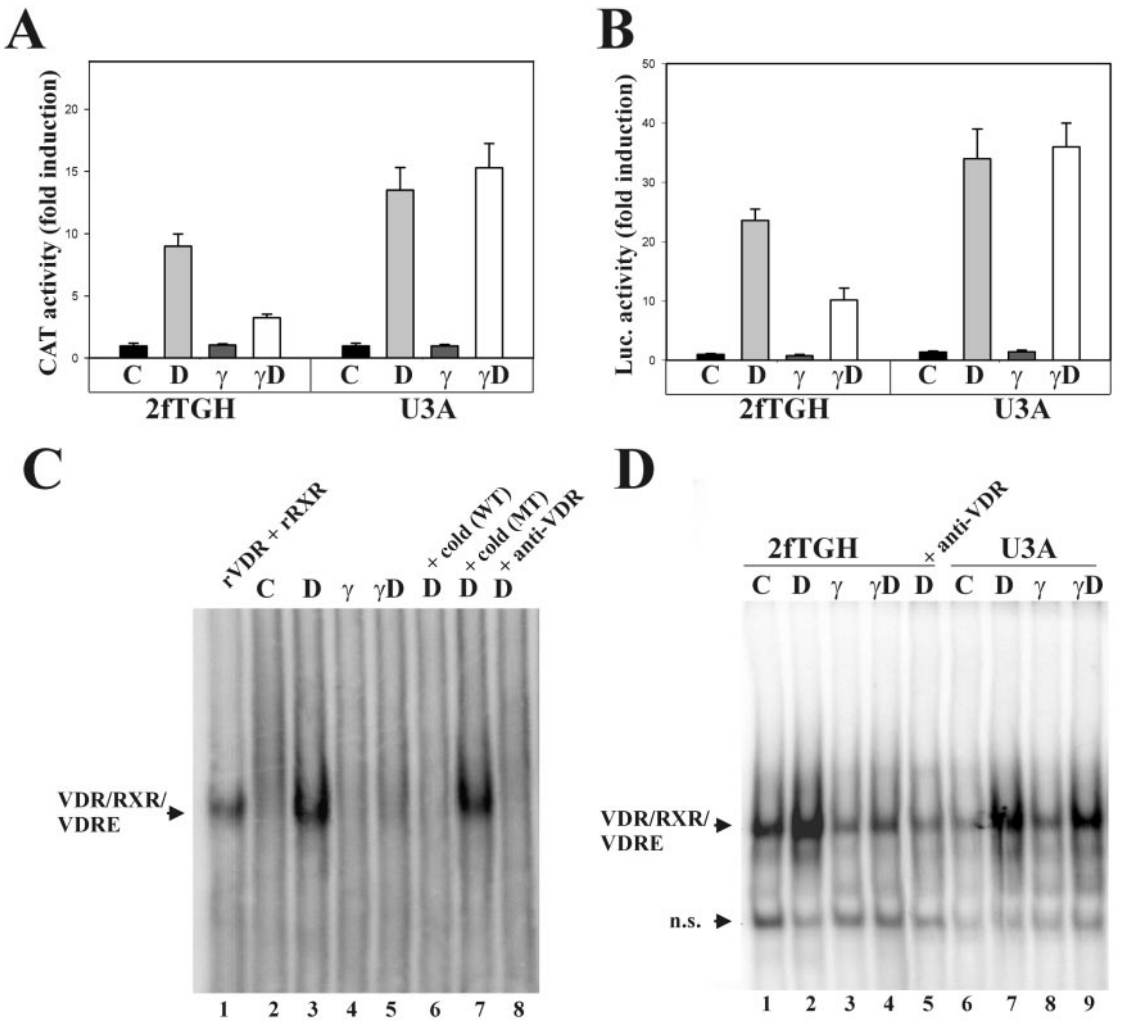

FIG. 1. IFN-γ antagonizes 1,25D transcriptional activity. (A) The human 24-hydroxylase promoter (−1262, +99) linked to CAT and a β-galactosidase expression plasmid were transiently transfected into wild-type 2fTGH or Stat1-null (U3A) cells. CAT activity was measured in cell lysates from untreated cells (C) or cells treated with 50 nM 1,25D (D), 1,000 U of IFN-γ/ml (γ), or both (γD) for 16 h. Results represent the means ± standard errors of the means of duplicate measurements from four independent experiments. (B) $(VDRE)_4$-Luc and β-galactosidase expression plasmid were transiently transfected into 2fTGH or U3A cells. Luciferase activity was measured in cell lysates from cells treated as described for panel A. Results represent the means ± standard errors of the means of triplicate measurements from two independent experiments. (C) EMSA using the proximal VDRE of the human 24-hydroxylase promoter as a probe and nuclear extracts from THP-1 cells, treated for 4 h as described for panel A. Recombinant VDR and RXR (lane 1), incubation with a 300 M excess of cold wild-type (lane 6) or mutant (lane 7) VDRE, and incubation with anti-VDR 9A7 antibody (lane 8) served as controls. (D) EMSA using nuclear extracts from 2fTGH or U3A cells treated as described for panel C. n.s., nonspecific band.

endogenous VDR-RXR bound to VDRE as judged by (i) comigration with recombinant VDR-RXR bound to VDRE (lane 1), (ii) competition by an excess of cold VDRE but not mutant VDRE (lanes 6 and 7), and (iii) inhibition of binding by incubation with anti-VDR 9A7 antibody (lane 8). VDR/RXR binding to VDRE was almost completely abolished by simultaneous treatment with 1,25D and IFN-γ (compare lanes 3 and 5). When nuclear extracts from 2fTGH cells were utilized, a similar pattern was found (Fig. 1D, lanes 1 to 4). In contrast, in Stat1-null U3A cells, there was no difference in endogenous VDR-RXR binding to VDRE between nuclear extracts from cells treated with 1,25D and extracts from cells treated with 1,25D plus IFN-γ (Fig. 1D, compare lanes 7 and 9). Taken together, these findings demonstrate that IFN-γ impairs VDR-RXR binding to VDRE through a Stat1-mediated mechanism.

VDR interacts with Stat1. Immunoprecipitation studies examined VDR-Stat1 interactions. VDR-overexpressing 2fTGH and U3A cells were either untreated or treated with 1,25D, IFN-γ, or both for 4 h. Stat1 coprecipitated with the VDR when 2fTGH extracts were immunoprecipitated with anti-VDR but not with nonspecific IgG or when extracts from U3A cells were used (Fig. 2A). These results demonstrate that VDR and Stat1 interact in vivo. Stat1 coprecipitated with VDR in every experimental condition tested (lanes 4 to 7), indicating that VDR and Stat1 interact independently of 1,25D or IFN-γ activation.

Similar results were obtained when THP-1 cells, expressing

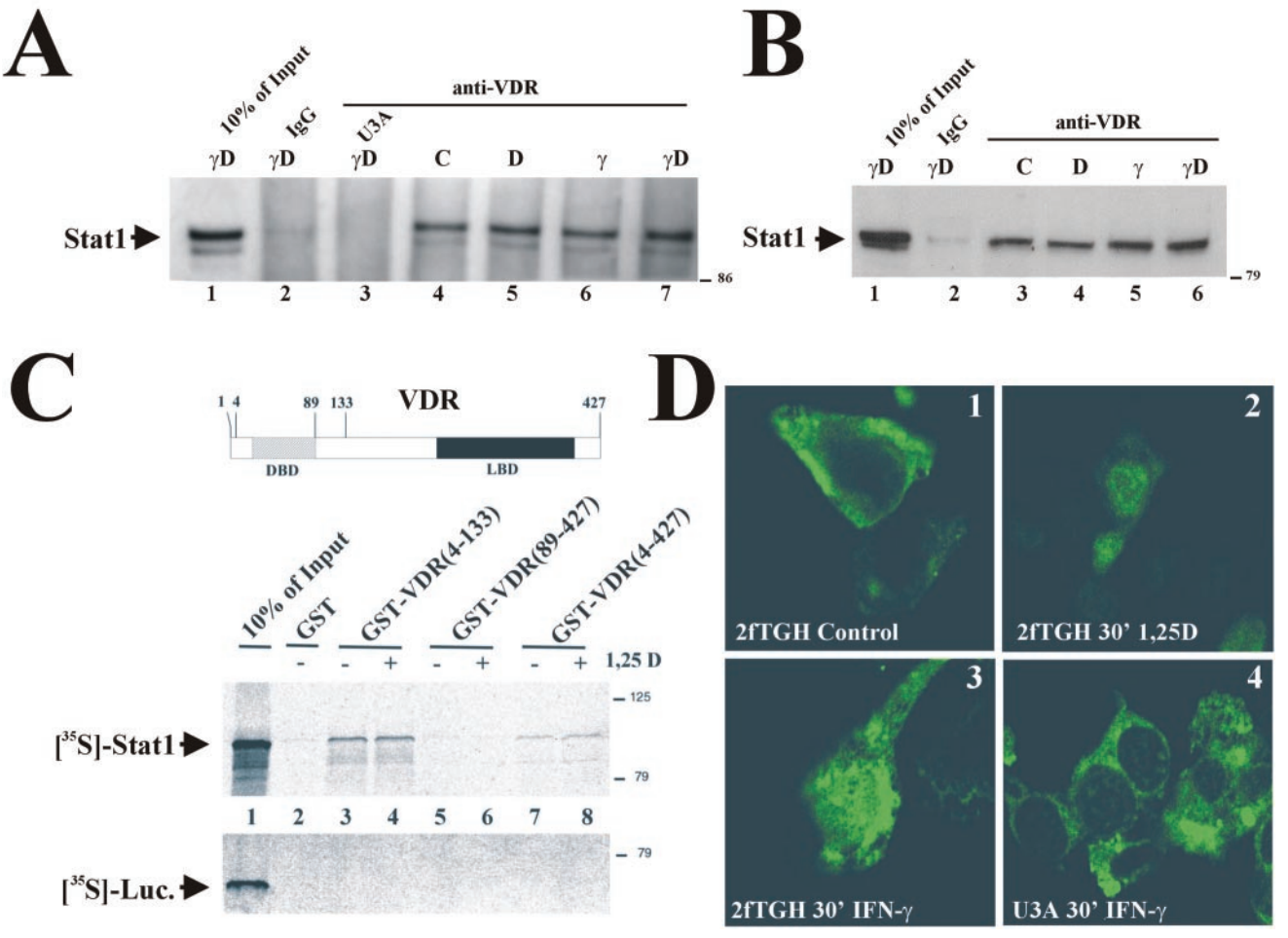

FIG. 2. VDR-Stat1 interaction. (A and B) Whole-cell extracts (600 μg of total protein) from VDR-transfected 2fTGH and U3A cells (A) or untransfected THP-1 (1 mg of total protein) (B) cells that were either untreated or treated with 50 nM 1,25D, 1,000 U of IFN-γ/ml, or both for 4 h were immunoprecipitated with anti-VDR 9A7 or nonspecific IgG and analyzed by Western blotting with anti-Stat1. (C) (Top) Schematic representation of the VDR. (Bottom) GST pull-down assay utilizing different GST-VDR fusion proteins purified from *E. coli* and bound to glutathione-agarose beads. Added was 1 μM 1,25D (+ lanes) or ethanol vehicle (− lanes). ^{35}S-labeled Stat1 and luciferase were synthesized in vitro and incubated with the beads. After washing and elution, samples were subjected to SDS–10% polyacrylamide gel electrophoresis and autoradiographed. Numbers at right show molecular mass in kilodaltons. (D) Indirect VDR immunofluorescence in 2fTGH or U3A cells transiently transfected with VDR, either untreated or exposed to 50 nM 1,25D or 1,000 U of IFN-γ/ml for 30 min at 37°C, using a mouse monoclonal antibody against the VDR and detected with fluorescein isothiocyanate-conjugated anti-mouse antibody.

endogenous levels of VDR, were used (Fig. 2B). Clearly, IFN-γ-mediated Stat1 phosphorylation at tyrosine 701 was not required for Stat1 to interact with the VDR. Consequently, in vitro-transcribed and -translated Stat1 was tested in GST pull-down assays using different GST-VDR fusion proteins to map Stat1-VDR interactions in vitro. GST alone did not interact with Stat1 (Fig. 2C, lane 2). GST fused with full VDR (amino acids 4 to 427) interacted with Stat1 in a 1,25D-independent manner (Fig. 2C, lanes 7 and 8). A C-terminal fusion VDR, comprising amino acids 89 to 427, which contains the ligand binding domain of the VDR, did not interact with Stat1 (Fig. 2C, lanes 5 and 6). In contrast, an N-terminal fusion VDR, comprising amino acids 4 to 133, which contains the DBD, bound Stat1 with an affinity stronger than that of full-length VDR (lanes 3 and 4). In vitro-transcribed and -translated luciferase did not interact with any of the GST-VDR fusion proteins. These observations demonstrate direct interactions between Stat1 and the VDR, since no additional cellular factors were needed. The interaction maps to the DBD of the VDR, and no posttranslational modifications of Stat1, i.e., arginine 31 methylation (36) or serine 727 (52) or tyrosine 701 (43) phosphorylation, are required. The demonstration that the ligand binding domain of the VDR is not required is consistent with Stat1-VDR interaction being 1,25D independent.

IFN-γ treatment drives unliganded VDR into the nucleus. Because Stat1-VDR interaction was ligand independent, we analyzed whether IFN-γ treatment could affect VDR subcellular localization. 2fTGH or U3A cells were transiently transfected with VDR and treated with 1,25D or IFN-γ for 30 min. The subcellular localization of the VDR was examined by indirect immunofluorescence. In both 2fTGH (Fig. 2D1 and D2) and U3A (data not shown) cells, the VDR was predominantly cytosolic in untreated cells and translocated to the nu-

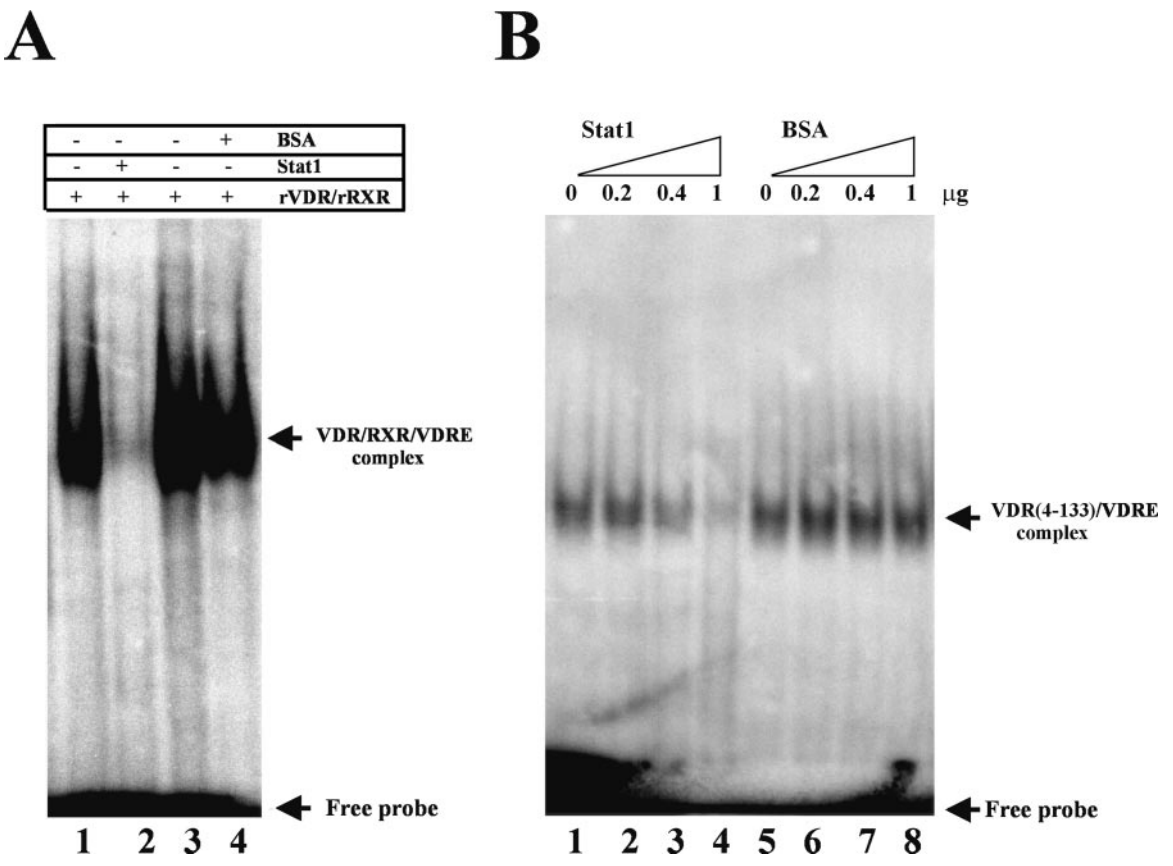

FIG. 3. Stat1 abrogates VDR binding to VDRE. (A) EMSA using the proximal VDRE of the human 24-hydroxylase promoter as a probe and recombinant VDR and RXR (0.1 μg each) with or without recombinant Stat1 or BSA (1 μg each). Every reaction mixture contained 1 μM 1,25D. (B) EMSA performed as described for panel A using the N-terminal (amino acids 4 to 133) portion of the VDR (0.1 μg). Binding reaction mixtures included the indicated amounts (micrograms) of Stat1 or BSA.

cleus within 30 min after 1,25D treatment, in agreement with previous reports (7). In 2fTGH cells treated with IFN-γ alone, a subset of the VDR was present in the nucleus in the absence of 1,25D stimulation of VDR translocation (Fig. 2D3). IFN-γ treatment was unable to translocate VDR to the nucleus in U3A cells (Fig. 2D4). Thus, IFN-γ-activated Stat1 can interact with and translocate unliganded VDR into the nucleus.

Stat1 abrogates VDR-RXR binding to VDRE. The findings that IFN-γ treatment impairs endogenous VDR-RXR binding to VDRE and that Stat1 and VDR interact in vivo and in vitro led us to determine whether Stat1 directly influenced VDR-RXR DNA binding. To this end, EMSAs were performed using the proximal VDRE of the human 24-hydroxylase promoter as a probe and recombinant proteins. Whereas VDR-RXR binding to VDRE was almost completely abolished in the presence of recombinant Stat1 (Fig. 3A, compare lanes 1 and 2), BSA, at the same concentration, had no significant effect (Fig. 3A, lanes 3 and 4).

Since the Stat1-interacting domain of the VDR mapped to the N-terminal region containing the DBD, the possibility that Stat1 impaired VDR-DNA binding by blocking the DBD of the VDR was next tested. Figure 3B shows that the N terminus of the VDR binds VDRE, presumably as a monomer in the 3′-half site (24, 25). Stat1 inhibited the binding to DNA of this recombinant protein in a dose-dependent fashion (Fig. 3B, lanes 1 to 4). The effect was Stat1 specific, since BSA (Fig. 3B, lanes 4 to 8), tested at identical concentrations, had no effect. These results indicate that IFN-γ, by activating Stat1 and inducing its nuclear translocation, promotes Stat1 interaction with the DBD of the VDR, which blocks VDR-RXR binding to 24-hydroxylase VDRE, therefore inhibiting 1,25D induction of 24-hydroxylase gene transcription. These findings could also explain the slight but significant increase in 1,25D-induced transcription in U3A cells over that in 2fTGH cells (Fig. 1A and B). Possibly, in 2fTGH cells, small amounts of inactive Stat1 in the nucleus bind VDR-DBD, slightly decreasing 1,25D-mediated transcription, an inhibition that cannot occur in U3A cells.

1,25D has a synergistic effect on IFN-γ-mediated transcription. Given that Stat1-VDR interactions impaired VDR-mediated transcription, their impact on Stat1-mediated transcription was tested. 2fTGH and U3A cells were transiently transfected with a firefly luciferase reporter construct driven by a VDRE-less promoter containing eight copies of GAS. Cotransfection of a cytomegalovirus-*Renilla* luciferase reporter was used to assess transfection efficiency. In 2fTGH cells, IFN-γ treatment strongly induced transcription (Fig. 4A). As expected, U3A cells did not respond to IFN-γ. 1,25D, which had no effect on basal transcriptional activity by itself, enhanced IFN-γ-induced transcription. Furthermore, VDR over-

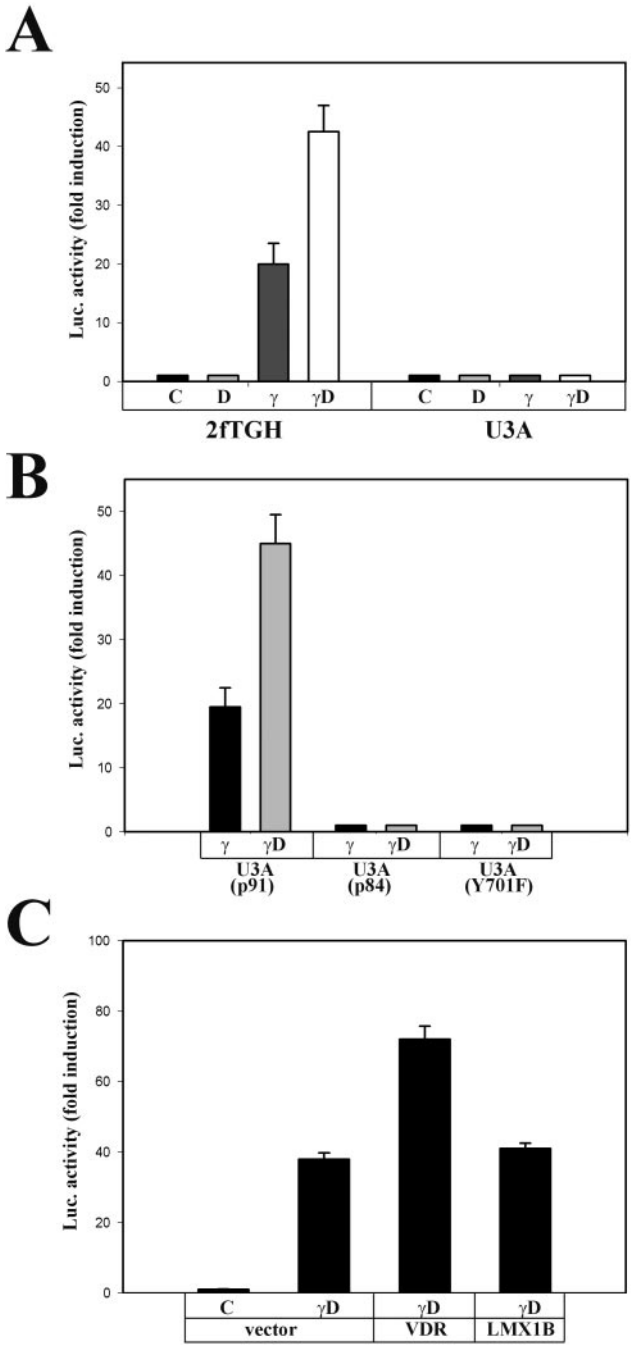

FIG. 4. 1,25D synergy on IFN-γ-mediated transcription. (A) Cooperative effects of 1,25D on Stat1-induced transcription. (B) Requirements of the Stat1 molecule in 1,25D–IFN-γ synergy. (C) VDR overexpression enhances Stat1-mediated transcription. 2fTGH cells, U3A cells, and U3A cells reconstituted with different Stat1 mutants were transiently transfected with a luciferase reporter construct driven by eight copies of the consensus GAS (TTCTCGGAA) and 0.1 μg of either human VDR expression vector, human LMX1B expression vector, or vector alone when indicated. Twenty-four hours after transfection, cells were either untreated (C) or exposed to 10 nM 1,25D (D), 1,000 IU of IFN-γ/ml (γ), or both (γD) as indicated for 4 h. Luciferase activity was determined in cell lysates. Bars and error bars represent means ± standard errors of the means from triplicate measurements from two (C) or three (A and B) independent experiments.

expression in 2fTGH cells increased the transcription by this GAS-driven promoter, whereas the unrelated developmental transcription factor LMX1B had no effect (Fig. 4C).

To further characterize domains and posttranslational modifications of the Stat1 molecule necessary for 1,25D–IFN-γ synergism, experiments were conducted with U3A cells stably transfected to rescue the expression of wild-type Stat1 (p91) or different Stat1 mutants (Fig. 4B). Transcriptional synergy was rescued in U3A cells reconstituted with wild-type Stat1 (p91) but not with either a C-terminal deletion mutant lacking 7 kDa in the transactivation domain (p84) or a tyrosine phosphorylation mutant, Y701F. These results indicate that both tyrosine phosphorylation and the presence of a functional Stat1 transactivation domain are required for the synergy.

VDR prolongs Stat1 activation. Nuclear Stat1 dephosphorylation by a yet-unknown phosphatase is one of the mechanisms by which the IFN-γ response is reduced (20, 21). The tyrosine phosphorylation of Stat1 reaches a maximum between 30 min and 1 h of IFN-γ treatment, and it starts to decrease by 2 h (38, 43). To gain some insight into potential mechanisms mediating VDR enhancement of Stat1-mediated transcription, EMSAs were conducted with a consensus GAS as probe and nuclear extracts from 2fTGH cells treated with 1,25D, IFN-γ, or both for 4 h. Simultaneous treatment with 1,25D increased Stat1 DNA binding (Fig. 5A, lanes 3 and 4). VDR overexpression further increased Stat1 binding to GAS (lanes 7 and 8). Western blot analysis showed that 1,25D treatment and/or VDR overexpression resulted in increased levels of tyrosine-phosphorylated Stat1, with no changes in total Stat1 (Fig. 5B). Time course experiments showed that simultaneous treatment with IFN-γ and 1,25D prolonged Stat1 DNA binding compared to that of IFN-γ treatment alone (Fig. 5C). These findings suggest that 1,25D-VDR could enhance IFN-γ transcriptional activity by prolonging the activated state of Stat1.

1,25D synergizes with IFN-γ in the induction of IP-10. 1,25D-VDR synergism on IFN-γ transcriptional activity was next tested in an endogenous gene, that for IP-10, a potent chemokine (33). RNase protection assays measured IP-10 steady-state mRNA levels in differentiated THP-1 cells (macrophage phenotype) and in peripheral blood monocytes from four healthy volunteers. In both THP-1 cells and normal monocytes, the induction of IP-10 by IFN-γ was further enhanced by simultaneous treatment with the cytokine and 1,25D (Fig. 6A, left and right panels, respectively). Densitometric quantitation of IP-10/GAPDH mRNA ratios showed that simultaneous treatment with 1,25D and IFN-γ increased by 60% the induction of IP-10 mRNA by IFN-γ alone (Fig. 6B). Transient-transfection studies of 2fTGH cells with a luciferase reporter construct driven by the human IP-10 promoter recapitulated the regulation found in mRNA levels (Fig. 6C). This synergy in the induction of IP-10 indicates that the effects observed in a GAS-driven artificial promoter take place, indeed, in an endogenous promoter.

DISCUSSION

The present study, designed to characterize the mechanisms mediating IFN-γ antagonism on 1,25D control of its own homeostasis, demonstrates a novel cross talk between the distinct signaling pathways of the cytokine IFN-γ and the hormonal

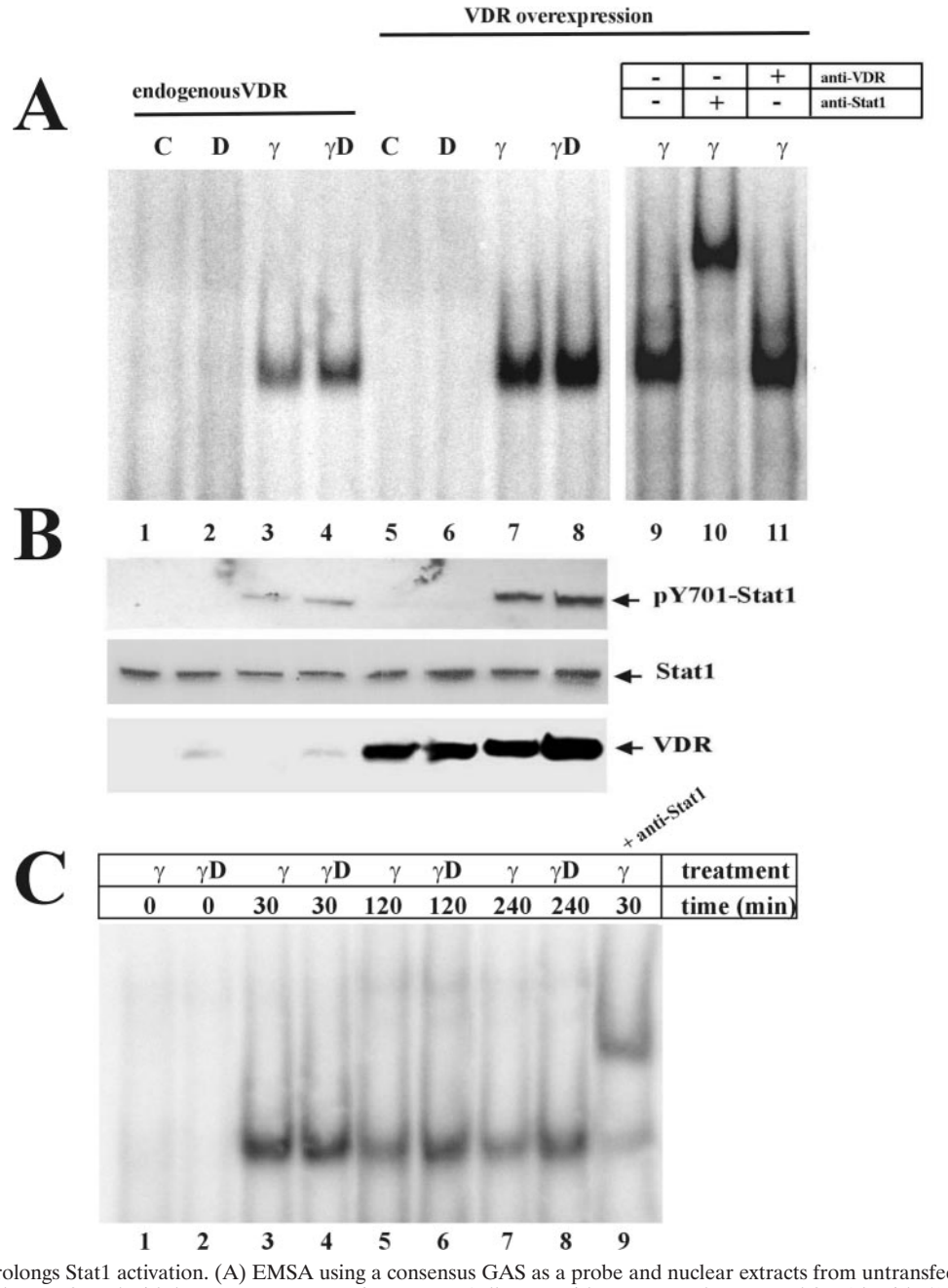

FIG. 5. VDR prolongs Stat1 activation. (A) EMSA using a consensus GAS as a probe and nuclear extracts from untransfected 2fTGH cells or from cells transiently transfected with human VDR expression vector. Cells were either untreated or treated for 4 h with 50 nM 1,25D, 1,000 U of IFN-γ/ml, or both. The right panel shows supershift analysis utilizing anti-VDR or anti-Stat1 antibodies. (B) Western blots from cell extracts treated as described for panel A, probed with anti-pY701 Stat1, anti-Stat1, and anti-VDR. (C) EMSA analysis was done as described for panel A with cell extracts from untransfected 2fTGH cells that were treated with 1,000 U of IFN-γ/ml or 1,000 U of IFN-γ/ml plus 50 nM 1,25D for the indicated periods of time.

form of vitamin D. Direct protein-protein interactions between Stat1 and the VDR, the transcription factors for IFN-γ and 1,25D, inhibit 1,25D transcriptional activity and enhance Stat1-mediated transcription.

In vitro studies using recombinant proteins addressed the mechanisms for IFN-γ inhibition of 1,25D transcriptional activity, demonstrating that (i) Stat1-VDR interactions also occur in the absence of ligand-mediated activation of either protein, (ii) Stat1 binds the DBD of the VDR, and (iii) Stat1 binding to the DBD of the VDR is sufficient to reduce VDR binding to VDRE. The biological relevance of these findings was confirmed in vivo. Coimmunoprecipitation experiments with THP-1 and 2fTGH cells showed that endogenous nuclear Stat1 and the VDR physically interact. In both cell types, Stat1 binds the VDR, reducing VDR-RXR binding to the 24-hydroxylase VDRE. In 2fTGH cells, IFN-γ-induced reduction in

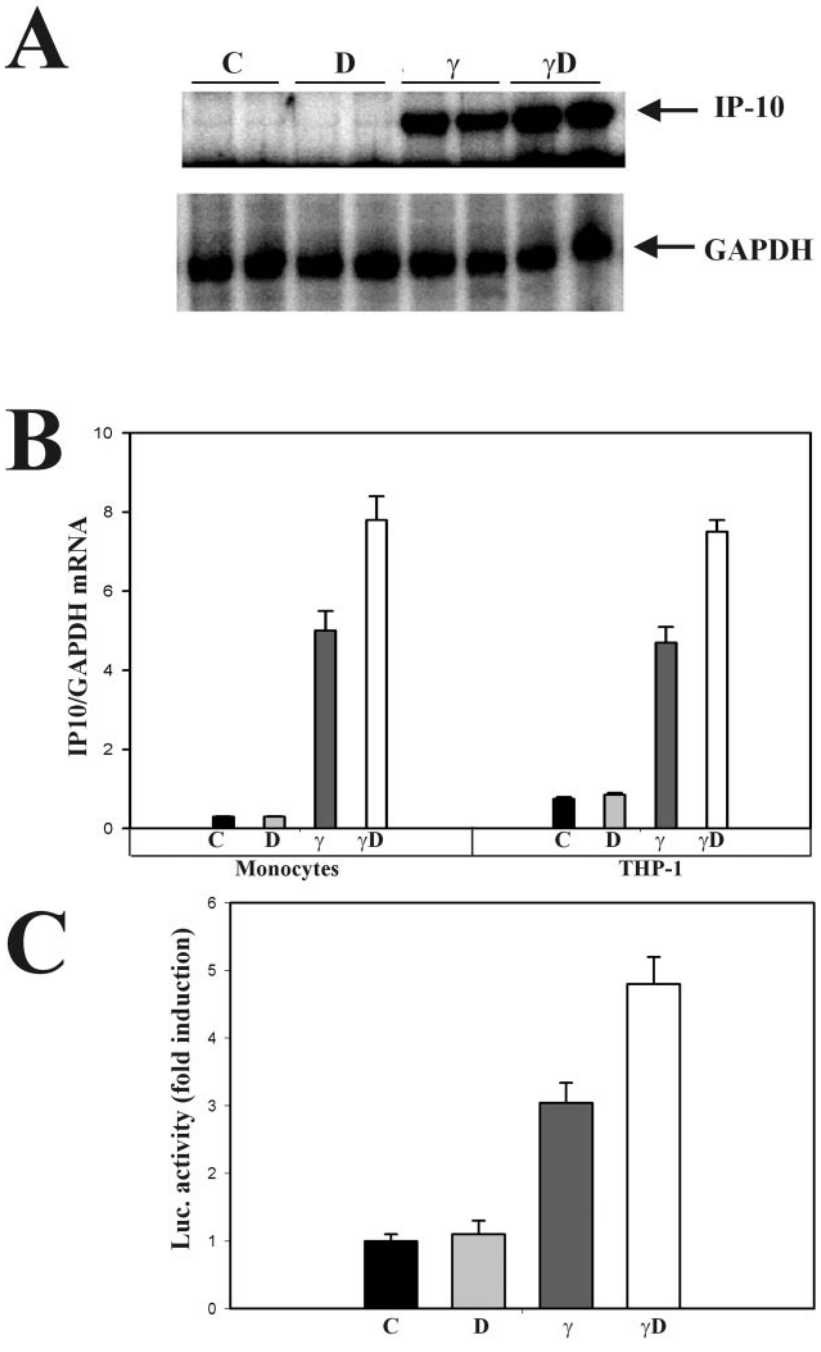

FIG. 6. 1,25D synergy on IFN-γ induction of the IP-10 gene. (A) Normal blood monocytes were left untreated or treated with 50 nM 1,25D, 150 U of IFN-γ/ml, or both for 18 h. IP-10 and GAPDH mRNAs were measured by RNase protection assays. (B) Relative IP-10/GAPDH mRNA levels in peripheral blood monocytes from four healthy volunteers (left graph) or differentiated THP-1 cells (right graph) from two independent experiments. Results represent the means ± standard errors of the means. (C) 2fTGH cells were transiently transfected with TGL-IP10. Twenty-four hours after transfection, cells were either untreated (C) or exposed to 10 nM 1,25D (D), 500 IU of IFN-γ/ml (γ), or both (γD) as indicated for 24 h. Luciferase activity was determined in cell lysates. Bars and error bars represent means ± standard errors of the means from triplicate measurements.

VDR/RXR binding to VDRE resulted in decreased 1,25D-VDR transcriptional activation of the 24-hydroxylase gene. In Stat1-null U3A cells, neither VDR-RXR binding to VDRE nor 1,25D transactivation of 24-hydroxylase was impaired by IFN-γ treatment. Since both proximal and distal VDREs in the human 24-hydroxylase promoter are typical VDRE sequences, inhibitory effects of IFN-γ identical to those exerted on 1,25D transactivation of human 24-hydroxylase could affect other genes induced by 1,25D through classical VDREs. In fact, IFN-γ antagonized 1,25D induction of a rat osteocalcin

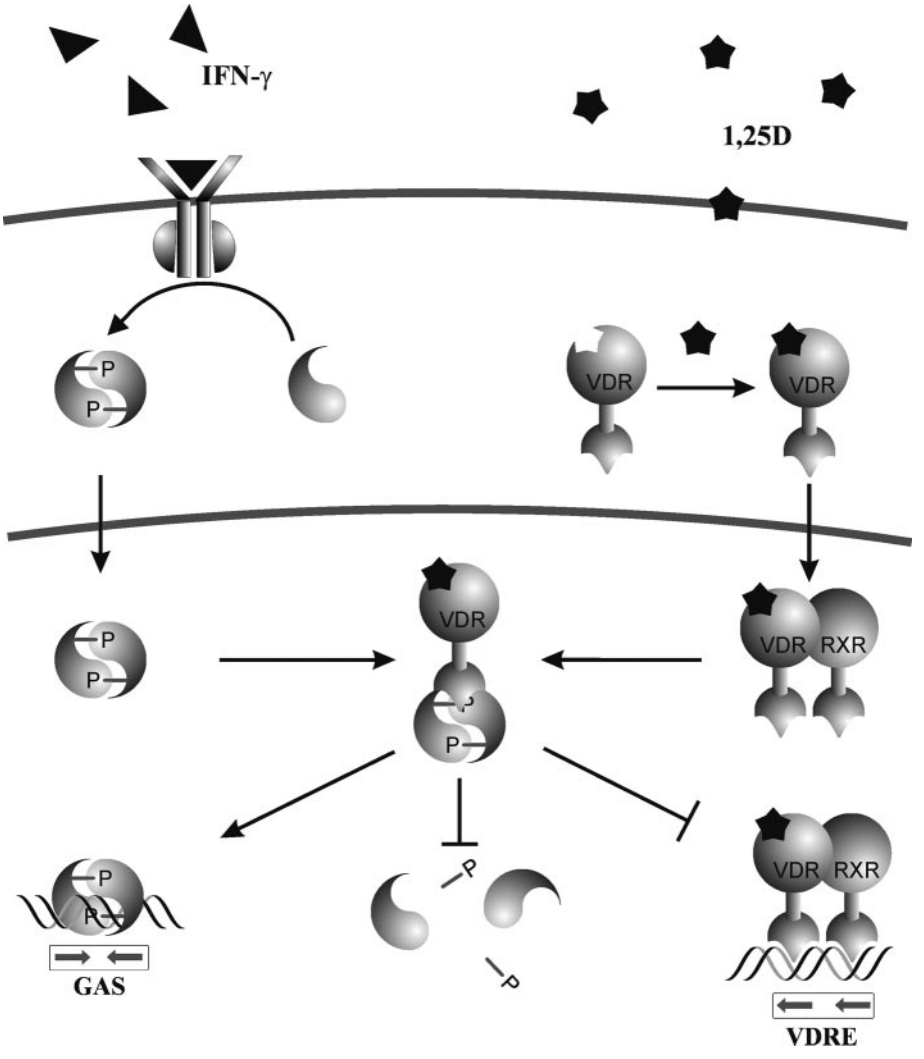

FIG. 7. Schematic diagram of functional Stat1-VDR interactions. Nuclear accumulation of Stat1 and the VDR by IFN-γ and 1,25D promotes physical interaction between Stat1 and the DBD of the VDR. Stat1-VDR complex formation inhibits VDR-RXR binding to VDRE and Stat1 deactivation by tyrosine dephosphorylation, thus resulting in IFN-γ antagonism on 1,25D-VDR transactivation and 1,25D cooperation on Stat1-mediated transcription.

VDRE-driven reporter (Fig. 1B). Thus, the mechanisms described by these studies may explain IFN-γ antagonism on 1,25D induction of the osteocalcin gene (39) and IFN-γ reduction of bone formation (44).

In contrast to the inhibitory effects of Stat1-VDR interactions on 1,25D action, 1,25D has cooperative effects on IFN-γ 47–Stat1 transcriptional activation. Specifically, 1,25D enhanced IFN-γ induction of the mRNA levels of the potent chemokine IP-10 in normal human monocytes and in differentiated THP-1 cells (macrophage phenotype), as well as IFN-γ transactivation of a human IP-10 promoter-driven reporter. 1,25D synergy on Stat1-induced transcription was reproduced in 2fTGH cells in a GAS-driven, VDRE-less artificial promoter. VDR-Stat1 synergy increased with VDR cotransfection.

Cross talk between Stat proteins and steroid receptors has been reported. Similar to the functional interactions between Stat1 and the VDR, Stat5 antagonizes transactivation of glucocorticoid receptor (GR)-responsive promoters through direct protein-protein interactions that also result in GR synergism on Stat5-mediated transcription of the β-casein gene (47). Further characterization of GR-Stat5 interactions revealed the DBD of the GR as dispensable for cooperation with Stat5, whereas the AF1 transactivation domain is required, thus suggesting that the GR acts as a ligand-dependent coactivator of Stat5. In contrast, the Stat5 transactivation domain is not required for GR cooperation (48).

Diverse cross-modulations occur between Stats and steroid receptors. Stat5 also antagonizes transcription by mineralocorticoid, progesterone, and estrogen receptors. However, only mineralocorticoid and progesterone receptors enhance Stat5-mediated transcription, which is inhibited by the estrogen receptor and unaffected by the androgen receptor (49). In contrast to Stat5 antagonism on transactivation by steroid receptors, interleukin-6-activated Stat3 acts as a GR transcriptional coactivator (55).

Physical Stat-steroid receptor interactions are not mandatory for cross talk between their cognate signaling pathways, since indirect mechanisms of cross-modulation also exist. In fact, there is no protein-protein interaction mediating Stat5b inhibition of peroxisome proliferator-activated receptor α transcriptional activity (56), GR enhancement of Stat1 activation of Fcγ receptor I in monocytes (4), or retinoic acid synergy on IFN-γ action (10).

The demonstration that 1,25D treatment and VDR overexpression markedly enhance Stat1 binding to GAS suggested that the VDR could operate as a coactivator of Stat1-mediated transcription. Although the possibility that active Stat1-VDR-containing complexes bind GAS in vivo cannot be completely ruled out, no complexes containing VDR and Stat1 bound to GAS were detected when supershifting EMSAs with an antibody against the VDR, even when overexpressing the VDR.

Tyrosine dephosphorylation of Stat1, by a yet-unknown nuclear phosphatase, deactivates IFN-γ signaling (20, 21). In fact, the vaccinia virus blocks IFN-γ action by dephosphorylating Stat1 through a virion-encoded phosphatase (38). Since 1,25D treatment prolonged the activated state of Stat1, we propose that Stat1-VDR interactions, by protecting Stat1 from inactivation by tyrosine dephosphorylation, enhance Stat1 binding to GAS and, consequently, Stat1-mediated induction of IFN-γ-regulated genes. Similarly, the GR prolongs the activated state of Stat5 (53), thus enhancing Stat5-mediated transcription.

In summary, among numerous potential mechanisms, our data suggest that, in human monocytes and macrophages, functional Stat1-VDR interactions result from the cross-modulation depicted in Fig. 7. Simultaneous exposure to 1,25D and IFN-γ increases nuclear VDR and Stat1 levels, thus promoting direct protein-protein interactions between the two transcription factors that prevent VDR-RXR binding to the VDRE and deactivation of Stat1 by tyrosine dephosphorylation. The biological consequences of nuclear VDR-Stat1 complex formation include IFN-γ antagonism on 1,25D-VDR transcriptional activity and 1,25D enhancement of IFN-γ–Stat1-induced transcription.

This model explains the pathogenesis of the abnormal 1,25D homeostasis and the resultant hypercalcemia in granulomatous processes. High concentrations of circulating 1,25D result from Stat1-VDR antagonism on 1,25D transactivation of 24-hydroxylase, the key enzyme for 1,25D inactivation. Reduction of 24-hydroxylase itself causes increased levels of 1,25D in serum (46). Also, Stat1-VDR synergy on IFN-γ action may contribute to increase serum 1,25D levels. In fact, 1α-hydroxylase mRNA levels in differentiated THP-1 cells exposed to 1,25D and IFN-γ are 45% higher than the levels in cells treated with IFN-γ alone (35).

Although the influence of different promoter architectures and/or cellular patterns of protein expression advises caution before generalizing the impact of this model to every 1,25D- and IFN-γ-regulated gene, VDR-Stat1 interactions may also provide new insights into the immunomodulatory properties of 1,25D.

1,25D is a potent immunosuppressor (31). The 1,25D produced by the activated macrophage exerts paracrine effects on surrounding activated T lymphocytes, inhibiting their production of interleukin-2 (5) and IFN-γ itself (14). Thus, 1,25D mediates a negative feedback loop to decrease inflammation (32). IFN-γ-induced Stat1-VDR antagonism on 1,25D action on macrophages, which cause high levels of 1,25D, is, however, unlikely to affect TH_1 lymphocytes, the main type responsible for inflammatory responses, because these cells lack the IFN-γ receptor β chain (41), mandatory for IFN-γ action.

VDR-Stat1 synergy may further enhance immunomodulation by 1,25D on monocytes/macrophages. In fact, synergistic effects of 1,25D and IFN-γ were reported previously for the induction of nonspecific esterase (51) and NADPH oxidase (its regulated subunits) (40) genes, two processes directly associated with terminal macrophage differentiation and enhanced antimicrobicidal and antiviral potential. Since both 1,25D and IFN-γ are activators of macrophage immune function, high levels of 1,25D could exert autocrine effects in activated macrophages by synergizing with IFN-γ, the most potent macrophage-activating cytokine.

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