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Inhibition of *Trail* Gene Expression by Cyclopentenonic Prostaglandin 15-Deoxy-$\Delta^{12,14}$- Prostaglandin J_2 in T Lymphocytes

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ABSTRACT

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a cyclopentenonic prostaglandin endowed with powerful anti-inflammatory activities, as shown in animal models of inflammatory/autoimmune diseases, where pharmacological administration of this prostanoid can ameliorate inflammation and local tissue damage via activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) and/or covalent modifications of cellular proteins. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily expressed in most of the cells, including those of immune system such as T lymphocytes, in which it is upregulated upon antigen-specific stimulation. This cytokine plays an important role in regulating various physiological and immunopathological processes, such as immunosurveillance of tumors and tissue destruction associated with different inflammatory and autoimmune diseases. Here, we demonstrate that 15d-PGJ_2 inhibits *trail* mRNA and protein expression by down-regulating the activity of its promoter in human T lymphocytes. Our data indicate that both the chemically reactive cyclopentenone moiety of 15d-PGJ_2 and the activation of PPARγ may be involved in this repressive mechanism. We identified nuclear factor κB (NF-κB) as a direct target of the prostanoid. 15d-PGJ_2 significantly decreases the expression and/or DNA binding of c-rel, RelA, and p50 transcription factors to the NF-κB1 site of *trail* promoter. Moreover, 15d-PGJ_2-mediated activation of the transcription factor heat shock factor-1 may contribute to inhibit *trail* promoter activity in transfected Jurkat T cells. These results suggest that modulation of TRAIL gene expression by 15d-PGJ_2 in T cells may provide a novel pharmacological tool to modify the onset and the progression of specific autoimmune and inflammatory disorders.

TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2 ligand is a member of the TNF superfamily and binds five receptors. TRAIL-R1 and TRAIL-R2 signal for apoptosis through cytoplasmic death domains (Pan et al., 1997), which are absent or nonfunctional in the two decoy receptors TRAIL-R3 and TRAIL-R4 (Sheridan et al., 1997); in addition, TRAIL interacts with a soluble receptor, called osteoprotegerin (Emery et al., 1998).

TRAIL is best known for its tumoricidal activity because initial studies were focused on its ability to induce apoptosis on transformed cells (Ashkenazi et al., 1999; Walczak et al., 1999); however, further research revealed that it can target normal primary cells too, and it is able to exert regulatory, prosurvival, and proliferative effects (Di Pietro and Zauli, 2004). In fact, TRAIL has been implicated in different aspects of immune cell regulation, such as intrathymic selection (Lamhamedi-Cherradi et al., 2003), secondary immune response of CD8 T cells (Janssen et al., 2005), and tumor immunity (Smyth et al., 2001; Takeda et al., 2001). In addi-

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ABBREVIATIONS: TNF, tumor necrosis factor; cyPG, cyclopentenone-type prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor; HSF-1, heat shock factor-1; HSP, heat shock protein; HSE, heat shock element; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Fas-L, Fas ligand; CAY10410, 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; NF-κB, nuclear factor κB; AP-1, activator protein 1; bp, base pair(s); PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; RT-PCR, reverse transcription-polymerase chain reaction; c.a., constitutively activated; EMSA, electrophoretic mobility shift assay; IL, interleukin; shRNA, short hairpin RNA; NF-AT, nuclear factor of activated T cells.

tion, TRAIL has been shown recently to be involved in local tissue damage caused by immune cell attack in several animal models of autoimmune diseases (Kaplan et al., 2002; Aktas et al., 2005; Huang et al., 2005; Sato et al., 2006). Apart from its cytotoxic effect, TRAIL can also play a role in these mechanisms by triggering a reverse signaling. In fact, TRAIL engagement has been demonstrated to enhance T-cell reactivity to autoantigens in terms of proliferation and Th1 cytokine production, functioning as a costimulator for autoreactive T lymphocytes (Chou et al., 2001; Tsai et al., 2004). These observations place TRAIL as a mediator of pathological processes associated with specific autoimmune diseases.

The 15-Deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) is a cyclopentenonic prostaglandin (cyPG) displaying strong anti-inflammatory properties for its regulatory effects on a variety of immune cells (Harris et al., 2002). Most actions of this prostanoid are related to its interaction with specific cellular proteins.

15d-PGJ$_2$ is a natural ligand of PPARγ (Forman et al., 1995), a nuclear receptor able to transrepress the expression of proinflammatory mediators by antagonizing the activity of different transcriptional factors, such as NF-κB, AP-1, signal transducer and activator of transcription-1, and NF-AT (Ricote et al., 1998; Yang et al., 2000; Chung et al., 2003; Cunard et al., 2004). Furthermore, the presence of an electrophilic center within the molecule allows 15d-PGJ$_2$ to react with different cellular proteins by means of Michael addition (Atsmon et al., 1990). As a consequence of such covalent interactions, the functions of target proteins may be altered, as described for different components of the NF-κB signaling and AP-1 nuclear factors (Rossi et al., 2000; Straus et al., 2000; Pérez-Sala et al., 2003). In addition, because of this cyclopentenonic structure, 15d-PGJ$_2$ can also trigger a stress response in cells by activation of the heat shock factor-1 (HSF-1) and induction of heat shock protein (HSP) expression (Santoro et al., 1989). These mechanisms have been shown to be specifically implicated in the 15d-PGJ$_2$-mediated regulation of immune cells. Moreover, they may account for the powerful anti-inflammatory activity of this prostaglandin described in animal models of experimental autoimmune diseases, where pharmacological administration of this prostanoid could ameliorate inflammation and local tissue damage (Kawahito et al., 2000; Cuzzocrea et al., 2002; Diab et al., 2002, 2004).

The purpose of this study was to analyze the pharmacological effect(s) of 15d-PGJ$_2$ on the activation of *trail* gene in T cells and the molecular mechanisms involved at the transcriptional level. Our data showed that 15d-PGJ$_2$ suppresses *trail* mRNA expression and promoter activity in activated T cells. We provide evidence that the reactive α,β-unsaturated carbonyl group in 15d-PGJ$_2$ structure plays an important role in this repressive mechanism, because a molecular analog (CAY10410) with modifications intended to maintain PPARγ ligand activity and to eliminate prostanoid metabolism via Michael addition to reactive nucleophiles does not inhibit *trail* gene expression in Jurkat T cells.

15d-PGJ$_2$ negatively interferes with the expression and the activity of NF-κB, a known inducer of *trail* gene expression in T cells (Baetu et al., 2001; Rivera-Walsh et al., 2001; Siegmund et al., 2001); furthermore, HSF-1 activation by 15d-PGJ$_2$ significantly contributes to block *trail* promoter activity and is able to bind to a putative HSE, located in the first -165 bp from the transcriptional start site of the gene. Moreover, we provide evidence that the activation of PPARγ can inhibit *trail* promoter activity and gene expression in T cells. These results demonstrate that TRAIL is a target of the complex immunosuppressive action of 15d-PGJ$_2$ and encourage the possible pharmacological use of this prostanoid (or derived molecules) for therapeutic intervention in the treatment of destructive inflammatory/autoimmune diseases.

Materials and Methods

Cell lines, Reagents, and Treatments. Jurkat T cells (a CD4$^+$ human lymphoblastoid T cell line) were maintained as described previously (Cippitelli and Santoni, 1998). Human enriched T cells were obtained from healthy donors as described in Cippitelli et al. (2003). Phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO). OKT3 anti-human CD3 mAb was purified from culture supernatant by protein A chromatography. Cyclopentenone (2-cyclopenten-1-one) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 15d-PGJ$_2$ cells were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Rosiglitazone and troglitazone were purchased from Alexis (Lausen, Switzerland). CAY10410 was purchased from Cayman Chemical (Ann Arbor, MI). For heat shock treatment, culture flasks were sealed with Parafilm and immersed in a water bath at 42°C for 1 h. Control cells were left in the incubator (sealed with Parafilm) at 37°C. After heat shock treatment, cells were returned to the incubator for appropriate stimulation.

Flow Cytometric Analysis. Jurkat cells (0.5×10^6) per experimental point were used to analyze surface expression of TRAIL by indirect staining with 0.5 μg of a mouse anti-human APO-2L/TRAIL (human mAb III6F; Alexis) followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG (MP Biomedicals, Irvine, CA). Nonspecific fluorescence was assessed by using an isotype-matched irrelevant mAb followed by the same secondary reagent. Fluorescence was analyzed with FACScan flow cytometer (BD Biosciences Pharmingen, San Diego, CA).

RNA Isolation and RT-PCR. Total RNA was extracted from Jurkat T cells or human T cells by TRIzol (Invitrogen, Carlsbad, CA). One to two micrograms of total RNA were reverse-transcribed (Promega, Madison, WI), and aliquots were used in subsequent polymerase chain reaction reactions. Primer sets are as follows: human *trail* sense, 5′-cttcacagtgctcctgcagt-3′, and human *trail* antisense, 5′-ttagccaactaaaaaggcccc-3′; human *c-rel* sense, 5′-agaggggaatgcgttttagataca-3′, and human *c-rel* antisense, 5′-caggaaggaaaaacatgaaaacaca-3′; human *ppar-γ1* sense, 5′-ggttgacacagagatgccattctg-3′, human *ppar-γ2* sense, 5′-gggtgaaactctgggagattctcc-3′, and human *ppar-γ1* antisense and human *ppar-γ2* antisense, 5′ gagttggaaggctcttcatgaggc-3′; *β-actin* sense, 5′-gtggggcgccccaggcacca-3′, and *β-actin* antisense, 5′-ctccttaatgtcacgcacgatttc-3′. Semiquantitative polymerase chain reaction conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction.

Plasmid Constructions. The different deletions of the human *trail* promoter, -1523 bp, -577 bp, -300 bp, -165 bp, and -35 bp (in pGL2-basic luciferase vector; Promega), were kindly provided by Dr. B. M. Evers (University of Texas Medical Branch, Galveston, TX). The human Fas-L promoter -453-bp Fas-L (in pGL2-basic luciferase vector; Promega) was kindly provided by Dr. C. V. Paya (Mayo Clinic, Rochester, MN).

The RSV-Gal expression vector and the reporter 3xNF-κB-Luc have been described previously (Cippitelli et al., 2003). Expression vector for human wild-type PPARγ2 (PSG5-PPARγ) was kindly provided by Dr. B. Staels (Institut Pasteur de Lille, Lille, France). The expression vectors for human constitutively activated (c.a.) HSF-1 S303A/S307A double mutant (pcDNA3-HSF-1-S303A/S307A) and HSF-1 deleted form HSF-1/203–503 (pcDNA3-HSF-1/203–503) were

kindly provided by Dr. R. I. Morimoto (Northwestern University, Evanston, IL). The retroviral vectors pSIREN-RetroQ and pSIREN-RetroQ/HSF-1 shRNA were kindly provided by Dr. M. Y. Sherman (University Medical School, Boston, MA).

DNA Transfections. Transfections of Jurkat cells were carried out by the diethylaminoethyl-dextran method as described in Cippitelli and Santoni (1998). To decrease variations due to different transfection efficiency, cells were transfected in single batches that were then separated into different drug treatment groups. An RSV-Gal expression vector was cotransfected each time to normalize DNA uptake. After 24 h, cells were treated with different combinations of stimuli, and after additional 8 h, cells were harvested and protein extracts were prepared for the luciferase and β-galactosidase assays as described in Cippitelli and Santoni (1998). Protein concentration was quantified by the BCA method (Pierce, Rockford, IL). Luciferase activity was read using the luciferase assay system (Promega) following the manufacturer's instructions. β-Galactosidase activity was determined as described in Cippitelli and Santoni (1998).

Virus Production and In Vitro Transduction. Phoenix retrovirus packaging cell lines were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Phoenix cells were transfected with viral DNA (5 μg of pSIREN-RetroQ or pSIREN-RetroQ/HSF-1 shRNA) at 50% confluence with Lipofectamine Plus (Invitrogen, Carlsbad, CA). After transfection, the cells were placed in fresh medium. After a further 24-h culture, virus-containing supernatant was harvested, filtered, and either stored at −20°C or used immediately for infection. Infection was performed on 0.5×10^6 Jurkat cells in 3 ml of complete medium with Polybrene (8 μg/ml) (hexadimethrine bromide; Sigma) for 8 to 12 h. After infection, cells were allowed to expand for 48 h and were then selected for puromycin resistance. The amount of puromycin used during selection was 2 μg/ml.

Electrophoretic Mobility Shift Assay. Nuclear proteins were prepared as described in Cippitelli and Santoni (1998). Protein concentration of extracts was determined by the BCA method (Pierce). The nuclear proteins (10 μg) were incubated with radiolabeled DNA probes in a 20-μl reaction mixture containing 20 mM Tris, pH 7.5, 60 mM KCl, 2 mM EDTA, 0.5 mM dithiothreitol, 1 to 2 μg of poly(dI-dC), and 4% Ficoll. Where indicated, a molar excess of double-stranded oligomer was added as an unlabeled competitor, and the mixture was incubated at room temperature for 10 min before adding the DNA probe. Nucleoprotein complexes were resolved as described in Cippitelli and Santoni (1998). Oligonucleotides were purchased by Invitrogen Life Technologies (CH Groningen, The Netherlands). Complementary strands were annealed and end-labeled as described in Cippitelli and Santoni (1998). Approximately 3×10^4 cpm of labeled DNA was used in a standard electrophoretic mobility shift assay reaction. In supershift analysis, the specific antibody was added to the binding reaction and the mixture was incubated for 30 min at room temperature before adding the labeled DNA probe. The antibodies against RelA, cRel, p50, and HSF-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

The following double-strand oligomers were used as specific labeled probes or unlabeled competitors (sense strand): TRAIL −143/−115, 5′-gcttctttcagtttccctcctttccaacg-3′; TRAIL NF-κB1, 5′-aaagcaaagaaaatccctcccct-3′; NF-κB Ig, 5′-gatcacaagggactttc-cgct-3′; octamer-(h-histone H2b), 5′-agctcttcaccttatttgcataagcgat-3′; and HSE, 5′-gctcctcgaatgttcgcgaagtttcg-3′.

Western Blot Analysis. For Western blot analysis, Jurkat T cells were pelleted, washed once with ice-cold phosphate-buffered saline, resuspended in lysis buffer [1% Nonidet P-40 (v/v), 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture (Roche, Indianapolis, IN) in phosphate-buffered saline] and subsequently incubated for 30 min on ice. The lysate was centrifuged at 14,000*g* for 15 min at 4°C, and the supernatant was collected as whole-cell extract. Nuclear proteins were prepared as described above. Protein concentration of nuclear and whole-cell extracts was determined by the BCA method (Pierce, Rockford, IL). Thirty to fifty micrograms of nuclear extract or whole-cell extract were run on 12% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted onto nitrocellulose membranes (Whatman Schleicher & Schuell, Keene, NJ) and blocked in 3% milk in Tris-buffered saline/Tween 20 buffer. Immunoreactive bands were visualized on the nitrocellulose membranes using horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse immunoglobulins and the enhanced chemiluminescence detection system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) following the manufacturer's instructions.

Antibodies against cRel, RelA, and Oct-1 were purchased from Santa Cruz Biotechnology. The antibody against β-actin was purchased by Sigma Chemical Co.

Results

15d-PGJ$_2$ Inhibited TRAIL Expression and Promoter Activation in T Cells. We have reported recently that 15d-PGJ$_2$ inhibits *fas-l* and *rankl* gene expression in activated T cells (Cippitelli et al., 2003; Fionda et al., 2007).

To analyze whether 15d-PGJ$_2$ may similarly affect TRAIL expression in these cells, a flow cytometric analysis was performed. As shown in Fig. 1A, surface TRAIL expression was induced by PMA plus ionomycin stimulation for 16 h in Jurkat T cells, and treatment of cells with 15d-PGJ$_2$ in the pharmacological micromolar range of concentration (from 10 to 1 μM) significantly blocked this induction. Thus, this prostaglandin inhibits TRAIL protein surface expression in Jurkat T cells.

We then examined whether this effect could be the consequence of a negative regulation of *trail* gene expression by RT-PCR analysis. In Jurkat cells, the combination of PMA and ionomycin induced *trail* mRNA expression, which was significantly suppressed by 10 μM 15d-PGJ$_2$ (Fig. 1B). Similar results were observed in fresh isolated enriched T cells after activation with plate-bound anti-CD3 antibody in the presence of 15d-PGJ$_2$ (Fig. 1C). In contrast, 15d-PGJ$_2$ did not affect β-actin mRNA expression, suggesting that the modulation of *trail* by this molecule was not the result of a generalized inhibition of cellular activation or of an unequal loading of mRNA samples. These results indicate that 15d-PGJ$_2$ represses *trail* gene expression in both activated Jurkat cells and human CD3-stimulated T cells.

15d-PGJ$_2$ is endowed with a peculiar molecular structure that let it link and activate the nuclear receptor PPARγ (Forman et al., 1995), and covalently bind to cellular proteins (Atsmon et al., 1990). In particular, its cyclopentenonic ring possesses an electrophilic-α,β-unsaturated ketone, which can react with the thiol groups of proteins by means of Michael addiction. We and others have described the role of cyclopentenonic structure in mediating many inhibitory effects of 15d-PGJ$_2$ in T cells (Cippitelli et al., 2003; Nencioni et al., 2003). To determine whether this reactive ring system may account for the inhibition of *trail* mRNA, we analyzed the effect on the expression of this gene of two different molecules, CAY10410 and cyclopentenone.

CAY10410 has the same structure as 15d-PGJ$_2$ except for the electrophilic carbon in the cyclopentenonic ring, so it can link and activate the nuclear receptor PPARγ, but it is not susceptible to nucleophilic addiction with thiols. Yet this molecule did not affect *trail* mRNA expression; indeed, it was strongly inhibited by cyclopentenone (Fig. 1B). Because this compound consists only of the cyclopentenonic ring of 15d-

PGJ_2, these results suggest that adduct(s) formation via Michael reaction has an important role in the inhibition of *trail* gene expression by 15d-PGJ_2.

We next examined the effect of this prostanoid on *trail* promoter, and transient transfection assays were performed in Jurkat T cells. As shown in Fig. 1D, the activity of a luciferase reporter vector driven by a −1523-bp 5′ fragment of the human *trail* promoter was induced by stimulation with PMA plus ionomycin for 8 h and was considerably decreased by 10 μM 15d-PGJ_2. These data demonstrate that the prostanoid inhibits the activity of *trail* gene promoter in activated T cells.

15d-PGJ_2 Inhibited Human *Trail* Promoter in Jurkat T Cells: Role of NF-κB. Although *trail* mRNA is detected in various cells and tissues, regulation of its expression remains largely unknown. Previous studies have demonstrated a pivotal role for the transcription factor NF-κB in the activation of *trail* gene expression in both Jurkat T cell line and primary T lymphocytes (Rivera-Walsh et al., 2001; Siegmund et al., 2001). In addition, the NF-κB-dependent expression of *trail* was shown to be associated with the presence of two different NF-κB binding sites in the promoter of this gene. In particular, the NF-κB1 site, located between −256 and −265 bp from the transcriptional start site, was described to bind the heterodimeric complex c-rel/p50 (Baetu et al., 2001).

Because NF-κB is one of the transcription factors strongly inhibited by 15d-PGJ_2 in many cellular types (Rossi et al., 2000; Straus et al., 2000; Piva et al., 2005) including T lymphocytes (Cippitelli et al., 2003; Fionda et al., 2007), we investigated whether it could be involved in the inhibitory action of the prostanoid on *trail* promoter. In this regard,

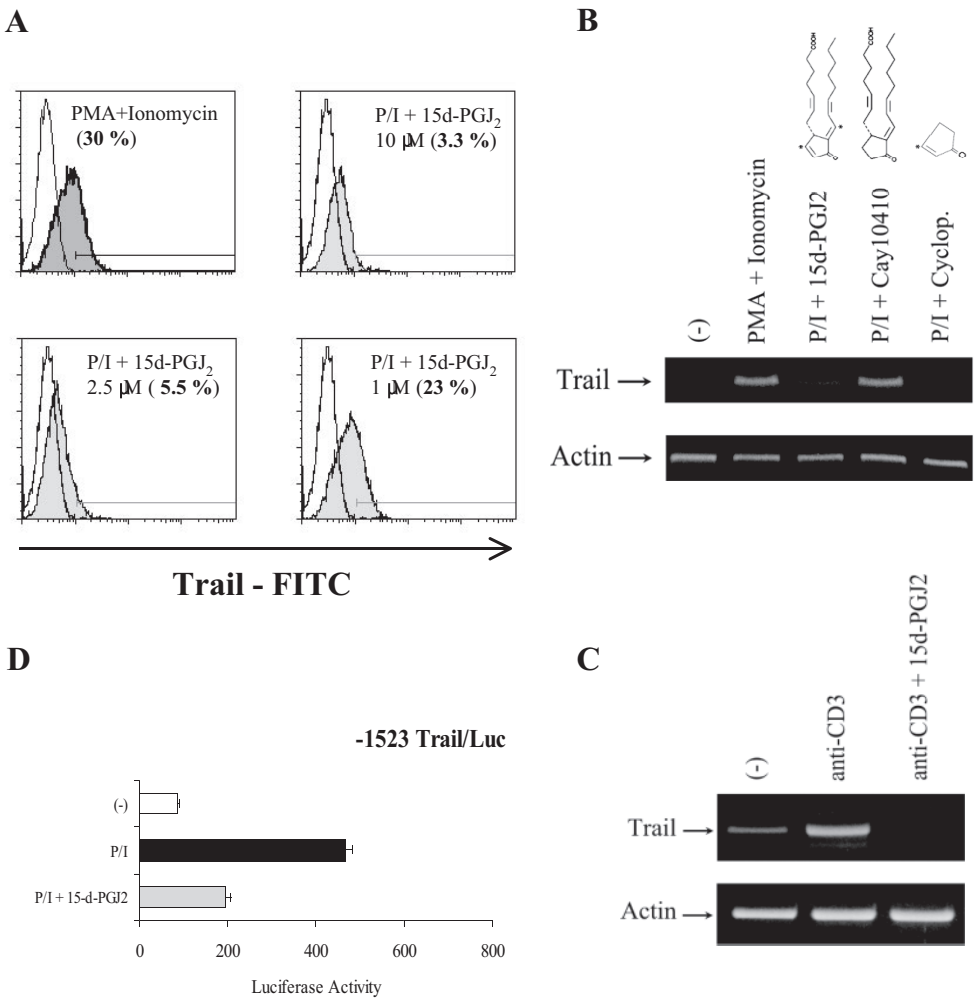

Fig. 1. 15d-PGJ_2 represses TRAIL protein, mRNA expression, and promoter activation in T cells: role of the cyclopentenone reactive ring. A, flow cytometric analysis of cell surface TRAIL expression. Jurkat T cells were untreated (−) or stimulated with 10 ng/ml PMA plus 0.5 μg/ml ionomycin for 16 h in the absence or presence of the indicated concentration of 15d-PGJ_2. The experiment shown is representative of various independent experiments all displaying similar results. B, RT-PCR analysis of total mRNA obtained from Jurkat T cells, untreated (−) or activated with PMA plus ionomycin for 6 h, in the absence or presence of 10 μM 15d-PGJ_2, 10 μM CAY10410, or 250 μM cyclopentenone (Cyclop). Molecular structures of 15d-PGJ_2, CAY10410, and cyclopentenone (2-cyclopenten-1-one) are shown. Asterisks indicate the position of chemically reactive electrophilic carbons. C, RT-PCR analysis of total mRNA obtained from enriched human T cells, untreated (−) or activated with plate-bound anti-CD3 mAb (OKT3) for 6 h in the absence or presence of 10 μM 15d-PGJ_2. RT-PCRs shown in the figure are representative of various independent experiments all displaying similar results. D, Jurkat cells were cotransfected with 10 μg of the −1523-bp Trail/Luc reporter plus 4 μg of RSV-Gal expression vector as described under *Materials and Methods*. Twenty-four hours after transfection, cells were left untreated (−) or were stimulated with 10 ng/ml PMA and 0.5 μg/ml ionomycin (P/I) in the absence or presence of 10 μM 15d-PGJ_2. After 8 h, cells were harvested, and protein extracts were prepared for the luciferase and β-galactosidase assays. Results are expressed as relative luciferase activity normalized to protein concentration and to β-galactosidase activity produced of the internal control plasmid and represent the mean value (X ± S.E.) from at least three experiments.

using mobility shift assays, we characterized the binding activity to the *trail* promoter NF-κB1 site of nuclear extracts obtained from Jurkat cells stimulated with PMA plus ionomycin. In accordance with Baetu et al. (2001), in activated cells, we observed the formation of a specific NF-κB complex (Fig. 2A), which contains the proteins c-rel, p50, and p65, as demonstrated by supershift analysis with specific antibodies (Fig. 2B). We then investigated whether 15d-PGJ_2 could affect the NF-κB activity and specific DNA binding to this promoter site. To this purpose, transient transfection assays were performed in Jurkat T cells, and it was confirmed that 15d-PGJ_2 significantly inhibited the transcriptional activity of NF-κB (Fig. 2C); we next performed electrophoretic mobility shift assay (EMSA) with the NF-κB1 site and nuclear extracts from activated Jurkat cells in the absence or presence of 10 μM 15d-PGJ_2. As shown in Fig. 2D, the prostaglandin is effective in reducing DNA binding of the NF-κB1 complex induced by the combination of PMA plus ionomycin; as control for an equal proteins loading, the same amount of nuclear proteins was run in the presence of a Octamer factor(s)-specific probe (Fig. 2E).

To further investigate the mechanism by which 15d-PGJ_2

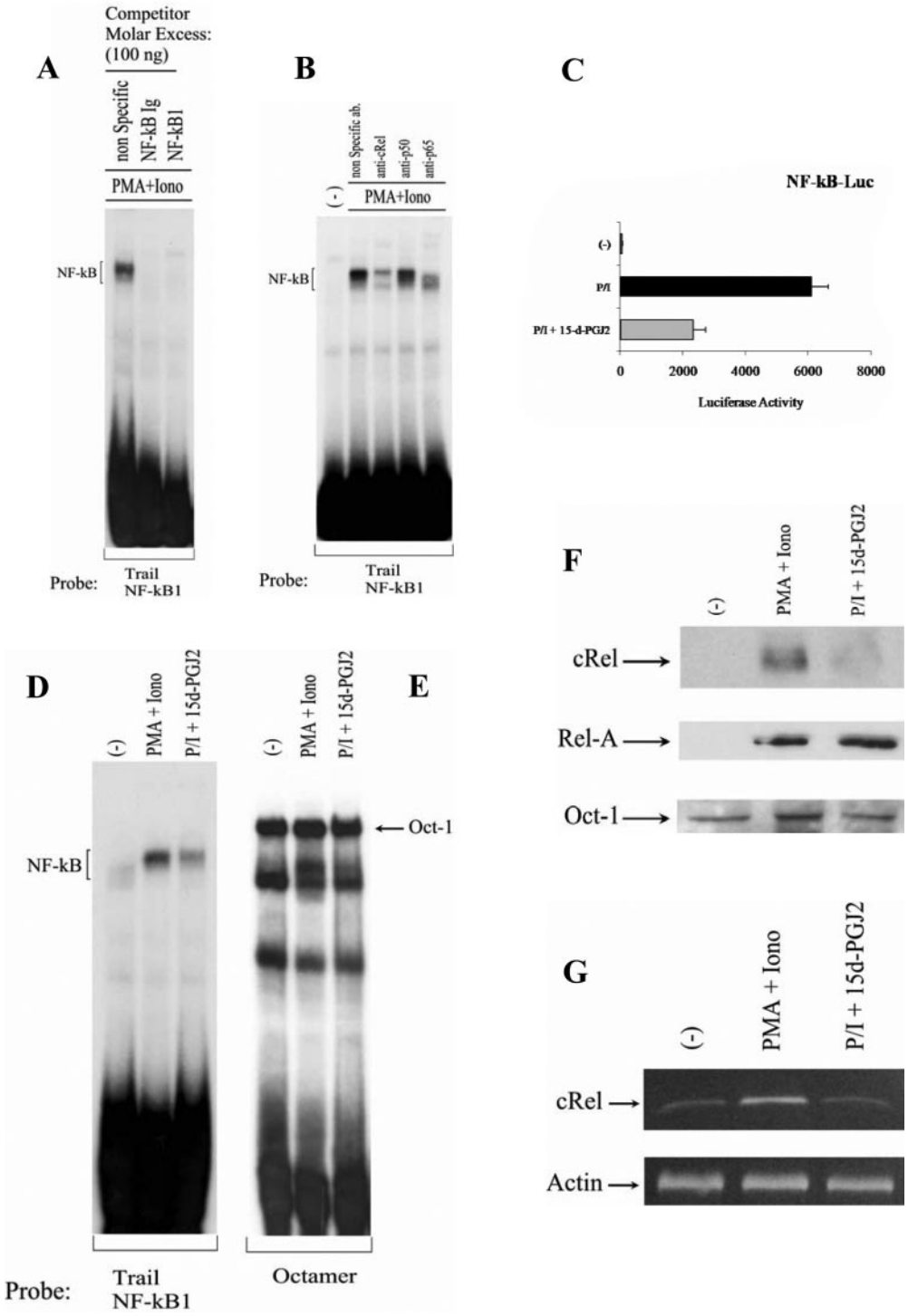

Fig. 2. 15d-PGJ_2 modulates NF-κB DNA binding on *trail* NF-κB1 binding site. A, EMSA was performed using the ^{32}P-labeled TRAIL NF-κB1 oligonucleotide as a probe in the presence of nuclear extracts (10 μg) from unstimulated (−) or PMA plus ionomycin-treated Jurkat cells (16 h). Where indicated, 100 ng of NF-κB Ig or TRAIL NF-κB1 unlabeled competitor was added to the reaction mixture to confirm specificity. B, supershift analysis of NF-κB complexes bound to the TRAIL NF-κB1 oligonucleotide: where indicated, purified anti-cRel, anti-p50, or anti-RelA (p65) was added to the reaction mixture. The same amount of a nonspecific antibody (anti-Octamer-1) did not supershift or inhibit NF-κB bound complexes (data not shown). C, Jurkat cells were transfected with 10 μg of the indicated reporter vector as described under *Materials and Methods*. Twenty-four hours after transfection, cells were left untreated (−) or were stimulated with 10 ng/ml PMA plus 0.5 μg/ml ionomycin (P/I) in the absence or presence of 15d-PGJ_2 treatment for 8 h. Cells were then harvested, and protein extracts were prepared for the luciferase and β-galactosidase assays. Results are expressed as relative luciferase activity normalized to protein concentration and represent the mean value (X ± S.E.) from at least three experiments. D and E, EMSA was performed using the ^{32}P-labeled TRAIL NF-κB1 or an Octamer oligonucleotide as a probe in the presence of nuclear extracts (10 μg) from unstimulated (−) or PMA plus ionomycin-treated Jurkat cells (16 h) in the absence or presence of 10 μM 15d-PGJ_2. The same nuclear extracts were also used with a Octamer factor(s)-specific probe as a control. The mobility of Octamer-1 is shown. F, Western blot assay of nuclear proteins from unstimulated (−) or PMA plus ionomycin-treated Jurkat cells (16 h) in the in the absence or presence of 10 μM 15d-PGJ_2. The different Western blots shown in the figure are representative of various independent experiments, all displaying similar results. G, RT-PCR analysis of total mRNA obtained from Jurkat cells, untreated (−) or activated with PMA plus ionomycin (6 h), in the absence or presence of 10 μM 15d-PGJ_2. The RT-PCR shown is representative of various independent experiments all displaying similar results.

interferes with the binding activity of NF-κB in our system, we verified the effect of the prostanoid on its nuclear translocation/expression. Western blot assays revealed the capability of 15d-PGJ$_2$ to strongly reduce the nuclear level of c-rel (but not p65) (Fig. 2F), and this effect correlated with a significant inhibition of its mRNA as analyzed in RT-PCR (Fig. 4G); as a control for equal samples loading, 15d-PGJ$_2$ did not affect the expression of the nuclear transcription factor Oct-1 or the β-actin mRNA, respectively (Fig. 2, F and G). These findings demonstrate that 15d-PGJ$_2$ can inhibit the activity of *trail* promoter by modulating the function and/or expression of the transcription factor NF-κB.

15d-PGJ$_2$-Mediated *Trail* Inhibition: Promoter Analysis. To better understand the mechanism(s) responsible for 15d-PGJ$_2$-mediated repression of *trail* promoter, we analyzed the activity of progressive deletions of *trail* promoter by transient transfection assays in Jurkat T cells. As shown in Fig. 3, the combination of PMA plus ionomycin differently induced the activation of various deletions of this promoter from −1523 to −35 bp immediately 5′ of the transcriptional start site, and a minimal active promoter fragment corresponding to the first −165-bp nucleotides was still sensitive to 15d-PGJ$_2$-mediated repression. Thus, in addition to the NF-κB1 site, the region spanning from −165 to −35 bp contains regulatory element(s) important for 15d-PGJ$_2$-mediated inhibition.

15d-PGJ$_2$ Inhibited Human *Trail* Promoter in Jurkat T Cells: Role of HSF-1. Cyclopentenone prostaglandins can activate HSF-1 (Koizumi et al., 1993), a transcription factor playing a key role as both a positive and negative regulator of several genes. In particular, HSF-1 was demonstrated to repress the expression of several proinflammatory genes, such as TNF-α and IL-1β (Cahill et al., 1996; Singh et al., 2002; Chou et al., 2005). As described previously (Cippitelli et al., 2003) and as confirmed in Fig. 4A, treatment of Jurkat cells with 10 μM 15d-PGJ$_2$ induces nuclear translocation and specific DNA binding of HSF-1.

We then examined a possible role for HSF-1 in the negative regulation of *trail* promoter by 15d-PGJ$_2$. To this purpose, we cotransfected the −1523-bp human *trail* promoter reporter and an expression vector encoding a constitutively active form of HSF-1 (HSF-1 c.a.) in Jurkat T cells. As shown in Fig. 4B, overexpression of HSF-1 c.a. repressed the activity of *trail* promoter induced by PMA plus ionomycin. This effect was specific for *trail* promoter because it had a strong enhancer effect on a *fas-l* promoter fragment cloned in the same luciferase reporter vector [as described in Cippitelli et al. (2003), and used as control (Fig. 4C)], indicating that the negative effect on *trail* promoter is specific and is not the result of a generalized inhibitory effect on transcription.

These data demonstrate that HSF-1 activation may contribute to *trail* promoter suppression by 15d-PGJ$_2$. Moreover, partial depletion of HSF-1 using a retrovirus encoding a shRNA against this transcription factor could attenuate the inhibition of *trail* mRNA expression of heat shock-treated Jurkat cells (Fig. 4, D and E), an experimental setting that activates HSF-1, avoiding other regulatory actions mediated by 15d-PGJ$_2$.

To identify putative elements important for HSF-1-mediated *trail* promoter inhibition, a series of 5′ deletions of the −1523 *trail* promoter, ranging from −1523 to −35 bp, were examined. As shown in Fig. 5, cotransfection of these pro-

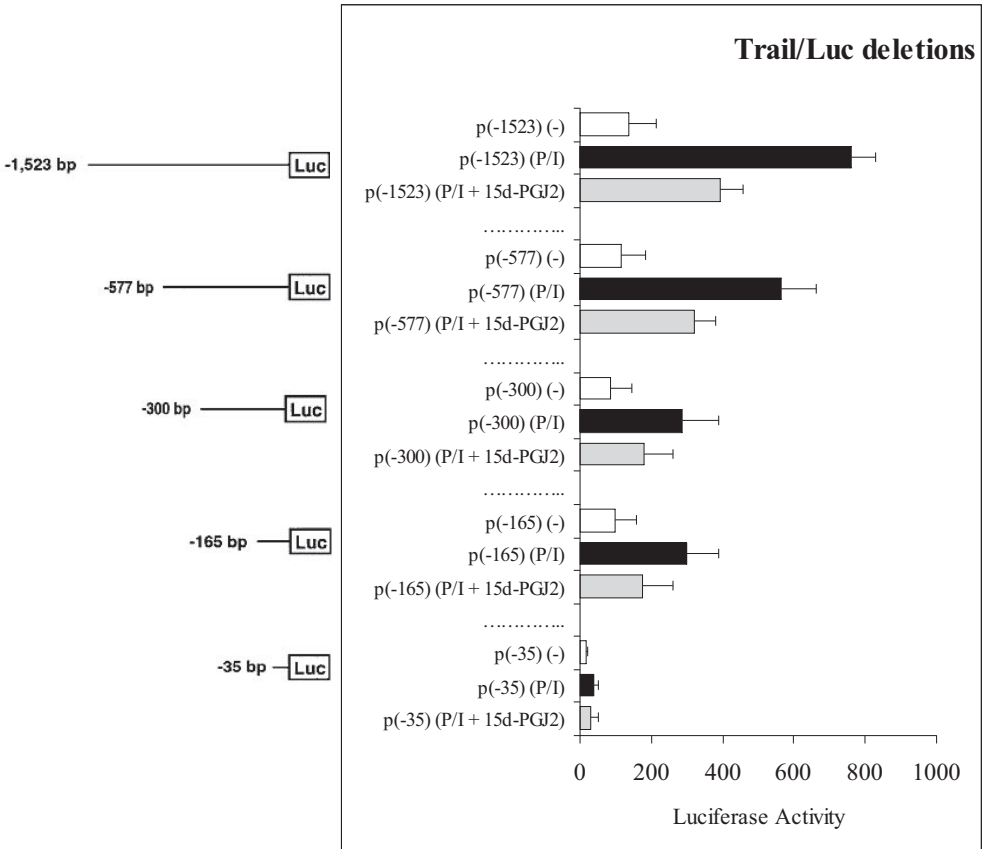

Fig. 3. A promoter analysis of 15d-PGJ$_2$-mediated inhibition. Jurkat cells were cotransfected with 10 μg of the indicated TRAIL/Luc reporter plus 4 μg of RSV-Gal expression vector. Activation and samples harvesting was carried out as described above. Twenty-four hours after transfection, cells were left untreated (−) or were stimulated with 10 ng/ml PMA plus 0.5 μg/ml ionomycin (P/I) in the absence or presence of 15d-PGJ$_2$. After 8 h, cells were harvested, and protein extracts were prepared for the luciferase and β-galactosidase assays. Results are expressed as relative luciferase activity as described above and represent the mean value (X ± S.E.) from at least three experiments.

moter reporters with a constitutively active form of HSF-1 showed that all but the −35-bp deletion recapitulated the HSF-1 mediated inhibition, observed when using the −1523 *trail* promoter. Through this analysis, the sequences important for *trail* repression by HSF-1 were localized to a region between −165 and −35 bp upstream of the transcriptional start site. This result suggests that HSF-1 is involved in the repression of the activity of this promoter fragment by 15d-PGJ_2.

Given that HSF-1 is capable of negatively regulating the expression of different genes by direct interaction with specific region(s) of their promoters, we analyzed the importance of this mechanism in the repression of *trail* promoter. A computer analysis of the first −165 bp in the human *trail* promoter identified putative HSF-1 binding elements spanning the position from −143 to −115 bp (Fig. 6A). Gel shift assays, performed with a synthetic double-stranded oligonucleotide for this promoter element (TRAIL −143/−115) and nuclear extracts from Jurkat cells treated with 15d-PGJ_2 demonstrated retardation in the mobility of this oligonucleotide due to a specific binding of HSF-1. The specificity of this interaction was confirmed by the addition of 100-fold excess of unlabeled TRAIL −143/−115 oligonucleotide or an HSE consensus sequence, which completely inhibited the formation of this complex (Fig. 6B); in addition, a specific anti-HSF-1 antibody (but not an irrelevant antibody) inhibited the HSF-1-specific complex in supershift assay (Fig. 6C).

Moreover, we examined the effect of a mutant HSF-1 (HSF-1 203/503) that lacks its DNA binding domain on *trail* promoter activity in transient transfection assays. As shown in Fig. 6D, overexpression of HSF-1 203/503 failed to repress *trail* promoter activity induced by PMA plus ionomycin, indicating that specific DNA binding activity is required for this inhibition.

Taken together, these data demonstrated that the first −165 bp from the transcriptional start site are still responsive both to 15d-PGJ_2 and to HSF-1, suggesting a role for this transcription factor in 15d-PGJ_2-mediated down-regulation of *trail* promoter. In this context, we identified an HSF-1

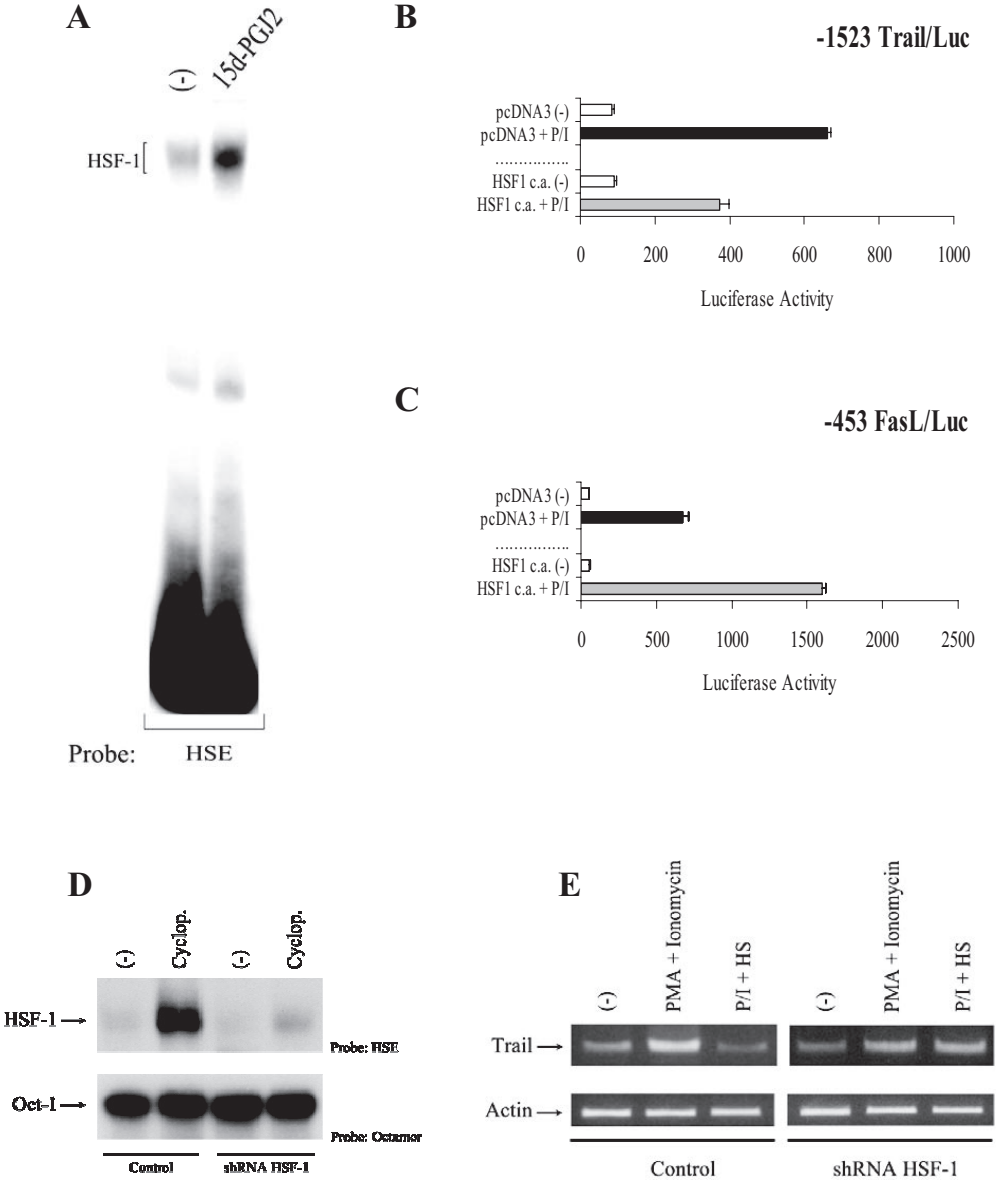

Fig. 4. Effect of HSF-1 activation on *trail* promoter activity. A, EMSA was performed using a ^{32}P-labeled HSE oligonucleotide as a probe in the presence of nuclear extracts (10 μg) from unstimulated (−) or 10 μM 15d-PGJ_2-treated cells. B and C, Jurkat cells were cotransfected with 10 μg of the indicated human TRAIL/Luc reporter or Fas-L/Luc reporter plus 4 μg of RSV-Gal expression vector. Where indicated, 5 μg of an expression vector encoding a constitutively active form of HSF-1 (HSF-1 c.a.) or a pcDNA3 empty control vector was added to the cotransfection setting. Activation and samples harvesting was carried out as described above. Twenty-four hours after transfection, cells were left untreated (−) or were stimulated with PMA plus ionomycin (P/I). After 8 h, cells were harvested, and protein extracts were prepared for the luciferase and β-galactosidase assays. Results are expressed as relative luciferase activity as described above and represent the mean value (X ± S.E.) from at least three experiments. D, EMSA was performed using a ^{32}P-labeled HSE or Octamer oligonucleotide as a probe in the presence of nuclear extracts from pSIREN-RetroQ (Control) or pSIREN-RetroQ/HSF-1 (shRNA HSF-1) retrovirus-infected Jurkat cells (10 μg), unstimulated (−) or treated with 250 μM cyclopentenone. E, RT-PCR analysis of total mRNA obtained from pSIREN-RetroQ (Control) or pSIREN-RetroQ/HSF-1 (shRNA HSF-1) retrovirus-infected Jurkat cells, untreated (−) or pretreated at 42°C for 1 h (HS) followed by activation with PMA plus ionomycin for 6 h.

binding site located between −143 and −115 bp of the *trail* promoter that could be involved in this repression.

15d-PGJ_2 Inhibited *Trail* Gene Promoter in Activated T Cells: Role of PPARγ. 15d-PGJ_2 is a natural ligand of PPARγ (Forman et al., 1995), a nuclear receptor able to regulate the expression of different genes in T lymphocytes by interfering with the transcriptional activity of their promoters (Yang et al., 2000; Cunard et al., 2002, 2004; Chung et al., 2003). In this regard, we assessed the role of PPARγ on *trail* promoter activation.

As we have described previously, our Jurkat cell line, unlike normal T cells, does not express this nuclear receptor either unstimulated or after activation (Cippitelli et al., 2003). We then analyzed its effect on *trail* promoter activation in experiments of cotransfection. We transiently transfected Jurkat T cells with the −1523-bp *trail* promoter reporter and a PPARγ expression vector or an empty vector as a control. As shown in Fig. 7, A and B, rosiglitazone, a specific PPARγ agonist, could reduce the activity of *trail* promoter induced by PMA plus ionomycin only in the presence of the nuclear receptor cotransfected. Moreover, in activated freshly isolated T cells (which express the nuclear receptor), troglitazone, another PPARγ activator, significantly reduced the induction of *trail* mRNA as detected by RT-PCR assay (Fig. 7, C and D). Thus, PPARγ activation may represent an additional parallel mechanism involved in the inhibition of *trail* gene expression mediated by 15d-PGJ_2.

Discussion

In this study, we described the inhibitory effect of the anti-inflammatory cyPG 15d-PGJ_2 on the expression of *trail* gene in activated T cells, explaining several mechanisms responsible for this repression. Prostaglandins are lipid molecules involved in the regulation of many processes, including inflammation. In this regard, a number of in vitro studies have described the modulation of different immune cells (Harris et al., 2002), including T lymphocytes (Yang et al., 2000; Chung et al., 2003; Cippitelli et al., 2003), by 15d-PGJ_2. Moreover, powerful anti-inflammatory activity of this prostaglandin have been described in animal models of experimental autoimmune diseases, in which pharmacological administration of this prostanoid could ameliorate local tissue damage via direct inhibition of signaling pathways/transcription factors, such as NF-κB or activation of the nuclear receptor PPARγ (Kawahito et al., 2000; Cuzzocrea et al., 2002; Diab et al., 2002, 2004).

TRAIL has been studied in cancer and autoimmune diseases because of its preferential toxicity for transformed cells and its capability of inhibiting autoimmune diseases in experimental animal models (Song et al., 2000; Cretney et al., 2006). However, recent findings have shown that, depending on the activation or differentiation status, also primary normal cells can be susceptible to TRAIL-mediated apoptosis (Corazza et al., 2004). Moreover, TRAIL has been demonstrated to be an effector molecule of T cell-mediated killing of oligodendrocytes in experimental autoimmune encephalomyelitis (Aktas et al., 2005), vascular smooth muscle cells in the atherosclerotic plaque (Sato et al., 2006), or monocytes and neutrophils in lupus erythematosus systemic (Kaplan et al., 2002; Matsuyama et al., 2004). In a different context, TRAIL can also stimulate apoptosis and the selection/proliferation of apoptosis-resistant fibroblast-like synoviocytes in rheuma-

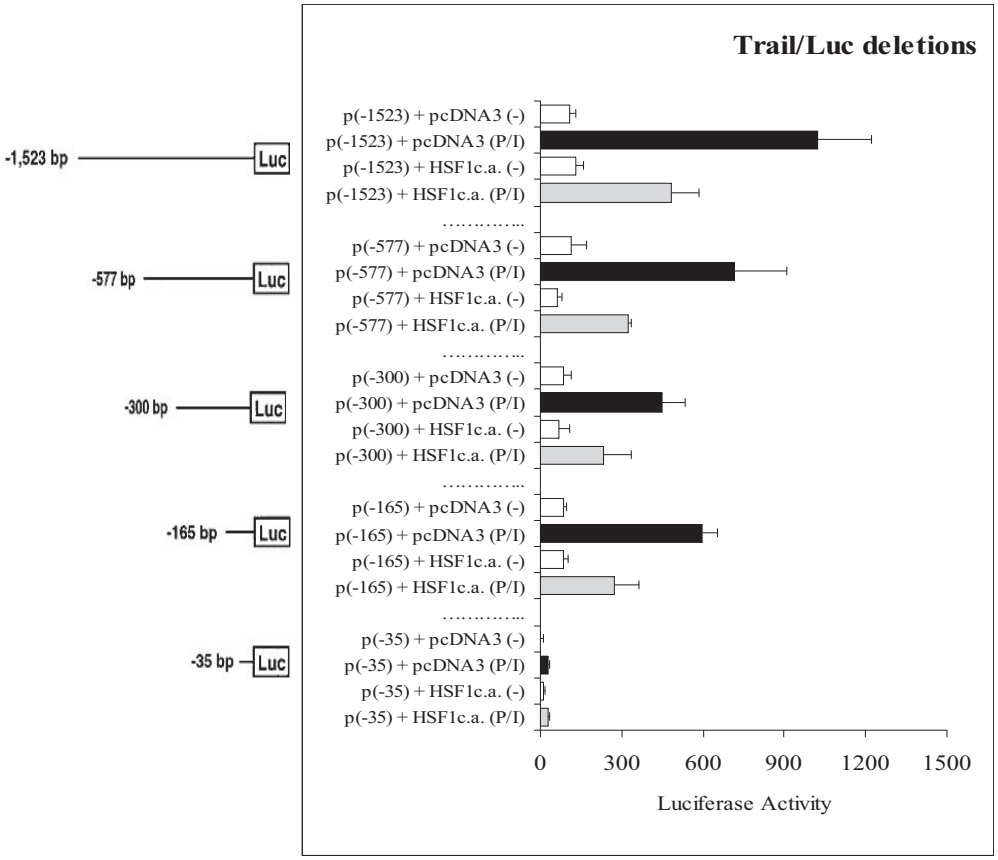

Fig. 5. A promoter analysis of HSF-1-mediated inhibition. Jurkat cells were cotransfected with 10 μg of the indicated human TRAIL/Luc reporter plus 4 μg of RSV-Gal expression vector. Five micrograms of an expression vector encoding a constitutive active form of HSF-1 (HSF-1 c.a.) or a pcDNA3 empty control vector were added to the cotransfection setting as indicated. Activation and samples harvesting was carried out as described above. Results are expressed as relative luciferase activity as described above and represent the mean value (X ± S.E.) from at least three experiments.

toid arthritis (Morel et al., 2005), indicating that a particular microenvironment can modify the specific response to this ligand. We have shown that human T cells and the Jurkat T cell line, activated in the presence of pharmacological concentrations of 15d-PGJ_2, have an impaired expression of *trail* mRNA and protein as a consequence of a reduced activity of its promoter.

Although *trail* mRNA is detected in various cells and tissues, the regulation of its expression remains largely unknown. However, it was demonstrated as the absolute requirement for NF-κB in Ag receptor-induced expression of *trail* in T lymphocytes (Baetu et al., 2001; Rivera-Walsh et al., 2001; Siegmund et al., 2001).

15d-PGJ_2 was shown to exert its anti-inflammatory activity through inhibition of critical steps in the activation of NF-κB, a transcription factor that has a critical role in the control of inflammatory responses (Hayden et al., 2006), or through the activation of PPARγ in different cells (Jiang et al., 1998; Ricote et al., 1998; Rossi et al., 2000; Straus et al., 2000).

In activated Jurkat cells, we observed the induction of NF-κB binding on the NF-κB1 site corresponding to the com-

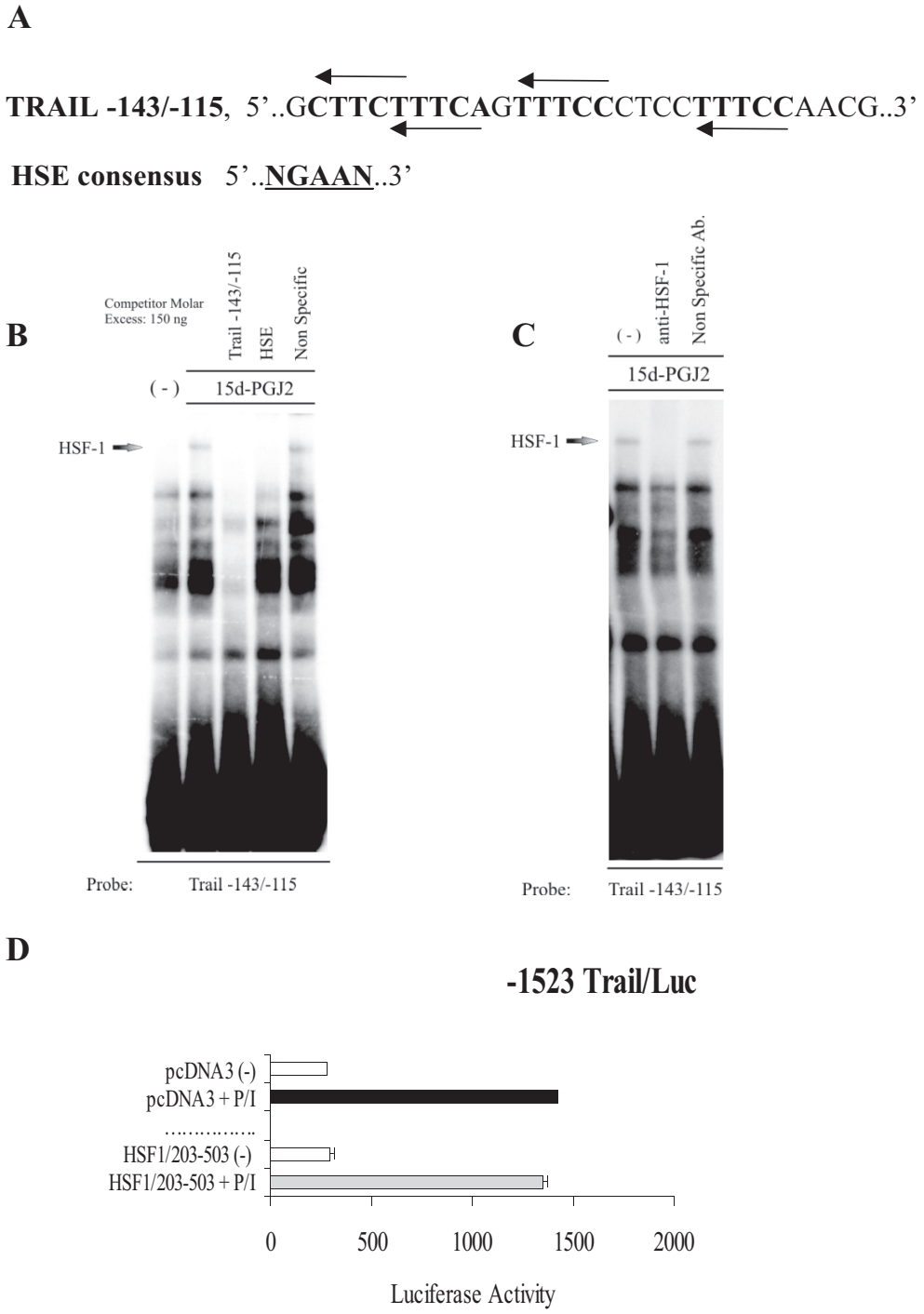

Fig. 6. HSF-1 binds *trail* promoter in 15d-PGJ_2-treated cells. A, schematic representation of the −143/−115 promoter region; the putative and the consensus HSE are indicated by arrows. B, EMSA was performed using a TRAIL −143/−115 ^{32}P-labeled oligonucleotide as a probe in the presence of nuclear extracts (10 μg) from unstimulated (−) or 10 μM 15d-PGJ_2-treated cells. Where indicated, 100 ng of the indicated unlabeled competitor was added to the reaction mixture to confirm specificity (nonspecific competitor is Octamer h-histone H2b). C, supershift analysis of HSF-1 complex bound to the TRAIL −143/−115 oligonucleotide: where indicated, purified anti-HSF-1 was added to the reaction mixture. The same amount of a nonspecific antibody (anti-Octamer-1) did not supershift or inhibit HSF-1-bound complex. D, Jurkat cells were cotransfected with 10 μg of the indicated human TRAIL/Luc reporter plus 4 μg of RSV-Gal expression vector. Where indicated, 5 μg of an expression vector encoding a DNA binding domain deleted form of HSF-1 (HSF-1/203–503) or a pcDNA3 empty control vector was added to the cotransfection setting. Activation and samples harvesting was carried out as described above. Results are expressed as relative luciferase activity and represent the mean value (X ± S.E.) from at least three experiments.

plex(es) p50/p65/c-rel strongly inhibited by the prostanoid (Fig. 2, B and D); moreover, the reduction of DNA binding activity of this transcriptional complex was correlated with the inhibition of c-rel (but not p65) nuclear translocation/expression (Fig. 2, F and G). These results suggest that the blockade of NF-κB activity may be one of the mechanisms responsible for the inhibition of *trail* expression by 15d-PGJ$_2$.

PPARγ is a nuclear receptor able to negatively interfere with the function of different transcription factors in T lymphocytes, such as c-Jun, NF-AT, and NF-κB, by regulating their DNA binding activity or specific recruitment of transcriptional coactivators (Yang et al., 2000; Chung et al., 2003; Cunard et al., 2004). As shown previously, Jurkat cells used in our experiments do not express detectable levels of PPARγ (Cippitelli et al., 2003), which is the reason we did not observe any effect of CAY10410 on *trail* mRNA expression in RT-PCR analysis (Fig. 1B). CAY10410 is a molecular analog of 15d-PGJ$_2$ whose structure compromises its capability of covalently interacting with cellular proteins without interfering with its property of PPARγ agonist. On the other hand, we evaluated the effect of this nuclear receptor on *trail* promoter in experiments of cotransfection and observed a significant repressive action of PPARγ in the presence of a specific agonist, rosiglitazone (Fig. 7, A and B). Our data indicate that in normal T lymphocytes that express PPARγ (Clark et al., 2000; Harris and Phipps, 2001) (Fig. 7D), activation of this nuclear receptor may contribute to the modulation of *trail* gene expression.

Given the role of NF-κB in the regulation of *trail* gene, it could be a target of the repressive action of PPARγ, even though *trail* promoter contains other putative sites for transcription factors inhibited by PPARγ, such as NF-AT and AP-1 (Gong and Almasan, 2000; Wang et al., 2000). Further experiments are needed to define the molecular mechanism(s) involved in this repression by PPARγ.

Most of the actions of 15d-PGJ$_2$ not related to PPARγ are dependent on its cyclopentenonic structure. We demonstrated that the compound cyclopentenone, which bears the chemically reactive $\alpha\beta$-unsaturated carbonyl group, could repress *trail* mRNA expression in activated Jurkat cells (Fig. 1B), indicating that adduct formation by Michael addition plays an important role for this inhibition.

The covalent interaction between the 15d-PGJ$_2$ and components of NF-κB signaling pathway (e.g., IκB kinase complex β, p50, and p65 subunits) via Michael reaction, results in an impaired nuclear entry, DNA binding activity, or transcriptional competence of NF-κB (Rossi et al., 2000; Straus et al., 2000; Cernuda-Morollón et al., 2001).

Our results suggest that the inhibition of this transcription factor by 15d-PGJ$_2$, through its electrophilic properties, is involved in the repression of *trail* gene expression in T cells. However, the cyclopentenonic ring may allow 15d-PGJ$_2$ to influence the activity of other possible transcriptional regulators of *trail* gene, and additional experiments will be necessary to define this possibility.

In this regard, analysis of progressive deletions of *trail* promoter delineated a minimal region of 165 bp from the transcriptional start site, which was lacking in NF-κB sites but still sensitive to the prostanoid (Fig. 3). This observation indicates that the repressive mechanism mediated by 15d-PGJ$_2$ on *trail* promoter may involve additional regulatory mechanisms other than NF-κB inhibition.

The anti-inflammatory activity of cyPGs was demonstrated to be dependent on their ability to activate the heat shock response and to induce the synthesis of cytoprotective HSPs in vivo (Ianaro et al., 2003), probably as a consequence of the covalent modification and alteration of cellular proteins (Straus and Glass, 2001). HSF-1 is the transcriptional factor mainly involved in the regulation of HSP expression; these proteins have direct anti-inflammatory and protective effects. However, it is increasingly evident that additional mechanisms, independent of activation of HSP expression, can mediate the action of heat shock response on inflammation and injury. In fact, HSF-1 can directly modulate the expression of several proinflammatory genes, such as TNF and IL-1β (Cahill et al., 1996; Singh et al., 2002; Chou et al.,

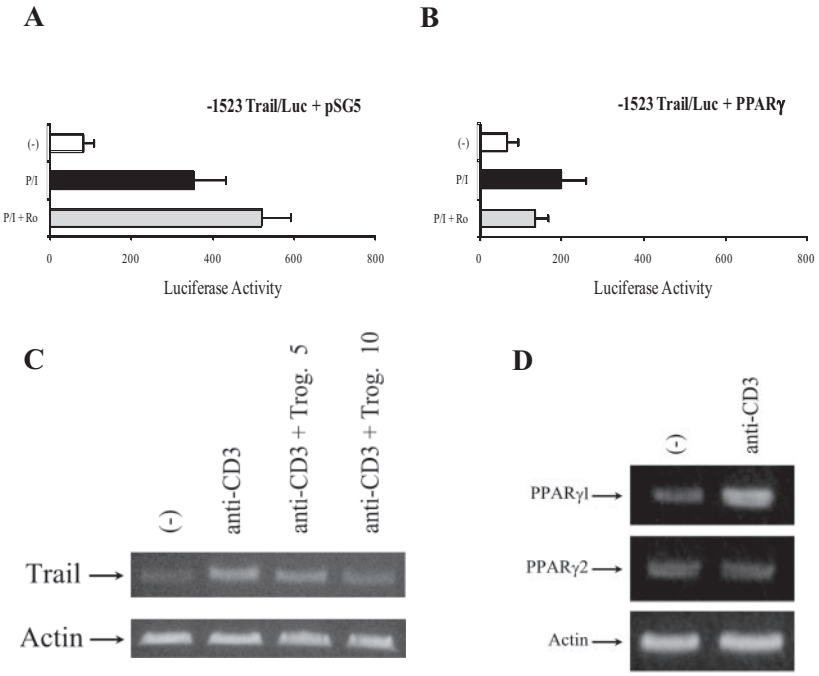

Fig. 7. PPARγ activation inhibits *trail* promoter activity. A and B, Jurkat cells were cotransfected with 10 μg of the indicated human TRAIL/Luc reporter plus 4 μg of RSV-Gal expression vector. Where indicated, 4 μg of an expression vector encoding a PPARγ or the PSG5 empty control vector was added to the cotransfection setting. Activation and samples harvesting was carried out as described above. Twenty-four hours after transfection, cells were left untreated (−) or were stimulated with PMA plus ionomycin (P/I) in the absence or in the presence of rosiglitazone (2.5 μM). After 8 h, cells were harvested, and protein extracts were prepared for the luciferase and β-galactosidase assays. Results are expressed as relative luciferase activity and represent the mean value (X ± S.E.) from at least three experiments. C and D, RT-PCR analysis of total mRNA obtained from enriched human T cells untreated (−) or activated with plate-bound anti-CD3 mAb (OKT3) for 6 h in the absence or in the presence of 5 and 10 μM troglitazone.

2005), whereas HSF-1 knockout is associated with a long-term increase in TNF-α levels and susceptibility to endotoxin (Xiao et al., 1999). More importantly, the activation of HSF-1 has been shown to occur in vivo in inflamed tissues and to be essential for the remission of the inflammatory reaction by 15d-PGJ_2 (Ianaro et al., 2003).

As reported previously (Cippitelli et al., 2003), in our experimental conditions, this prostanoid activated a strong heat shock response and induced HSF-1 DNA binding (Fig. 4A). Moreover, overexpression of an active form of HSF-1 (HSF-1 c.a.) significantly repressed *trail* promoter activity, whereas a mutant HSF-1 defective in DNA binding activity did not exert any effect. It is interesting that HSF c.a. significantly reduced the activity of all *trail* promoter deletions except for the −35-bp fragment (Fig. 5). These data indicated the presence of element(s) sensitive to HSF-1 within the first 165 bp upstream the transcriptional start site and the requirement of its binding to DNA for this repression. We also analyzed the possibility of a direct binding between this transcription factor and *trail* promoter and demonstrated that putative HSE sites, spanning from −143 to −115 bp (Fig. 6A), were able to bind HSF-1 in cells treated with 15d-PGJ_2 (Fig. 6, B and C).

The role of this promoter element in the regulation of *trail* transcription has never been studied in T lymphocytes. However, the inducibility of the −165-bp *trail* promoter deletion by PMA plus ionomycin suggests that activation-induced transcriptional factor(s) regulate this region. The inhibitory action of HSF-1 could be due to a mechanism of competitive binding to DNA with a transcriptional enhancer or to a direct repression, as described for the negative regulation of IL-1β (Cahill et al., 1996) and TNF-α (Singh et al., 2002) promoters, respectively. In addition, it could interfere with the expression of transcriptional component(s) important for *trail* promoter activity. Further studies are required to understand how this transcription factor may act in this context.

In conclusion, our study reveals that *trail* gene is a novel target of inhibitory mechanisms mediated by 15d-PGJ_2 in T cells. These data might help to better understand the complex range of action of this molecule that accounts for its anti-inflammatory activity and support the possible evaluation for its pharmacological use to treat destructive inflammatory and autoimmune pathologies.

Acknowledgments

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